Liver-x-Receptor-Mediated Expression of Key Genes in Macrophages Implicated in the Control of Cholesterol Homeostasis and Atherosclerosis

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Abstract

Atherosclerosis is a leading cause of morbidity and mortality in the western world. The deposition of lipoprotein cholesterol in the arterial wall is a critical early step in the pathogenesis of atherosclerosis. These atherogenic lipoproteins are then taken up by macrophages to transform into lipid-loaded foam cells. ATP-binding cassette transporter-A1 (ABCA1) is a membrane-bound protein that mediates efflux of cholesterol from cells, such as macrophages. Tangier disease, which arises due to mutations in the ABCA1 gene, is associated with foam cells in a number of tissues and premature atherosclerosis. Proteins in HDL, such as apolipoprotein E (apoE), act as acceptors of cholesterol released from macrophages via the action of ABCA1, and take it to the liver where it can be excreted through the bile system. Increasing the expression of both ABCA1 and apoE is therefore considered as a potential therapeutic approach for the prevention or treatment of atherosclerosis. Liver-X-receptors (LXRs) are members of a subfamily of nuclear receptors that are potent activators of ABCA1 and apoE expression. Agonists of LXRs inhibit macrophage foam cell formation in vitro and atherosclerosis in mouse models of the disease. The signalling pathways through which LXR agonists induce the expression of ABCA1 and apoE expression in macrophages are not known. The major aim of the studies presented in this thesis was to investigate such signalling pathways using the human macrophage THP-1 cell line as a model system with key findings confirmed in primary cultures.

Both natural LXR agonists, such as combinations of 22-(R)-hydroxycholesterol (22-(R)-HC) and 9-cis-retinoic acid (9CRA), and synthetic ligands induced the expression of ABCA1 and apoE. Such an induction of ABCA1 and apoE expression was attenuated by treatment of the cells with pharmacological inhibitors of c-Jun-N-terminal kinase/stress-activated kinase (JNK/SAPK) and phosphoinositide-3-kinase (PI3K) pathways. The action of 22-(R)-HC and 9CRA was associated with activation of JNK/SAPK, its upstream component SEK1/MKK4 and its down-stream target c-Jun along with the key target for PI3K, protein kinase B (PKB). The role of these pathways was confirmed further by analysing the action of expression of dominant negative forms of key proteins on the activation of ABCA1 promoter. In addition, small interfering RNA-mediated knockdown of JNK/SAPK was found to attenuate the induction of apoE expression. The action of these pathways culminated at the level of activator protein-1, a transcription factor that contains c-Jun, and whose binding sites are present in the regulatory regions of both the apoE and ABCA1 genes. Finally, a potential cross-talk between the JNK/SAPK and PI3K pathways was identified in which protein kinase C played an important role.

In conclusion, the studies presented in this thesis demonstrated, for the first time, an important role for a pathway involving PKB, PKC and JNK/SAPK cascade in the activation of ABCA1 and apoE expression by LXR agonists, which has implications to macrophage foam cell formation and atherosclerosis.

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Table of Contents

Abbreviations

Abbreviation	Full term
ABC	ATP binding cassette transporter
ABCA1	ATP binding cassette transporter A1
ACAT	Acyl CoA:cholesterolacyltransferase
AF-1	Activation function-1
AF-2	Activation function-2
AT1R	Angiotensin II type 1 receptor
AP-1	Activator protein-1
apoE	Apolipoprotein E
APS	Ammonium Persulphate
AR	Androgen receptor
ASC-2	Activating signal co-integrato-2
ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine serum albumin
CAD	Coronary artery diseases
CE	Cholesterol ester
CK2	Casein kinase II
CVD	Cardiovascular disease
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
CEPT	Cholesterol ester transfer protein
CHD	Coronary heart diseases
cdks	Cycline dependent kinases
CYP7A-1	Cholesterol 7α-hydroxylase
Da	Daltons
DAG	Diacylglycerol
DBD	DNA binding domain
dCTP	Deoxycytidine triphosphoate
DIPE	Diisopropyl ether
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DNAase	Deoxynuclease
ddH2o	Double distilled water
DN	Dominant negative
dNTP	Deoxyribonucleotide triphosphate

DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EMSA	Electrophoretic mobility shift assay
eNOS	Endothelial NOS
ER	Estrogen receptor
ERK	Extracellular regulated kinase
EtBr	Ethidium bromide
ECL	Enhanced chemi-luminescence
FXR	Farnesoid X receptor
FCS	Foetal calf serum
FGF	Fibroblast growth factor
g	Gram(s)
g	Gravity
GAPDH	Glyceraldehydes 3-phosphate dehydrogenase
GM-CSF	Granulocyte/macrophage-colony stimulating factor
G-PCR	G-protein coupled receptors
GR	Glucocorticoid receptor
GRIP-1	Glucocorticoid receptor interacting protein-1
GSK-3	Glycogen synthase kinase-3
h	Hour(s)
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HDL	High density lipoprotein
HI-FCS	Heat-inactivated foetal calf serum
HNF-4	Hepatic nuclear factor-4
HREs	Hormone response elements
ICAM	Intracellular adhesion molecule
IFN-γ	Interferon-γ
IL	Interleukin
IRS	Insulin receptor substrate
JAK2	Janus kinase 2
JNK	c-Jun N- terminal kinase
Kb	Kilo bases
kDa	Kilo daltons
L	Liter
LBD	Ligand binding protein
LCAT	Lecithin:cholesterol acyltraferase
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LPDS	Lipoprotein deficient serum

LPL	Lipoprotein lipase
Luc	Luciferase
LXR	Liver x receptor
LXRE	LXR response element
M	Molar
MAPK	Mitogen activated kinase
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony stimulating factor
β-ME	β-mercaptoethanol
min	Minutes
MMP-9	Matrix metalloproteinase-9
MMLV	Moloney Murine Leukaemia Virus
mRNA	Mesenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NC	Nuclear receptor
NCoR	Nuclear receptor corepressor
NF-κB	Nuclear factor for kappa light chain in B cells
OD	Optical density
oxLDL	Oxidised density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
P/CAF	P300/CBP associated factor
PCR	Polymerase chain reaction
PDGF	Patelet derived growth factor
PEI	Polyethelenimine
Pen/strep	Penicillin/streptomycin
PH	Pleckstrin homology (domain)
PI	Phosphatidyl-inositol
PI3K	phosphoinositide-3-kinase
PIP	Phosphatidylinositol phosphate
PKA	Protein kinase A
PKB	Protein kinase B (AKT)
PKC	Protein kinase C
PLTP	Phospholipid transfer protein
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulphonyl fluoride
PPAR	Peroxisome proliferator activated factor
PGC-1α	PPAR γ coactivator 1 α
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PVDE	Polyvinylidene fluoride

RAR	Retinoic acid receptor
RCT	Reverse cholesterol transport
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
RNAase	Ribonuclease
rpm	Revolutions per minute
RTK	Receptor tyrosine kinases
RT	Reverse transcriptase
RT	Room temperature
RXR	Retinoid X receptor
SAPK	Stress activated protein kinase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Sec	Seconds
SEK-1	SAPK/ERK kinase
siRNA	Small interfering RNA
S6K	Ribosomal protein S6 kinase (p70)
SMCs	Smooth muscle cells
SMRT	Silencing mediator for retinoid and thyroid hormone receptors
SR	Scavenger receptor
SR-A	Scavenger receptor class A
SR-B1	Scavenger receptor class B type 1
SRC	Steroid receptor coactivator
SREBP	Sterol regulatory element binding protein
SWI/SNF	Switch/sucrose non-fermenting
T	Treated
TAE	Tris-acetate-EDTA
Taq	Thermos aquaticus
TBE	Tris-borate-EDTA
TD	Tangier disease
TE	Tris- EDTA
TEMED	N, N, N, N-tetra methyl ethylene diamine
TF	Transcription factor
TG	Triglycerides
TGF-β	Transforming growth factor-β
TNF	Tumour necrosis factor
TR	Thyroid receptor
TRAP	Thyroid hormone receptor- associated proteins
U	Unit
UC	Unesterified cholesterol

UT	Untreated
UV	Ultra violet
V	Volts
VCAM-1	Vascular cell adhesion molecule- 1
VDR	Vitamin D receptor
v/v	Volume to volume
VLDL	Very low density lipoprotein
WCE	Whole cell extract
WHAM	Wisconsin hypoalpha mutant
w/v	Weight to volume

CHAPTER ONE:

GENERAL INTRODUCTION

Chapter 1: General Introduction

Cholesterol is vital for diverse cellular functions and plays several structural and metabolic roles. Cholesterol distributes along the entire plasma membrane of cells, where it modulates its fluidity and permeability. Cholesterol is also required for the regulation of the function of integral membrane proteins, transcriptional regulation and for the formation of lipid rafts (Tabas, 2002). Cholesterol concentrates in specialized sphingolipid-rich domains of the plasma membrane, called rafts and caveolae (Anderson, 1998). These contain a variety of signalling molecules that depend on the well-maintained cholesterol content for normal activity. In addition, cholesterol is a substrate for the production of steroids and covalently links to a protein involved in limb development (Lewis et al., 2001). However, elevated cellular levels of free cholesterol are toxic and can have pathological effects, in particular in cells of the arterial wall, where its accumulation initiates atherosclerotic cardiovascular disease (CVD) (Glass and Witztum, 2001; Guyton and Klemp, 1996; Lusis, 2000). The body therefore depends on a complex homeostatic system to modulate the availability of cholesterol to tissues, which operates at both the cellular level and within the plasma component of the serum (Oram and Heinecke, 2005).

In the human plasma, two-thirds of the cholesterol is carried by a class of lipoprotein particles called low-density lipoproteins (LDL), which provide a source of cholesterol for steroidogenesis and cellular membranes. The uptake of cellular cholesterol occurs through the interaction of LDL with a cell-surface receptor (LDL receptor or LDLR) that mediates internalization and degradation of the lipoprotein particles (Oram and Heinecke, 2005). The hepatic LDLR is responsible for clearing most of the LDL cholesterol from the plasma. Cells other than those in steroidogenic tissues and the liver (e.g. macrophages and smooth muscle cells) cannot metabolize cholesterol. Instead, they modulate their membrane cholesterol content by a feedback system that controls the rate of cholesterol biosynthesis and uptake by the LDL receptor (Brown and Goldstein, 1999). This system in most cell types is sufficient to provide cells with enough cholesterol to maintain membrane integrity and function without cholesterol overloading. Some cells, specifically macrophages, can take up cholesterol by other endocytic and phagocytic pathways that are not under feedback regulation by this sterol (Osterud and Bjorklid, 2003). Such cells must either store this excess cholesterol as esters or secrete it (Oram and Heinecke, 2005).

High-density lipoproteins (HDL), which carries about one-third of the cholesterol in the human plasma, are involved in the removal of excess cholesterol from cells. HDL is a multifunctional and heterogeneous class of particle that transports a variety of lipids and lipophilic molecules between tissues and other lipoproteins. One of the major functions of HDL is to transport cholesterol from peripheral tissues to the liver for elimination via the bile system (Glomset, 1968; Oram and Yokoyama, 1996), a process called reverse cholesterol transport (RCT). HDL, or its components, can remove cellular cholesterol by multiple mechanisms (Oram and Yokoyama, 1996). For example, the phospholipids in HDL absorb cholesterol that has diffused from the plasma membrane into the aqueous phase by a passive process, which is facilitated by the interaction of HDL particles with scavenger receptor BI (SR-BI) (Oram and Heinecke, 2005). In addition, four transporters have been identified in the cell membrane that mediate cholesterol efflux to HDL components by metabolically active pathways. All these transporters belong to a superfamily of ATP-binding cassette transporters (ABCs). ABCA1 mediates the transport of cellular cholesterol, phospholipids and other metabolites to HDL proteins (apolipoproteins) that are associated with little or no lipid (lipid-free apolipoproteins) (Wang and Tall, 2003). ABCA7 is a close homolog of ABCA1 and selectively transports phospholipids to lipid-depleted apolipoproteins (Abe-Dohmae et al., 2004). ABCG1 and ABCG4, which are highly expressed in tissue macrophages and hepatocytes, mediate cholesterol transport from cells to HDL particles (Nakamura et al., 2004; Neufeld et al., 2001; Schmitz et al., 2001; Wellington et al., 2002). From these four ABC transporters, ABCA1 has been extensively characterized. Several studies using cultured cells or specific animal models have shown that ABCA1 is an effective athero-protective agent and a major determinant of plasma HDL levels (Aiello et al., 2003; Oram and Heinecke, 2005; Singaraja et al., 2003; Wang and Tall, 2003). Therefore, this transporter has become an important new therapeutic target for clearing cholesterol from arterial macrophages and, thereby, preventing the development of atherosclerosis, which is addressed below in more detail.

1.1 Atherosclerosis

The term atherosclerosis, which is a slow progressive disorder, was coined in 1904, and came from the Greek words "athero", meaning porridge or gruel that refers to the soft consistency of the core plaque, and "sclerosis" meaning hardening (Gurr et al., 2002). Atherosclerotic CVD is the leading cause of all deaths in industrialized countries,

accounting for nearly 40% of mortality (Glass and Witztum, 2001; Hansson and Libby, 2006; Lusis, 2000). Atherosclerosis is a form of arteriosclerosis in which a plaque builds up in the inner lining of arteries. A plaque consists of a combination of lipids, cholesterol, cellular debris, fibrin, calcium and other substances, and usually develops in the inner lining of medium and large arteries. Accumulation of these substances leads to the thickening of the arterial wall and causes them to lose elasticity and so become less resilient. Overtime, these plaques, which are known as atherosclerotic lesion or atheroma, mature and gain new characteristics. The development of such atherosclerotic lesions involves a complicated sequence of events in which various cell types, such as monocyte-derived macrophages, T cells and the normal cellular elements of the arterial wall (e.g. smooth muscle cells (SMCs) and collagen), contribute to form a complicated atherosclerotic plaque (Glass and Witztum, 2001; Hansson and Libby, 2006; Lusis, 2000; Ross, 1999).

Atherosclerosis begins early in life, sometimes in late childhood, but causes no symptoms. It usually becomes symptomatic when it interferes with the coronary or cerebral circulation. If a blood clot (thrombus) forms inside a cerebral artery, it cuts off the blood supply to certain areas of the brain and an ischaemic stroke may occur. If it blocks a blood vessel that feeds the heart, it causes a heart attack or myocardial infarction (Gurr et al., 2002). If atherosclerosis narrows other arterial branches, for example, the arteries supplying blood to the intestines, abdominal angina occurs, and sudden, complete blockage of the blood supply to the intestine can cause bowel infarction. In the extremities, atherosclerosis can narrow the major arterial supply to the legs. The reduced blood flow results in cramps and pain during exercise, known as intermittent claudication. Severely compromised blood supply to the legs leads to pale or cyanotic, cold legs which eventually may develop gangrenes (Hansson and Libby, 2006).

Systematic investigation of the mechanisms that initiate atherosclerosis have relied on animal models of this disease. In this regard, two strains of genetically altered mice have been particularly fruitful. ApoE-/- mice, which are deficient in the gene coding for apolipoprotein E (apoE), a key component of plasma lipoproteins and a regulator of overall cholesterol metabolism, develop spontaneous hypercholesterolemia and atherosclerotic disease that progresses to myocardial infarction and stroke (Piedrahita

et al., 1992; Plump et al., 1992; Zhang et al., 1992a). The development of atherosclerosis in these mice can be "speeded-up" by feeding them a high fat diet. Mice deficient in the gene encoding the LDL receptor (LDLR-/-) develop hypercholesterolemia and atherosclerotic plaques when fed a high fat diet (Ishibashi et al., 1994). Crossbreeding of these deficient mice with those that carry deletion in other genes or transplantation of bone marrow from mice that are wildtype or deficient in a particular protein into irradiated apoE-/- or LDLR-/- mice have provided important information on the role of specific genes or population of cells in the development of this disease.

1.1.1 Risk factors for atherosclerosis

Epidemiological studies have identified numerous risk factors (e.g. environmental/ dietary and genetic factors) that contribute to the development of atherosclerosis, such as hypercholesterolemia, hypertension, diabetes mellitus, obesity, male sex, smoking, age, family medical history, physical inactivity, dietary habits, infections and stress, which all work synergistically (Glass and Witztum, 2001; Lusis, 2000). Two genetic disorders, Tangier disease (TD) and familial hypoalphalipoproteinemia (FHA) have been found to be quite frequent and contribute to the development of atherosclerosis (Medh, 2000). Tangier disease is characterised by severe deficiency of HDL caused by a mutation in the ABCA1 gene, which plays a critical role in regulating cellular cholesterol efflux and RCT (Van Eck et al., 2002) (see section 1.2). FHA is a condition that leads to high levels of lipids in the plasma due to their impaired removal. FHA is caused by a mutation in the ABCA1 gene, resulting in decreased cellular cholesterol efflux and degradation of HDL (Brooks-Wilson et al., 1999). Amongst the many genetic and environmental risk factors, elevated levels of serum cholesterol is probably unique in being sufficient to drive the development of atherosclerosis in humans and experimental animals in the absence of other risk factors (Glass and Witztum, 2001). The greatest risk is in individuals that have high levels of serum LDL cholesterol and low levels of HDL cholesterol (Repa and Mangelsdorf, 2002; Ross, 1999). Although the exact athero-protective mechanism of HDL is not known, it is speculated that its ability to stimulate RCT may be a major reason (Repa and Mangelsdorf, 2002).

1.1.2 Lesions of atherosclerosis

Atherosclerosis is perceived to be an inflammatory response of macrophages and lymphocytes to pathogenic lipoproteins in the endothelial lining of the arterial wall (Li

and Glass, 2002; Ross, 1999). The limited development of atherosclerosis in hypercholesterolemic apoE-/- or LDLR-/- mice that are also deficient in macrophage colony stimulating factor (M-CSF), which is required for differentiation of monocytes into macrophages, represents one of many lines of evidence that demonstrate the importance of immune cells in this disease (Smith et al., 1995). The lesions formed as a response to activation of endothelial cells via a variety of insults (e.g. modified LDL) can be divided into three stages; the fatty streak, the mature plaque and the fibrous plaque (Lusis, 2000; Ross, 1999).

Fatty streaks, which represent the earliest visible atherosclerotic lesions, consist mainly of macrophages that have taken up massive amounts of cholesterol to form lipid-loaded foam cells (Li and Glass, 2002). The formation of fatty streaks is initiated by the adherence of circulating monocytes and T cells to activated endothelial cells at lesion prone sites within large arteries (Hansson and Libby, 2006). The activated endothelial cells in the arteries express leukocyte adhesion molecules, specifically vascular cell adhesion molecule 1 (VCAM1), as part of the initial response to cholesterol accumulation in the intima (Cybulsky and Gimbrone, 1991). The adherent monocytes and T cells migrate into the subendothelial space. Under the influence of M-CSF, produced by endothelial cells and SMCs (Rajavashisth et al., 1990), the monocytes differentiate into macrophages (Smith et al., 1995). The macrophages then accumulate massive amounts of cholesterol and become foam cells (Li and Glass, 2002) (Figure 1.1). Although the expression of VCAM1 by the endothelium ceases after a few weeks, SMCs begin to express this adhesion molecule (Li et al., 1993). Expression of VCAM1 and other adhesion molecules by SMCs might promote further recruitment of, and retention of, mononuclear cells in the arterial intima (Hansson and Libby, 2006). This is also accompanied by an influx of T cells, which can undergo antigen-dependent activation and produce cytokines and other regulatory molecules that influence the functional properties of the nearby endothelial cells, SMCs and macrophages (Hansson, 2001). The progression of fatty streaks to more complex lesions (mature atherosclerotic plaques, also known as atheromas) involves the migration of SMCs from the media into the intima, where they accumulate cholesterol and become SMC-derived foam cells (Li and Glass, 2002).

The death of foam cells leads to the formation of a necrotic, cholesterol-rich core region that is surrounded by a fibrous cap of extracellular, collagen-rich matrix proteins secreted by SMCs (Jonasson et al., 1986). The shoulder region of the plaque, which is where it grows further, and the interface between the fibrous cap and the lipid core have high accumulation of activated T cells and macrophages (Jonasson et al., 1986). These produce pro-inflammatory cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) (Hansson and Libby, 2006). With time, the mature plaques can progress into an even more complex lesion.

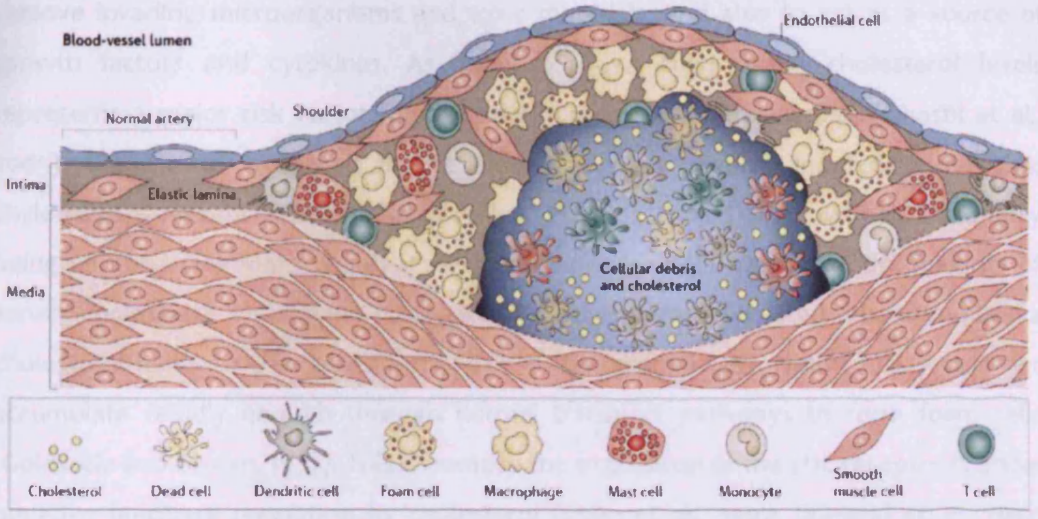


Figure 1.1 Cellular composition of atherosclerotic plaques.

The atherosclerotic plaque has a core containing lipids, which include esterified cholesterol and debris from dead cells. Surrounding it, a fibrous cap, containing SMCs and collagen fibres, stabilizes the plaque. Immune cells, including macrophages, T cells and mast cells, populate the plaque, and are frequently in an activated state. They produce cytokines which can affect plaque inflammation and vascular function. Until complications occur, an intact endothelium covers the plaque. Figure taken from Hansson and Libby, 2006.

As the lesion grows, those regions that contain a high concentration of lipids are particularly prone to rupture (Li and Glass, 2002). Such unstable plaques also contain necrotic debris, a thin fibrous cap and numerous macrophages in the shoulder regions where rupture most often occurs. The fibrous cap consists of a dense collagen-rich extracellular matrix with SMCs, collagen fibres, macrophages and T cells, which protrude

into the arterial lumen, impeding the flow of blood and stabilizing the plaque in the lesion. Eventually, the growth of the lesion extends inward, causing narrowing of the vessel lumen and the clinical symptoms of angina. The rupture of the fibrous cap that overlies the lipid core of the plaque (Figure 1.2) can lead to thrombus formation, which occludes the vessel lumen and results in acute myocardial infarction (Glass and Witztum, 2001; Hansson and Libby, 2006).

1.1.3 Macrophages and cellular cholesterol accumulation

Macrophages are an essential part of the body's host defence system (Li and Glass, 2002). The normal function of macrophages is to act as an antigen-presenting cell to remove invading microorganisms and toxic materials, and also to act as a source of growth factors and cytokines. As detailed above, high serum cholesterol levels represents a major risk factor for the development of atherosclerosis (Ohashi et al., 2005). Cholesterol is carried in the bloodstream by several lipoprotein particles: chylomicrons, very low-density lipoproteins (VLDL), LDL and HDL, with HDL primarily being an anti-inflammatory, anti-atherogenic molecule. LDL transports the majority of serum cholesterol in humans (Glass and Witztum, 2001). Elevated levels of plasma cholesterol leads to the deposition of LDL in the arterial wall. Native LDL would not accumulate rapidly enough through normal transport pathways to form foam cells (Goldstein and Brown, 1977). This is because the expression of the LDL receptor is under negative feedback regulation by cholesterol (Chen et al., 2007; Dueland et al., 1992; Spitsen et al., 2000). It is the oxidation of LDL once it has entered the intima, and its subsequent uncontrolled uptake by a number of scavenger receptors, that is thought to be the primary initiating event in the development of atherosclerosis (Cyrus et al., 1999). When there is damage to the arterial wall, an inflammatory response occurs in which monocytes enter the arterial wall and differentiate into macrophages. These macrophages then take up oxidized LDL (oxLDL) to form foam cells (Vainio and Ikonen, 2003). This inflammatory response of macrophages and lymphocytes to pathogenic lipoproteins in the arterial wall therefore contributes to the development of atherosclerosis (Glass and Witztum, 2001; Li and Glass, 2002; Libby et al., 2002; Ross, 1999).

Macrophages have essential functions in all phases of atherosclerosis, firstly in the development of the fatty streak, and ultimately on processes that cause the unstable plaque to rupture (Li and Glass, 2002; Smith et al., 1995). A study by Watanabe et al.

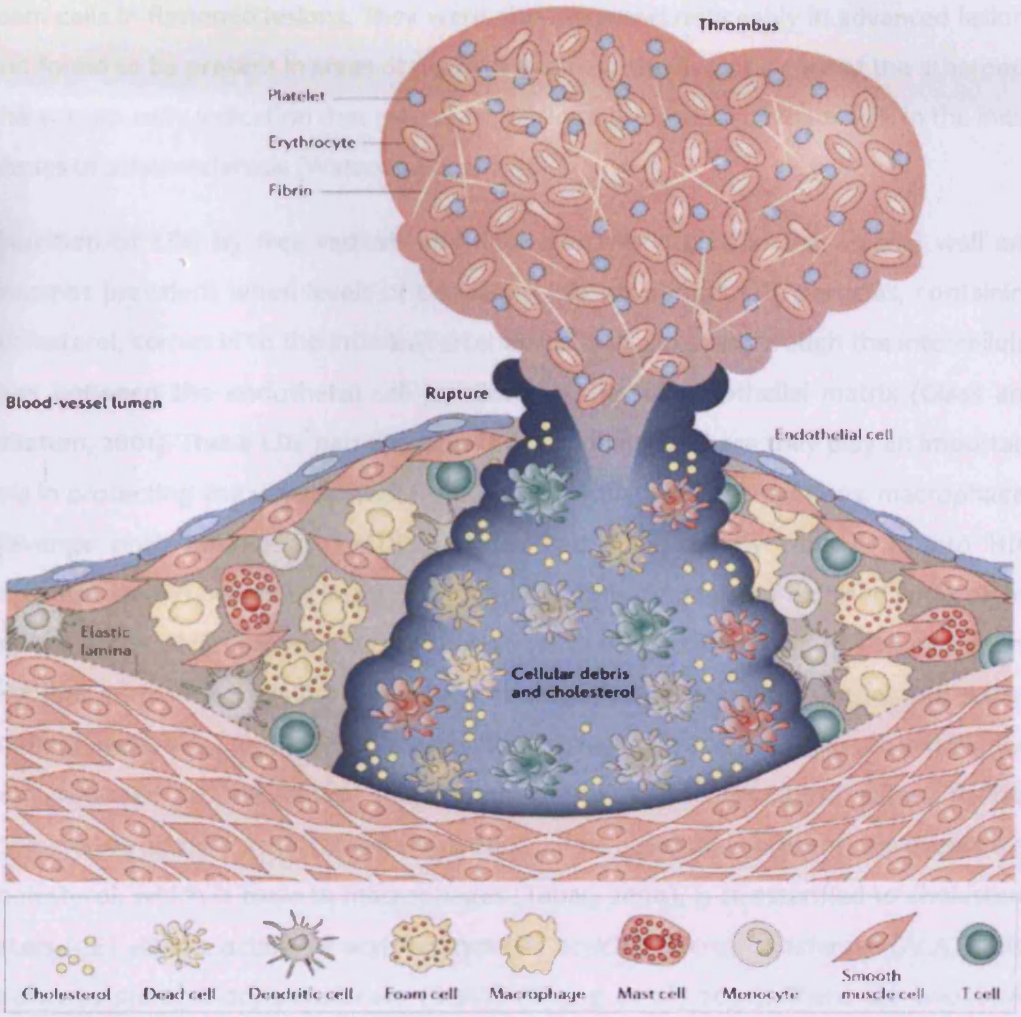


Figure 1.2 Plaque activation, rupture and thrombosis.

When activated, immune cells, including macrophages, T cells and mast cells, relealse pro-inflammatory cytokines, this reduces collagen formation and induces the expression of tissue factor. The weakened plaque might fissure when subjected to the forces of arterial blood pressure. Exposure of subendothelial structures promotes platelet aggregation and thrombosis. A thrombus forms and might occlude the lumen of the artery, leading to acute myocardial infarction. Figure taken from Hansson and Libby, 2006.

(1985) showed increased accumulation of lipid laden macrophages in the intima and their "clinging" to the endothelial surface of the arterial intima even when there were no grossly observable alterations in animals on high cholesterol diets for eight weeks or longer. Lipid-laden macrophages were the predominant cell type seen in new layers of foam cells in flattened lesions. They were also increased noticeably in advanced lesions and found to be present in areas of necrosis and near the necrotic core of the atheroma. This was an early indication that macrophages were the source of foam cells in the initial phases of atherosclerosis (Watanabe et al., 1985).

Oxidation of LDL by free radicals and lipoxygenases occurs in the arterial wall and becomes prevalent when levels of circulating LDL are raised. LDL particles, containing cholesterol, comes in to the intima of arterial wall and can pass through the intercellular gaps between the endothelial cell junctions to the subendothelial matrix (Glass and Witztum, 2001). These LDL particles attract macrophages, where they play an important role in protecting the vascular wall from injury. In inflammatory reactions, macrophages scavenge proinflammatory oxLDL particles and subsequently remove it into HDL particles to avoid becoming foam cells and dying. This clearance of oxidized lipoproteins and the efflux of lipoprotein-derived cholesterol to HDL acceptors for RCT represent the first line of defence against cholesterol toxicity in macrophages (Cuchel and Rader, 2006; Li and Glass, 2002). When macrophage scavenger receptors take up oxLDL, it is first delivered to lysosomes. These then hydrolyze its cholesterol esters to free cholesterol and fatty acids (Li and Glass, 2002). If no acceptor is present, the excess free cholesterol, which is toxic to macrophages (Tabas, 2004), is re-esterified to cholesterol esters (CE) via the action of acylcoenzyme A: acylcholesterol transferase (ACAT), also known as sterol O-acyltransferase (SOAT) (Chang et al., 2001). There are two SOAT genes that have been identified, SOAT1, which is expressed by a variety of cells including macrophages, and SOAT2, which is expressed in the liver and the intestine (Brewer, 2000). The functions of SOAT are opposed by neutral cholesterol esterase, exemplified by hormone sensitive lipase, which hydrolyses cholesterol esters and also functions as the rate limiting enzyme for the hydrolysis of triglycerides in adipocytes (Brewer, 2000). Several studies have indicated that cholesterol esterification is a protective response of macrophages to excess free cholesterol in conditions in which cholesterol efflux pathways become saturated (Accad et al., 2000; Fazio et al., 2001). Although these findings argue against the use of SOAT inhibitors for the prevention or treatment of

atherosclerosis, a recent study using an inhibitor of SOAT1 and SOAT2 in apoE-/- mice demonstrated a significant decrease in serum lipoprotein levels and extent of atherosclerosis without any evidence of toxicity (Kusunoki et al., 2001), indicating that partial inhibition of these pathways may be beneficial.

1.1.4 Reverse cholesterol transport

Glomset in 1968 was the first to introduce the concept of RCT to illustrate the mechanism by which peripheral cholesterol is returned to the liver for excretion in the bile system (Glomset, 1968). Ross and Glomset (1973) hypothesized that atherosclerotic lesions develop when an imbalance occurs between removal of arterial cholesterol and its deposition after endothelial injury, and suggested the relationship of RCT to atherosclerosis. Later in 1975, this relationship was developed further by Miller and Miller who suggested that, on the basis of an inverse relationship between HDL levels and atherosclerosis, special importance should be placed on increasing circulating levels of HDL as a way to improve the clearance of cholesterol from the arterial wall to prevent the development of this disease (Miller and Miller, 1975). The physiological need for this process is clear as mammalian cells do not have the ability to catabolize the sterol ring and most excess sterols can only be eliminated from the body by biliary excretion. Excess unesterified cholesterol (UC) is toxic to cells, which have therefore developed several ways to protect against its toxicity (Cuchel and Rader, 2006; Ohashi et al., 2005).

1.1.5 Macrophage reverse cholesterol transport

As the intracellular levels of cholesterol increase, endogenous cholesterol biosynthesis and LDL-R expression are repressed by inhibition of the sterol regulatory element-binding protein (SREBP) pathway (Brown and Goldstein, 1999). In this pathway, the precursor SREBP protein is associated with the rough endoplasmic reticulum (RER). When the cell content of cholesterol is low, a protease cleaves SREBP to release the transcriptionally active N-terminal portion, which migrates to the nucleus and binds to specific promoter sequences in target genes; for example, LDL-R and enzymes involved in cholesterol synthesis (e.g. HMG-CoA synthase). When the cell content of cholesterol is high, this pathway does not operate and the transcription of target genes is low (Edwards and Davies, 1996). However, this mechanism is not sufficient to maintain cholesterol homeostasis in the face of continued cholesterol uptake by scavenger receptor-dependent mechanisms. Therefore, macrophages and other cell types must

export cholesterol to extracellular acceptors for transport to the liver for protection against cholesterol toxicity (Brown and Goldstein, 1999; Cuchel and Rader, 2006; Li and Glass, 2002). The return of this extrahepatic cholesterol to the liver is necessary to maintain a balance between cholesterol uptake and *de novo* synthesis. The efflux of cholesterol represents the second line of defence against cholesterol toxicity by macrophages (Cuchel and Rader, 2006).

The protective effects of RCT are particularly important for the removal of cholesterol from the intimal space of the blood vessel wall (Li and Glass, 2002). A critical step in this process is the efflux of free cholesterol from these cells to acceptor apolipoproteins, such as apoAI, which are initially synthesized and secreted by the liver (Oram and Yokoyama, 1996), to form a pre-HDL particle. Then, cholesterol in pre-HDL is esterified and transported to the liver (Figure 1.3) (Cuchel and Rader, 2006). This RCT function is thought to be mediated mainly by HDL, which can deliver cholesterol esters to the liver directly through a selective uptake process involving SR-BI, or indirectly through transfer of cholesterol esters in HDL to other lipoproteins, such as VLDL, intermediate density lipoproteins (IDL) or LDL through the action of the cholesterol ester transfer protein (CETP) (Acton et al., 1996; Repa and Mangelsdorf, 2002).

Macrophages have two potential mechanisms for disposing of excess cholesterol: enzymatic modification to more soluble forms and efflux via membrane transporters. The major mechanism for cholesterol efflux is RCT through the ABC family of transporters where HDL serves as the primary extracellular acceptor. In addition to ABCA1, other mechanisms/proteins are known to be involved, including sterol 27-hydroxylase (CYP27A1), SR-BI and passive diffusion (Ohashi et al., 2005). CYP27A1 is expressed in macrophages at relatively high levels and may play a role in the excretion of cholesterol by converting it to the more soluble form 27-OH (Bjorkhem, 1992; Escher et al., 2003). In addition, cholesterol efflux can occur via passive diffusion. SR-BI can also induce cholesterol efflux by enabling HDL to bind to cells and recognize lipids within cholesterol-rich domains in the plasma membrane (Williams et al., 1999). This explains why the risk of atherosclerosis is inversely correlated with levels of HDL cholesterol. There is also evidence that macrophages may contribute directly to the availability of extracellular cholesterol levels through the secretion of apoE which contributes to the formation of HDL particles (Linton et al., 1995).

The apoE synthesized by macrophages is expressed at high levels in atherosclerotic lesions, particularly in regions association with macrophage–derived foam cells (Greenow et al., 2005). Several studies have shown that the expression of apoE by macrophages has potent anti-atherogenic effects because of its ability to act both as a

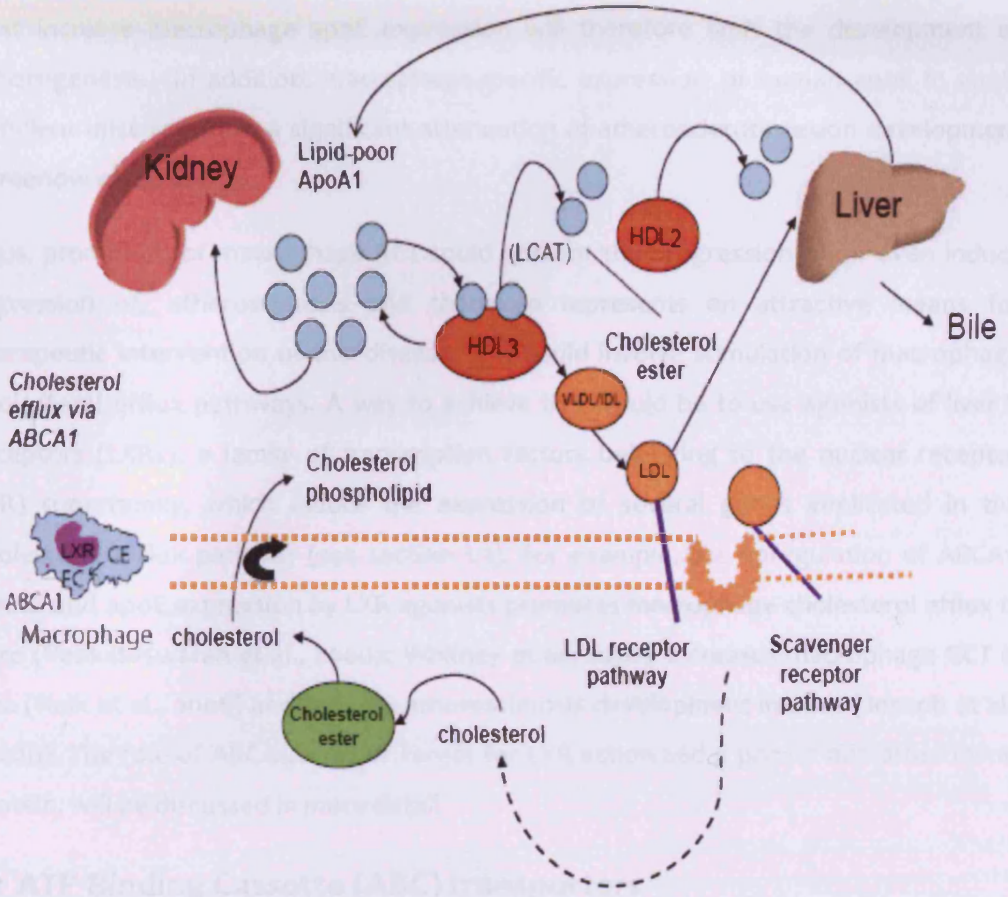


Figure 1.3 Reverse cholesterol transport.

Lipid-poor apoA-I particles, which are secreted by the liver and the intestine, are a potent acceptor of free cholesterol and phospholipids from the liver and peripheral cells. Nonlipidated apoA-I is cleared by the kidney. nHDL mature into small spherical HDL particles through the action of the cholesterol esterifying enzyme LCAT, and the CE are transferred to the liver by SR-BI and to other lipoproteins by CETP. LDL CE is delivered into hepatocytes after endocytosis of LDL particles by the LDL-R. The lipoprotein-derived CE is hydrolyzed in the liver to cholesterol, which is secreted in the bile as bile acids, or is reassembled into lipoproteins that are secreted into the circulation (not shown). nHDL, nascent HDL particles; LCAT, lysolecithin:cholesterol acyltransferase; CE, cholesterol ester; CETP, cholesterol ester transfer protein; SR-BI, scavenger receptor BI. Adopted from Cuchel and Rader, 2006.

powerful antioxidant and to promote cellular cholesterol efflux and RCT (Greenow et al., 2005; Thorngate et al., 2003). For example, increased expression of endogenous apoE in macrophages results in enhanced cholesterol efflux, which attenuates the deleterious effects of cholesterol overload on macrophage function, reduces foam cell formation of macrophages, and promotes removal of cholesterol from the vessel wall. Approaches that increase macrophage apoE expression will therefore limit the development of atherogenesis. In addition, macrophage-specific expression of human apoE in apoE-deficient mice results in a significant attenuation of atherosclerotic lesion development (Greenow et al., 2005).

Thus, promotion of macrophage RCT could prevent the progression of, or even induce regression of, atherosclerosis and therefore represents an attractive means for therapeutic intervention of this disease. This could involve stimulation of macrophage cholesterol efflux pathways. A way to achieve this would be to use agonists of liver X receptors (LXRs), a family of transcription factors belonging to the nuclear receptor (NR) superfamily, which induce the expression of several genes implicated in the cholesterol efflux pathway (see section 1.3). For example, the upregulation of ABCA1, ABCG1 and apoE expression by LXR agonists promotes macrophage cholesterol efflux *in vitro* (Venkateswaran et al., 2000a; Whitney et al., 2001), increases macrophage RCT *in vivo* (Naik et al., 2006) and reduces atherosclerosis development in mice (Joseph et al., 2002b). The role of ABCA1, a major target for LXR action and a potent anti-atherogenic protein, will be discussed in more detail.

1.2 ATP Binding Cassette (ABC) transporters

The term "ABC transporter" was first applied by Christopher Higgins in 1992 in order to unite the relevant members of this gene family (Higgins, 1992). ABC transporter genes code for the largest family of *trans*-membrane (TM) proteins (Dean et al., 2001). Proteins are classified as ABC transporters based on the sequence and organization of their ATP-binding domains, also known as nucleotide-binding folds (NBFs) (Figure 1.4A). The NBFs contain characteristic motifs (Walker A and B) separated by approximately 90-120 amino acids. ABC transporter proteins also contain an additional element, the signature C motif, located just upstream of the Walker B site (Hyde et al., 1990). The TM domain contains 6-11 membrane spanning α-helices and provides the specificity for the substrate (Dean, 2002). In eukaryotes, all ABC transporters bind and hydrolyze ATP to generate

the energy needed and use it to drive the transport of various metabolites across all cell membranes from the cytoplasm to the outside of the cell or into an intracellular compartment such as endoplasmic reticulum (ER), mitochondria and peroxisomes (Dean and Allikmets, 1995).

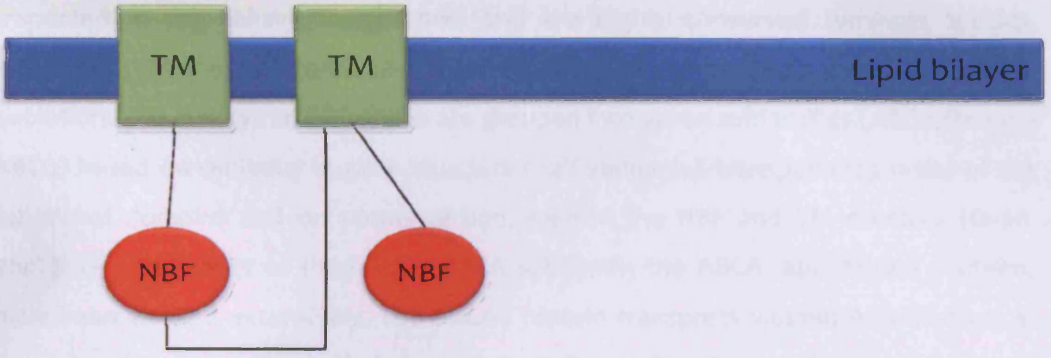


Figure 1.4A A typical ABC transporter protein.

The structure of a representative ABC transporter protein containing TM domains and the NBF. This schematic diagram of ABC transporter is revised from Dean, 2002.

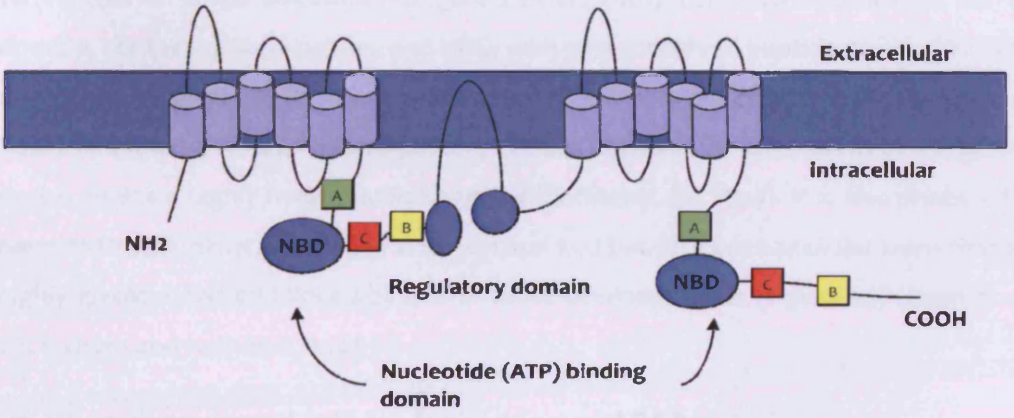


Figure 1.4B The predicted structure of ABCA1.

ABCA1 is a full length transporter with two TM domains and two NBD arrangement. It also has a regulatory domain between the two halves of the protein that contains highly hydrophobic segments. The two NBD contain the highly conserved Walker A and Walker B domains. In addition, a signature or C motif is present. The intracellular position of the NH_2 and COOH terminus is also indicated. The schematic diagram of ABCA1 is revised from Oram and Heinecke, 2005.

The eukaryotic ABC proteins fall into two groups, full transporters containing two halves of similar structural units, TMs and two NBFs joined covalently (e.g. ABCA1 and ABCA7), or as half transporters of single structural units. The latter must form either active homodimers or heterodimers to create functional transporters (e.g. ABCG1 and ABCG4) (Dean et al., 2001; Hyde et al., 1990). The genes that encode ABC proteins are widely dispersed in the eukaryotic genomes and are highly conserved between species, indicating that most of these genes have existed since the beginning of eukaryotic evolution. The eukaryotic ABC genes are grouped into seven subfamilies (ABCA through ABCG) based on similarity in gene structure (half versus full transporters), order of the functional domains and on sequence homology in the NBF and TM domains (Dean, 2002). Two members of the human ABCA subfamily, the ABCA1 and ABCA4 proteins, have been studied extensively. The ABCA4 protein transports vitamin A derivatives in the outer segments of the rod photoreceptor cells and therefore performs a crucial step in the vision cycle. The ABCA1 protein is involved in disorders of cholesterol transport and HDL biosynthesis.

1.2.1 Biological structure of ABCA1

The human ABCA1 is a single polypeptide chain of 2,261 amino acids and comprises of two halves of similar structure (Fitzgerald et al., 2001). Each half encodes for the TM domain, containing six α-helices, and NBD, with two conserved peptide motifs (Walker A and B), and a Walker C signature unique to ABC transporters (Figure 1.4B) (Dean et al., 2001). In addition, ABCA1 has a regulatory domain between the two halves of the protein that contains a highly hydrophobic segment (Luciani et al., 1994). It is also predicted to have its NH_2 terminus oriented into the cytosol and two large extracellular loops that are highly glycosylated and linked by one or more disulfide bonds (Figure 1.5) (Dean et al., 2001; Oram and Heinecke, 2005).

1.2.2 Localization and cellular trafficking of ABCA1

The cellular localization of ABCA1 and its potential sites of action are critical to understanding the process of cellular lipid efflux. Initial immunocytochemical studies suggested that endogenously expressed human ABCA1 was localized on the plasma membrane (Lawn et al., 1999; Orso et al., 2000). Subsequent studies established that the presence of ABCA1 on the plasma cell membrane and its movement to the cell surface was required for the transporter to function in apoA-I-dependent cellular lipid efflux

(Neufeld et al., 2001). Furthermore, studies by Hamon et al. (2000) have established that the ABCA1 transporter also resides in intracellular endocytic compartments, which are membrane-bound compartments inside cells of roughly 300-400 nm in diameter when fully mature (Fitzgerald et al., 2001; Hamon et al., 2000). In addition to its movement to the cell surface, trafficking of the ABCA1 transporter in endocytic compartments may also play an important role in apoA-I-mediated efflux of cellular lipids (Neufeld et al., 2001). The early endosomes, containing the ABCA1 transporter, were found to shuttle between the plasma membrane and intracellular endocytic compartments. The delivery of ABCA1 to lysosomes for degradation has also been suggested to potentially serve as a mechanism to decrease the surface expression of ABCA1, and hence, modulate apoA-I-mediated cellular lipid efflux (Neufeld et al., 2001; Neufeld et al., 2004).

1.2.3 Biological functions of ABCA1

Most of the known functions of eukaryotic ABC transporters involve the shuttling of hydrophobic compounds either within the cell, as part of a metabolic process, or outside the cell for transport to other organs or for secretion from the body (Dean, 2002). The function of ABCA1 is to mediate the extrusion of membrane phospholipids, unesterified cholesterol and other lipophilic molecules across cellular membranes towards specific extracellular acceptors, such as lipid-poor HDL apolipoproteins (Oram and Heinecke, 2005; Repa and Mangelsdorf, 2002). ABCA1, like other ABC transporters, forms a channel in the membrane that promotes flipping of lipids from the inner to the outer membrane leaflet by an ATPase-dependent process (Oram and Heinecke, 2005). It has also been reported to promote secretion of apoE (Von Eckardstein et al., 2001a). In addition, there is evidence that ABCA1 promotes engulfment of apoptotic cells by macrophages (Hamon et al., 2000) and generates microparticles (MPs) that bleb from the plasma membranes. MPs are submicron membrane elements, mainly expressing phosphatidylserine at the plasma membrane. MPs are found in the circulation of healthy subjects but their levels can increase in various pathological conditions such as thrombosis (Combes et al., 2005).

ABCA1 appears to target specific membrane domains for lipid secretion, which are likely to be regions that are sensitive to accumulation of cholesterol and other lipophilic compounds (Yamauchi et al., 2004). Thus, ABCA1 removes cholesterol that accumulates as cytosolic cholesteryl ester lipid droplets. The ABCA1-dependent control of the lipid content of the membrane dramatically influences the plasticity and fluidity of the

membrane itself. Two models have been proposed for the ability of ABCA1 to target specific lipid domains (Oram and Heinecke, 2005). The exocytosis model suggests that excess intracellular cholesterol is packaged into transport vesicles or rafts, perhaps in the Golgi apparatus, which then translocates to domains in the plasma membrane containing ABCA1 (Oram and Heinecke, 2005; Oram and Lawn, 2001). In support of this mechanism, a recent study showed that overexpression of ABCA1 in the absence of apolipoproteins increases the appearance of cholesterol on the cell surface (Vaughan and Oram, 2003). The other model, retro-endocytosis, suggests that ABCA1- and apolipoprotein-containing vesicles endocytose extracellular material (e.g. cholesterol) to intracellular lipid deposits, where ABCA1 pumps lipids into the vesicle lumen for release by exocytosis (Santamarina-Fojo et al., 2001; Takahashi and Smith, 1999). In support of this model, ABCA1 has been shown to recycle rapidly between the plasma membrane and the late endosomal/lysosomal compartments and that these compartments accumulate cholesterol in cells with dysfunctional ABCA1, and that ABCA1 containing intracellular vesicles also contain apolipoproteins (Neufeld et al., 2001; Neufeld et al., 2004).

Although the structure of ABCA1 has not been determined, electron microscopy and X-ray crystallography of other ABC transporters, which translocate lipids from the inner to the outer membrane leaflets and which extrude a variety of lipophilic compounds from cells, have generated molecular models that may apply to ABCA1. It has been suggested that the two symmetrical *trans*-membrane bundles come together to form a chamber that scans the inner leaflet of the membrane for substrates, incorporates them into the chamber, and flips them to the outer membrane leaflet for extrusion from the cell (Oram and Heinecke, 2005). This involves a series of conformational changes in the ABCA1 protein that are probably driven by the NBD domains (Oram and Heinecke, 2005).

The structural studies suggest the following model for the ABCA1 pathway. Excess cellular cholesterol along with phospholipids accumulate within domains of the cytosolic leaflet of the plasma membrane or intracellular vesicle membranes. This cholesterol is not accessible to apolipoproteins and therefore must be translocated to the cell surface or into the vesicle lumen for removal. These lipid domains may assemble around ABCA1 molecules or ABCA1 may migrate to these domains after they are formed. Other lipophilic molecules may also accumulate in these domains (Oram and Heinecke, 2005).

In this model, the TM chamber of ABCA1 is initially open at the bottom. Excess cellular cholesterol, along with phospholipids that have accumulated in the cytosolic leaflet of membrane, are laterally transported into the chamber by a process that is facilitated by high-affinity phospholipid binding sites. This phospholipid recognition induces ATP binding to the NBDs, which promotes their dimerization and thus closes the chamber. The trapped lipids are then flipped to the outer membrane leaflet. The hydrolysis of ATP by the NBDs forms an ADP-bound intermediate that changes the conformation of the TM domains, opening the chamber at the membrane outer leaflet. Lipids are extruded from the chamber into cholesterol-rich domains on the cell surface, where they are removed by apolipoproteins. The structure of the ABCA1 chamber reverts back to its substrate uptake conformation after ADP dissociates from the NBDs. The removal of lipids by apolipoproteins is believed to consist of a two step process: first apolipoproteins bind to ABCA1 and then "solubilize" the ABCA1 transported lipids (Wang et al., 2001b). It has been proposed that ABCA1 carries out the flipping of membrane phospholipids, principally phosphatidylcholine, and cholesterol towards the lipid-poor apoAI in nascent HDL particle, which can now accept cholesterol to initiate RCT (Ishii et al., 2002).

1.2.4 Tissue specific expression

ABCA1 mRNA is widely distributed among multiple tissues with variation in abundance at specific sites. The highest mRNA expression levels of ABCA1 are detected in liver, heart, lung, placental trophoblasts, kidney, adrenal gland and small intestine. The lowest expression is found in the pancreas, ovary, colon, skeletal muscle, bone marrow and mammary glands. At the cellular level, tissue macrophages as well as macrophage-like cell lines of mouse or human origin express high levels of ABCA1. The progression of foam cell formation and atheroma development are consistently influenced by the expression of ABCA1 in macrophages and other cell types. Early studies suggested that inactivation of the ABCA1 gene increases absorption of dietary cholesterol, suggesting that ABCA1 may facilitate the re-secretion of sterols back into the intestinal lumen across the apical membrane. Thus, ABCA1 may also suppress the flux of dietary cholesterol into the body. This was largely based on the observation that LXR/RXR agonists inhibit absorption of dietary cholesterol in mice that is associated with an induction of intestinal ABCA1 expression (Knight et al., 2003; Repa et al., 2000b). A lack of ABCA1 in the Wisconsin Hypoalpha Mutant (WHAM) chicken does not impede LXR agonist-induced

reduction in dietary cholesterol absorption but suppresses cholesterol secretion from the basolateral side of the intestine (Mulligan et al., 2003). It is therefore likely that intestinal ABCA1 functions to generate HDL particles that transport dietary cholesterol to the liver, providing another protective mechanism against excess cholesterol overload (Mulligan et al., 2003; Oram and Heinecke, 2005; Repa et al., 2000b).

1.2.5 Polymorphisms of ABCA1

Mutations in ABCA1 cause a severe HDL deficiency syndrome, called TD, that is characterized by deposition of sterols in tissue macrophages (Assmann et al., 1995; Hayden et al., 2001). TD was first identified in the 1960s as an HDL deficiency syndrome affecting families in the Tangier Island in the Chesapeake Bay in USA (Fredrickson et al., 1961). In the mid 1990s, It was discovered that the ability of purified apoA-I to remove cholesterol and phospholipids from fibroblasts isolated from TD patients was severely impaired (Francis et al., 1995), consistent with a defective ABCA1. In 1999, four studies independently identified the defective TD gene as ABCA1 (Bodzioch, 1999; Brooks-Wilson et al., 1999; Lawn et al., 1999; Rust, 1999). The clinical manifestation of TD include premature coronary artery disease (Oram, 2000; Singaraja et al., 2003). The individuals are characterized by an accumulation of cholesteryl esters in the reticulo-endothelial cells of several tissues, including tonsils, thymus, lymph nodes, bone marrow, spleen, liver, gall bladder and intestinal mucosa. Many patients also have lipid deposits in SMCs and fibroblasts.

Over 70 mutations in the ABCA1 gene have been identified in subjects with low plasma HDL levels, one-third of which are missense mutations (Cohen et al., 2004; Frikke-Schmidt et al., 2004; Singaraja et al., 2003). Although these mutations occur throughout the gene, they tend to cluster in regions specifying for the extracellular loops, the NBD domains and the C-terminal region. The functional effect of such missense mutations has been studied in cultured cells. When most of these mutants are expressed in cells, they appear in the plasma membrane but have severely impaired lipid transport and apolipoprotein binding activities (Fitzgerald et al., 2002; Lawn et al., 1999). There are, however, some substitution mutations, such as Q597R and R587W, that do not localize to the plasma membrane (Rigot et al., 2002; Tanaka et al., 2003). In addition, some of the missense mutations prevent ABCA1 trafficking to the plasma membrane and cause severe HDL deficiency (Albrechta et al., 2004). Furthermore, one mutant, W590S, has

been described that has normal apolipoprotein binding activity but defective lipid transport (Fitzgerald et al., 2002).

Three independent studies have shown that ABCA1 is responsible for the rate-limiting step in the efflux of cholesterol from peripheral cells (Bodzioch, 1999; Brooks-Wilson et al., 1999; Rust, 1999). Because of the important role of ABCA1 in cholesterol transport, several groups have examined the ABCA1 gene for polymorphisms that might be associated with plasma lipid levels and CVD. Polymorphisms in the ABCA1 gene are associated with either increased or decreased CVD (Singaraja et al., 2003). Several of these common polymorphisms in the coding, promoter and 5'- upstream regions are associated with either low or high plasma HDL levels. For example, a common variant of ABCA1, the R219K variant, which is found in 46% of Europeans, lowers the risk of coronary artery disease (Trivedi, 2001). In the R219K variant, the amino acid arginine is replaced by lysine and this is associated with lower triglyceride levels and raised HDL cholesterol levels (up to 15 percent higher than normal) (Singaraja et al., 2003). Carriers of this variant have 29% fewer coronary events and have less bypass surgery. The variants, V771M and V825I, have also been reported to be associated with increased HDL levels. Other ABCA1 mutations, such as the R1587K variant, is associated with low levels of HDL and causes familial hypoalphalipoproteinemia (Frikke-Schmidt et al., 2004; Singaraja et al., 2003).

The only naturally occurring animal model for TD is the WHAM chicken. ABCA1 in these chickens has a missense mutation near the N-terminus that produces a defective protein (Attie et al., 2000). Similar to human TD patients and ABCA1 knockout mice, the WHAM chicken has very low levels of HDL, due to hypercatabolism of lipid poor apoA-I, and accumulates cholesteryl esters in tissues. The most severe lipid accumulation occurs particularly in hepatic parenchymal and intestinal epithelial cells (Schreyer et al., 1994).

Further advances on the role of ABCA1 have emerged from the development of ABCA1-/- mice. Studies in these mice have provided additional evidence that ABCA1 is a major determinant of plasma HDL levels. The phenotype in these mice is similar to that of human TD (Mcneish et al., 2000; Orso et al., 2000). Thus, the absence of ABCA1 in mice leads to accumulation of sterols in some tissues and deficiency of HDL, consistent with the function of ABCA1 in cholesterol trafficking across the plasma membrane. Conversely, overexpression of human ABCA1 in transgenic C57BL/6 mice results in

elevation of HDL levels (Joyce et al., 2002; Singaraja et al., 2002). In general, ABCA1 mutations that impair its function are associated with premature CVD (Cohen et al., 2004; Frikke-Schmidt et al., 2004; Singaraja et al., 2003).

Such mouse models have provided support for the atheroprotective effects of ABCA1. Deletion of mouse ABCA1 results in a considerable reduction in the levels of plasma HDL (Orso et al., 2000). However, there is no apparent change in arterial lesion formation when these mice are crossed with an atherogenic strain, such as apoE-/- or LDLR-/- (Aiello, 2002). This is probably because humans carry most of their serum cholesterol in LDL rather than HDL (as mice do). If ABCA1 is eliminated only from macrophages (by bone marrow transplantation), serum HDL levels are relatively unaffected and this leads to enhanced lesion formation in recipient mice (Aiello, 2002; Van Eck et al., 2002). In contrast, overexpression of human ABCA1 in transgenic mice results in protection against an atherogeneic diet (Singaraja et al., 2002).

Studies on heterozygous TD patients have also demonstrated the importance of ABCA1 in RCT and atherosclerosis. These studies have estimated that individuals lacking functional ABCA1 have a prevalence of CVD that is at least six fold higher than those with a normal protein (Serfaty-Lacrosniere et al., 1994). This moderately high risk for atherosclerosis is not as dramatic as would be expected for individuals with the absence of HDL, a well known atheroprotective lipoprotein. The low levels of LDL may protect these TD heterozygotes from atherogenesis. Studies of TD patients who tend to have more normal levels of LDL show a significant inverse correlation between ABCA1 and the prevalancy and severity of atherosclerosis (Clee et al., 2000). It has been shown that individuals who carry a single functional allele of ABCA1 show increase in the intima media thickness of the arterial wall, a marker for atherosclerosis (Van Dam, 2002). Overall, all these studies suggest the importance of macrophage ABCA1 as a target for therapeutic interventions for treating atherosclerosis.

1.2.6 Regulation of gene expression

This section will start with an overview of eukaryotic gene expression and its regulation. The process of differential gene expression or the selective activation of different subsets of genes underlies processes such as differentiation, development and disease. Selective activation of gene expression is carefully regulated and, ultimately, controls all functions of cells, tissues and organs. Gene expression occurs in two steps: (i) the

transcription of the information encoded in genomic DNA into a molecule of RNA; (ii) translation of the information encoded in the nucleotides of mRNA into a defined sequence of amino acids in a protein.

1.2.6.1 Transcription

The transcription of a DNA molecule into an RNA copy is achieved by a DNA-dependent RNA polymerase enzyme. Essentially, this enzyme catalyzes the joining of a single-stranded chain of ribonucleotides via phosphodiester bonds, with information concerning the order of the ribonucleotides being provided by the sequence of the DNA molecule being transcribed (Gelles and Landick, 1998). In eukaryotic cells RNA is synthesized by three polymerases that are responsible for transcribing the different types of genes. These are: (i) RNA polymerase I, a nuclear enzyme that transcribes ribosomal RNA (rRNA) (18S, 5.8S and 28S) genes; (ii) RNA polymerase II that is located in the nucleoplasm and transcribes the genes that code for messenger RNA (mRNA); and (iii) RNA Polymerase III, which is also nucleoplasmic and transcribes the genes encoding transfer RNA (tRNA) and small RNAs that play structural and catalytic roles in the cell. RNA transcribed by RNA polymerase I and III are not translated into proteins but are involved in protein synthesis. The DNA-dependent RNA polymerase which transcribes genes coding for proteins is RNA polymerase II, a large multi-subunit enzyme. This process is described below in more detail.

Transcription of mRNA is initiated through the recruitment of RNA polymerase II (Pol II) to the promoters of target genes. The transcription of all such genes requires the activity of critical core promoter elements that initiate the transcription and elongation of RNA. In addition, upstream regulatory elements, enhancer and silencer regions are required for regulated and/or cell-type-specific gene transcription (See section 1.2.6.3). The core promoter includes the TATA box, the TFIIB recognition element (BRE), the initiator (Inr) or the downstream promoter element (DPE). Most, but not all, genes have a TATA box located 25 base pairs upstream or downstream of the transcriptional initiation site (also called CAP site). This element helps to specify the precise site at which transcription is initiated by interaction with the TATA box- binding protein (TBP). The exact sequence of the TATA box is variable, and a number of related thymine- and adenine-rich sequences all able to confer TATA box function. To initiate transcription, RNA polymerase II also requires various additional proteins, called general transcription

factors (GTF) (Orphanides et al., 1996; Roeder 1996). This occurs in conjunction with the assembly of multiple components of the basal transcription machinery, including GTFs, namely TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH, which bind in a series of ordered steps to specific recognition sequences on the promoter of a gene close to the transcription initiation sites in order for RNA polymerase to bind and start transcription (Orphanides et al., 1996; Roeder 1996).

Transcription can be divided into three phases: initiation, elongation and termination. The initiation phase concerns RNA polymerase-mediated starting of transcription on a DNA molecule (Gelles and Landick, 1998). For genes transcribed by RNA polymerase II, the initial contact is made by TFIID, which is a protein complex made up of TBP and at least 12 TBP-associated factors (TAFs), to the TATA box. TBP is a sequence-specific protein that binds to DNA via a specific domain that recognises the TATA box (Hernandez, 1993). During transcription, TAFs assist in the attachment of TFIID to the TATA box. After TFIID has bound to the core promoter, the pre-initiation complex (PIC) is formed by attachment of the remaining GTFs (Green, 2000). These GTFs bind to the complex in the order TFIIA, TFIIB, TFIIF/ RNA polymerase II, TFIIE and TFIIH. The TFIIA binds and stabilizes the TFIID-TATA box complex. Next, TFIIB binds to TFIID thus providing a physical link between TFIID and the RNA polymerase II which has already complexed with TFIIF at the promoter. TFIIB is important in ensuring the correct positioning of RNA polymerase II relative to the transcription start site. Then, binding of TFIIE and TFIIH complete the transcription initiation complex so that RNA polymerase II can initiate transcription. TFIIH, which is a protein kinase and has an ATP-dependent helicase activity, is responsible for the unwinding of the DNA helix, thereby allowing transcription to begin. In addition, it will specifically hyperphosphorylate the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II. The CTD is that portion of the polymerase which is involved in the initiation of DNA transcription. Initiation of RNA synthesis is accompanied by extensive phosphorylation of the CTD. RNA polymerase II containing nonphosphorylated CTD is recruited to the PIC whereas the hyperphosphorylated CTD form is involved in active transcription. This addition of phosphate groups to CTD of RNA polymerase II is the final step in the assembly of the initiation complex, thereby causing RNA polymerase II to change its confirmation and dissociate from the initiation site and begin synthesizing RNA.

Once a pre-initiation complex has been formed and an appropriate RNA polymerase has attached to it in a functionally active state, transcription is initiated by separating the DNA strands to form an open complex. Eventually, transcription elongation begins. The RNA polymerase II engages in elongation by moving along the DNA template, synthesizing RNA by covalently adding ribonucleotide triphosphates to the 3' end of the growing RNA chain. Therefore, the polymerase extends the growing RNA chain in a 5' to 3' direction. As the enzyme moves in a 3' to 5' direction along the antisense DNA strand, it unwinds the DNA and separates the DNA strands to expose the template strand for ribonucleotide base pairing, with the helix being reformed behind the polymerase (Gelles and Landick, 1998). In recent years, it has become apparent that transcription elongation is also a highly regulated process which can control multiple stages required for the maturation of mRNA. These pre-mRNA processing events are tightly coupled to transcription and take place with the elongation process. Such processing includes pre-mRNA capping at the 5' end and the addition of a poly-A tail at the 3' end as well as removal of introns by splicing and mRNA export. Capping and polyadenylation are thought to increase the stability of mRNA, to aid its export from the nucleus and transportation to the cytoplasm and to generally identify the RNA molecule as an mRNA. This maturation of mRNA is used by the protein synthesis machinery as an indication that both ends of the mRNA are present (Shatkin and Manley, 2000; Daneholt 1997).

The termination of transcription, namely the dissociation of the transcription complex from the DNA template to release the newly transcribed mRNA and the ending of RNA synthesis, occurs at a specific DNA sequence known as the terminator. The exact molecular mechanisms controlling the termination process are still under investigation. This transcriptional termination is necessary for maintaining active cellular RNA polymerase II and to prevent erroneous transcription of downstream genes (Sims et al., 2004).

1.2.6.2 Translation

Translation of an mRNA into protein is a cyclic process that requires a large amount of energy in the form of ATP and GTP hydrolysis. Translation also occurs in three phases; initiation, elongation and termination. Sequences within RNA provide structures to which ribosomes associate and so initiate translation. These sequences come into two parts: the ribosome binding sequence and the initiation codon. The ribosome binding

sequence positions the ribosome so that translation can be initiated if there is a start codon on the mRNA at an appropriate position relative to where the ribosome binds. Translation can be initiated once these two parts come together. Although, the full details of the initiation process are not fully understood, the following process is known to occur. The first step involves the formation of the pre-initiation complex that consists of the small 40S ribosomal subunit, the initiation factor eIF-2 bound to the initiator Met-$tRNA_i^{met}$,a molecule of GTP and three additional initiation factors, eIF-1, eIF-1A and eIF-3. After assembly, the pre-initiation complex associates with the 5'end of the mRNA. This step requires eIF-4F, also called the Cap Binding Complex, which consists of the initiation factors eIF-4A, eIF-4E and eIF-4G. The factor eIF-4G acts as a bridge between eIF-4E, attached to the CAP, and eIF-3 bound to the pre-initiation complex (Hentze 1997). The result is that the pre-initiation complex becomes attached to the 5'region of the mRNA. This attachment is also influenced by the poly (A) tail at the distant 3' end of the mRNA. Thus, the CAP structure and poly (A) tail probably work together (Preiss and Hentze 1998). The poly (A) tail could also have an important regulatory role, as the length of the tail appears to correlate with the extent of the initiation that occurs with a particular mRNA. After becoming attached to the 5' end of the mRNA, the initiation complex has to scan along the molecule and find the initiation codon. The start codon, which is usually 5'-AUG-3' is recognizable because it is contained in a short sequence called the Kozak consensus. Once the initiation complex is positioned over the initiation codon, the large 60S subunit of the ribosome attaches to form an 80S initiation complex. This attachment requires hydrolysis of GTP and leads to the release of the initiation factors. Two final initiation factors are involved in this stage, eIF-5 and eIF-6. EIF-5 aids the release of the other factors and eIF-6 is associated with the unbound large subunit and prevents it from attaching to a small subunit in the cytoplasm (Sachs and Varani 2000).

The ribosome binds to the mRNA at the start AUG codon that is recognized only by the initiator tRNA. This then leads to the elongation stage of translation, which requires three elongation factors, eEF-1A, eEF-IB and eEF-2. Essentially eEF-1 controls the insertion of new charged tRNA and eEF-2 controls translocation of the ribosome following peptide bond formation (Green, 2000). During this stage, complexes composed of an amino acid linked to tRNA sequentially bind to the appropriate codon in the mRNA by forming complementary base pairs with the tRNA anticodon. The ribosome moves from codon to codon along the mRNA. Amino acids are added one by one into a polypeptide

sequence that is dictated by DNA and represented by mRNA. The genetic code in the mRNA molecule is thus used to build up a polypeptide chain. Protein synthesis ends when one of the three termination codon is reached on the RNA (Frank, 2000). Two release factors, eRF-1 and eRF-3 recognize the termination codons (UAA, UAG and UGA). The release factors terminate translation and release the complete polypeptide chain from the ribosome. The dissociated ribosome subunits enter the cytoplasm where they remain until used again in another round of translation (Frank, 2000).

Translation is not the final stage in the regulation of gene expression. The polypeptide that dissociates from the ribosome is usually inactive and, before taking on its functional role in the cell, may undergo one of the following four types of post-translational processing (Paulus, 2000; Wickner et.al. 1999). The first type, protein folding, involves folding of the polypeptide chain into its correct tertiary structure. The second type, proteolytic cleavage, involves cleavage of the polypeptide at specific sites by proteases. In the third type, chemical modification, individual amino acids in the polypeptides or the amino or carboxy groups of the terminal amino acids might be modified by attachment of new, small chemical groups (e.g. an acetyl, methyl or phosphate group). Such chemical modifications often play an important role in determining the precise activity of the target protein and have important regulatory roles, such as activation of signal transduction. The fourth type, intein splicing involves removal of inteins, intervening sequences in some proteins, by an endogenous activity and joining of exteins in order for the protein to become active.

Cell must also ensure that the newly synthesized protein is transported to its correct location where it can carry out its function. This process is called protein targeting. In a cell, the protein may be destined to stay in the cytoplasm (e.g. enzymes involved in glycolysis) or, alternatively, it may need to be targeted to an organelle such as mitochondria, peroxisome, lysosome or the nucleus, or even be inserted into the plasma membrane or exported out of the cell. Essentially, if a protein is destined for the cytoplasm, it is made on free ribosomes in the cytoplasm and released directly. If it is destined for other final locations, specific protein-targeting mechanisms are involved (Bernstein, 2000; Bukau et al., 1996). For example, those destined for the nucleus have one or more internal nuclear localization sequences. Specific proteins bind to the

sequence once the protein has been synthesized by free ribosomes and target it to the nucleus via a process that requires energy (Johnson, et al., 2002).

The secretory proteins have an N-terminal signal peptide that directs the secretory protein to the rough endoplasmic reticulum (RER) membrane and so targets the protein to cross into the RER lumen and be exported. The signal peptide opens the translocon channel and leads the nascent polypeptide through it as it is synthesized. On reaching the ER lumen, the signal peptide is cleaved off and the polypeptide moves to the Golgi complex that is near the RER by vesicular transport. The polypeptide then moves through the Golgi complex to the *trans* compartment, being processed en route by, for example, attachment of carbohydrate residues (glycosylation). The vesicles bud from the *trans* compartment and carry the glycosylated, secretory proteins to the plasma membrane where the vesicle fuse, thereby releasing their content to the cell exterior (Bernstein, 2000; Bukau et al., 1996).

The synthesis of integral membrane proteins is also mediated by ribosomes on the RER, but the polypeptide chain becomes inserted in the RER membrane rather than being transported into the lumen. During transport to the Golgi and then to the cell surface, these proteins stay anchored in the membrane. In this case, in addition to the signal peptide, the polypeptide chain contains a stop transfer or anchor signal that fixes the protein in the membrane and the final vesicle which fuses with the plasma membrane becomes part of it. This transfer of proteins across the ER membrane occurs during synthesis by a mechanism similar to that for secretory proteins. GTP hydrolysis plays an important part in these processes with GTP-binding proteins acting as molecular switches. The proteins undergo allosteric changes when GTP replaces GDP or bound GTP is hydrolysed (Eicher and Irihimovitch, 2003).

1.2.6.3 Transcriptional control of gene expression

Gene expression can be regulated at many of the steps in the pathway from DNA to RNA to protein (Figure 1. 5). Thus, a cell can control the levels of protein present by regulating the transcription of DNA to mRNA, modification of mRNA, export of the mRNA from nucleus to the cytoplasm and, finally, by selective mRNA translation to a specific protein. In addition, the regulation of gene expression is subject to mRNA degradation and protein activity control. For most genes, transcriptional control plays a major role in

processes such as development and differentiation along with a cell's response to metabolic needs and environmental stimuli.

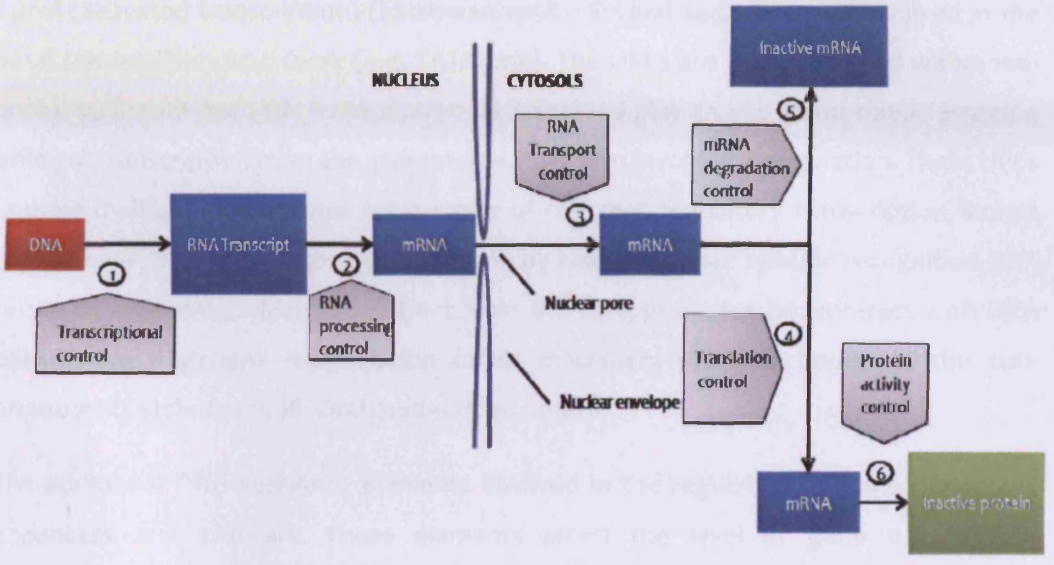


Figure 1.5 Regulation of gene expression.

Gene expression can be controlled at several different steps, although for most genes the main site of control is transcription of DNA into RNA.

Gene transcription is generally controlled by interaction of DNA binding proteins (*cis*-acting elements) to specific regulatory sequences (*trans*-acting factors) in target genes. These DNA sequence elements are usually located in the 5' region of a gene upstream from the transcriptional initiation site. These DNA sequence involved include the promoter region, which consists of core promoter and upstream regulatory elements (UPEs), and the enhancer elements and silencer regions (Latchman 1998). Transcriptional regulation occurs via transcription factors other than GTFs that bind to short control elements associated with the regulatory regions of target genes, and then interact with each other and with the transcription initiation complex by protein-protein interaction to activate or repress the rate of transcription of the target gene (Orphanides et al., 1996; Roeder, 1996). Recruitment of specific co-activator or co-repressor proteins are required to aid transcription.

Upstream promoter elements (UPEs) contain two types of sequences. The first type are those sequences that are found in many genes that exhibit distinct patterns of

regulation, and are therefore likely to be involved in the basic process of transcription (often referred to as the general transcription factor machinery). The second type is those that are only in genes transcribed in a particular tissue or in response to a specific signal (activated transcription) (Latchman 1998). Several sequences are involved in the basal transcription machinery (e.g. TATA box). The UPEs are usually located within 100-200bp upstream from the transcription start site and play an important role in ensuring efficient transcription from the promoter and are also involved in regulation. These UPEs contain multiple binding sites for a range of different regulatory transcription factors (TFs). These TFs regulate gene transcription by binding to their specific recognition DNA sequence elements which are distinct from the core promoter and interact with RNA polymerase II general transcription factor machinery which is bound at the core promoter (Latchman 1998; Orphanides et al., 1996).

The additional DNA sequence elements involved in the regulation of transcription are enhancers and silencers. These elements affect the level of gene transcription irrespective of whether they are in an upstream position, downstream position or within the coding region in any orientation relative to the transcriptional start site. Enhancers also contain multiple binding sequences for TFs that act to alter the rate of gene transcription either positively or negatively. Such binding of TF provides a mechanism for tissue- and stimulus-specific gene expression.

In most cases, the TFs that bind to enhancer or promoter sequences are activator proteins that induce gene transcription. These proteins have at least two distinct domains, the DNA binding domain, that recognizes the specific DNA sequence to bind to, and the activation domain, that is responsible for bringing transcriptional activation by interaction with other TFs and/or the RNA polymerase complex. Many TFs operate as dimers, either homodimers or heterodimers, with subunits held together via dimerization domains. Finally some TFs (e.g. NR) are responsive to specific small molecules (ligands) which regulate their activity. In these cases, the ligand binds at a ligand binding domain in the TF (See section 1.3).

DNA binding domains contain characteristic protein motifs. For example, the helix-turn-helix motif contains two α-helices separated by a short β-turn. When the TF binds to DNA, the recognition helix lies in the major groove of the DNA double helix. The second type of DNA binding domain is the zinc finger motif, which consists of a peptide loop

with either two cysteines and two histidines (the C_2H_2 finger) found, for example, in GTFs or four cysteins (the C_4) finger (e.g. NRs) that coordinates a zinc ion. A particularly important difference between the two fingers is that the C_2H_2 proteins generally contain three or more repeating finger units and bind as monomers. In contrast, the C4 zinc finger proteins generally contain only two finger units and generally bind to DNA as homodimers or heterodimers. The zinc finger motif secondary structure consists of two antiparallel β-strands and one α-helix. TF often contain several zinc fingers, in each case the α-helix binds to the major groove of the DNA double helix. Some TFs (e.g. the basic leucine zipper proteins (bZIP)) contain basic domains that interact with the acidic target DNA (Latchman 1998). The Leucine zipper has a sequence consisting of a leucine residue at every seventh amino acid and forms an α- helix with the leucines presented on the same side of the helix every second turn, thereby giving a hydrophobic surface. Two TF monomers can interact via the hydrophobic faces of their leucine zipper motifs to form a dimer. The preceding regions are rich in basic amino acids and interact with the acidic DNA. Finally, the Helix-loop-helix (HLH) motif contains two potential α-helices connected by a non-helical loop of variable length. The carboxy-terminal α-helix has a hydrophobic face and two TF monomers, each with an HLH motif, can dimerize through this (Latchman 1998). Interaction with DNA is again achieved through clusters of basic amino acids.

Unlike DNA binding domains and dimerization domains, common structural motifs have not yet been identified in the activation domains of TFs. A transcription factor may contain 2 to 5 activation domains in its coding region, either the same type or different. Many different types of activation domains have been identified and include: (i) acidic activation domains, which are rich in acidic amino acids (e.g. aspartic and glutamic acids); (ii) glutamine-rich domains (such as SP-1 transcription factor); and (iii) proline-rich domains (such as c-Jun transcription factor). Transcriptional repressor proteins that inhibit the transcription of specific genes also exist. They may act by binding either to control elements within the promoter region near the gene or at sites located a long distance away from the gene, called silencers. The repressor protein may inhibit transcription directly. However, other repressors inhibit transcription by blocking activation (Brivanlou and Darnell, 2002). The next section will discuss the analysis of ABCA1 gene promoter and the regulation of ABCA1 expression.

1.2.7 Analysis of the ABCA1 gene promoter

The ABCA1 gene in the human and mouse genomes has been mapped to human chromosome 9q31 and mouse chromosome 4q23.1 (Luciani et al., 1994). The human ABCA1 gene comprises 50 exons spanning 150 kb (Santamarina-Fojo et al., 2000). Comparative analyses of the mouse and human ABCA1 promoters have identified important functional motifs that are strongly conserved between the two species. The human ABCA1 promoter region contains multiple binding motifs for transcription factors with roles in lipid metabolism, such as SP1, NF-κB and AP-1, as well as a TATA box, a CAAT box and three E-box motifs (Figure 1.6) (Santamarina-Fojo et al., 2001). In addition, the ABCA1 gene promoter contains multiple binding motifs for the liver-enriched transcription factor hepatocytes nuclear factor (HNF)-3β. Potential binding sites for transcription factors known to play a role in monocyte/macrophage differentiation, including STATs, c-Myb and GATA were also identified (Santamarina-Fojo et al., 2000). Moreover, initial studies on the human ABCA1 promoter in RAW264 cells localized a cholesterol response element within the first 990 bp upstream of the transcriptional start site (Santamarina-Fojo et al., 2000). Subsequent studies led to the identification of specific motifs that modulate ABCA1 gene expression in response to oxysterol-induced transcription through the LXRs (Costet et al., 2000; Schwartz et al., 2000). A major finding was the presence of a direct repeat of the NR half-site TGACCT, separated by four bases (DR4), as the element responsible for the LXR-dependent *trans*-activation of the human ABCA1 gene promoter (Costet et al., 2000; Schwartz et al., 2000). This sequence binds the heterodimer of LXR with the obligate partner retinoid X receptors (RXR) (Costet et al., 2000; Schwartz et al., 2000; Venkateswaran et al., 2000a).

Such sterol-mediated upregulation of ABCA1 expression requires activation of the LXR/RXR heterodimer, as indicated by the finding that it can be reproduced by a synthetic, non-steroidal LXR ligand along with the absence of such regulation in cells derived from mice lacking LXRα and LXRβ (Costet et al., 2000; Repa et al., 2000b). Incubation of human embryonal kidney 293 cells and CV-1 cells (Costet et al., 2000), along with RAW264 macrophages (Schmitz and Langmann, 2001), with 9-*cis*-retinoic acid (9CRA), 20-(S)-hydroxy cholesterol or 22-(R)-hydroxy cholesterol (22(R)-HC) resulted in

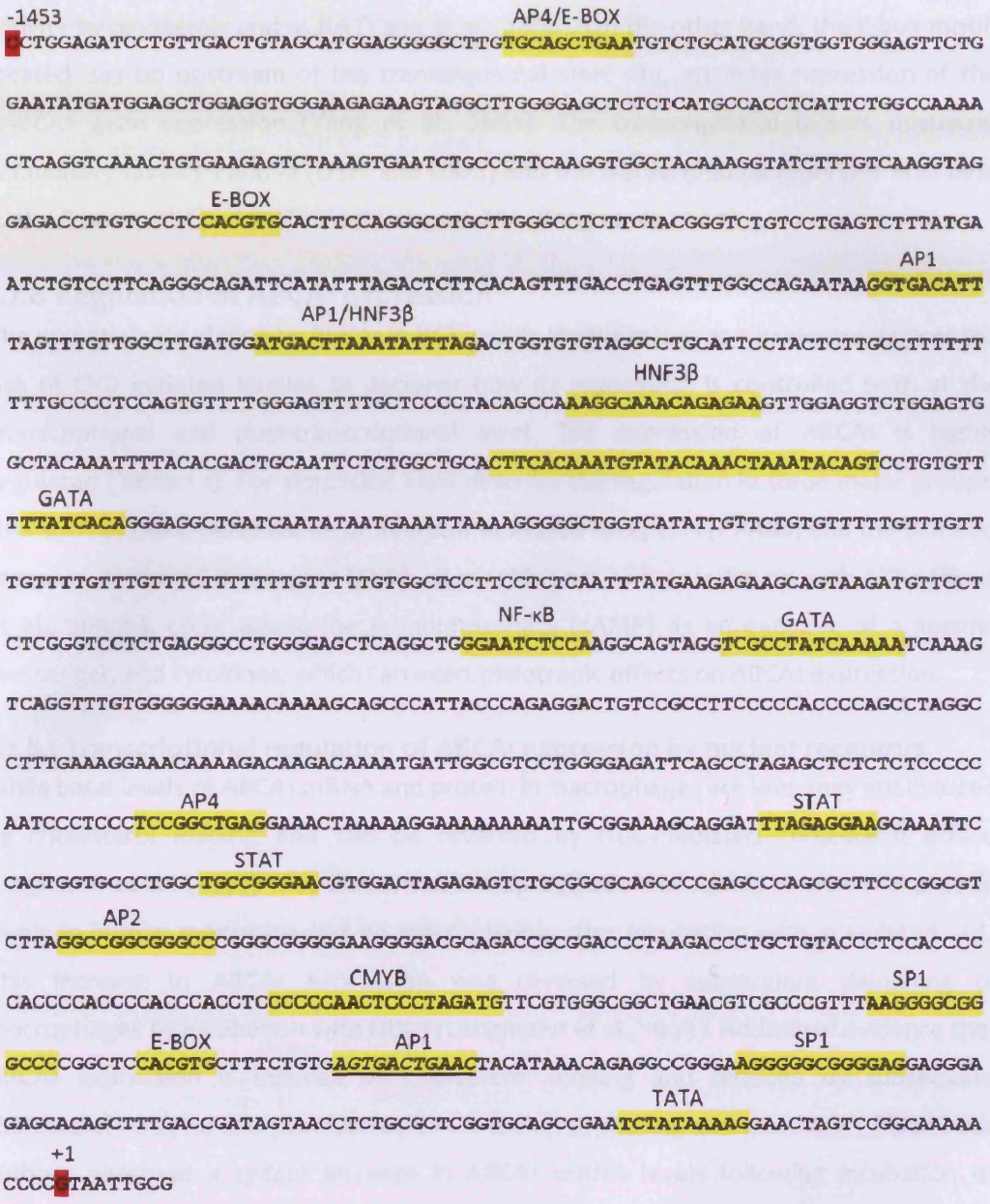


Figure 1.6 The sequence of the human ABCA1 promoter.

The sequence of the human ABCA1 promoter region is shown. The location of some potential binding sites for transcription factors in the distal and proximal promoter regions are highlighted. The transcription start site (G) is indicated as +1. Adapted from Santamarina-Fojo et al., 2000.

up to 9-fold induction in the expression of a luciferase reporter construct driven by the ABCA1 gene promoter. Addition of both RXR agonist 9CRA and LXR agonist (oxysterols) resulted in further induction of the expression of the gene (up to 37-fold). Interestingly, mutation of this LXR motif markedly diminished the induction of the ABCA1 promoter

activity by oxysterols and 9CRA (Yang et al., 2000). On the other hand, the E-box motif, located -147 bp upstream of the transcriptional start site, mediates repression of the hABCA1 gene expression (Yang et al., 2002). The transcriptional factors upstream stimulatory factors-1 and -2 (USF1 and USF2) and the transcriptional repressor Fra2 bind to the E-box motif and facilitate this repression (Yang et al., 2000).

1.2.8 Regulation of ABCA1 expression

The essential role played by ABCA1 in RCT and its identification as a protector against the risk of CVD initiated studies to decipher how its expression is controlled both at the transcriptional and post-transcriptional level. The expression of ABCA1 is highly regulated (Table 1.1). For simplicity, I will describe the regulation in three major groups: NRs such as LXRs, peroxisome proliferator activated receptors (PPARs) and the bile acid receptor, farnesoid X receptor (FXR), all of which act as heterodimers with RXRs (Repa et al., 2000b), cyclic adenosine monophosphate (cAMP) as an example of a second messenger, and cytokines, which can exert pleiotropic effects on ABCA1 expression.

1.2.8.1 Transcriptional regulation of ABCA1 expression by nuclear receptors

While basal levels of ABCA1 mRNA and protein in macrophages are low, they are induced by cholesterol loading and can be reversed by HDL-mediated cholesterol efflux. Langmann et al. (1999) first demonstrated upregulation of ABCA1 mRNA and protein levels in human monocyte-derived macrophages after incubation with acetylated LDL. This increase in ABCA1 expression was reversed by subsequent deloading of macrophages by incubation with HDL3 (Langmann et al., 1999). Additional evidence that ABCA1 expression is induced by cholesterol loading and reduced by subsequent cholesterol removal was provided by Lawn et al. (1999) using quantitative RT-PCR. These authors observed a 17-fold increase in ABCA1 mRNA levels following incubation of fibroblasts in serum-free medium containing cholesterol (Lawn et al., 1999). In addition, cell culture conditions that suppress cell growth, such as serum deprivation, enhances ABCA1 gene expression, whereas growth of fibroblasts in serum-containing media suppresses ABCA1 expression (Lawn et al., 1999). Thus, it appears that ABCA1-mediated cellular lipid efflux requires cell quiescence. The activation of LXR/RXR induces transcription of ABCA1 which helps reduce the cholesterol content of macrophage foam cells and plays a significant role in the RCT pathway (Costet et al., 2003; Langmann et al., 2005; Venkateswaran et al., 2000a; Zhang and Mangelsdorf, 2002).

Table 1.1: Transcriptional regulation of ABCA1. Table derived from Schmitz and Langmann, 2005; Zarubica et al., 2007. (+) Induction, (-) Repression

Substances	Cell types/ tissues	Factors	ABCA1 expression	References
Secondary messengers				
Nucleotide analogs	RAW264.7, J774	cAMP	+	Le Goff et al., 2006; Oram et al., 2000
Lipids				
Oxysterols, LXR agonists	Macrophages, intestine, liver, Sertoli cells, neuronal cells	LXRα, LXRβ	+	Costet et al., 2000; Koldamova et al., 2003; Mascrez et al., 2004
Retinoids	Macrophages	RXRα, RARγ, LXRα	+	Costet et al., 2003; Wagsater et al., 2003
PPARα agonists	Macrophages, intestine	PPAR α	+	Chinetti et al., 2001
PPARγ agonists	Macrophages	PPAR γ	+	Chawla et al., 2001a; Chinetti et al., 2001; Li et al., 2004
PPARδ agonists	THP-1	PPAR δ	+	Oliver et al., 2001
PXR-agonist	HepG2, rat hepatocytes	Rifampicin, LCA, PCN	+	Schmitz and Langmann, 2005
Polyunsaturated fatty acids	J774, RAW264.7	?	–	Wang and Oram, 2002
Cholesterol depletion	HUVEC	SREBP2	–	Zeng et al., 2004
ggPP	THP-1, CaCo-2	ggPP, Rho	–	Gan et al., 2001
Hormones				
Estrogen	Liver, intestine	ERα	+	Srivastava, 2002b
Androgen	LNCaP	?	–	Fukuchi et al., 2004
Thyroid hormone	Fibroblast, 293T	TRα	–	Huuskonen et al., 2004b
Cytokines				
TNFα	J774	?	–	Khovidhunkit et al., 2003
IL-1β	J774	?	–	Khovidhunkit et al., 2003
LPS	RAW 64.7, J774	NF-κB	–	Khovidhunkit et al., 2003
IFNγ	Macrophages	STAT1	–	Panousis and Zuckerman, 2000
TGFβ	Macrophages	?	+	Panousis et al., 2001

PPAR-α and -γ also participate in the upreguation of ABCA1 expression and RCT indirectly via enhanced transcription of LXRα (Chawla et al., 2001a; Chinetti et al., 2001; Chinetti-Gbaguidi et al., 2005). Chinetti et al. (2001) reported that PPARα and PPARγ agonists induce ABCA1 mRNA expression and apoA-I-mediated cholesterol efflux in normal

macrophages but not in macrophages from patients with TD (Chinetti et al., 2001). The expression of LXRα mRNA was also induced by these agents. Furthermore, the addition of both PPAR and LXR activators had an additive effect on ABCA1 expression. Around the same time, Chawla et al. (2001a) demonstrated that ligand-mediated activation of PPARγ leads to induction of LXRα expression and enhanced macrophage expression of mouse ABCA1 (Chawla et al., 2001a). However, no functional PPAR response element has been identified in the ABCA1 promoter. The results of these findings indicate that PPAR agonists might indirectly modulate ABCA1 gene expression by activation of the LXRα pathway, and thereby illustrate a complex interaction between PPARα, PPARγ and LXRα in the cellular regulation of ABCA1 gene expression.

The downregulation of ABCA1 transcription can be achieved through pregnane X receptor (PXR) activated by a wide variety of compounds including natural and synthetic androgens (Schmitz and Langmann, 2005; Sporstol et al., 2005) or through thyroid hormone receptor (TR), TR/RXR dimers and geranylgeranyl pyrophosphate (ggPP), an intermediate in the endogenous mevalonate pathway (Gan et al., 2001).

1.2.8.2 Transcriptional regulation of ABCA1 expression by cyclic AMP

cAMP is a ubiquitous second messenger involved in the control of a variety of physiological events from muscle contraction to memory, and in cellular functions such as growth (Cooper, 2003). cAMP also plays an important role in the upregulation of ABCA1 gene expression by acting both at the transcriptional and translational level. Lawn et al. (1999) showed a 10-fold increase in ABCA1 mRNA expression in fibroblasts incubated with a cell permeable and non-hydrolysable cAMP analog, 8-Br-cAMP (Lawn et al., 1999). Abe-Dohmae et al. (2000) also demonstrated that pre-incubation of RAW264 macrophages with dibutyryl cAMP induces specific apoA-I binding to the cell surface and apoA-I-mediated cholesterol efflux as well as a 9- to 13-fold increase in ABCA1 mRNA levels (Abe-Dohmae et al., 2000). In addition, Takahashi et al. (2000) identified the ABCA1 gene as one of the targets up-regulated by treatment of RAW264 cells with cAMP (Takahashi et al., 2000). In separate studies, treatment of mouse macrophages (RAW264 and J774 cells) with 8-Br-cAMP also caused increases in apoA-I-mediated cholesterol efflux, ABCA1 mRNA and protein levels, as well as the incorporation of ABCA1 into the plasma membrane and the binding of apoA-I to cell surface ABCA1, whereas it had little or no effect on ABCA1 mRNA expression in human tissues, arguing for different

regulation between cell types and species (Oram et al., 2000). This effect has been linked to an increased stability of ABCA1 mRNA upon stimulation of the cells with cAMP. However, the identification of a cAMP-responsive element essential for the induction of ABCA1 gene expression has recently been reported (Le Goff et al., 2006). This acts in conjunction with a nearby signal transducers and activators of transcription protein (STAT) 3/4 element and is not conserved in the human ABCA1 gene, explaining the lack of cAMP stimulation of the human ABCA1 gene (Le Goff et al., 2006). The outcome of these combined cell culture studies have provided evidence that ABCA1 is a cAMP-inducible transporter in mice that promotes cellular efflux of lipids (Le Goff et al., 2006).

1.2.8.3 Transcriptional regulation of ABCA1 expression by cytokines

Cytokines have been shown to exert pleiotropic effects on ABCA1 gene transcription. In general, pro-inflammatory cytokines, TNF-α, IFN-γ and interleukin-1β (IL-1β) down-regulate the LXR-mediated enhancement of ABCA1 gene transcription and protein expression (Lusis, 2000; Panousis and Zuckerman, 2000). IFN-γ also reduces ABCA1 mRNA expression as well as cholesterol and phospholipid efflux to apoA-I in mouse peritoneal macrophages and foam cells (Panousis and Zuckerman, 2000). These authors have suggested that by decreasing cellular cholesterol efflux through pathways that include the upregulation of ACAT expression and the downregulation of ABCA1 expression, IFN-γ may promote the conversion of macrophages into foam cells, thereby accelerating the progression of atherosclerosis. In contrast, transforming growth factor-β (TGF-β) has the reverse effect and induces ABCA1 expression (Panousis et al., 2001). These results reinforce the recent findings of multiple interactions between cellular handling of cholesterol and inflammatory responses mostly mediated at the LXR level. The activation of LXR indeed exerts a global anti-inflammatory effect and promotes macrophage survival (Castrillo et al., 2003b; Chawla et al., 2001a).

1.2.8.4 Post-transcriptional modulation of ABCA1 expression and activity

After cells, particularly macrophages, are loaded with cholesterol, several protein kinases influence the expression and activity of ABCA1 (Oram and Heinecke, 2005; Schmitz and Langmann, 2005). There is emerging recent evidence that ABCA1 expression and function is regulated at a post-transcriptional level, via control of either protein stability and turnover or its activity (Llaverias et al., 2005). The basal cellular levels of ABCA1 transporter are controlled by calpain-mediated degradation (Trompier

and Chimini, 2005). It has been shown that ABCA1 contains so called PEST (Pro-Glu-Ser-Thr) sequences required for proteolysis through the calpain pathway. The calpain mediated proteolytic degradation of ABCA1 is mainly regulated by protein kinase A (PKA)-dependent phosphorylation of the PEST sequence (Martinez et al., 2003; Wang et al., 2003a). Binding of apoA-I also greatly stabilizes ABCA1 by inhibiting calpain-mediated degradation (Martinez et al., 2003; Wang et al., 2003a). Polyunsaturated fatty acids, in addition to acting as inhibitors of LXR-stimulated gene expression, increase the already rapid turnover of ABCA1 in macrophages and thereby diminish the cell surface expression of this protein (Wang and Oram, 2002).

ABCA1 activity is under the control of diverse protein kinases (Table 1.2). Activation of PKA by cAMP is required for optimum lipid transport activity of ABCA1 (See et al., 2002; Tang et al., 2004a). ABCA1 is constitutively phosphorylated by PKA at serines 1042 and 2054 in RAW 264.7 macrophages. Such ABCA1 phosphorylation directly modulates its activity and the downstream efflux of phospholipids and cholesterol to the acceptor ApoA-I (See et al., 2002). It has also been reported that ApoA-I docking at the cell surface also induces ABCA1 phosphorylation through the cAMP/PKA dependent pathway (Haidar et al., 2004; Zarubica et al., 2007). Casein kinase2 (CK2), conversely acts as a down-regulator of ABCA1 activity by phosphorylating amino acid residues located downstream of the first NBD (Roosbeek et al., 2004). In addition, other regulatory kinases have been reported to contribute to ABCA1-dependent efflux of lipids by acting on targets other than the transporter itself (Tang et al., 2004a). For example, Janus kinase 2 (JAK2) is required for the interaction of apolipoproteins with ABCA1 but has little effect on the intrinsic cholesterol flipase activity (Tang et al., 2004a). Moreover, there is evidence that protein kinase C (PKC) may play a role in modulating the lipid transport activity of ABCA1. Several studies have shown that inhibition or activation of PKCs decrease or increase respectively, cholesterol efflux from cells to HDL or purified apoA-I (Li et al., 1997; Mendez et al., 1991; Theret et al., 1990; Walter et al., 1995), implicating PKC isoforms as modulators of ABCA1 activity. However, short term treatment of ABCA1 transfected cells with different PKC inhibitors has been shown to have no effect on apoA-I-mediated phospholipid or cholesterol efflux (Tang et al., 2004a), thereby suggesting that PKCs do not directly activate ABCA1 but modulate trafficking of lipid substrates to ABCA1.

Table 1.2: Regulation of ABCA1 activity. Table derived from Oram and Heinecke, 2005. (+) increase; (-) decrease.

Effector	Mediator	ABCA1	Mechanism	Reference
Signalling				
Apolipoproteins?	PKA	+ Lipid transport	ABCA1 phosphorylation	Haidar et al., 2004
?	CK2	- Lipid transport	ABCA1 phosphorylation	Repa et al., 2000b
Apolipoproteins	JAK2	+ApoA-I binding	?	Takai et al., 2001
Apolipoproteins	PKC	+ Lipid transport	ABCA1 phosphorylation?	Lewis et al., 2001
Substrate trafficking				
Apolipoproteins	PKC	+Cholesterol transport	Cholesterol trafficking	Wu et al., 2004a
Partner proteins				
?	Cdc42	+ Lipid transport	ABCA1 binding	Tontonoz and Mangelsdorf, 2003

Abbreviation: Cdc42, cell division cycle 42; CK2, casein kinase2; JAK2, janus kinase2; PKA, protein kinase A; PKC, protein kinase C.

The activity of the ABCA1 pathway is influenced by factors that control intracellular trafficking of lipids (Table 1.2). Trafficking of lipid substrates to ABCA1 may also be mediated by signalling processes elicited by the interaction of apolipoproteins with ABCA1-expressing cells (Oram and Heinecke, 2005). Yamauchi et al. (2004) has reported that the ability of apolipoproteins to remove cholesterol selectively from sites of cholesterol esterification involves activation of PKC by apolipoproteins (Yamauchi et al., 2004). There is also growing evidence that the activity of ABCA1 is modulated by its interaction with a diverse group of proteins (Table 1.2). For example, cdc42, a member of the Rho GTPase family, directly interacts with ABCA1 and forms complexes with proteins that control cytoskeletal elements and intracellular lipid transport (Diederich et al., 2001; Nofer et al., 2003; Nofer et al., 2006).

Collectively, the previous section has given a brief overview of the various factors that are able to modulate ABCA1 expression. The major focus of this study is to investigate the regulation of ABCA1 by the principal controller LXRs. Therefore, the next section will discuss these members of the NR family in more detail.

1.3 Liver X receptors

NRs are members of a superfamily of transcription factors that are regulated by small lipophilic molecules such as steroids, thyroid hormones (TH), retinoids (vitamin A metabolites) and vitamin D3 (Aranda and Pascual, 2001; Crestani et al., 2004b; Mckenna and O'Malley, 2001, , 2002b). At least 48 types of NRs have been identified in the human and mouse genomes and are divided into two general subfamilies (Escriva et al., 2004; Geyeregger et al., 2006). Type 1 receptors include steroid hormone receptors, which in unstimulated cells are usually sequestered in the cytoplasm by heat shock proteins. Upon ligand binding, these receptors form homodimers and migrate into the nucleus where they regulate the transcription of their target genes. Examples of such receptors include the estrogen receptor (ER), androgen receptor (AR), progesterone receptor (PR) and glucocorticoid receptors (GR), as well as RXR (Crestani et al., 2004b). Type 2 receptors include a large number of receptors that are mainly localized in the nucleus even in the absence of their ligands. These receptors are characterized by the formation of heterodimers with RXRs and include: LXR (Lehmann et al., 1997; Willy et al., 1995), PPAR, TR, Vitamin D receptor (VDR) and retinoic acid receptor (RAR) (Crestani et al., 2004b). In addition, the NR superfamily also includes a large number of orphan receptors for which the ligands have, as yet, not been identified (Aranda and Pascual, 2001; Evans, 1988; Mandelsdorf et al., 1995). In addition to direct ligand-mediated activation of NRs, they are also regulated at a post-translational level that may be brought about by interactions with diverse signal transduction pathways, including mitogen activated protein kinases (MAPKs), PKA and PKC (Rochette-Egly, 2003; Tata, 2002). NRs and their cognate ligands serve as potent regulators of expression of key genes involved in diverse aspects of development, reproduction, immune function and homeostasis (Chawla et al., 2001b; Francis et al., 2003). Several NRs have been shown to play important physiological roles in macrophages. In particular, LXRs, which recognise oxysterol ligands, are key regulators of lipid metabolism.

Two LXR members have been identified, LXRα and LXRβ (also known as NR1H3 and NR1H2 respectively). These share a high degree of amino acid similarity (78% in both their DNA-and ligand-binding domains) and appear to respond to the same endogenous ligands (Alberti et al., 2000) but differ in their tissue distribution. LXRα was first identified in the liver (hence the name liver X receptor) but is now known to be expressed at high levels in other metabolically active tissues/cells, particularly those

associated with lipid metabolism, such as macrophages, intestine and adipose tissue, where its expression is induced in response to cholesterol accumulation (Willy et al., 1995). In contrast, LXRβ is expressed in a ubiquitous manner (Song et al., 1995). A dozen or more LXR target genes have been identified and the majority of them appear to have one of two biological functions; firstly, removal of excess cholesterol through efflux, catabolism or decreased absorption; and secondly, synthesis of fatty acids (Chawla et al., 2001b; Joseph and Tontonoz, 2003).

1.3.1 LXR protein structure

The LXR subfamily are considered as 'metabolic receptors', which includes PPARs and FXR, and they are key "actors" in the integrated regulation of lipid and glucose metabolism (Edwards et al., 2002a). Like other members of this NR class, LXRs have a conserved modular structure with six different regions, commonly labelled A to F, corresponding to autonomous functional domains that can be interchanged between related proteins without loss of function (Figure 1.7) (Aranda and Pascual, 2001; Geyeregger et al., 2006; Gronemeyer et al., 2004; Rochette-Egly, 2003). These domains are described below in detail

The DNA binding domain (DBD) (region C in Figure 1.7) is the most conserved region and consists of two highly conserved zinc finger motifs that allows the receptor to recognize specific DNA consensus sequences known as hormone response elements (HREs). In addition to the zinc finger motifs, this domain is composed of two α-helices and a C-terminal extension (CTE). Helix 1 and Helix 2 cross at right angles to form the core of the DBD folding that recognizes a hemi-site of the response element (Aranda and Pascual, 2001; Bastien and Rochette-Egly, 2004; Rochette-Egly, 2003).

The ligand binding domain (LBD) (region E in Figure 1.7) at the C-terminus of the receptor is the second most conserved region. This domain consists of a LBD hydrophobic pocket, the main dimerization interface, and contains a region involved in ligand-dependent transcription activation function (AF-2) (Aranda and Pascual, 2001; Rosenfeld et al., 2006). LBD is a large multifunctional domain with a lipophilic core that binds specific small lipid molecules. AF-2 determines the specific ligand binding properties of each receptor and mediates ligand regulated interactions with other proteins that act as effectors of transcriptional activation and/or repression

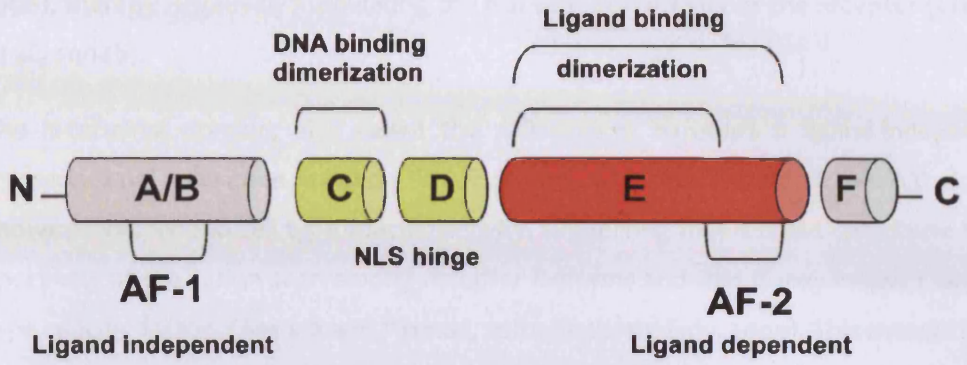


Figure 1.7 Structural and functional domain organization of a typical NR.

The typical structure of a NR is composed of several functional domains A to F based on regions of conserved sequence and function. The DNA binding domain (DBD; region C) is the most highly conserved domain and contains two zinc finger motifs. The ligand binding domain (LBD; region E) is less conserved than DBD and mediates ligand binding, dimerization and a ligand-dependent *trans*-activation function (AF-2). Within the AF-2, the integrity of a conserved amphipathic α-helix, termed AF-2 activation domain, has been shown to be required for ligand-dependent *trans*-activation. The N-terminal A/B region contains a cell- and promoter-specific *trans*-activation function (AF-1). The region D, called the hinge domain, contains nuclear localization signals (NLS hinge); The F domain is not present in all NRs and its function is poorly understood. Figure adapted from Aranda and Pascual, 2001.

(Gronemeyer et al., 2004). The LBD is formed by 12 conserved α-helical regions (H1 to H12) with a conserved β-turn situated between helices H5 and H6. The helices are folded into a three layered, anti-parallel α-helical sandwich, in which a central core layer of three helices is packed between two additional layers of helices to create a cavity, the ligand binding pocket, which accommodates the ligand. This cavity is mainly hydrophobic and is buried within the bottom half of the LBD (Aranda and Pascual, 2001; Bastien and Rochette-Egly, 2004).

The F domain is present in some NRs. This domain may be involved in providing additional discrimination between receptor agonists and antagonists (Smirnov, 2002). For example, removal of this domain from NR converts the action of antagonists into those for agonists. It is possible that this domain can bind some corepressor (Xu et al.,

1996), thereby negatively modulating the transcription activity of the receptor (Crestani et al., 2004b).

The N-terminal domain, also called the A/B region, harbours a ligand-independent transcriptional activation function (AF-1) (Figure 1.7). The action of the A/B domain shows promoter and cell type specific activity, suggesting that it could contribute to the specificity of the action seen among receptor isoforms and that it may interact with cell type specific factors (Aranda and Pascual, 2001; Rochette-Egly, 2003). This domain is also a target for phosphorylation mediated by different signalling pathways which include cyclin dependent kinases (cdks) and MAP Kinases (Davis, 2000; Morgan, 1997). Such modifications can affect transcriptional activity of the receptors (Rochette-Egly, 2003; Shao and Lazar, 1999). Indeed, several studies have shown that such phosphorylation is able to affect transcriptional activity of NRs, including LXRs (Bastien and Rochette-Egly, 2004; Chen et al., 2006; Juge-Aubry et al., 1999; Kato et al., 1995; Rochette-Egly, 2003; Yamamoto et al., 2007).

The short D region is not well conserved among the different receptors and serves as a hinge region between the DBD and LBD, allowing rotation of the DBD, and protein flexibility for simultaneous receptor dimerization and interaction to the DNA consensus sequences located in the regulatory regions of target genes (Figure 1.7). In many NRs, the D domain contains nuclear localization signals (NLS) and also contains residues whose mutation abolishes the interaction of the NRs with specific corepressors (Aranda and Pascual, 2001; Bastien and Rochette-Egly, 2004; Chen and Evans, 1995; Horlein et al., 1995).

The structure of ligand-bound receptors is much more compact than unliganded receptors (Aranda and Pascual, 2001). After ligand binding, the NRs undergo allosteric conformational changes in the LBD that promotes interactions with coactivator proteins, thus facilitating transcriptional regulation of target genes (Aranda and Pascual, 2001; Mckenna and O'Malley, 2002a; Rosenfeld and Glass, 2001; Svensson et al., 2003). The recruitment of coactivators is the second essential step in the action of NRs. This recruitment is the direct consequence of the ligand-induced conformational changes that generates the surface to which the NR-interacting domain (NID) of coactivators bind. This recruitment of coactivators appears to depend on specific interactions between the receptors and the coactivators, which requires multiple copies of a

common, highly conserved LxxLL motifs (Rosenfeld et al., 2006; Shao et al., 2000). The ligand-induced recruitment of coactivators to NRs are dependent on AF-2, which consists of a short LxxLL helical motif within the LBD (Bourguet et al., 2000), although a distinct set of coactivators can also associate with the AF-1 domain. Point mutations in the AF-2 domain has no effect on the binding of regulatory ligands, dimerisation or DNA binding but abolishes transcriptional activation (Svensson et al., 2003). This domain is therefore predicted to serve as an adapter surface for interactions with other molecules necessary for transcriptional activation (Barettino et al., 1994).

1.3.2 LXR/RXR heterodimers

LXRs are localized in the nucleus and form obligate heterodimers with RXR proteins (Willy et al., 1995). LXR/RXR heterodimers bind the promoters of target genes via DNA sequences composed of two direct hexameric repeats of the consensus motif AGGTCA, separated by four nucleotides (DR4 element), also termed LXR response elements or LXR-REs (Chawla et al., 2001b; Edwards et al., 2002a; Willy et al., 1995; Zhang et al., 2001). While other RXR heterodimers, such as RXR/RAR, RXR/TR, PPAR/RXR and RXR/VDR are considered non permissive due to their inability to respond to RXR selective ligands (retinoids such as 9CRA) independently of the partner ligand (Aranda and Pascual, 2001; Forman et al., 1995), the LXR/RXR forms a permissive heterodimer and can be activated by ligands for both LXR and RXR receptors, either separately or in synergy (Aranda and Pascual, 2001; Janowski et al., 1996; Repa and Mangelsdorf, 2002). Thus, under certain conditions both ligands in combination can activate LXR/RXR heterodimer in an additive or sometimes synergistic manner. Interestingly, stimulation by 9CRA requires the AF-2 domain of LXR, but not RXR, to become transcriptionally active. This requirement demonstrates that binding of the RXR ligand leads to a conformation change in LXR resulting in transcriptional activation (Aranda and Pascual, 2001). The ligands can also play a potential role in dimerization and DNA binding. For example, 9CRA in some cases can increase the binding of RXR homodimers to the DR1 sequence (Zhang et al., 1992b), which can lead to its unavailability for heterodimer formation with other receptors and, thereby, decreased transcription of genes dependent on such heterodimers (Aranda and Pascual, 2001).

1.3.3 LXR agonists/antagonists

The putative endogenous activators of LXRs consist of a specific group of naturally occurring, oxidized derivatives of cholesterol (oxysterols) (Forman et al., 1997; Janowski et al., 1996; Lehmann et al., 1997). In contrast to steroid hormones that bind their respective receptors with high affinity (binding constants in the nanomolar range), natural ligands for LXRs appear to consist of cholesterol metabolites that bind with relatively low affinities (binding constants in the micromolar range) which is consistent with the higher physiological concentration of these agonists (Janowski et al., 1999; Peet et al., 1988). Although sterol loading of cells induces the expression of LXR target genes (Repa and Mangelsdorf, 1999), neither cholesterol esters nor free cholesterol itself can act as LXR activators and their conversion to oxysterols is required for transcriptional activity (Ory, 2004). These oxysterols serve as intermediary substrates or end products of various metabolic pathways including cholesterol biosynthesis, bile acid synthesis in the liver and steroid hormone synthesis in the adrenal glands. The introduction of an epoxide, hydroxyl or keto group to such derivatives of cholesterol on side chain renders the compounds biologically active inducers of LXRs (Janowski et al., 1996; Lehmann et al., 1997). The most potent natural activators are 22(R)-, 20(S)-, 24(S)-hydroxycholesterol and 24(S), 25-epoxycholesterol (Janowski et al., 1999; Janowski et al., 1996; Lehmann et al., 1997). Importantly, these oxysterols exist in tissues that express LXRs and at concentrations that activate LXRs with Kd values between 0.1μM to 0.4μM *in vitro*, suggesting they are physiologically relevant ligands (Janowski et al., 1999). 27 hydroxycholesterol, which is formed via the action of the enzyme sterol 27-hydroxylase, has been suggested to be the endogenous LXR ligand in cholesterol-loaded human macrophages, where the action of LXR is of particular importance in relation to atherogenesis (Fu et al., 2001). The same author has also found that 24(S), 25-epoxycholesterol is particularly abundant in the liver, where both cholesterol metabolism and LXR expression are high. Thus, certain oxysterols may be cell-specific physiological ligands for the LXRs (Fu et al., 2001).

Most of the endogenous LXR ligands that have been identified so far activate both LXRα and LXRβ with the exception of the oxysterol, 5,6-24(S),25-diepoxycholesterol, which has been shown to be relatively selective for LXRα. This raises the possibility of developing subtype-selective, synthetic LXR ligands for pharmacological applications (Janowski et al., 1999). Also, cholestenoic acid, a metabolite of 27-hydroxycholesterol, is

a naturally occurring ligand for LXRα, but not for LXRβ (EC50 for LXRα is 200nM and for LXRβ is ≥5μM). Similarly, natural and synthetic 6α-hydroxy bile acids are more selective activators of LXRα than LXRβ (Song et al., 2000).

In addition to the endogenous ligands, a number of synthetic pharmacological LXR agonists have been reported, which are structurally unrelated to oxysterols. The increased potency of these compounds compared to physiological ligands makes them useful tools for the study of LXR function. The first such synthetic LXR ligand was the compound T0901317, which binds to and activates LXRs specifically with a Kd value around 20nM (Repa et al., 2000b; Schultz et al., 2000) compared to 300nM for 24(S), 25-epoxycholesterol (Schultz et al., 2000). Another compound, GW3965, a tertiary amine, has been found to be an orally active LXR ligand (Collins et al., 2002). More recently, another synthetic compound, acetyl podocarpic (ADP), has been shown to be 1000-fold more potent than endogenous ligands (e.g. 22(R)-HC) in the activation of LXRα/β and their target genes (e.g. ABCA1) (Sparrow et al., 2002). All these ligands have been shown to promote cellular cholesterol efflux and to inhibit atherosclerosis in animal models of this disease, which makes them potentially attractive agents for the modulation of human lipid metabolism (Joseph et al., 2002b).

In contrast to oxysterols that enhance transcriptional activity of LXRs, the activation of these receptors can also be antagonized by other small lipophilic agents (Chawla et al., 2001b). For example, geranylgeranyl-pyrophosphate, an intermediate in the cholesterol biosynthesis pathway, negatively regulates LXRα and LXRβ transcriptional activity by inhibiting the interaction of the LXRs with nuclear coactivators (Forman et al., 1997). Similarly, unsaturated fatty acids antagonize LXR activity but by acting as competitive inhibitors for LXR ligands, thereby preventing the binding of LXR/RXR heterodimers to the LXR-RE (Yoshikawa et al., 2002). Additionally, human blood plasma contains natural antagonists for LXRα and LXRβ (Song et al., 2001). Such antagonists were identified as specific 3-sulfate derivatives of oxysterols and might be formed by the oxidation of cholesterol 3-sulfate, which is present at elevated levels in individuals with hypercholesterolemia (Song et al., 2001; Tamasawa et al., 1993). Thus, endogenous LXR antagonists can counteract LXR agonist actions in the control of cholesterol homeostasis and atherogenesis (Geyeregger et al., 2006).

1.3.4 LXR and coregulators

Initiation of transcription in eukaryotic cells is a complicated multi-step process involving a large number of cofactors that exert functions in remodelling of the chromatin and/or recruitment of RNA polymerase II to the promoters of target genes (Lemon and Tjian, 2000). Because packaging of eukaryotic DNA into chromatin has a generally repressive effect on transcription, the enzymes that alter chromatin structure have critical roles in the regulation of gene expression (Narlikar et al., 2002). Transcriptional regulation by LXRs is achieved when they are activated by the interacting ligand and bind to the DR4 element in target gene promoters. As with other transcriptional regulatory proteins, the mechanism by which LXR heterodimers affect the rate of RNA polymerase II-directed transcription involves the interaction of receptors with components of the transcription pre-initiation complex. This interaction could occur directly or indirectly through the action of coregulators. Coregulators are coactivators or corepressors, which interact with NRs and enhance or lower the transcription rate of their target genes respectively. These coregulators are required for efficient transcriptional regulation. In general, coregulators play one or more of the following roles in regulating the transcriptional activity of NRs. First, coregulators function as bridging factors to recruit coactivators to DNA bound NRs, such as the steroid receptor coactivator (SRC) proteins, which can recruit p300/CREB binding protein (CPB) to DNA bound receptors. Second, coregulators can acetylate nucleosomal histones and various transcription factors at the promoters of target genes (e.g. p300/CBP and p300/CBP associated factor (P/CAF)), which have potent nucleosomal histone acetyltransferase (HAT) activity. Acetylation of histones by HAT causes an expansion of the chromatin structure, thereby allowing transcription to take place. Conversely, removal of the acetyl group by histone deacetylases (HDACs) condenses the DNA structure, thereby preventing transcription (North and Verdin, 2004). Third, coregulators function as bridging factors between DNA bound receptors and components of the basal transcriptional machinery (e.g. transcription factor IIA, IIB and IID). Although, the precise activation mechanisms and coregulators used by LXRs to activate the transcription of target genes in a ligand-dependent manner are currently poorly understood (Glass and Rosenfeld, 2000; Mckenna and O'Malley, 2002b; Rosenfeld and Glass, 2001), a putative model has been derived from studies on some LXR responsive promoters and extensive research on other NRs (Bastien and Rochette-Egly, 2004; Edwards et al., 2002b; Huuskonen et al., 2004a).

In this model, LXR, like other NRs, such as RAR, TR and PPARδ that form heterodimers with RXRs, interact with corepressor proteins, such as the NR corepressor (NCoR) (Horlein et al., 1995) and the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), in the absence of agonists (Chen and Evans, 1995; Hu et al., 2003; Wagner et al., 2003). These corepressor interactions are maintained in the absence of the ligands and the transcriptional activity of LXR target genes is repressed (Bastien and Rochette-Egly, 2004; Hu et al., 2003; Wagner et al., 2003). Upon ligand binding, the basal rate of transcription occurs when the corepressors start to dissociate from the LXR/RXR heterodimer, and the transcription of the target genes becomes fully activated when coactivators are recruited to the ligand-activated LXR/RXR heterodimer (Bastien and Rochette-Egly, 2004; Glass and Rosenfeld, 2000; Rosenfeld et al., 2006). The transition from active repression to ligand-dependent transcriptional activation requires dissociation of corepressors and recruitment of coactivators. Perissi et al. (2004) suggested that the ligand-dependent corepressor to coactivator exchange requires the ubiquitinylation machinery that targets the corepressor complex for proteosome-dependent degradation. Coactivator proteins implicated in *trans*-activation by LXRs include glucocorticoid receptor-interacting protein 1 (GRIP1), a member of the p160 co-activator family (Huuskonen et al., 2004a) and PPAR γ coactivator 1 α (PGC-1α) (Geyeregger et al., 2006; Mckenna and O'Malley, 2002a; Oberkofler et al., 2003; Rosenfeld and Glass, 2001). Many of these are components of large multi-protein complexes with associated enzymatic activities, including nucleosome remodelling activities, histone methyltransferase activity, HAT activity and the ability to recruit core transcription factors. This link is sufficient to cause maximal LXR-mediated transcriptional activation (Huuskonen et al., 2004a). The second step in this process is the recruitment of associated proteins such as P/CAF, and via their HAT activities, modification of the chromatin structure so that the mediator multi-polypeptide complex, the human mediator-like thyroid hormone receptor-associated proteins/vitamin D receptor-interacting proteins (TRAP/DRIP) (Fondell et al., 1996; Rachez et al., 1999) can replace SRC-1/CBP. Subsequent recruitment of RNA polymerase II complex to TRAP/DRIP completes the second step in NR *trans*-activation. It is possible that p300/CBP interacts directly with the basal transcription machinery (Felzien et al., 1999) or that p300/CBP acetylates histones and recruits switch/sucrose non-fermenting (SWI/SNF) chromatin remodelling complexes to the NRs, thereby facilitating the

interaction with basal transcription machinery (Huang et al., 2003). Therefore, these interactions between LXRs, chromatin remodelling complexes and coactivators at oxysterol-activated promoters lead to the stimulation of gene transcription.

LXR-mediated *trans*-activation can also be regulated by at least two other mechanisms. Activating signal co-integrator-2 (ASC-2) has been shown to interact specifically with LXRs and stimulate gene transcription (Kim et al., 2003). Although ASC-2 exists in a complex containing methyltransferases, the precise mechanism is not known. Moreover, LXRs can interact with multi-protein bridging factor-1 (MBF-1) (Brendel et al., 2002), which directly interacts with the general transcription factor TFIID (TFIID), making it a possible bridging molecule between LXR and basal transcription machinery.

Although, in the absence of ligands, LXRs, like other NRs, may actively repress gene transcription by recruiting corepressor proteins (NCoR and SMRT) (Hu et al., 2003; Repa et al., 2000b; Wagner et al., 2003), they also have the ability to negatively regulate gene expression in a ligand-dependent manner by antagonizing the activities of other classes of signal-dependent transcription factors, such as activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB). This phenomenon, referred to as *trans*-repression, is thought to be the primary mechanism by which LXRs inhibit the expression of pro-inflammatory genes in macrophages (De Bosscher et al., 2003; Joseph et al., 2003; Zelcer and Tontonoz, 2006). LXR-dependent repression of cytokine-induced expression of matrix metalloproteinase-9 (MMP-9) and osteopontin (OPN) genes has been demonstrated in macrophages, and this is through antagonism of the NF-κB signalling pathway (Castrillo et al., 2003a) and interference with the AP-1 signalling pathway (Ogawa et al., 2005) respectively. The molecular mechanisms responsible for such *trans*-repression are less well understood than the mechanisms responsible for transcriptional activation except that they do not require sequence-specific DNA binding to response elements in promoters of target genes (Li and Glass, 2004; Pascual and Glass, 2006).

Post-translational modifications, such as phosphorylation, triggered by diverse signal transduction pathways, have also been shown to be an important regulatory mechanism for a number of NRs (Crestani et al., 2004a; Hu et al., 1996; Juge-Aubry et al., 1999; Rosenfeld et al., 2006). Indeed, phosphorylation of NRs or other factors required for their actions, such as coactivators, have been reported previously (Bastien and Rochette-Egly, 2004; Blanquart et al., 2004; Hu et al., 1996; Kato et al., 1995; Lee et al.,

2006b; Orti et al., 1992; Rochette-Egly, 2003; Wagner et al., 2003). Although phosphorylation can be induced by the ligand, constitutive phosphorylation has also been found to be present *in vivo* (Orti et al., 1992). Phosphorylation modulates the transcriptional activity of some of these receptors (Blanquart et al., 2004; Hu et al., 1996).

The involvement of cell signalling pathways in the regulation of target gene expression by NRs have previously been described. For example, the MAPK pathways have been shown to affect NR activity via phosphorylation of either the NR itself or co-regulator proteins (Rochette-Egly, 2003). It is well known that several NRs (e.g. ER, PR, AR, TR, PPARs, RAR and RXR) are phosphorylated by MAPKs leading to modification of their transcriptional activities via diverse mechanisms (Banfi et al., 2003; Chen et al., 2003; Gianni et al., 2002a; Kato et al., 1995; Lange et al., 2000; Shen et al., 2001; Yeh et al., 1999). In addition, Chen et al. (2003) reported that all the three MAPK pathways are able to modulate the activities of transcription coregulators, such as p300 and SMRT. In particular, the c-Jun N-terminal kinases, also known as stress activated protein kinases (JNK/SAPK), have been reported to potentiate the activation function of ER via phosphorylation of ER associated proteins, CBP and GRIP1 (Feng et al., 2001). The JNK/SAPK pathway has also been shown to directly phosphorylate several NRs such as PPARγ (Adams et al., 1997; Camp et al., 1999; Hu et al., 1996), PR (Faivre et al., 2005) and GR (Rogatsky et al., 1998a). The JNK/SAPK has also been shown to target the AF-1 domain of RXRα (Gianni et al., 2002b; Lee et al., 2000; Matsushima-Nishiwaki et al., 2001), and thereby modulate its function.

The phosphoinositide 3-kinase pathway (PI3K) has also been shown to play a vital role in NR signalling with various components of the pathway being involved in the regulation of the *trans*-activation function of these DNA binding proteins. The primary role for this pathway seems to be in the phosphorylation of NRs. For instance, protein kinase B (PKB), a downstream target for PI3K, is known to translocate to the nucleus upon stimulation of the cells with particular ligands, and phosphorylate certain NRs (Rochette-Egly, 2003; Vanhaesebroeck et al., 1997; Wymann and Pirola, 1998). For example, PKB has been shown to phosphorylate ER-α (Campbell et al., 2001) and AR (Lin et al., 2001) in their N-terminal regions. PKB also plays an important role in RAR signalling (Gianni et al., 2002b), where it phosphorylates RARα at serine 96 in its DNA binding domain and

inhibits transcriptional activation by this receptor (Srinivas et al., 2006). PKC also phosphorylates RARα and strongly reduces its *trans*-activation by inhibiting its dimerization with RXRα (Delmotte et al., 1999) or VDR (Hsieh et al., 1991; Hsieh et al., 1993). PKC has also been shown to regulate the transcriptional activation function of PPAR (Blanquart et al., 2004). In the light of such previous findings on the control of NRs, it is possible that cell signalling pathways could regulate LXR-mediated gene transcription via phosphorylation of the LXR protein or one of the coregulator components of the transcription complex. In this context, Chen et al. (2006) showed for the first time that LXRs are phospho proteins. Moreover, they showed that LXRα is phosphorylated at a single site (serine 198) in the hinge region of the protein and this is a consensus site for MAPK phosphorylation. A brief description of the two common signalling pathways which have so far been found to be involved in NR signalling is detailed below.

1.3.4.1 Mitogen activated protein kinase pathway

MAPKs belong to a family of serine/threonine specific protein kinases that play an important role in several cellular processes such as growth, differentiation, proliferation and cell survival/apoptosis (Chang and Karin, 2001). MAPKs are intracellular signal transducing molecules expressed in all eukaryotic cells that modulate their basic cellular events by responding to extracellular signals (Morrison and Davis, 2003). Activated MAPKs indirectly regulate the expression of multiple genes through the phosphorylation and subsequent activation of transcription factors that regulate them. The MAPKs are "proline-directed" kinases and thus phosphorylate Ser/Thr residues in target proteins only if it is immediately followed by a proline residue (Kyriakis and Avruch, 2001; Roux and Blenis, 2004). All MAPK pathways include a central three-tiered core signalling system (Figure 1.8). MAPKs are activated by dual phosphorylation of Thr/Tyr residues in a conserved tri-peptide motif (Thr-X-Tyr), which is located in the activation loop (Kyriakis and Avruch, 2001; Roux and Blenis, 2004). Such activation occurs via signalling cascades involving MAPK kinases (MAPKKs) that are in turn activated by MAPK kinase kinase (MAPKKK) (see Figure 1.8) (Morrison and Davis, 2003; Strniskova et al., 2002; Weston and Davis, 2002). There are five subgroups of MAPKs that have been identified so far, namely extracellular signal regulated kinases (ERK1/2), ERK5, ERK7, JNK/SAPK and p38 isoforms (Johnson et al., 2005).

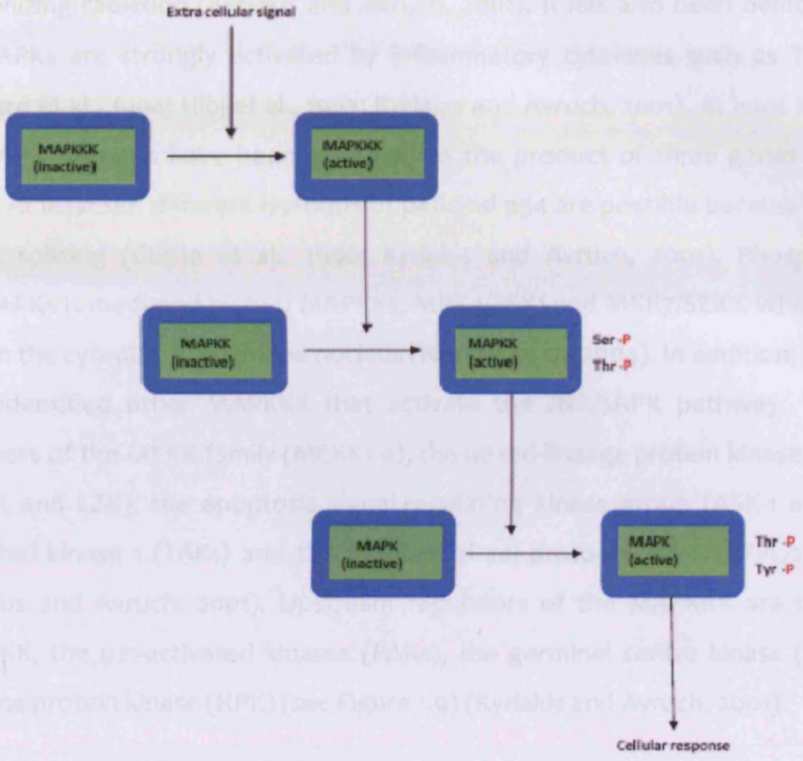


Figure 1.8 The core MAPK kinase signalling module.

Various extracellular signals "feed" into MAPKKK to MAPKK to MAPK, the core module, through several upstream regulators. Each MAPK is activated by dual Thr/Tyr phosphorylation catalysed by a MAPKK, which is in turn regulated by Ser/Thr phosphorylation by MAPKKK. The downstream targets of this signalling pathway then mediate the appropriate cellular responses. See text for further explanation. Figure adapted from Kyriakis and Avruch 2001.

Of these major subfamilies of MAPKs that are present in all eukaryotic organisms, three subfamilies have been extensively characterized. These have been integrated into different signal transduction pathways and also show differences in substrate specificity. The ERKs and the p38 groups of MAPKs are related to enzymes found in budding yeast and contain the dual phosphorylation motifs Thr-Glu-Tyr and Thr-Gly-Tyr respectively. The third group of MAPKs, the JNK/SAPK, contain the dual phosphorylation motif Thr-Pro-Tyr.

JNK/SAPKs were originally identified by their ability to bind to and phosphorylate the amino-terminal activation domain of the c-Jun transcription factor in response to a variety of stress-inducing signals, including UV stimulation, heat shock, oxidant stress

and ionizing radiation (Kyriakis and Avruch, 2001). It has also been demonstrated that JNK/SAPKs are strongly activated by inflammatory cytokines such as TNF-α and IL-1 (Derijard et al., 1994; Hibi et al., 1993; Kyriakis and Avruch, 2001). At least twelve distinct JNK/SAPK isoforms have been identified as the product of three genes (Gupta et al., 1996). At least ten different isoforms of p46 and p54 are possible because of differential mRNA splicing (Gupta et al., 1996; Kyriakis and Avruch, 2001). Phosphorylation of JNK/SAPKs is mediated by two MAPKKs, MKK4/SEK1 and MKK7/SEK2, which are present both in the cytoplasm and in the nucleus (Nishina et al., 2004). In addition, recent studies have identified other MAPKKK that activate the JNK/SAPK pathway. These include members of the MEKK family (MEKK1-4), the mixed-lineage protein kinase family (MLK1-3, DLK and LZK), the apoptosis signal-regulating kinase group (ASK-1 and -2), TGF-β-activated kinase 1 (TAK1) and the product of *col* proto-oncogene (TPL-2) (Figure 1.9), (Kyriakis and Avruch, 2001). Upstream regulators of the MAPKKK are the Ste-20-like MAPKKK, the p21-activated kinases (PAKs), the germinal centre kinase (GCK) and the histidine protein kinase (HPK) (see Figure 1.9) (Kyriakis and Avruch, 2001).

JNK/SAPKs activate c-Jun in response to extracellular stimuli by phosphorylating the N-terminal activation domain of this factor (Ser63 and Ser73) resulting in increased transcriptional activity (Hibi et al., 1993). c-Jun is a major component of the AP-1 family, which typically consists of a heterodimer of bZIP transcription factors, typically c-Jun, Jun-D, and members of the fos family (usually c-fos) and activating transcription factor-2 (ATF-2) family. Although homodimers of the Jun family are possible, this cannot occur for the fos family. AP-1 therefore consists of Jun-Jun, Jun-Fos or Jun-ATF dimers (Karin et al., 1997; Kyriakis and Avruch, 2001). Transcriptional activation by AP-1 is mediated through both direct phosphorylation and dephosphorylation of AP-1 components, as well as the phosphorylation and activation of transcription factors that regulate the expression of c-Jun and c-Fos (Kyriakis and Avruch, 2001). Whilst c-Jun is a well known substrate of JNK/SAPK, there are other targets such as ATF-2 and the ETS domain transcription factor Elk-1 (Gupta et al., 1995). Activation of JNK/SAPKs results in increased phosphorylation of ATF-2, and thereby stimulation of its transcriptional activity (Gupta et al., 1995). Like c-Jun and ATF-2, phosphorylation of the Elk-1 activation domain by JNK/SAPK increases its transcriptional activity (Kyriakis and Avruch, 2001; Shaulian and Karin, 2002).

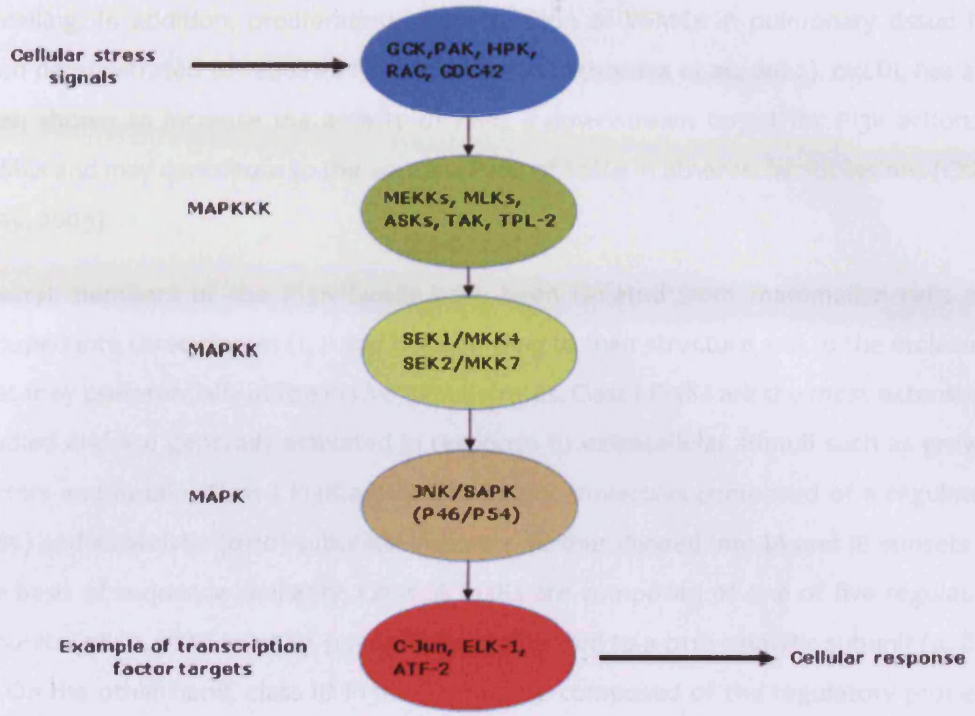


Figure 1.9 A schematic representation of the JNK/SAPK signalling cascade.

Abbreviations: GCK, germinal centre kinase; PAK, p21-activated kinase; HPK, histidine protein kinase; MEKKs, MAPK/ERK kinase kinase; MLKs, mixed-lineage protein kinases; ASKs, apoptosis signal-regulating kinases; TAK, TGF-β activated kinase; SEK, SAPK/ERK kinase. See text for further details. Adapted from Greenow, K., 2004.

1.3.4.2 Phosphoinositide 3- kinase pathway

PI3K is a member of a large family of lipid kinases which modulate a variety of cellular processes, including growth, proliferation, differentiation, metabolism and cell survival/apoptosis (Krasilnikov, 2000). Several recent reviews have discussed the function of PI3K in CVD (Alloatti et al., 2004; Oudit et al., 2004). For example, roles for PI3K have been proposed in macrophage accumulation in inflammation. Indeed, monocytes from PI3Kγ knockout mice have decreased ability of migration and recruitment to the inflamed peritoneum (Hirsch et al., 2000). A pro-atherogenic role for PI3K is also indicated by the finding that apoE-knockout mice receiving transplanted bone marrow from mice that are deficient in p110δ, a catalytic subunit of PI3K (see below), display reduced atherosclerosis in comparison to those receiving wild type bone marrow (Chang et al., 2007). This is potentially due to decreased accumulation of macrophage foam cells mediated by the proinflammatory effects of PI3K dependent

signalling. In addition, proliferation and migration of VSMCs in pulmonary tissue has been demonstrated to require PI3K activation (Gonchorova et al., 2002). oxLDL has also been shown to increase the activity of PKB, a downstream target for PI3k action, in VSMCs and may contribute to the accumulation of SMCs in atherosclerotic lesions (Chien et al., 2003).

Several members of the PI3K family have been isolated from mammalian cells and grouped into three classes (I, II and III) according to their structure and to the molecules that they preferentially utilize *in vivo* as substrates. Class I PI3Ks are the most extensively studied and are generally activated in response to extracellular stimuli such as growth factors and insulin. Class I PI3K are heterodimeric molecules composed of a regulatory (p85) and a catalytic (p110) subunits which are further divided into IA and IB subsets on the basis of sequence similarity. Class IA PI3Ks are composed of one of five regulatory subunits (p85α, p85β or p55γ, p55α or p50α) attached to a p110 catalytic subunit (α, β or δ). On the other hand, class IB PI3Ks (PI3Kγ) are composed of the regulatory p101 and the catalytic p110γ subunits. Class I PI3Ks are often implicated in the control of cellular functions based on an inhibitory effect of the pharmacological agent LY294002. This and wortmannin are broad inhibitors of all PI3kinases. Class II and III PI3Ks are different from Class I in structure and function. Class II PI3K comprises of three catalytic isoforms (C2α, β and γ), but unlike class I and III, have no regulatory proteins. These are therefore monomeric enzymes that catalyse the production of phosphatidylinositol 3-phosphate (PtdIns3P) from phosphatidylinositol (PtdIns) (may also produce phosphatidylinositol (3,4)-bisphosphate (PtdIns(3,4)P2) from phosphatidylinositol 4-phosphate (PtdIns4P)). However, little is known about their function. Class III PI3Ks are similar to class II in that they utilise only PtdIns, but are more similar to class I in structure as they exist as heterodimers with a catalytic subunit (Vps34) and a regulatory subunit (p150). Class III PI3K seems to be primarily involved in the regulation of intracellular trafficking of proteins and vesicles (Hawkins et al., 2006; Hirsch et al., 2007). However, there is evidence that they are also able to contribute to the effectiveness of several processes important to immune cells, such as phagocytosis.

In the inner leaflet of the plasma membrane of eurkaryotic cells, PI3Ks are kept generally at a steady state level (Payrastre et al., 2001). Activation of PI3Ks requires tyrosine phosphorylation by an activated receptor associated with protein tyrosine kinases (e.g.

granulocyte/macrophage-colony stimulating factor (GM-CSF)) or receptors with intrinsic tyrosine kinase activity (e.g. platelet-derived growth factor (PDGF) and insulin). Class I subfamily of PI3Ks can be activated by either receptor tyrosine kinases (RTKs) or G-protein coupled receptors (GPCR). Once activated, the active PI3K enzyme catalyzes the addition of a phosphate group specifically to the 3-OH position of the inositol ring of phosphoinositides (Rameh and Cantly, 1999). These then serve to activate downstream signalling targets. The most common reaction is the phosphorylation of phosphatidylinositol-4, 5- biphosphate (PtdIns(4,5)P2) to produce phosphophatidylinositol 3,4,5, triphosphate (PtdIns(3,4,5)P3) by class I PI3Ks, leading to the recruitment and activation of protein kinases that contain specialised lipid-binding domains such as the pleckstrin homology (PH) domains. Downstream protein kinases are subsequently recruited to the membrane through the binding of its PH domain to PtdIns(3,4,5)P3, where they are brought into close proximity with their targets (Hawkins et al., 2006). The most widely studied of these are 3-phosphoinositide-dependent protein kinase-1 (PDK)-1 and its substrate PKB (also referred to as AKT). This then regulates the action of several downstream effectors, including glycogen synthase kinase-3 (GSK-3) and the mammalian target of rapamycin (mTOR) (Figure 1.10). PDK-1 also activates the kinases p70(S6K) and PKCs (Bjornsti and Houghton, 2004; Cantly, 2002; Vanhaesenbroeck and Alessi, 2000; Yang et al., 2004). Conversely, the activated PI3K complex is down-regulated by PTEN. An attractive hypothesis for such negative regulation of PI3K signalling is that tyrosine phosphorylation of p85, which occurs after the p85-p110 complex formation has been recruited to the active RTK, serves as a negative regulatory signal that leads to a reduction in p110 catalytic activity (Cuevas et al., 2001). The other hypothesis is that PI3K production of phosphoinositides is antagonized by protein phosphatases and tensin homologue (PTEN), which is a dual specificity phosphatase that has activity against lipid and protein substrates. This PTEN dephosphorylates PtdIns(3,4,5)P3 to PtdIns(4,5)P2 both *in vitro* and *in vivo*, thereby resulting in the down-regulation of PI3K signalling pathways (Igor and Charles, 2002).

1.3.5 Role of LXRs in cholesterol homeostasis

Under physiological conditions, cholesterol homeostasis is maintained via dietary intake by intestinal absorption and the regulated pathways of *de novo* synthesis and catabolism. Cholesterol biosynthesis is controlled by the accumulation of sterols, which

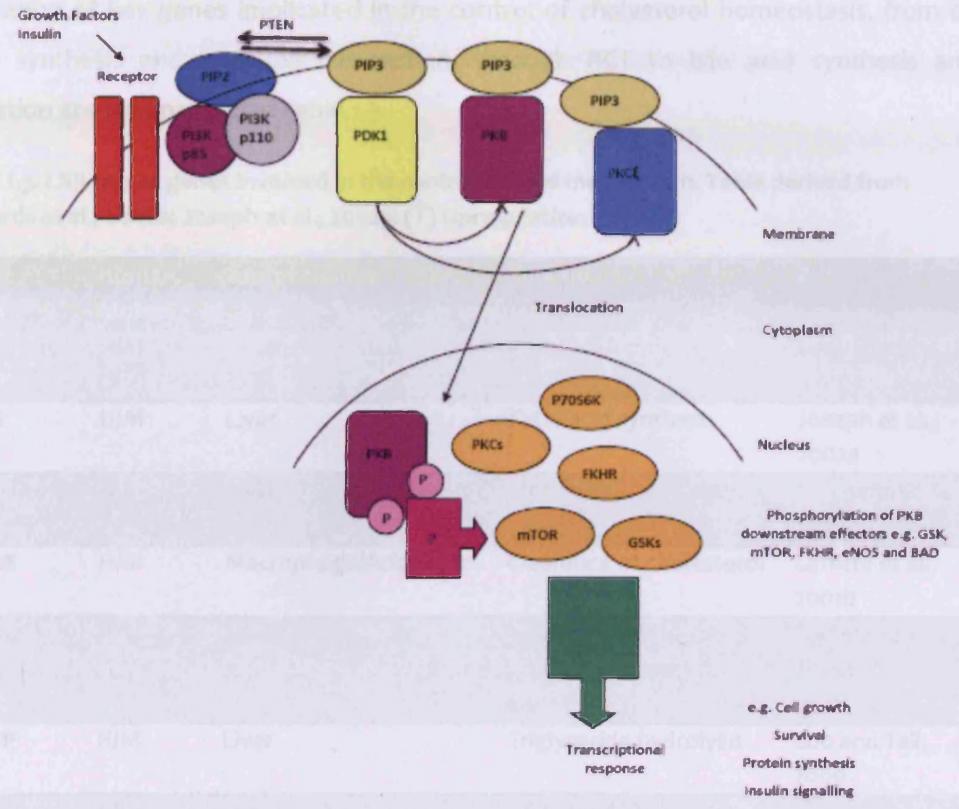


Figure 1.10 Scematic repesentation of the PI3K pathway.

The conversion of PIP2 to PIP3 is catalysed by PI3K, providing a docking site for downstream kinases such as PDK-1 and PKB. PTEN antagonises PI3K signalling by converting PIP3 to PIP2. PKB is activated by phosphorylation by PDK-1 and regulates a number of other signalling molecules including PKCs, GSK-3 and mTOR. This leads, either directly or indirectly, to the regulation of transcription factors and gene expression. Abbreviations: FKHR, forkhead in rhabdomysarcoma transcription factor; GSK3, glycogen synthase kinase 3; mTOR, mammalian target of rapamycin; PDK1, 3'-phosphoinositide-dependent kinase-1; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol bisphosphate; PIP3, phosphatidylinositol triphosphate; PKB, protein kinase B; PKC, protein kinase C; PTEN, phosphatase and tensin homologue.

by negative feedback prevent further synthesis via a pathway involving the inhibition of expression of the rate limiting enzyme, HMG-CoA reductase, via regulated proteolysis of the SREBP family of transcription factors (Goldstein and Brown, 1977). Excess cholesterol also activates a feed-forward pathway that results in the conversion of cholesterol into bile acids. The primary function of LXRs in macrophages is to maintain cellular cholesterol homeostasis. Alterations in cholesterol and fatty acid metabolism each have the potential to influence the development of CVD. The role of LXRs in the

regulation of key genes implicated in the control of cholesterol homeostasis, from *de novo* synthesis and intestinal absorption, through RCT to bile acid synthesis and excretion are summarized in Table 1.3.

Table 1.3: LXR target genes involved in the control of lipid metabolism. Table derived from Edwards et al., 2002b; Joseph et al., 2002a. (↑) Upregulation

Target genes	Human/ Mouse	Target tissues	Function	References
↑ SREBP-1c	H/M	Liver/Intestine	Fatty acid synthesis	Repa et al., 2000a
↑ FAS	H/M	Liver	Fatty acid synthesis	Joseph et al., 2002a
↑ CYP7A1	M	Liver	Clearance of cholesterol	Lehmann et al., 1997
↑ apoE	H/M	Macrophage/Adipocyte	Clearance of cholesterol	Laffitte et al., 2001b
↑ LXRα	H	Macrophage	Transcriptional control of lipid homeostasis genes	Laffitte et al., 2001a; Whitney et al., 2001
↑ CETP	H/M	Liver	Triglyceride hydrolysis	Luo and Tall, 2000
↑ LPL	H/M	Liver/Macrophage	Triglyceride hydrolysis	Zhang et al., 2001
↑ ABCA1	H/M	Macrophage/Intestine	Cholesterol efflux	Costet et al., 2000
↑ ABCG1	H/M	Macrophage	Cholesterol efflux	Kennedy et al., 2001
↑ABCG4	H	Macrophage	Cholesterol efflux	Engel et al., 2001; Wang et al., 2004b
↑ ABCG5/8	H	Liver/Intestine	Cholesterol efflux/ sterol transport	Berge et al., 2000

Abbreviation: ABCA1, ATP-binding cassette transporter, subfamily A, member 1; ABCG1/4/5/8, ATP-binding cassette transporter, subfamily G, members 1, 4, 5 or 8; apoE, apolipoprotein E; CETP, cholesteryl ester transfer protein; CYP7A1, cholesterol 7 alpha-hydroxylase; FAS, fatty acid synthetase; LXR, Liver X receptor; SREBP-1c, sterol regulatory element binding protein-1c.

Three important classes of LXR target genes influencing cholesterol homeostasis have been identified (Figure. 1.11). First, LXRs induce the expression of ABC transporters that have been linked to cholesterol efflux in macrophages and other cell types (Chawla et al., 2001b). Second, LXRs induce the expression of apoE in macrophages, which can potentially serve as an acceptor of cholesterol transported by ABCA1-dependent processes (Laffitte et al., 2001b). Third, LXRα seems to induce the synthesis of fatty acids

that are preferential substrates of ACAT in cholesterol esterification reactions (Repa et al., 2000a). Thus, ABCA1 and ACAT act in concert to reduce free cholesterol levels and protect cells from its cytotoxic effects.

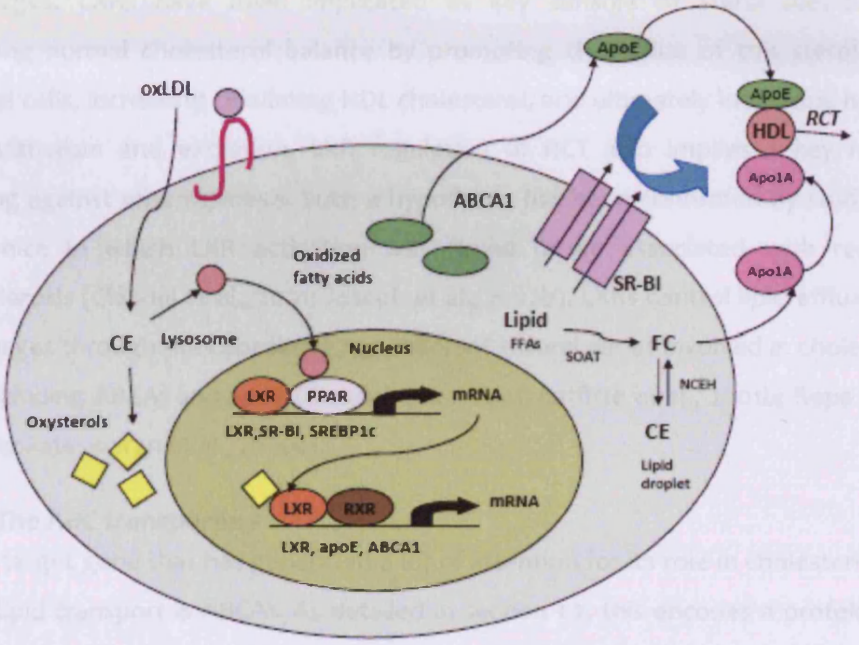


Figure 1.11 Schematic illustration of the mechanisms by which LXRs enhance cholesterol efflux from macrophages.

Oxysterols generated from free cholesterol (FC) activate LXR-RXR heterodimers, resulting in increased transcription of target genes. These genes include those encoding ABCA1, ABCG1 and apoE, which are linked to the efflux of cholesterol to extracellular acceptors, and fatty acid synthetase (FAS), which leads to the synthesis of free fatty acids (FFAs) used for cholesterol esterification by SOAT. PPARs may promote cholesterol efflux by inducing LXRα expression. CE, cholesterol ester; LXR-RE, liver X receptor response element; SOAT, acyl coenzyme A:acylcholesterol transferase; NCEH, neutral cholesterol ester hydrolase. Figure revised from Li and Glass, 2002.

1.3.5.1 Role of LXRs in reverse cholesterol transport

Virtually all animal cells synthesize cholesterol and import cholesterol from plasma lipoproteins. To achieve neutral cholesterol balance and prevent cholesterol overload, cells must export excess cholesterol. The only quantitatively significant sink for excess cholesterol is the liver, owing to its unique ability to synthesize bile acids and to

transport cholesterol into bile (Glomset, 1968). In addition, the RCT pathway enables excess cholesterol to be actively exported from lipid-laden cells, such as macrophages, and transported back to the liver for excretion (Brown and Goldstein, 1997). As described previously in this chapter, this process is particularly important in macrophages. LXRs have been implicated as key sensors of sterol metabolism, maintaining normal cholesterol balance by promoting the efflux of this sterol from peripheral cells, increasing circulating HDL cholesterol, and ultimately increasing hepatic sterol catabolism and excretion. LXR regulation of RCT also implies a key role in protecting against atherogenesis. Such a hypothesis has been confirmed by studies on apoE-/- mice in which LXR activation was found to be associated with reduced atherosclerosis (Claudel et al., 2001; Joseph et al., 2002b). LXRs control lipid efflux from macrophages through the coordinate regulation of several genes involved in cholesterol efflux, including ABCA1 and apoE (Claudel et al., 2001; Laffitte et al., 2001b; Repa et al., 2000b; Venkateswaran et al., 2000a).

1.3.5.1.1 The ABC transporters

One LXR target gene that has generated a lot of attention for its role in cholesterol and phospholipid transport is ABCA1. As detailed in section 1.2, this encodes a protein that pumps cholesterol and phospholipids from macrophages and other cells to apoAI, apoE or HDL vesicles, the first step in RCT, for transport to the liver. A number of studies, summarised in section 1.2 above have demonstrated a potent role for LXRs in the regulation of ABCA1 expression and cholesterol efflux (Claudel et al., 2001; Joseph and Tontonoz, 2003; Laffitte et al., 2001b; Repa et al., 2000b; Venkateswaran et al., 2000a).

Besides ABCA1, four other ABC transporters have also been identified as LXR targets, all of which are expressed in macrophages and contribute to the control of cholesterol homeostasis: ABCG1, ABCG4, ABCG5 and ABCG8. The expression of ABCG1 and ABCG4 is also induced in macrophages by lipid loading and treatment with oxysterols in a LXR-dependent manner (Kennedy et al., 2001; Venkateswaran et al., 2000b; Wang et al., 2004b). The function of ABCG1 and ABCG4 is to facilitate cholesterol efflux, perhaps by working in concert with ABCA1 (Kennedy et al., 2005; Klucken et al., 2000; Wang et al., 2004b; Wang et al., 2007). Macrophages lacking ABCG1 show a diminished cholesterol efflux capacity to HDL, suggesting that like ABCA1, ABCG1 will be anti-atherogenic (Kennedy et al., 2005). The role of ABCG5/G8 is to mediate the efflux of plant sitosterols.

Another rare genetic disorder of lipid metabolism, sitosterolemia (Salen et al., 1997), in which hyperlipidemia results from impaired efflux of sitosterol and related compounds to the intestinal lumen and to the bile (Schmitz et al., 2001) involves mutations in ABCG5/G8 (Hubacek et al., 2001; Lee et al., 2001). Genetic deficiency of these transporters leads to abnormal absorption of sitosterols and a hyperabsorption of cholesterol. In addition, treatment of mice with LXR agonists results in the stimulation of hepatobiliary excretion of cholesterol, which is linked to the activation of ABCG5/G8 expression in the liver and intestinal cells (Berge et al., 2000; Yu et al., 2003). These finding further strengthen the role of these transporters in dietary and biliary sterol trafficking, in particular in response to LXR agonists (Crestani et al., 2004b).

1.3.5.1.2 Apolipoprotein E

Another LXR target gene that is important in cholesterol homeostasis is apoE. This was first described as a lipoprotein constituent of triglyceride-rich VLDL. However, apoE is now known as a major protein constituent of several plasma lipoproteins that carry dietary and liver-derived cholesterol such as chylomicron remnants, IDL and HDL. Recognition of apoE by LDL receptors mediates hepatic uptake of these particles (Curtiss and Boisvert, 2000). Indeed, apoE has been implicated in the maintenance of overall plasma cholesterol homeostasis and stimulation of cholesterol efflux from macrophages (Greenow et al., 2005). ApoE is a 34kDa protein that is synthesized primarily in the liver with significant amounts being produced by several peripheral tissues and cell types, including macrophages, adipose tissue and the brain (Newman et al., 1985). In addition, the secretion of apoE can be regulated by a number of factors such as cholesterol loading of the cells (Mazzone, 1996).

A potent anti-atherogenic role for apoE has been demonstrated by several lines of evidence. In humans, it has been found that inhibition of apoE expression is associated with a pro-atherogenic lipoprotein profile and diffuse atherosclerotic disease (Greenow et al., 2005; Schaefer et al., 1986). In addition, apoE-/- mice are severely hypercholesterolaemic compared to wildtype mice and the increase in cholesterol is distributed in lower LDL fraction due to their impaired clearance (Zhang et al., 1992a). Although apoE expression is absent in normal vessels, it is present at high levels in atherosclerotic lesions, where it is associated with macrophage-derived foam cells (Rosenfeld et al., 1993). The anti-atherogenic effect of apoE is due to several properties

of the protein: its antioxidant properties; its modulation of the function of platelets, SMCs and lymphocytes; and its promotion of cholesterol efflux and RCT (Larkin et al., 2000). The beneficial nature of apoE production by macrophages is clear from several animal studies. Bone marrow transplantation have shown that macrophage-derived apoE exerts anti-atherogenic properties independently of its effects on plasma lipid levels (Fazio et al., 1997). Mice expressing apoE only in macrophages are protected against atherosclerosis whereas those specifically lacking its expression in these cells are more susceptible to the development of this disease (Fazio et al., 1997). In addition, transfer of human apoE to apoE-deficient mice induces complete regression of atherosclerotic lesions in the absence of any changes in plasma cholesterol and lipoprotein profile (Shimano et al., 1995). By increasing cholesterol efflux from macrophages, apoE attenuates the deleterious effects of excess cholesterol in these cells, restricts the transformation of macrophages into foam cells, and improves the elimination of cholesterol from the vessel wall.

Zhang et al. (1996) analysed the function of apoE in mediating cholesterol efflux from monocyte-derived macrophages in the absence of added cholesterol acceptors, such as HDL and apolipoproteins (Zhang et al., 1996). They found that apoE produced by monocyte-derived macrophages mediates most of the cholesterol efflux from these cells and that cholesterol regulates the association of apoE with phospholipids. They also found that apoE associates with lipids after it has been secreted from macrophages and the level of secreted apoE is the rate-limiting step in RCT. Net synthesis of phospholipids by macrophages occurs secondary to apoE-mediated loss of macrophage phospholipids rather than due to cholesterol enrichment.

ApoE secreted from human monocyte-derived macrophages is responsible for cholesterol efflux from these cells and for the decrease in their cholesterol content (Zhang et al., 1996). Antibody to apoE can decrease this efflux by about two-thirds, showing that apoE does indeed cause cholesterol efflux from these macrophages. Enriching the monocytes with cholesterol did not, however, increase the levels of secreted apoE. Instead, it induced the complexing of apoE with phospholipids and cholesterol. Cholesterol alters the physical and chemical properties of the cell membrane in a way that favours the interaction of amphipathic apolipoproteins with phospholipids. ApoE picks up lipids after it is secreted from macrophages. ApoE thus

acts is an autocrine pathway to mediate cholesterol efflux from human monocyte-derived macrophages.

ApoE gene expression is complex in relation to regulation by cellular changes and extracellular or intracellular factors (Greenow et al., 2005). Hepatic apoE expression is controlled by a distal enhancer known as the hepatic control region (Alla et al., 1997). The expression of apoE in adipocytes and macrophages is directed by a distinct sequence termed the multiple enhancer region (Shih et al., 2000). Interestingly, this enhancer along with the promoter also contains conserved LXR-REs that mediate activation of gene transcription in response to oxysterol ligands in macrophages but not in monocytes (Laffitte et al., 2001b). A recent study by Laffitte and colleagues demonstrated the involvement of LXRs in the regulation of apoE expression (Laffitte et al., 2001b). In macrophages, apoE gene transcription is increased by cholesterol loading. Oxysterols and the synthetic agonist T0901317 also enhance apoE expression and this is abolished in LXRα-/- and LXRβ-/- mice even in the presence of high concentrations of the ligand (Laffitte et al., 2001b). The apoE gene has been mapped to chromosome 19 and is present in a gene cluster that contains apoCI, apoCII and apoCIV. It has recently been demonstrated that this entire gene cluster responds to LXR activation in both human and murine macrophages (Mak et al., 2002b). The induction of this gene cluster by LXR ligands was reduced in LXRαβ-/- mice, but unchanged in LXRα-/- or LXRβ-/- mice, thereby suggesting that both LXR isoforms are functional regulators of lipid-inducible apolipoprotein expression (Mak et al., 2002b). In contrast to ABCA1, regulation of apoE by LXR and RXR is tissue/cell type-specific, occurring only in macrophages and adipose tissue but not in the liver (Laffitte et al., 2001b; Mak et al., 2002b). Similar to the ABCA1 gene, apoE expression is regulated by the PPARγ/LXRα regulatory circuit (Akiyama et al., 2002).

LXRs induce the expression of multiple apolipoproteins such as apoA1, apoB and apoE that could then serve as cholesterol acceptors in the context of an atherosclerotic lesion. Interestingly, like apoE, all of these α-helical secreted apolipoproteins have been shown to serve as extracellular acceptors for cholesterol in the ABCA1-mediated efflux pathway (Bortnick et al., 2000; Curtiss and Boisvert, 2000). The induction of these acceptors within the arterial wall in macrophages would be expected to promote RCT and cholesterol efflux. Previous studies have shown that ABCA1 is involved in the

secretion of apoE from human macrophages (Von Eckardstein et al., 2001a). In brain of ABCA1 deficient mice, apoE levels were reduced, thus indicating that ABCA1 not only mediates cholesterol efflux but also facilitates apoE synthesis and secretion from glial cells (Hirsch-Reinshagen et al., 2004). In addition, apoE was capable of stimulating efflux of cholesterol from cells via the ABCA1 dependent pathway (Krimbou et al., 2004).

1.3.5.1.3 Lipoprotein remodelling enzymes

LXRs have also been shown to positively regulate the expression of several lipoprotein remodelling enzymes that transfer lipids between lipoproteins, including the human CETP, the phospholipid transfer protein (PLTP) and lipoprotein lipase (LPL) (Laffitte et al., 2003; Luo and Tall, 2000). The remodelling enzyme CETP mediates the transfer of cholesterol esters from HDL to triglyceride-rich lipoproteins (apoB-containing particles), which are subsequently cleared by the liver, and in exchange HDL receives triglycerides. This modification of HDL by CETP makes HDL more susceptible to hydrolysis by hepatic lipase at the hepatocyte surface, which is an important component in the regeneration of small HDL particles and free apoAI that can re-circulate in the RCT pathway. Genetic CETP deficiency in humans cause increased atherosclerosis while overexpression of CETP in mice reduces coronary heart disease (Hayek et al., 1995). The promoter of the CETP gene contains a functional DR4 LXR-RE, which mediates the upregulation of expression of this gene by sterols in response to a high fat diet (Luo and Tall, 2000). The CETP gene can be *trans*-activated by both LXRα and LXRβ.

PLTP has been identified as a key modulator of HDL metabolism in the plasma by its ability to remodel HDL particles into large α-HDL and small pre-β-HDL particles, leading to a reduction in plasma HDL levels (Huuskonen et al., 2004a). The pre-β-HDL particles are efficient acceptors of cholesterol from peripheral cells and might also be involved in RCT (Van Tol, 2002). In addition, PLTP has recently been shown to regulate VLDL secretion from the liver (Jiang et al., 2001; Laffitte et al., 2003). PLTP-deficient mice exhibit decreased levels of VLDL and LDL in an apoE-deficient or apoB-transgenic background (Jiang et al., 1999). Therefore, some of the actions of LXR agonists on HDL and VLDL levels are consistent with the known roles of PLTP in lipoprotein metabolism. In particular, it seems likely that the ability of LXR agonists to raise plasma VLDL and triglyceride levels may involve PLTP. The expression of PLTP is induced by LXR agonists in hepatic cells and in macrophages (Cao et al., 2002; Laffitte et al., 2003) leading, *in vivo*,

to the formation of large HDL particles that are enriched in cholesterol, apoA-I, apoE and phospholipids (Cao et al., 2002). Also, PLTP expression and activity is increased in mice treated with LXR ligands and this is lost in LXRα/β-/- mice (Laffitte et al., 2003). A functional LXR-RE was mapped in the human PLTP promoter and found to be responsible for the transcriptional activation of this gene by LXR/RXR heterodimers in transient transfection assays (Laffitte et al., 2003; Mak et al., 2002a).

LPL is the rate-limiting enzyme in the hydrolysis of lipoprotein triglycerides (Goldberg, 1996). It is highly expressed in adipose and muscle tissues and is also produced by macrophages. LXR ligands also induce the expression of LPL only in liver and macrophages but not in adipose tissue (Zhang et al., 2001). LXRα is thought to be a more selective regulator of LPL than LXRβ due to a higher affinity of LXRα for a DR4 like LXR-RE that has been identified within the regulatory region of the murine LPL gene, which is also conserved in the human LPL gene (Zhang et al., 2001).

1.3.5.2 Role of LXRs in lipogenesis

In addition to the role of LXRs in RCT, they have been implicated in the control of fatty acid metabolism. The lipogenic activity of LXRs results from the upregulation of the master regulator of hepatic lipogenesis, SREBP-1c (Repa et al., 2000a; Schultz et al., 2000), and thereby its downstream targets such as fatty acid synthase (FAS) (Joseph et al., 2002a), steroyl CoA desaturase I (SCD-1) and acyl CoA carboxylase (ACC) seen in response to LXR ligands such as T0901317 (Tontonoz and Mangelsdorf, 2003). Mice carrying a targeted disruption of the LXRα gene were found to be deficient in the hepatic expression of SREBP-1c, FAS, SCD-1 and ACC along with defects in cholesterol metabolism (Peet et al., 1998). The demonstration that the SREBP-1c promoter is a direct target for the LXRs provides a straight forward explanation for the ability of LXR ligands to induce hepatic lipogenesis (Repa et al., 2000a). Thus, pharmacological activation of LXRs increases lipogenesis to potentially harmful levels, causing hypertriglyceridemia (Yoshikawa et al., 2002), which is clearly a major current limitation of using LXR agonists in the treatment of atherosclerosis. However, a newly characterized synthetic oxysterol, N,N-dimethyl-3β-hydroxycholenamide (DMHCA), has recently been reported to have a gene-selective LXR modulatory activity (Quinet et al., 2004). Both *In vitro* and *in vivo* studies have shown that DMHCA mediates potent transcriptional activation of most LXR target genes while exhibiting minimal effects on SREBP-1c expression. DMHCA therefore

does not alter circulating plasma triglycerides compared to nonsteroidal LXR agonists but still has the potential to stimulate cholesterol transport through the upregulation of LXR target genes, including ABCA1, in peritoneal macrophages, liver and small intestine.

It is worth noting that the regulation of lipid homeostasis by LXRs may also contribute to their action in cholesterol homeostasis. Recent data has revealed that LXR-/- mice are defective in hepatic lipid metabolism and are resistant to obesity when challenged with a diet containing both high fat and cholesterol (Kalaany et al., 2005). This study indicates an interaction between dietary cholesterol and lipid homeostasis in that LXRs selectively sense the cholesterol component of a lipid-rich diet to control the balance between storage and oxidation of dietary fat (Kalaany et al., 2005). Moreover, regulation of lipogenesis may function to coordinate the levels of fatty acids and phospholipids, both of which are essential for the mobilisation of free cholesterol (Zhang and Mangelsdorf, 2002). For example, oleoyl-CoA, a product of SCD-1 catalysis, is a substrate for cholesterol esterification. Therefore, by increasing SCD-1 activity via SREBP-1c, the LXRs would ultimately promote the esterification and storage of free cholesterol within the cell and protect the organism from toxic accumulation of this sterol. In addition, phospholipids facilitate bile acid flow from liver into bile for eventual excretion and lipoprotein transport of excess cholesterol, and thereby help to maintain the ratio of cholesterol to other lipids in plasma membranes (Repa et al., 2000a). LXRα also induces synthesis of fatty acids which are preferred substrates for SOAT in cholesterol esterification reactions (Li and Glass, 2002). ABCA1 and SOAT act together to reduce free cholesterol levels and protect cells from the cytotoxic effects of excess cholesterol. Therefore, it is possible to recognize why such a transcription factor that upregulates cholesterol efflux and downregulates cholesterol synthesis also upregulates fatty acid synthesis. Finally, it should also be taken into consideration that the effects of LXRs on lipogenesis may be tissue specific since it has been observed that in skeletal muscles, LXR ligands increase cholesterol efflux and do not seem to activate lipid deposition (Muscat et al., 2002).

1.3.6 Autoregulation of the human LXRα gene

Recently, three independent studies have identified that the expression of the human LXRα gene is maintained by an auto-regulatory mechanism in human cells (Laffitte et al., 2001a; Li et al., 2002; Whitney et al., 2001). In multiple human cell types, including

primary macrophages, the expression of LXRα could be induced by natural and synthetic LXR agonists. This autoregulation occurs less than 4h upon addition of the ligands to the cells. Both LXRα and LXRβ can activate the transcription of the promoter when linked to a reporter gene. The autoregulation occurs via LXR-REs present in the human LXRα promoter (Hu and Lala, 2002; Li et al., 2002; Whitney et al., 2001). The autoinduction of LXRα expression provides a unique sensory mechanism utilized by cells to respond to increasing cholesterol levels. The generation of LXR ligands leads not only to the activation of the receptor but also to increased receptor levels within the cell. This potentially leads to an amplification of the response to oxysterols and the LXR/ABCA1 cholesterol efflux pathway. Interestingly, LXRα autoregulation appears to be limited to human cell types such as macrophages and hepatoma cells and does not occur in murine cell lines or primary murine macrophages (Laffitte et al., 2001a). This dissimilarity in response suggests that mice may be less responsive than humans to treatment with LXR agonists.

1.3.6.1 LXR regulation by PPARs

In addition to autoregulation, PPARs have also been shown to cause a marked increase in LXRα expression. The observation that the expression of the LXRα gene is responsive to PPARγ provides evidence for substantial crosstalk between the PPAR and LXR pathways. The PPARs are another NR subfamily expressed in human macrophages and can be activated by certain lipid components of oxLDL. PPARα and PPARγ have been well characterised and are known to be involved in lipid metabolism and found to be present in macrophages in atherosclerotic lesions (Akiyama et al., 2002; Chawla et al., 2001a). Agonists of PPARα and γ induce the expression of LXRα in human and murine macrophages (Chawla et al., 2001a; Chinetti et al., 2001). Consistent with this, PPARγ deficiency results in a marked reduction of LXRα expression and macrophage cholesterol efflux (Akiyama et al., 2002; Chawla et al., 2001a). It has been reported that in rat hepatic cells (Tobin et al., 2000) and in human primary macrophages (Chinetti et al., 2001), fatty acids, in particular unsaturated fatty acids, induce LXRα but not LXRβ in a PPARα-dependent manner (Tobin et al., 2000). In a parallel study, it has also been demonstrated that agonist-mediated activation of PPARγ in human macrophages leads to increased expression of both human and mouse LXRα via a PPAR binding site in the LXRα promoter (Chawla et al., 2001a).

Beside the activation of the LXRα gene by PPAR agonists, it has been reported that a regulatory cascade exists in which PPARγ and PPARα stimulates ABCA1 function by activating LXRα transcription (Chawla et al., 2001a). The same study has shown that mice which lack PPARγ activator in their macrophages have atherosclerosis. In addition, it has been shown that the apoE gene is also regulated by PPARγ and LXR agonists, and that macrophage specific inhibition of PPARγ expression reduces levels of apoE and drastically decreases basal cholesterol efflux (Akiyama et al., 2002). As a result of this cross-regulation, LXR and PPARγ ligands (i.e. thiozolidinediones (TZD)) have additive effects on the expression of LXRα target genes such as ABCA1 and apoE. Thus, mature macrophages express more ABCA1 and export more cholesterol when exposed to the factors that activate PPARγ or LXRα (Chawla et al., 2001a; Chinetti et al., 2001; Laffitte et al., 2001a).

1.3.7 LXRs and atherosclerosis

As mentioned earlier, the activation of LXRs and their target genes stimulates cholesterol efflux in macrophages, inhibits intestinal cholesterol absorption and promotes bile acid synthesis in the liver. The importance of these receptors in physiological lipid metabolism suggests that they may also influence the development of metabolic disorders such as hyperlipidemia and atherosclerosis. Recently, a number of studies *in vitro* and *in vivo* have indicated that activation of the LXR pathway is anti-atherogenic (Joseph and Tontonoz, 2003; Repa and Mangelsdorf, 2002; Zelcer and Tontonoz, 2006). Over-expression of LXRα or their ligand-dependent activation stimulates cholesterol efflux in macrophages loaded with acetylated LDL *in vitro* (Joseph and Tontonoz, 2003). The first direct link between LXR activity and the pathogenesis of atherosclerosis came with the demonstration that activation of the LXRs inhibited the development of atherosclerosis in mouse models of this disease (Joseph et al., 2002b; Joseph and Tontonoz, 2003; Schuster et al., 2002; Tangirala et al., 2002). Thus, treatment of apoE-/- or LDLR-/- mice with the two synthetic LXR agonists, GW3965 and T0901317, was found to induce the expression of ABCA1 and ABCG1, and led to a marked reduction (approximately 50%) in the size of atherosclerotic lesion development in the aortas of hyperlipidemic mice (Joseph et al., 2002b; Terasaka et al., 2003). In contrast, loss of LXRs from the haematopoietic compartment (e.g. macrophages) by bone marrow transplantation resulted in a significant increase in atherosclerotic lesion formation in both LDLR-/- (more than 3 fold increase) and apoE-/- (3-8 fold increase) mice (Tangirala et

al., 2002). In addition, LXRαβ-/- mice show severe defects in hepatic cholesterol metabolism and also develop splenomegaly and accumulate foam cells in multiple peripheral tissues (Schuster et al., 2002). A recent study further established that treatment of LDLR-/- mice with T0901317 reduces the size of pre-existing lesions and that this reduction is dependent on LXR activity in macrophages (Levin et al., 2005).

Two other NRs, PPARγ and RXR, have also been implicated in the pathogenesis of atherosclerosis, and treatment of atherogenic mouse models with RXR or PPARγ agonists results in significant decrease in lesion development (Claudel et al., 2001; Li et al., 2000a). For example, a reduction in atherosclerosis was seen with RXR agonist LG268 (Claudel et al., 2001). Also in these mouse models, PPARγ ligands have been reported to reduce atherosclerosis, which is most likely a consequence of indirect LXR-mediated effects (Claudel et al., 2001; Li et al., 2000a). In addition, transplantation of PPARγ-/- bone marrow into LDLR knockout mice also increases atherosclerosis (Chawla et al., 2001a). Collectively, these studies strongly support the hypothesis that macrophage LXR pathway is an important homeostatic mechanism that helps to protect against cholesterol overload, and they point to the potential usefulness of LXR, RXR and PPAR agonists as attractive targets for the intervention of CVD.

1.3.8 LXRs and inflammation

Atherosclerosis has long been known as a disorder of lipid metabolism as well as a chronic inflammatory disease. Recently, several studies have explored the role of LXRs in macrophage inflammatory pathways and reported a relationship between inflammatory gene expression and lipid metabolism in activated macrophages treated with LXR agonists (Castrillo and Tontonoz, 2004). In addition to inducing genes involved in RCT, LXR agonists were found to inhibit the innate immune response and the expression of a cluster of genes involved in inflammation (Castrillo et al., 2003a; Joseph et al., 2003). The importance of inflammation in atherosclerosis is well established. Certain inflammatory mediators such as monocyte chemotactic protein-1 (MCP-1), IL-1β and IL-6 promote monocyte recruitment in the atherosclerotic plaques, stimulate SMC proliferation and increase extracellular matrix production (Hansson, 1999). Metalloproteinases, such as MMP-9, are highly expressed by macrophages and SMCs and have been implicated in both lesion remodelling and plaque rupture (Pasterkamp et al., 2000). The induction of MCP-1, IL-6, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), IL-1β and

MMP-9 in response to bacterial pathogens and pro-inflammatory cytokines was inhibited by LXR activation (Joseph et al., 2003). The repression of expression of these genes was observed in LXRα-/- or LXRβ-/- macrophages but was completely abolished in LXRα/β null cells, indicating that either LXRα or LXRβ can mediate the anti-inflammatory activity of LXR agonists (Castrillo et al., 2003a). In addition, treatment of apoE-/- mice with LXR ligands reduced the expression of the inflammatory mediator MMP-9 in atherosclerotic aortas (Joseph et al., 2003). Both studies demonstrated that this regulation was LXR-dependent and the mechanism whereby LXRs exert their inhibitory effects on inflammatory genes appears to involve antagonism of the NF-κB pathway (Castrillo et al., 2003a). Collectively, these data suggest that LXR ligands may exert their anti-atherogenic effects not only by promoting cholesterol efflux but also by acting to limit the production of inflammatory mediators in the artery wall.

Reciprocally, inflammatory pathways might also alter the LXR-mediated cholesterol metabolism, contribute to foam cell formation and accelerate lesion development (Zelcer and Tontonoz, 2006). Recently, it has been reported that bacterial and viral pathogens antagonise LXR transcriptional activity and cholesterol efflux. For example, activation of toll like receptors (TLRs) such as TLR3 or TLR4, which recognize conserved motifs on microbes and induce inflammatory signals during bacterial or viral infection of macrophages severely compromise the expression of ABCA1/G1, apoE and other LXR target genes both *in vitro* and *in vivo* (Castrillo et al., 2003b). Consistent with these effects on LXR-dependent gene expression, activation of TLR3 or TLR4 potently inhibits cholesterol efflux from macrophages. This effect is accomplished through activation of the viral response transcription factor IFN regulatory factor 3 (IRF3). However, the mechanism by which this factor blocks LXR actions is unclear. This LXR/TLR cross-talk explains how bacterial and viral infections interfere with cholesterol metabolism and modulate CVD (Zelcer and Tontonoz, 2006).

Recently, another function of LXR signalling in the innate immune response has been reported in which mice lacking LXRs were found to be susceptible to infection with intracellular pathogens (Joseph et al., 2004). The authors were able to show that altered macrophage function was a major contributor to pathological susceptibility to microbial infection using bone marrow transplantation from LXRα/β-/- mice into wild type mice. The inability of LXR-/- to mount response to microbial infection correlated with

accelerated rates of macrophage apoptosis. The increased susceptibility of LXR-/- macrophages to pathogen-induced apoptosis results from the loss of regulation of the anti-apoptotic gene scavenger receptor cystine-rich repeat protein (SPα) by LXRα. SPα, which is also known as apoptosis inhibitor 6 (API6) or as apoptosis inhibitor of macrophages (AIM), is a member of the scavenger receptor cystine-rich repeat (SRCR) family (Gebe et al., 1997). This activity was attributed to the induction of SPα and other anti-apoptotic factors as well as to the inhibition of pro-apoptotic genes. SPα is the first LXR isoform-selective target gene and its selective regulation by LXRα and the induction of LXRα mRNA during infection, suggest that this isoform may have functions in innate immunity (Zelcer and Tontonoz, 2006). Similarly, Valledor et al. (2004) showed that activation of LXR/RXR heterodimers by natural and synthetic agonists inhibits macrophage apoptosis in response to apoptotic stimuli (e.g. cycloheximide) (Valledor et al., 2004).

The ability of the LXR pathway to enhance macrophage survival through induction of the anti-apoptotic Spα gene highlights a common pathway used for both metabolic and immune control (Zelcer and Tontonoz, 2006). In addition to being induced in the setting of bacterial infections, Spα is also upregulated during macrophage lipid loading. Recently, the importance of this macrophage survival pathway in atherogenesis was explained (Arai, 2005). Macrophages from Spα-/- mice were highly susceptible to oxLDL loading-induced apoptosis *in vitro* and *in vivo* within atherosclerotic lesions. As a result, early atherosclerotic lesions in Spα-/- LDLR-/- mice are reduced compared with those in Spα+/+ LDLR-/- mice (Arai, 2005; Zelcer and Tontonoz, 2006).

1.4 Aims of the project

LXRs, members of the NR family, have emerged as key factors in the control of cholesterol homeostasis. Cholesterol efflux is a critical process in atherogenesis and is affected by LXR mediators. The evidence presented above clearly indicates that the activation of LXR leads to changes in lipid metabolism, including promotion of RCT from macrophages to the liver, where it can be converted to bile acids or eliminated in the bile excretion system. The relevance of this effect for the development of atherosclerosis is clear from studies showing that synthetic agonists inhibit the development of this disease in animal models (Joseph et al., 2002b). These findings define LXRs as potential therapeutic targets for the treatment of lipid disorders. LXRs regulate the expression of many genes involved in RCT and lipid loading of macrophages. The role of LXR target genes, such as ABCA1 and apoE, in RCT and atherosclerosis is well established as described previously in this chapter. Future therapeutic approaches should therefore seek to increase ABCA1 and apoE expression in macrophages to enhance cholesterol removal from these cells and to prevent foam cell formation in atherosclerotic lesion development. Previous work in our laboratory has shown that the LXR ligand, 22(R)-HC, has a stimulatory effect on apoE gene expression in macrophages (Greenow, K., 2004). It is important to understand comprehensively the mechanisms by which LXR agonists exert these inducible effects on the expression of genes involved in cholesterol efflux such as ABCA1 and apoE as this will:

1. Contribute to the understanding of the mechanisms by which LXRs upregulate the expression of such genes in macrophages.
2. Identify potentially novel targets for therapeutic intervention of atherosclerosis.

The signal transduction pathways underlying such an action of LXRs remain poorly understood. However, previous work in our laboratory has shown that JNK/SAPK and PI3K are required for the upregulation of apoE gene expression by 22(R)-HC (Greenow, K., 2004). These results suggest regulation of the function of LXRs by phosphorylation of either the receptors themselves or specific cofactors that interact with them (Bastien and Rochette-Egly, 2004; Huuskonen et al., 2004a; Rochette-Egly, 2003; Shao and Lazar,

1999). Paying attention to these two pathways, the initial aim of the present study was to elucidate the regulatory mechanisms by which LXR agonists, induce ABCA1 expression in macrophages.

The main aims of the project were to:

1. Determine whether the JNK/SAPK and PI3K signal transduction pathways are also involved in the LXR-mediated regulation of ABCA1 expression in macrophages.
2. Investigate the role of key components in these pathways and the potential mechanisms underlying their actions.

To achieve these overall goals, studies presented in chapter 3 investigated the effect of LXR agonists on ABCA1 mRNA expression in murine J774.2 macrophages, a well established mouse model for differentiated macrophages (Ralph and Nakoinz, 1975; Ralph et al., 1975). In addition, the potential role of the JNK/SAPK and PI3K pathways, which had previously been identified in our laboratory to be required for the 22(R)-HC-induced apoE gene expression in THP-1 cells, was studied by the use of RT-PCR, pharmacological agents and transient transfection assays with LXR-responsive promoter and DNA constructs specifying for dominant negative (DN) forms of key components of the JNK/SAPK and PI3K pathways. On the basis of the outcome from these studies and, as atherosclerosis is in essence a human disease, the initial aims of the project were modified slightly to understand the regulation of ABCA1 transcription by LXR/RXR in human macrophages with apoE included in representative, comparative experiments.

Studies presented in chapter 4 explored the effect of natural LXR agonists on ABCA1 and apoE protein levels in THP-1 macrophages, a well established human model for differentiated macrophages (Tsuchiya et al., 1982; Tsuchiya et al., 1980), using western blot analysis. In addition, the action of synthetic LXR agonists, which are known to stimulate RCT and inhibit the development of atherosclerosis, on the expression of the ABCA1 and apoE genes was studied. Furthermore, the potential role of the JNK/SAPK and PI3K pathways in the responses was investigated using pharmacological agents.

Studies presented in chapters 5 and 6 focussed on the JNK/SAPK and the P3K pathways respectively in the regulation of ABCA1 expression by several complementary approaches, such as monitoring the activation of key components of the pathways, transient transfection assays with ABCA1 promoter and DNA constructs specifying for DN forms of various proteins, and electrophoretic mobility shift assays (EMSA) for evaluating DNA-protein interactions. Studies presented in chapter 7 investigated any potential cross talk between these two pathways.

CHAPTER TWO:

MATERIALS AND METHODS

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Suppliers

The suppliers from which the materials/chemicals used in this research project were purchased from are listed below in Table 2.1. Any reagents that are not listed were obtained from Sigma Chemical Corporation Ltd.

Table 2.1: The chemical reagents/materials and their suppliers

Supplier	Materials
Abcam, Cambridgeshire, UK	ABCA1 antibody
Autogen Bioclear, Wiltshire, UK	DMEM Tissue culture medium, RPMI 1640 Tissue culture medium, INTERFERin™ siRNA transfection reagent
GE Healthcare, Buckinghamshire, UK	Rainbow full range protein size markers, Random hexamers (pdN6), ECL-Western blotting detection reagent, X-ray film, Nick columns, Megaprime DNA labelling kit, α^{32}P-dCTP
Anachem, Luton, UK	Acrylamide: bisacrylamide 29:1 Acrylamide: bisacrylamide 37.5:1
Boehringer Mannheim Ltd, East Sussex, UK	Positively charged Nylon membrane, Ribonucleoside triphosphate set
Bioline, London, UK	Taq DNA polymerase (10xNH_4 reaction buffer), Agarose, Magnesium chloride
Biogenesis Ltd, Poole, UK	Goat polyclonal apoE antibody
Corning Costar, Netherlands	Falcon 15ml and 50ml polypropylene tubes
Calbiochem, Nottingham, UK	LY294002, Curcumin, SP600125, T0901317, Rapamycin, Bisindolylmaleimide, G06983, G06976, Rotllerin
DIFCO Biosciences, Surrey, UK	LB agar capsules, LB medium capsules
European Collection of Animal Cell Culture (ECACC),Salisbury, UK	Cell lines (J774.2, THP-1 and Hep3B)
Fisher Scientific, Loughborough, UK	β-mercaptoethanol, EDTA, Ethanol, Glycerol, Hydrochloric acid, Industrial methylated spirit, Isopropanol, Sodium dodecyl sulphate, Methanol, Butan-1-ol, Sodium chloride, Sodium hydroxide, Tris
Genetic Research Instrumentation, Essex, UK	Saran Wrap
Greiner, Gloucestershire, UK	Tissue culture flasks, 96-well plates, 6- and 12-well plates, Cell scrapers
Gibco BRL Life Technologies Ltd, Paisley, UK	Fetal calf serum, Trypsin-EDTA, Ammonium persulphate
Helena Biosciences, Sunderland, UK	Cell scrapers
Millipore Ltd., Gloucestershire, UK	PVDF membrane
Marligen Biosciences, Sunderland, UK	High purity plasmid Maxi Prep system
National Diagnostics, Atlanta, USA	Agarose, 10 x TBE

New England Biolabs, Hertfordshire, UK	Restriction endonucleases, DNA molecular weight markers, Anti-PKB, Anti-PKB (pSer473), PKB kinase assay activity kit, Anti SEK1/MKK4, Anti pSEK1/MKK4 (pSer257/Thr261), Anti-SAPK/JNK, Anti SAPK/JNK (pThr183/Tyr185) and SAPK/JNK assay kit
Nycomed Pharma, Bridport, UK	Lymphoprep™
Oxoid Ltd, Hampshire, UK	Phosphate-buffered saline tablets
Pierce, Chester, UK	Micro BCA protein assay kit
Promega Ltd, Southampton, UK	Rnasin™ ribonuclease inhibitor, Passive lysis buffer (5x), Firefly luciferase assay reagent, MMLV reverse transcriptase and 10xbuffer of MMLV, dNTPs, 5X Reverse transcriptase buffer and Wizard plus SVTM Miniprep kit
PeproTech EC Ltd, London, UK	TGF-β
Qiagen Ltd, Crawley, UK	RNeasy™ total RNA isolation kit, Superfect™ transfection reagent, Effectene™ transfection reagent, JNK1, JNK2 and GAPDH siRNA
Santa-Cruz Biotechnology Inc., California, USA	Anti-total-c-Jun (H-79), Anti-phospho-c-Jun (KM-1; pSer63)
Sigma Aldrich, Steinheim, Germany	Poly-ethyleneimine (PEI), DIPE, SB216763, SB415286
Sigma Genosys, Cambridgeshire, UK	DNA oligonucleotides
Sigma, Poole, UK	Anti-β-actin, Ampicillin, Aproptonin, Benzamidine, Bovine serum albumin, Bromophenol blue, Ethidium bromide, Ficoll (Type 400), Glycerol, Leupeptin, Mineral oil, Molecular biology grade water, PEI, Penicillin/Streptomycin, PMA, PMSF, 10x TBE, TEMED, Tissue culture grade DMSO, Trypan blue solution, Tween 20, 22(R)-and 22(S)- hydroxycholesterol, 9-cis -retinoic acid
Schleicher and Schuell, London, UK	Tissue culture filters (0.2μm)

2.1.2 Culture media and stock solutions

2.1.2.1 Media

The LB medium and LB agar were all supplied in a capsule form and made up according to the manufacturer's instruction (DIFCO Biosciences).

2.1.2.2 Stock solutions

The solutions were prepared to the specifications described in Tables 2.2, 2.3 and 2.4, are categorised according to the procedure in which they were used. The solutions were prepared using sterile double distilled water and were of molecular biology grade. All

stock solutions used for tissue culture were autoclaved for 20min at a pressure of 975kPa.

Table 2.2: Composition of stock solutions for the electrophoresis of RNA/DNA

Solution	Composition
5x DNA loading dye	0.5x TBE 40% (v/v) Glycerol 0.25% (w/v) Bromophenol blue

Table 2.3: Solutions for the isolation of proteins and Western blot analysis

Solution	Composition
Whole cell extraction buffer	10mM HEPES (pH 7.9) 400mM NaCl 0.5mM DTT 5% (v/v) Glycerol 0.5mM PMSF 10µg/ml Aprotinin 0.5M Benzamidine 0.1mM EDTA
Phosphatase-free whole cell extraction buffer	10mM Tris-HCl (pH 7.05) 50mM NaCl 50mM NaF 1% (v/v) Triton x-100 30mM Sodium pyrophosphate 5µM $ZnCl_2$ 100µM Sodium orthovanadate 1mM DTT 2.8µg/ml Aprotinin 2.5µg/ml each of Leupeptin and Pepstatin 0.5mM Benzamidine 0.5mM PMSF
2x SDS gel-loading buffer (Laemmli buffer)	0.125M Tris-HCl (pH 6.8) 4% (v/v) SDS 10% (v/v) glycerol 10% (v/v) β-mercaptoethanol 0.2% (w/v) bromophenol blue 1mM DTT 2.8µg/ml Aprotinin 2.5µg/ml each of Leupeptin and Pepstatin 0.5mM Benzamidine 0.5mM PMSF
Nuclei extraction buffer A	10mM Hepes (pH 7.9) 10mM KCl 1.5mM $MgCl_2$ 0.5mM PMSF 0.5mM DTT 1µg/ml Pepstatin A 10µg/ml Aprotinin 10µg/ml Leupeptin 10µg/ml Type I-S soybean trypsin inhibitor
Nuclei extraction buffer C	20mM Hepes (pH 7.9)

	420mM NaCl 1.5mM $MgCl_2$ 0.2mM EDTA 25% (v/v) glycerol 0.5mM DTT 0.5mM PMSF 1µg/ml Pepstatin A 10µg/ml Aprotinin 10µg/ml Leupeptin 10µg/ml Type I-S soybean trypsin inhibitor
Upper gel buffer for SDS-PAGE	1M Tris-HCl 10% (w/v) SDS pH 6.8
Lower gel buffer for SDS-PAGE	1.5M Tris-HCl 10% (w/v) SDS pH 8.8
10xRunning buffer for SDS-PAGE	25mM Tris-HCl 250mM glycine 0.1% (w/v) SDS pH 8.3
Reducing solubilising solution	50mM Tris-HCl 20% (w/v) sucrose 2% (w/v) SDS 0.1% (w/v) bromophenol blue 10% (v/v) glycerol 5% (v/v) β-mercaptoethanol pH 6.8
Western blot transfer buffer	25mM Tris 192mM glycine 20% (v/v) methanol
10x Tris buffered saline (TBS)	10mM Tris-HCl 200mM NaCl pH 7.4

Table 2.4: Stock solutions used for the preparation of binding reactions for EMSA

Solution	Composition
10x Binding buffer	340mM KCl 50mM $MgCl_2$ 1mM DTT
Dilution buffer	40mM KCl 0.1mM EDTA

2.1.2.3 Antibiotics

A stock solution of ampicillin (100mg/ml) was filter sterilised using 0.22µm filters and stored in aliquots at -20°C.

2.2 Methods

2.2.1 Preparations of plastics and glassware

All glassware and plastics (e.g. test tubes, pipette tips and Eppendorf tubes) for the isolation and manipulation of DNA, RNA and proteins were autoclaved for 20min at a

pressure of 975 kPa at 121°C. This procedure was also carried out on solutions required for methods involving the manipulation of RNA or DNA.

2.2.2 Tissue culture

The following cell lines were used in this study: J774.2, an adherent murine monocyte-derived macrophage cell line; THP-1, a human monocytic cell line derived from a human leukemia cell; U937, a human monocytic cell line derived from a human histiocytic lymphoma and Hep3B, an adherent human hepatocarcinoma cell line derived from a human carcinoma. J774.2 and Hep3B were grown as adherent cells whereas THP-1 and U937 were maintained as undifferentiated monocytes grown in suspension. Differentiation of these cells was achieved by adding phorbol 12-myristate 13-acetate (PMA).

2.2.2.1 Maintenance of cells in culture

The cells were maintained either in RPMI 1640 (THP-1; U937) or Dulbecco's Modified Eagle's medium (DMEM) (J774.2; Hep3B). Both medium contained stabilized glutamine and were supplemented with penicillin (100U/ml), streptomycin (100U/ml) (designated pen/strep here after) and 10% (v/v) heat-inactivated (30min, 56°C) fetal calf serum (HI-FCS).

All the components used to supplement the media were sterilized using a 0.2µm sterile filter. The cells were maintained in a humid incubator at 37°C and 5% (v/v) CO_2 atmosphere. The culture medium was replaced every two days.

2.2.2.2 Subculturing of cells

The sub-culturing of J774.2 cells was carried out by detaching the cells (when they were 60-70% confluent) from the growth surface using a sterile disposable cell scraper and collecting them in 30 ml Universal tubes. The cells were then centrifuged at 800g for 5min at 4°C and resuspended in fresh medium containing 10% (v/v) HI-FCS and 1% (v/v) pen/strep. The cells were then plated out again at a ratio of 1:6 (e.g. cells from one flask were sub-cultured into six new flasks of the same size).

THP-1 and U937 cells were sub-cultured when they reached approximately 60-70% confluency ($1x10^6$cells/ml). The cells were centrifuged at 800g for 5min at 4°C and the pellet was resuspended in medium containing 10% (v/v) HI-FCS and 1% (v/v) pen/strep.

The cells were then plated into fresh tissue culture flasks at a ratio of 1:4 and incubated as above.

The adherent Hep3B cell line was sub-cultured when it reached between 50% and 60% confluency. The cells were washed with fresh medium and covered with a volume of trypsin/EDTA solution that was sufficient to cover the cell monolayer (e.g. 1.5ml for 125cm^2 large flask) and incubated at 37°C for 5-6min or until the cells had detached from the plastic. Cell culture medium containing HI-FCS was then added to the flasks to inactivate the action of trypsin/EDTA and the cell suspension was transferred to a sterile 50ml polypropylene tube and centrifuged at 800g for 5min. The resulting pellet was resuspended in medium containing 10% (v/v) HI-FCS and 1% (v/v) pen/strep and the cells were plated out into new fresh tissue culture flasks at a ratio of 1:4.

2.2.2.3 Preserving and storing of cells

Cells at early passage (2-4) were centrifuged at 800g for 5min. The supernatant was removed and the cells were resuspended in FCS containing 10% (v/v) glycerol for THP-1 or 10% (v/v) DMSO for J774.2, U937, and Hep3B cells. The mixture was then aliquoted in to 1ml sterile cryo-vials, covered with thick layers of tissue paper, and placed in a polystyrene box overnight at -70°C. The cryo-vials were then stored in liquid nitrogen.

2.2.2.4 Thawing frozen cells

Frozen cryo-vials stored in liquid nitrogen were placed in water bath at 37°C until the contents had thawed. The outside of the cryo-vials were cleaned with 70% (v/v) ethanol and the contents transferred into a Universal tube containing 10ml of HI-FCS. After centrifugation for 5min at 800g, the supernatant was discarded and the cell pellet was resuspended in complete medium containing 10% (v/v) HI-FCS and plated out in tissue culture flasks.

2.2.2.5 Counting Cells

A haemocytometer (Neubauer chamber) was used to count the number of cells for subculturing and transfection purposes. The haemocytometer was covered with a precision ground cover-slip that was gently pressed on it until Newton rings were visible. Then, 10µl of cell suspension was placed at the edge of the cover-slip. The chamber was placed under the microscope and all the cells in its large square were counted.

2.2.2.6 Differentiation of THP-1 monocytes

The differentiation of THP-1 monocytes was initiated by adding 0.16μM of PMA to the cells ($1x10^6$/ml). Differentiation was completed after 24h of PMA addition when the cells lost their round morphology, became elongated and adhered to the bottom of the flask.

2.2.2.7 Delipidation of HI-FCS

The study of LXR-mediated regulation of genes implicated in the control of cholesterol homeostasis required all experiments to use delipidated HI-FCS. Lipids were removed from HI-FCS according to the method of Cham and Knowles (1976) with minor modifications. To 10ml of HI-FCS, 20ml of 2:3 (v/v) butanol:DIPE was added and mixed by rotation for 30min at room temperature. The phases were then separated by centrifugation at 4,000g for 5min and the serum removed into fresh tube. In order to remove any residual solvent, nitrogen was bubbled through the serum for 2-3h.

2.2.2.8 Treatment of cells with oxysterol ligands

For certain experiments, the addition of the LXR ligands 22(R)-HC and 22(S)-HC and/or the RXR ligand 9CRA was necessary. For J774.2 macrophages, the cells were first incubated for 4h in 10% (v/v) lipoprotein deficient serum (LPDS). The medium was then removed and replaced with fresh medium (of the same serum content and composition) containing the ligands or the vehicle (i.e. DMSO) and incubated at 37°C in a humid atmosphere of air containing 5% (v/v) CO_2 for the requisite time. For THP-1 cells, these were seeded in RPMI media containing 10% LPDS for 4h followed by the addition of 0.16μM PMA and incubation for 24h (Tsuchiya et al., 1982). The ligands or vehicle (i.e. DMSO) were then added and the incubation continued for the requisite time. These conditions are routinely used for experiments with such ligands.

2.2.2.9 Treatment of cells with inhibitors

For experiments with pharmacological inhibitors, the appropriate concentration of the inhibitors were added directly to the medium 1h before the addition of the mediators (i.e. pre-treatment). Table 2.5 details the pharmacological inhibitors used in this study and their mode of action.

2.2.2.10 Trypan blue exclusion test

The trypan blue exclusion assay was used to determine cell viability. For this, 0.4% (w/v) of trypan blue solution was added to the cell culture media at a ratio of 1:100 (v/v) and incubated at 37°C for 5-10min in an incubator. The cells were then washed with PBS and viewed under a microscope.

Table 2.5: Action of pharmacological inhibitors used

Inhibitor	Formula	Target	Mechanism	Reference
LY294002	$C_{19}H_{17}NO_3$	PI3K	Reversible ATP competitive inhibitor of PI3K	Vlahos et al., 1995
SB216763	$C_{19}H_{12}N_2O_2Cl_2$	GSK-3	Glycogen synthase kinase-3 inhibitor	Smith et al., 2001
SB415286	$C_{16}H_{10}N_3O_5Cl$	GSK-3	Glycogen synthase kinase-3 inhibitor	Smith et al., 2001
Rapamycin	$C_{51}H_{79}NO_{13}$	mTOR	Selective inhibitor of p70 S6 kinase	Gottschalk et al., 1994
SP600125	$C_{14}H_8N_2O$	JNK/SAPK	Reversible ATP competitive inhibitor of JNK	Bennett et al., 2001
Curcumin	$C_{21}H_{20}O_6$	JNK/SAPK	Inhibits an upstream kinase of the JNK pathway	Brouet et al., 1995
Bisindolylmaleimide I	$C_{25}H_{24}N_4O_2$	PKC	Reversible ATP competitive inhibitor of PKC	Hers et al., 1999
Gö 6976	$C_{24}H_{18}N_4O$	PKC	Selective inhibitor of Ca^{+2} dependent PKCα-isozyme	Martiny-Baron et al., 1993
Gö 7983	$C_{26}H_{26}N_4O_3$	PKC	Selective inhibitor of several PKC-isozymes	Wang et al., 1998
Rottlerin	$C_{30}H_{28}O_8$	PKC	Selective inhibitor of PKCδ-isozyme	Gschwendt et al., 1994

PI3K, Phosphoinositide 3-kinases; GSK, Glycogen synthase kinase-3; mTOR, mammalian target of rapamycin; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; PKC, Protein kinase C.

2.2.2.11 Human primary monocyte-derived macrophage cell cultures

Human primary monocytes/macrophages were isolated from buffy coats obtained from the National Blood Service, Wales. The isolation was carried out using Accuspin™ centrifuge tubes and the separation medium used was Lymphoprep™. The separation medium comprises of an aqueous solution of a high molecular weight polysaccharide dextran and sodium diatrizoate that causes erythrocyte aggregation. Accuspin™ centrifuge tubes were layered on top of the polyethylene barrier (frit) with 15ml of Lymphoprep™ that had previously been warmed to room temperature. Subsequently, these were centrifuged at 800g for 1min in order to place the Lymphoprep™ in the tube below the frit. Then, 30ml of blood was layered over the frit and centrifuged at 800g for 30min at room temperature to allow sedimintation of erythrocytes. The resultant mononuclear cells were collected and transferred to a new centrifuge tube and an equal volume of ice cold PBS-0.4% (w/v) tri-sodium citrate was added. Cells were pelleted at 800g for 5min at 4°C. The pelleted cells were resuspended in 10ml of 0.2% (v/v) saline solution and incubated on ice for 30sec followed by the addition of 10ml of 1.6% (v/v) saline solution and immediate centrifugation at 800g for 5min at 4°C. The resultant interface was collected and washed 6-8 times with ice cold PBS-0.4% (w/v) tri-sodium citrate to remove contaminating platelets. Primary monocytes were plated out in flasks ($10x10^6$ cells/ml) in complete culture medium containing 5% (v/v) HIFCS and 1% (v/v) pen/strep. The cells were allowed to adhere for 4h in an incubator at 37°C, containing 5% (v/v) CO2, and then the media was replaced with fresh culture medium. The human primary monocyte-derived macrophages were left to differentiate for 7-10 days before use in experiments. Every 2 days, half of the volume of the medium was removed and replaced with fresh culture medium containing 5% (v/v) HI-FCS, and 1% (v/v) pen/strep. For stimulation with LXR agonists, the medium was removed and replaced with fresh culture medium containing 5% (v/v) LPDS and 1% (v/v) pen/strep.

2.2.3 Isolation of total RNA

Total RNA was isolated using the Rneasy™ mini kit (Qiagen). The method relies on the selective binding properties of RNA to a silica gel-based membrane (Suppliers information, 1997). The disruption of the cells was carried out in the presence of guanidinium isothiocyanate, which is an effective deproteinising agent that removes the proteins which complex with the RNA. Additionally, guanidinium isothiocyanate is a strong inhibitor of ribonucleases (RNases), which are liberated from organelles such as

nuclei and lysosomes when the cells are disrupted. This inhibition results in the isolation of intact, undegraded RNA (Chomczynski and Sacchi, 1987).

2.2.3.1 Procedure

Following incubation with the ligands, the cells were removed from the flasks, transferred to a 20ml Universal tube and centrifuged at 800g for 5min. The cells in the pellet were lysed by the addition of 600µl of lysis buffer RLT (provided in the kit) followed by passaging for about 10 times through a 0.9mm needle fitted to a 2ml syringe. Once this homogenisation step was complete, 600µl of 70% (v/v) ethanol was added to the lysate and then 700µl of the sample was applied to an RNeasy column (provided in the kit), which was then centrifuged for 800g for 15sec. This process was repeated until the entire sample had been applied to the column (the flow through was discarded). The column was then washed once with 700µl of wash buffer RW1 (provided in the kit), centrifuged as above and then washed twice with 500µl of wash buffer RPE (provided in the kit). The column was centrifuged for 800g for 2min in order to remove the residual ethanol. The RNA was then eluted by placing the column in a fresh microcentrifuge tube and then applying 50µl of RNase-free water (provided in the kit) directly to the membrane. Following centrifugation for 1min at 800g, the RNA was collected in an Eppendorf tube.

2.2.3.2 Quantitation and assessment of the purity of total cellular RNA

The concentration of RNA was determined spectrophotometrically at 260nm (OD260) and at 280nm (OD280) using a U-1800 spectrophotometer (Hitachi). The concentration was calculated by multiplying the absorbance by 40 (the absorbance of 1µg/ml of RNA) and by the appropriate dilution factor. The purity was assessed by calculating the ratio of the absorbance at 260 and 280nm. Pure RNA has an OD260:OD280nm ratio of approximately 2 (impurities may either increase or decrease this ratio). To be suitable for RT-PCR, a ratio between 1.8 and 2.2 was required, otherwise there was a possibility that the contamination may interfere with the assessment of the concentration and with the reverse transcription reaction.

The RNA (1µg) was also subjected to electrophoresis to verify the quantification and to determine the quality of the RNA. Electrophoresis was carried out on a 1% (w/v) agarose gel. The RNA was visualised under an ultraviolet transilluminator using a Syngene Gel

documentation system. Intact undegraded RNA should show a 28S:18S rRNA ratio of approximately 2:1.

2.2.4 Semi quantitative Reverse transcriptase Polymerase Chain Reaction (RT-PCR)

2.2.4.1 Introduction

RT-PCR is a two-step process, the reverse transcription of RNA into cDNA followed by the amplification of the cDNA by the polymerase chain reaction (PCR). As PCR can amplify specific DNA molecules in an exponential manner, it is an ideal technique for the semi quantitative or even quantitative analysis of RNA and DNA molecules. The amplification allows RT-PCR to be used to measure short lived and low abundance mRNA transcripts that may not be detected by Northern blotting and is also less time consuming (Wang et al., 1989).

When the cDNA of interest is amplified, it is compared against a standard. The expression of the standard should remain constant in each sample of RNA. Therefore, the levels of the target gene in each sample can be normalized to take into account factors such as the varying efficiencies of the RT reaction. Such standards include housekeeping genes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.2.4.2 Reverse Transcription (RT) of RNA to cDNA

A mixture, containing 1µg of total RNA, 200pmoles of random hexamer primers (PdN6) and tissue culture grade water, made up to a final volume of 12.5µl was incubated at 70°C for 5min and then chilled on ice for 5min. The mixture was then made up to 20µl with 1µl of deoxyribonucleosidetriphosphate mix (dNTPs) [10mM of each], 4µl of 5x reverse transcriptase NH_4 reaction buffer (Promega), 0.5µl of 200U of recombinant RNase inhibitor and 1µl of 200U/µl Moloney Murine Leukaemia Virus (M-MLV) reverse transcriptase (Promega). The mixture was then incubated at 37°C for 1h followed by 2min at 90°C. The cDNA product was diluted to 100µl with tissue culture grade water and stored at -20°C.

2.2.4.3 PCR reactions

All PCR reactions were carried out in a final volume of 50µl using primers and optimised conditions shown in Tables 2.6 and 2.7 respectively. All PCR reactions were overlaid with mineral oil and carried out using a Biometra TRIO Thermoblock. The optimised cycle number for each primer set was chosen so that it corresponded to the exponential

phase of amplification and, therefore, provided a direct correlation between the amount of product and the amount of the original cDNA template used.

The primer sequences were obtained from the following references: ABCA1 (Klucken et al., 2000); apoE, LPL and GAPDH (Wang et al., 1989); and 28S rRNA (Kong et al., 1999).

Table 2.6: The PCR primer sequences used in this study

mRNA species	Specific primers	Product size (bp)
hABCA1	For:5'GTTGGAAAGATTCTCTATACACCTGA-3' Rev:5'CGTCAGCACTCTGATGATGGCCTG-3'	690
hapoE	For:5'TTCCTGGCAGGATGCCAGGC-3' Rev:5'GGTCAGTTGTTCCTCCAGTTC-3'	270
hLPL	For:5'GAGATTTCTCTGTATGGCACC-3' Rev:5'CTGCAAATGAGACACTTTCTC-3'	276
mMCP-1	For:5'CTTCTATGCCTCCTGCTCATAGCT-3' Rev:5'CTTGGGGTCAGCACAGATCTCCTT-3'	219
m/hGAPDH	For:5'CCCTTCATTGACCTCAACTACATGG-3' Rev:5'AGTCTTCTGGGTGGCAGTGATGG-3'	456
mβ-actin	For:5'TGGAGAAGAGCTATGAGCTGCCTG-3' Rev:5'GTGCCACCAGACAGCACTGTGTTG-3'	204
28S rRNA	For:5'TGAACTATGCTTGGGCAGGG-3' Rev:5'AGCGCCATCCATTTTCAGGG-3'	513

2.2.4.4 Agarose gel electrophoresis

The amplification products were size-fractionated by electrophoresis on a 1.5% (w/v) agarose gel and the fragment sizes determined by comparison to standard DNA molecular weight markers. Gels were made using 0.25x TBE and contained ethidium bromide (0.5μg/ml). Electrophoresis was carried out on a Horizontal Gel Unit (Fisher) at 100V with 0.5x TBE as a "running" buffer. Samples were mixed with DNA loading dye before adding to the wells. DNA bands were visualised under UV using a Syngene Gel Documentation System as described in section 2.2.4.5.

2.2.4.5 Densitometric scanning of gels

The image of the gel on a UV transilluminator was recorded as a computer file using GeneTools™ software (Syngene). This allowed analysis to be carried out with the densitometry software package Quantiscan (Biosoft, Cambridge, UK). The intensity of the signals from the target gene and the control gene were calculated and a background for the gel was subtracted from each value. A ratio of the target gene: control gene was then determined.

Table 2.7: The optimised PCR protocols for each primer set

Reagent	hABCA1	apoE	mMCP-1	hLPL	GAPDH	28S rRNA	mβ-actin
forward primer (100μM)	0.25μl	0.25μl	0.25μl	0.5μl	0.5μl	1μl	1μl
Reverse primer (100μM)	0.25μl	0.25μl	0.25μl	0.5μl	0.5μl	1μl	1μl
dNTPs (100μM)	0.5μl	0.5μl	0.5μl	1μl	0.5μl	1μl	2μl
$MgCl_2$ (50mM)	1μl	1.5μl	2μl	2μl	2μl	1μl	1μl
DMSO (%v/v)	-	2.5	-	-	-	-	2.5μl
10x PCR buffer	5μl	5μl	5μl	5μl	5μl	5μl	5μl
Taq polymerase (5U/μl)	0.25μl	0.25μl	0.25μl	0.25μl	0.25μl	0.5μl	0.25μl
cDNA	10μl	10μl	10μl	10μl	10μl	5μl	10μl
ddH_2O	32.75μl	29.75μl	31.75μl	30.75μl	31.25μl	35.5μl	27.25μl
Total volume (μl)	50	50	50	50	50	50	50
PCR programme							
Initial melting	95°C 5min	94°C 2min	95°C 5min	96°C 5min	96°C 5min	95°C 5min	95°C 2min
Annealing	64°C 1min	64°C 30sec	72°C 2min	72°C 2min	62°C 1min	62°C 1min	58°C 2min
Extension	72°C 1min	72°C 2.5min	72°C 2min	72°C 2min	72°C 2min	72°C 2min	72°C 2min
Melting	95°C 1min	93°C 30sec	94°C 30sec	93°C 30sec	93°C 30sec	93°C 30sec	94°C 2min
Final long extension step	72°C 10min	72°C 10min	72°C 10min	72°C 10min	72°C 10min	72°C 10min	72°C 5min
Number of cycles	24	24	25	17	22	11	18

M, mouse; H, human

2.2.5 Western blot analysis

2.2.5.1 Preparation of whole cell protein extracts

Total cellular protein was isolated from THP-1 macrophages (grown to about 70% confluence in 75cm² tissue culture flasks) using either whole cell extraction buffer, phosphatase-free whole cell protein extraction buffer or Laemmli buffer (Table 2.3). The cells were scraped and then transferred with the media to a 20ml Universal tube and centrifuged at 800g for 5min. The cells were resuspended in 1ml of ice cold PBS, transferred to a 1.5ml Eppendorf tube and centrifuged at a maximum speed (13,000rpm) in a microcentrifuge for 1min at 4°C. The cells were then washed twice with 1ml of ice cold PBS containing NaF (10mM) and Na3V04 (sodium orthovanadate) (100μM) and the pellet was immediately frozen on dry ice. The pellet was resuspended by adding 5 volumes of phosphatase-free whole cell extraction buffer (approximately 150μl) or Laemmli buffer and the cells were then lysed by vigorous pippetting followed by vortexing (45sec). The mixture was centrifuged again and the lysates were transferred to new tubes and stored at -80°C until required.

2.2.5.2 Preparation of nuclear protein extracts

Isolation of nuclear extracts was carried out according to the protocol described by {Ramji et al., 1993) with minor modifications. Briefly, cells grown in 75cm² tissue culture flasks were scraped in media and pelleted by centrifugation at 1000g for 5min. The cells were washed twice with 1ml of ice cold PBS, transferred to an Eppendorf tube and pelleted by centrifugation at 13,000rpm in a microcentrifuge for 5min. The pellet was resuspended in 50μl of ice cold nuclei extraction buffer A (Table 2.3), left on ice for 15min and then vortexed for 10 sec. The cells were lysed by drawing the mixture five times through a 100μl Hamilton syringe. The mixture was centrifuged again in a microcentrifuge at 13,000rpm for 20sec and the supernatant, containing the cytosolic proteins, was stored at -70°C. The pellet, containing the nuclei was resuspended in 60 μl of ice cold nuclei isolation buffer C (Table 2.3) and incubated on ice for 30min. Following centrifugation in a microcentrifuge at 13,000rpm at 4°C for 5min, the supernatant, containing the nuclear extracts, was removed and stored at -80°C.

2.2.5.3 Micro BCA protein assay

The concentration of total proteins in extracts was determined using the Micro BCA™ protein assay reagent kit. The procedures were performed as described in the manufacturer's instructions (Pierce). A standard curve was prepared for each assay

using suitable serial dilutions of a 2mg/ml bovine serum albumin (BSA) solution to give concentrations of 5µg/ml, 10µg/ml, 15µg/ml, 20µg/ml and 25µg/ml. The standard and test samples were diluted in PBS. Then, 100µl of each sample was placed in a 96 well micro-titer plate (Greiner) followed by the addition of 100µl of protein assay reagent (provided in the kit). After mixing and incubation at 37°C for 4h, the absorbance was read at 595nm using a titertek Multiscan MCC/340 Dynex Technologies MRX microplate reader. The protein concentration of the experimental samples was calculated from the standard curve.

2.2.5.4 JNK kinase assay

The JNK kinase activity was assayed using a non-radioactive JNK kinase assay kit from Cell Signalling Technology (CST) with a slight modification to the manufacturer's protocol. To 250µg of whole cell extracts, 20µl of c-Jun fusion protein beads were added and left to incubate overnight at 4°C with gentle rocking. The mixture was then centrifuged in a microcentrifuge for 1min at 13,000rpm and the pellet was washed twice with 300µl of phosphatase-free whole cell extraction buffer (Table 2.3) without Triton X-100 and twice with 300µl of 1X JNK kinase buffer (provided in the kit). The pellet was then resuspended in 50µl of 1XJNK kinase buffer supplemented with 200µM ATP and incubated for 30min at 30°C. The kinase reaction was then terminated by the addition of 25µl of solubilising solution (provided in the kit). The sample was then boiled for 5min and loaded onto an SDS-PAGE gel. Electrophoresis and Western blotting were carried out as described in sections 2.2.5.6 and 2.2.5.7, respectively and the immunodetection of proteins was carried out using the phospho c-Jun (Ser63) antibody.

2.2.5.5 PKB kinase assay

The PKB kinase assay was carried out using a non-radioactive kinase assay kit from CST with a slight modification to the manufacturer's protocol. To 250µg of whole cell extracts, 20µl of immobilised PKB antibody was added and then incubated overnight at 4°C with gentle rocking. The mixture was then centrifuged for 1min at 13,000g and the pellet was washed twice with 300µl of phosphatase-free whole cell extraction buffer (Table 2.3) without Triton X-100 and twice with 300µl of 1XPKB kinase buffer (provided in the kit). The pellet was resuspended in 40µl of 1XPKB kinase buffer supplemented with 200µM ATP and 1µg of substrate (GSK-3 fusion protein). The mixture was then incubated for 30min at 30°C and the kinase reaction was then terminated by the addition of 20µl

solubilising solution (provided in the kit). The sample was then boiled for 5min and loaded onto an SDS-PAGE gel. Electrophoresis and Western blotting were carried out as described in sections 2.2.5.6 and 2.2.5.7, respectively and the immunodetection of the protein was carried out using the phospho GSK-3a/β (Ser21/29) antibody.

2.2.5.6 Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed under reducing conditions according to the method of {Laemmli, 1970). Typically, separating gels and stacking gels were prepared from stock solutions as shown in Table 2.8.

Table 2.8: Composition of stacking and separation gels/20ml for SDS-PAGE

Gel component	7.5% (w/v) Separating gel	10% (w/v) Separating gel	5% (w/v) Stacking gel
Upper buffer	-	-	2.5ml
Lower buffer	5ml	5ml	-
Acrylamide:bisacylamide (37.5:1)	3.75ml	5ml	1.25ml
ddH2O	11.25ml	10ml	6.14ml
10% (w/v) ammonium perslphate	200µl	200µl	100µl
TEMED	20µl	20µl	10µl

The gels were prepared using the Mini-PROTEAN II™ Slab Electrophoresis Cell apparatus from Bio-Rad Laboratories according to the manufacturer's instructions. The separation gel was poured to within 1.5cm of the top of the inner glass plate. Butanol was layered on top of the gel solution to exclude air bubbles and the gel was allowed to polymerise for 30-40min. Once the gel had set, the butanol was washed off with ddH2O and the excess liquid removed using Whatman 3MM Paper. The stacking gel was then poured on top and the well-forming comb inserted. After polymerisation of the stacking gel, the comb was removed and the wells washed with ddH2O. The gel was then placed in the electrophoresis tank and the upper and lower chambers were filled with 1x "running" buffer (see Table 2.3)

Protein samples (20-40µg) were mixed with an equal volume of gel loading buffer or bromophenol blue (BPB) and then incubated at room temperature for 10min or boiled for 5-8min. The samples were then allowed to cool and then loaded onto the gel (10% for Western blots with all antibodies except ABCA1 where a 7.5% separating gel was used). Rainbow protein size markers (7-10µl) (GE Healthcare) were loaded into the first lane of each gel (see Appendix III). Electrophoresis was carried out at 200V until the BPB marker dye had reached within 0.5cm of the lower end of the gel (approximately 50min to 1h for

ABCA1). The gels were then removed from the glass plates and subjected to Western blotting.

2.2.5.7 Western blotting

Electrophoretic transfer of proteins from the gel to PVDF membranes (Millipore) was performed using a Bio-Rad Trans Blot Electrophoretic transfer Cell as described in the manufacturer's instructions. Briefly, the gel was removed from the glass plates and the stacking gel cut away. The gel was then equilibrated by incubation in transfer buffer (Table 2.3) for 10min. The membrane, Whatman 3MM paper (pre-cut to the gel size) and the sponge pads of the transfer apparatus were also prepared by pre soaking in transfer buffer. The activated membrane (soaked in methanol for 1min) was then placed on the gel and sandwiched between the Whatman paper and the sponge pads before being placed in the blotting cassette. The cassette was then subjected to electro-blotting in a tank containing transfer buffer at 4°C at a constant voltage of 15V (overnight) or for 1h at 150V. Proteins transferred to the PVDF membranes were immunodetected as described in section 2.2.58.

2.2.5.8 Immunoprobing of the blots

Following electrophoretic transfer, the rainbow size markers were cut out from the PVDF membrane. The proteins that had been transferred from the gel were probed immunochemically using the ECL detection kit (GE Healthcare). First non-specific protein binding sites on the membrane were blocked by incubation, with shaking, in blocking buffer [5-10% (w/v) non-fat milk and 0.1% (v/v) Tween-20 in 1x TBS] for 1h at room temperature. After three incubations with washing buffer [1x TBS containing 0.1% (v/v) Tween-20 (TBS-T)] for 5min each, the membrane was incubated with the primary antibody, diluted in 1xTBS-T containing 5% (w/v) non-fat milk or BSA, for 1h at room temperature (see Table 2.9 for the dilution factor for each antibody used in this study). The membrane was then washed three times for 15min each using washing buffer (TBS-T) and then immersed in secondary antibody solution [horseradish peroxidase-conjugated goat anti-rabbit IgG diluted in 1xTBS-T containing 5-10% (w/v) non-fat milk or 5% BSA]. The membrane was then washed with washing buffer (TBS-T) a further three times for 15min each. Detection of membrane bound antigen-antibody complexes was then carried out using the ECL detection reagent as described by the manufacturer. The films were then developed (section 2.2.5.9).

Table 2.9: Primary antibody binding conditions

Primary Antibody	Dilution	%(w/v) Milk	%(w/v) BSA	Incubation Time (h)	Size (kDa)
Goat anti-apoE	1/1000	5	-	1	34
Rabbit anti-ABCA1	1/1000	3	-	O/N	220
Rabbit anti-phospho JNK/SAPK	1/1000	-	5	O/N	46/54
Rabbit anti-total JNK/SAPK	1/1000	-	5	O/N	46/54
Rabbit anti-phospho c-Jun (JNK/SAPK Assay)	1/1000	-	5	O/N	35/37
Rabbit anti-phospho SEK1/MKK4 (Ser257/Thr261)	1/1000	-	5	O/N	44
Rabbit anti-total SEK1/MKK4 (Ser257/Thr261)	1/1000	-	5	O/N	44
Mouse anti-phospho c-Jun (Ser63)	1/1000	-	5	O/N	47
Rabbit anti-total c-jun (Ser63)	1/1000	-	5	O/N	43
Rabbit anti-phospho PKB (Ser473)	1/1000	-	5	O/N	60
Rabbit anti-total PKB	1/1000	-	5	O/N	60
Phospho GSK3 (PKB Assay)	1/1000	-	5	O/N	30
Mouse anti-β-actin	1/12000	5	-	1	42

O/N, overnight

2.2.5.9 Autoradiography

The membranes were wrapped in Saran Wrap and exposed for the requisite time (10sec-5min) to a Kodak X-ray film in a Hi-Speed-X light proof autoradiography cassette (with two intensifying screens on either side of the cassette) (Genetic Research Instrumentation). Further exposure times depended on the strength of the signals obtained. The exposed film was then developed in an Agfa-Gevaert automatic developer. The expected protein size was validated by comparison to standard rainbow markers.

2.2.6 Isolation and manipulation of DNA

2.2.6.1 Purification of DNA spotted onto filter papers

The region on the filter paper, containing the recombinant plasmid DNA, was cut and placed in a 1.5ml Eppendorf tube and 50µl of TE buffer was added. Following 5min incubation at room temperature, the mixture was subjected to centrifugation in a microcentrifuge for 5min at maximum speed. The supernatant, containing the plasmid DNA, was stored at -20°C until required.

2.2.6.2 Bacterial strains and vectors

Table 2.10 shows the genotype of the *Escherichia Coli (E. Coli)* strains used in this study. The maps of the recombinant plasmids for all expression and reporter vectors used in this study are shown in the Appendix III.

Table 2.10: Genotype of E Coli strains used

Bacterial Strain	Genotype	Reference
DH5-α	supE44 ΔlacU169 (Φ80 lacZΔM15) hsd17 recA1 endA1 gyrA96 thi- 1 recA1	Hanahan, 1983a; Hanahan, 1983b

2.2.6.3 Preparation of competent cells

For the preparation of competent bacterial cells, a modified version of the method described by Mandel and Higa (1970) was used. LB-medium (5ml, pre-heated to 37°C) was inoculated with a single bacterial colony of the *E. coli* DH5-α strain and incubated for 12-18h at 37°C with moderate shaking. Then, 0.1ml of this overnight culture was used to inoculate 9.9ml of fresh LB-medium, which was then incubated with moderate shaking at 37°C until it reached an OD550 of 0.5-0.6. The cells were then pelleted by centrifugation at 3000g at 4°C for 5min, resuspended in half their original volume (5ml) of ice-cold 50mM $CaCl_2$ and incubated on ice for 25min. The cells were again pelleted by centrifugation, as above, and resuspended in 1/10 of their original volume (1ml) of ice-cold 50mM $CaCl_2$. Competent Cells were kept on ice until used for transformation or mixed with an equal volume of 40% (v/v) sterile glycerol, dispensed into 1ml aliquots in pre-chilled Eppendorf tubes and stored at -80°C.

2.2.6.4 Transformation of competent cells

For each transformation, approximately 1-5-µl of recombinant plasmid DNA was added to 200µl of competent cells and incubated on ice for 40min. The cells were then heat-shocked at 42°C for 90sec and placed immediately on ice for a further 2min. Then, 800µl of LB medium (pre warmed to 37°C) was added to the cells and the mixture incubated for 1h at 37°C with moderate shaking. After the incubation, 200µl of transformed bacteria were spread on LB-agar plates containing 100µg/ml ampicillin. Remaining cells were pelleted by centrifugation in a microfuge at 13,000rpm for 3min, resuspended in 100µl of LB-medium (pre warmed to 37°C) and spread onto a separate plate. The plates were incubated overnight at 37°C. An ampicillin plate spread with untransformed cells was used as a control for each experiment.

2.2.6.5 Small-scale preparation of plasmid DNA (Mini-prep protocol)

Small-scale preparation of plasmid DNA was carried out using the Wizard® plus SV DNA mini-prep kit (Promega) according to the manufacturer's instructions. A single colony of transformed bacteria was used to inoculate 10ml of LB-medium containing 100µg/ml of ampicillin and incubated overnight at 37°C with moderate shaking. The culture was then centrifuged at 800g for 5min and the resulting bacterial pellet was resuspended in 250µl of Cell Resuspension solution (provided in the kit).The suspension was then transferred to a microcentrifuge tube and 250µl of Cell Lysis solution (provided in the kit) was added. The suspension was mixed by inversion and clearing was observed confirming that lysis was complete. Alkaline Protease (10µl) (provided in the kit) was then added to the solution and, after inversion, the solution was incubated at room temperature for 5min. Then, 350µl of Neutralisation solution (provided in the kit) was added, mixed by inversion and centrifuged in a microfuge at 13,000rpm for 10min. The clear supernatant was transferred to a spin column (provided in the kit) inserted into a collection tube and subjected to centrifugation in a microfuge at 13,000rpm for 1min. The DNA was eluted from the column by adding 50µl of Nuclease-Free water and centrifuging in a microfuge at 13,000rpm for 1min.

2.2.6.6 Large-scale preparation of plasmid DNA (Maxi-prep protocol)

The High Purity Plasmid Maxi Prep System was used for the large-scale preparation of plasmid DNA according to the manufacturer's instructions (Marligen Biosciences). A single colony of transformed bacteria was used to inoculate 10ml of LB-medium containing 100µg/ml of ampicillin and incubated for 14-16h at 37°C with moderate shaking. The entire culture was then added to 250ml of LB-ampicillin (100µg/ml) medium and left to grow overnight with shaking at 37°C. The cells were then pelleted by centrifugation at 16,000g for 30min at 4°C. All medium was thoroughly removed and the resulting pellet was resuspended using 10ml of Cell Suspension buffer (provided in the kit). The cell suspension was then lysed using 10ml of Cell Lysis solution (provided in the kit). The mixture was left to incubate at room temperature for 5min, after which, 10ml of Neutralisation solution (provided in the kit) was added. The solution was mixed by inversion and centrifuged at 15,000g at room temperature for 30min.The supernatant was then transferred to a column (provided in the kit), which had previously been equilibrated by allowing 30ml of Equilibration buffer (provided in the kit) to drain through the column by gravity flow. The resulting flow through was discarded and the

column was washed with 60ml of Wash buffer (provided in the kit). The DNA was then eluted by adding 15ml of Elution buffer (in the kit) to the column. Once all the solution had drained from the column by gravity flow, 10.5ml of isopropanol was added to precipitate the DNA. The solution was mixed and centrifuged at 15,000g for 30min at room temperature. The pellet was washed two times with 2ml of 70% (v/v) ethanol by centrifugation at 15,000g at 4°C for 5min. The ethanol was removed and the pellet was left to air-dry for 10min. The DNA pellet was then dissolved in 500µl of TE buffer.

2.2.6.7 Quantification of DNA

The concentration and the efficiency of purification of recombinant plasmid DNA were assessed by measuring the Optical Density (OD) at 260nm and 280nm using a U-1800 Hitachi spectrophotometer. The concentration was calculated by multiplying the absorbance by 50 (the absorbance of 1µg/ml of DNA) and by the appropriate dilution factor.

2.2.6.8 Restriction endonuclease digestion of recombinant plasmid DNA

Restriction endonuclease digestion of DNA was performed using buffers and conditions recommended by the suppliers. The incubation times varied depending on the enzymes with the majority of the digest reactions being carried out for at least 3h at 37°C except SmaI for which the incubation temperature was 25°C. The digests were typically carried out using 10U of each enzyme with the exception of KpnI where 20U were used due to its low activity. All double digests were performed sequentially where the first digest step was followed by the addition of a single volume of sterile water, the second enzyme and the recommended buffer. For some digests performed in this study, the reaction mixture was supplemented with 100µg/ml BSA. All reactions were stopped by incubation at 65°C for 10min. DNA fragments were analysed by agarose gel electrophoresis. Table 2.11 details the restriction endonuclease digestion used to analyse each preparation of recombinant plasmid DNA before transfection based experiments.

2.2.6.9 Gel electrophoresis of DNA

Size-fractionation of products of the digestion reactions was carried out by electrophoresis using 0.8% (w/v) agarose gels in 1x TBE buffer containing 0.5µg/ml ethidium bromide. The samples were mixed in a 1 to 10 ratio with 5x DNA loading buffer (Table 2.2), where BPB was used as the tracking dye, before pipetting into the wells of the gel along with 5-10µl of the 1kb molecular size standards (see the Appendix I).

Table 2.11: Restriction endonuclease digestion of plasmid constructs

Plasmid construct	Vector	Resrtiction endonucleases	Fragment (kb)	Lab. Source
LXR	pGL3- Basic	KpnI, NheI	4.8,2.8,1.2	M. Watson (California U.)
LXR-DN	pGL3- Basic	KpnI, NheI		T. Kocarek (Wayne State U.)
ABCA1	pGL3- Basic	SmaI	4.8,0.6,0.3	P. Costet (Columbia U.)
ABCA1 DR4Mut	pGL3- Basic	SmaI	4.8,0.6,0.3	P. Costet (Columbia U.)
PKB AAA-DN	pcDNA3	*	*	B. Hemmings (Basel)
p110-DN	pcDNA3	*	*	B. Hemmings (Basel)
JNK/SAPK-DN	pcDNA3	*	*	E. Nishida (Kyto U.)
Tam67-DN	pcDNA3	*	*	P. Brown (National Cancer I.)
SEK-1-DN	pcDNA3	*	*	J.R. Woodgett (Ontario Cancer I.)
PKCε-DN	pcDNA3	EcoRI	2.7	S. Ohno (Yokohama City U.)
PKCλ-DN	pcDNA3	EcoRI, SalI	2	S. Ohno (Yokohama City U.)
PKCα-DN	pcDNA3	XhoI	2	JW Soh (Inha U.)
PKCδ-DN	pcDNA3	XhoI	2	JW Soh (Inha U.)
PKCη-DN	pcDNA3	XhoI	2	JW Soh (Inha U.)
PKCξ-DN	pcDNA3	XhoI	2	JW Soh (Inha U.)

* Already checked by other researchers in the laboratory.

Electrophoresis was carried out in a horizontal gel apparatus (Fisher) at a constant voltage of 100 volts for approximately 1h in 1x TBE containing 0.5µg/ml of ethidium bromide. The DNA was visualised using an ultraviolet transilluminator and the image was photographed using Genetools (Syngene). The sizes of the fragments were determined by comparison with the DNA size standards.

2.2.7 Cell transfection

2.2.7.1 Transient transfection of Hep3B cells using 25kDa polyethylenimine (PEI)

The transient transfection of the Hep3B cell line was carried out using PEI according to the method of Dixon et al. (2000) with minor modifications. Hep3B cells that were about 60% confluent were seeded 24h prior to transfection onto each well of a 6-well plate in 2ml of DMEM containing 10% (v/v) HI-FCS per well and left overnight at 37°C with 5% CO_2. On the day of tranfection, the cell culture medium was removed and the cells were washed in HI-FCS- and antibiotic-free media. Then, 2ml of fresh media supplemented with 10% (v/v) HI-FCS was added to the cells and incubated for 4h. A transfection mixture containing 5µg of recombinant plasmid DNA in 5% (w/v) glucose and 1.5µl of 25KDa PEI solution [5.625mg/ml PEI dissolved in 8ml ddH2O, pH7.2] was made. The resulting complex was immediately suspended in 1ml of DMEM with 10% (v/v) HI-FCS and added drop wise to the cells. The plates were swirled to ensure uniform distribution of the

complexes. The cells were returned to the incubator for a further 24h. In the experiments examining the effects of LXR/RXR ligands, the medium was replaced 24h after transfection with fresh DMEM containing 10% delipidated HI-FCS and the ligands were then added as usual and left in the incubator for a further 24h.

2.2.7.2 Transient transfection of U937 cells using SuperFect™ transfection method

This method was carried out as described by the manufacturer (Qiagen) for suspension cells. Twenty four hours prior to transfection, the cells were split in a ratio of 1 to 2. On the day of transfection, cells were suspended at a density of $6x10^5$ cells/ml in RPMI 1640 medium supplemented with 3% (v/v) LPDS and plated out at 0.5ml/well in 12-well plates, which were then incubated for 4h at 37 °C in air containing 5% (v/v) CO_2. The DNA/ Superfect™ complex was prepared by diluting DNA (1µg ABCA1 promoter construct/$6x10^5$ cells) to 50µl/µg of DNA with antibiotic- and serum-free RPMI 1640 medium. Superfect™ solution (3µl/µg DNA) was added and the mixture was vortexed for 10sec and incubated for 10min at room temperature. The complex was then diluted with complete media containing 3% LPDS (200µl/µg of DNA). Subsequently, the mixture was added drop wise to the cells and the plates were centrifuged (800g, 5min) to ensure uniform distribution of the complexes. PMA (0.16µM) along with the ligands were added to each well following the transfection and the cells were returned to the incubator for a further 18h. For co-transfection experiments, the cells were transfected with 1µg of expression plasmid and 5µg of promoter-reporter DNA plasmid as described above.

2.2.7.3 Transient transfection of THP-1 cells using Effectene™ transfection method

Transient transfection with Effectene™ was carried out as described by the manufacturer (Qiagen) for suspension cells. For each transfection, 0.4µg of the ABCA1 promoter construct was made up to 100µl with EC buffer along with 3.2µl of the Enhancer reagent (both provided in the kit). The solution was mixed by vortexing and incubated at room temperature for 5min. The DNA-Enhancer complex solution was then centrifuged (800g, 30sec) and 10µl of Effectene™ reagent was added and mixed by pipetting. Then, 600µl of complete medium plus 10% (v/v) LPDS was added to the transfection complex and left to incubate for 10min at room temperature. The mixture was added drop wise to the cells and the plates were swirled to ensure uniform distribution of the complexes. PMA (0.16µM) was added to each well following the

transfection and the cells were returned to the incubator for a further 24h. The ligands were then added as usual and the cell left in the incubator for a further 24h and then harvested.

2.2.7.4 Transient transfection of THP-1 cells using Interferin™ small interfering RNA (siRNA) transfection method

siRNA interacts selectively with a single target sequence within mRNA, thereby providing sequence specific mRNA degradation and inhibition of protein production. The transfection with Interferin™ was carried out as described by the manufacturer's protocol (Autogen Bioclear) for suspension cells. Twenty four hours prior to transfection, the cells were split in a ratio of 1 to two. On the day of transfection, cells were suspended at a density of 2-3x10^5 cells/ml in RPMI 1640 medium supplemented with 10% (v/v) LPDS and plated out at 0.5ml/well in 12-well plates, and then incubated for 4h at 37°C in air containing 5% (v/v) CO_2. For THP-1 cells, 50-60% approximately silencing was typically observed with Interferin™ using 5nM siRNA concentration. Thus, 5nM of siRNA duplexes were diluted into 200µl of serum free medium. The siRNA solution (200µl) was then vortexed gently and 10µl of Interferin™ was added. The mixture was then homogenized for 10sec and then incubated for 10min at room temperature to allow complex formation to take place. The Interferin™/siRNA mixture was added dropwise to the cells and the plates were swirled to ensure uniform distribution of the complexes. The cells were returned to the incubator for a further 24h. Twelve hour post transfection, 1.3ml of complete medium supplemented with 10% LPDS (v/v) was added. PMA (0.16µM) along with the diluted mixture was added to each well. Twenty four hours post PMA addition, the ligands were added as usual and the incubation contained for a further 24h.

2.2.7.5 Preparation of cell extracts for the determination of reporter gene activity

The cells were harvested by scraping and washed once with PBS. They were then resuspended in 120µl of 1x passive cell lysis buffer (Promega) by vortexing and then left on ice for 10min. Following centrifugation in a microfuge at 13,000rpm for 2min, the supernatant was transferred to a fresh microcentrifuge tube and either stored at -80°C until required or used immediately for the measurement of reporter gene activity.

2.2.7.6 Measurement of luciferase activity

Cell extracts and firefly luciferase assay reagent (Promega) were both equilibrated to room temperature. The luciferase activity was determined by mixing 20µl of cell extracts with 100µl of luciferase assay reagent in a luminometer tube. The luciferase activity was measured using a Turner Designs (TD-50/50) Luminometer set at a sensitivity value of 70% with a 2sec delay period and a 20sec integrate period. The number of counts recorded by this instrument correspond to the amount of luciferase expressed from the reporter plasmids. All measurements were performed in duplicate and the average counts in cell extracts were normalised to the amount of protein in each lysate (determined as described in section 2.2.5.3.). This approach was used rather than the use of a control promoter plasmid linked to another reporter gene since most such promoters are themselves regulated by lipid metabolite products and cytokines (Foka, P., personal communication).

2.2.8 Electrophoretic Mobility Shift Assays (EMSA)

2.2.8.1 Generation of double-stranded oligonucleotides

The sequences of the oligonucleotides used for EMSA are shown in Table 2.12. Each single-stranded oligonucleotide was designed in such a way so that following annealing with the complementary oligonucleotide, 5' overhangs, with at least one G residue remained on either side of the double-stranded pair. This allowed radiolabelling by "fill in" reaction using Klenow polymerase and [α-^{32}P] dCTP.

For annealing, 200ng of each forward and the corresponding reverse oligonucleotides were incubated at 100°C for 10min in the presence of a medium salt buffer (1x NEB Buffer 3) in a final volume of 100µl. The mixture was allowed to cool down slowly to room temperature. The double stranded-oligonucleotides were stored at -20°C or radiolabelled immediately.

2.2.8.2 Preparation of radiolabelled probe of double-stranded oligonucleotides

The annealed double-stranded oligonucleotides with 5' overhangs (10µl) were diluted to 35µl with sterile water. Then, 10µl of labelling buffer and 2µl of Klenow polymerase (both provided in Megaprime™ labelling kit, GE Healthcare) along with 3µl of [α-^{32}P] dCTP (GE Healthcare) was added and the mixture incubated for 30min at 37°C. The reaction was stopped by the addition of 350µl of 1xTE buffer. The labelled probe was then separated

Table 2.12: Sequences of oligonucleotide probes for EMSA analysis

Probe	Sequence of Oligonucleotides
AP-1	5' CGCTTGATGAGTCAGCCGGAA 3' 3' GCGAACTACTCAGTCGGCCTT 5'
ABCA1	5' GCTGAGTGACTGAACTACATAAA 3' 3' GACTCACTGACTTGATGTATTTGG 5'
DR4	5' TTTGACCGATAGTAACCTC 3' 3' GGCTATCATTGGAGACGCG 5'
apoE	5' GGGTTCAAGCGATTCTCCTGCCTCAGCCTCCCAA 3' 3'AGTTCGCTAAGAGGACGGAGTCGGAGGGTTCATCG 5'
C/EBP	5'CAGTGTTTCCAGAC 3' 3' ACAAAGGTCTGGTT 5'
NFκB	5'AGTTGAGGGGACTTTCCCAGG 3' 3'TCAACTCCCCTGAAAGGGTCCG 5'
NF-1	5' GCCTTGGCATTA 3' 3' GAACCGTAATCG 5'

from unincorporated nucleotides by passing the mixture through a pre-packed 1ml Sephadex G50 column (Nick column, Pharmacia), which had been pre-equilibrated with 9ml of 1xTE buffer. Then, 400µl of 1xTE buffer was added on top of the column to elute the DNA. The second fraction which contained the radiolabelled probe was collected in an Eppendorf tube. The later fractions, containing unincorporated radioisotope, were discarded. The probe was stored at -20°C for a week.

2.2.8.3 DNA/protein binding reactions for EMSA

The binding of the [α-^{32}P] labelled probes to the proteins in whole cell or nuclear extracts was carried out according to Ramji et al. (1993). Briefly, 5-10µg of whole cell extracts or 3-5 µg of nuclear extracts were mixed with dilution buffer (Table 2.4) to a final volume of 26µl. Then, 2µl of 10x binding buffer (Table 2.4) and 2µl of poly dI-dc (1µg/ml) were added to the reaction mixture, which was incubated on ice for 10min to allow any non-specific DNA binding to occur. Following addition of 3µl of labelled probe (60,000-100,000 c.p.m.), the mixture was left for 20min at room temperature. Subsequently, 12µl of 20% (w/v) Ficoll was added and the mixture was subjected to electrophoresis (section 2.2.8.6).

2.2.8.4 Competition binding assays

In order to determine the specificity of DNA-protein interactions, an excess quantity of unlabelled specific- and non-specific-oligonucleotides were added to the whole cell or nuclear extracts during the binding reaction. The specific oligonucleotide contained the

identical sequence to that of the radiolabelled probe and the non-specific oligonucleotide contained an unrelated sequence. The procedure was similar to that described for EMSA with the exception of the presence of a 200-fold molar excess of the competitor oligonucleotide prior to the addition of the radiolabelled probe.

2.2.8.5 Antibody supershift assays

Appropriate antibody (2µl) was added to whole cell or nuclear extracts during the binding reactions and prior to the addition of the radiolabelled probe. The reaction mixture was incubated on ice for 30min and subjected to electrophoresis following the addition of 12µl of 20% (w/v) Ficoll as described in section 2.2.8.6. Rabbit pre-immune serum was used as a control for the antibody.

2.2.8.6 Electrophoresis of DNA-protein complexes

DNA-protein complexes were separated from the DNA probe by electrophoresis on a 4-6% (v/v) non-denaturing polyacrylamide gels containing 0.5xTBE (Table 2.13). The vertical gel electophoresis was performed in a cold cabinet at 4°C either overnight at 40V or for 4h at 150V in 0.5xTBE "running" buffer. The progress of electrophoresis was monitored by loading 50µl of 10x DNA loading dye (Table 2.2) to a lane in the gel. The gel was "pre-run" for 30min prior to the loading of the samples. Following electrophoresis, the gel was removed from between the glass plates, transferred to a Whatman 3MM paper, covered with Saran Wrap and dried under vacuum using a gel dryer (model 583 Gel Dryer (Bio-Rad)) for 1h at 80°C. The dried gel was subjected to autoradiography.

Table 2.13: Composition of non-denaturing polyacrylamide gels for EMSA analysis

Component	4% (w/v) Acrylamide	6% (w/v) Acrylamide
Acrylamide:bisacrylamide (29:1)	5ml	7.5ml
10x TBE	2.5ml	2.5ml
ddH2O	42.5ml	40ml
10% APS	500µl	500µl
TEMED	50µl	50µl

2.2.8.7 Autoradiogaphy

The dried gel was placed in contact with Kodak X-ray film in a Hi-speed-X light proof cassette with an intensifying screen (Genetic Research Instrumentation) and stored at -80°C for varying exposure times (6-72h), depending on the strength of the signal. The film was developed in a Gevamatic 60 automatic developer (Agfa-Gevaert).

2.2.9 Densitometric analysis of the data

The intensity of bands from agarose gel images and immunoblots were analysed using GeneTools™ (Syngene) software as described by Harvey et al. (2007). Also, see section 2.2.4.5 for a detailed account on the densitometric analysis of data generated by RT-PCR.

2.2.10 Statistical analysis of the data

To assess data for statistical significance a standard student's t-test was carried out, the details of which are in the Appendix IV.

CHAPTER THREE:

MECHANISMS UNDERLYING THE REGULATION OF ABCA1 GENE EXPRESSION BY LXR AGONISTS IN J774.2 MACROPHAGES

Chapter 3: Mechanisms underlying the regulation of ABCA1 gene expression by LXR agonists in J774.2 macrophages

3.1 Introduction

The aim of the work presented in this chapter was to understand the mechanisms underlying the regulation of ABCA1 expression by LXR agonists. Such agonists reduce the transformation of macrophages into foam cells by increasing cellular cholesterol efflux. Specifically in mouse models of atherosclerosis, inactivation of LXRs promote the development of this disease whereas treatment with LXR agonists reduces lesion formation (Aiello, 2002; Van Eck et al., 2002). Little is known about the mechanisms underlying LXR-regulated expression of this gene, especially the signalling pathways. Therefore, the potential signalling pathways involved in LXR-mediated regulation of ABCA1 expression were studied.

Previous studies in the laboratory on LXR-mediated regulation of gene expression were carried out on the THP-1 cell line. A major problem with these cells is that THP-1 monocytes have to be differentiated into macrophages using PMA, thereby adding an additional effector in the analysis. The murine macrophage cell line, J774.2, already consists of adherent macrophages. These cells have therefore been used extensively to investigate macrophage gene expression relevant to atherosclerosis (Charriere et al., 2003; Harvey et al., 2007; Mead et al., 2002; Mitchell et al., 1993; Wang et al., 2007). The J774.2 cell line was therefore selected as a model system for initial studies in this thesis as it most closely represents differentiated macrophages (Ralph and Nakoinz, 1975; Ralph et al., 1975). The pattern of gene expression in these cells are well-conserved with human macrophages (Mcknight et al., 1996; Mead et al., 2002; Naureckiene et al., 2007; Preiss-Landl et al., 2002) In addition, studying signalling mechanisms in a murine cell line allows direct comparisons with the widely used *in vivo* mammalian model for atherosclerosis development (the apoE-null and LDLR-null mice).

There is good evidence that ABCA1 mRNA levels are induced in response to treatment of different cell lines with natural or synthetic LXR agonists (Joseph et al., 2002b; Repa et al., 2002; Repa et al., 2000a; Repa et al., 2000b; Tangirala et al., 2002). The primary aim of the research presented in this chapter was to investigate whether this response could be reproduced in the laboratory using J774.2 macrophages. The secondary aim was to

delineate the cell signalling pathways in LXR-mediated activation of ABCA1 expression in macrophages. This aim was achieved initially by investigating the effect of specific pharmacological inhibitors of various signalling pathways on the induction of ABCA1 gene expression by LXR agonists. Key findings from these experiments were then confirmed with the use of DN mutants against central components of the identified signalling pathways. A summary of the experimental strategy used is shown in Figure 3.1.

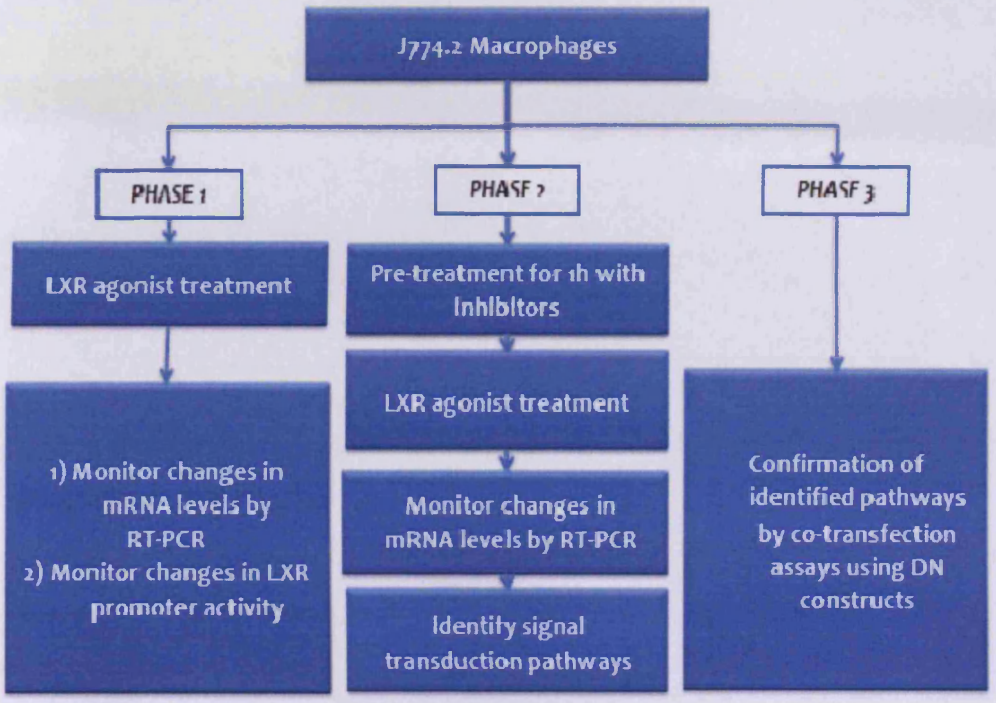


Figure 3.1 Summary of the experimental strategy used to delineate the signal transduction pathways involved in the LXR agonist-mediated ABCA1 gene expression in J774.2 macrophages.

3.2 Results

3.2.1 Experimental design

3.2.1.1 Cell culture

In order to investigate the regulation of ABCA1 gene expression by LXR agonists, J774.2 and Hep3B cell lines were used as model systems in the studies presented in this chapter. Both cell lines have previously been used widely as *in vitro* models for studying the mechanisms involved in atherogenesis (Kennedy et al., 2001; Ralph and Nakoinz, 1975; Ralph et al., 1975; Singaraja et al., 2001; Tengku-Muhammad et al., 1996). J774.2

macrophages were grown until they were about 60-70% confluent and, prior to stimulation with ligands for 12h and 24h, they were cultured for 4h in DMEM medium containing 10% (v/v) LPDS. For all experiments, it was necessary to use 10% (v/v) delipidated serum (employed by numerous laboratories with such studies) (Beyea et al., 2007; Costet et al., 2003; Gerbod-Giannone et al., 2006; Quinn et al., 2005) to reduce the potential contribution of lipids present in the HI-FCS to LXR-induced expression of the genes analysed. For studies with pharmacological inhibitors, these were added to the cells 1h prior to treatment with LXR agonists in order to ensure cellular uptake and inhibition of the target enzymes. The action of the inhibitors was not due to any cytotoxic effect as judged by analysis of cell viability using the trypan blue exclusion assay. The concentration of the ligands used was 2μg/ml for 22(R)-hydroxycholesterol (denoted 22(R)-HC) (Laffitte et al., 2001b) and 10μM for 9-*cis*-retinoic acid (denoted 9CRA) (Costet et al., 2000). In addition cells in some experiments were treated with the ligand 22(S)-hydroxycholesterol (denoted 22(S)-HC) (2μg/ml) (Laffitte et al., 2001b), an inactive enantiomer of 22(R)-HC, as a control as it binds to but does not activate the LXRs. The ligands were dissolved in DMSO in such a way so that the final concentration of this vehicle was less than 0.1% (v/v) to ensure that it would not affect cell viability. Also, as an additional control, all control samples were treated with an equal volume of DMSO to ensure that the observed effects were due to the ligands and not the DMSO vehicle. After this incubation, the cells were harvested and total RNA was isolated and used for RT-PCR analysis. The integrity of total RNA preparation was analysed by resolving an aliquot on 1% (w/v) agarose gels. As expected for total RNA, the relative intensity of the 28SrRNA band was approximately twice the intensity of the 18SrRNA band, for each sample.

3.2.1.2 RT-PCR

The PCR technology originated in 1986 as a non-cloning technique for direct amplification of a specific short segment of DNA. In the exponential phase, the amount of amplified product is proportional to the amount of template DNA sequence. The value of PCR is that it can use tiny amounts of various types of DNA, such as genomic DNA. To carry out PCR, the target DNA is mixed with a thermostable DNA polymerase such as *Taq* DNA polymerase. In addition to a supply of deoxyribonucleotide triphosphates, two specific oligonucleotide primers, one that complements the 3' end on one DNA strand and another that complements the 3' end on the opposite strand,

are used. The PCR product and appropriate size markers are subjected to electrophoresis. The resulting ethidium bromide stained bands on an agarose gel are placed under UV light for viewing.

A PCR cycle consist of three steps; denaturation, primer annealing and elongation. The first step is denaturation, where the reaction mixture is heated to typically 94 °C for a short time period to denature the target DNA into single strands that can act as templates for DNA synthesis. The second step is primer annealing, where the mixture is rapidly cooled to a defined temperature which allows the two primers to bind to the sequences on each of the two strands flanking the target DNA. The third step is elongation, where the temperature of the mixture is raised to ~72 °C and kept at this temperature for a pre-set period of time to allow DNA polymerase to elongate each primer by copying the single stranded templates. These three steps of PCR cycle are repeated for a set number of times depending on the degree of amplification required. As more and more reaction cycles are carried out, the original DNA is amplified and at this point the vast majority of the products are identical; in that the DNA amplified is only that between the two primer sites.

PCR can also be used for amplifying specific RNA molecules following conversion of total or poly (A)+ RNA to cDNA using reverse transcription (called reverse transcription polymerase chain reaction or RT-PCR). The test PCR product along with that for the control gene (such as GAPDH and 28SrRNA) are subjected to electrophoresis on an agarose gel. The images from ethidium bromide-stained gels are captured as a digital image. GeneTools ™ Band analysis software (Syngene) is used to quantify the intensities of the signal from the test PCR bands and the control gene PCR product, and a ratio of the target gene to control gene is determined. Although, this technique is rapid, it does require extensive optimisation to ensure that the PCR product is terminated when both the gene of interest and the internal control are in the exponential phase of amplification.

An improvement on PCR technology is real-time PCR (also abbreviated as RT-PCR). With this, the PCR sensitivity is enhanced by the addition of a fluorogenic probe that emits fluorescence that accumulates in the mixture and can be detected and quantified in real time (Higuchi et al., 1992; 1993). The greater the amount of target DNA that accumulates during the PCR cycle, the more intense the fluorescence which can be measured by

using a charge-coupled device camera (Higuchi et al., 1992; 1993). Another type of basic real-time PCR works by monitoring the hybridization of a set concentration of fluorescently labelled probe oligonucleotide to the PCR product. The more the PCR product, the more the hybridization occurs and so the more fluorescence is observed that is dependent on the specific method utilized. Real time PCR has provided sensitivity, reproducibility and considerably reduced risk of carryover contamination (Mackay et al., 2007).

Real time PCR has also given rise to related technologies that make it even more valuable than before. As detailed above, Real time PCR can make use of cDNA, for example, and thus apply the PCR technology to mRNA and even make the analysis quantitative, an approach known as Q-PCR (Cooper, et al., 2003; Bustin et al., 2005). Q-PCR allows reliable detection and measurement of products generated during each cycle of the PCR process, which are directly proportional to the amount of template prior to the start of the PCR process (Ginzinger, 2002). Some Q-PCR applications include measuring mRNA expression levels, DNA copy number, transgene copy number and gene expression analysis, allelic discrimination and measuring viral titers (Ginzinger, 2002; Ding et al., 2004).

In general quantitative PCR methods tend to be cumbersome and, additionally, require the use of a specialised real time PCR machine that may not be accessible to all scientists. It must also be accompanied by a method for detecting PCR product accumulation and an instrument to perform thermocycling and record the results during each PCR cycle in real time (Ginzinger, 2002). At present, real-time Q-PCR is beginning to be used a lot in a wide range of applications because of reduction in cost of reagents and the instrumentation required. Researchers are using the technology for measuring gene expression in cells that are sparse in numbers and difficult to isolate, as well as for analysis of clinical samples as an aid to treating patients (Ginzinger, 2002). As new developments in Q-PCR and real time PCR arise, they should allow greater advances in science and medicine.

It should be noted that RT-PCR quantifies steady state mRNA levels and hence the changes observed could be either due to gene transcription or mRNA stability. RNA levels may not reflect the level of protein produced by the cell (Gygi et al., 1999) as many types of regulation occur at the post-transcriptional level (Bustin, 2002). In general, to

investigate the physiological changes in gene expression, the relative expression ratio is often adequate as it is based on the relative expression of a target gene versus a reference gene (Pfaffl 2001). Therefore, the use of semi-quantitative RT-PCR was justified in the studies presented in this chapter as the overall trend in the changes of ABCA1 mRNA expression was confirmed and extended by analysis of the changes in steady state protein levels using Western blotting (See chapter 4). Additionally, the conditions for semi-quantitative RT-PCR had already been optimised when the studies were carried out and the procedures used standard equipment already present in the laboratory with an overall low cost.

The induction of ABCA1 specific mRNA was measured by semi-quantitative RT-PCR using GAPDH or 28SrRNA as a control for cDNA input. Previous optimisation experiments in the laboratory, involving titration of the number of amplification cycles, showed that the chosen conditions (i.e. 24 cycles for ABCA1 and 17 cycles for GAPDH) were within the exponential phase of amplification and, therefore, provided a direct correlation between the amount of amplification product and RNA template abundance in the sample. The PCR products were size-fractionated by electrophoresis on 1.5% (w/v) agarose gels and the signals were quantified using Syngene System Tools software (see Materials and Methods). The software assigns numerical values to each of the PCR products under analysis relating it directly to the intensity of the PCR product. The signals from the ABCA1 gene were normalised to that for GAPDH or 28SrRNA, with the ratio in vehicle-treated cells being arbitrarily assigned as 1 in each case.

3.2.1.3 Cell transfection

For transfection experiments, the Hep3B cells were split 24 hours prior to the experiment. On the day of the transfection, the cells were suspended in 6-well plates containing 2ml of fresh medium containing 10% (v/v) HI-FCS. For each well, 1ml of PEI-DNA complex was added (see Materials and Methods). Following transfection, cells were incubated for 24h in DMEM supplemented with 10% LPDS and either 10μM of 9CRA plus 2μg/ml of 22(R)-HC or DMSO as a vehicle control. Cells were then harvested and extracts prepared for luciferase assays. The luciferase activity was normalized to the concentration of the cellular proteins. All transfections were carried out in triplicate and repeated at least three times. Results are presented as mean fold induction (mean ±SD),

with basal level arbitrarily assigned as 1. A standard statistical t-test was carried out on the data and changes in expression were found to be significant to the level indicated.

3.2.2 The effect of oxysterols on ABCA1 mRNA expression in J774.2 cells

Before carrying out detailed studies on the actions of LXR agonists in J774.2 macrophages, it was necessary to perform some experiments to confirm typical LXR responses in these cells. The first response that was studied was the induction of ABCA1 expression by the oxysterol 22(R)-HC using the inactive enantiomer, 22(S)-HC, for comparison. As shown in Figure 3.2, the mRNA levels of ABCA1 were induced in a statistically significant manner in response to 22(R)-HC treatment at both 12h and 24h. The maximal increase in mRNA levels was observed at 12h following incubation with this ligand. Unlike 22(R)-HC, the inactive enantiomer 22(S)-HC, which is not able to activate the LXRs, did not induce ABCA1 mRNA levels (Figure 3.2). Relative fold induction of ABCA1 mRNA expression after normalization to the 28S rRNA levels was approximately 4-fold following 12h incubation of the cells with 22(R)-HC. A similar induction has previously been reported in the murine RAW 264.7 cell line (Claudel et al., 2001).

As described in the General Introduction, LXR functions in cells as a heterodimer with its obligate partner RXR on target gene promoters. Therefore, we decided to investigate whether the endogenous ABCA1 gene can be activated by the oxysterol 22(R)-HC and 9CRA, either alone or in combination, in J774.2 macrophages. The RT-PCR data shown in Figure 3.3 demonstrates a significant increase in ABCA1 mRNA expression in cells treated with 22(R)-HC (approximately 2-fold induction) or 9CRA (about 2-fold induction). A more pronounced induction in expression of about 5-fold was obtained with combined treatment of both ligands. A similar trend has been seen in THP-1 macrophages (Costet et al., 2000).

3.2.3 Transient transfection of Hep3B cells using LXR-promoter luciferase DNA constructs

In order to delineate the mechanisms by which LXR/RXR heterodimer regulates gene expression (e.g. signal transduction pathways, coactivator/corepressor requirements), it is useful to have a functional *trans*-activation assay. Such an assay can be used to delineate sequence requirements for particular responses and also allows the use of DNA constructs specifying for DN or constitutively active forms of key components of

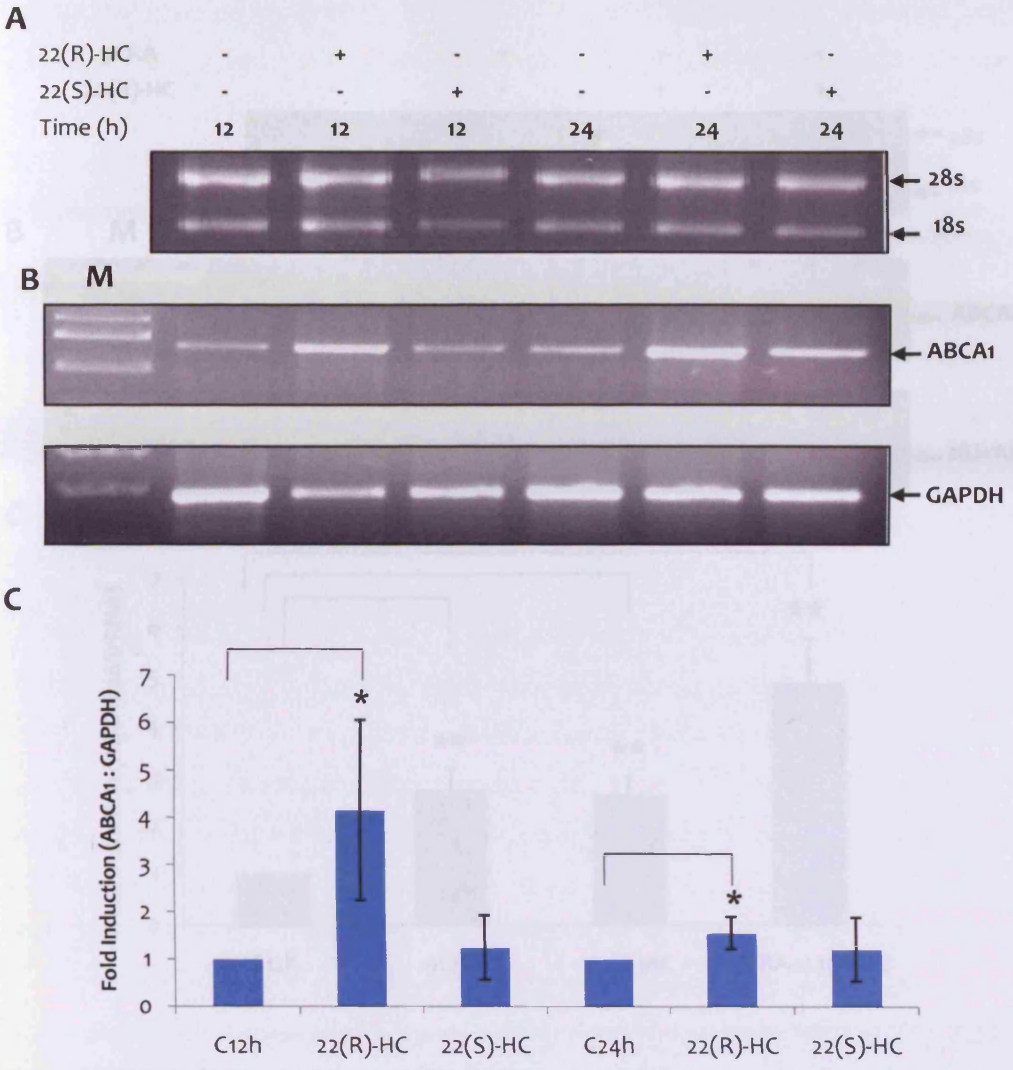


Figure 3.2 RT-PCR analysis of ABCA1 mRNA expression in J774 macrophages following exposure to oxysterols.

The cells were incubated with DMEM containing 10% (v/v) LPDS for 4h and then either treated with vehicle DMSO (C12h and C24h) or exposed to the oxysterols, 22(R)-hydroxycholesterol (donated 22(R)HC) [2μg/ml] and 22(S)-hydroxycholesterol (donated 22(S)HC) [2μg/ml)] for 12h and 24h. The quality of total cellular RNA was assessed by electrophoresis on 1% (w/v) agarose gel (panel A). Then, cDNA was prepared against total cellular RNA and used in PCR reactions in which primers against ABCA1 or GAPDH were present. The amplification products were size-fractionated by electrophoresis on a 1.5% (w/v) agarose gel (panel B), M corresponds to the 1 kbp molecular weight markers. The ABCA1 and GAPDH signals at each point were determined by densitometric analysis. The ABCA1:GAPDH ratio for the C12h and C24h samples were arbitrarily assigned as 1 with those from the other samples shown as fold induction (mean ±SD) from four independent experiments (panel C; *P<0.05 compared with control)

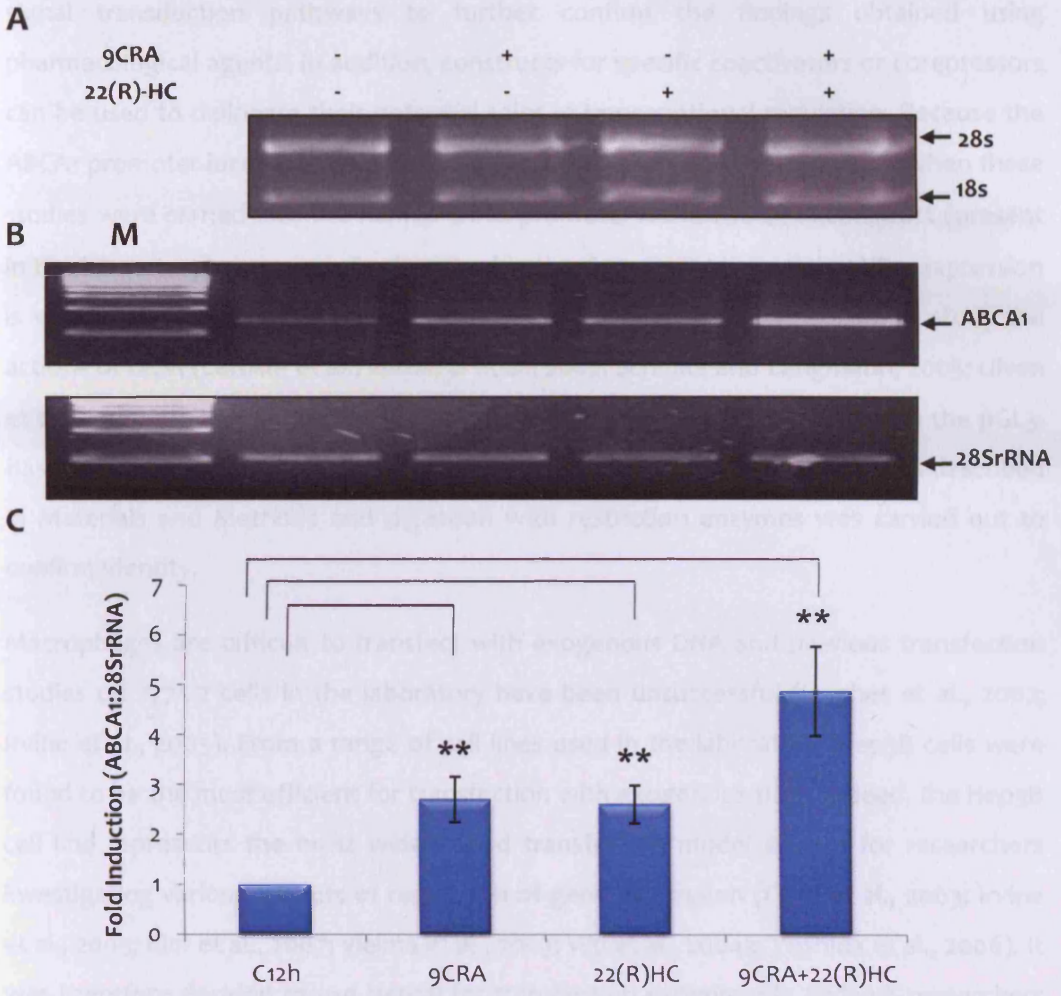


Figure 3.3 Effect of LXR agonists on ABCA1 mRNA expression in J774 macrophages.

The cells were incubated with DMEM containing 10% (v/v) LPDS for 4h and then either treated with the vehicle DMSO (C12h) or exposed to the RXR ligand 9CRA [(10μM)] or the LXR ligand 22(R)HC (2μg/ml) for 12h. The quality of total cellular RNA was assessed by electrophoresis on 1% (w/v) agarose gel (panel A). Then, cDNA was prepared against total cellular RNA and used in PCR reactions in which primers against ABCA1 or 28SrRNA were present. The amplification products were size–fractionated by electrophoresis on a 1.5% (w/v) agarose gel (panel B), M corresponds to the 1 kbp molecular weight markers. The ABCA1 and 28SrRNA signals at each point were determined by densitometric analysis. The ABCA1:28SrRNA ratio for the C12h sample was arbitrarily assigned as 1 with those from the other samples shown as fold induction (mean ±SD) from three independent experiments (panel C; $^{**}P<0.01$ compared with C12h).

signal transduction pathways to further confirm the findings obtained using pharmacological agents. In addition, constructs for specific coactivators or corepressors can be used to delineate their potential roles in transcriptional regulation. Because the ABCA1 promoter-luciferase DNA construct was unavailable in the laboratory when these studies were carried out, the human LXRα promoter-luciferase DNA construct (present in the laboratory) was used. As described in the General Introduction, LXRα expression is subject to autoregulation and its promoter has been used previously to study the actions of LXRs (Laffitte et al., 2001a; Li et al., 2002; Schmitz and Langmann, 2005; Ulven et al., 2004; Whitney et al., 2001). The -2625hLXRα-Luc DNA construct was in the pGL3-Basic cloning vector (Appendix III). Recombinant plasmid DNA was purified as described in Materials and Methods and digestion with restriction enzymes was carried out to confirm identity.

Macrophages are difficult to transfect with exogenous DNA and previous transfection studies on J774.2 cells in the laboratory have been unsuccessful (Hughes et al., 2002; Irvine et al., 2005). From a range of cell lines used in the laboratory, Hep3B cells were found to be the most efficient for transfection with exogenous DNA. Indeed, the Hep3B cell line represents the most widely used transfection model system for researchers investigating various aspects of regulation of gene expression (Foka et al., 2003; Irvine et al., 2005; Kim et al., 2007; Vielma et al., 2003; Wu et al., 2004a; Yoshida et al., 2006). It was therefore decided to use Hep3B for transfection experiments. Indeed, researchers in the LXR field commonly use these cells or other hepatoma cell lines for analysis (Aravindhan et al., 2006; Jakel et al., 2004; Jaye et al., 2005; Kennedy et al., 2001; Liao et al., 2002; Martin et al., 2000; Menke et al., 2002; Oberkofler et al., 2004; Singaraja et al., 2001).

The transfection was facilitated by the use of the PEI reagent. The amino group in this reagent interacts with negatively charged DNA but leaves the molecule with a net positive charge that binds to the negatively charged cell membranes. The -2625hLXRα-Luc was transiently transfected into Hep3B cells as described in Materials and Methods. As shown in Figure 3.4, combinations of 22(R)-HC plus 9CRA activated the LXRα promoter activity by approximately four fold. In contrast, no such effect was seen with the pGL3-Luc parent reporter plasmid only, which produced low, background activity

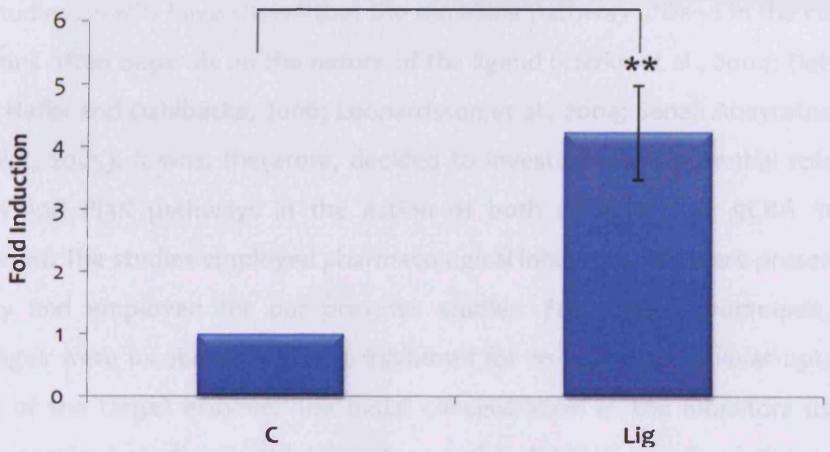


Figure 3.4 LXR/RXR heterodimers activate the LXR gene promoter in a heptoma cell line.

Hep3B cells were transfected with 5μg of the LXRα promoter-luciferase DNA construct using the PEI reagent. Following transfection, cells were incubated for 24h in DMEM supplemented with 10% LPDS containing either the DMSO vehicle (C) or 22(R)-HC plus 9CRA (2μg/ml and 10μM, respectively; Lig). The cells were then harvested and luciferase activity and protein concentration was determined as described in Materials and Methods. Relative luciferase activity was normalised to protein concentration and values are expressed as mean fold induction (the value in vehicle-treated cells has arbitrarily been assigned 1). Bars indicate mean ±SD with significance of induction versus control shown by $^{**}P<0.01$. The results represent the outcome of three independent experiments each of which was carried out in triplicate.

(data not shown). This result confirms that the LXRα promoter is activated by LXR agonists and that Hep3B cells represent an efficient system to study promoters regulated by this NR

3.2.4 Effect of pharmacological inhibitors of known signal transduction pathways on the LXR/RXR-mediated induction of ABCA1 mRNA expression

Previous studies in our laboratory have established a role for the JNK/SAPK and PI3K pathways in the 22(R)-HC-mediated regulation of apoE gene expression (Greenow, K., 2004). A major aim of the studies presented in this thesis was to investigate the signalling pathways involved in the LXR agonist-induced expression of the ABCA1 gene. Several studies on NRs have shown that the signalling pathway utilised in the control of their actions often depends on the nature of the ligand (Clarke et al., 2004; Debevec et al., 2007; Hafizi and Dahlbäcka, 2006; Leonardsson et al., 2004; Senali Abayratna Wansa and Muscat, 2005). It was, therefore, decided to investigate the potential role of the JNK/SAPK and PI3K pathways in the action of both 22(R)-HC and 9CRA in J774.2 macrophages. The studies employed pharmacological inhibitors that were present in the laboratory and employed for our previous studies. For these experiments, J774.2 macrophages were incubated with the inhibitors for 1h to ensure cellular uptake and inhibition of the target enzyme. The initial concentration of the inhibitors used was based on previous studies in the laboratory and a detailed search of the published literature. Where DMSO was used for dissolving the inhibitors, as recommended by the manufacturer, it was ensured that its final concentration that was added to the cells was less than 0.1% (v/v) to avoid a potential effect on cell viability. The untreated cells were also exposed to media containing this amount of DMSO as a vehicle control. The incubation period with the ligands was 12h because maximal induction of ABCA1 expression was seen at this time point (Figure 3.2). The inhibitors used were LY294002 for the PI3K pathway and curcumin and SP600125 for the JNK/SAPK pathway (Bennett et al., 2001; Brouet and Ohshima, 1995; Chen and Tan, 1998; Dorai et al., 2000; Nakahara and Carthew, 2004; Nauc et al., 2004; Sood et al., 2001; Tamagno et al., 2005; Vlahos et al., 1995).

3.2.4.1 Effect of the PI3K inhibitor on LXR/RXR-mediated induction of ABCA1 expression in J774.2 cells

The inhibitor LY294002 prevents PI3K function by acting as a competitor for the ATP-binding site of the enzyme (Vlahos et al., 1995). As shown in Figure 3.5, LY294002

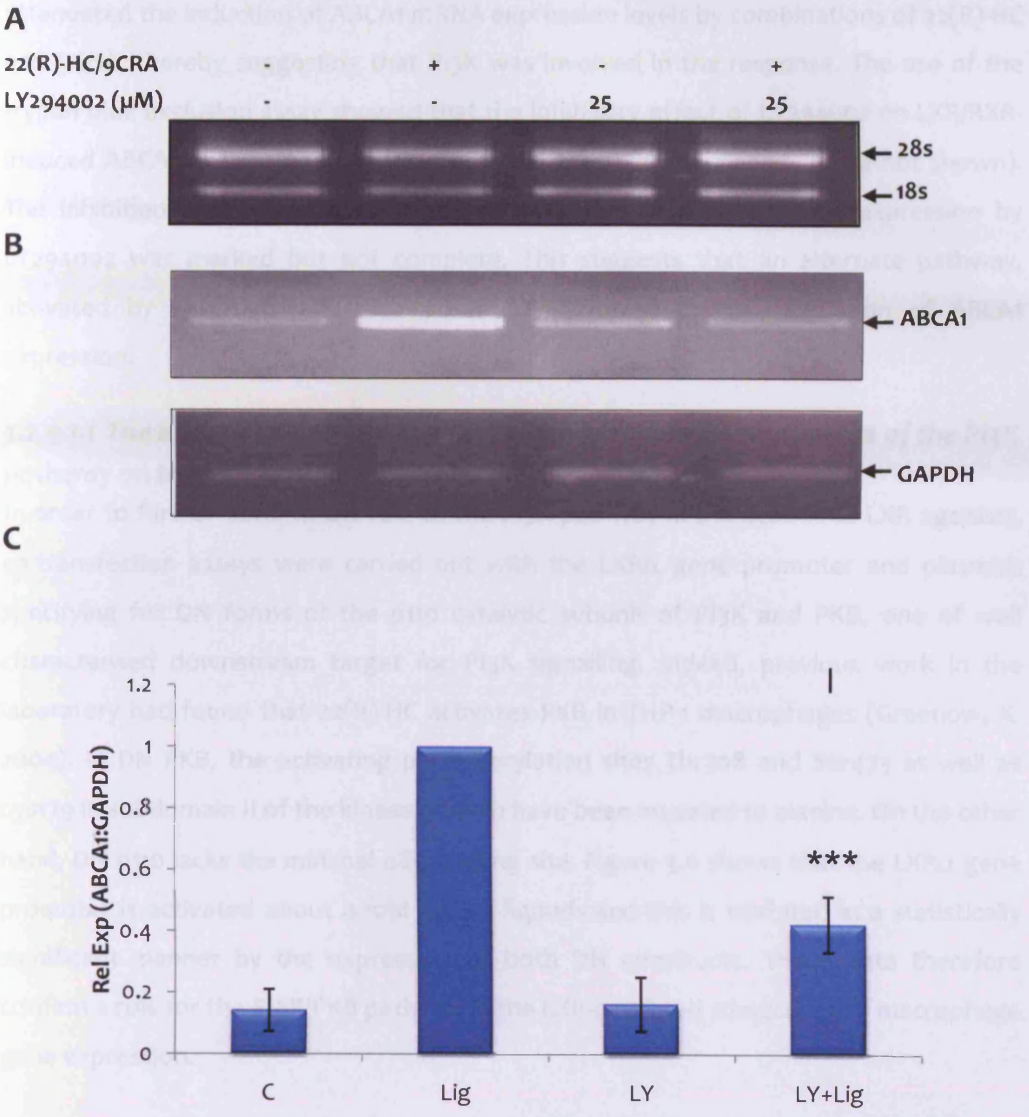


Figure 3.5 The effect of the LY294002 inhibitor on the induction of ABCA1 mRNA expression in J774.2 cells.

The cells were incubated with DMEM containing 10% (v/v) LPDS for 4h and then either treated with the vehicle DMSO (C) or exposed to 22(R)-HC (2μg/ml) and 9CRA (10μM) (Lig) for 12h. The inhibitor LY294002 (LY) was added 1h before the addition of the ligands at a concentration of 25μM. The quality of total cellular RNA was assessed by electrophoresis on 1% (w/v) agarose gel (panel A). Then, cDNA was prepared against total cellular RNA and used in PCR reactions in which primers against ABCA1 or GAPDH were present. The amplification products were size-fractionated by electrophoresis on a 1.5% (w/v) agarose gel (panel B). The ABCA1 and GAPDH signals at each point were determined by densitometric analysis and presented as relative expression (mean ±SD) normalised to the expression of GAPDH from four independent experiments. The relative expression in the presence of the ligand alone (Lig) has been assigned as 1 as shown in panel C. (***$P<0.001$).

attenuated the induction of ABCA1 mRNA expression levels by combinations of 22(R)-HC and 9CRA, thereby suggesting that PI3K was involved in the response. The use of the trypan blue exclusion assay showed that the inhibitory effect of LY294002 on LXR/RXR-induced ABCA1 expression was not due to an effect of cell viability (data not shown). The inhibition of 22(R)-HC/9CRA-mediated induction of ABCA1 mRNA expression by LY294002 was marked but not complete. This suggests that an alternate pathway, activated by LXR/RXR, was also potentially involved in the regulation of ABCA1 expression.

3.2.4.1.1 The effect of dominant negative plasmids against components of the PI3K pathway on the LXR promoter activity

In order to further confirm the role of the PI3K pathway in the actions of LXR agonists, co-transfection assays were carried out with the LXRα gene promoter and plasmids specifying for DN forms of the p110 catalytic subunit of PI3K and PKB, one of well characterised downstream target for PI3K signalling. Indeed, previous work in the laboratory had found that 22(R)-HC activates PKB in THP-1 macrophages (Greenow, K. 2004). In DN PKB, the activating phosphorylation sites Thr308 and Ser473 as well as Lys179 in subdomain II of the kinase domain have been mutated to alanine. On the other hand, DN p110 lacks the minimal p85 binding site. Figure 3.6 shows that the LXRα gene promoter is activated about 4-fold by the ligands and this is inhibited in a statistically significant manner by the expression of both DN constructs. These data therefore confirm a role for the PI3K/PKB pathway in the LXR-mediated stimulation of macrophage gene expression.

3.2.4.1.2 The effect of GSK-3 inhibitors on the LXR/RXR-mediated induction of ABCA1 expression in J774.2 cells

GSK-3 is a well characterized downstream target for PKB actions. The PKB-mediated phosphorylation of serine 21 in GSK-3α and serine 9 in GSK-3β acts to inhibit their enzymatic activity resulting in their deactivation (Cross et al., 1995; Srivastava and Pandey, 1998). GSK-3α/β have been shown to be involved in signalling by several NRs (Salas et al., 2004). Therefore, inhibitors of GSK-3, maleimide derivatives SB216763 and SB415286 (Alonso and Martinez, 2004; Smith et al., 2001), were employed to delineate the role of this enzyme in the regulation of ABCA1 expression by LXR agonists. These two potent and selective GSK-3α/β inhibitors function by competing for ATP binding

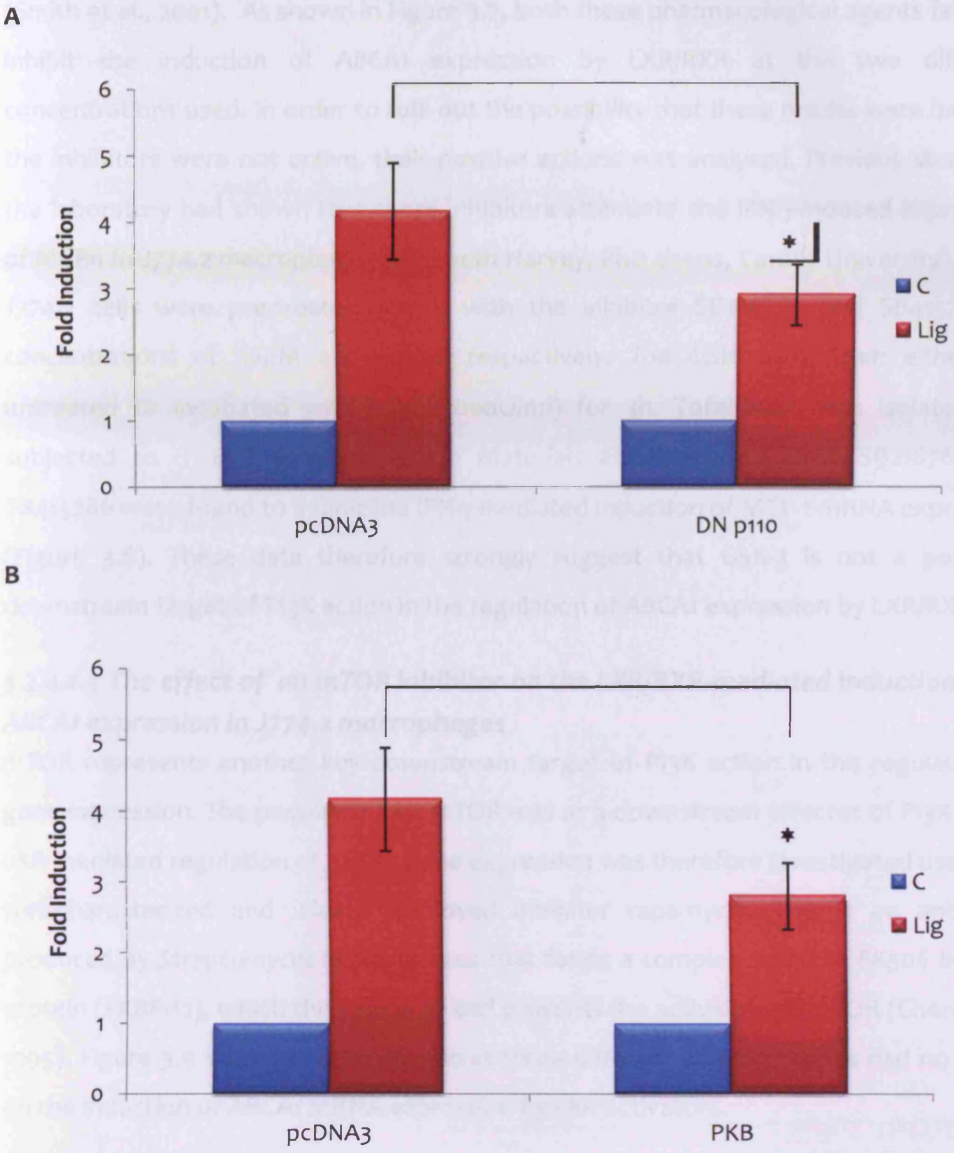


Figure 3.6 Effect of DN constructs against the PI3K pathway on the activity of the LXR promoter.

Hep3B cells were co-transfected with 5μg of the LXRα promoter-luciferase DNA construct and a plasmid construct specifying for a mutant kinase dead form of PI3K p110 (A), a plasmid construct specifying for a DN mutant form of PKB (B) or the vector pcDNA3, using the PEI reagent. Following transfection, cells were incubated for 24h in DMEM supplemented with 10% LPDS containing either the DMSO vehicle (C) or 22(R)-HC (2μg/ml) and 9CRA (10μM) (Lig). The cells were then harvested and luciferase activity and protein concentration determined as described in Materials and Methods. Relative luciferase activity was normalised to protein concentration and values are expressed as mean fold induction (the value in vehicle-treated cells has arbitrarily been assigned 1). Bars indicate mean ±SD with significance of induction versus control shown by *P<0.05. The results represent the outcome of four independent experiments each of which was carried out in triplicate.

(Smith et al., 2001). As shown in Figure 3.7, both these pharmacological agents failed to inhibit the induction of ABCA1 expression by LXR/RXR at the two different concentrations used. In order to rule-out the possibility that these results were because the inhibitors were not active, their positive actions was analysed. Previous studies in the laboratory had shown that these inhibitors attenuate the IFN-γ-induced expression of MCP-1 in J774.2 macrophages (Elizabeth Harvey, PhD thesis, Cardiff University). Thus, J774.2 cells were pre-treated for 1h with the inhibitor SB216763 and SB415286 at concentrations of 30μM and 50μM, respectively. The cells were then either left untreated or incubated with IFNγ (1000U/ml) for 3h. Total RNA was isolated and subjected to RT-PCR as described in Materials and Methods. Both SB216763 and SB415286 were found to inhibit the IFN-γ-mediated induction of MCP-1 mRNA expression (Figure 3.8). These data therefore strongly suggest that GSK-3 is not a potential downstream target of PI3K action in the regulation of ABCA1 expression by LXR/RXR.

3.2.4.1.3 The effect of an mTOR inhibitor on the LXR/RXR-mediated induction of ABCA1 expression in J774.2 macrophages

mTOR represents another key downstream target of PI3K action in the regulation of gene expression. The possibility that mTOR acts as a downstream effector of PI3K in the LXR-mediated regulation of ABCA1 gene expression was therefore investigated using the well-characterized and widely employed inhibitor rapamycin. This is an antibiotic produced by *Streptomyces hygroscopicus* that forms a complex with the FK506 binding protein (FKBP-12), which then binds to and prevents the activation of mTOR (Chen et al., 1995). Figure 3.9 shows that rapamycin at three different concentrations had no effect on the induction of ABCA1 mRNA expression by LXR activators.

In order to rule out the possibility that these results were because the inhibitor was not active, a positive control for its action was performed. A previous study in the laboratory had shown that rapamycin prevents, at least in part, the IFN-γ-mediated inhibition of LPL expression in THP-1 macrophages (Sandra Evans, PhD thesis, Cardiff University). THP-1 monocytes were differentiated with PMA for 24h. The cells were then pre-treated with rapamycin at two different concentrations (150nM and 500nM) for 1h. They were then cultured for 24h in the presence of IFN-γ (1000U/ml) or DMSO (vehicle control). Total

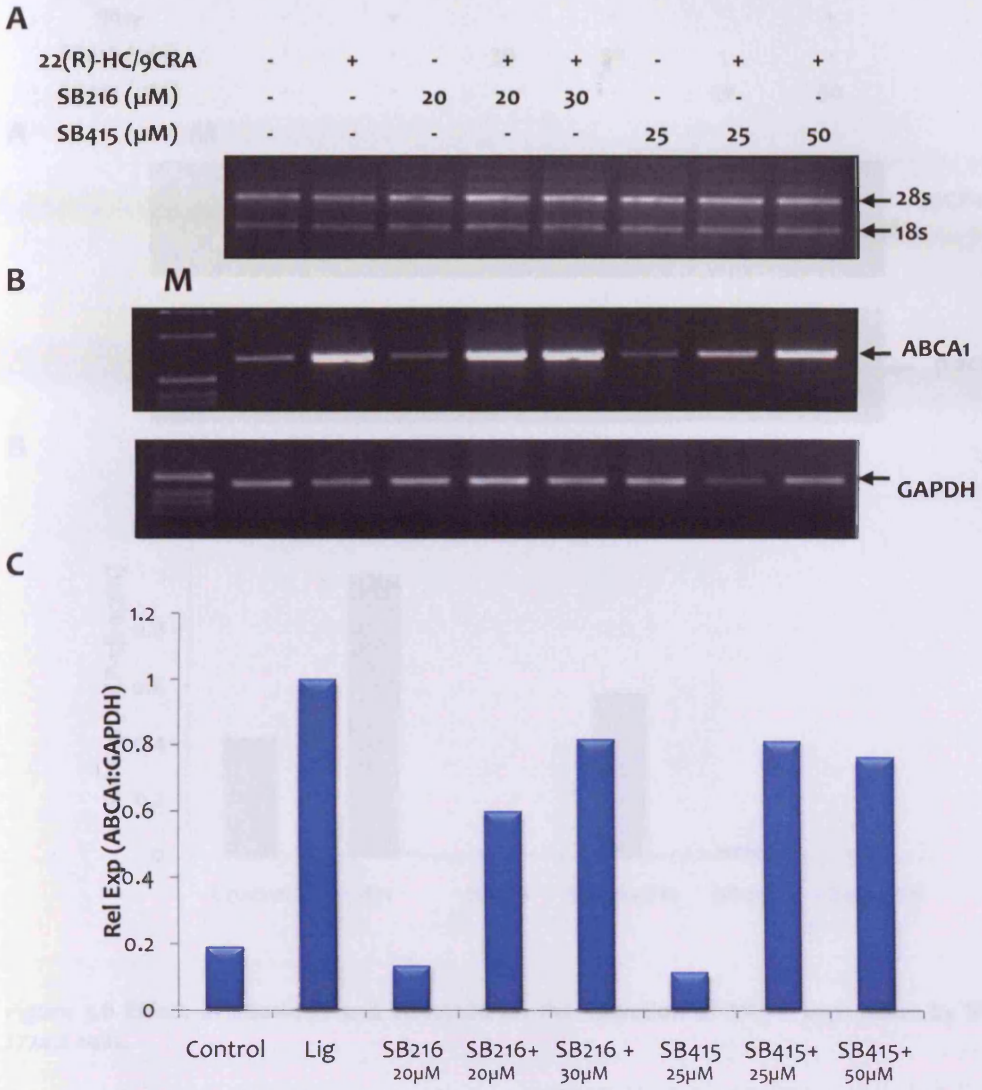


Figure 3.7 The effect of SB216763 and SB415286 on the induction of ABCA1 expression by LXR/RXR in J774.2 cells.

J774.2 cells were incubated with DMEM containing 10% (v/v) LPDS for 4h and then either treated with the vehicle DMSO (control) or exposed to 22(R)-HC plus 9CRA [2μg/ml and 10μM] (Lig) for 12h. The inhibitors SB216763 (SB216) or SB415286 (SB415) were added 1h before the treatment with the ligands at the indicated concentrations. The quality of total cellular RNA was assessed by electrophoresis on 1% (w/v) agarose gel (panel A). Then, cDNA was prepared against total cellular RNA and used in PCR reactions in which primers against ABCA1 or GAPDH were present. The amplification products were size-fractionated by electrophoresis on a 1.5% (w/v) agarose gel (panel B). M corresponds to the 1 kbp molecular weight markers. The ABCA1 and GAPDH signals at each point were determined by densitometric analysis. The ABCA1:GAPDH ratio for the ligand sample (Lig) was arbitrarily assigned as 1 with those from the other samples shown as relative expression (Rel Exp) (average) from two independent experiments.

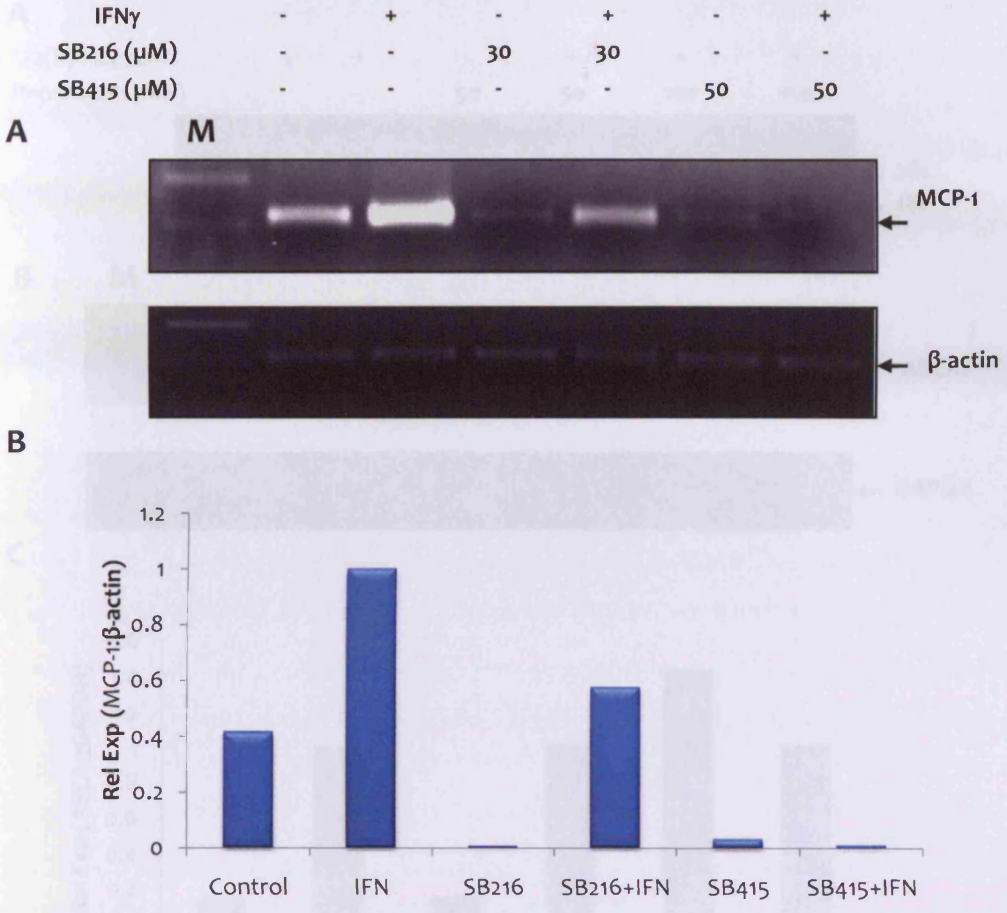


Figure 3.8 Effect of SB216763 and SB415286 on the induction of MCP-1 expression by IFNγ in J774.2 cells.

J774.2 cells were either treated with the vehicle DMSO (control) or exposed to IFNγ (1000U/ml) (IFN) for 3h. The inhibitors SB216763 (SB216) or SB415286 (SB415) were added 1h before the treatment with the ligands at the indicated concentrations. The cDNA prepared against total cellular RNA was used in PCR reactions in which primers against MCP-1 or β-actin were present. The amplification products were size–fractionated by electrophoresis on a 1.5% (w/v) agarose gel (panel A), M corresponds to the 1 kbp molecular weight markers. The MCP-1 and β-actin signals at each point were determined by densitometric analysis. The MCP-1:β-actin ratio for cells treated with IFN-γ was arbitrarily assigned as 1 with those from the other samples shown as relative expression (Rel Exp) (panel B).

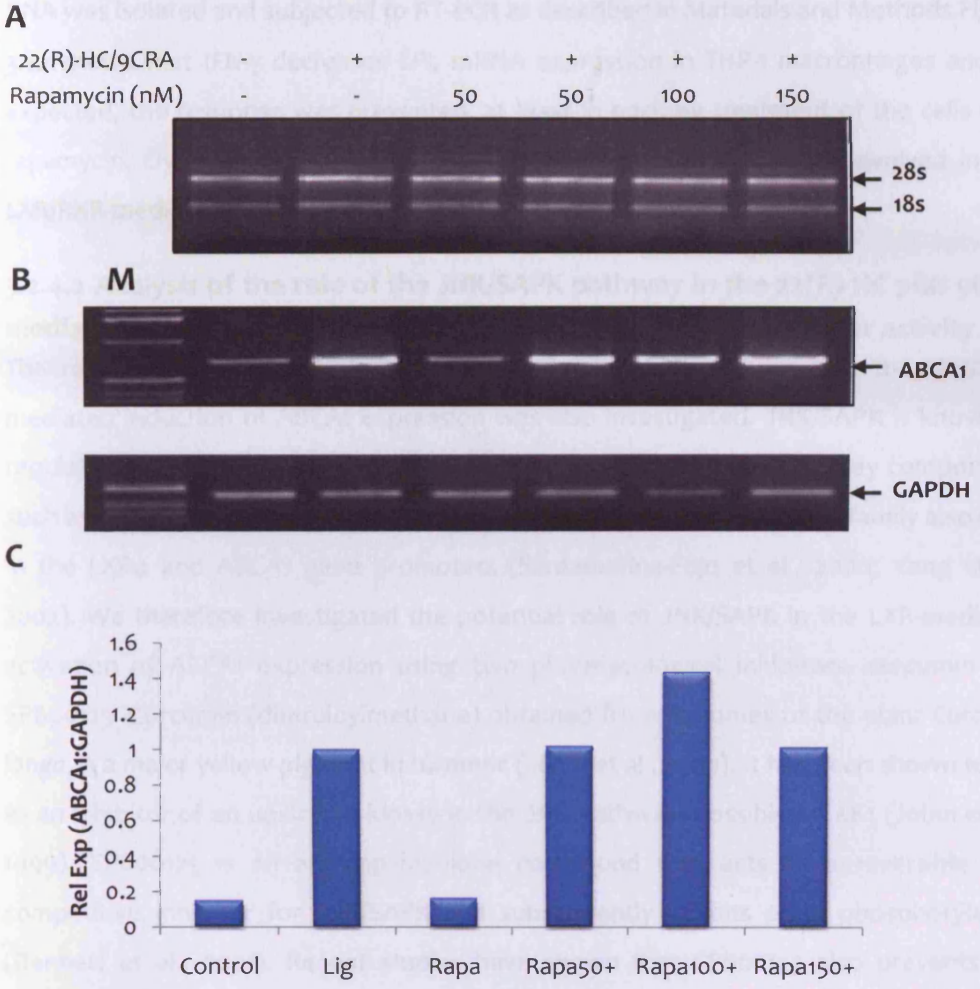


Figure 3.9 The effect of rapamycin on the induction of ABCA1 expression by LXR/RXR in J774.2 cells.

J774.2 cells were incubated with DMEM containing 10% (v/v) LPDS for 4h and then either treated with the vehicle DMSO (control) or exposed to 22(R)-HC plus 9CRA [2μg/ml and 10μM] (Lig) for 12h. The inhibitor rapamycin (Rapa) was added 1h before treatment with the ligand at the indicated concentrations. The quality of total cellular RNA was assessed by electrophoresis on 1% (w/v) agarose gel (panel A). Then, cDNA was prepared against total cellular RNA and used in PCR reactions in which primers against ABCA1 or GAPDH were present. The amplification products were size-fractionated by electrophoresis on a 1.5% (w/v) agarose gel (panel B). M corresponds to the 1 kbp molecular weight markers. The ABCA1 and GAPDH signals at each point were determined by densitometric analysis. The ABCA1:GAPDH ratio for the ligand sample (Lig) was arbitrarily assigned as 1 with those from the other samples shown as relative expression (Rel Exp) (average) from two independent experiments.

RNA was isolated and subjected to RT-PCR as described in Materials and Methods.Figure 3.10 shows that IFN-γ decreases LPL mRNA expression in THP-1 macrophages and, as expected, the response was prevented, at least in part, by treatment of the cells with rapamycin. Overall, these results strongly suggest that mTOR is not involved in the LXR/RXR-mediated induction of ABCA1 mRNA expression in J774.2 cells.

3.2.4.2 Analysis of the role of the JNK/SAPK pathway in the 22(R) HC plus 9CRA-mediated induction of ABCA1 mRNA expression and LXRα promoter activity

The role of JNK/SAPK, which is a branch of the MAPK pathways, in the LXR/RXR-mediated induction of ABCA1 expression was also investigated. JNK/SAPK is known to regulate the AP-1 family of transcription factors by phosphorylation of key components such as c-Jun (Davis, 2000). The binding motif of this transcription factor family also exist in the LXRα and ABCA1 gene promoters (Santamarina-Fojo et al., 2000; Yang et al., 2002). We therefore investigated the potential role of JNK/SAPK in the LXR-mediated activation of ABCA1 expression using two pharmacological inhibitors, curcumin and SP600125. Curcumin (diferuloylmethane) obtained from rhizomes of the plant *Curcuma longa*, is a major yellow pigment in turmeric (Jobin et al., 1999). It has been shown to act as an inhibitor of an upstream kinase in the JNK pathway, possibly MEKK1 (Jobin et al., 1999). SP600125 is an anthrapyrazolone compound that acts as a reversible ATP competitive inhibitor for JNK/SAPK and subsequently inhibits c-Jun phosphorylation (Bennett et al., 2001). Recent studies have shown that SP600125 also prevents the activation of JNK/SAPK itself, thereby acting upstream of this kinase (Nakahara and Carthew, 2004; Tamagno et al., 2005).

As shown in Figure 3.11, two different concentration of SP600125 (50 μM and 100 μM) failed to attenuate the induction of ABCA1 mRNA expression by LXR/RXR ligands. This is in contrast to previous studies demonstrating inhibitory actions at the same concentrations on the induction of apoE expression in THP-1 macrophages by the oxysterol 22(R)-HC (Kirsty Greenow, PhD thesis, Cardiff University). In order to rule-out the possibility that SP600125 was not active, its positive action was analysed. Previous studies in the laboratory had shown that SP600125 inhibits the TGF-β-mediated activation of apoE expression in THP-1 monocytes (Nishi Singh, PhD thesis, Cardiff

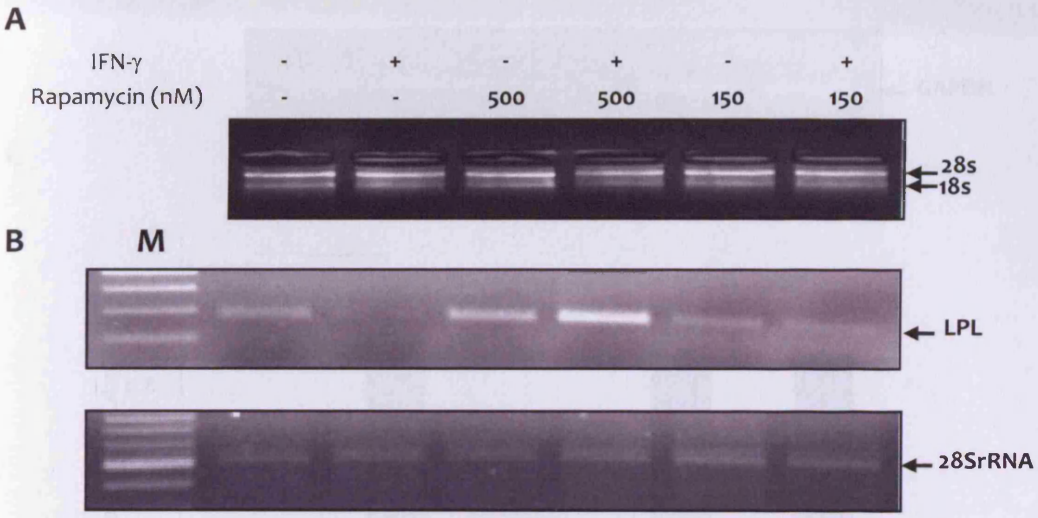


Figure 3.10 The effect of rapamycin on the IFN-γ-mediated decrease in LPL mRNA expression in THP-1 cells.

THP-1 cells were either treated with the vehicle DMSO (control) or exposed to IFNγ (1000U/ml) for 24h. The inhibitor rapamycin (Rapa) was added 1h before the IFNγ treatment at the indicated concentrations. The quality of total cellular RNA was assessed by electrophoresis on 1% (w/v) agarose gel (panel A). Then, cDNA was prepared against total cellular RNA and used in PCR reactions in which primers against LPL or 28SrRNA were present. The amplification products were size-fractionated by electrophoresis on a 1.5% (w/v) agarose gel (panel B). M corresponds to the 1 kbp molecular weight markers.

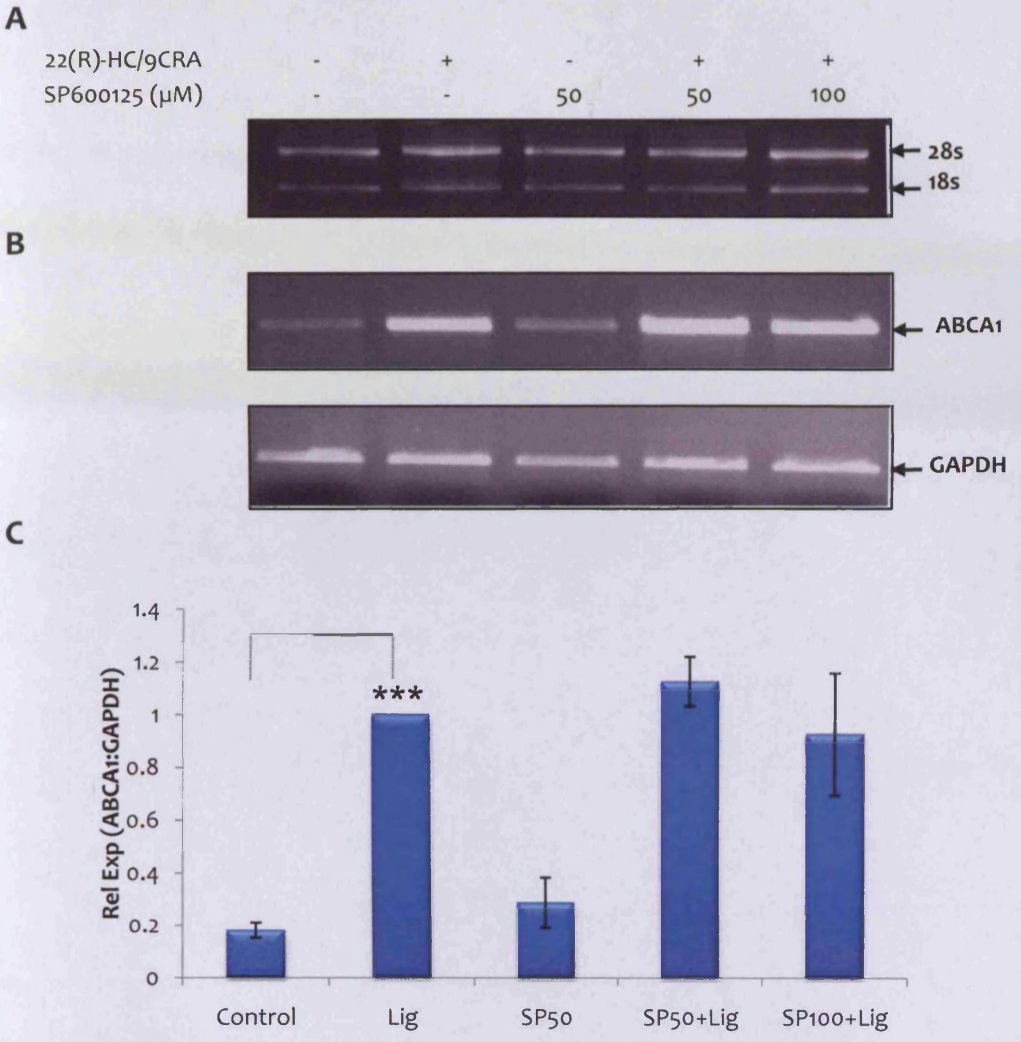


Figure 3.11 The effect of SP600125 on the induction of ABCA1 mRNA expression by LXR activators in J774.2 cells.

The cells were incubated with DMEM containing 10% (v/v) LPDS for 4h and then either treated with the vehicle DMSO (control) or 22(R)-HC plus 9CRA [2μg/ml and 10μM] (Lig) for 12h. The inhibitor SP600125 (SP) was added 1h before the addition of the ligands at the indicated concentrations (50 and 100μM). The quality of total cellular RNA was assessed by electrophoresis on 1% (w/v) agarose gel (panel A). Then, cDNA was prepared against total cellular RNA and used in PCR reactions in which primers against ABCA1 or GAPDH were present. The amplification products were size-fractionated by electrophoresis on a 1.5% (w/v) agarose gel (panel B). The ABCA1 and GAPDH signals at each point were determined by densitometric analysis. The ABCA1:GAPDH ratio for the ligand sample (Lig) was arbitrarily assigned as 1 with those from the other samples shown as relative expression (Rel Exp) (mean ±SD) from three independent experiments (panel C; ***$P<0.001$).

University). As shown in Figure 3.12, this was indeed found to be the case in this study, thereby showing that SP600125 was functional. Initial experiments with curcumin at two different concentrations (15μM and 30μM) showed that the inhibitor was toxic to the cells at 12h (data not shown). A similar toxicity was also seen with several concentrations of the inhibitor at <15μM. It was therefore not possible to investigate the action of this inhibitor on ABCA1 expression.

We also investigated the effect of DN JNK/SAPK on the activation of LXR promoter activity by the ligands. The DN JNK/SAPK plasmid was a kind gift from E. Nishida of Kyoto University. This plasmid has been used successfully in the laboratory and by other researchers in the field (Deng et al., 2001; Desbois-Mouthon et al., 2000; Gao et al., 2006; Vuong et al., 2000; Wang et al., 2003b; Wang et al., 2000). It specifies for a protein in which the phosphorylation site, Thr-Pro-Tyr, has been altered to Val-Pro-Ala. As shown in Figure 3.13, the induction of LXRα promoter activity by LXR/RXR agonists was not prevented but increased slightly by expression of DN JNK/SAPK.

3.3 Discussion

A number of proteins in the ABC transporter super family have recently been discovered to play a key role in cholesterol efflux and RCT (Lawn et al., 1999). ABCA1 is a transporter on the plasma membrane that translocates cholesterol and phospholipids out of the cells. Mutations of the ABCA1 gene results in defects in apolipoprotein-mediated cholesterol efflux as found in TD (Bortnick et al., 2000). ABCA1 expression levels are typically low or undetectable in normal cells (Langmann et al., 1999) but can be induced by variety of activators such as sterols, cAMP, 9CRA and PPAR agonists. In addition, studies by Laffitte et al. (2001b) demonstrated that ABCA1 expression is positively regulated by activation of LXRs.

Recent work analyzing the effect of synthetic LXR ligands in murine models of atherosclerosis provide direct evidence for an athero-protective effect of LXR activators (Hu et al., 2003; Joseph et al., 2002b; Levin et al., 2005; Schuster et al., 2002; Tangirala et al., 2002; Terasaka et al., 2003), thereby indicating that agonists of this NR represent promising agents for therapeutic intervention of CVD (Joseph et al., 2002b; Lund et al., 2003). The molecular mechanisms underlying such regulation of ABCA1 expression by activation of LXRs is at present poorly understood and form the focus of studies in this thesis.

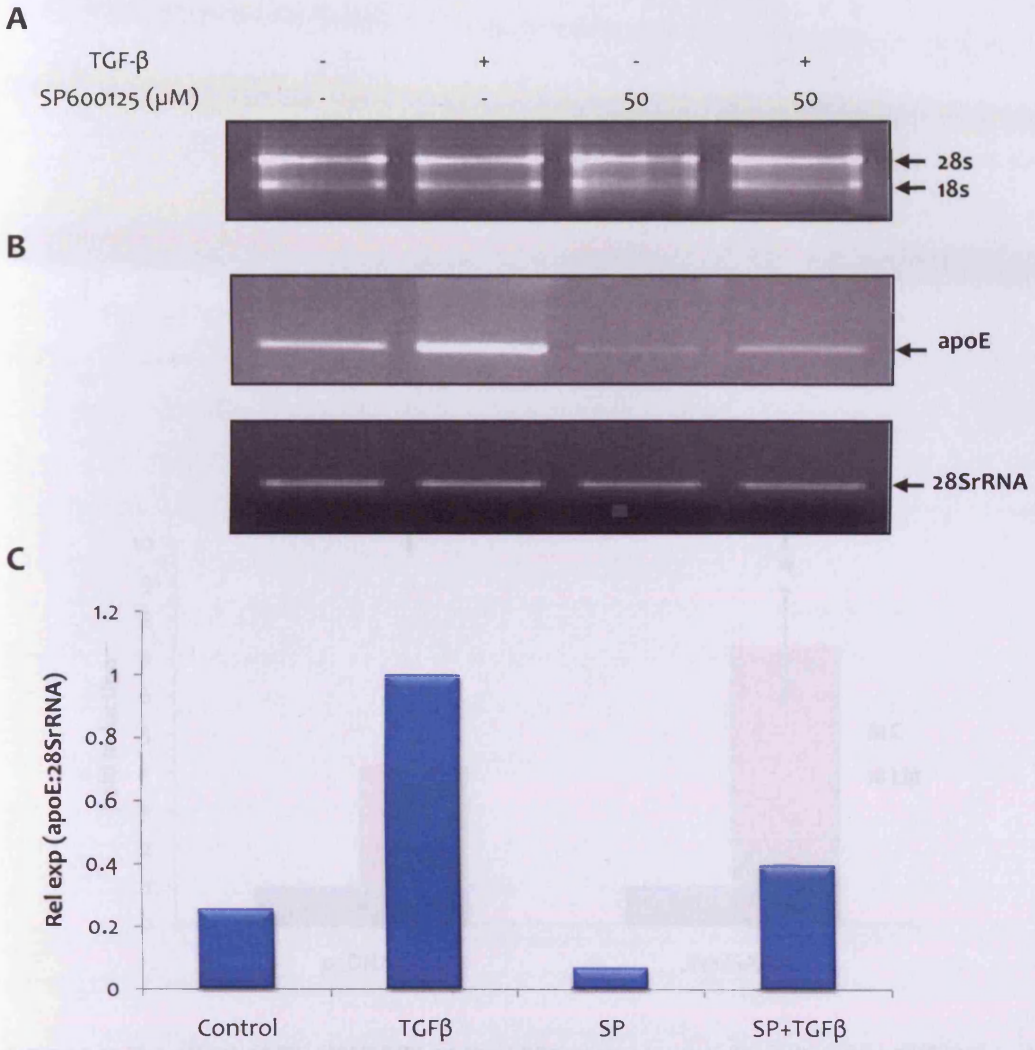


Figure 3.12 The effect of SP600125 on the TGF-β-induced apoE mRNA expression in THP-1 cells.

THP-1 cells were either treated with the vehicle DMSO (control) or TGF-β (30ng/ml) for 24h. The inhibitor SP600125 (SP) was added 1h before treatment with TGF-β at the indicated concentration (50μM). The quality of total cellular RNA was assessed by electrophoresis on 1% (w/v) agarose gel (panel A). Then, cDNA was prepared against total cellular RNA and used in PCR reactions in which primers against apoE or 28SrRNA were present. The amplification products were size–fractionated by electrophoresis on a 1.5% (w/v) agarose gel (panel B). The apoE and 28SrRNA signals at each point were determined by densitometric analysis. The apoE:28SrRNA ratio for cells treated with TGF-β was arbitrarily assigned as 1 with those from the other samples shown as relative expression (Rel Exp) (panel C).

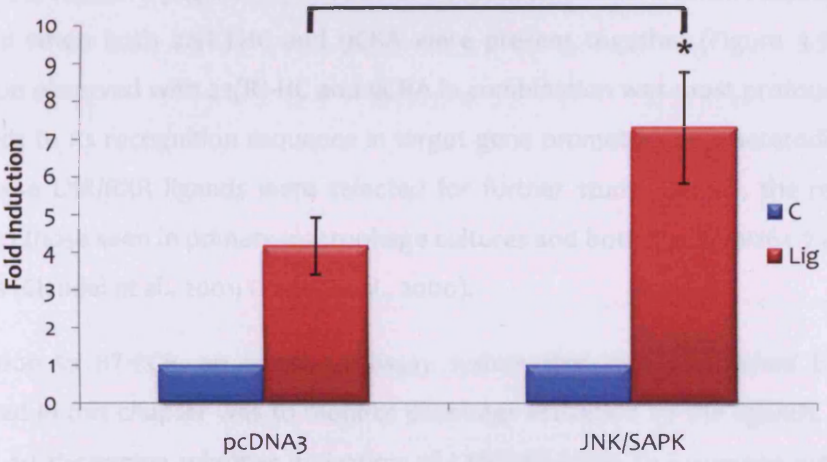


Figure 3.13 The effect of DN JNK/SAPK on the LXRα promoter activity induced by 22(R)-HC and 9CRA.

Hep3B cells were co-transfected with 5μg of the LXRα promoter-luciferase DNA construct and a plasmid construct specifying for DN JNK/SAPK or the vector pcDNA3, using the PEI reagent. Following transfection, cells were incubated for 24h in DMEM supplemented with 10% LPDS containing either the DMSO vehicle (C) or 22(R)-HC plus 9CRA [2μg/ml and 10μM] (Lig). The cells were then harvested and luciferase activity and protein concentration determined as described in Materials and Methods. Relative luciferase activity was normalised to protein concentration and values are expressed as mean fold induction (the value in vehicle-treated cells has arbitrarily been assigned 1). Bars indicate mean ±SD with significant effect shown by *$P<0.05$. The results represent the outcome of three independent experiments each of which was carried out in triplicate.

In the studies presented in this chapter, an *in vitro* system, J774.2 macrophages, was chosen for the analysis of LXR-mediated induction of ABCA1 gene expression. Several studies have shown that oxysterols and synthetic LXR ligands rapidly induce the expression of ABCA1 and apoE mRNA in different macrophage cell lines (Costet et al., 2000; Joseph et al., 2002b; Laffitte et al., 2001b; Mak et al., 2002b; Repa et al., 2000b; Schultz et al., 2000; Schwartz et al., 2000; Sparrow et al., 2002; Venkateswaran et al., 2000a). It was important to first optimise and validate the use of the RT-PCR technique for the determination of the effects of LXR activators on ABCA1 gene expression in J774.2 macrophages. The results from RT-PCR analysis showed that 22(R)-HC induces the expression of ABCA1. As expected, no such induction was seen with the inactive enantiomer 22(S)-HC (Figure 3.2). An additive activation of ABCA1 mRNA expression was obtained when both 22(R)-HC and 9CRA were present together (Figure 3.3). As the activation observed with 22(R)-HC and 9CRA in combination was most profound and as LXR binds to its recognition sequence in target gene promoters as a heterodimer with RXR, these LXR/RXR ligands were selected for further study. Overall, the results are similar to those seen in primary macrophage cultures and both the RAW264.7 and THP-1 cell lines (Claudel et al., 2001; Costet et al., 2000).

In addition to RT-PCR, an additional assay system that was established by studies presented in this chapter was to monitor promoter activation by the ligands. It was of interest to determine whether activation of LXR/RXR leads to increased activity of a promoter regulated by the ligands in a transient transfection assay. The -2625hLXRα-Luc reporter construct contains the sequence for the human LXRα promoter linked to the luciferase gene. Because of the difficulties experienced in the laboratory, and others in the field, with the efficient transfection of J774.2 macrophages with exogenous DNA (Hughes et al., 2002; Irvine et al., 2005), such assays were performed in human Hep3B cells. Indeed, human hepatoma cell lines have been used extensively to study cytokine-mediated gene expression relevant to atherosclerosis (Blaschke et al., 2006; Coulouarn et al., 2005; Khovidhunkit et al., 2003; Miyake et al., 2000). The results shown in Figure 3.4 indicate that the activity of the LXRα promoter was induced by LXR/RXR ligands in Hep3B cell transfected using the PEI reagent.

It was next decided to identify the potential signalling pathways that might be involved in mediating LXR/RXR induction of ABCA1 expression. For this purpose, a number of

pharmacological inhibitors against various components of JNK/SAPK and PI3K signalling pathways and DN plasmids were used. The results showed that there was a potentially important role for the PI3K pathway in the activation of ABCA1 expression by LXR agonists. Thus, LXR/RXR-mediated induction of ABCA1 expression was significantly attenuated by LY294002 (Figure 3.5). As mentioned earlier in General Introduction, the predominant form of PI3K is a member of the class I subfamily of PI3K enzymes and consists of the p85α regulatory subunit and the p110 catalytic subunit (Carpenter and Cantley, 1996; Cuevas et al., 2001). A number of signalling effectors function downstream of PI3K (Figure 1.10) and one of the best characterised targets is PKB. Having determined that PI3K was involved in the induction of ABCA1 expression by activation of LXRs by the use of a pharmacological inhibitor, transient co-transfection assays were carried out using DN constructs specifying for the catalytic subunit p110 and the downstream target PKB. Both DN constructs attenuated the activation of the LXRα gene promoter by the ligands (Figure 3.6). These results also suggest that PKB is a potential downstream effector of PI3K action in the up regulation of ABCA1 gene expression by 22(R)-HC and 9CRA. It is therefore important to substantiate this link further by monitoring, for example, the activity of PKB in response to LXR activators.

GSK-3 belongs to a family of conserved Ser/Thr kinases present in all eukaryotic organisms and one of its primary function is to phosphorylate and inactivate glycogen synthase in response to insulin (Cross et al., 1995). In addition to its role in metabolic and neurological abnormalities, GSK-3 is also implicated in the regulation of many transcription factors. These include cAMP response element binding protein, nuclear factor of activated T cells, heat shock factor-1, AP-1 and NF-κB (Tavares et al., 2004). mTOR is a Ser/ Thr protein kinase with highly conserved homologues that are found in all eukaryotic organisms. The enzyme plays a key role in the control of protein synthesis activated by metabolic agents (e.g. glucose and amino acids), growth factors and cytokines (Fingar and Blenis, 2004; Manning, 2004). GSK and mTOR are important downstream targets of PKB actions in the PI3K pathway and involved in NR signalling (Choi et al., 2004; Doronzo et al., 2006; Krasilnikov, 2000). The phosphorylation of NRs, such as GR and AR, by GSK-3 in response to a variety of signals reduces their transcriptional activity (Hirota et al., 2003; Moeller et al., 2006; Salas et al., 2004). PKB phosphorylation of GSK-3 results in the activation of these NRs by inhibition of GSK-3. Thus, as GSK-3 and mTOR are potential candidates that may link the PI3K pathway with

LXR transcriptional regulation of genes, its potential role was investigated further by the use of pharmacological inhibitors. The GSK inhibitors, SB216763 and SB415286, nor rapamycin, an inhibitor of the p70S6 kinase that binds and prevents the function of mTOR, had any effect on the induction of ABCA1 expression by LXR activation (Figures 3.7 and 3.9). These results therefore rule out GSK and mTOR as downstream targets for PKB in the regulation of ABCA1 expression. However, the possibility that the PI3K pathway is mediating its effects on the LXR regulation of ABCA1 expression through another downstream component of PKB cannot be ruled out.

Because of the importance of the PI3K pathway in the regulation of LXRs and the previously noted phosphorylation of NRs, a search was carried out on the presence of potential consensus sites for components of the PI3K pathway in LXRs using Scan site database (Obenauer et al., 2003). The results of this analysis indicated several PI3K pathway motifs, including PI3K p85 and PDK1 binding sites and putative phosphorylation sites for PKB and GSK-3 (Obenauer et al., 2003). For example, a number of putative consensus sites for PKB exist in both LXRα (Ser198 and Ser230) and LXRβ (Ser244). Thus, PKB may directly phosphorylate LXRα and/or LXRβ (Greenow, K., 2004). These results indicate that the necessary sequences for PI3K binding exist in this NR. Indeed, recent data show that PI3K may affect several other key genes of LXR actions such as SREBP-1c and apoA5 (Ballerini et al., 2006; Barthel and Schmoll, 2003; Chen et al., 2004; Doronzo et al., 2006; Jakel et al., 2004; Nowak et al., 2005; Taniguchi et al., 2005; Wellen and Hotamisligil, 2005).

The role of the JNK/SAPK pathway was also investigated via the use of two structurally distinct inhibitors, curcumin and SP600125. SP600125 failed to inhibit the LXR/RXR-induced ABCA1 mRNA expression (Figure 3.11). DN constructs against JNK/SAPK were used to further investigate the role of this pathway. Again, the expression of such a DN form failed to prevent the ligand-mediated activation of the LXRα promoter in transfected cells. In contrast to SP600125, the inhibitor curcumin was found to be toxic to the cells even at very low concentrations. Overall, these results are in contrast to previous studies that showed a role for the JNK/SAPK signal transduction pathway in the 22(R)-HC-mediated induction of apoE expression in THP-1 macrophages (Greenow, K., 2004). This response was affected by both SP600125 and curcumin. The exact reasons for such a discrepancy are currently unclear but may be due to the use of human

macrophages in the previous study and murine macrophages in the present study. Indeed, differences between macrophages of human and mouse origins have been described previously (Chen et al., 2002; Cook et al., 2001; Kiss et al., 2005; Menke et al., 2002; Vu-Dac et al., 1998; Wagner, 2000; Yanai et al., 2004). For example, Whitney et al. (2001) reported that natural and synthetic LXR ligands induce the expression of the LXRα gene in primary human macrophages and differentiated THP-1 macrophages but not in any mouse tissues or cell lines examined. In contrast to ABCA1, the expression of a number of other LXR target genes, such as ABCG1, LXRα, SREBP-1c and apoE, show weak or no induction in mouse macrophages but stronger induction in human macrophages (Costet et al., 2003). In addition, Kiss et al. (2005) showed a unique regulation of cholesterol efflux by cAMP and 9CRA in human macrophages that is different from that seen in murine macrophages.

It was worth noting that the inhibitors used in this study have been employed successfully for a number of projects in the laboratory (Singh, N. 2003; Greenow, K. 2004). For example, it has already been shown that both GSK inhibitors, SB216763 and SB415286, inhibit the induction of MCP-1 expression by IFN-γ in the J774.2 cell line (Harvey, E. 2005). In addition, rapamycin has been found to prevent the IFN-γ-mediated suppression of LPL mRNA expression in THP-1 macrophages (Evans, S. 2005) and SP600125 was shown to inhibit the TGF-β-mediated activation of apoE mRNA and protein expression in THP-1 monocytes and macrophages (Singh and Ramji, 2006). These studies along with experiments carried out in this study (Figures 3.8; 3.10; and 3.12) prove the effectiveness of these three inhibitors.

The J774.2 cell line chosen for this study has been used widely to investigate regulation of gene expression in differentiated macrophages that is relevant to atherosclerosis (Hughes et al., 2002; Kiss et al., 2005; Mead et al., 2002; Piraino et al., 2006; Tengku-Muhammad et al., 1996). It has a major advantage over the use of monocytic cell lines in that there is no requirement of additional stimuli to induce differentiation, such as PMA in the case of THP-1 cells, which might complicate the analysis (Auwerx et al., 1988; Perez et al., 2003). However, there are several limitations to the use of J774.2 cells as a model system. For example, these cells do not express the apoE gene (Mazzone et al., 1987), which plays an important role in the pathogenesis of atherosclerosis (Ilveskoski et al., 1999). ApoE is a lipid transport protein with a variety of physiological functions and has

potent anti-atherogenic activities (Curtiss and Boisvert, 2000; Fazio et al., 1997; Larkin et al., 2000). For example, apoE within HDL plays a key role in RCT from peripheral tissues to the liver (Von Eckardstein et al., 2001b). Moreover, apoE knock-out mice develop atherosclerosis even when fed a diet that is low in cholesterol (Linton and Fazio, 1999; Perez et al., 2003). In addition, response elements for LXRα and PPARγ have been identified in the apoE gene promoter (Laffitte et al., 2001b), thereby showing that NRs are regulators of apoE expression. Finally, species-dependent metabolic differences cannot be overlooked (Mead et al., 2002; Schmitz and Langmann, 2001). In addition to differences in gene regulation by mouse and human LXRs, mice also lack plasma CETP activity that is present in humans (Zelcer and Tontonoz, 2006). A recent study demonstrated that in two CETP containing animal models, Syrian hamsters and cynomolgus monkeys, activation of LXRs induced a significant increase in LDL cholesterol levels that was not previously observed in mice (Groot et al., 2005). Direct induction of CETP by LXRs, which has been demonstrated in human cell lines, may contribute to this increase (Luo and Tall, 2000).

Although the use of J774.2 macrophages confirmed the role of the PI3K pathway in the action of LXR activators, definitive conclusions on the JNK/SAPK pathway could not be made because SP600125 had no effect and curcumin was found to be toxic. Because J774.2 macrophages are difficult to transfect with exogenous DNA, Hep3B cells were used to investigate the action of the DN JNK/SAPK plasmid. However, no attenuation of the LXR agonist-induced LXRα promoter activity was seen. Because of various difficulties detailed above and as it was not possible to rule-out that the JNK/SAPK pathway was not required for the response, it was decided to use of THP-1 cells for subsequent studies. This was because of previous studies in the laboratory on the action of 22(R)-HC on apoE expression had identified a conclusive role for JNK/SAPK and PI3K pathways and the findings correlated with primary cultures of human monocyte-derived macrophages (Greenow, K., 2004). The THP-1 cell line also offers a number of other advantages. Firstly, the action of ligands and signalling pathways on apoE expression can be investigated along with ABCA1. Secondly, these cells could be transfected with exogenous DNA. Thirdly, they are human in origins.

CHAPTER FOUR:

EFFECT OF PHARMACOLOGICAL INHIBITORS ON THE LXR AGONIST-INDUCED EXPRESSION OF KEY GENES IMPLICATED IN THE CONTROL OF CHOLESTEROL HOMEOSTASIS IN THP-1 MACROPHAGES

Chapter 4: Effect of pharmacological inhibitors on the LXR agonist-induced expression of key genes implicated in the control of cholesterol homeostasis in THP-1 macrophages

4.1 Introduction

Previous studies in the laboratory had shown that 22(R)-HC induces apoE expression in THP-1 macrophages and this requires the JNK/SAPK and PI3K pathways (Greenow, K., 2004). The main aim of the work presented in this thesis is to extend these findings to other LXR regulated genes (e.g. ABCA1) and other activators of these NRs (e.g. combinations of 22(R)-HC and 9CRA, synthetic ligands). As THP-1 monocytes require differentiation into macrophages, it was decided to use J774.2 macrophages (an adherent cell line) in the studies described in chapter 3. These studies showed a dramatic induction of ABCA1 mRNA expression by combinations of 22(R)-HC plus 9CRA than that seen with either of the ligand. Whilst the previously noted role of the PI3K pathway in this response was confirmed, a role for JNK/SAPK could not be verified as curcumin was toxic to these cells and SP600125 had no effect.

It was decided to carry out experiments for subsequent studies in human THP-1 macrophages because of the problems detailed above and in chapter 3 in relation to mouse J774.2 macrophages (e.g. transfection with exogenous DNA) and given that atherosclerosis is in essence a human disease. Indeed, several recent studies have demonstrated species-specific differences in NR signalling (Schmitz and Langmann, 2005). For example, the Laffitte group have shown that human LXRα is subject to positive autoregulation and the ability of LXRα to regulate its own promoter is species specific (Laffitte et al., 2001a). Such species-specific differences in the ability to amplify the LXR response (i.e. autoregulation of LXRα) raises the possibility that humans may be more responsive than mice to LXR agonists (Laffitte et al., 2001a). In addition, different groups have demonstrated differential induction of LXR regulated gene expression in human and murine macrophages (Laffitte et al., 2001a; Li et al., 2004; Repa et al., 2000b). Furthermore, murine and human LXRs appear to direct distinct transcriptional activities (Lee and Plutzky, 2006). For example, certain LXR target genes, such as CETP, CYP7A1 and ABCG1, are regulated by LXRs in a species specific manner (Lund et al., 2003). The expression of CETP is induced by LXRs in humans as it contains a functional

LXR-RE in its promoter region whereas mice do not express this gene (Luo and Tall, 2000). On the other hand, the expression of the mouse Cyp7a gene is induced by LXR activation but no such effect is seen in humans (Chen et al., 2002; Lehmann et al., 1997; Tontonoz and Mangelsdorf, 2003). In addition, although the regulatory regions of both the human and murine ABCG1 gene contain LXR-RE, the magnitude of the induction of human ABCG1 expression by LXR agonists is much greater than the murine counterpart (Kennedy et al., 2001). Moreover, the apoE gene is significantly more responsive to LXR ligands in human macrophages than in murine macrophages (Laffitte et al., 2001a). Finally, substantial difference in lipid metabolism exist between mice and humans (Lee and Plutzky, 2006; Repa et al., 2000a; Tangirala et al., 2002; Zelcer and Tontonoz, 2006).

In order to investigate the effect of LXR agonists on ABCA1 and apoE protein levels, THP-1 and U937 macrophages were used as model systems during the rest of this study. Both cell lines have previously been widely used as *in vitro* models for studying the mechanisms involved in the regulation of macrophage gene expression relevant to atherogenesis (Auwerx, 1991; Kohro et al., 2004; Larigauderie et al., 2004; Olsson et al., 1983; Ricote et al., 1998; Via et al., 1989; Wang et al., 2003b; Wu et al., 1994). Several studies have shown similarities in responses in these cells with those observed in primary cultures of human monocyte-derived macrophages (Matheson et al., 2002). Both these cell lines can be considered as monoblasts as they represent a relatively immature cell of the monocyte-macrophage cell lineage. However, these cell lines are blocked at certain steps in the differentiation process but they can be induced to differentiate into macrophages by treatment with phorbol esters, such as PMA (Auwerx, 1991; Koren et al., 1979; Tsuchiya et al., 1982; Tsuchiya et al., 1980). This differentiation process is associated with a dramatic change in cell morphology and membrane expression of antigens and receptors associated with native monocyte-derived macrophages (Auwerx, 1991; Tsuchiya et al., 1982).

LXRs regulate the levels of the ABCA1 and ABCG1 cholesterol transporters as well as apoE in various cells, thereby affecting cholesterol transport and metabolism. ApoE, ABCA1 and ABCG1 proteins are major players in mediating cellular efflux of cholesterol and phospholipids from macrophages to apoA-I containing lipoproteins including preβ-HDL and, thereby, exert anti-atherogenic activities (Venkateswaran et al., 2000b; Perez et al., 2003; Lawn et al., 1999; Bortnick et al., 2000; Chawla et al., 2001b). Previous work

has shown that the expression of LXR target genes apoE, ABCA1 and ABCG1 is induced in human and murine macrophages by agonists of this NR (Laffitte et al., 2001a; Laffitte et al., 2001b; Venkateswaran et al., 2000a). The studies presented in this chapter therefore aimed to investigate the action of natural and synthetic LXR activators on apoE and ABCA1 expression in THP-1 macrophages. A number of synthetic LXR agonists, which are structurally unrelated to oxysterols, have been developed. The increased potency of these compounds compared to physiological ligands makes them useful tools for the study of LXR function. The first such synthetic LXR ligands were the compounds T0901317 (Schultz et al., 2000) and GW3965 (Collins et al., 2002). These were shown to promote cellular cholesterol efflux and to inhibit atherosclerosis in animal models of the disease (Joseph et al., 2002b). The secondary aim of the research presented in this chapter was to further delineate whether the same signalling pathways were involved in LXR-mediated activation of ABCA1 and apoE genes. This was achieved initially by studying the effects of specific pharmacological inhibitors of signalling pathways on the induction of ABCA1 and apoE expression by either natural or synthetic LXR agonists. Data from these experiments were then confirmed by co-transfection assays using ABCA1 promoter-reporter DNA constructs and plasmids specifying for DN forms of specific signalling proteins. Additionally, key finding were confirmed in human primary macrophages to rule out the possibility that the results obtained were peculiar to the transformed cell line. Figure 4.1 illustrates the overall experimental strategy for the work presented in this chapter.

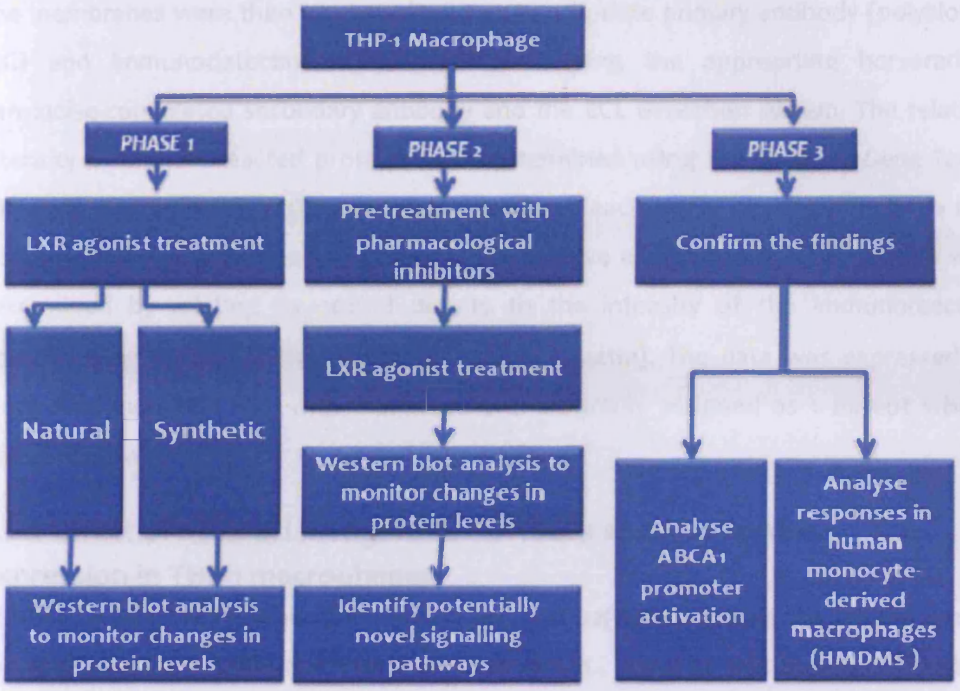


Figure 4.1 Summary of the experimental strategy used to delineate the signal transduction pathways underlying LXR agonist-mediated activation of genes involved in the cholesterol homeostasis in THP-1 macrophages.

4.2 Results

4.2.1 Experimental design

In order to achieve the aims detailed above, THP-1 monocytes were differentiated for 24h with 0.16μM PMA in RPMI medium 1640 containing 10% (v/v) LPDS. After this differentiation period, LXR agonists at appropriate concentration were added and the cells left to incubate for a further 24h. Additionally, all control samples were treated with an equal volume of DMSO. In addition to LXR agonists, THP-1 cells were also treated in some experiments with the inactive ligand 22(S)-HC (2μg/ml). Because the function of apoE and ABCA1 in the control of cholesterol homeostasis is carried out by proteins, it was decided to apply western blot analysis for subsequent work to examine the effect of LXR agonists on ABCA1 and apoE protein levels in differentiated THP-1 macrophages. For this, total protein from THP-1 macrophages was isolated, quantified and subjected to western blot analysis using conditions shown in Tables 2.8 and 2.9. Thus, equal amount of total protein was subjected to SDS-PAGE and then transferred to PVDF membranes.

The membranes were then incubated with an appropriate primary antibody (polyclonal IgG) and immunodetection was carried out using the appropriate horseradish peroxidise-conjugated secondary antibody and the ECL detection system. The relative intensity of immunoreacted proteins were determined using the Syngene Gene Tools software. The software assigns numerical values to each signal relating directly to the intensity of the immunoreacted proteins. The relative expression of each protein was determined by relating its optical density to the intensity of the immunoreacted housekeeping protein in the equivalent sample (β-actin). The data was expressed as mean fold induction ±SD with the basal level arbitrarily assigned as 1 except where stated otherwise.

4.2.2 Effect of natural LXR agonists on ABCA1 and apoE protein expression in THP-1 macrophages

Previous studies in the laboratory had shown that 22(R)-HC induces the expression of the apoE protein in THP-1 macrophages (Greenow, K., 2004). It was decided to extend this finding by investigating the ability of 22(R)-HC or 9CRA either alone or in combination to modulate apoE and ABCA1 expression in THP-1 macrophages by Western blot analysis. As shown in Figure 4.2, 22(R)-HC or 9CRA, either alone or in combination, induced the expression of the apoE and ABCA1 proteins in THP-1 macrophages whereas the inactive enantiomer, 22(S)-HC, had no such effect. In the case of ABCA1 but not apoE, the induction in expression by combinations of 22(R)-HC and 9CRA was much greater than that produced by the individual ligands.

4.2.3 Effect of synthetic LXR agonists on ABCA1 and apoE protein expression in THP-1 macrophages

The next line of investigation involved determining the effect of synthetic LXR ligands on the expression of ABCA1 and apoE protein levels in THP-1 cells using Western blotting. The non-steroidal LXR agonists, GW3965 and To901317 (denoted T1317 hereafter), have potent anti-atherogenic activities in two different murine models of this disease [LDLR(-/-) and apoE(-/-) mice] (Joseph et al., 2002b; Tangirala et al., 2002; Terasaka et al., 2003). Figures 4.3 and 4.4 show that GW3965 and To901317, at two different concentrations, induce the expression of ABCA1 and apoE proteins. Whilst the induction of apoE and ABCA1 expression by GW3965 was of a similar extent, the degree of induction of ABCA1 by To901317 was much greater than that seen with GW3965 (Figures 4.3-4.4). To further

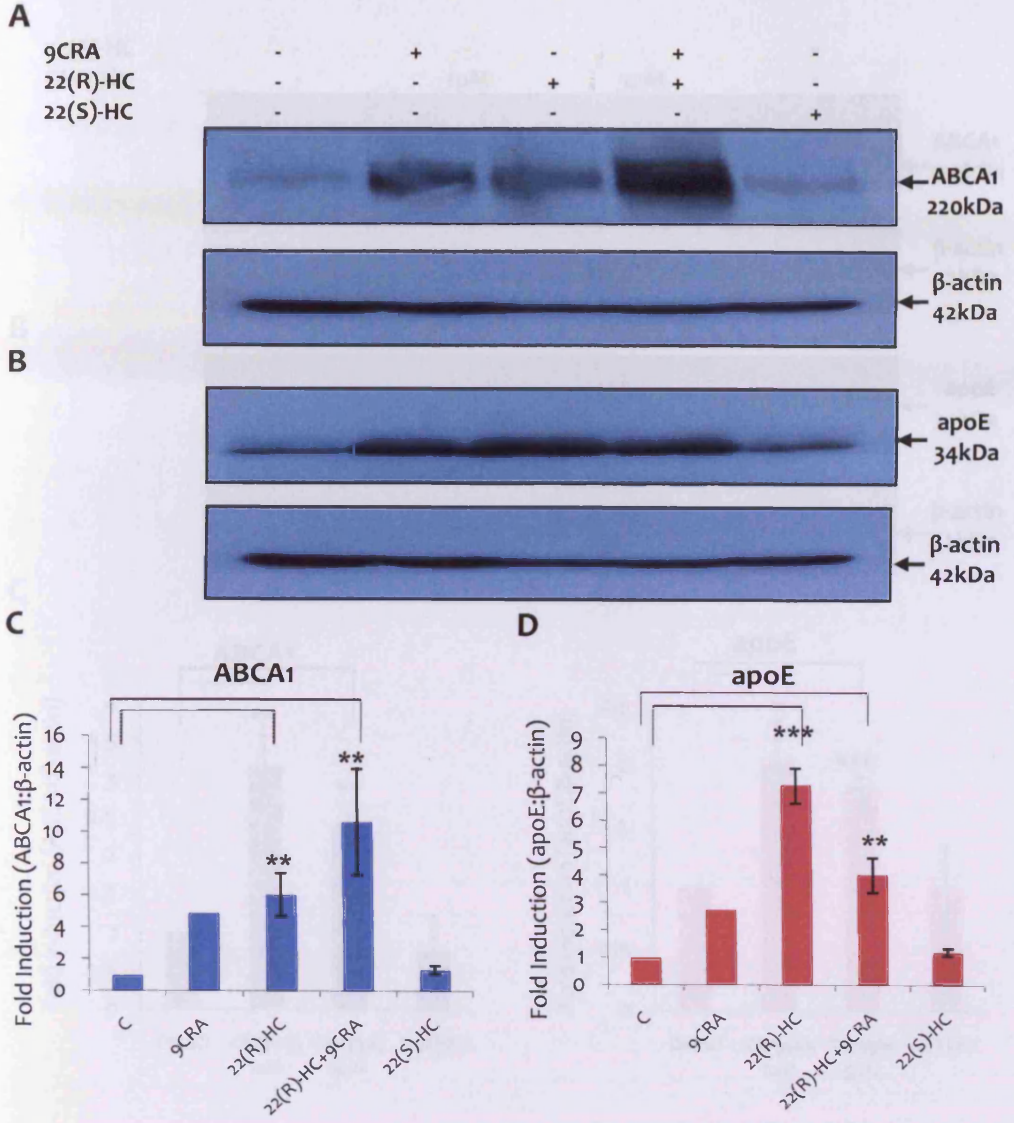


Figure 4.2 Effect of natural LXR agonists on ABCA1 and apoE protein expression in THP-1 macrophages.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (2μg/ml) or 9CRA (10μM) either alone or in combination. In addition, cells were treated with 22(S)-HC (2μg/ml) or DMSO as a vehicle control (C). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against apoE, ABCA1 or the β-actin control. Antigen-antibody complexes were detected using the ECL detection system. Results shown in panels A and B are representative of four and three independent experiments respectively except for 9CRA treatment which has carried out twice. Densitometric analysis was carried out on the data and presented as mean fold induction (±SD) in relation to basal levels (in the presence of vehicle alone; C) assigned as 1 as shown in panels C and D (**P<0.01, ***P<0.001 compared to controls).

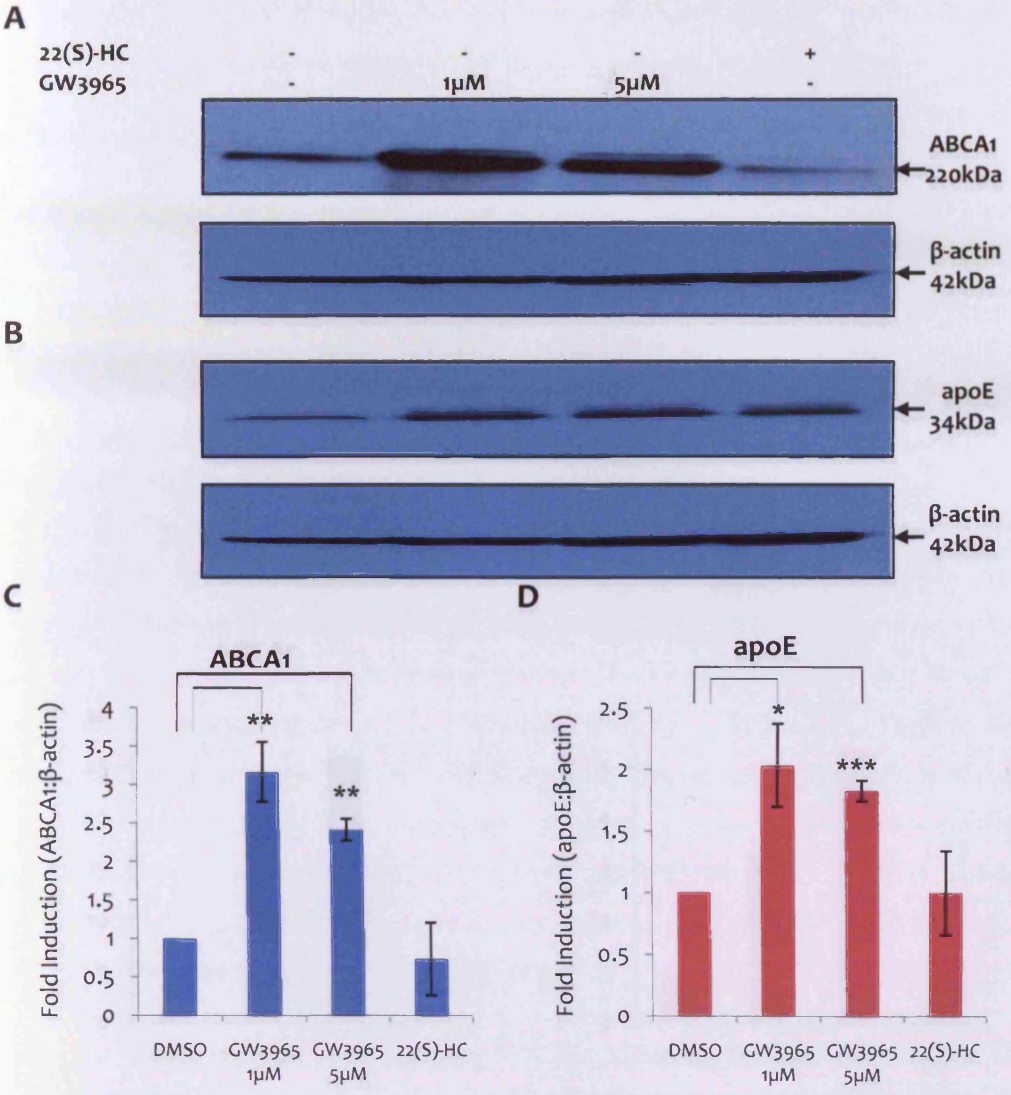


Figure 4.3 Induction of ABCA1 and apoE protein levels by GW3965 in THP-1 macrophages.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with synthetic LXR agonist GW3965 at the indicated concentrations. In addition, cells were treated with 22(S)-HC (2μg/ml) or DMSO as a vehicle control. Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE or the β-actin control. Antigen-antibody complexes were detected using the ECL detection system. Results shown in panels A and B are representative of three independent experiments. Densitometric analysis was carried out on the data and presented as mean fold induction (±SD) in relation to basal levels (i.e. DMSO treated cells) assigned as 1 as shown in panels C and D (*$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to controls).

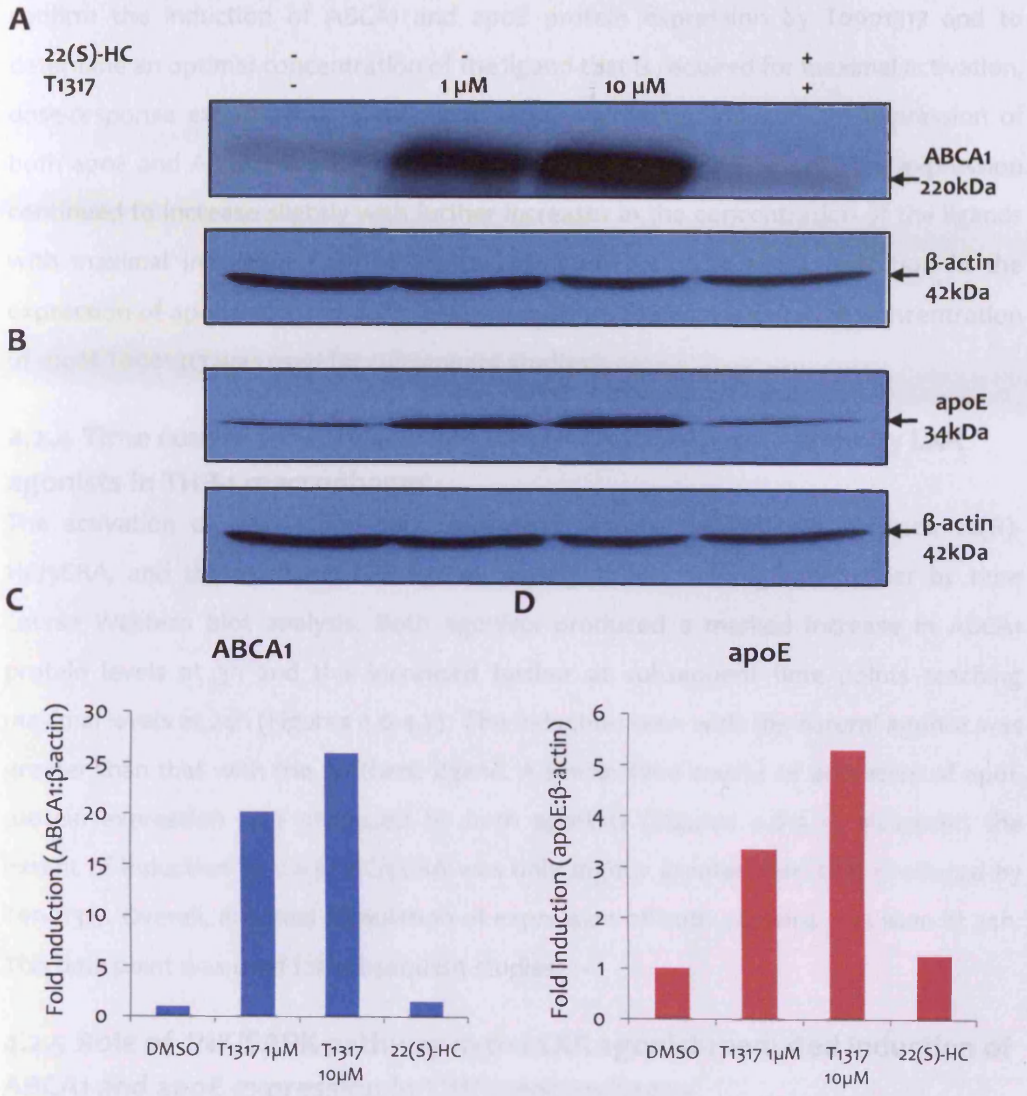


Figure 4.4 Induction of ABCA1 and apoE protein levels by T0901317 in THP-1 macrophages.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with the indicated concentrations of the synthetic LXR agonist T0901317 (T1317). In addition, cells were treated with 22(S)-HC (2μg/ml) or DMSO as a vehicle control. Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE or the β-actin control. Antigen-antibody complexes were detected using the ECL detection system. Results shown in panels A and B are representative of two independent experiments. Densitometric analysis was carried out on the data and presented as average fold induction in relation to basal levels (i.e. in the presence of DMSO alone) assigned as 1 as shown in panels C and D.

confirm the induction of ABCA1 and apoE protein expression by T0901317 and to determine an optimal concentration of the ligand that is required for maximal activation, dose-response experiments were carried out. A dramatic induction of expression of both apoE and ABCA1 was seen with 0.5μM of the ligand (Figure 4.5). The expression continued to increase slightly with further increases in the concentration of the ligands with maximal induction seen at 7.5-10μM (Figure 4.5). The slight reduction in the expression of apoE seen with 1μM T0901317 appears to be an anomaly. A concentration of 10μM T0901317 was used for subsequent studies.

4.2.4 Time course for activation of ABCA1 and apoE expression by LXR agonists in THP-1 macrophages

The activation of ABCA1 and apoE expression by the natural LXR agonists, 22(R)-HC/9CRA, and the synthetic LXR ligand, T0901317, was investigated further by time course Western blot analysis. Both agonists produced a marked increase in ABCA1 protein levels at 3h and this increased further at subsequent time points reaching maximal levels at 24h (Figures 4.6-4.7). The induction seen with the natural agonist was greater than that with the synthetic ligand. A similar time course of activation of apoE protein expression was produced by both agonists (Figures 4.6-4.7). However, the extent of induction by 22(R)-HC/9CRA was only slightly greater than that produced by T0901317. Overall, maximal stimulation of expression of both proteins was seen at 24h. This time point was used for subsequent studies.

4.2.5 Role of JNK/SAPK pathway in the LXR agonist-mediated induction of ABCA1 and apoE expression in THP-1 macrophages

Previous studies in the laboratory had shown that 22(R)-HC activates JNK/SAPK in THP-1 macrophages and indicated a role for this kinase in the stimulation of apoE gene expression by this ligand (Greenow, K., 2004). However, the role of this pathway in the actions of combinations of 22(R)-HC and 9CRA or the synthetic ligands in these cells was not analysed. In addition, the potential role for this pathway in the activation of ABCA1 expression was not studied. It was therefore decided to investigate these aspects using the two pharmacological inhibitors of this pathway, SP600125 and curcumin. Initial experiments aimed to confirm the previously noted attenuation of 22(R)-HC-induced apoE expression and extend the analysis to ABCA1. The expression of apoE and ABCA1 was monitored by Western blot analysis. As expected, the 22(R)-HC-induced apoE protein expression was significantly attenuated by treatment of the cells with SP600125

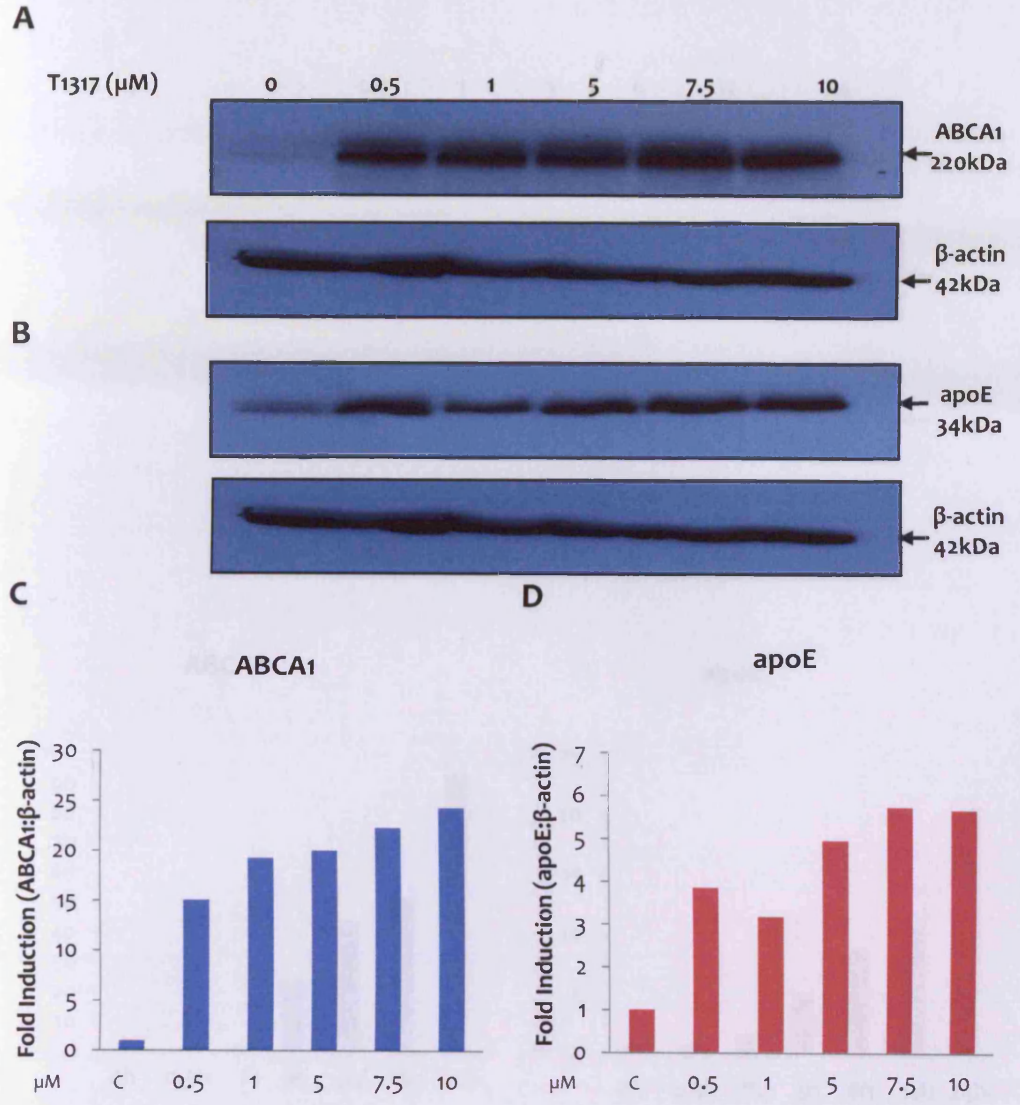


Figure 4.5 Concentration-dependent increase in ABCA1 and apoE protein expression by T0901317.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with the indicated concentrations of T0901317 (T1317). In addition, cells were treated with DMSO as a vehicle control (C). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE or the β-actin control. Antigen-antibody complexes were detected using the ECL detection system (panels A and B). Densitometric analysis was carried out on the data and presented as fold induction in relation to basal levels (in the presence of vehicle alone; C) assigned as 1 as shown in panels C and D.

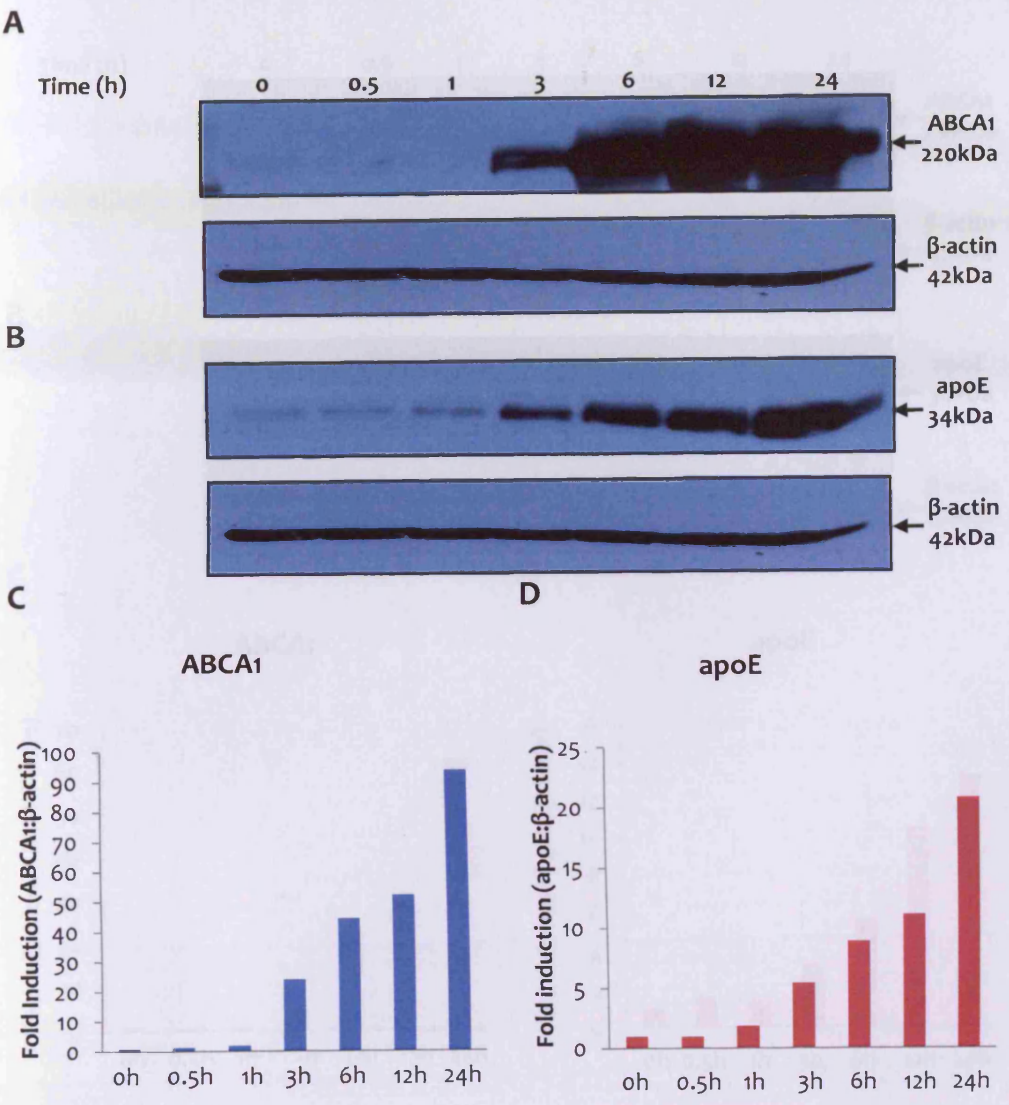


Figure 4.6 Time course action of natural LXR agonist on ABCA1 and apoE expression in THP-1 macrophages.

THP-1 macrophages were incubated for the time indicated in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (2μg/ml) in combination with 9CRA (10μM). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE or the β-actin control. Antigen-antibody complexes were detected using the ECL detection system (panels A and B). Densitometric analysis was carried out on the data and presented as fold induction in relation to basal levels (0h) assigned as 1 as shown in panels C and D.

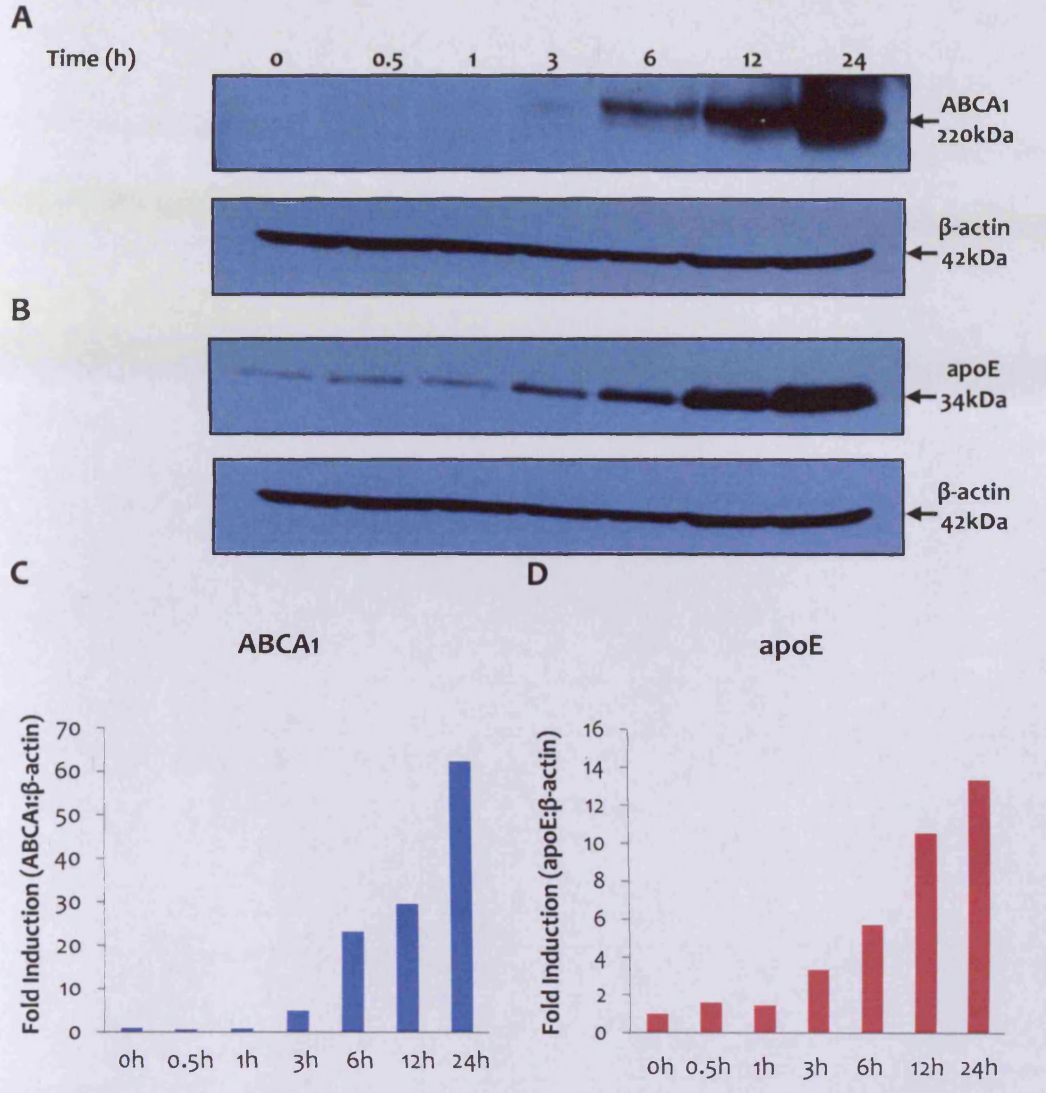


Figure 4.7 Time course action of synthetic LXR agonist T0901317 on ABCA1 and apoE protein expression in THP-1 macrophages.

THP-1 macrophages were incubated for the time indicated in RPMI medium 1640 containing 10% (v/v) LPDS with T0901317 (10μM). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE or the β-actin control. Antigen-antibody complexes were detected using the ECL detection system (panels A and B). Densitometric analysis was carried out on the data and presented as fold induction in relation to basal levels (0h) assigned as 1 as shown in panels C and D.

or curcumin (Figure 4.8). Similarly, the induction of ABCA1 expression by this ligand was attenuated in the presence of both these inhibitors. The effect of curcumin and SP600125 on the induction of ABCA1 and apoE expression by combinations of 22(R)-HC and 9CRA or the synthetic ligands was next investigated. As shown in Figures 4.9-4.11, both inhibitors attenuated, in a statistically significant manner, the induction of ABCA1 and apoE protein expression by 22(R)-HC plus 9CRA or the synthetic ligands GW3965 except for T0901317 treatment which was carried out twice. Overall, these results strongly suggest that the JNK/SAPK pathway is involved in the induction of ABCA1 and apoE expression by both natural and synthetic LXR agonists.

4.2.6 Concentration-dependent effects of JNK/SAPK inhibitors on LXR agonist-induced ABCA1 and apoE expression in THP-1macrophages

The experiments with the JNK/SAPK inhibitors shown in Figures 4.8-4.11 used a single concentration of SP600125 and curcumin (100μM and 25μM, respectively). It was decided to extend these findings by concentration-response experiments. Because combinations of 22(R)-HC and 9CRA and/or the synthetic ligand T0901317 are used extensively in LXR research (Ballerini et al., 2006; Chen et al., 2004; Costet et al., 2000; Crestani et al., 2004b; Hu et al., 2003; Jakel et al., 2004; Laffitte et al., 2001a; Laffitte et al., 2001b; Liao et al., 2002; Perez et al., 2003; Schmuth et al., 2004; Schultz et al., 2000; Tang et al., 2004b; Wagner et al., 2003), it was decided to restrict the analysis to these two activators. As shown in Figures 4.12-4.15, 22(R)-HC/9CRA or T0901317 produced a dramatic increase in the expression of ABCA1 and apoE proteins, which was attenuated by both inhibitors. Thus, the inhibition of ABCA1 expression induced by 22(R)-HC plus 9CRA or T0901317 was seen with 25μM SP600125 and this was attenuated further as the concentration of this pharmacological agent was increased. For apoE, no inhibition was obtained with 15μM SP600125 (Figures 4.12-4.13). However, there was a concentration-dependent attenuation of the response at subsequent concentration of the inhibitor used (Figure 4.12-4.13). Similarly, curcumin produced a concentration-dependent attenuation of ABCA1 and apoE expression that was induced in the presence of 22(R)-HC plus 9CRA or T0901317 (Figures 4.14-4.15). However, whereas a marked inhibition of 22(R)-HC plus 9CRA-induced expression of both proteins was obtained with 15μM of the inhibitor, higher concentrations were required in the case of T0901317 (Figures 4.14-4.15). Overall, these results show that SP600125 and curcumin inhibit the LXR agonist-induced expression of ABCA1 and apoE proteins at several different concentrations.

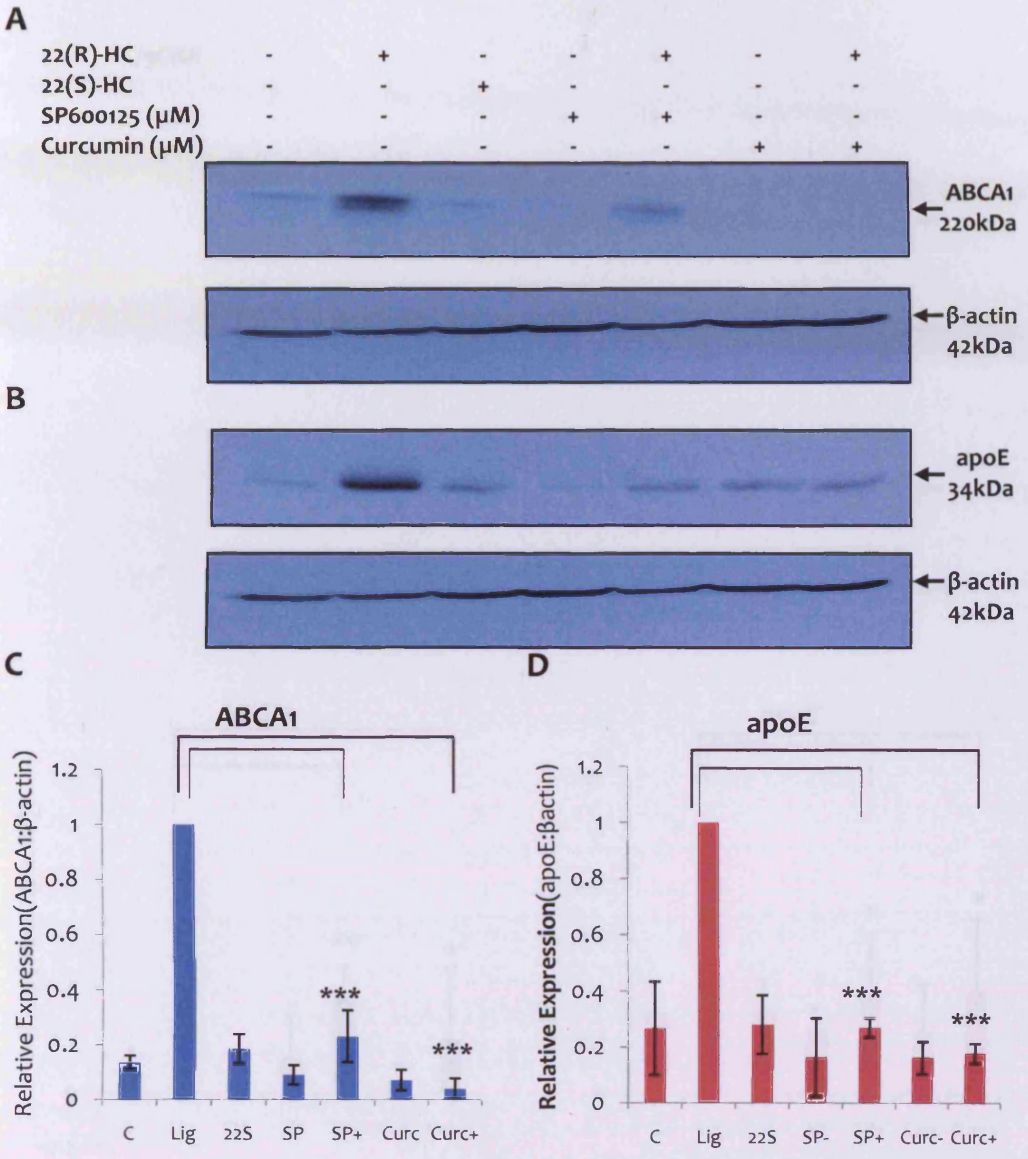


Figure 4.8 Effect of JNK/SAPK inhibitors on 22(R)-HC-mediated induction of ABCA1 and apoE expression.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (Lig) (2μg/ml) in the absence or the presence of SP600125 (SP) (100μM) or curcumin (Curc) (25μM). In addition, cells were treated with 22(S)-HC (2μg/ml) or DMSO as a vehicle control (C). The inhibitors were added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE and the β-actin control. Antigen-antibody complexes were detected using the ECL detection system. Results shown in panels A and B are representative of three independent experiments. Densitometric analysis was carried out on the data and presented as relative expression (mean ±SD) normalised to the expression of β-actin. The relative expression in the presence of the Ligand alone (Lig) has been assigned as 1 as shown in panels C and D (***P<0.001).

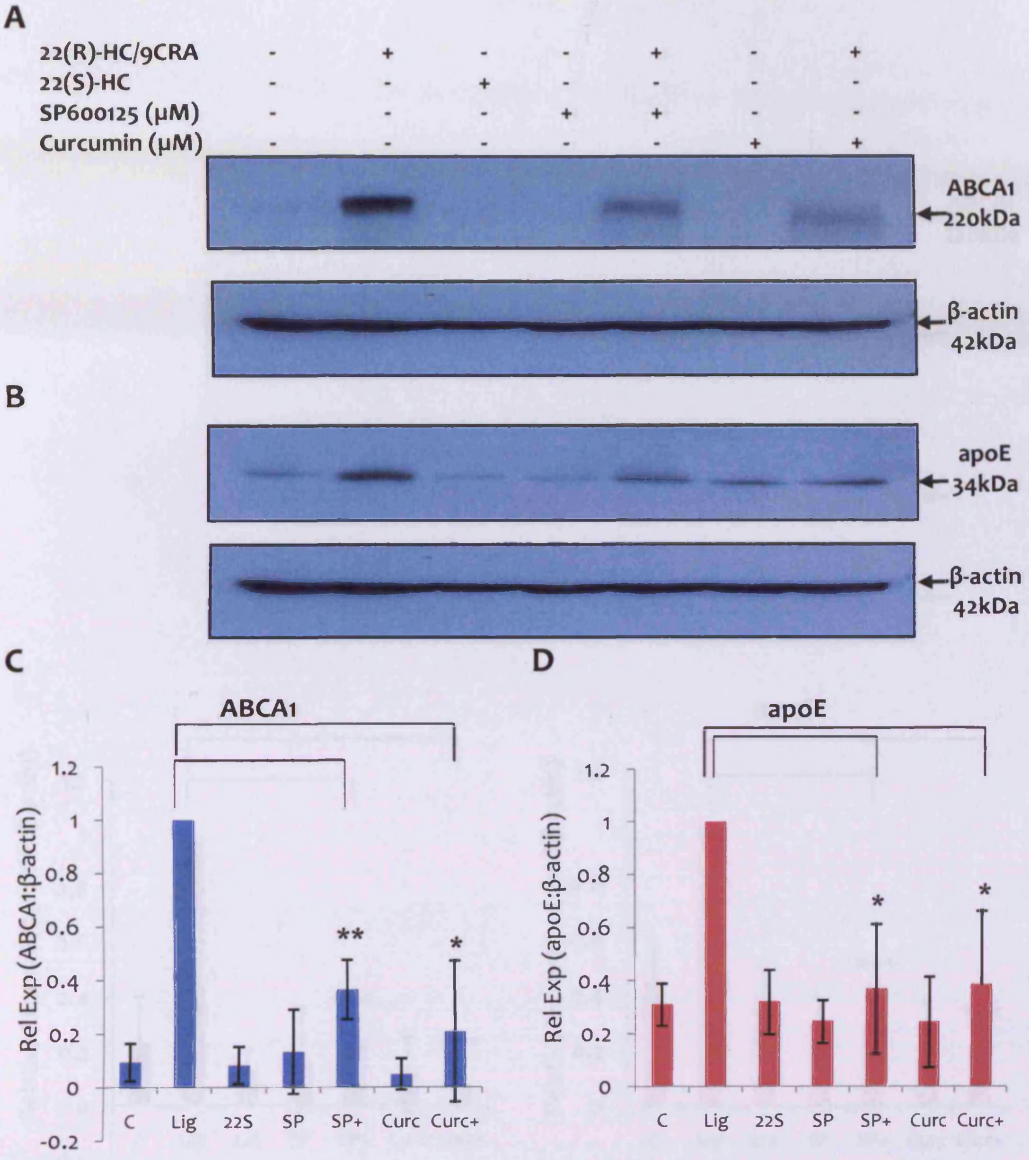


Figure 4.9 Effect of JNK/SAPK inhibitors on 22(R)-HC/9CRA-mediated induction of ABCA1 and apoE expression in THP-1 macrophages.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (2μg/ml) and 9CRA (10μM)] (Lig) in the absence or the presence of SP600125 (SP) (100μM) or curcumin (Curc) (25μM). In addition, cells were treated with 22(S)-HC (2μg/ml) or DMSO as a vehicle control (C). The inhibitors were added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE and the β-actin control. Antigen-antibody complexes were detected using the ECL detection system. Results shown in panels A and B are representative of three independent experiments. Densitometric analysis was carried out on the data and presented as relative expression (mean ±SD) normalised to the expression of β-actin. The relative expression (Rel Exp) in the presence of the ligand alone (Lig) has been assigned as 1 as shown in panels C and D (*P<0.05, **P<0.01 compared to controls).

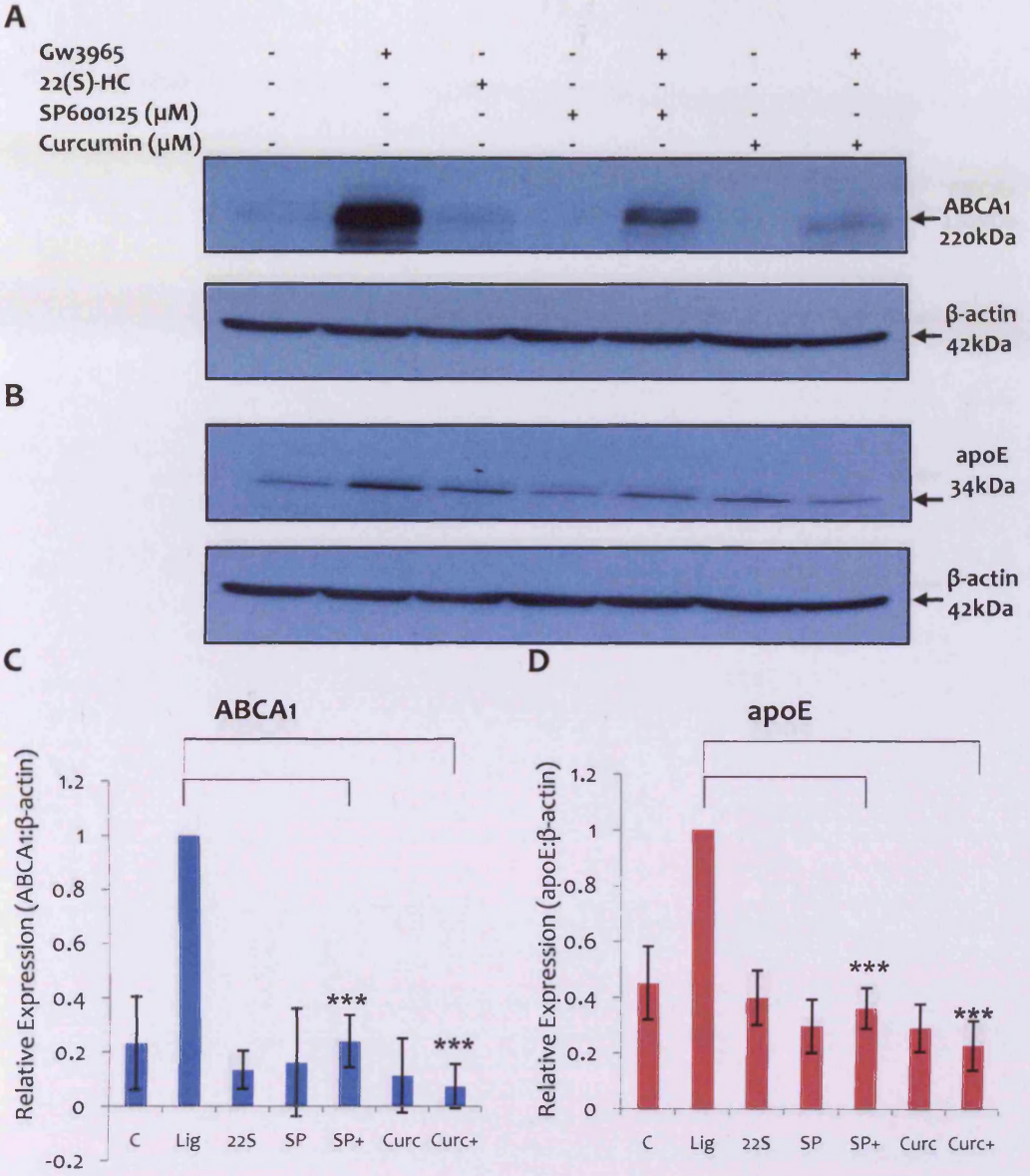


Figure 4.10 Effect of JNK/SAPK inhibitors on GW3965-mediated induction of ABCA1 and apoE expression.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with GW3965 (Lig) (1μM) in the absence or the presence of SP600125 (SP) (100μM) or curcumin (Curc) (25μM). In addition, cells were treated with 22(S)-HC (2μg/ml) or DMSO as a vehicle control (C). The inhibitors were added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE and the β-actin control. Antigen-antibody complexes were detected using the ECL detection system. Results shown in panels A and B are representative of three independent experiments. Densitometric analysis was carried out on the data and presented as relative expression (mean ±SD) normalised to the expression of β-actin. The relative expression in the presence of the ligand alone (Lig) has been assigned as 1 as shown in panels C and D (***P<0.001).

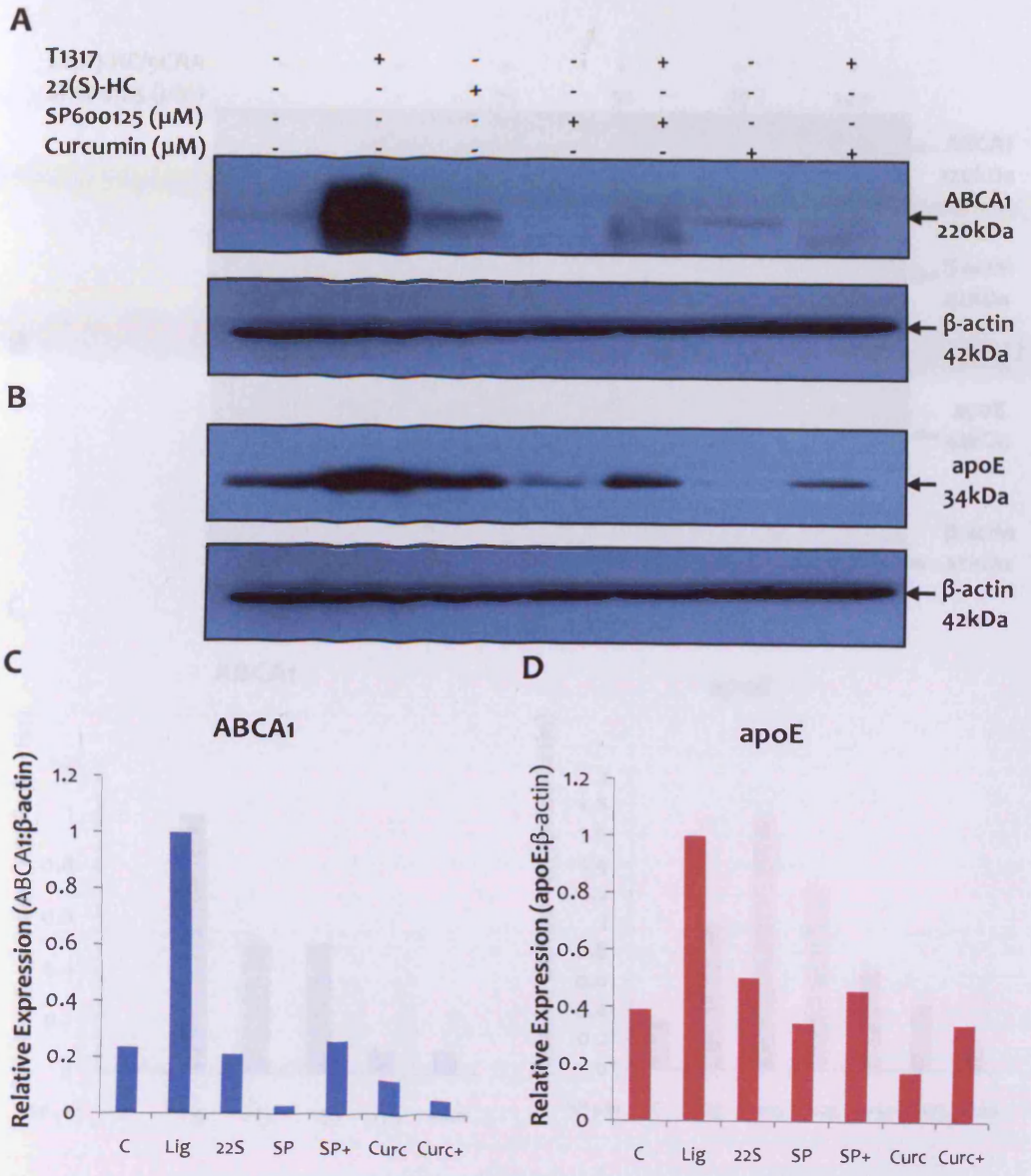


Figure 4.11 Effect of JNK/SAPK inhibitors on T0901317-mediated induction of ABCA1 and apoE expression.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with T0901317 (T1317) (Lig) (10μM) in the absence or the presence of SP600125 (SP) (100μM) or curcumin (Curc) (25μM). In addition, cells were treated with 22(S)-HC (2μg/ml) or DMSO as a vehicle control (C). The inhibitors were added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE and the β-actin control. Antigen-antibody complexes were detected using the ECL detection system. Results shown in panels A and B are representative of two independent experiments. Densitometric analysis was carried out on the data and presented as relative expression (average) normalised to the expression of β-actin. The relative expression in the presence of the ligand alone (Lig) has been assigned as 1 (panels C and D).

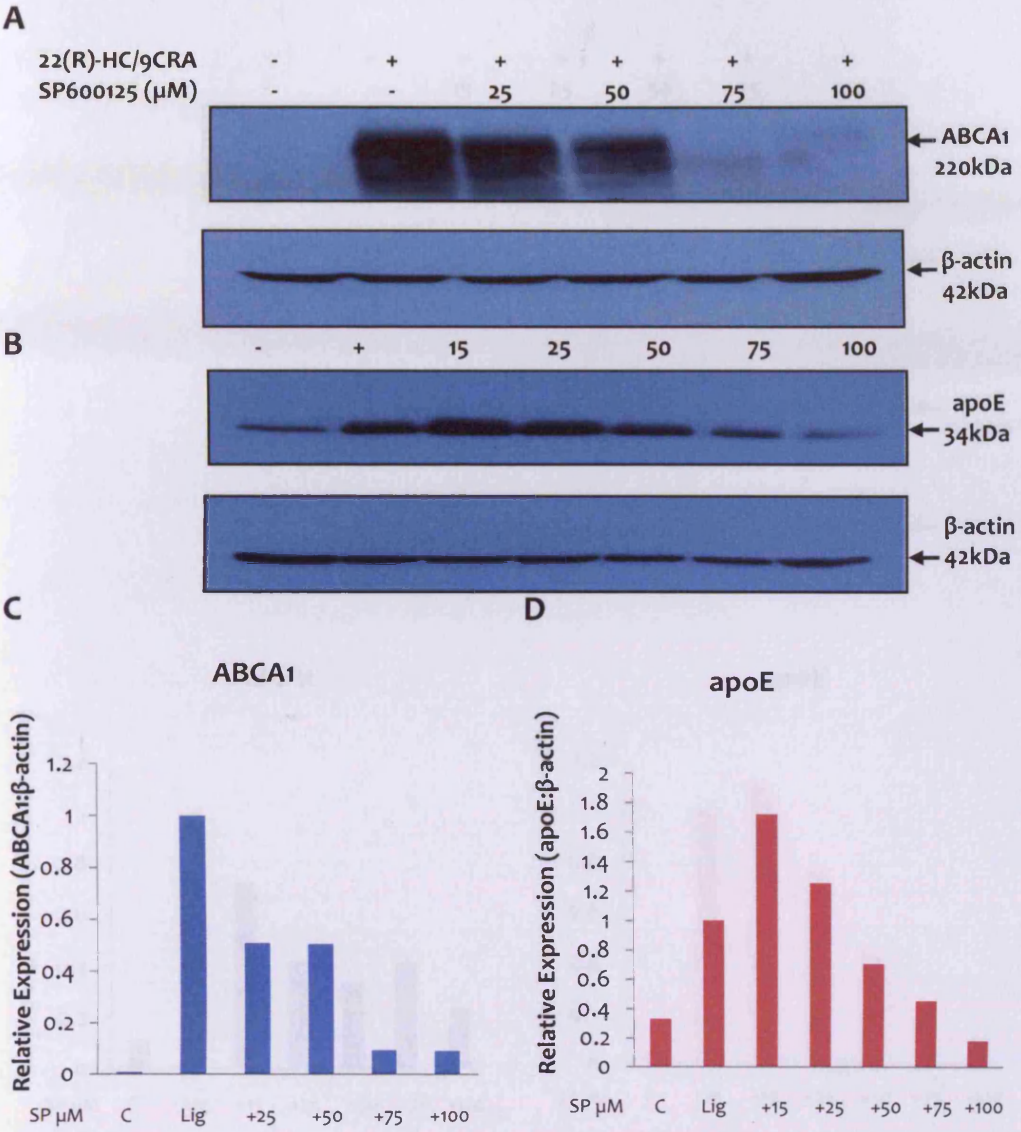


Figure 4.12 Concentration-dependent inhibition of 22(R)-HC/9CRA-mediated induction of ABCA1 and apoE expression by SP600125.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (2μg/ml) and 9CRA (10μM) (Lig). In addition, cells were treated with DMSO as a vehicle control (C). The inhibitor, SP600125 (SP), at the indicated concentrations was added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE or the β-actin control. Antigen-antibody complexes were detected using the ECL detection system (panels A and B). Densitometric analysis was carried out on the data and presented as relative expression (average) normalised to the expression of β-actin. The relative expression in the presence of the ligand alone (Lig) has been assigned as 1 as shown in panels C and D.

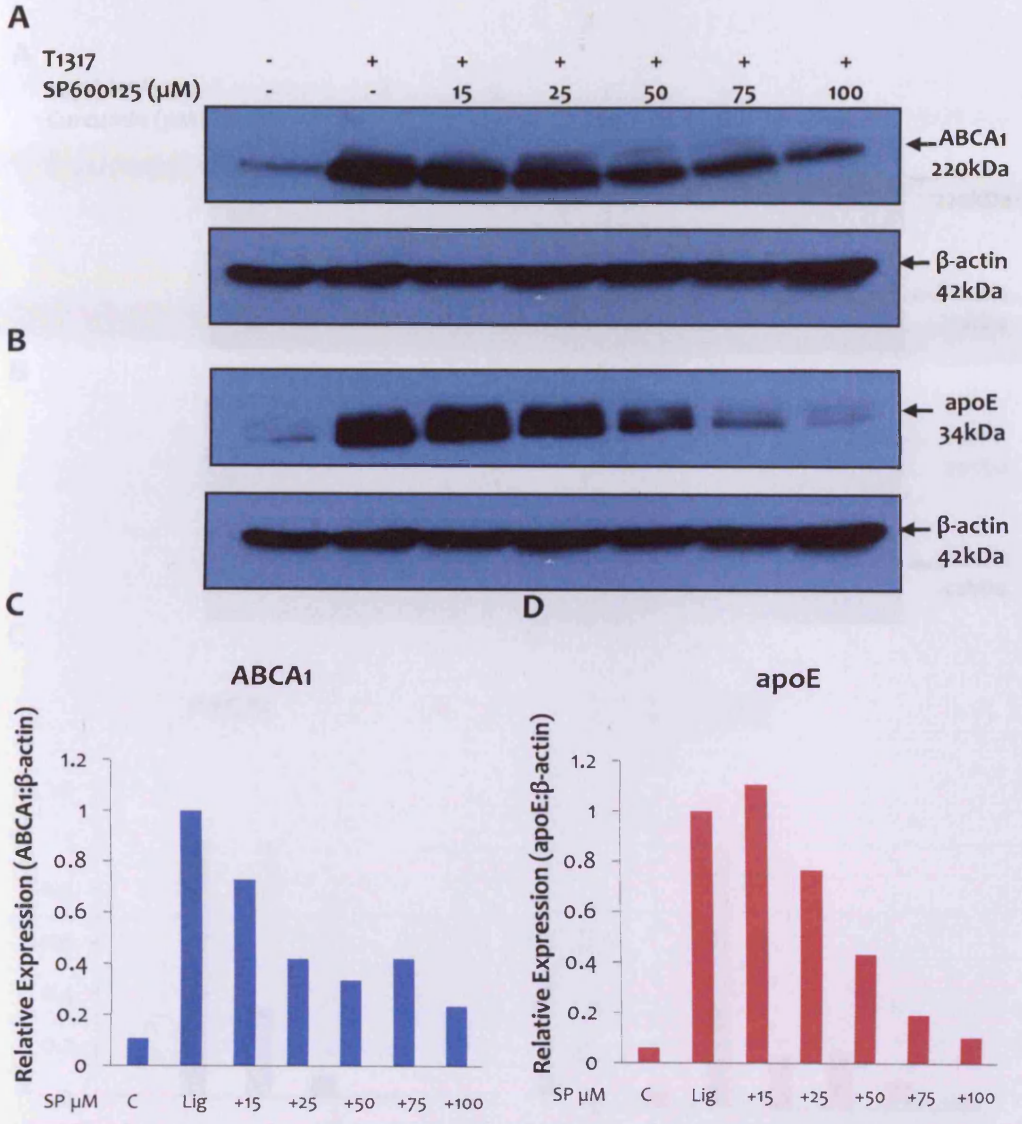


Figure 4.13 Concentration-dependent inhibition of T0901317-mediated induction of ABCA1 and apoE expression by SP600125.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with T0901317 (T1317) (Lig) (10μM). In addition, cells were treated with DMSO as a vehicle control (C). The inhibitor, SP600125 (SP), at the indicated concentrations was added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE or the β-actin control. Antigen-antibody complexes were detected using the ECL detection system (panels A and B). Densitometric analysis was carried out on the data and presented as relative expression (average) normalised to the expression of β-actin. The relative expression in the presence of the ligand alone (Lig) has been assigned as 1 as shown in panels C and D.

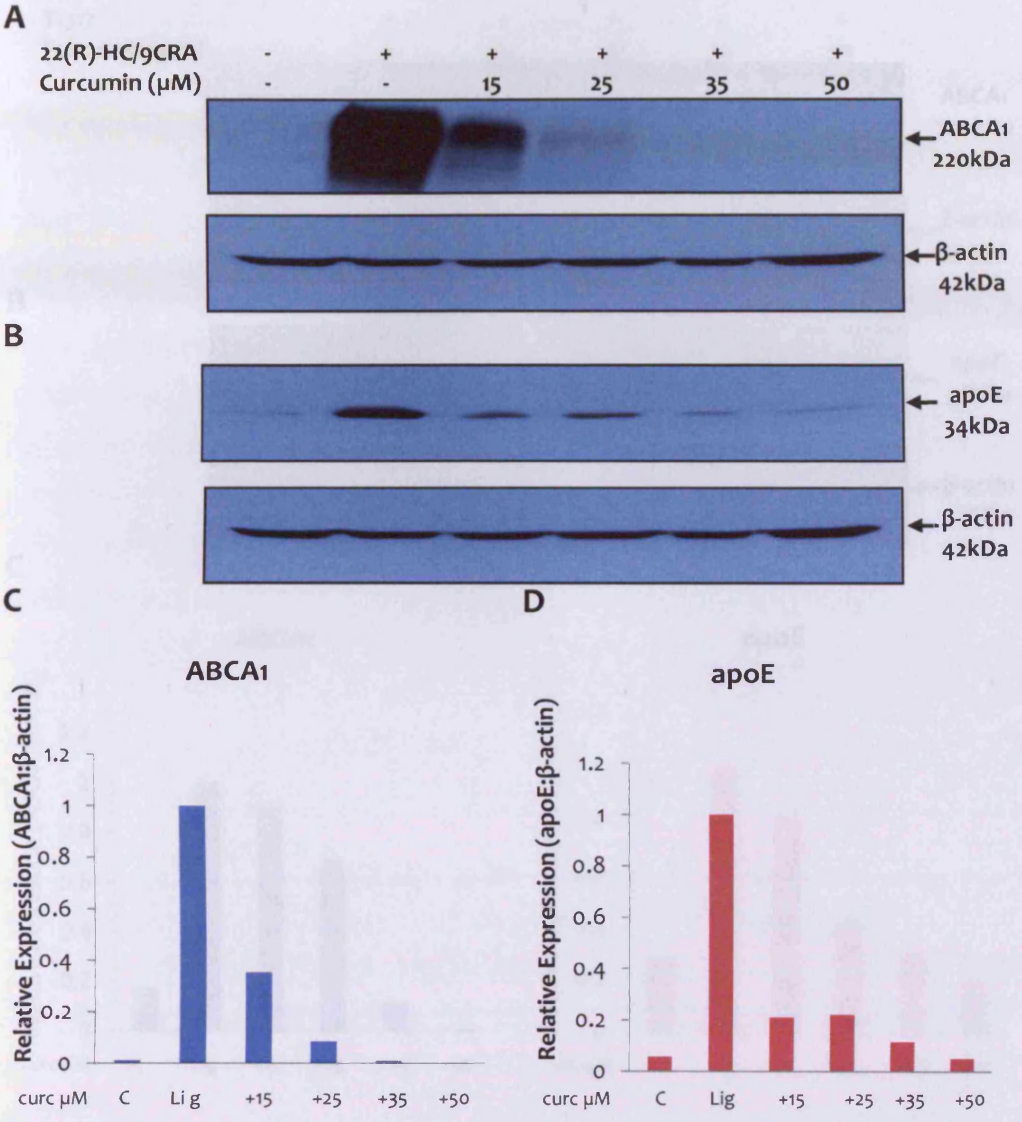


Figure 4.14 Concentration-dependent inhibition of 22(R)-HC/9CRA-mediated induction of ABCA1 and apoE expression by curcumin.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (2μg/ml) and 9CRA (10μM) (Lig). In addition, cells were treated with DMSO as a vehicle control (C). The inhibitor, curcumin (curc), at the indicated concentrations was added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE or the β-actin control. Antigen-antibody complexes were detected using the ECL detection system (panels A and B). Densitometric analysis was carried out on the data and presented as relative expression (average) normalised to the expression of β-actin. The relative expression in the presence of the ligand alone (Lig) has been assigned as 1 as shown in panels C and D.

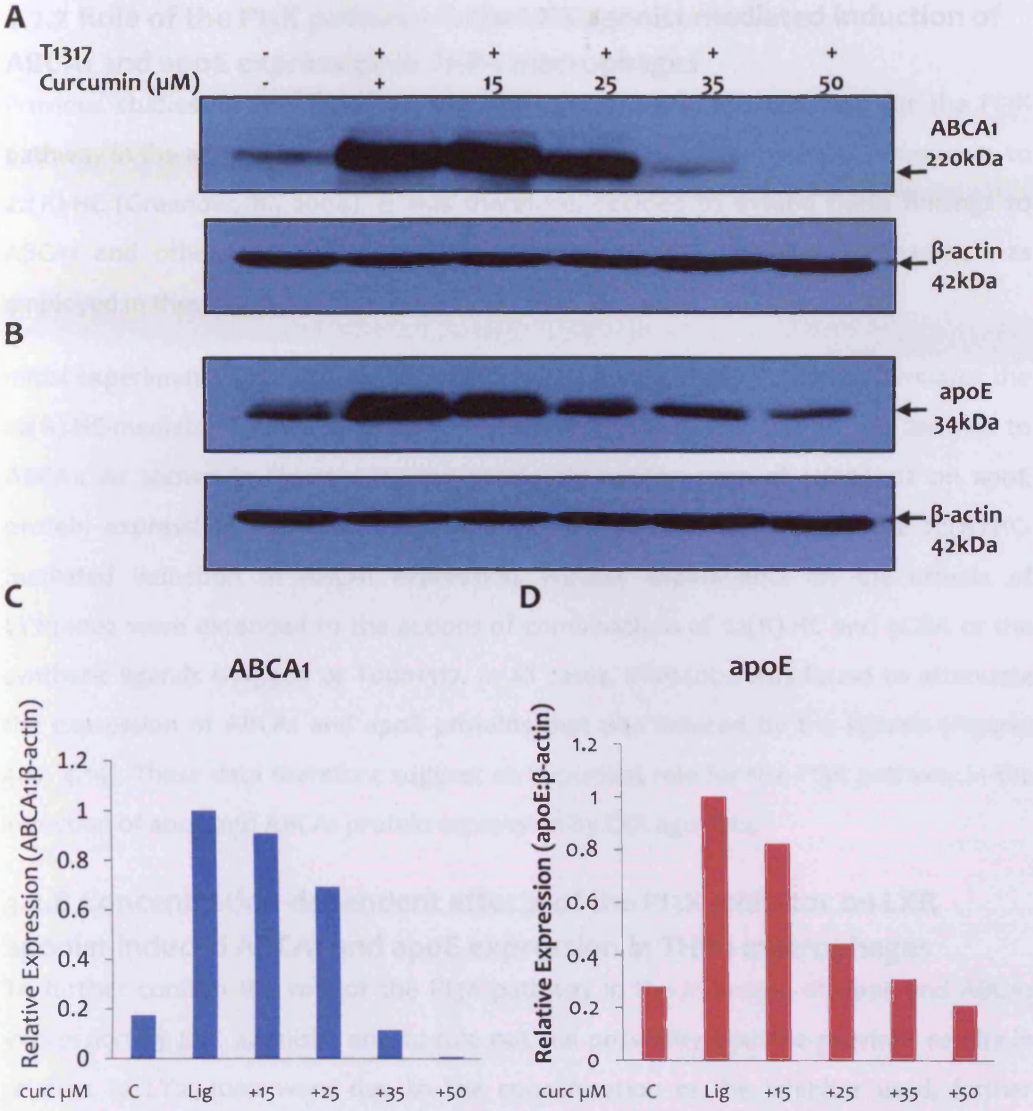


Figure 4.15 Concentration-dependent inhibition of T0901317-mediated induction of ABCA1 and apoE expression by curcumin.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with T0901317 (T1317) (Lig) (10μM). In addition, cells were treated with DMSO as a vehicle control (C). The inhibitor, curcumin (curc), at the indicated concentrations was added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE or the β-actin control. Antigen-antibody complexes were detected using the ECL detection system (panels A and B). Densitometric analysis was carried out on the data and presented as relative expression (average) normalised to the expression of β-actin. The relative expression in the presence of the ligand alone (Lig) has been assigned as 1 as shown in panels C and D.

4.2.7 Role of the PI3K pathway in the LXR agonist-mediated induction of ABCA1 and apoE expression in THP-1 macrophages

Previous studies in the laboratory had also revealed an important role for the PI3K pathway in the activation of apoE gene expression in THP-1 macrophages in response to 22(R)-HC (Greenow, K., 2004). It was therefore, decided to extend these findings to ABCA1 and other LXR activators. The widely used PI3K inhibitor, LY294002, was employed in these studies.

Initial experiments aimed to confirm the previous finding that LY294002 attenuates the 22(R)-HC-mediated induction of apoE protein expression and extend the analysis to ABCA1. As shown in Figure 4.16, the previously noted action of LY294002 on apoE protein expression was confirmed. In addition, LY294002 attenuated the 22(R)-HC-mediated induction of ABCA1 expression. Further experiments on the effects of LY294002 were extended to the actions of combinations of 22(R)-HC and 9CRA or the synthetic ligands GW3965 or T0901317. In all cases, LY294002 was found to attenuate the expression of ABCA1 and apoE proteins that was induced by the ligands (Figures 4.17- 4.19). These data therefore suggest an important role for the PI3K pathway in the induction of apoE and ABCA1 protein expression by LXR agonists.

4.2.8 Concentration-dependent effects of the PI3K inhibitor on LXR agonist-induced ABCA1 and apoE expression in THP-1 macrophages

To further confirm the role of the PI3K pathway in the induction of apoE and ABCA1 expression by LXR agonists, and to rule out the possibility that the previous results in relation to LY294002 were due to the concentration of the inhibitor used, further experiments were carried out using a range of concentrations of this pharmacological agent. Similar to such experiments with the JNK/SAPK inhibitors SP600125 and curcumin, the analysis was restricted to combinations of 22(R)-HC and 9CRA or T0901317. In both cases, LY294002 was found to attenuate the expression of ABCA1 and apoE proteins that was induced by the ligands (Figures 4.20-4.21).

4.2.9 Effect of LXR agonists on ABCA1 and apoE expression in human primary macrophages

In order to rule out the possibility that the results obtained are peculiar to the transformed THP-1 cell line, the effects of LXR agonists on the expression of the ABCA1 and apoE genes in human primary monocyte-derived macrophages (HMDMs) were

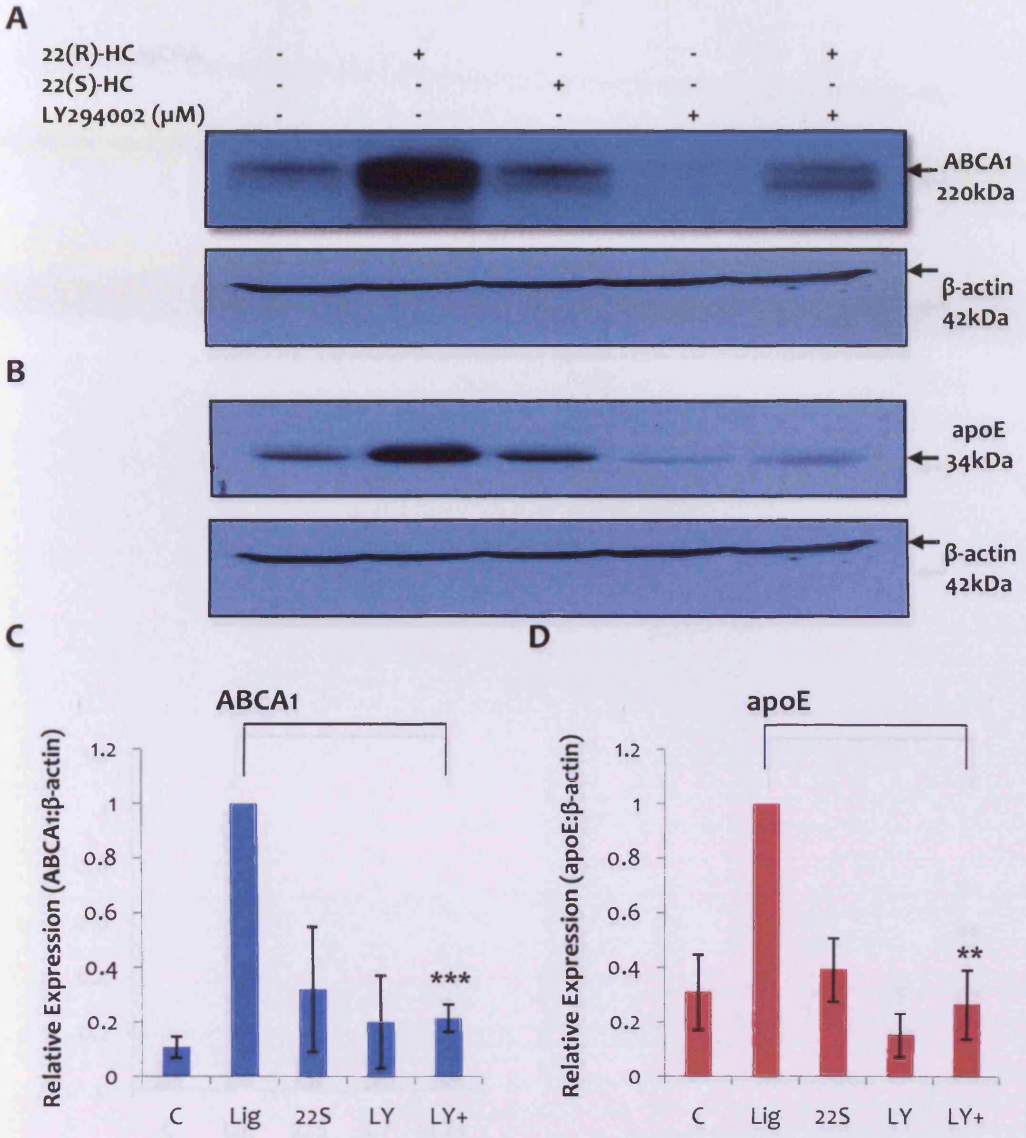


Figure 4.16 Effect of the PI3K inhibitor LY294002 on the 22(R)-HC-mediated induction of ABCA1 and apoE expression.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (2μg/ml) (Lig) in the absence or the presence of LY294002 (LY) (100μM). In addition, cells were treated with 22(S)-HC (2μg/ml) or DMSO as a vehicle control (C). The inhibitor was added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE and the β-actin control. Antigen-antibody complexes were detected using the ECL detection system. Results shown in panels A and B are representative of three independent experiments. Densitometric analysis was carried out on the data and presented as relative expression (mean ±SD) normalised to the expression of β-actin. The relative expression in the presence of the ligand (Lig) alone has been assigned as 1 as shown in panels C and D (**P<0.01, ***P<0.001).

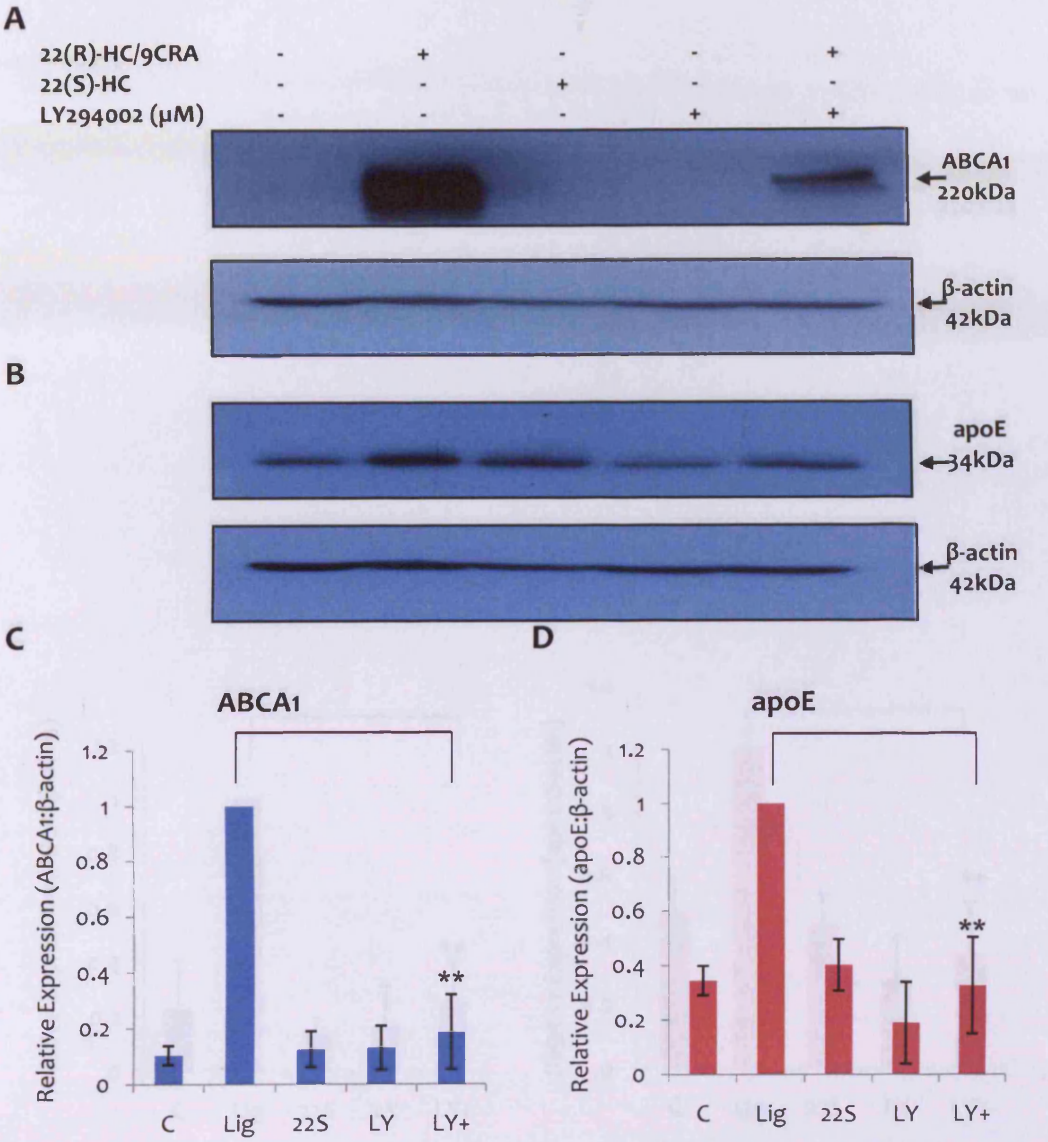


Figure 4.17 Effect of the PI3K inhibitor LY294002 on the 22(R)-HC/9CRA-mediated induction of ABCA1 and apoE expression.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (2μg/ml) and 9CRA (10μM) (Lig) in the absence or the presence of LY294002 (LY) (100μM). In addition, cells were treated with 22(S)-HC (2μg/ml) or DMSO as a vehicle control (C). The inhibitor was added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE and the β-actin control. Antigen-antibody complexes were detected using the ECL detection system. Results shown in panels A and B are representative of three independent experiments. Densitometric analysis was carried out on the data and presented as relative expression (mean ±SD) normalised to the expression of β-actin. The relative expression in the presence of the ligand (Lig) alone has been assigned as 1 as shown in panels C and D (**P<0.01).

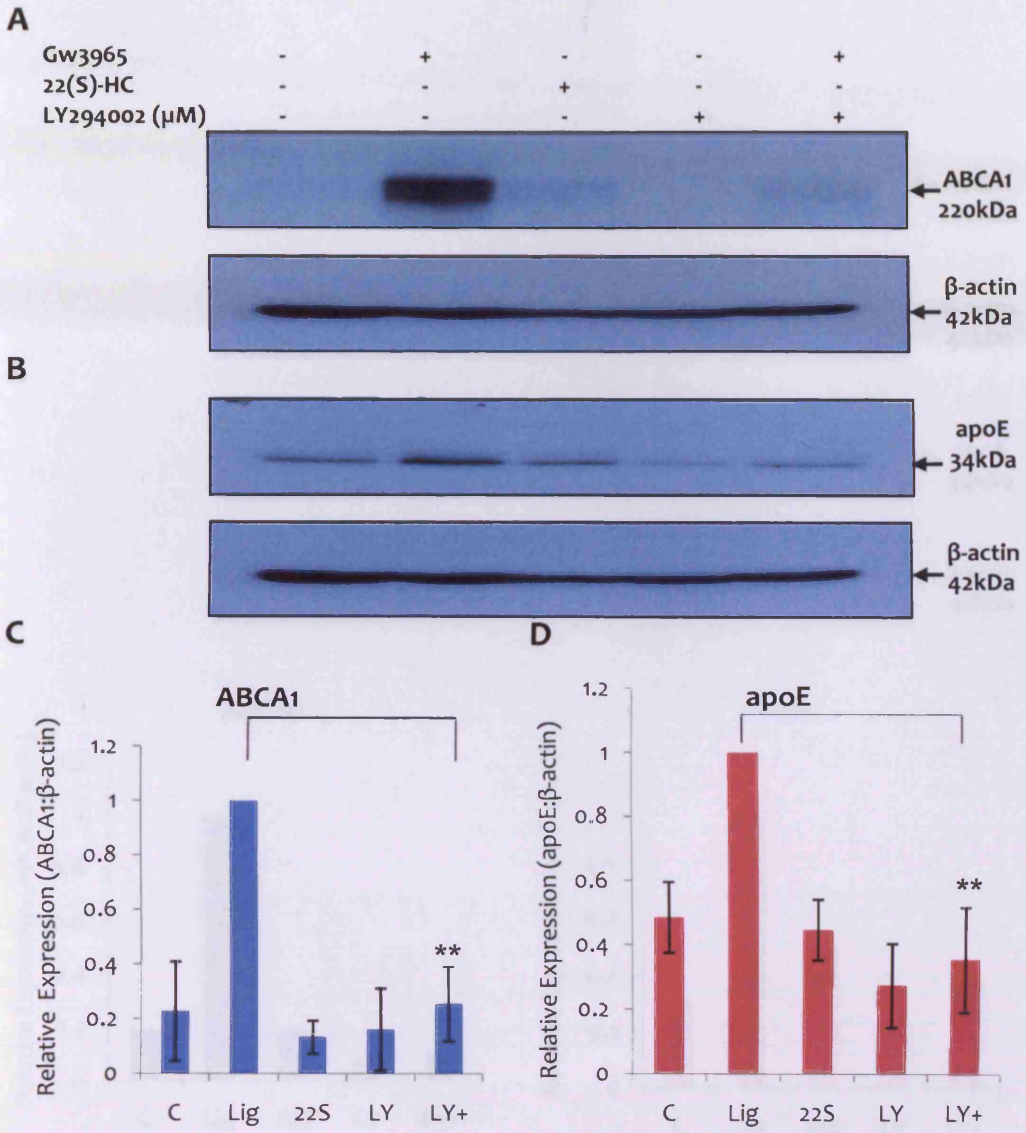


Figure 4.18 Effect of the PI3K inhibitor LY294002 on the GW3965-mediated induction of ABCA1 and apoE expression.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with GW3965 (1μM) (Lig) in the absence or the presence of LY294002 (LY) (100μM). In addition, cells were treated with 22(S)-HC (2μg/ml) (22S) or DMSO as a vehicle control (C). The inhibitor was added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE and the β-actin control. Antigen-antibody complexes were detected using the ECL detection system. Results shown in panels A and B are representative of three independent experiments. Densitometric analysis was carried out on the data and presented as relative expression (mean ±SD) normalised to the expression of β-actin. The relative expression in the presence of the ligand alone (Lig) has been assigned as 1 as shown in panels C and D (**P<0.01).

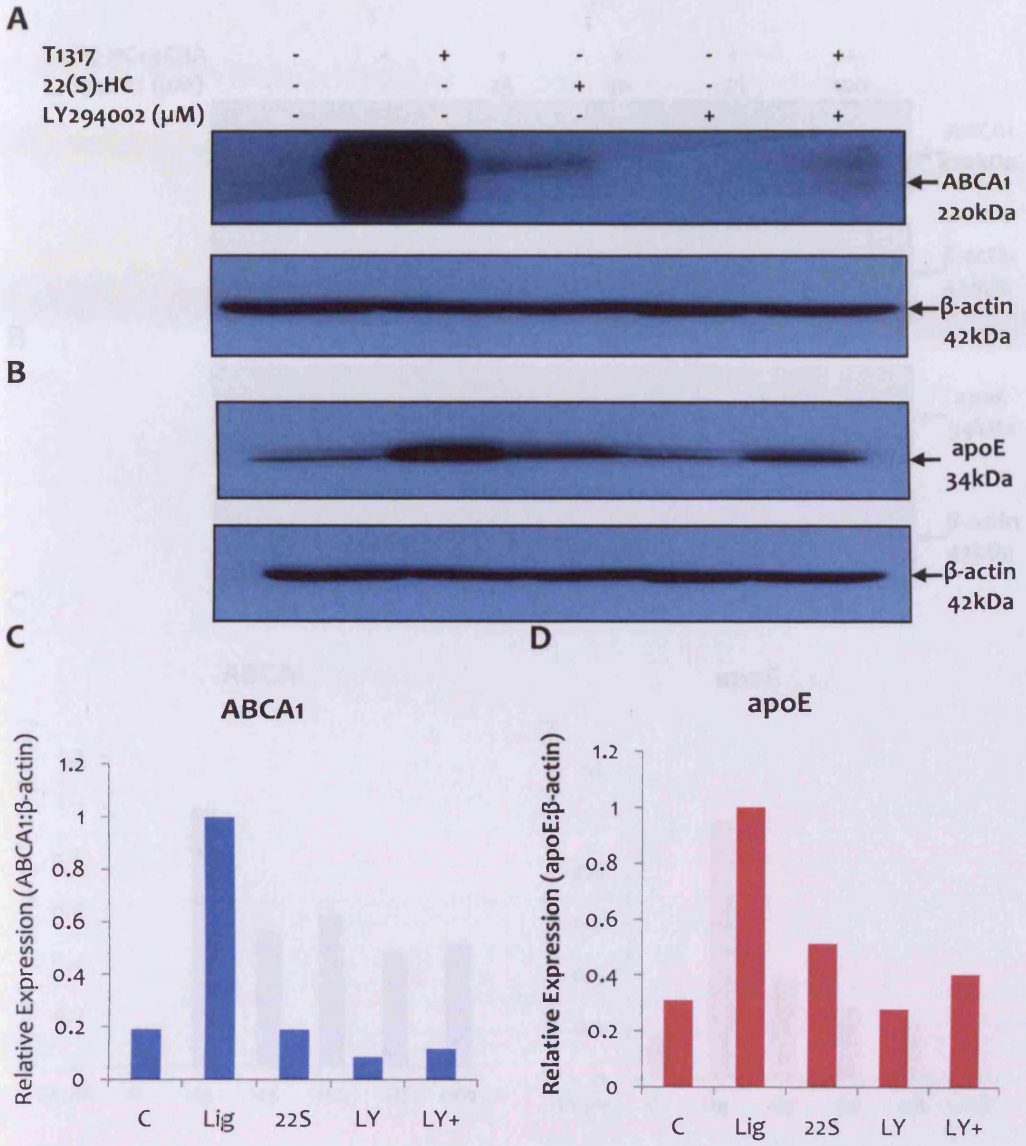


Figure 4.19 Effect of the PI3K inhibitor LY294002 on the T0901317-mediated induction of ABCA1 and apoE expression.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with T0901317 (T1317) (10μM) (Lig) in the absence or the presence of LY294002 (LY) (100μM). In addition, cells were treated with 22(S)-HC (2μg/ml) (22S) or DMSO as a vehicle control (C). The inhibitor was added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE and the β-actin control. Antigen-antibody complexes were detected using the ECL detection system. Results shown in panels A and B are representative of two independent experiments. Densitometric analysis was carried out on the data and presented as relative expression (average) normalised to the expression of β-actin. The relative expression in the presence of the ligand alone (Lig) has been assigned as 1 as shown in panels C and D.

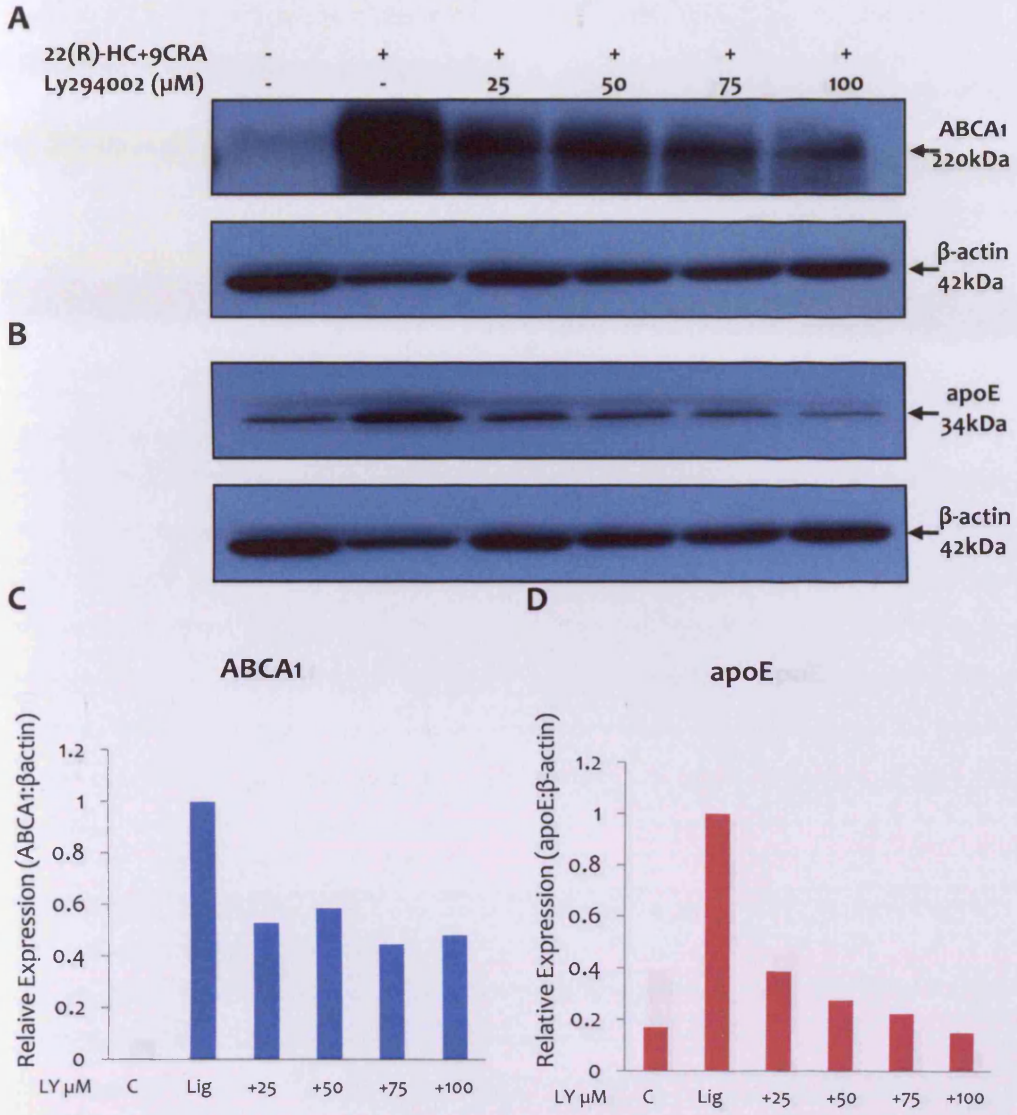


Figure 4.20 Concentration-dependent inhibition of 22(R)-HC/9CRA-mediated induction of ABCA1 and apoE expression by LY294002.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (2μg/ml) and 9CRA (10μM) (Lig). In addition, cells were treated with DMSO as a vehicle control (C). The inhibitor, LY294002 (LY), at the indicated concentrations was added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE and the β-actin control. Antigen-antibody complexes were detected using the ECL detection system (panels A and B). Densitometric analysis was carried out on the data and presented as relative expression normalised to the expression of β-actin. The relative expression in the presence of the ligand alone (Lig) has been assigned as 1 as shown in panels C and D.

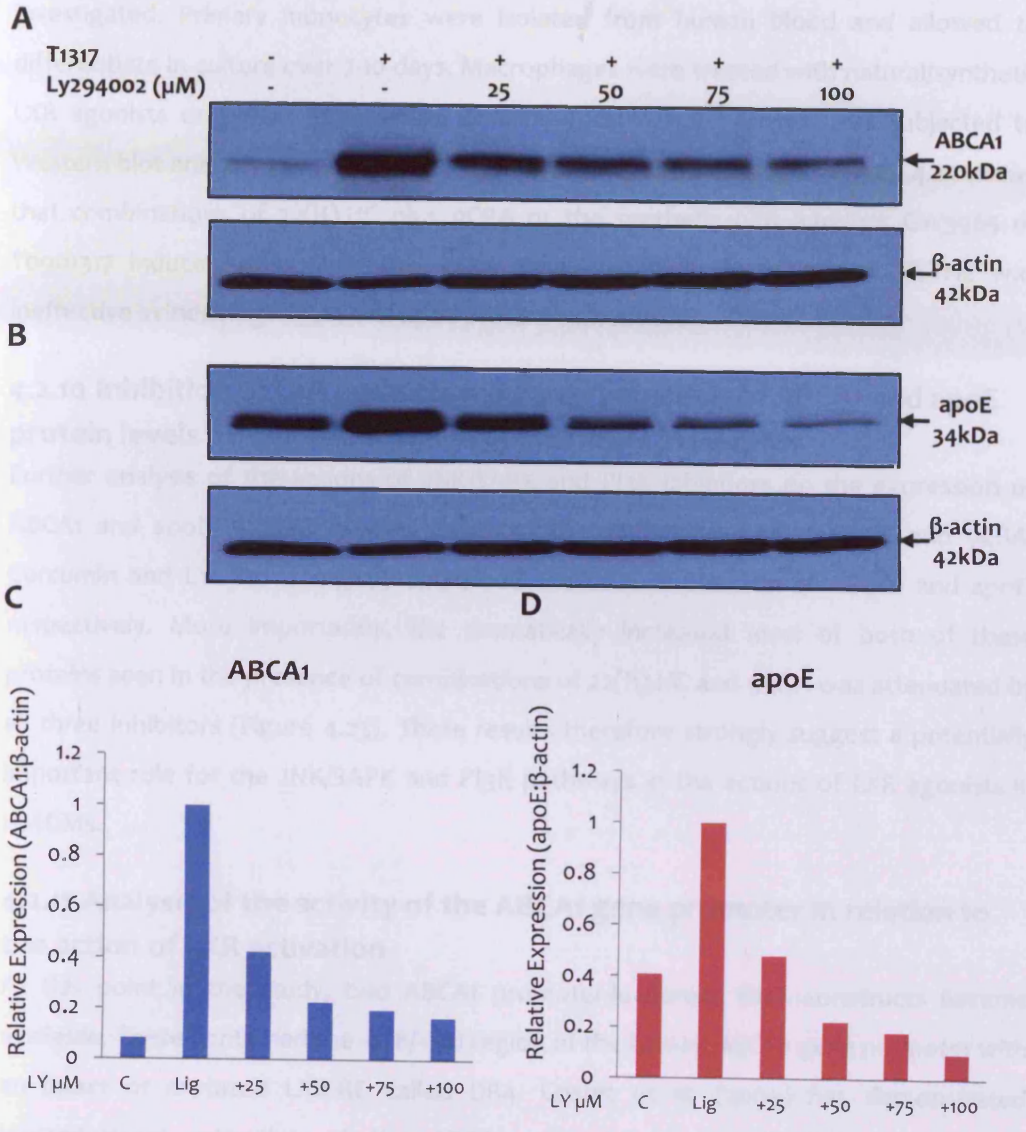


Figure 4.21 Concentration-dependent inhibition of T0901317-mediated induction of ABCA1 and apoE expression by LY294002.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with T0901317 (T1317) (Lig) (10μM). In addition, cells were treated with DMSO as a vehicle control (C). The inhibitor, LY294002 (LY), at the indicated concentrations was added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE and the β-actin control. Antigen-antibody complexes were detected using the ECL detection system (panels A and B). Densitometric analysis was carried out on the data and presented as relative expression normalised to the expression of β-actin. The relative expression in the presence of the ligand alone (Lig) has been assigned as 1 as shown in panels C and D.

investigated. Primary monocytes were isolated from human blood and allowed to differentiate in culture over 7-10 days. Macrophages were treated with natural/synthetic LXR agonists or DMSO as a vehicle control. Total cellular protein was subjected to Western blot analysis using antibodies against ABCA1, apoE or β-actin. Figure 4.22 shows that combinations of 22(R)-HC plus 9CRA or the synthetic LXR agonists GW3965 or T0901317 induce ABCA1 and apoE expression in HMDMs. In contrast, 22(S)-HC was ineffective in inducing ABCA1 and apoE protein expression.

4.2.10 Inhibition of LXR agonists-mediated induction of ABCA1 and apoE protein levels by JNK/SAPK and PI3K inhibitors in HMDMs

Further analysis of the actions of JNK/SAPK and PI3K inhibitors on the expression of ABCA1 and apoE in HMDMs was restricted to combinations of 22(R)-HC and 9CRA. Curcumin and LY294002 slightly decreased the basal expression of ABCA1 and apoE, respectively. More importantly, the dramatically increased level of both of these proteins seen in the presence of combinations of 22(R)-HC and 9CRA was attenuated by all three inhibitors (Figure 4.23). These results therefore strongly suggest a potentially important role for the JNK/SAPK and PI3K pathways in the actions of LXR agonists in HMDMs.

4.2.11 Analysis of the activity of the ABCA1 gene promoter in relation to the action of LXR activation

At this point in the study, two ABCA1 promoter-luciferase DNA constructs became available. These contained the -928/+101 region of the human ABCA1 gene promoter with an intact or mutated LXR-RE called DR4. Costet et al. (2000) has demonstrated transcriptional activation of the ABCA1 promoter by oxysterols in the murine macrophage RAW264.7 cell line. Mutation of this DR4 element abolished the oxysterol-mediated induction of the hABCA1 promoter (Costet et al., 2003; Costet et al., 2000) in RAW264.7 macrophages. However, similar studies have not been carried out in human macrophages. Therefore, it was decided to use these DNA constructs as additional tools for the investigation of the signal transduction pathways activated by LXR agonists.

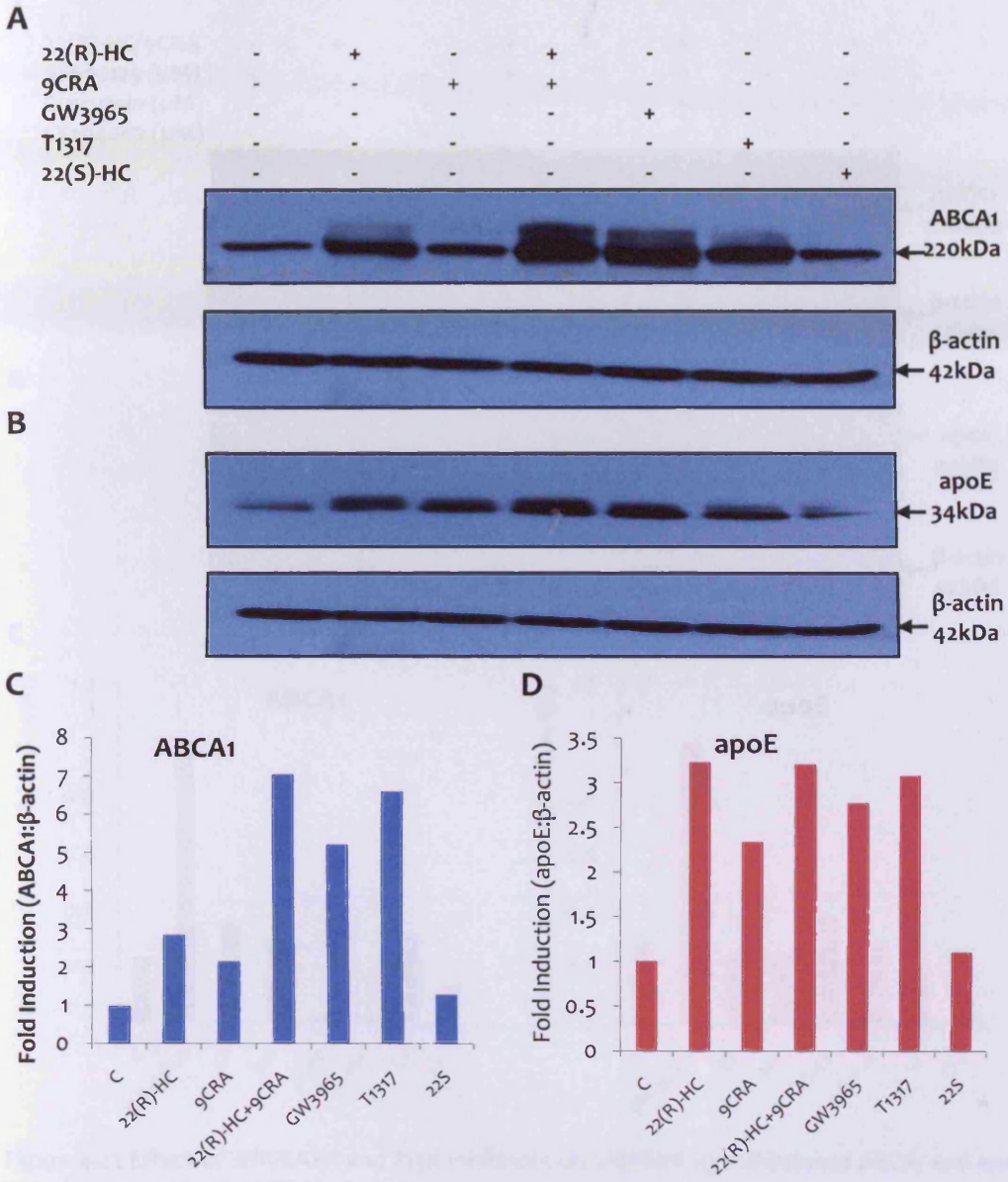


Figure 4.22 Effect of LXR agonists on ABCA1 and apoE protein expresstion in HMDMs.

Human monocytes were isolated and cultured for 7-10 days in RPMI medium 1640 supplemented with 5% (v/v) FCS. Differentiated macrophages were incubated for 24h in medium containing 10% LPDS with 22(R)-HC (3μg/ml), 22(S)-HC (3μg/ml), 9CRA (10μM), GW3965 (1μM) or T1317 (10μM). In addition, cells were treated with DMSO as a vehicle control (C). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against apoE, ABCA1 or the β-actin control. Antigen-antibody complexes were detected using the ECL detection system. Results shown in panels A and B are representative of two independent experiments except for 9CRA treatment alone, which has been carried out once. Densitometric analysis was carried out on the data and presented as fold induction (average) in relation to basal levels (in the presence of vehicle alone; C) assigned as 1 as shown in panels C and D.

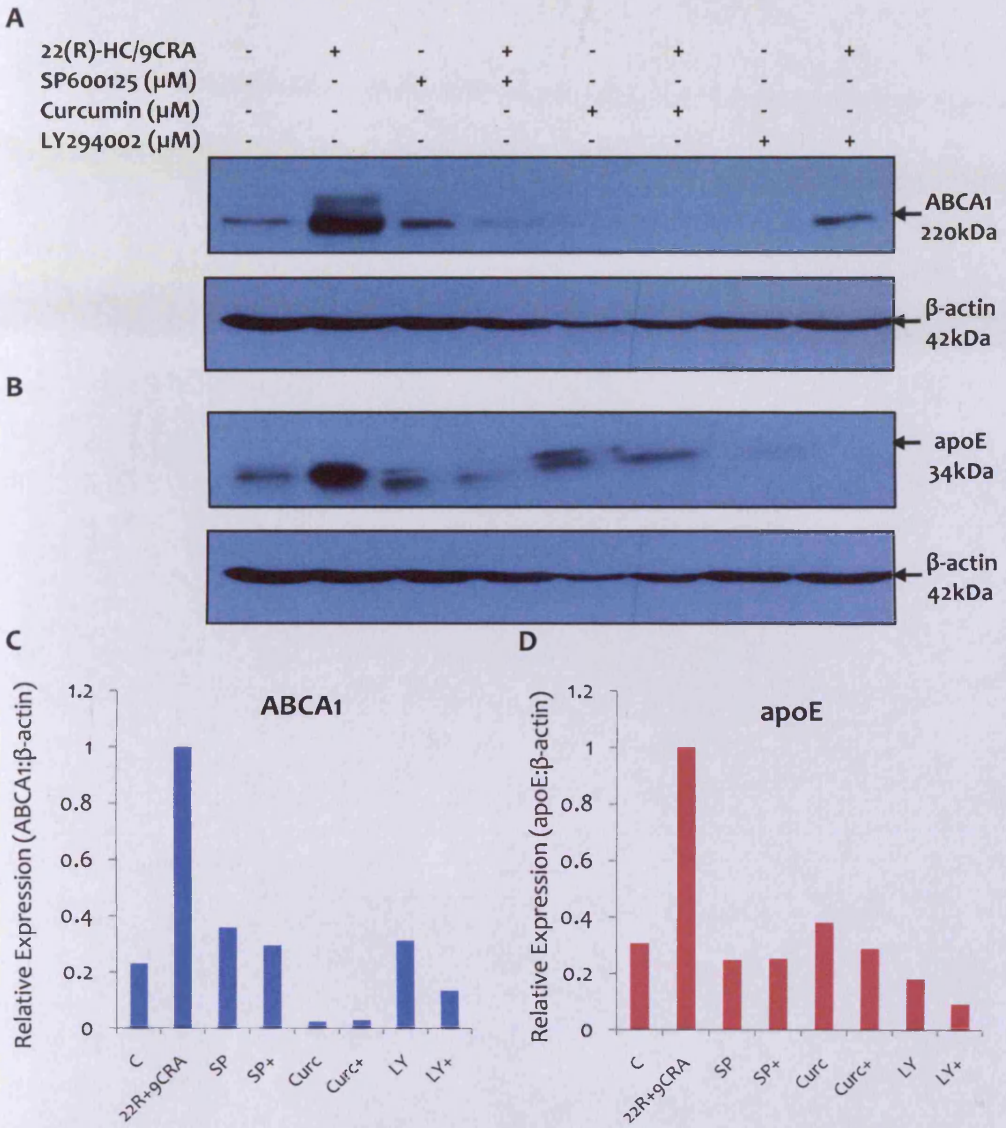


Figure 4.23 Effect of JNK/SAPK and PI3K inhibitors on LXR/RXR ligand-induced ABCA1 and apoE protein expresstion in HMDMs.

Human monocytes were isolated and cultured for 7-10 days in RPMI medium 1640 supplemented with 5% (v/v) FCS. Differentiated macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPSD with 22(R)-HC (3μg/ml) and 9CRA (10μM) in the absence or the presence of SP600125 (SP) (50μM), curcumin (curc) (25μM) and LY294002 (LY) (100μM). The inhibitors were added 1h before the ligand (pre-treatment). In addition, cells were treated with DMSO as a vehicle control (C). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against apoE, ABCA1 or the β-actin control. Antigen-antibody complexes were detected using the ECL detection system. Results shown in panels A and B are representative of two independent experiments. Densitometric analysis was carried out on the data and presented as relative expression normalised to that for β-actin. The Relative expression in the presence of the ligand alone (Lig) has been assigned as 1 as shown in panels C and D.

The studies presented in the previous chapter showed that the LXRα gene promoter was regulated by LXR agonists in human hepatoma Hep3B cells. It was, however, decided to use a homologous macrophage system for subsequent studies. Previous studies in the laboratory have shown that THP-1 macrophages are difficult to transfect at high efficiencies with exogenous DNA. On the other hand, U937 macrophages can be transfected with high efficiency and have therefore been used for numerous studies analysing promoter regions in relation to macrophage gene expression (Hughes et al., 2002; Irvine et al., 2005; Koren et al., 1979; Olsson et al., 1983; Wang et al., 2003b). Similar to THP-1 cells, U937 are cultured as monocytes but can be differentiated into macrophages with PMA (Rao, 2001).

U937 cells were transfected with the hABCA1 promoter using the Superfect™ transfection method. Following differentiation in the presence of PMA, transfected cells were treated with combinations of 22(R)-HC and 9CRA or the synthetic ligand T0901317. Hep3B cells were included in some experiments for comparative purposes. In addition, the ABCA1 promoter construct containing mutations in the DR4 element was included in some experiments. As shown in Figure 4.24A, combinations of 22(R)-HC and 9CRA produced an approximate 6-fold induction of ABCA1 promoter activity in Hep3B cells. Such an induction was not seen with the DR4 mutant construct (Figure 4.24A). Having confirmed that the ABCA1 promoter behaved in an expected manner in Hep3B cells, which were used for all transfection studies in chapter 3, further experiments were carried out on U937 cells. The ABCA1 promoter activity was induced by combinations of 22(R)-HC and 9CRA or T0901317 in U937 macrophages (Figure 4.24B). However, the induction by combinations of 22(R)-HC and 9CRA (about 37-fold) was more dramatic than that seen with T0901317 (approximately 10-fold) (Figure 4.24B).

In order to further confirm that the induction of ABCA1 promoter activity by the LXR agonists was indeed mediated through LXRs, experiments were carried out using a DN form of LXRα. The empty plasmid vector pcDNA3 was used as a control. The induction of ABCA1 promoter activity in cells transfected with the control plasmid and then treated with combinations of 22(R)-HC and 9CRA or T0901317 was decreased by approximately 75% in cells expressing DN LXRα (Figure 4.25). These results therefore show that the ABCA1 gene promoter is regulated by LXR agonists in the U937 transfection system in a similar manner to the endogenous gene in THP-1 macrophages.

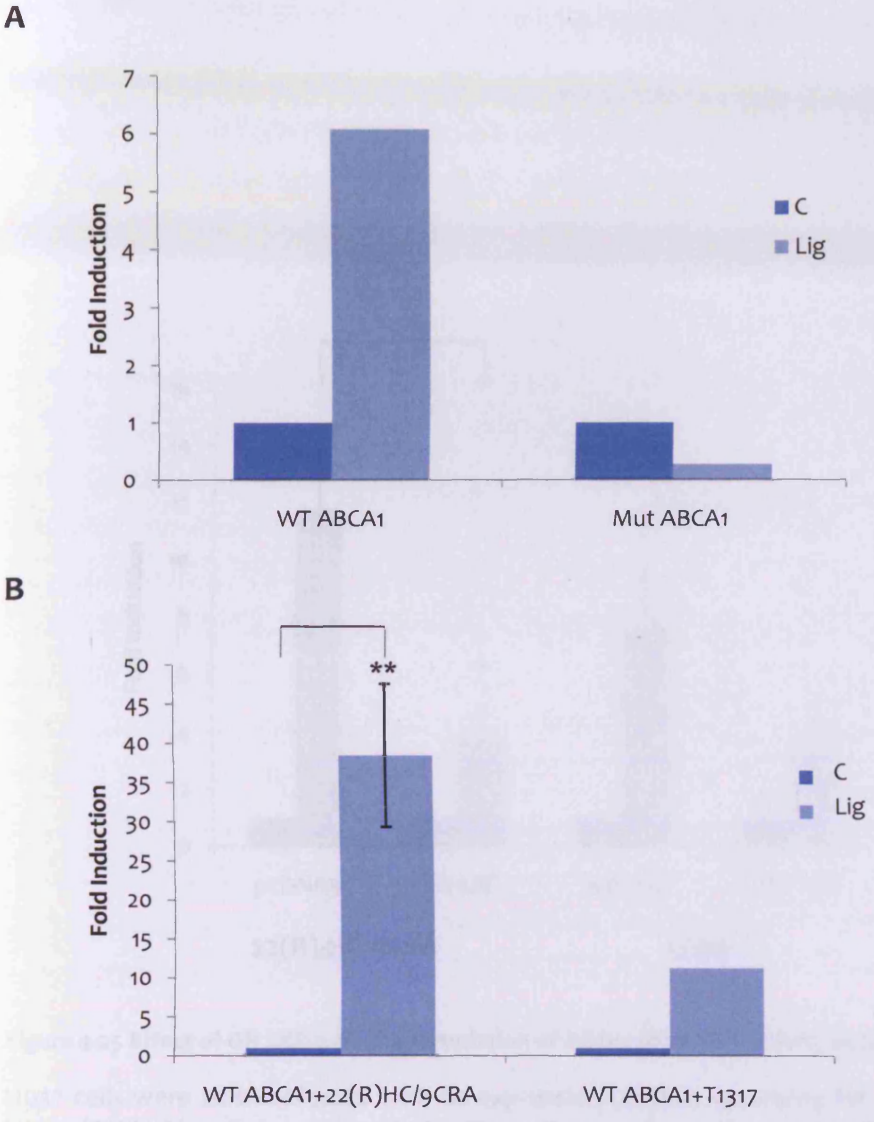


Figure 4.24 Effect of LXR agonists on the activity of ABCA1 promoter in transfected cells.

Hep3B cells (A) or U937 cells (B) were transfected with the indicated ABCA1 promoter constructs. The cells were then treated with either DMSO as a vehicle control (C) or 22(R)-HC (2μg/ml) and 9CRA (10μM) (Lig) for 18h (U937 cells) and 24h (Hep3B cells). PMA (0.16μM/ml) was included for U937 cells in order to initiate differentiation. The cells were then harvested and luciferase activity and protein assays were carried out as described in Materials and Methods. Relative counts were normalised to protein concentration and values are expressed as fold induction (average, panel A; mean ±SD, panel B) (the value in cells treated with vehicle alone has been arbitrarily assigned as 1). The results represent the outcome of two (panel A) to five (panel B, except for T1317 treatment, which has been carried out twice) independent experiments carried out in triplicate. The data was analysed by student's t-test (**P<0.01 compared to untreated cells).

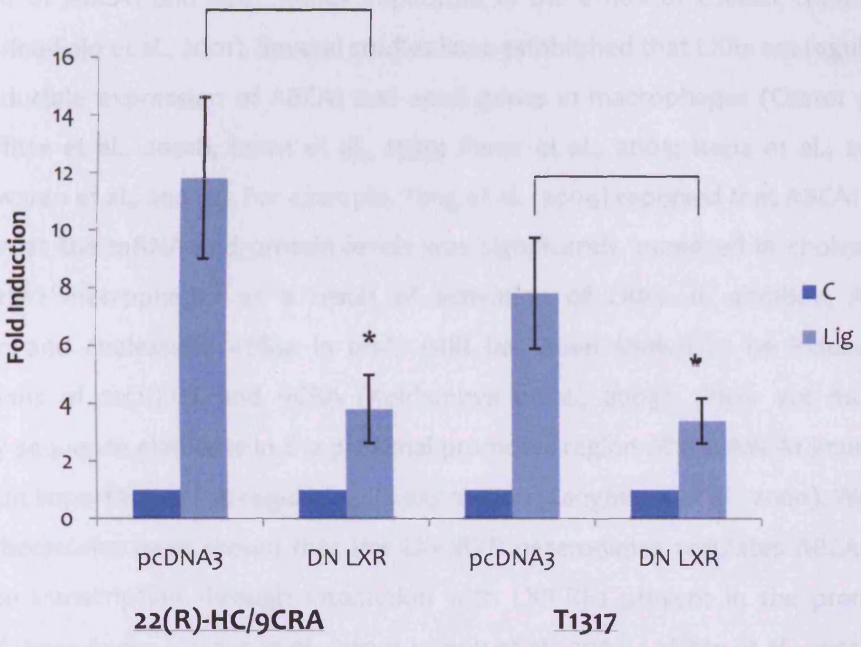


Figure 4.25 Effect of DN LXR-α on the stimulation of ABCA1 promoter activity by LXR agonists.

U937 cells were co-transfected with an expression plasmid specifying for a DN form of LXR-α (DN LXR) or the vector pcDNA3. The cells were then treated with either DMSO as a vehicle control (C) or the indicated LXR ligands (Lig) [22(R)-HC (2μg/ml) and 9CRA (10μM) or T1317 (10μM)] for 18h. PMA (0.16μM/ml) was included in order to initiate differentiation. The cells were then harvested and luciferase activity and protein assays were carried out as described in Materials and Methods. Relative counts were normalised to protein concentration and values are expressed as mean fold induction (±SD) (the value in cells treated with vehicle alone has been arbitrarily assigned as 1). The results represent the outcome of three independent experiments carried out in triplicate. The data was analysed by student's t-test (*$P<0.05$ compared to pcDNA3 transfected cells treated with the indicated LXR agonists).

4.3 Discussion

Macrophages contribute to the formation of arterial atherosclerotic lesions by accumulating excessive amounts of lipids, mainly CE, through the uptake of modified lipoproteins via scavenger receptors (Brown and Goldstein, 1983; Febbraio et al., 2001; Steinberg et al., 1989; Vainio and Ikonen, 2003; Yamada et al., 1998). Thus, elimination of accumulated CE from macrophage foam cells represents a promising therapeutic approach to prevent the development of atherosclerosis. Indeed, different strategies have been reported to reduce cellular CE content, one of which is to increase the expression of ABCA1 and apoE genes implicated in the efflux of cellular cholesterol (Santamarina-Fojo et al., 2001). Several studies have established that LXRs are regulators of lipid-inducible expression of ABCA1 and apoE genes in macrophages (Costet et al., 2000; Laffitte et al., 2001b; Lawn et al., 1999; Perez et al., 2003; Repa et al., 2000b; Venkateswaran et al., 2000a). For example, Tang et al. (2004) reported that ABCA1 gene expression at the mRNA and protein levels was significantly increased in cholesterol-loaded THP-1 macrophages as a result of activation of LXRs. In addition, ABCA1 expression and cholesterol efflux in brain cells has been shown to be induced by combinations of 22(R)-HC and 9CRA (Koldamova et al., 2003). There are multiple regulatory sequence elements in the proximal promoter region of the ABCA1 gene that may play an important role in regulating its expression (Langmann et al., 2000). Work in several laboratories have shown that the LXR/RXR heterodimer regulates ABCA1 and apoE gene transcription through interaction with LXR-REs present in the promoter regions of these genes (Costet et al., 2000; Joseph et al., 2003; Laffitte et al., 2001b; Lin et al., 2005; Repa et al., 2000b; Schwartz et al., 2000; Venkateswaran et al., 2000a). Therefore, initial studies were conducted to ascertain whether this response could be reproduced in the THP-1 model system. Hence it was first decided to study the ability of natural or synthetic LXR agonists to increase ABCA1 and apoE protein expression in THP-1 macrophages. To our knowledge no studies have previously been carried out in relation to signalling pathways involved in the LXR-mediated regulation of gene expression. The aim of the subsequent studies presented in this thesis was therefore to investigate this key aspect using ABCA1 and apoE as model genes.

The studies showed that natural LXR/RXR ligands, 22(R)-HC and 9CRA, and the synthetic agonists, GW3965 and T0901317, induce ABCA1 and apoE expression in THP-1 macrophages (Figures 4.2-4.4). In general, combinations of 22(R)-HC and 9CRA produced

the most extensive activation of expression of both proteins followed by T0901317 and GW3965. We also showed that this response requires the activation of the JNK/SAPK and PI3K signalling pathways, as specific pharmacological inhibitors against the two pathways attenuated the induction of ABCA1 and apoE protein expression by the ligands in a concentration-dependent manner (Figures 4.8-4.21). Such an attenuation of the responses by the JNK/SAPK and PI3K inhibitors was seen in both THP-1 cells and HMDMs. These findings are in agreement with previous work in the laboratory that showed an important role for JNK/SAPK and PI3K pathways in the 22(R)-HC-induced expression of apoE in THP-1 macrophages (Greenow, K., 2004). Further studies on the ABCA1 promoter showed that its activity was induced by combinations of 22(R)-HC and 9CRA in Hep3B and U937 cells (Figure 4.24). However, the 22(R)-HC and 9CRA-mediated activation of the ABCA1 promoter activity was much greater in U937 cells than Hep3B cells (Figure 4.24). Because the previously noted induction of ABCA1 promoter activity in murine RAW264.7 macrophages was also high (37 fold) (Costet et al., 2000), it appears that the activation of ABCA1 promoter by LXR agonists is greater in macrophages than in hepatocytes. The activation of ABCA1 promoter by 22(R)-HC and 9CRA was abrogated when the LXR-binding DR4 element was mutated (Figure 4.24). In addition, the expression of a DN form of LXR-α attenuated the induction of ABCA1 promoter activity by combinations of 22(R)-HC and 9CRA (Figure 4.25). Furthermore, the ABCA1 promoter was also activated by the synthetic ligand T0901317 (Figure 4.24).

This study has investigated the effects of natural and two different synthetic LXR agonists on ABCA1 and apoE gene expression and the corresponding signalling pathways in THP-1 macrophages. However, our results show differential quantitative effect of natural and synthetic LXR agonists on the induction of ABCA1 and apoE expression. In addition, the results suggest the potential involvement of the same cell signalling pathways, JNK/SAPK and PI3K, in the activation of ABCA1 and apoE expression by natural and synthetic LXR ligands. However, recent work has reported differential modulation of COX-2 expression in a human epithelial cell line by structurally distinct PPARγ agonists (Li et al., 2004; Patel et al., 2005). Thus, Patel et al. (2005) reported the involvement of PI3K and ERK pathways in the induction of COX-2 expression by the synthetic PPARγ ligand troglitazone. On the other hand, 15-deoxy-PGJ2, a natural PPARγ agonist, activated only the PI3K pathway and had no effect on COX-2 expression (Park et al., 2004; Patel et al., 2005). These data are in contrast with our finding where it seems

very likely that ABCA1 and apoE expression is activated by diverse LXR agonists via similar signal transduction pathways.

NRs can positively or negatively regulate gene expression by several mechanisms. They regulate transcription by binding to specific DNA sequences in the promoter or enhancer regions of target genes known as hormone response element (HRE). The DNA bound NRs recruit coactivator proteins in a ligand-dependent manner (Aranda and Pascual, 2001; Glass, 2006). The binding of the ligand leads to a conformational change, which alters the affinity of the NRs for coregulator proteins, and results in the dissociation of corepressors and the recruitment of coactivators. The new complex attracts a large number of proteins which engages the RNA polymerase enzyme II in the transcription of the target genes (Bastien and Rochette-Egly, 2004). In addition, NRs can also modulate gene expression by mechanisms independent of binding to HRE. For example, they can alter expression of genes that do not contain a HRE through positive or negative interference with the activity of other transcription factors, a mechanism generally referred to as transcriptional cross-talk (Aranda and Pascual, 2001; Gottlicher et al., 1998; Gronemeyer et al., 2004). Such cross-talk can occur in three ways. The first way is based on the interference between the transcriptional activities of certain NRs and other transcription factors (e.g. AP-1 and NF-κB). For example, ERs utilize protein-protein interactions to enhance transcription of genes that contain AP-1 sites (Gaub et al., 1990). The second way arises from the NRs themselves being the target of other signalling pathways that modify the receptors post-translationally (e.g. phosphorylation, ubiquitylation and acetylation) (Fu et al., 2002; Wang et al., 2001a) and alter their function. The phosphorylation of NRs by signalling pathways may occur in response to the ligand or in the absence of the ligand, and individual phosphorylation can act either to enhance or to inhibit the *trans*-activation potential of the receptor (Rochette-Egly, 2003). The third way of NR cross-talk is called a non-genomic action of ligands that has been seen for several NRs, which is mediated through putative membrane receptors (Gronemeyer et al., 2004; Losel and Wehling, 2003; Schmidt et al., 2000; Wehling, 1997). It is becoming increasingly clear that non-genomic signalling by NR agonists is an important aspect of NR-regulated activation of gene expression. The non-genomic action of NRs is rapid and inhibitors of transcription and protein synthesis have no effect (Gronemeyer et al., 2004; Hafezi-Moghadam et al., 2002; Schmidt et al., 2000; Simoncini

et al., 2000). This non-genomic function is thought to be the mechanism responsible for the recently observed effects of NR agonists on intracellular signalling pathways.

Cell signalling pathways have been implicated in the *trans*-activation of several NRs (Aranda and Pascual, 2001; Gronemeyer et al., 2004; Rochette-Egly, 2003). Multiple signal-dependent kinases activated by extracellular signals that bind to cell surface receptors (e.g. MAPKs, cell cycle-dependent kinases (CDKs), casein kinases and PKA) affect receptor activity (Shao and Lazar, 1999). This effect is mediated either through direct phosphorylation of the NR itself or through the modification of coregulator proteins required by them (Rochette-Egly, 2003). Phosphorylation can lead to changes in DNA binding, ligand binding or interaction with coactivator proteins. The exact mechanism used often depends on the specific kinase, the NR and the domain in the receptor that is phosphorylated (Shao and Lazar, 1999). For example, the ER is phosphorylated at specific serine or threonine residues by MAPK *in vitro* and in cells treated with growth factors that stimulate the Ras-MAPK cascade, leading to enhanced transcriptional activity (Aranda and Pascual, 2001; Kato et al., 1995). Phosphorylation events can also inactivate NRs, probably by switching off their activity. Recently, the transcriptional activity of NRs that bind to DNA as a heterodimer with RXRs (e.g. RAR/RXR) (Adam-Stitah et al., 1999; Lee et al., 2000; Matsushima-Nishiwaki et al., 2001) and VDR/RXR (Solomon et al., 1999) have been shown to be negatively modulated by phosphorylation of the RXRα heterodimerization partner by JNK/SAPK (Lee et al., 2000; Rochette-Egly, 2003). Furthermore, Sugawara et al. (2003) showed that the PPARγ-mediated suppression of angiotensin II type 1 receptor (ATIR) gene transcription is augmented by treatment of the cells with the ERK inhibitor PD98059 but not with the p38 kinase inhibitor SB203580 (Sugawara et al., 2003). These results suggest that potential phosphorylation of PPARγ by the ERK pathway attenuates PPARγ-mediated ATIR transcriptional suppression possibly by inhibiting PPARγ activity (Sugawara et al., 2003). In addition, inhibition of the transcriptional activity of other NRs, such as GR (Rogatsky et al., 1998a) and PPARγ (Adams et al., 1997; Camp and Tafuri, 1997; Camp et al., 1999; Hu et al., 1996), has been correlated with the phosphorylation of their N-terminal domain by MAPK (ERK and/or JNK/SAPK). This negative regulation of PPARγ results from a reduced ligand binding affinity due to intramolecular communication between the phosphorylated AF-1 domain and the COOH-terminus of the ligand binding pocket (Rochette-Egly, 2003; Shao et al., 1998).

Our results demonstrate that a similar mechanism of regulation may occur in the LXR-mediated induction of ABCA1 and apoE expression in THP-1 macrophages and primary HMDMs as the pharmacological inhibitors of JNK/SAPK and PI3K abolished the induction of ABCA1 and apoE expression by 22(R)-HC and 9CRA in both cellular systems. These findings are similar to those seen for PPARs as Chinetti et al. (2001) demonstrated that PPARα and PPARγ activators induce ABCA1 gene expression in human primary macrophages and in differentiated THP-1 cells (Chinetti et al., 2001). Such confirmation of the results seen in THP-1 macrophages was necessary as there are a few instances where differences in responses to mediators have been seen between primary monocytes or macrophages and transformed cell lines (Pei et al., 2005; Quinn et al., 2005; Vosper et al., 2001). For example, Quinn et al. (2005) showed that the induction of CYP27A1 expression by PPARγ and RXR ligands was greater in HMDMs than in THP-1 macrophages. With respect to the cell signalling pathways, Rao et al. (2001) reported that LPS stimulates JNK/SAPK activity in THP-1 macrophages whereas all three branches of the MAPK cascade are activated by LPS in primary human macrophages. Similar differences between primary cultures and transformed cell lines have been found in relation to PI3K and the p70S6 kinase (Rao, 2001). In addition, inhibition of DNA synthesis by rapamycin in bone marrow-derived macrophages was much less than that observed in a mouse macrophage BAC1.2F5 cell line (Hamilton et al., 1998). The similarity between the mechanisms underlying LXR-mediated regulation of expression of ABCA1 and apoE genes in human primary macrophages and THP-1 macrophages indicates that, consistent with over hundred publications in the field, the THP-1 cell line represents a good model system to study the regulation of gene expression relevant to atherosclerosis. The use of THP-1 cells also overcomes the donor-specific variability that is seen with HMDMs.

4.3.1 Selective role of JNK/SAPK in the regulation of macrophage gene expression by LXRs

The JNK/SAPK pathway has been found in the laboratory to be involved in the induction of apoE and ABCA1 expression by TGF-β (Singh and Ramji 2006; Singh, N., 2003). Thus, the regulation of two important genes involved in RCT by TGF-β requires the JNK/SAPK pathway. This pathway may also play a potential role in regulating cholesterol efflux. Recently, Witting et al. (2003) reported that ceramide induces ABCA1-dependent cholesterol efflux through JNK/SAPK (Chung et al., 2003; Willaime-Morawek et al., 2003;

Witting et al., 2003). In addition, work by Nofer et al. (2003) demonstrated that the apoA-I-induced cholesterol efflux was reduced in cells pre-treated with inhibitors of the JNK/SAPK pathway. ABCA1 is a major player in the control of cholesterol efflux and would therefore be an obvious target for the action of JNK/SAPK (Nofer et al., 2003). Moreover, a recent study has demonstrated that the PPARγ agonist, 15-deoxy-PGJ2, stimulates differentiation of embryonic midbrain cell in a PPARγ-dependent manner that requires activation of the JNK/SAPK pathway (Park et al., 2004). In addition, another study has also shown that the induction of ABCA1 protein and mRNA expression by the 22(R)-HC/9CRA combination was decreased by treatment of the cells with As_2O_3, which inhibits NR function via SEK1/MKK4- and JNK/SAPK-mediated phosphorylation of RXRα (Mann et al., 2005). Indeed, previous work in our laboratory has shown that 22(R)-HC induces JNK/SAPK activity in macrophages and that this is inhibited by the JNK/SAPK inhibitors SP600125 and curcumin (Greenow, K., 2004). Maximal activation of JNK/SAPK was observed following stimulation of the cells with 22(R)-HC for 30min, which may be due to a non-genomic action of the ligand.

4.3.2 Role of PI3K in the regulation of macrophage gene expression by LXRs

Data regarding the possible involvement of PI3K in LXR-mediated regulation of target gene expression is still limited. Recent studies in the laboratory have shown that the PI3K inhibitor, LY294002, inhibits the 22(R)-HC-mediated induction of apoE gene expression in macrophages (Greenow, K., 2004). Indeed, it has been reported that the synthetic LXR agonists, GW3965 and T0901317, decrease blood glucose levels in rodent models of diabetes, which suggests that LXR may be involved in physiological mechanisms which control the homeostasis of glucose metabolism (Barthel and Schmoll, 2003; Cao et al., 2003). The decrease in blood glucose levels brought about by LXR agonist treatment is due, in part, because of the suppression of expression of genes for the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK), fructose 2, 6-bisphosphatase (FBPase) and glucose-6-phosphatase (G-6-Pase) (Cao et al., 2003; Stulnig et al., 2002). PI3K signalling pathway also suppresses the expression of these genes and is activated by insulin (Desbois-Mouthon et al., 2000; Lizcano and Alessi, 2002; Nowak et al., 2005; Taniguchi et al., 2005), so both LXRs and insulin may work through the same signalling mechanism to suppress gluconeogenesis. It is not known if the downregulation of gluconeogenesis enzymes by LXR agonists involves the binding of

LXR/RXR heterodimers to regulatory regions of genes for PEPCK, FBPase and G-6-Pase, or is mediated by an indirect mechanism, maybe through PI3K (Barthel and Schmoll, 2003). Taniguchi et al. (2005) found that LXRα mRNA levels were unchanged after treatment with recombinant adenovirus expressing RNA interference (RNAi) against insulin receptor substrate (IRS)-1 or IRS-2 proteins or both, but the expression of the downstream LXRα genes, including ABCA1 and CYP7A1 isoforms was increased three-fold. This increase in lipogenic gene expression correlated with increased triglyceride accumulation in the serum and the liver. Changes were also noted in liver morphology, with the animals exhibiting a microvascular hepatic steatosis resembling fatty hepatic lesions seen in human patients with type 2 diabetes mellitus. This, the increased LXR activity suggested by the elevated expression of LXR downstream genes probably contributed significantly to the increased expression of SREBP1-c, which has been reported to affect the expression of PEPCK. Because insulin induces the expression of LXR itself (Edwards et al., 2002a), LXRs may contribute to the regulation of gluconeogenesis through insulin (Cao et al., 2003; Stulnig et al., 2002). It has been shown that the increase in SREBP1-c transcription could be mediated by LXRs binding to the LXR-REs in the promoter of these gene (Chen et al., 2004), and that the downstream target of PI3K, PKB, could be a potential regulator of SREBP-1c transcription (Ono et al., 2003).

In summary, the outcome of the studies presented in this chapter show that natural and synthetic LXR agonists upregulate the expression of ABCA1 and apoE genes in THP-1 cells and HMDMs. A novel involvement of JNK/SAPK and PI3K signal transduction pathways in the response was also identified, and allows to propose a potential model shown in Figure 4.26. These novel results provide the basis for further investigation of these pathways in the regulation of ABCA1 and apoE expression by 22(R)-HC and 9CRA. The following chapters will look at each of these pathways in detail.

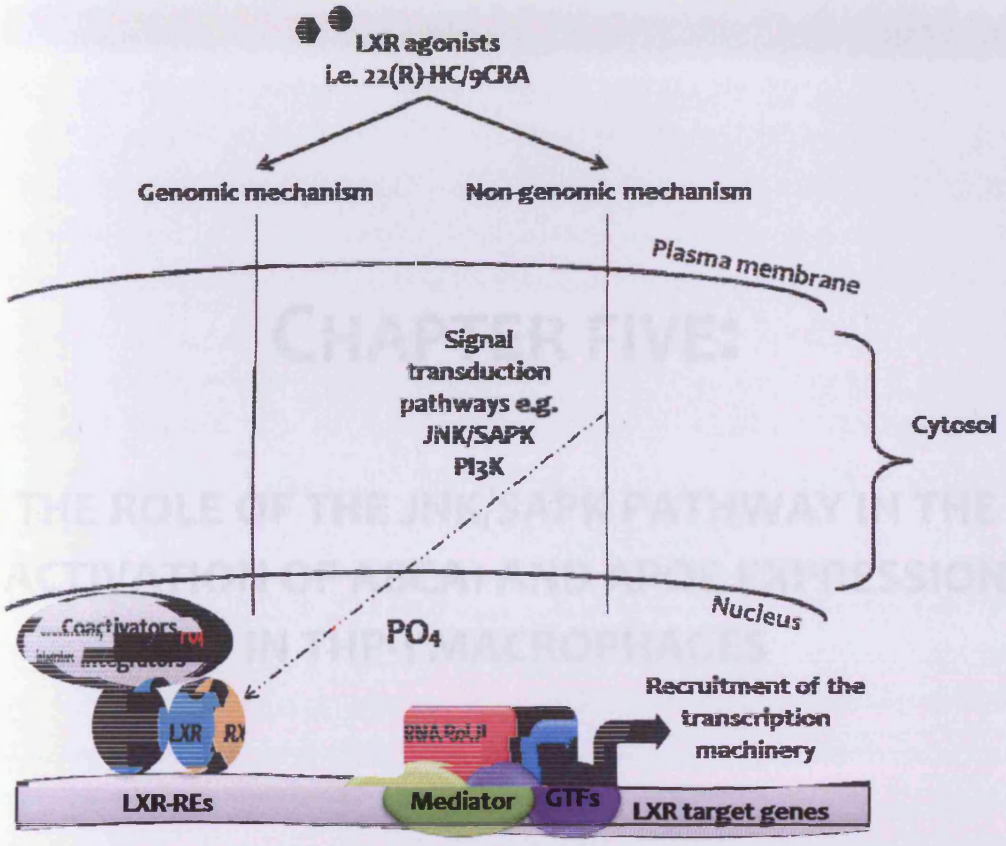


Figure 4.26 Schematic illustration of the involvement of signal transduction pathways in the LXR-mediated induction of ABCA1 and apoE expression.

CHAPTER FIVE:

THE ROLE OF THE JNK/SAPK PATHWAY IN THE ACTIVATION OF ABCA1 AND APOE EXPRESSION IN THP-1 MACROPHAGES

Chapter 5: The role of the JNK/SAPK pathway in the activation of ABCA1 and apoE expression in THP-1 macrophages

5.1 Introduction

Regulation of gene expression by LXR agonists is of particular therapeutic interest in the light of an important role for this NR in inhibiting macrophage foam cell formation and atherosclerosis (Joseph et al., 2002b; Joseph and Tontonoz, 2003; Levin et al., 2005; Li et al., 2004; Li and Glass, 2002; Tangirala et al., 2002; Venkateswaran et al., 2000a). Both ABCA1 and apoE stimulate macrophage cholesterol efflux. It has been shown that in ABCA1 transgenic mice, cholesterol efflux from macrophages was enhanced and this resulted in decreased atherosclerotic lesion formation (Srivastava, 2002a). In addition, deficiency of ABCA1 in macrophages of LDLR-/- mice increases the development of atherosclerosis, thereby suggesting that this protein plays a key role in the regulation of cholesterol homeostasis and function of macrophages (Francone et al., 2005).

Studies presented in chapter 4 showed a critical role for the JNK/SAPK and PI3K signalling pathways in the induction of ABCA1 and apoE protein expression by LXR agonists. The potential involvement of these pathways at this stage was restricted to the use of pharmacological inhibitors. It was therefore decided to confirm and extend these findings by alternative, complimentary approaches. The aims of the studies presented in this chapter were to investigate the role of the JNK/SAPK pathway in detail. Firstly, the activation of JNK/SAPK phosphorylation was monitored by Western blot analysis of cellular proteins isolated at various time points following exposure of THP-1 cells with combinations of 22(R)-HC and 9CRA. Secondly, the effect of these ligands on JNK/SAPK activity was determined. Thirdly, the effect of curcumin and SP600125 to inhibit any changes in activity and/or phosphorylation of JNK/SAPK was studied. Fourthly, the effect of DN constructs against key components of the JNK/SAPK pathway on the induction of ABCA1 promoter activity by LXR agonists was investigated. Fifthly, RNA interference (RNAi) assays were used to further confirm the role of JNK/SAPK in the response. Finally, EMSA was also employed in order to study the activation of AP-1 binding by treatment of the cells with 22(R)-HC and 9CRA. Figure 5.1 illustrates the overall experimental strategy for the work presented in this chapter.

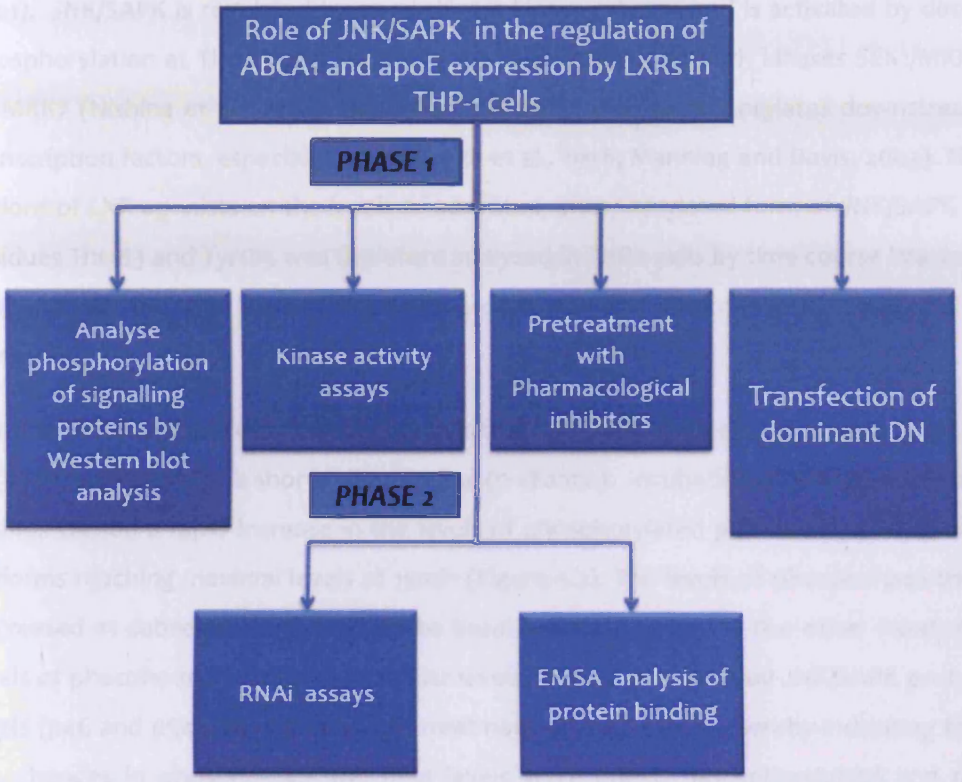


Figure 5.1 Summary of the experimental strategy used for further analysis of the role of JNK/SAPK pathways in the activation of gene expression by LXR agonists in THP-1 macrophages.

5.2 Results

5.2.1 Time course of JNK/SAPK activation by LXR agonists in THP-1 macrophages

JNK/SAPK was originally identified by its ability to bind to and phosphorylate the amino-terminal activation domain of the c-Jun transcription factor in response to a variety of stress-inducing signals. These included UV stimulation, heat shock, oxidant stress, ionizing radiation, DNA damaging chemicals (e.g. alkylating agents) and exposure to protein synthesis inhibitors such as anisomycin and cycloheximide (Derijard et al., 1994; Hibi et al., 1993; Kyriakis and Avruch, 2001). At least twelve distinct JNK/SAPK isoforms have been identified as the product of three genes JNK-1, -2 or -3 (Dreskin et al., 2001; Gupta et al., 1996; Kallunki et al., 1994; Waetzig and Herdegen, 2005). At least ten different isoforms of p46 and p54 are possible because of differential mRNA splicing (Dreskin et al., 2001; Gupta et al., 1996; Kyriakis and Avruch, 2001). JNK-1 and JNK-2 are ubiquitously expressed whereas JNK-3 is found mainly in neuronal tissues (Dreskin et al.,

2001). JNK/SAPK is regulated by an upstream kinase cascade and is activated by direct phosphorylation at Thr183 and Tyr185 by the dual specificity MAPK kinases SEK1/MKK4 or MKK7 (Nishina et al., 2004). Activated JNK/SAPK then phosphorylates downstream transcription factors, especially c-Jun (Gupta et al., 1996; Manning and Davis, 2003). The actions of LXR agonists on the levels of activated, phosphorylated form of JNK/SAPK at residues Thr183 and Tyr185 was therefore analysed in THP-1 cells by time course Western blot analysis. The total level of JNK/SAPK protein was also determined for comparative purposes.

Initial experiments were carried out on cells that had been treated with combinations of 22(R)-HC and 9CRA for a shorter time course (0-180min). Incubation of the cells with the ligands caused a rapid increase in the levels of phosphorylated p46 and p54 JNK/SAPK isoforms reaching maximal levels at 15min (Figure 5.2). The levels of phospho-p46 then decreased at subsequent time points to basal levels (i.e. 0h). On the other hand, the levels of phospho-p54 remained at similar levels. The amount of total JNK/SAPK protein levels (p46 and p54) did not vary with treatment of the ligands, thereby indicating that the changes in phospho-p46 and p-54 levels were due to phosphorylation and not increased expression of the JNK/SAPK proteins.

As detailed above, JNK/SAPK is an important upstream activator of c-Jun. The JNK/SAPK activity can therefore be determined by a non-radioactive kit from Cell Signalling Technology which analyses the ability of this kinase to phosphorylate the recombinant c-Jun protein. This kit was therefore used to investigate if, similar to the changes in JNK/SAPK phosphorylation (Figure 5.2), combinations of 22(R)-HC and 9CRA also induce the activity of the enzyme. For this, THP-1 macrophages were treated with 22(R)-HC and 9CRA as Figure 5.2 (i.e. 0, 30, 45, 60, 90 and 180min). An antibody to the c-Jun fusion protein linked to agarose beads (provided in the kit) was then used to selectively immunoprecipitate the active JNK/SAPK enzyme from cell lysates.

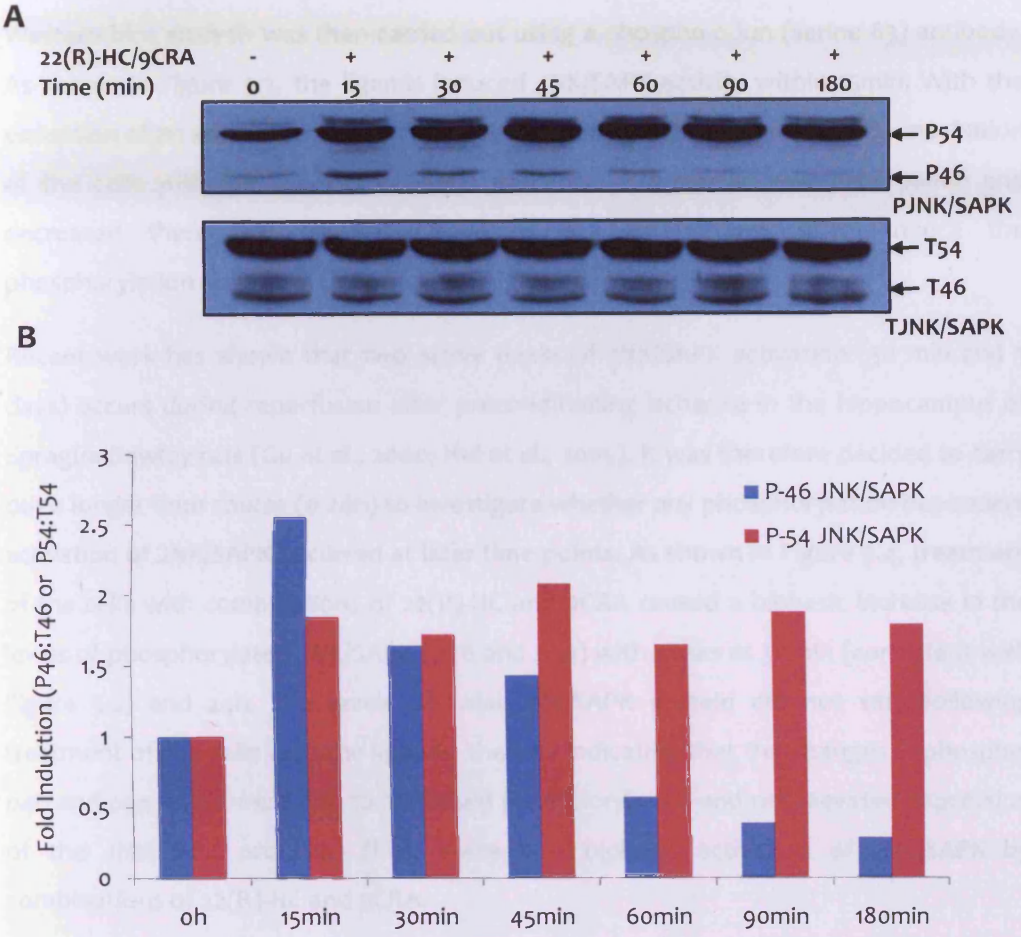


Figure 5.2 Time course action of 22(R)-HC and 9CRA on JNK/SAPK phosphorylation in THP-1 macrophages.

Differentiated THP-1 macrophages were incubated for the indicated period of time with combinations of 22(R)-HC (2μg/ml) and 9CRA (10μM). Western blot analysis was carried out using 80μg of whole cell extracts. Blotted membranes were incubated with anti-phospho-JNK/SAPK (Thr183/Tyr185) or anti-total-JNK/SAPK primary antibodies as indicated (rabbit polyclonal IgG) and detected with anti-rabbit HRP-conjugated secondary antibodies. The image shown in panel A is representative of two independent experiments. Densitometric analysis was carried out on the data and presented as fold induction (average) in relation to basal expression at 0h (assigned as 1).

The immunoprecipitate was then incubated in the presence of ATP and kinase buffer, which allows the immunoprecipitated JNK/SAPK to phosphorylate the c-Jun substrate. Western blot analysis was then carried out using a phospho-c-Jun (serine 63) antibody. As shown in Figure 5.3, the ligands induced JNK/SAPK activity within 15min. With the exception of an anomaly at 45min, the JNK/SAPK activity that was induced by incubation of the cells with the ligands for 15min remained at a similar level until 90min and decreased thereafter. Thus, combinations of 22(R)-HC and 9CRA induce the phosphorylation and the activity of JNK/SAPK.

Recent work has shown that two active peaks of JNK/SAPK activation (30 min and 3 days) occurs during reperfusion after preconditioning ischemia in the hippocampus of Sprague-Dawley rats (Gu et al., 2000; Hui et al., 2005). It was therefore decided to carry out a longer time course (0-24h) to investigate whether any phosphorylation-dependent activation of JNK/SAPK occurred at later time points. As shown in Figure 5.4, treatment of the cells with combinations of 22(R)-HC and 9CRA caused a biphasic increase in the levels of phosphorylated JNK/SAPK (p46 and p54) with peaks at 30min (consistent with Figure 5.2) and 24h. The levels of total JNK/SAPK protein did not vary following treatment of the cells with the ligands, thereby indicating that the changes in phospho-p46 and p54 levels were due to increased phosphorylation and not elevated expression of the JNK/SAPK proteins. Thus, there is a biphasic activation of JNK/SAPK by combinations of 22(R)-HC and 9CRA.

5.2.2 Time course action of ligands on phosphorylation of SEK1/MKK4 at serine 257 and threonine 261 in THP-1 macrophages

Previous studies have implicated SEK1/MKK4 as an immediate upstream kinase for the activation of JNK/SAPK (Derijard et al., 1995; Sanchez et al., 1994). Activation of SEK1/MKK4 occurs through phosphorylation of two residues in this protein, serine 257 and threonine 261 by the protein kinase MEKK1. The activation of SEK1/MKK4 by phosphorylation following stimulation of the cells with the ligands was therefore monitored by time course Western blot analysis. Similar analysis with an antibody against total SEK1/MKK4 was also included for comparative purposes. According to the data sheet provided by the manufacturer (Cell Signalling Technology), the phospho- and total-SEK1/MKK4 antibodies should detect two closely migrating polypeptides of similar molecular weight (44kDa) that could produce a single signal. Figure 5.5 shows that

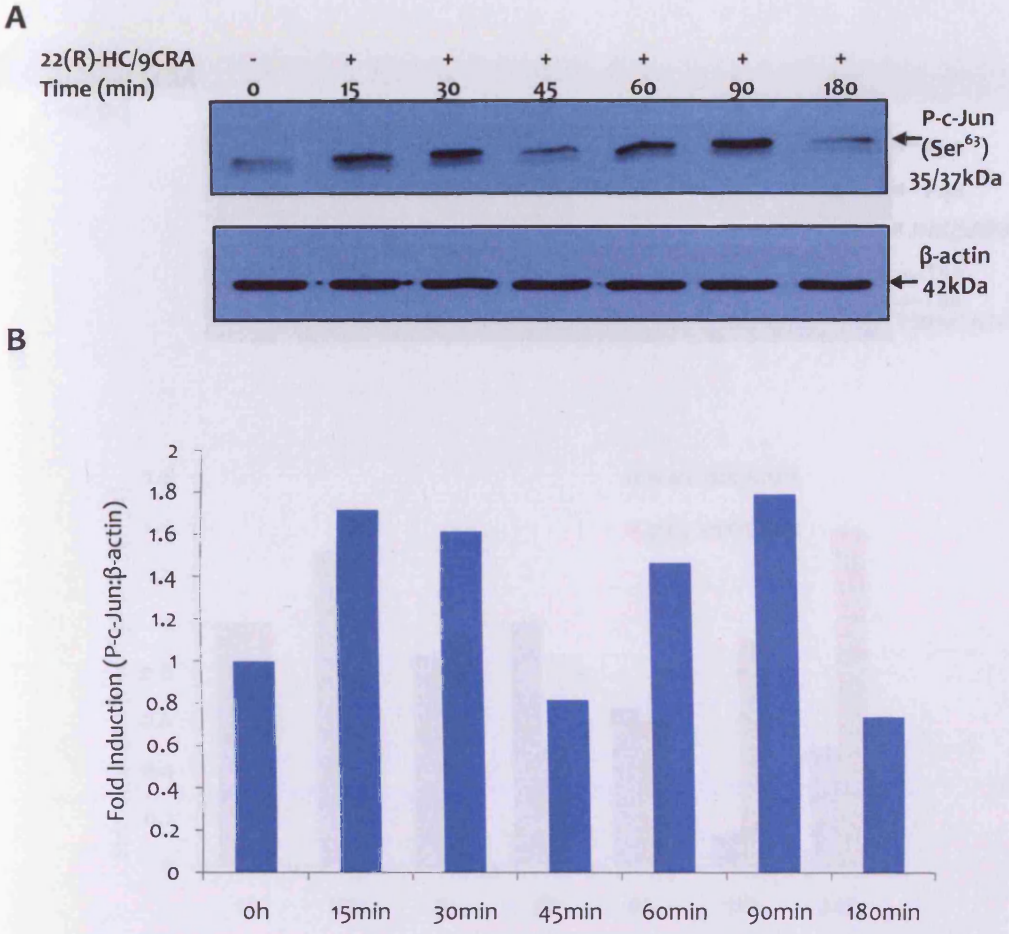
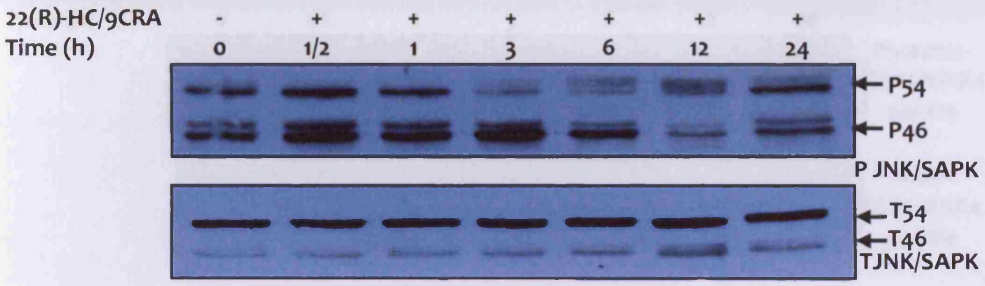


Figure 5.3 The effect of 22(R)-HC and 9CRA on JNK/SAPK activity in THP-1 macrophages.

The cells were treated for the indicated time with 22(R)-HC (2μg/ml) and 9CRA (10μM). The JNK/SAPK activity assay was then carried out on whole cell extracts (200 μg) as described in Materials and Methods. Phosphorylated c-Jun (Ser63) was detected by Western blot analysis. Equal amount of proteins in the different samples was verified by Western blot analysis using a β-actin antibody. Antigen-antibody complexes were detected using the ECL detection system. The image shown in panel A is representative of two independent experiments. Densitometric analysis was carried out on the data and presented as average of fold induction of c-Jun phosphorylation normalised to the expression of β-actin in relation to basal expression at 0h (arbitrarily assigned as 1).

A



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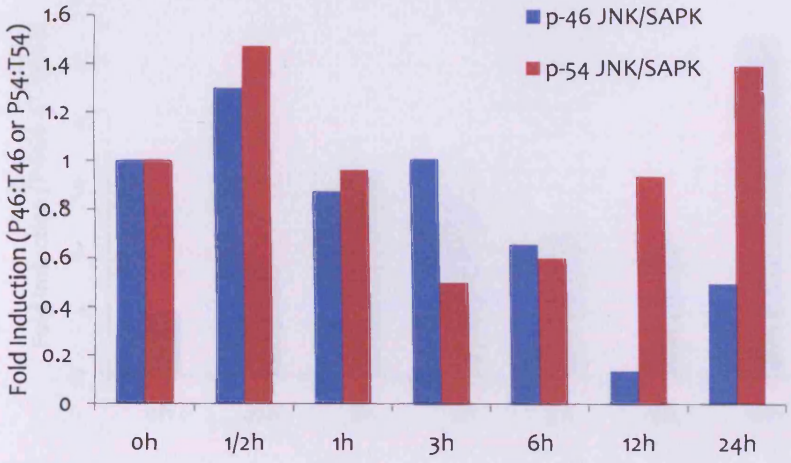


Figure 5.4 Time course of JNK/SAPK phosphorylation by LXR agonists in THP-1 macrophages.

The cells were treated for the indicated time with combinations of 22(R)-HC (2μg/ml) and 9CRA (10μM). Western blot analysis was carried out using equal volume of lysates (50μl). Blotted membranes were incubated with anti-phospho-JNK/SAPK (Thr183/Tyr185) or anti-total-JNK/SAPK primary antibodies as indicated (rabbit polyclonal IgG) and detected with an anti-rabbit HRP-conjugated secondary antibody. The image shown in panel A is representative of two independent experiments. Densitometric analysis was carried out on the data and presented as average fold induction in relation to basal expression at 0h (assigned as 1) (panel B).

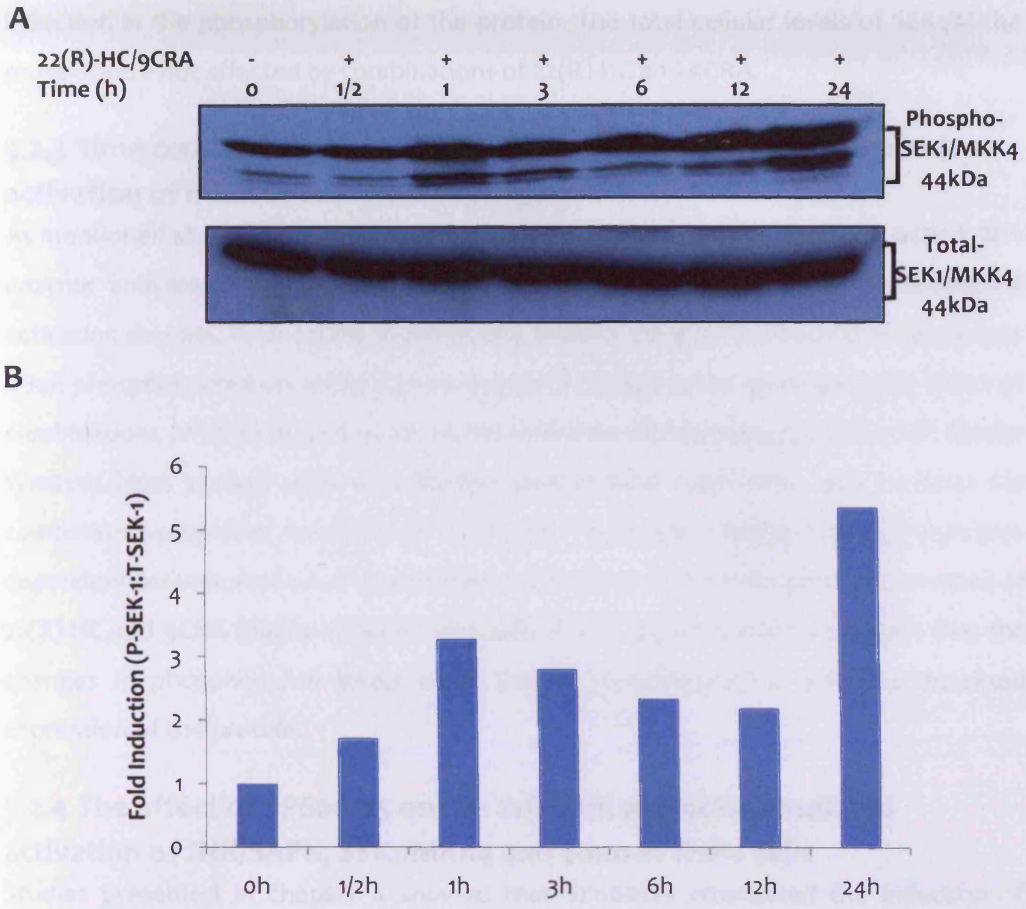


Figure 5.5 Time course of SEK1/MKK4 phosphorylation by LXR agonists in THP-1 macrophages.

The cells were treated for the indicated time with combinations of 22(R)-HC (2μg/ml) and 9CRA (10μM). Western blot analysis was carried out using equal volume of lysates (50μl). Blotted membranes were incubated with anti-phospho-SEK1/MKK4 (Ser257/Thr261) or with anti-total-SEK1/MKK4 primary antibodies as indicated (rabbit polyclonal IgG) and detected with anti-rabbit HRP-conjugated secondary antibodies. The image shown in panel A is representative of two independent experiments. Densitometric analysis was carried out on the data and presented as average fold induction in relation to basal expression at 0h (assigned as 1) (panel B).

treatment of the cells with 22(R)-HC and 9CRA causes a biphasic increase in the levels of phosphorylated SEK1/MKK4 with peaks at 1h and at 24h. This is particularly apparent with the faster migrating "band" in panel A. Overall, there was an approximate 4-5-fold induction in the phosphorylation of the protein. The total cellular levels of SEK1/MKK4 protein were not affected by combinations of 22(R)-HC and 9CRA.

5.2.3 Time course action of LXR agonists on phosphorylation-dependent activation of c-Jun in THP-1 macrophages

As mentioned above, c-Jun is an important downstream target for JNK/SAPK action. The enzyme activates it by phosphorylation of serines 63 and 73 in the transcriptional activation domain. Time course Western blot analysis using an antibody that recognises c-Jun phosphorylated on serine 63 was therefore carried out to investigate the effect of combinations of 22(R)-HC and 9CRA on the activation of this transcription factor. Similar Western blots probed with an antibody against total c-Jun were also included for comparative purposes. As shown in Figure 5.6, there was a biphasic phosphorylation-dependent activation of c-Jun in response to treatment of the cells with combinations of 22(R)-HC and 9CRA (peaks at 30min and 24h). These results therefore suggest that the changes in phospho-c-Jun levels were due to phosphorylation and not increased expression of the protein.

5.2.4 The effect of SP600125 on the 22(R)-HC and 9CRA-mediated activation of JNK/SAPK, SEK1/MKK4 and c-Jun in THP-1 cells

Studies presented in chapter 4 showed that SP600125 attenuated the induction of ABCA1 and apoE expression by combinations of 22(R)-HC and 9CRA. In order to confirm that SP600125 indeed inhibited JNK/SAPK activation, the effect of this inhibitor on the 22(R)-HC and 9CRA-mediated increase in the levels of phospho-JNK/SAPK was analysed. In addition, the effect of this inhibitor on the activation of SEK1/MKK4 and c-Jun was studied. The 24h incubation period was chosen as it corresponds to the activation of all three proteins by the ligands along with the induction of ABCA1 and apoE expression. Thus, differentiated THP-1 macrophages were pre-treated with SP600125 for 1h prior to stimulation of the cells with 22(R)-HC and 9CRA for 24h. The cellular proteins were

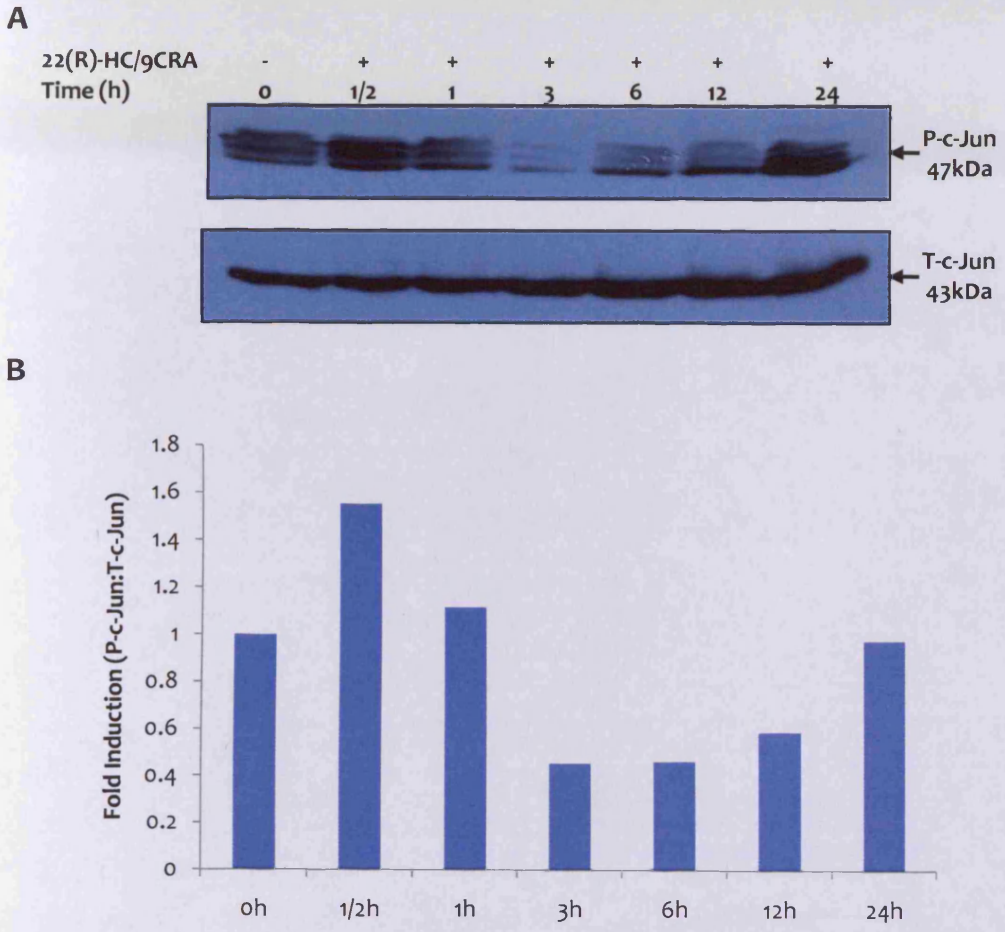


Figure 5.6 Time course of c-Jun phosphorylation by LXR agonists in THP-1 macrophages.

The cells were treated for the indicated time with combinations of 22(R)-HC (2μg/ml) and 9CRA (10μM). Western blot analysis was carried out using equal volume of lysates (25μl). Blotted membranes were incubated with anti-phospho-c-Jun or anti-total-c-Jun primary antibodies as indicated. Antigen-antibody complexes were detected using the ECL detection system. The image shown in panel A is representative of two independent experiments. Densitometric analysis was carried out on the data and presented as average fold induction in relation to basal expression at 0h (assigned as 1) (panel B).

subjected to Western blot analysis using antibodies against phosphorylated and total JNK/SAPK, SEK1/MKK4 and c-Jun. As expected, combinations of 22(R)-HC and 9CRA induced the levels of phosphorylated proteins at 24h without having any effect on the total levels of these proteins (Figure 5.7). The vehicle control in these experiments was at the same time point as that for the ligands (i.e. 24h), thereby showing that the observed changes in phosphorylation were not because of culturing of the cells for this time period. Inclusion of SP600125 at two different concentrations (50 and 100μM) attenuated both the basal and ligand-induced phosphorylation of phospho-p46 and phospho-p54 JNK/SAPK proteins without affecting the total protein levels (Figure 5.7). In addition, inclusion of SP600125 attenuated both the basal and ligand-induced levels of phospho-SEK1/MKK4 and c-Jun proteins. Overall, these results confirm the positive action of SP600125 in THP-1 macrophages.

5.2.5 The effect of curcumin on the 22(R)-HC and 9CRA-mediated phosphorylation of JNK/SAPK, SEK1/MKK4 or c-Jun in THP-1 cells

Curcumin also inhibited the 22(R)-HC and 9CRA-mediated induction of ABCA1 and apoE expression (See chapter 4). Experiments were therefore carried out to investigate whether, similar to SP600125, it also affected the increased phosphorylation of JNK/SAPK, SEK1/MKK4 or c-Jun seen in the presence of the ligands. The concentration of curcumin used (30μg) corresponded to that which produced inhibition of ABCA1 and apoE expression by the ligands. As shown in Figure 5.8, combinations of 22(R)-HC and 9CRA induced the phosphorylation of JNK/SAPK, SEK1/MKK4 and c-Jun, as expected. However, inclusion of curcumin had no effect on this ligand-induced phosphorylation of these proteins.

Analysis of JNK/SAPK activity by the use of non-radioactive, *in vitro* kinase assay had shown that combinations of 22(R)-HC and 9CRA induces JNK/SAPK activity (Figure 5.3). In order to clarify the action of curcumin further, the effect of this inhibitor on JNK/SAPK activity was investigated. As shown in Figure 5.9, inclusion of 30μM of curcumin attenuated the basal and the induced JNK/SAPK activity seen in the presence of 22(R)-HC and 9CRA. This result therefore suggests that curcumin inhibits the 22(R)-HC and 9CRA-mediated stimulation of JNK/SAPK enzymatic activity without affecting the increased phosphorylation of JNK/SAPK, SEK1/MKK4 or c-Jun.

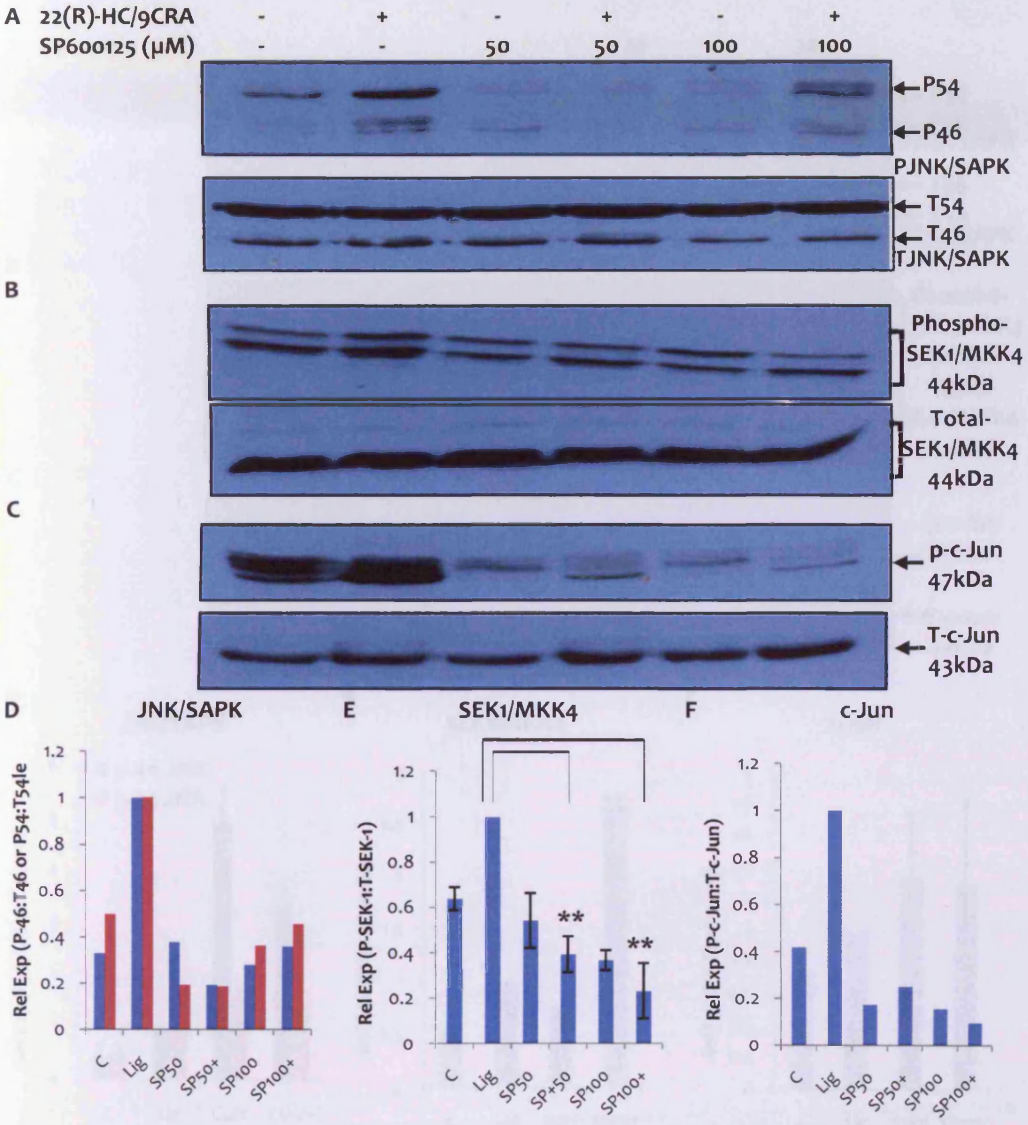


Figure 5.7 The effect of SP600125 on LXR agonist-mediated phosphorylation of JNK/SAPK, SEK1/MKK4 and c-Jun in THP-1 macrophages.

Differentiated THP-1 macrophages were treated for 24h with 22(R)-HC (2μg/ml) and 9CRA (10μM) in the absence or the presence of SP600125 at the indicated concentrations. In addition, cells were treated with DMSO as a vehicle control (C). The inhibitor was added 1h before the ligand (pre-treatment). Western blot analysis was carried out using equal amount of the lysates. Blotted membranes were incubated with primary antibodies against phospho-JNK/SAPK or total-JNK/SAPK (A), phospho-SEK1/MKK4 or total-SEK1/MKK4 (B) and phospho-c-Jun or total-c-Jun (C). Antigen-antibody complexes were detected using the ECL detection system. The images shown in panels A and C are representative of two independent experiments whereas the image in panel B is representative of three independent experiments. Densitometric analysis was carried out on the data and presented as relative expression (mean ±SD, panel E; average panels D and F) in relation to basal expression (C). The relative expression in the presence of the ligand alone (Lig) has been arbitrarily assigned as 1 (panel E; **$P<0.01$).

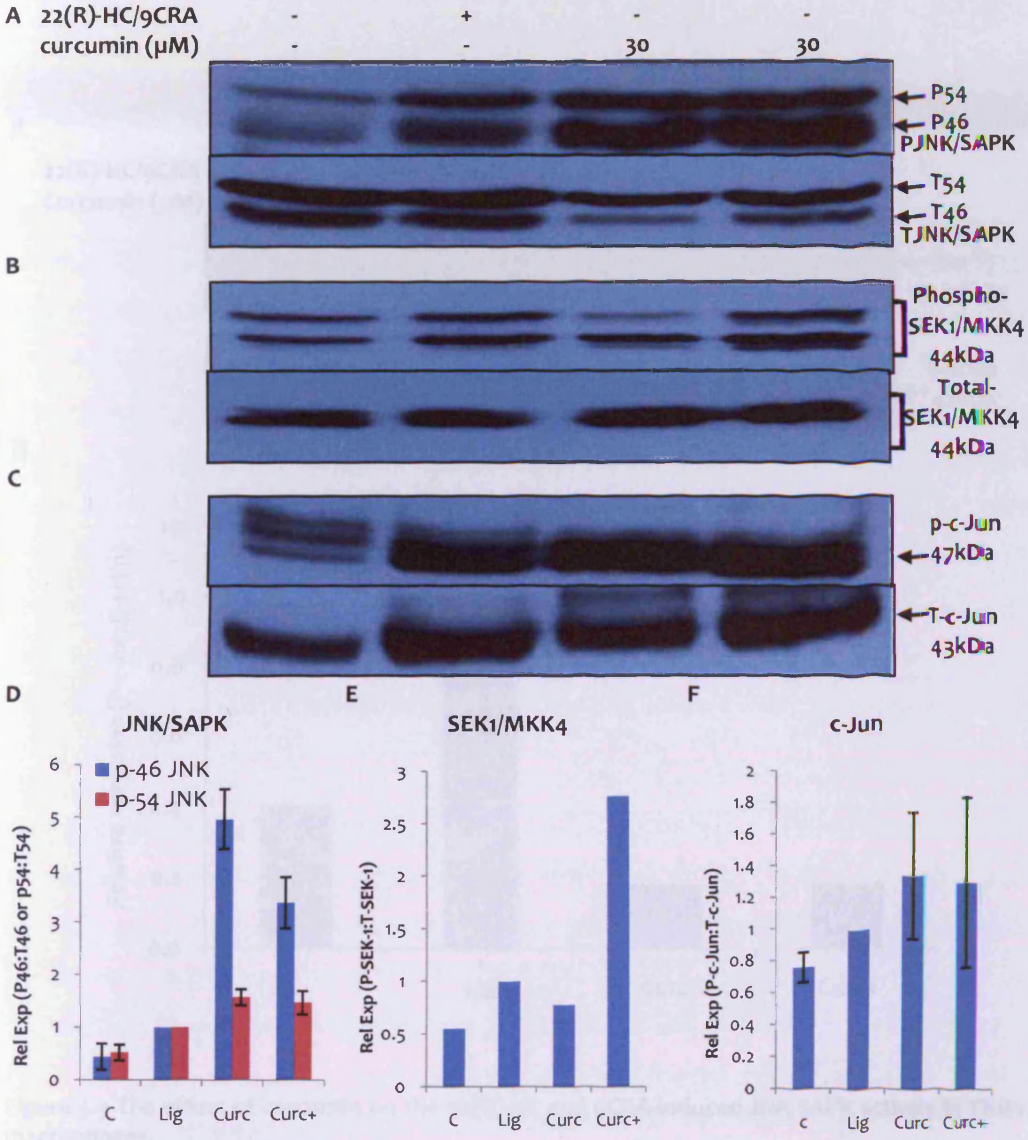


Figure 5.8 The effect of curcumin on the 22(R)-HC and 9CRA-mediated phosphorylation of JNK/SAPK, SEK1/KK4 and c-Jun in THP-1 macrophages.

Differentiated THP-1 macrophages were treated for 24h with 22(R)-HC (2μg/ml) and 9CRA (10μM) in the absence or the presence of 30μM of curcumin. In addition, cells were treated with DMSO as a vehicle control (C). The inhibitor was added 1h before the ligand (pre-treatment). Western blot analysis was carried out using equal amount of the lysates. Blotted membranes were incubated with primary antibodies against phospho-JNK/SAPK or total-JNK/SAPK (A), phospho-SEK1/MKK4 or total-SEK1/MKK4 (B) and phospho-c-Jun or total-c-Jun (C). Antigen-antibody complexes were detected using the ECL detection system. The images shown in panels A, B and C are representative of three, two and four independent experiments respectively. Densitometric analysis was carried out on the data and presented as relative expression (C) (mean ±SD, panels D and F; average, panel E) in relation to basal expression. The relative expression in the presence of the ligand alone (Lig) has been arbitrarily assigned as 1 (panels D–F).

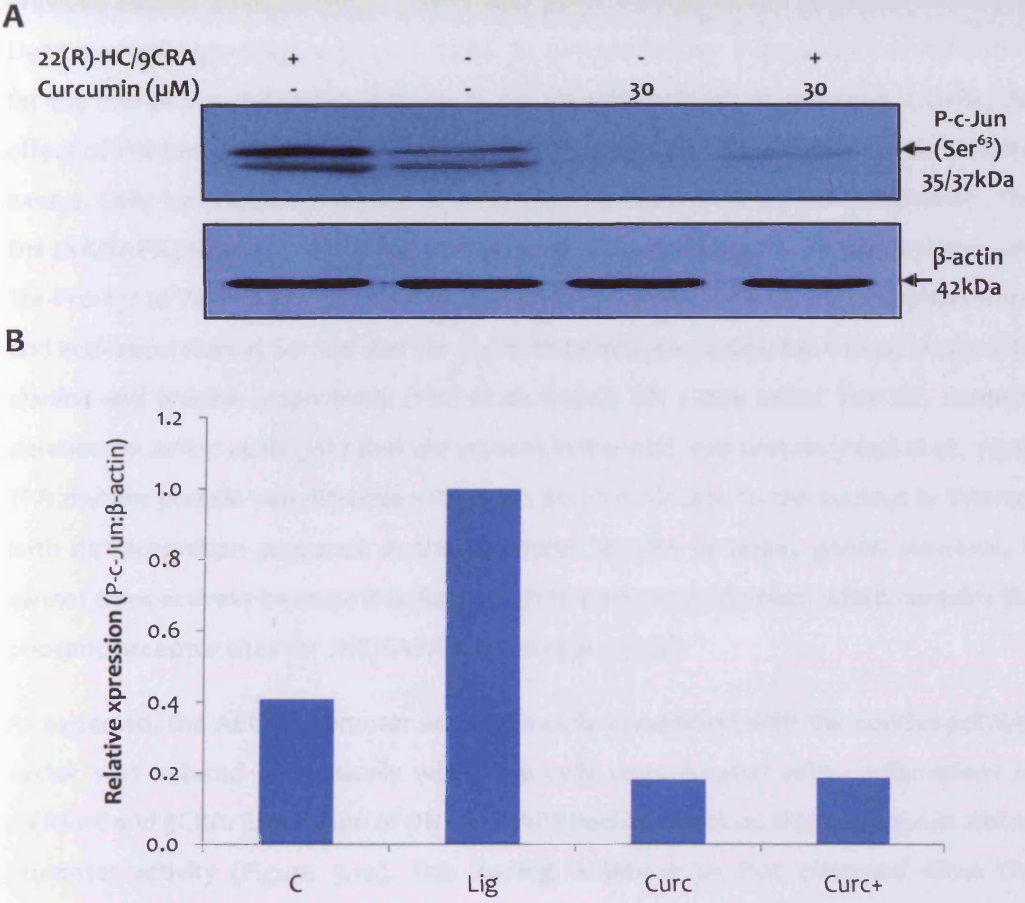


Figure 5.9 The effect of curcumin on the 22(R)-HC and 9CRA-induced JNK/SAPK activity in THP-1 macrophages.

Differentiated THP-1 macrophages were treated for 24h with 22(R)-HC (2μg/ml) and 9CRA (10μM) in the absence or the presence of curcumin as indicated. In addition, cells were treated with DMSO as a vehicle control (C). The inhibitor was added 1h before the ligand (pre-treatment). The JNK/SAPK activity assay was then carried out on whole cell extracts (200 μg) as described in Materials and Methods. Phosphorylated c-Jun (Ser63) was detected by Western blot analysis. Equal amount of proteins in the different samples was verified by Western blot analysis using a β-actin antibody. Antigen-antibody complexes were detected using the ECL detection system. The image shown in panel A is representative of two independent experiments. Densitometric analysis was carried out on the data and presented as relative expression (average) normalised to the expression of β-actin. The relative expression in the presence of the ligand alone (Lig) has been assigned as 1 as shown in panel B.

5.2.6 Effect of DN forms of JNK/SAPK, SEK1/MKK4 and c-Jun on LXR agonist-induced activation of ABCA1 promoter activity

Previous studies showed that 22(R)-HC and 9CRA induces ABCA1 promoter activity in U937 macrophages (Figure 4.24). In order to provide further independent confirmation for the role of the JNK/SAPK pathway in the activation of ABCA1 promoter activity, the effect of DN forms of JNK/SAPK, SEK1/MKK4 and c-Jun was analysed by co-transfection assays. Cells transfected with the pcDNA3 plasmid were included for comparison. The DN JNK/SAPK plasmid, SAPKα-VPA, was generated by changing the phosphorylation site Thr-Pro-Tyr to Val-Pro-Ala (Kawasaki et al., 1996). In DN SEK1/MKK4, the phosphorylation and activation sites at Ser 220 and Thr 224 in the wild type protein have been changed to alanine and leucine respectively (Yan et al., 1994). DN c-Jun, called Tam 67, contains deletions in amino acids 3-122 that are present in the wild type protein (Alani et al., 1991). This mutant protein can dimerize with c-Jun and translocate to the nucleus to interact with its recognition sequence in the promoter regions of target genes. However, it cannot *trans*-activate because it lacks most of the activation domain, which contains the phospho-acceptor sites for JNK/SAPK (Brown et al., 1994).

As expected, the ABCA1 promoter activity in cells transfected with the control pcDNA3 vector was induced dramatically when the cells were treated with combinations of 22(R)-HC and 9CRA. Expression of DN JNK/SAPK had no effect on this induction of ABCA1 promoter activity (Figure 5.10). This finding is similar to that observed when the transfection experiments were carried out in Hep3B cells (See chapter 3). On the other hand, DN forms of both SEK1/MKK4 and c-Jun attenuated the induction of ABCA1 promoter by the ligands.

5.2.7 Further analysis of the role of the JNK/SAPK pathway in the actions of 22(R)-HC and 9CRA on apoE expression

In contrast to co-transfection assays with the ABCA1 gene promoter, previous studies have shown that the DN form of JNK/SAPK is able to attenuate the 22(R)-HC-induced expression of the endogenous apoE protein in THP-1 macrophages (Greenow, K., 2004). It was therefore decided to investigate if DN JNK/SAPK could also act in a similar manner in relation to the induction of apoE expression by combinations of 22(R)-HC and 9CRA. For this, THP-1 monocytes were transfected with the control pcDNA3 plasmid or DN

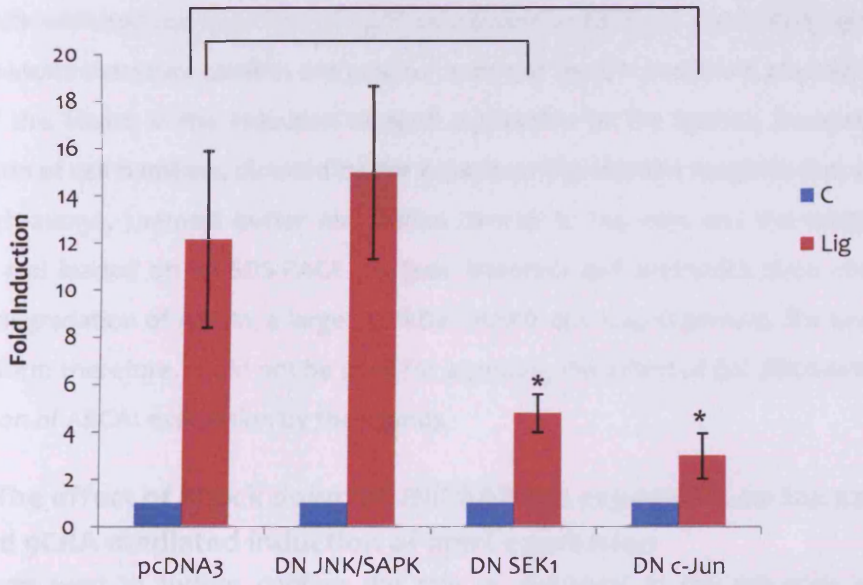


Figure 5.10 The effect of DN constructs on the 22(R)-HC and 9CRA-mediated induction of ABCA1 promoter activity.

U937 cells were co-transfected with the human ABCA1 promoter [from -928 to +101bp] construct and expression plasmids for DN forms of JNK/SAPK, SEK1/MKK4 or c-Jun (Tam67). Cells transfected with the control pcDNA3 plasmid were included for comparison. The cells were then treated with DMSO as a vehicle control (C) or combinations of 22(R)-HC (2μg/ml) and 9CRA (10μM) (Lig) for 18h. The transfected cells were then harvested and luciferase reporter activity was determined. Relative counts were normalised to protein concentration and values are expressed as mean fold induction (±SD) in the presence of the ligands in relation to basal levels (assigned as 1). The results are from three independent experiments carried out in triplicate (*$P<0.01$).

JNK/SAPK and then differentiated for 24h in the presence of PMA (0.16μM). The differentiated cells were then cultured for 24h in the presence of combinations of 22(R)-HC and 9CRA (DMSO vehicle- and 22(S)-HC-treated cells were used as controls). Equal amount of whole cell extracts were then subjected to Western blot analysis using antibodies against apoE or β-actin. The expression of apoE was induced by combinations of 22(R)-HC and 9CRA but not by the inactive enantiomer 22(S)-HC (Figure 5.11). DN JNK/SAPK inhibited this induction of apoE expression by 22(R)-HC and 9CRA (Figure 5.11). These results therefore confirm the positive action of the DN JNK/SAPK plasmid and the role of this kinase in the induction of apoE expression by the ligands. Because of the limitation of cell numbers, dictated by the expensive transfection reagents that are used for such assays, Laemmli buffer was added directly to the cells and the mixture was boiled and loaded on an SDS-PAGE gel (see Materials and Methods). Such conditions cause degradation of ABCA1, a large (220kDa) membrane-bound protein. The procedure in this form therefore, could not be used for analysing the effect of DN JNK/SAPK on the induction of ABCA1 expression by the ligands.

5.2.8 The effect of knock down of JNK/SAPK1/2 expression on the 22(R)-HC and 9CRA-mediated induction of apoE expression

RNAi was used to further confirm the role of JNK/SAPK in the induction of apoE expression by combinations of 22(R)-HC and 9CRA. For this, THP-1 cells were transfected with small interfering RNA (siRNA) targeted against JNK/SAPK1/2 or the control GAPDH using the INTERFERin™ transfection reagent (Invitrogen). Equal amount of cellular proteins were then subjected to Western blot analysis using antisera against JNK/SAPK, apoE or β-actin. As shown in Figure 5.12, the levels of endogeneous JNK/SAPK1/2 were reduced to approximately 50-60% in cells transfected with the corresponding siRNA compared to that present in cells expressing the control GAPDH siRNA. Combinations of 22(R)-HC and 9CRA induced apoE protein expression in cells expressing GAPDH siRNA (Figure 5.12). This induced expression of apoE was attenuated by knockdown of JNK/SAPK1/2 (Figure 5.12). These results therefore further substantiate an important role for JNK/SAPK in the induction of apoE expression by LXR agonists. Again, because of restrictions on cell numbers, dictated by the expensive siRNA and associated transfection reagents, and the method used for preparing cellular extracts, it was not possible to investigate ABCA1 protein expression.

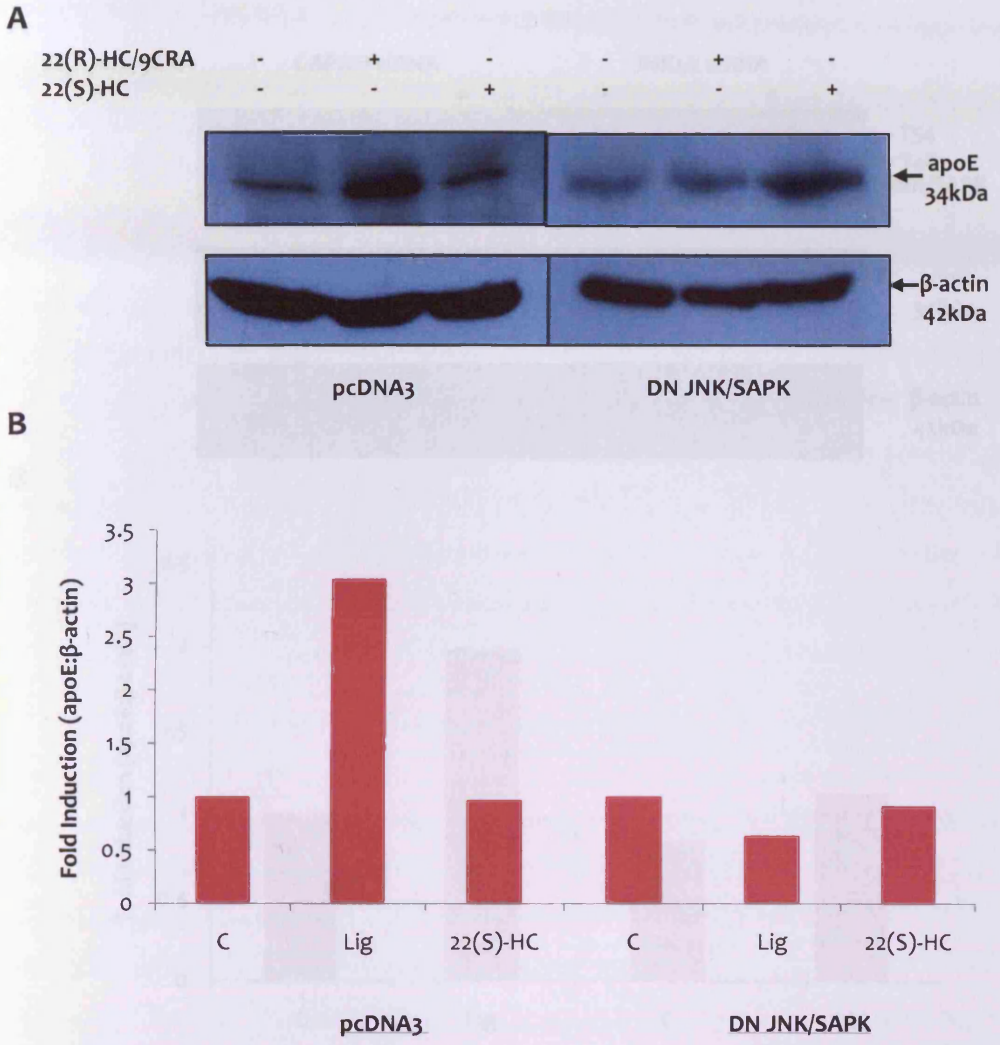


Figure 5.11 Effect of DN JNK/SAPK on the induction of apoE expression by LXR agonists.

THP-cells were transfected with the expression plasmid specifying for DN JNK/SAPK or the vector pcDNA3. The transfected cells were treated with either DMSO as a vehicle control (C), combinations of 22(R)-HC and 9CRA (2μg/ml and 10μM respectively) (Lig) or 22(S)-HC (2μg/ml) for a further 24h. Whole cell protein extracts were prepared and equal amount were subjected to Western blot analysis using antibodies against apoE and β-actin as indicated (panel A). The apoE protein levels were normalised to β-actin and the values obtained from densitometric analysis of the data is shown in panel B. The value for cells incubated in the presence of the DMSO vehicle has arbitrarily been assigned as 1.

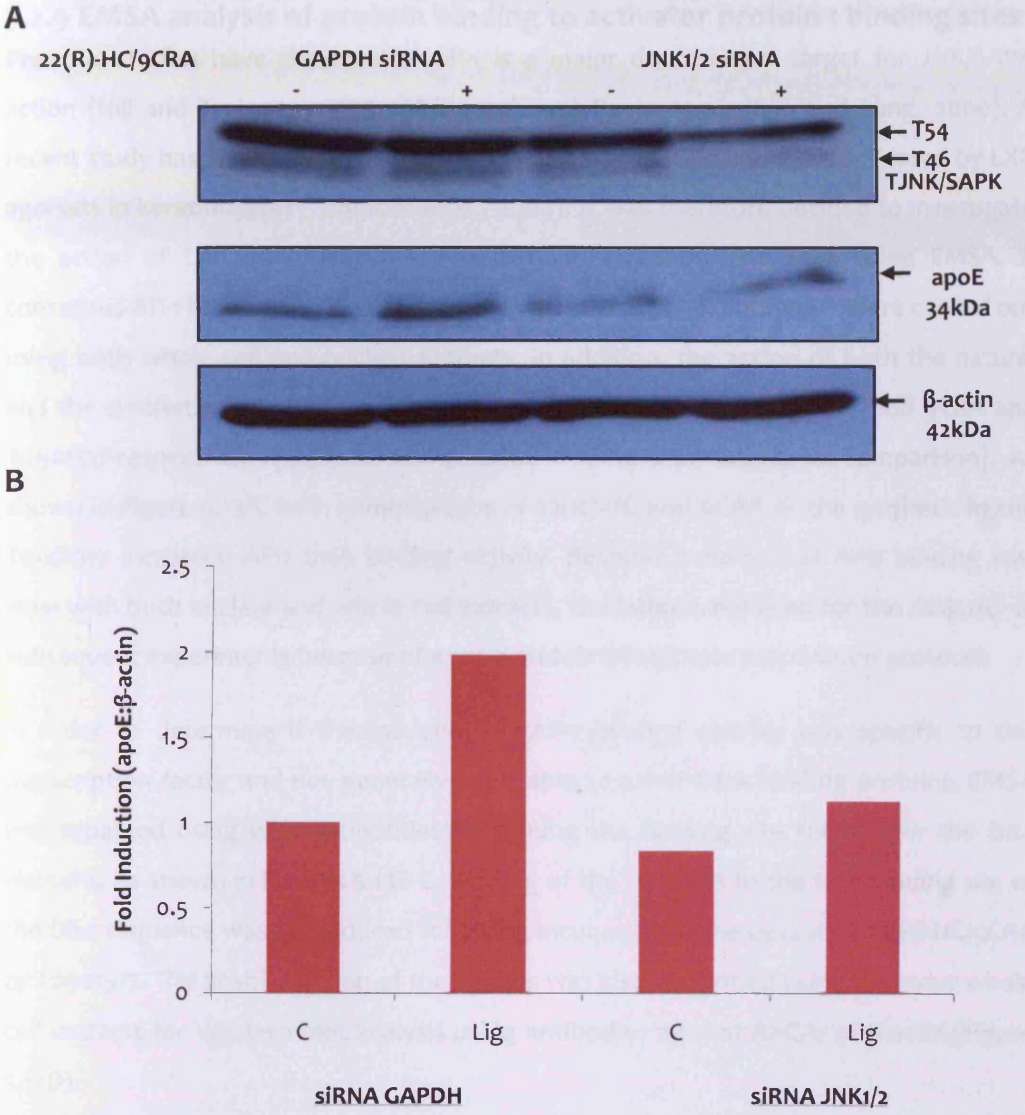


Figure 5.12 Effect of knock down of JNK/SAPK1/2 expression on LXR agonist-mediated activation of apoE expression.

THP-cells were transfected with the indicated siRNAs using INTERFERin™ siRNA transfection reagent. At 48h post-transfection, the cells were treated with 22(R)-HC (2μg/ml) and 9CRA (10μM) (Lig) or DMSO as a vehicle control (C) for a further 24h. The total cell lysates were prepared and subjected to Western blot analysis using antibodies against JNK/SAPK, apoE and the β-actin control. The image shown in panel A is representative of two independent experiments. Densitometric analysis was carried out on the data and presented as average of fold induction in relation to basal expression seen in the presence of DMSO control (assigned as 1).

5.2.9 EMSA analysis of protein binding to activator protein-1 binding sites

Previous studies have shown that AP-1 is a major downstream target for JNK/SAPK action (Hill and Treisman, 1995; Whitmarsh and Davis, 1996; Xiao and Lang, 2000). A recent study has shown that the expression of AP-1-regulated genes is activated by LXR agonists in keratinocytes (Schmuth et al., 2004). It was therefore decided to investigate the action of LXR agonists on AP-1 activity in THP-1 macrophages using EMSA. A consensus AP-1 binding site was used as a probe and initial experiments were carried out using both whole cell and nuclear extracts. In addition, the action of both the natural and the synthetic LXR agonists was analysed (combinations of 22(R)-HC and 9CRA and T0901317 respectively) [22(S)-HC was included in some experiments for comparison]. As shown in Figure 5.13A, both combinations of 22(R)-HC and 9CRA or the synthetic ligand T0901317 increased AP-1 DNA binding activity. Because induction of AP-1 binding was seen with both nuclear and whole cell extracts, the latter were used for the majority of subsequent experiments because of a rapid and simple sample preparation protocol.

In order to determine if the induction of AP-1 binding activity was specific to this transcription factor and not generally applicable to other DNA binding proteins, EMSA was repeated using oligonucleotides containing the binding site for NF-1 or the DR4 element. As shown in Figures 5.13B-C, binding of the proteins to the NF-1 binding site or the DR4 sequence was not induced following incubation of the cells with 22(R)-HC/9CRA or T0901317. The positive action of the ligands was also confirmed using the same whole cell extracts for Western blot analysis using antibodies against ABCA1 or β-actin (Figure 5.13D).

Competition experiments were carried out in order to confirm specific binding of AP-1 in THP-1 extracts to the probe used for EMSA. For such competition assays, extracts were pre-incubated for 10min in the presence or absence of 200-fold molar excess of double stranded DNA competitor (AP-1 and C/EBP or NF-κB binding sites as specific and non-specific sequences, respectively) before addition of the radiolabelled probe. As shown in Figure 5.14, AP-1 binding was induced by combinations of 22(R)-HC and 9CRA or T0901317. This increase in binding was almost totally inhibited by the inclusion of 200-fold molar excess of oligonucleotides containing the specific AP-1 binding sequenc but not by those containing the unrelated C/EBP or NF-κB recognition sequence.

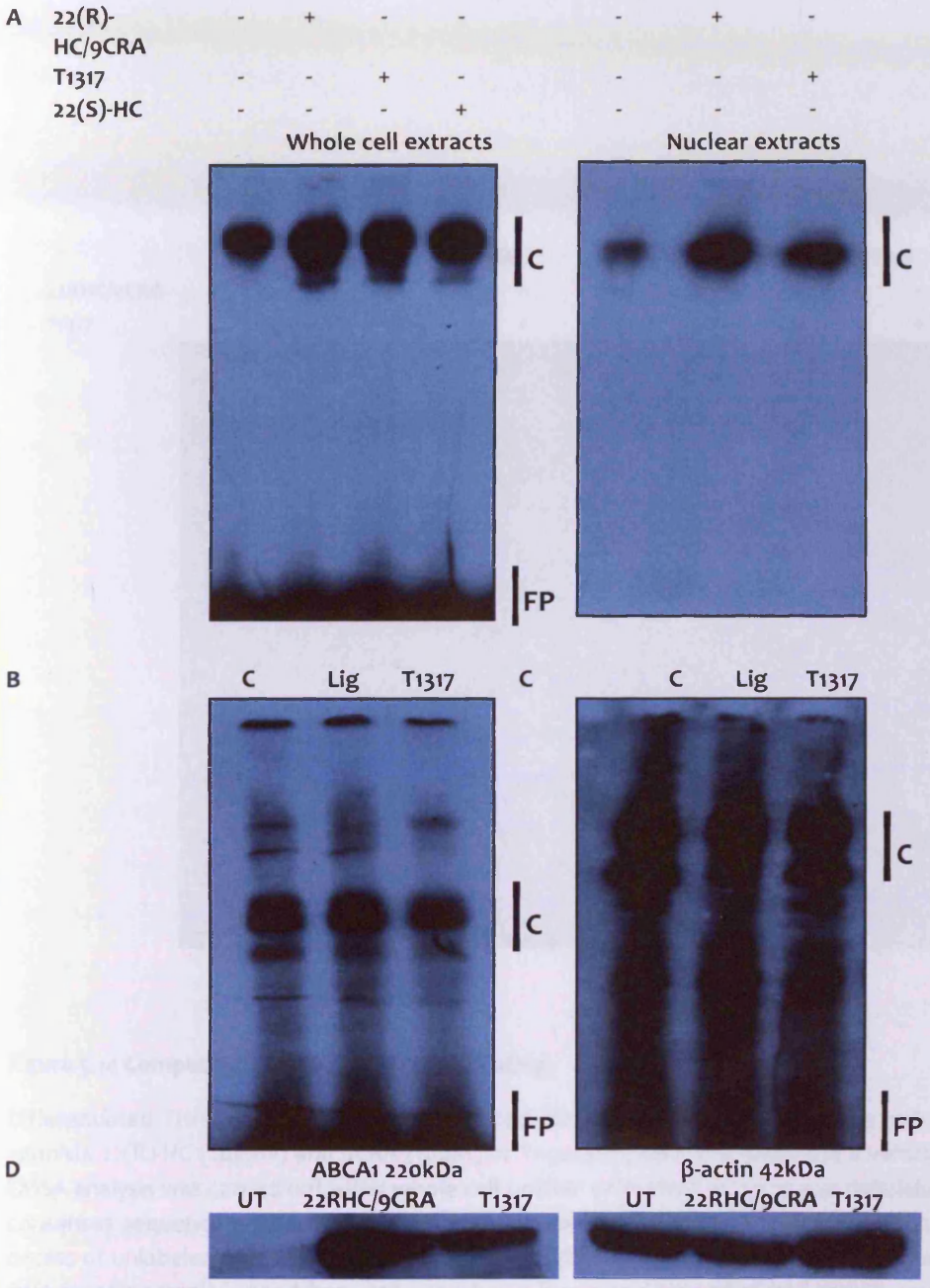


Figure 5.13 The effect of LXR agonists on AP-1 DNA binding and ABCA1 expression.

Differentiated THP-1 macrophages were cultured for 24h in the presence of the indicated LXR agonists 22(R)-HC (2μg/ml) and 9CRA (10μM) or T0901317 (10μM); referred to as Lig and T1317, respectively in panels B and C. In addition, cells were treated with DMSO as a vehicle control (C) or 22(S)-HC (2μg/ml) as shown. Whole cell protein or nuclear extracts were prepared and then EMSA were carried out using radiolabelled probe AP-1 (A), NF-1 (B) and DR4 (C). The protein extracts were also used to determine the expression of ABCA1 and β-actin by Western blot analysis using antibodies against ABCA1 or β-actin (D). The major DNA-protein complexes and free probe are shown by a vertical line labelled C and FP respectively. The free probe has migrated off the gel in EMSA using nuclear extracts. The results shown are representative of three (panel A) and two (panel B) independent experiments.

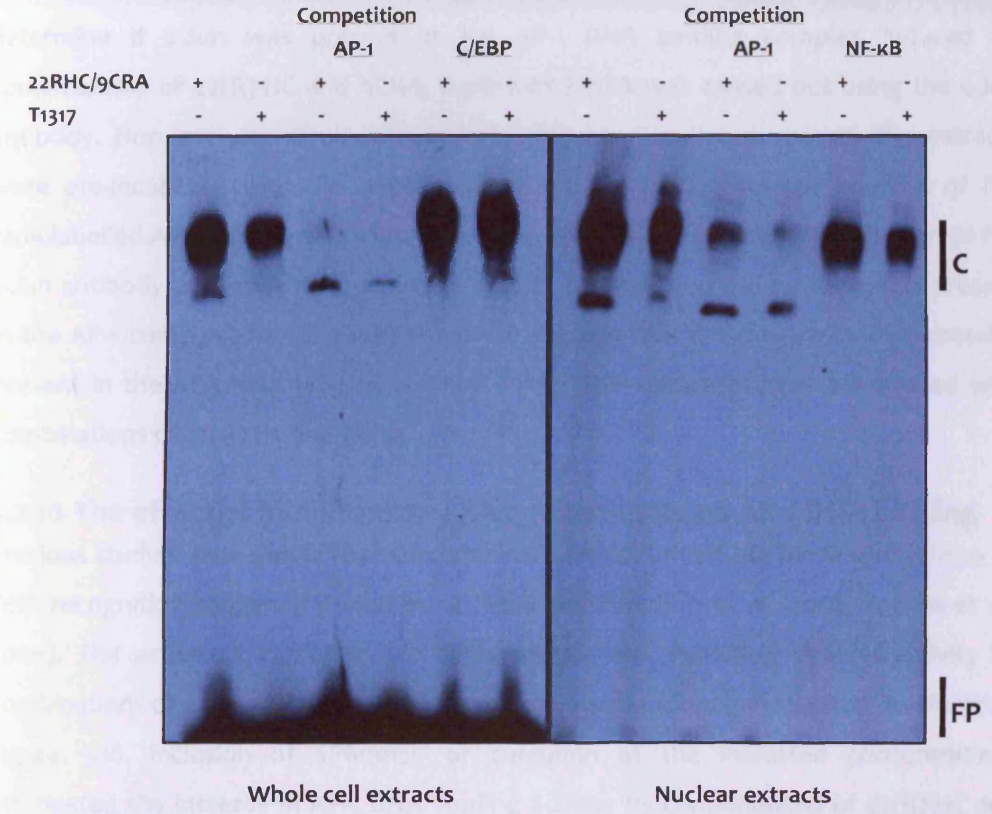


Figure 5.14 Competition EMSA on AP-1 DNA binding.

Differentiated THP-1 macrophages were cultured for 24h in the presence of the indicated LXR agonists 22(R)-HC (2μg/ml) and 9CRA (10μM) or T0901317 (10μM), or DMSO as a vehicle control. EMSA analysis was carried out using whole cell protein or nuclear extracts and radiolabelled AP-1 consensus sequence probe. Competition assays were carried out in the presence of 200 molar excess of unlabeled specific (AP-1) or nonspecific (C/EBP or NF-κB) competitor oligonucleotides. DNA-protein complexes and free probe are shown by vertical lines labelled C and FP, respectively. The free probe has migrated off the gel in the case of EMSA using nuclear extracts. The results shown are representative of two independent experiments (whole cell extract) and one independent experiments (nuclear extract).

These results therefore confirm the specificity of protein binding to the AP-1 recognition sequence.

c-Jun is a major member of the AP-1 family of transcriptional regulators and a well established key target for JNK/SAPK action (Kyriakis and Avruch, 2001). In order to determine if c-Jun was present in the AP-1 DNA binding complex induced by combinations of 22(R)-HC and 9CRA, supershift EMSA was carried out using the c-Jun antibody. Non immune serum was included for comparative purposes. The extracts were pre-incubated with the antisera for 30min on ice before the addition of the radiolabelled AP-1 binding site oligonucleotide. As shown in Figure 5.15, inclusion of the c-Jun antibody, but not the non-immune serum, supershifted the c-Jun protein present in the AP-1 complex. These results therefore suggest that c-Jun is one of the proteins present in the AP-1 DNA binding complex when THP-1 macrophages are treated with combinations of 22(R)-HC and 9CRA.

5.2.10 The effect of inhibitors on LXR agonist-induced AP-1 DNA binding

Previous studies have shown that curcumin can directly inhibit the binding of AP-1 to its DNA recognition sequence (Hahm et al., 2002; Hergenhahn et al., 2002; Tomita et al., 2006). The action of curcumin and SP600125 on the induction of AP-1 activity by combinations of 22(R)-HC and 9CRA or T0901317 was therefore investigated. As shown in Figure 5.16, inclusion of SP600125 or curcumin at the indicated concentrations attenuated the increase in AP-1 DNA binding activity by combinations of 22(R)-HC and 9CRA or by the synthetic ligand T0901317.

5.3 Discussion

Mammalian cells have developed complex feedback mechanisms to ensure sufficient supply of cholesterol is available to them and excessive accumulation is inhibited. These homeostatic mechanisms probably fail in macrophages during atherogenesis. Uncontrolled cholesterol accumulation is promoted by scavenger receptors of macrophages leading to the formation of lipid-loaded foam cells. In this study, the cell signalling pathways that are potentially involved in the expression of key genes implicated in the control of foam cell formation and atherosclerosis by LXR agonists were investigated. Studies in chapter 4 showed a potential role for the JNK/SAPK and PI3K pathways in such an action of LXR agonists. Therefore, the primary aim of the

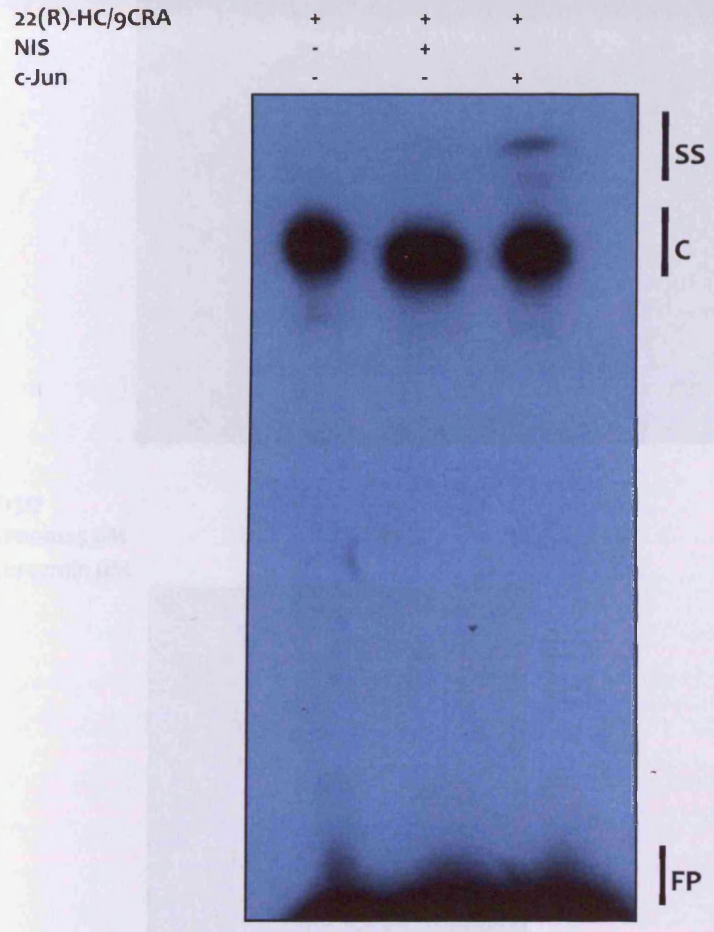


Figure 5.15 Supershift EMSA using c-Jun antibody.

Differentiated THP-1 macrophages were incubated for 24h in the presence of combinations of 22(R)-HC (2μg/ml) and 9CRA (10μM). Whole cell protein extracts were prepared and pre-incubated for 30min with non-immune serum (NIS) or c-Jun antibody as shown. EMSA was carried out using radiolabelled AP-1 consensus sequence probe. The DNA-protein complex, the DNA-protein- antibody supershift complex and the free probe are shown by vertical lines labelled C, SS and FP, respectively. Results shown are representative of two independent experiments.

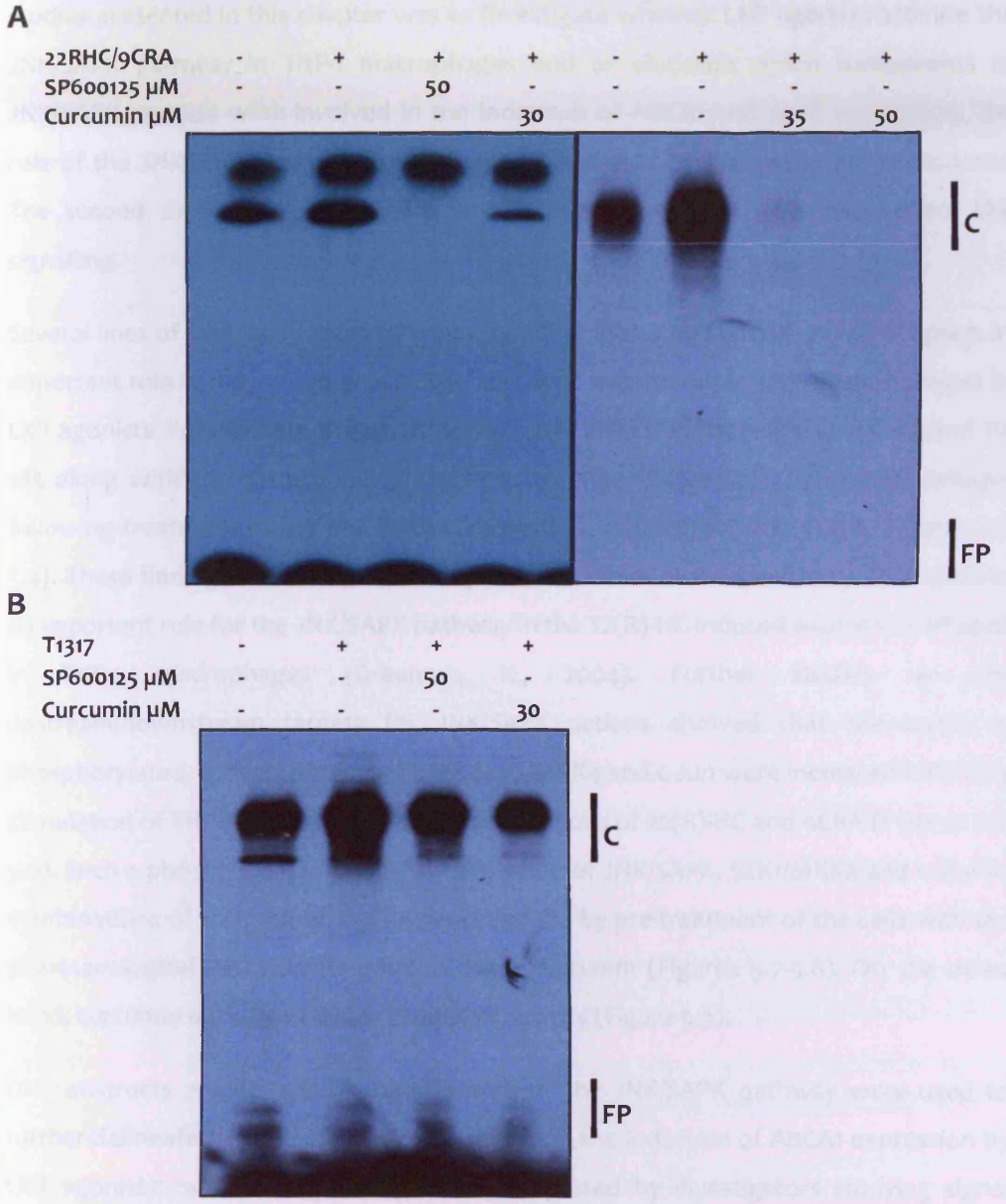


Figure 5.16 The effect of inhibitors on the LXR ligand-induced binding of AP-1.

Differentiated THP-1 macrophages were treated for 24h with combination of 22(R)-HC (2μg/ml) and 9CRA (10μM), T0901317 (10μM) or DMSO as a vehicle control in the absence or the presence of the inhibitors SP600125 and curcumin at the indicated concentrations. The inhibitors were added 1h before the ligand (pre-treatment). EMSA was carried out using radiolabelled AP-1 consensus probe. DNA-protein complex and free probe are shown by vertical lines labelled C and FP, respectively (the free probe has migrated off the gel on the right side of panel A).

studies presented in this chapter was to investigate whether LXR agonists activate the JNK/SAPK pathway in THP-1 macrophages and to elucidate which components of JNK/SAPK cascade were involved in the induction of ABCA1 and apoE expression. The role of the JNK/SAPK pathway could then be confirmed by expression of DN mutants. The second aim was to investigate how this pathway may potentially affect LXR signalling.

Several lines of evidence support the novel finding that activation of JNK/SAPK plays an important role in the induction of ABCA1 and apoE expression in THP-1 macrophages by LXR agonists. For example, the phosphorylation of JNK/SAPK at residues Thr 183 and Tyr 185 along with the corresponding kinase activity was induced in THP-1 macrophages following treatment of the cells with combinations of 22(R)-HC and 9CRA (Figures 5.2-5.4). These findings are consistent with previous studies in the laboratory that showed an important role for the JNK/SAPK pathway in the 22(R)-HC-induced expression of apoE in THP-1 macrophages (Greenow, K., 2004). Further studies on the upstream/downstream targets for JNK/SAPK actions showed that the levels of phosphorylated, activated forms of both SEK1/MKK4 and c-Jun were increased following stimulation of THP-1 macrophages with combinations of 22(R)-HC and 9CRA (Figures 5.5-5.6). Such a phosphorylation-mediated activation of JNK/SAPK, SEK1/MKK4 and c-Jun by combinations of 22(R)-HC and 9CRA was inhibited by pre-treatment of the cells with the pharmacological inhibitor SP600125 but not curcumin (Figures 5.7-5.8). On the other hand, curcumin did indeed inhibit JNK/SAPK activity (Figure 5.9).

DN constructs against specific components of the JNK/SAPK pathway were used to further delineate the role of individual proteins in the induction of ABCA1 expression by LXR agonists. Such an approach is commonly used by investigators studying signal transduction pathways (Gilchrist et al., 1999; Izumi et al., 2001). Expression of a DN form of SEK-1 or c-Jun (Tam67) attenuated the induction of ABCA1 promoter activity by combinations of 22(R)-HC and 9CRA (Figure 5.10). The data on the action of DN c-Jun are similar to previous studies in the laboratory where it inhibited the induction of apoE expression in response to TGF-β in THP-1 monocytes (Singh and Ramji, 2006). DN SEK1/MKK4 has also been shown previously to significantly inhibit the 22(R)-HC activated expression of the apoE gene in THP-1 macrophages (Greenow, K., 2004). In contrast to SEK1/MKK4 or c-Jun, the expression of DN JNK/SAPK had no effect on the induction of

ABCA1 promoter activity by 22(R)-HC and 9CRA (Figure 5.10). However, the potential role for JNK/SAPK in the induction of apoE expression by the ligands was confirmed by RNAi (Figures 5.11- 5.12). The precise reasons why the expression of DN JNK/SAPK had no effect on the activation of ABCA1 promoter activity by LXR agonists is currently unclear. Functional redundancy between JNK/SAPK and another pathway required for the full activation of the ABCA1 promoter by LXR agonists is a possible reason. It is also possible that the action of JNK/SAPK on ABCA1 mRNA or protein expression requires a particular chromatin configuration that is absent in the transfected plasmids.

Our findings show that whereas both p46 and p54 JNK/SAPK are phosphorylated after treatment of THP-1 cells with 22(R)-HC and 9CRA, the p54 isoform is more activated by these ligand at 24h (second peak) compared to 0h (Figure 5.4). As with the ERK subfamily of MAP kinases, emerging data suggest that p46 and p54 JNK/SAPKs, whilst showing a number of overlapping features, may also have distinct substrate preferences (Chan et al., 1997). For example, Kallunki et al. (1994) have shown p54 JNK/SAPK binds c-Jun with greater affinity than the p46 isoform. They suggest that at the concentration of c-Jun encountered intracellularly, the p54 subunit is more likely to phosphorylate it than p46, thereby raising the question of alternative roles for p54 and p46 JNK/SAPK. It has also been shown that specific activation of p54 in COS cells by UV radiation and pro-inflammatory cytokines was approximately 10-fold greater than that of the p46 isoform (Sluss et al., 1994). Thus, these issues raise the question of the role that signalling heterogeneity within the JNK/SAPK subfamily may play in the varied functional responses in macrophages (Riches, 1995), in particular with respect to activation of the AP-1 family.

Previous work has shown that curcumin can block cytokine- and phorbol ester-stimulated JNK/SAPK activation, c-Jun phosphorylation and AP-1 transcriptional activity (Chen and Tan, 1998; Han et al., 2002; Squires et al., 2003). In addition, it has been shown that curcumin can act as an inhibitor of AP-1 DNA binding (Hahm et al., 2002; Hergenhahn et al., 2002). On this basis, curcumin has been used as an inhibitor for JNK/SAPK and AP-1. However, other studies have shown that curcumin inhibits an upstream kinase of the JNK/SAPK pathway, most likely MEKK1 (Jobin et al., 1999). There are also studies that show that curcumin does not directly inhibit the kinase function of JNK/SAPK, SEK1/MKK4 or MEKK1 activity and suggest a more complex mode of action

(Chen and Tan, 1998). Some of these discrepancies could reflect differences in cell types and/or mediators used. Our results show that curcumin has no effect on the phosphorylation of JNK/SAPK, SEK1/MKK4 or c-Jun at specific sites when a concentration of 30μM was used (Figure 5.8). However, JNK/SAPK activity, which could potentially be dependent on multiple phosphorylation, was attenuated and also there was a concentration-dependent decrease in AP-1 DNA binding activity by curcumin (Figures 5.9-5.16). Our data are in agreement with a study by Singh and Ramji (2006) that showed that curcumin had no effect on JNK/SAPK activation by TGF-β in THP-1 monocytes whilst JNK activity was inhibited in concentration-dependent manner (Singh and Ramji, 2006). In contrast, work in our laboratory showed that curcumin inhibits JNK/SAPK phosphorylation and kinase activity in THP-1 macrophages induced by 22(R)-HC and in Hep3B cells stimulated with IL1 (Greenow, K., 2004, Ali, S., personal communication). Indeed, these findings are in agreement with a previous study that showed that curcumin prevents JNK/SAPK activation by various agonists, including anisomycin, UV, gamma radiation and TNFα (Chen and Tan, 1998).

An attractive hypothesis on the mechanisms underlying the involvement of cell signalling pathways, such as JNK/SAPK, in NR-mediated regulation of target genes is that the NR protein or one of the coregulator components of the transcription complex is being targeted for phosphorylation (See previous chapter for more detail). Therefore, according to the results in the present chapter, the possibility that any one of the components of the JNK/SAPK pathway may be affecting the LXR transcriptional complex (e.g. LXR itself or one or more of the coregulators) is the most likely hypothesis for the involvement of the JNK/SAPK pathway in LXR-regulated gene transcription. Such phosphorylation would result in an increase in transcriptional activation of the target genes.

Further studies focussed on the major downstream targets of JNK/SAPK pathway involved in the LXR-mediated regulation of ABCA1 and apoE gene expression. EMSA showed an increase in protein binding to AP-1 recognition sequence when extracts were used from cells that had been treated with either combinations of 22(R)-HC and 9CRA or T0901317 (Figure 5.13). Such an activation of AP-1 DNA binding activity was inhibited by pre-treatment of the cells with SP600125 and curcumin (Figure 5.16). The AP-1 family mainly consists of members of the Jun and Fos families. The Jun family includes c-Jun,

JunB and JunD whereas the Fos family includes c-Fos, Fos-B, Fra-1 and Fra-2 (Wisdom, 1999). The AP-1 DNA binding complex consists of various combinations of Fos and Jun family members (homodimers between the Jun family members or heterodimers between the Jun and Fos family members) (Hess et al., 2004; Karin et al., 1997). AP1 dimers stimulate transcription by binding to a palindromic DNA sequence (5-TGAG/CTCA-3; often called Tumour Promoter Activator element) usually present in the enhancer regions of many genes implicated in the control of cell division, growth and differentiation (Shaulian and Karin, 2001). Regulation of AP-1 activity is complex and involves transcriptional and post-transcriptional mechanisms (Hess et al., 2004; Karin, 1995; Tomita et al., 2006). An important post-transcriptional control mechanism is the direct phosphorylation of AP-1 components (Whitmarsh and Davis, 1996), thereby leading to changes in DNA binding and/or *trans*-activation potential. For example, c-Jun is efficiently phosphorylated by JNK/SAPK at sites within its N-terminal *trans*-activation domain and by ERKs at an inhibitory site located within the C-terminal DNA-binding domain (Chou et al., 1992; Hess et al., 2004; Karin, 1995; Karin et al., 1997). By contrast, the kinases that regulate the activity of c-Fos are not yet fully understood. Potential candidates are, as yet, undefined Fos-related kinase (Deng and Karin, 1994) and ERK (Chen et al., 1996). However, the significance of these kinases in the control of Fos activity and function remains elusive (Hess et al., 2004).

NRs can also cross-talk with other transcription factors and, thereby, interfere with their regulation of target gene expression. Schmuth et al. (2004) first demonstrated that oxysterols, via activation of LXRs, induce a general increase in expression of AP-1 regulated genes during keratinocyte differentiation, and this effect can be abolished by mutation of the distal AP-1 response element in the involucrin promoter. In addition, AP-1 binding activity was shown by EMSA to be induced by oxysterols along with an increase in the activity of an AP-1 reporter plasmid. Furthermore, Fra-1 was identified as a key component in the AP-1 DNA binding complex by antibody interference/supershift EMSA using nuclear extracts from oxysterol treated keratinocytes. Additionally, oxysterol treatment increased both the binding and the expression of two other AP-1 proteins, JunD and c-Fos, whereas Fra-2, Jun B and c-Jun were not affected (Schmuth et al., 2004). Similar alterations in AP-1 proteins were also seen when natural (25-OH-cholesterol) or non-steroidal LXR agonists (GW3965 or T0901317) were used (Schmuth et al., 2004).

The influence of cross-talk by LXR, ERs, GR and PPARs on cytokine signal transduction has been reviewed previously (Wang et al., 2004a). Ogawa et al. (2005) showed that two synthetic agonists for LXRs (GW3965 and T0901317) inhibit cytokine-induced expression of osteopontin in RAW 264.7 macrophages, and this inhibition was mediated through interference with AP-1 signalling pathways (Ogawa et al., 2005). In further support for the role of the AP-1 family in signalling by NRs, Patel et al. (2005) have reported that the regulation of COX-2 expression by PPARγ agonist, troglitazone, involves the activation of AP-1. In addition, a cross-talk between GR and AP-1 or NF-κB results in the transcriptional repression of their target genes (De Bosscher et al., 2003; Liberman et al., 2007). Although, in most cases, the cross-talk results in repression, there is also data showing the cross-talk between NRs and AP-1 promotes transcription. for example, ligand-bound ERs induce AP-1 activity (Bjornstrom and Sjoberg, 2002).

Several NRs have also been shown to alter transcription through the AP-1 response element in various cell types (Uht et al., 1997). The involvement of the AP-1 family in NR signalling may occur through several mechanisms, including direct interaction with NRs or coregulators, binding to AP-1 elements in the promoters of the NR genes or even through the binding to AP-1 elements in the regulatory regions of target genes (Uht et al., 1997). Previous studies have demonstrated that the major sequence elements required for the transcriptional regulation of the ABCA1 gene include binding sites for LXRs, AP-1, -2 and -4, SP1, NF-κB as well as three E-box motifs (Costet et al., 2000; Santamarina-Fojo et al., 2000; Yang et al., 2002). Yang et al. (2002) reported that the E-box motif present 147bp upstream of the transcriptional start site of the hABCA1 gene promoter binds to the transcription factors USF1 and USF2 as well as Fra-2 (Yang et al., 2002).

From the results presented here, it is possible to suggest that the regulation of ABCA1 and apoE expression by LXR agonists in THP-1 macrophages may involve the activation of the AP-1 pathway. A possible mechanism is that c-Jun, as a member of AP-1 complex, may be interacting with the LXR transcriptional complex at the ABCA1 promoter. Such a mechanism has been shown previously to occur in estrogen signalling, whereby ERα, c-Jun and the transcriptional coactivator, p160/GRIP1, form a multiprotein complex at the promoter *in vitro* and *in vivo*, and the ERα-c-Jun interaction is crucial for the stability of this complex (Teyssier et al., 2001; Webb et al., 1999). Furthermore, the PPARγ ligand,

15d-PGJ_2 has been shown to directly inhibit AP-1 DNA binding by forming a covalent adduct with c-Jun (Pérez-Sala et al., 2003). The second and the most likely potential mechanism for the role of c-Jun in LXR signalling is that, as a part of the AP-1 complex, it is activating ABCA1 through the AP-1 motif present in its promoter region. Therefore, it is possible that AP-1 may be required for the full stimulatory response of LXR agonists on promoters regulated by this NR. Such a requirement for additional transcription factors have been seen in other studies. For example, full activation of the SREBP-1c promoter by insulin requires LXR-REs and Sp1 binding sites (Cagen et al., 2004). It is also worth noting that the AP-1 element has already been shown to be essential for the induction of apoE expression during macrophage differentiation (Basheeruddin et al., 1994). The third possibility is that c-Jun is up-regulating the expression of genes encoding coactivator proteins necessary for the LXR activation of ABCA1. Recently, a model for the regulation of CYP7A1 gene transcription by FXR has been proposed by Gupta et al. (2001), which also involves the JNK/SAPK-c-Jun pathway. In this model, bile acids activate PKC leading to phosphorylation and activation of JNK/SAPK1/2 and c-Jun in addition to FXR (Chiang John, 2002). Both c-Jun and FXR bind to different elements (AP-1 and IR-1 respectively) in the CYP7A1 promoter (Gupta et al., 2001). In addition, the same study showed that phosphorylated c-Jun could also induce the transcription of the non-specific small heterodimer partner (SHP)-1 corepressor by binding to an AP-1 site in its promoter, which in turn represses CYP7A1 transcription (Gupta et al., 2001). This model could potentially be applied to the LXR regulation of genes implicated in the control of macrophage cholesterol homeostasis such as ABCA1 and apoE, with the JNK/SAPK pathway inducing activation of a coactivator protein. This mechanism may also provide explanation for the potential role for the second peak of induction of JNK/SAPK and c-Jun phosphorylation seen in the studies.

The rapid activation of the JNK/SAPK pathway by LXR ligands (first peak at 30min) suggests that the initial activation of this pathway by LXR agonists is probably not dependent on gene activation. This ability of a NR agonist to rapidly activate a cell signalling pathway independent of gene transcription is just beginning to be recognized. Actually, the importance of the so called non-genomic or extranuclear signalling action of NR ligands that does not involve its cognate receptor have been recently identified and well described for several members of the NR family (Aranda and Pascual, 2001; Germain et al., 2006; Gronemeyer et al., 2004; Moeller et al., 2006; Simoncini et al.,

2000). The definition of non-genomic effects given by Losel and Wehling is "any action that does not directly and initially influence gene expression, but rather drives more rapid effects such as the activation of signalling cascades" (Losel and Wehling, 2003). These rapid actions of agonists have been subsequently described to generate intracellular second messengers to activate or inhibit cell signalling pathways (Davis and Davis, 2002; Davis et al., 2002). Recent studies have identified that this initiation of intracellular signalling mechanisms occurs through the NR ligand binding to a receptor present at the plasma membrane or in the cytoplasm (Boonyaratanakornkit and Edwards, 2004; Schmidt et al., 2000). To include this alternative mode of action in the definition of non-genomic effects, the term membrane-initiated NR signalling has been proposed (Schmidt et al., 2000). However, the identities or nature of these receptors have been elusive so far. In addition, it is likely that a subclass of NRs mediate these actions by somehow associating with the cell membrane and/or signalling complexes in the cytoplasm (Schmidt et al., 2000). However, studies have also suggested the potential existence of separate membrane receptors unrelated to the classical NRs which are responsible for this non-genomic action of NR ligands (Boonyaratanakornkit and Edwards, 2004; Gronemeyer et al., 2004).

Steroids can induce an increase in several second messengers such as inositol triphosphotes, cAMP, Ca^{2+} and the activation of MAPK and PI3K pathways, which occurs very rapidly within seconds or minutes and is receptor-mediated (Aranda and Pascual, 2001; Germain et al., 2006). The ERK1/2 MAPK pathway can be activated by thyroid hormone binding to the integrin αVβ3, located in the cell membrane, without entering the cell. Thus, this mechanism leads to phosphorylation of NRs which can promote cell growth (Bergh et al., 2005; Moeller et al., 2006; Tang et al., 2004c). This non-genomic action of TH is mostly extranuclear, appears to be independent of TH receptors and has rapid effects on proteins rather than the regulation of gene expression (Moeller et al., 2006). Consistent with these findings, recent work has also reported a non-genomic action for PPARγ-mediated gene regulation, where its ligand, 15-d-PGJ2, causes enhanced AP-1 binding activity in VSMCs. This induction of AP-1 activity occurs within 30 min and was shown to be due to activation of the ERK pathway and was partially dependent on PI3K (Takeda et al., 2001). Therefore, in addition to the 22(R)-HC and 9CRA-mediated activation of the LXR/RXR heterodimer at the ABCA1 promoter, classical genomic action of LXR agonist (See chapter 4), it is also likely that the LXR-mediated

regulation of ABCA1 and apoE expression requires the non-genomic activation of the JNK/SAPK pathway by 22(R)-HC and 9CRA or T0901317.

As no previous work has explored a non-genomic action for LXR agonists, it is unclear how the ligand is activating the JNK/SAPK pathway. One possible way is that a membrane bound LXR receptor exists. Another possibility is that the ligands may be acting independently of the LXRs as signalling molecules. Regulation via membrane-bound receptor is the most likely possibility as our studies have demonstrated that the induced expression of ABCA1 and apoE by LXR is ligand specific as 22(S)-HC, which does bind to, but not activate the receptor, did not induce ABCA1 and apoE expression. Indeed, several unrelated membrane receptors contribute to a large diversity of rapid responses (Picard, 1998; Wehling, 1997). In addition, the existence of binding sites for TH on the cell surface has been known for many years (Giguere et al., 1996; Schwartz et al., 1967). Thus, on the basis of published literature and the present results, a hypothetical scheme can be proposed to explain, at least partially, the mechanisms by which LXR agonists induce ABCA1 and apoE expression in THP-1 cells by stimulating LXR activity via the JNK/SAPK pathway (Figure 5.17).

In summary, the work presented in this chapter implicates activation of SEK1/MKK4-JNK/SAPK and c-Jun/AP-1 signalling as an important signal transduction pathway in LXR/RXR-inducible expression of apoE and ABCA1 in macrophages. To our knowledge, this is the first study to demonstrate that LXR agonists induce SEK1/MKK4-JNK/SAPK-c-Jun pathway. Collectively, this novel study demonstrates that the JNK/SAPK signalling pathway plays important roles in LXR-mediated regulation of key genes involved in the control of cholesterol homeostasis. Our finding provides new potential targets for therapeutic intervention of atherosclerosis.

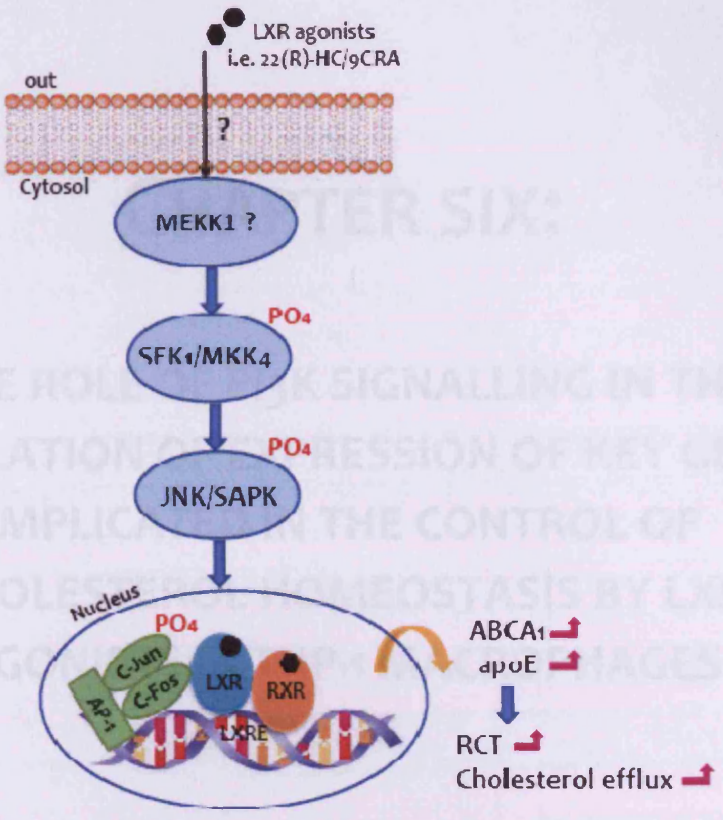


Figure 5.17 Hypothetical scheme of the signal transduction pathway activated by LXR agonists leading to increased expression of ABCA1 and apoE in THP-1 macrophages.

CHAPTER SIX:

THE ROLE OF PI3K SIGNALLING IN THE REGULATION OF EXPRESSION OF KEY GENES IMPLICATED IN THE CONTROL OF CHOLESTEROL HOMEOSTASIS BY LXR AGONISTS IN THP-1 MACROPHAGES

Chapter 6: The role of PI3K signalling in the regulation of expression of key genes implicated in the control of cholesterol homeostasis by LXR agonists in THP-1 macrophages

6.1 Introduction

The data presented in chapters 3 and 4 revealed a potentially important role for the PI3K pathway in the LXR agonist-induced expression of ABCA1 and apoE in murine and human macrophages. As discussed earlier, the roles of ABCA1 and apoE in the control of cholesterol efflux and the prevention of atherosclerosis are well established. The regulation of ABCA1 and apoE expression by LXR agonists therefore represents a potentially important target for therapeutic intervention of atherosclerosis. The aim of the studies presented in this chapter was to carry out further investigation of the role of the PI3K signalling pathway in the regulation of ABCA1 and apoE expression by LXR agonists.

PI3K is a heterodimeric enzyme composed of a regulatory polypeptide p85 and a catalytic subunit p110 (Carpenter and Cantley, 1996; Hawkins et al., 2006; Hirsch et al., 2007; Rameh and Cantly, 1999). PI3K catalyses the synthesis of 3-phosphorylated phosphoinositides and affects cell survival, metabolism and membrane trafficking. One of the best characterized downstream targets of the lipid metabolites produced by the action of PI3K is the Ser/Thr protein kinase PKB. This plays an important role in a variety of biological processes, including the control of cell survival, cell growth and regulation of gene expression. Various peptide growth factors, including insulin and insulin-like growth factor I, are known activators of PKB (Downward, 1998). The binding of such growth factors to their cell surface receptors results in the recruitment of PI3K to the plasma membrane. There are two main stages involved in the activation of PKB: the binding of the main lipid products of PI3K actions [PtdIns(3)P and/or PtdIns(3,4)P_2] to the pleckstrin homology domain present in the amino terminus of PKB, and the phosphorylation of PKB at Thr308 and Ser473 residues by PDK-1 or -2 leading to full activation of enzyme activity. These changes cause its translocation from the cytoplasm to the nucleus (Hresko et al., 2003; Krasilnikov, 2000; Toker and Newton, 2000; Vanhaesebroeck et al., 1997). Several downstream targets of PKB have been identified.

The majority of these have been implicated in insulin signalling and the promotion of cell survival by phosphorylation and inactivation of various proteins, such as GSK-3β, mTOR, the transcription factor forkhead (FKHR), endothelial nitric oxide synthase (eNOS) and several anti-apoptotic effectors, such as Bcl-2-associated death promoter (BAD) (Krasilnikov, 2000; Rameh and Cantly, 1999; Toker, 2000). PDK1 also activates the kinase p70(S6K) and PKC isoforms (Cantly, 2002; Toker, 2000; Vanhaesenbroeck and Alessi, 2000; Yang et al., 2004).

As the phosphorylated lipid products of PI3K activate various isoforms of PKC *in vitro* (Toker, 2000), PKC may lie downstream of PI3K (Reddy et al., 1997). PKC is a Ser/Thr-specific protein kinase that is involved in a number of important biological processes, such as cell cycle progression, apoptosis, differentiation and immune responses (Saijo et al., 2003). At present, 11 different PKC isoforms have been identified and grouped into three subsets based on their ability to respond to Ca^{2+} and/or diacylglycerol, a lipid produced by the action of phospholipase C (PLC) (Nishizuka, 1992, 1995). Both the classical PKC isoforms (α, βI, βII and γ) and the novel PKC isoforms, including δ, ε, θ and η are activated by diacyglycerols or PMA. The classical PKCs, but not the novel PKCs, also respond to a change in the intracellular concentration of Ca^{2+} ions. Unlike the classical and novel PKC isoforms, atypical PKC isoforms, such as ξ, λ and τ do not respond to either Ca^{2+} or PMA. Each of these isoforms are organized into two domains, a C-terminal catalytic domain and an N-terminal regulatory domain. The different PKC isoform are expressed in a ubiquitous manner (Delmotte et al., 1999). Activation of PKC often leads to the expression of the Jun and fos transcription factors that then interact with an AP-1 recognition site.

The primary aim of the studies presented in this chapter was to confirm whether the LXR agonists activate the PI3K/PKB pathway in THP-1 macrophages, and to elucidate which components of the PKB signalling cascade were involved in the induction of ABCA1 and apoE expression by such agonists. The secondary aim was to investigate exactly how this cellular signalling pathway may potentially affect LXR-mediated regulation of ABCA1 and apoE expression. As the increase in ABCA1 expression is likely to enhance mobilization of cholesterol across cell membranes and, thereby, regulate cellular cholesterol homeostasis (Wagner et al., 2003), such studies might identify potentially novel therapeutic targets for the treatment of atherosclerosis. Therefore, the overall

experimental strategy was to first investigate the activation of PKB by phosphorylation in THP-1 cells stimulated with LXR agonists via time course Western blot analysis using phospho-specific antibodies. In addition, non-radioactive, *in vitro* kinase assays were carried out to monitor changes in PKB activity. Furthermore, DNA constructs specifying for DN mutant proteins were used in transfection assays to confirm an important role for components of the PI3K pathway in the action of LXR activators. EMSA was also employed in order to study the activation of AP-1 binding by treatment of the cells with 22(R)-HC and 9CRA. Figure 6.1 illustrates the overall experimental strategy for the work presented in this chapter.

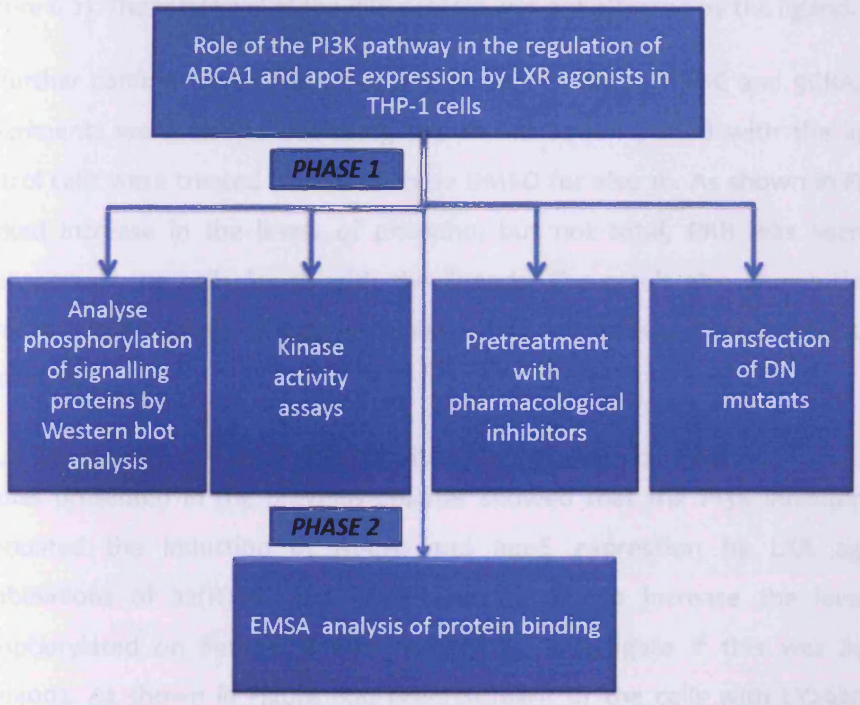


Figure 6.1 Summary of the experimental strategy used for further analysis of the role of the PI3K pathway in the activation of gene expression by LXR agonists in THP-1 macrophages.

6.2 Results

6.2.1 Time course of PKB phosphorylation by LXR agonists in THP-1 macrophages

Stimulation of cell surface RTK by growth factors leads to activation of PKB (Wymann et al., 2003). Full activation of PKB requires phosphorylation at two sites (Wymann and

Pirola, 1998), one in its catalytic domain (Thr 308) by PDK1 (Bayascas and Alessi, 2005) and another in the hydrophobic carboxyl terminal regulatory region (Ser 473) by the putative kinase PDK2 (Wymann et al., 1998; Yang et al., 2004; Lessmann et al., 2006). Previous work in the laboratory showed that phosphorylation of PKB on Ser 473 but not on Thr 308 is induced by stimulation of THP-1 macrophages with 22(R)-HC (Greenow, K., 2004). However, the action of combinations of 22(R)-HC and 9CRA on PKB phosphorylation on Ser 473 had not been determined and was therefore analysed by time course Western blot analysis. Treatment of THP-1 macrophages with 22-(R)-HC plus 9CRA resulted in a marked increase in the levels of phospho-PKB (Ser 473) within 15min, reaching maximal levels at 30-60min, and declining gradually at subsequent time points (Figure 6.2). The total level of the PKB protein was not affected by the ligand.

To further confirm the activation of PKB at Ser 473 by 22(R)-HC and 9CRA, additional experiments were carried out using the 1h incubation period with the ligands. The control cells were treated with the vehicle DMSO for also 1h. As shown in Figure 6.3, a marked increase in the levels of phospho, but not total, PKB was seen following incubation of the cells for 1h with the ligands. The result also shows that such an increase in PKB phosphorylation was specific to the ligands and not due to culturing the cells for 1h.

6.2.2 Effect of LY294002 on the phosphorylation of PKB

Studies presented in the previous chapter showed that the PI3K inhibitor LY294002 attenuated the induction of ABCA1 and apoE expression by LXR agonists. As combinations of 22(R)-HC and 9CRA were found to increase the levels of PKB phosphorylated on Ser473, it was decided to investigate if this was inhibited by LY294002. As shown in Figure 6.4, pre-treatment of the cells with LY294002 indeed inhibited the increase in the levels of phospho-PKB seen in the presence of combinations of 22(R)-HC plus 9CRA. In contrast, LY294002 had no effect on the levels of total PKB (Figure 6.4).

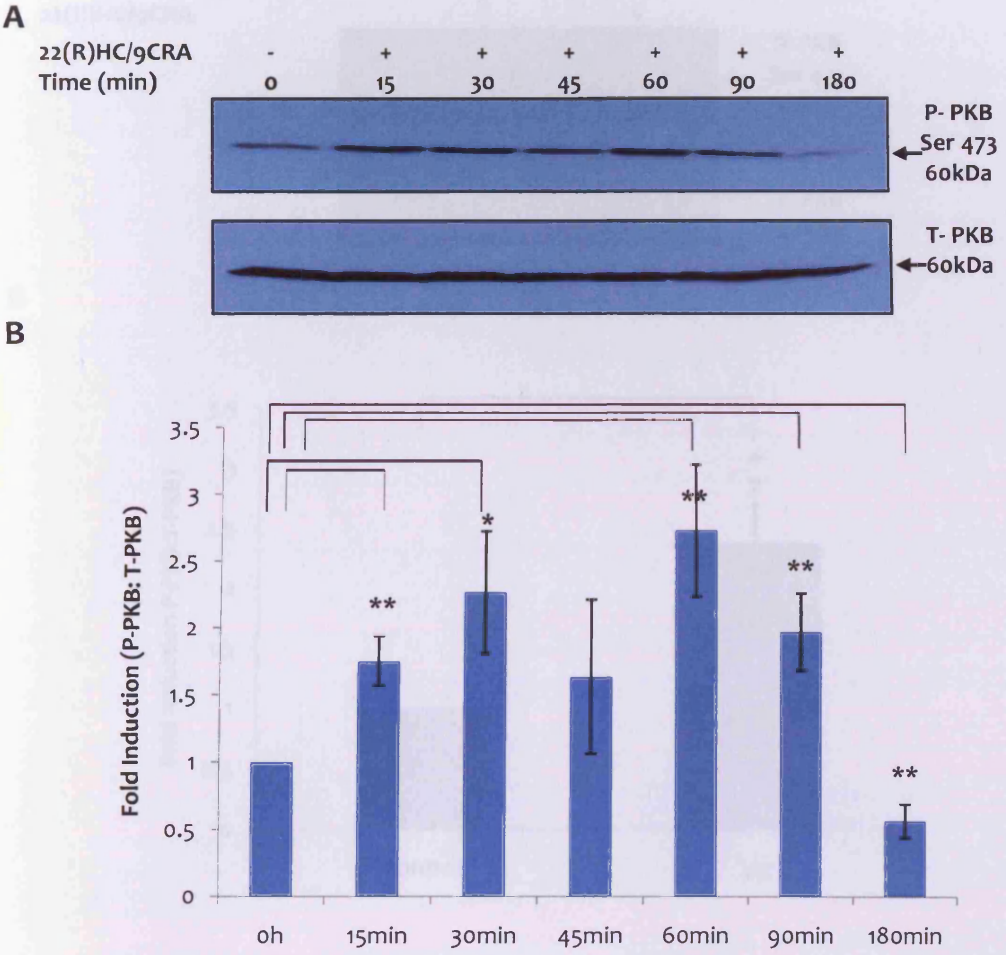


Figure 6.2 Effect of 22(R)-HC and 9CRA on PKB posphorylation at Ser 473 in THP-1 macrophages.

Differentiated THP-1 macrophages were incubated for the indicated time points with combinations of 22(R)-HC (2µg/ml) and 9CRA (10µM). Western blot analysis was carried out using 80µg of whole cell extracts. Blotted membranes were incubated with phospho-PKB (Ser473) and total-PKB primary antibodies. Antigen-antibody complexes were detected using the ECL detection system. The image shown in panel A is representative of three independent experiments. Densitometric analysis was carried out on the data and presented as mean fold induction (±SD) in relation to basal expression at 0h (assigned as 1) (panel B; *P<0.05, **P<0.01 compared to control 0h).

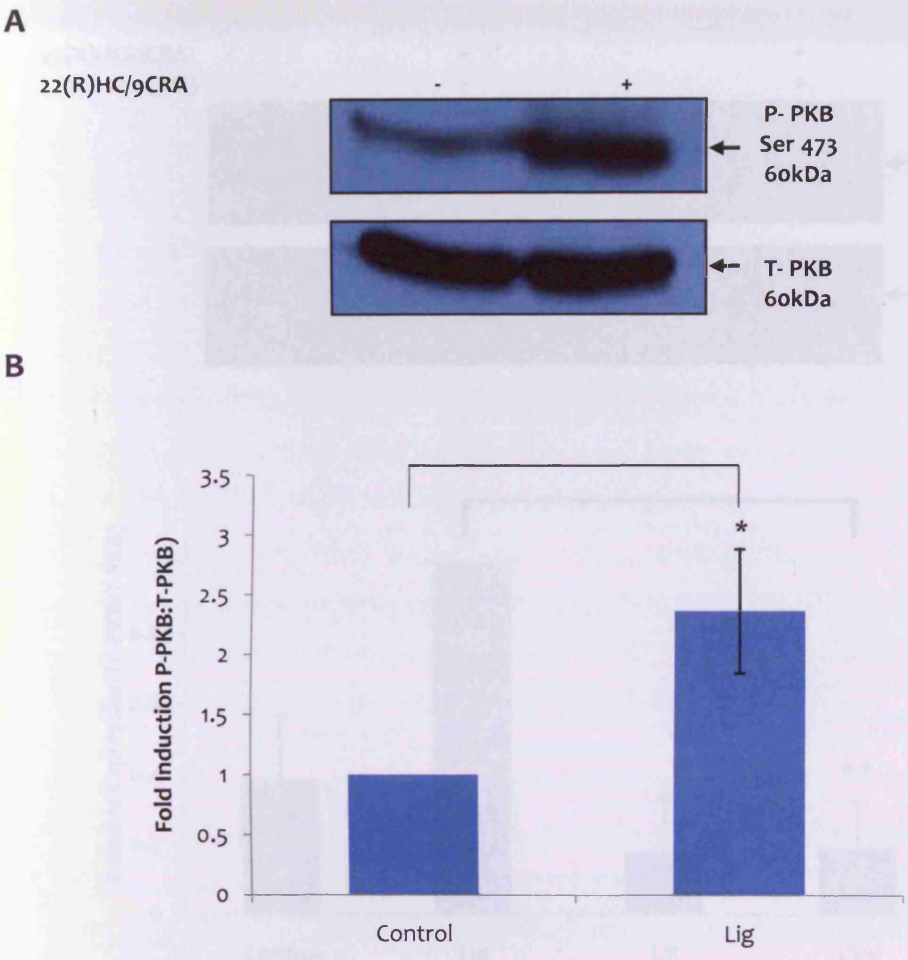


Figure 6.3 Activation of PKB by phosphorylation on Ser 473 after 1h treatment of THP-1 macrophages with 22(R)-HC and 9CRA.

Differentiated THP-1 macrophages were incubated for 1h with combinations of 22(R)-HC (2µg/ml) and 9CRA (10µM) or DMSO as vehicle control. Western blot analysis was carried out using equal volume of lysates (50µl). Blotted membranes were incubated with phospho-PKB (Ser473) and total-PKB primary antibodies. The image shown in panel A is representative of three independent experiments. Densitometric analysis was carried out on the data and presented as mean fold induction (±SD) in relation to basal expression at 1h (assigned as 1) (panel B; *P<0.05 compared to control).

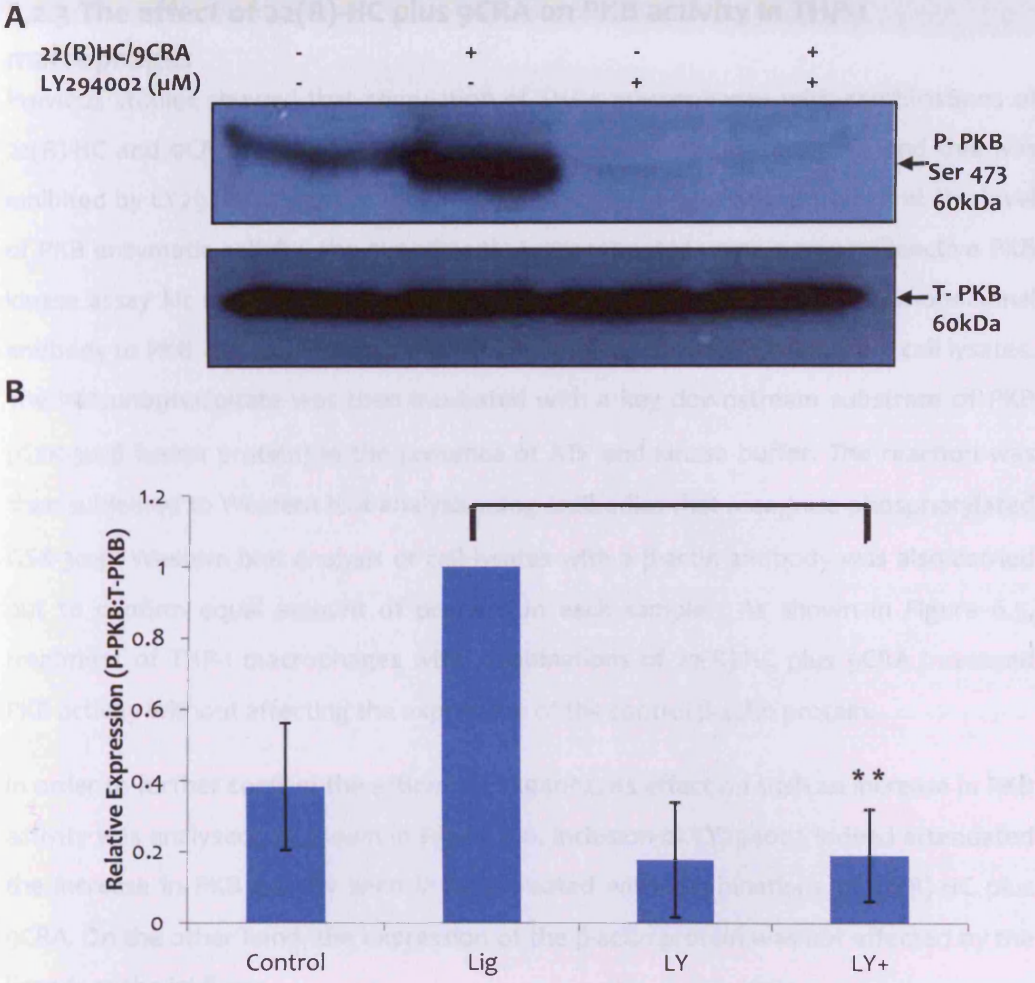


Figure 6.4 inhibition of 22(R)-HC and 9CRA-induced posphorylation of PKB at Ser 473 by LY294002 in THP-1 macrophages.

Differentiated THP-1 macrophages were treated for 1h with combinations of 22(R)-HC (2μg/ml) and 9CRA (10μM) or DMSO as a vehicle control in the absence or the presence 100μM of LY294002. The inhibitor was added 1h before the ligand (pre-treatment). Western blot analysis was carried out using equal volume of lysates (50μl). Blotted membranes were incubated with anti-phospho-PKB and anti-total-PKB primary antibodies. Antigen-antibody complexes were detected using the ECL detection system. The image shown in panel A is representative of three independent experiments. Densitometric analysis was carried out on the data and presented as relative expression (mean ±SD) normalised to the expression of T-PKB. The relative expression in the presence of the ligand alone (Lig) has been arbitrarily assigned as 1 (panel B; **P<0.01 compared to control at 1h).

6.2.3 The effect of 22(R)-HC plus 9CRA on PKB activity in THP-1 macrophages

Previous studies showed that stimulation of THP-1 macrophages with combinations of 22(R)-HC and 9CRA resulted in an increase in the levels of phospho-PKB and this was inhibited by LY294002. In order to confirm that such changes also occurred at the level of PKB enzymatic activity, the experiments were repeated using a non-radioactive PKB kinase assay kit from Cell Signalling Technology. For this, an immobilised monoclonal antibody to PKB was used to selectively immunoprecipitate the protein from cell lysates. The immunoprecipitate was then incubated with a key downstream substrate of PKB (GSK-3α/β fusion protein) in the presence of ATP and kinase buffer. The reaction was then subjected to Western blot analysis using antibodies that recognise phosphorylated GSK-3α/β. Western blot analysis of cell lysates with a β-actin antibody was also carried out to confirm equal amount of proteins in each sample. As shown in Figure 6.5, treatment of THP-1 macrophages with combinations of 22(R)-HC plus 9CRA increased PKB activity without affecting the expression of the control β-actin protein.

In order to further confirm the action of LY294002, its effect on such an increase in PKB activity was analysed. As shown in Figure 6.6, inclusion of LY294002 indeed attenuated the increase in PKB activity seen in cells treated with combinations of 22(R)-HC plus 9CRA. On the other hand, the expression of the β-actin protein was not affected by the ligands or the inhibitor.

6.2.4 Effect of DN PKB on the activation of ABCA1 promoter by LXR agonists

In order to further confirm the role of PKB in the induction of ABCA1 gene expression by combinations of 22(R)-HC plus 9CRA, the effect of a DNA construct specifying for a DN form of PKB on ABCA1 promoter activity was analysed. For this, U937 cells were co-transfected with the ABCA1 promoter plasmid and either DN PKB or the control pcDNA3 vector. The transfected cells were then treated with combinations of 22(R)-HC plus 9CRA or the DMSO vehicle control. As shown in Figure 6.7, the ligands indeed stimulated ABCA1 promoter activity when the cells were transfected with the control pcDNA3 plasmid and this was attenuated in a statistically significant manner by expression of DN PKB.

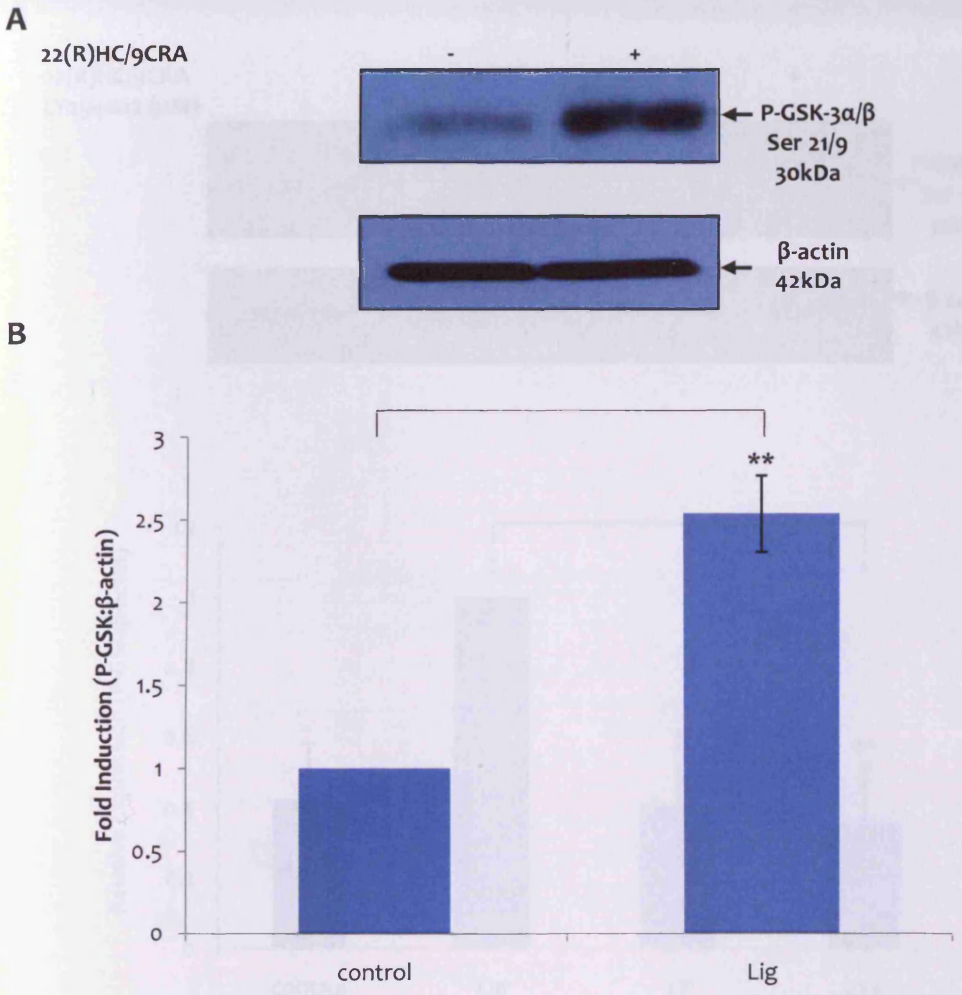


Figure 6.5 Stimulation of PKB activity by 22(R)-HC and 9CRA in THP-1 macrophages.

Differentiated THP-1 macrophages were incubated for 1h with combinations of 22(R)-HC (2μg/ml) and 9CRA (10μM) or the DMSO vehicle control. The PKB activity was analysed using a non- radioactive kit as described in Materials and Methods. Western blot analysis was carried out using anti-phospho-GSK-3α/β primary antibody. The cell extracts were also subjected to Western blotting with β-actin antibody as a loading control. The image shown in panel A is representative of three independent experiments. Densitometric analysis was carried out on the data and presented as mean fold induction (±SD) in relation to basal expression (assigned as 1) (panel B; $^{**}P<0.01$ compared to control).

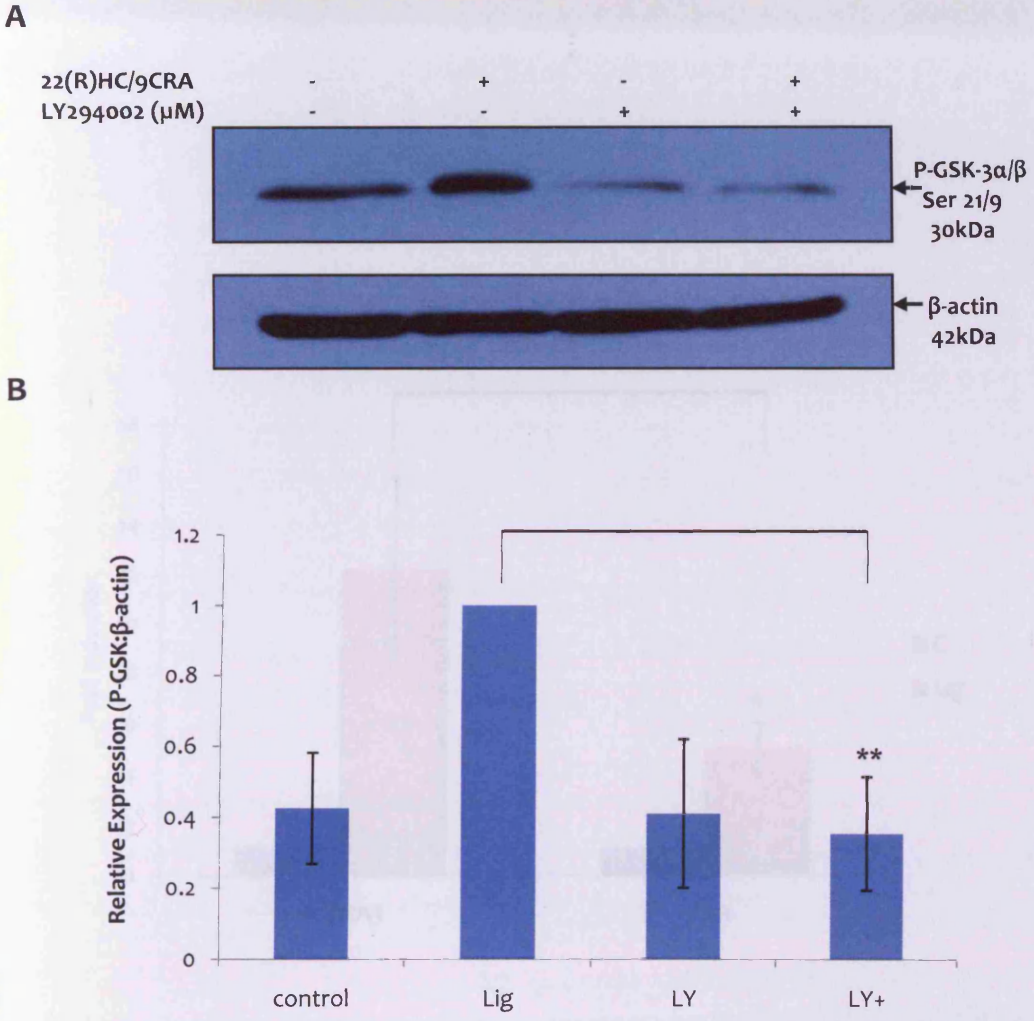


Figure 6.6 The effect of LY294002 on 22(R)-HC and 9CRA-induced PKB activity in THP-1 macrophages.

Differentiated THP-1 macrophages were treated for 1h with 22(R)-HC (2μg/ml) and 9CRA (10μM) in the absence or the presence of 100μM LY294002 (LY). The inhibitor was added 1h before the ligand (pre-treatment). The PKB activity was analysed using a non radioactive kit as described in Materials and Methods. Western blot analysis was carried out and blotted membranes were incubated with primary antibodies against anti-phospho-GSK-3α/β and β-actin. Antigen-antibody complexes were detected using the ECL detection system. The image shown in panel A is representative of three independent experiments. Panel B shows the relative expression from three experiments (mean ±SD) normalised to the expression of β-actin a determined by densitometric analysis (**$P<0.01$). The relative expression in the presence of the ligand alone (Lig) has been arbitrarily assigned as 1.

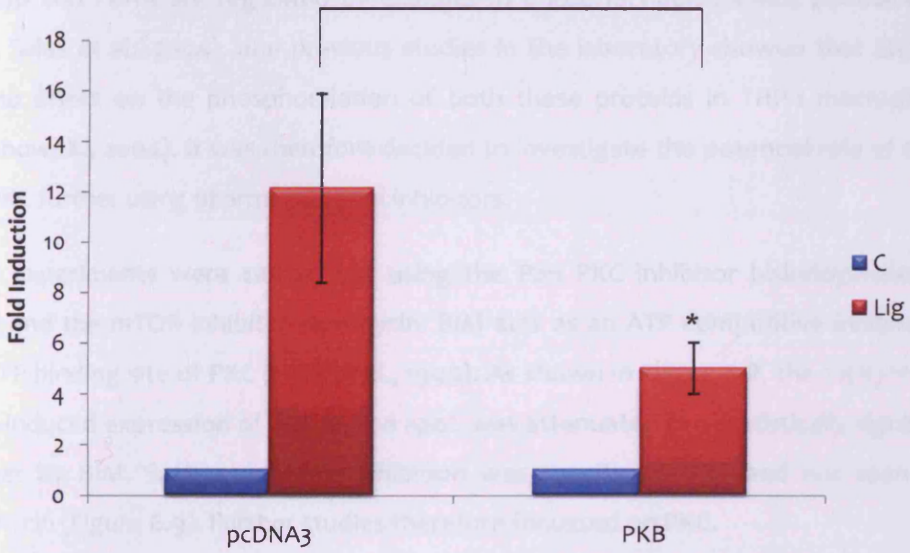


Figure 6.7 The effect of DN PKB on 22(R)-HC and 9CRA-induced ABCA1 promoter activity.

U937 cells were co-transfected with human ABCA1 promoter [from -928 to +101bp] construct and the expression plasmid for DN form of PKB. Cells transfected with the control pcDNA3 plasmid were included for comparison. The cells were then treated with DMSO as a vehicle control (C) or combinations of 22(R)-HC (2μg/ml) and 9CRA (10μM) (Lig) for 18h. The transfected cells were then harvested and luciferase reporter activity was determined. Relative counts were normalised to protein concentration and values are expressed as mean fold induction (±SD) in the presence of the ligands in relation to basal levels (assigned as 1). The results were from three independent experiments each carried out in triplicate (*$P<0.01$).

These results therefore lend further support to PKB as a key downstream component of the PI3K pathway involved in the activation of ABCA1 expression by LXR agonists.

6.2.5 Potential downstream targets for PKB actions in the induction of ABCA1 and apoE expression by LXR agonists

A number of downstream targets for PKB actions have been identified, including GSK-α/β, p70S6K, which is a target of mTOR, inhibitor kappaB (IκB), FKHR and isoforms of PKC (Bjornsti and Houghton, 2004; Cantly, 2002; Krasilnikov, 2000; Yang et al., 2004). GSK-α/β and FKHR are regulated by changes in phosphorylation status (Hirota et al., 2003; Salas et al., 2004) and previous studies in the laboratory showed that 22(R)-HC had no effect on the phosphorylation of both these proteins in THP-1 macrophages (Greenow, K., 2004). It was therefore decided to investigate the potential role of mTOR and PKC further using pharmacological inhibitors.

Initial experiments were carried out using the Pan PKC inhibitor bisindoylmaleimide (BIM) and the mTOR inhibitor rapamycin. BIM acts as an ATP competitive inhibitor for the ATP binding site of PKC (Hers et al., 1999). As shown in Figure 6.8, the 22(R)-HC and 9CRA-induced expression of ABCA1 and apoE was attenuated in a statistically significant manner by BIM. Such a dramatic inhibition was specific to BIM and not seen with rapamycin (Figure 6.9). Further studies therefore focussed on PKC.

6.2.6 Further analysis of the role of PKC in the 22(R)-HC plus 9CRA-induced expression of apoE and ABCA1 in THP-1 macrophages

Studies presented in the previous section showed that BIM inhibited the 22(R)-HC plus 9CRA-induced expression of ABCA1 and apoE in THP-1 macrophages. As mentioned above, BIM is a pan inhibitor of PKC. There is increasing evidence that individual PKC isoforms are involved in different signal transduction pathways (Valledor et al., 1999). Further experiments were therefore carried out using more selective PKC inhibitors Gö6983, Gö6976 and rottlerin.

Gö6983 affects classical, novel and atypical PKC isoenzymes in a concentration dependent manner (Gschwendt et al., 1996), with IC_{50} value less than 10nM for conventional PKC, 10nM for PKCδ and 60nM for PKCζ. Thus, novel and classical PKC isoforms will be mostly inhibited at a concentration of 10nM and atypical PKCζ isoenzymes will be affected mainly at a concentration of 60nM. Thus, the use of Gö6983

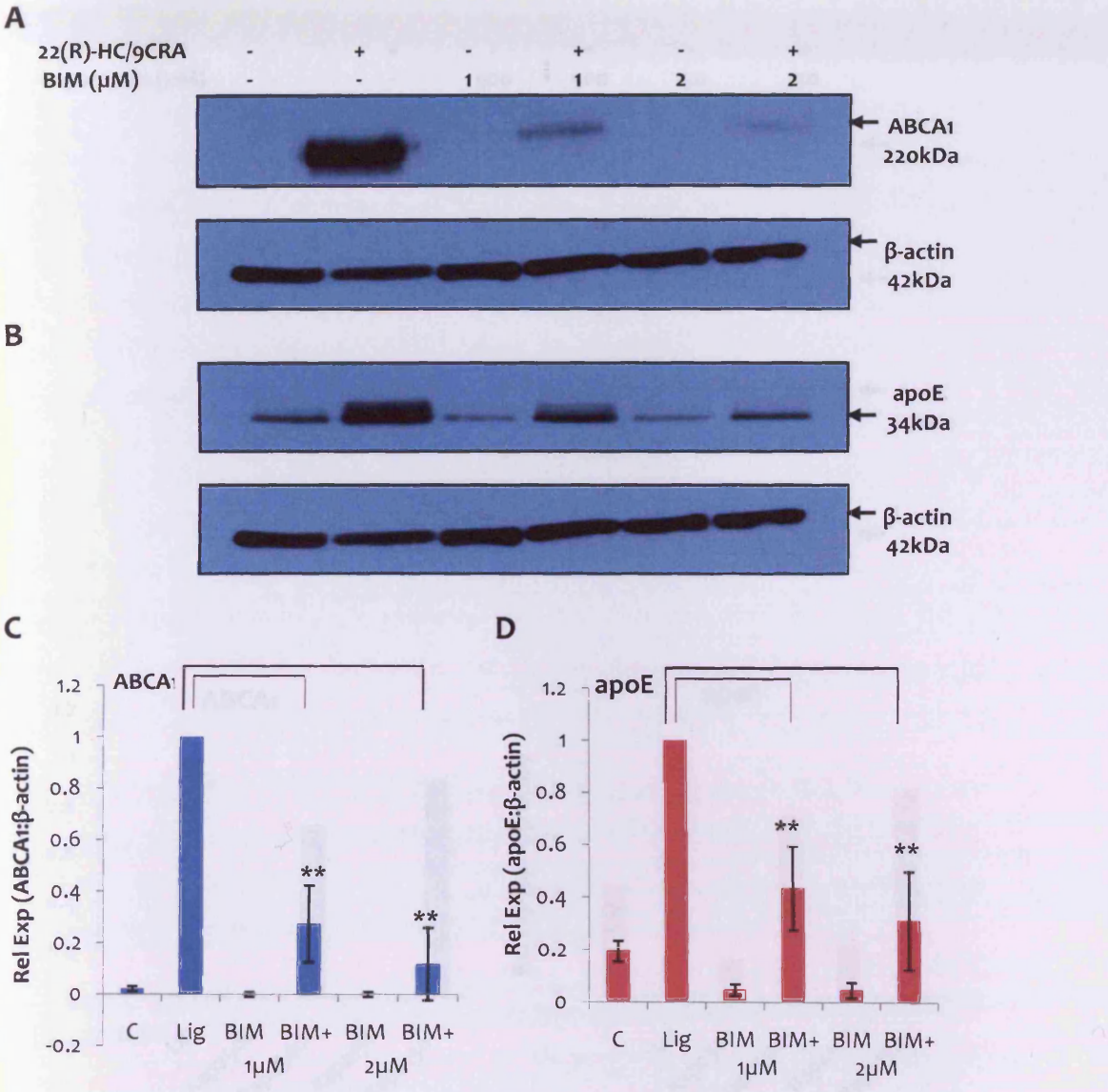


Figure 6.8 Effect of PKC Inhibitor bisindolylmaleimide on the induction of ABCA1 and apoE protein expression by 22(R)-HC and 9CRA in THP-1 macrophages.

Differentiated THP-1 macrophages were treated for 24h with 22(R)-HC (2μg/ml) and 9CRA (10μM) in the absence or the presence of BIM at the indicated concentrations. The inhibitor was added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE or the β-actin control as shown. Antigen-antibody complexes were detected using the ECL detection system. The image shown in panels A and B is representative of three independent experiments. The histogram C and D show relative expression (Rel Exp) from three experiments (mean ±SD) normalised to the expression of β-actin. The relative expression in the presence of the ligand alone (Lig) has been arbitrarily assigned as 1, as determined by densitometric analysis (**P<0.01).

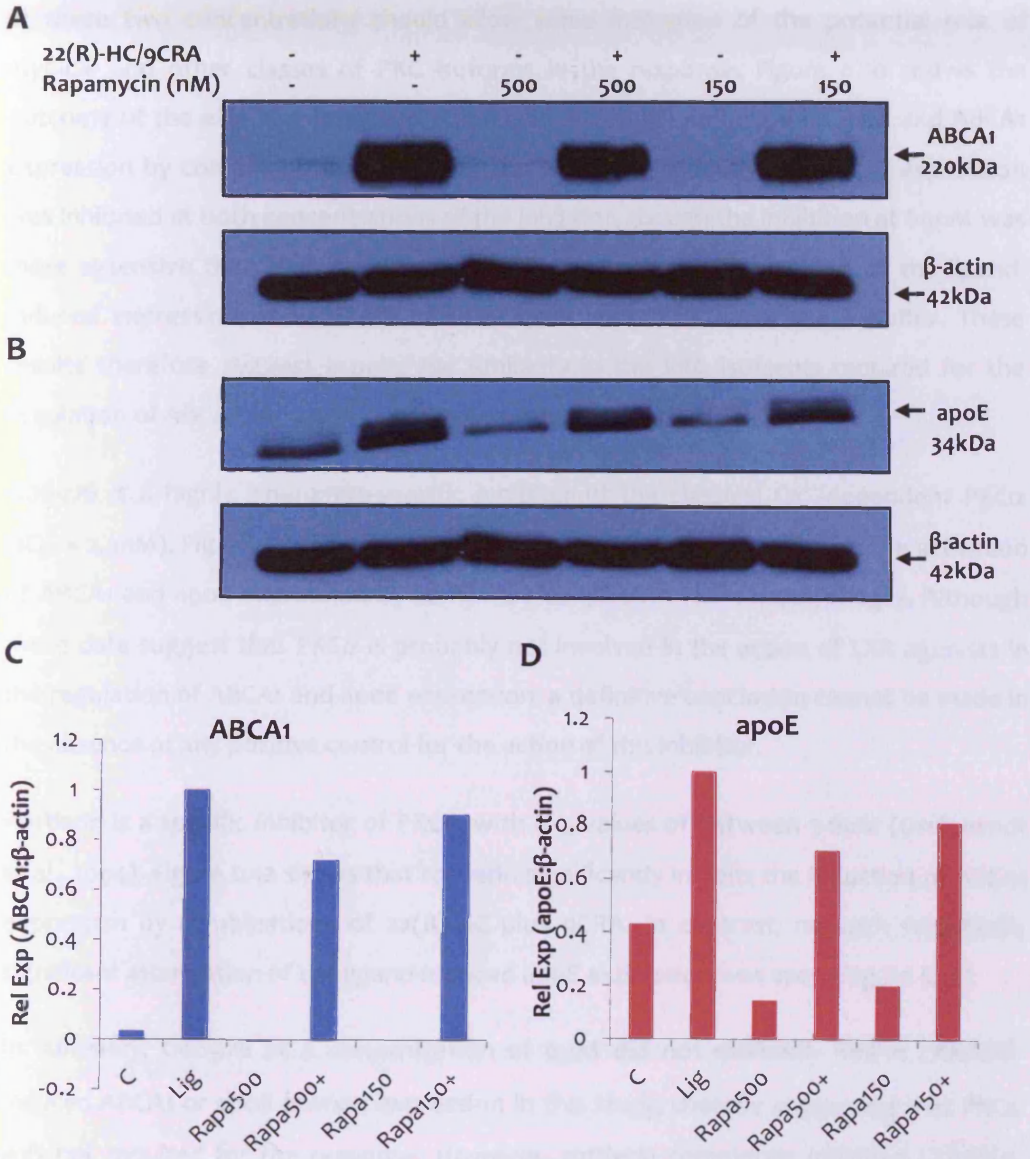


Figure 6.9 Effect of rapamycin on the induction of ABCA1 and apoE protein expression by combinations of 22(R)-HC and 9CRA in THP-1 macrophages.

THP-1 macrophages were treated for 24h with 22(R)-HC (2μg/ml) and 9CRA 10μM) in the absence or the presence of rapamycin at the indicated concentrations. The inhibitor was added 1h before the ligand (pre-treatment). Equal amount of protein (20-40μg) was subjected to SDS-PAGE and Western blot analysis using antibodies against ABCA1, apoE and the β-actin control as shown (panels A and B). The results in the histogram in panels C and D show the relative expression (Rel Exp) normalised to the expression of β-actin. The relative expression in the presence of the ligand alone (Lig) has been arbitrarily assigned as 1.

at these two concentrations should allow some indication of the potential role of atypical and other classes of PKC isoforms in the response. Figure 6.10 shows the outcome of the effect of 10nM and 60nM Gö6983 on the induction of apoE and ABCA1 expression by combinations of 22(R)-HC and 9CRA. The induction of ABCA1 expression was inhibited at both concentrations of the inhibitor, though the inhibition at 60nM was more extensive than that seen at 10nM. On the other hand, inhibition of the ligand-induced expression of apoE was seen at both concentration of the inhibitor. These results therefore suggest a potential similarity in the PKC isoforms required for the regulation of ABCA1 and apoE expression in response to the ligands.

Gö6976 is a highly isoenzyme-specific inhibitor of the classical Ca^{2+}-dependent PKCα (IC_{50} = 2.3nM). Figure 6.11 shows that Gö6976 has no significant effect on the activation of ABCA1 and apoE expression by 22(R)-HC plus 9CRA in THP-1 macrophages. Although these data suggest that PKCα is probably not involved in the action of LXR agonists in the regulation of ABCA1 and apoE expression, a definitive conclusion cannot be made in the absence of any positive control for the action of this inhibitor.

Rottlerin is a specific inhibitor of PKCδ, with IC_{50} values of between 3-6μM (Gschwendt et al., 1994). Figure 6.12 shows that rottlerin significantly inhibits the induction of ABCA1 expression by combinations of 22(R)-HC plus 9CRA. In contrast, no such statistically significant attenuation of the ligand-induced apoE expression was seen (Figure 6.12).

In summary, Gö6976 at a concentration of 6μM did not markedly inhibit LXR/RXR-induced ABCA1 or apoE protein expression in this study, thereby suggesting that PKCα was not required for the response. However, rottlerin completely inhibited LXR/RXR-induced expression of ABCA1, but not apoE, in THP-1 cells, thereby suggesting that a novel PKC isoform might be important for the induced expression of ABCA1 expression by LXR agonists.

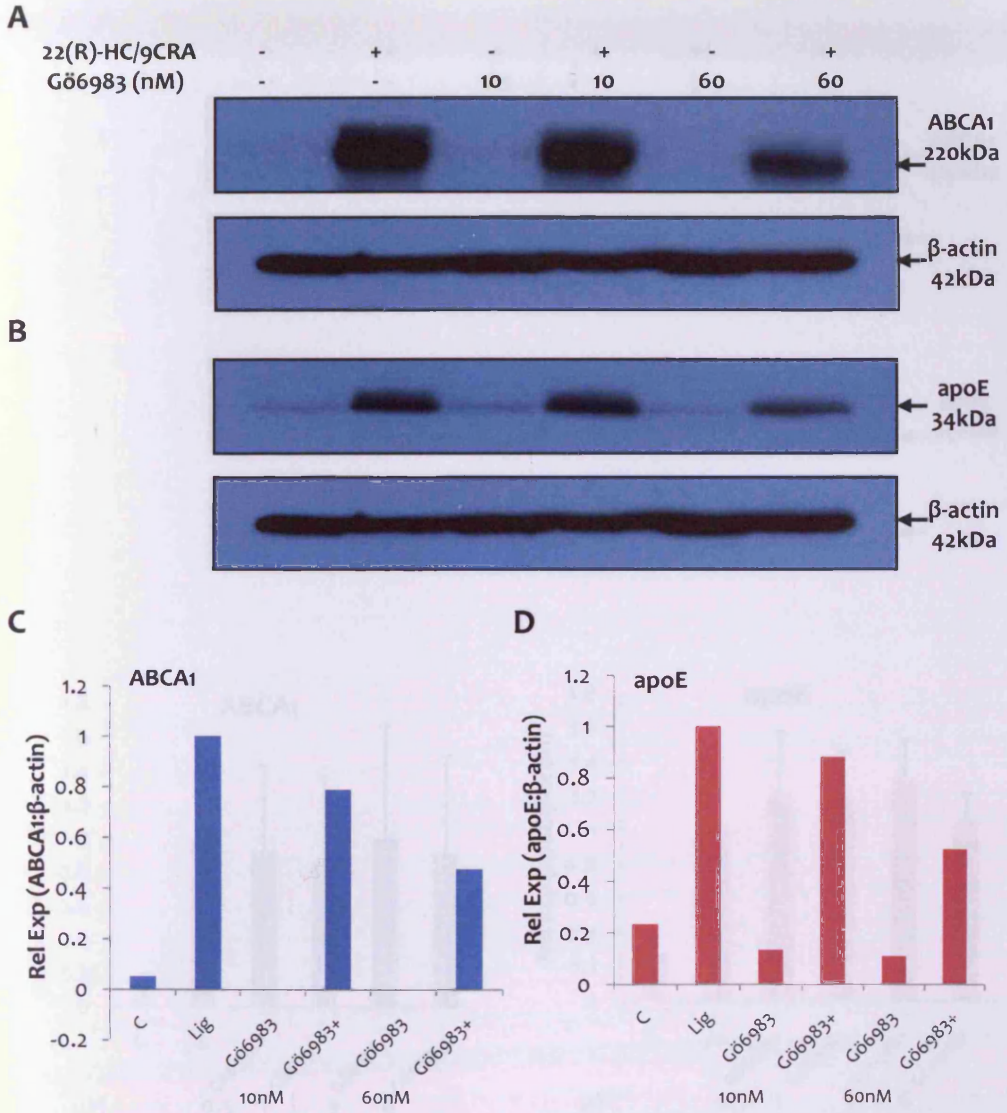


Figure 6.10 Effect of Gö6983 on the induction of ABCA1 and apoE protein expression by 22(R)-HC and 9CRA in THP-1 macrophages.

THP-1 macrophages were treated for 24h with 22(R)-HC (2μg/ml) and 9CRA (10μM) in the absence or the presence of Gö6983 at the indicated concentrations. The inhibitor was added 1h before the ligand (pre-treatment). Equal amount of protein (20-40μg) was subjected to SDS-PAGE and Western blot analysis using antibodies against ABCA1, apoE and the β-actin control as shown (panels A and B). The results in the histogram in panels C and D show relative expression (Rel Exp) from two independent experiments (average) normalised to the expression of β-actin. The relative expression in the presence of the ligand alone (Lig) has been arbitrarily assigned as 1.

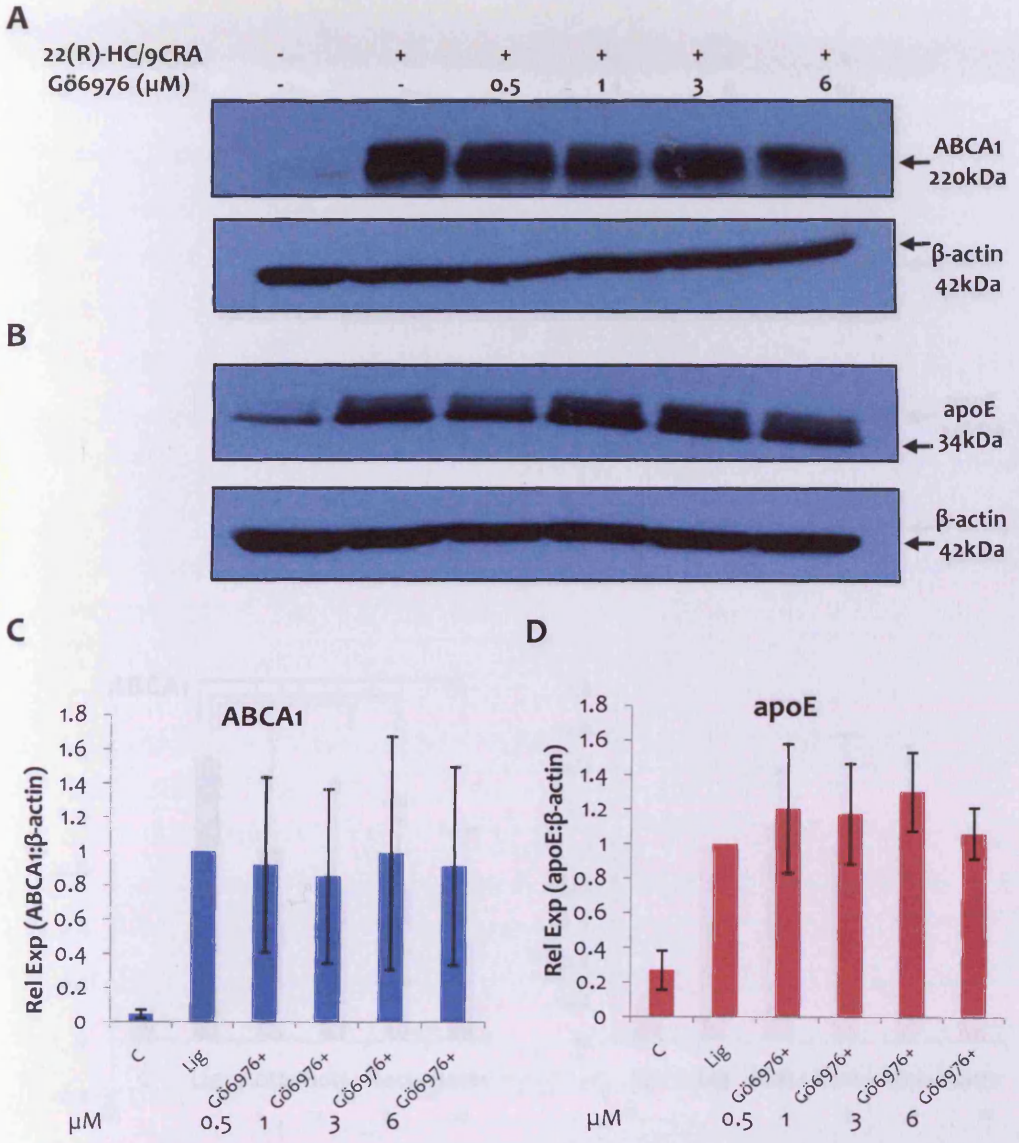


Figure 6.11 The effect of Gö6976 on the induction of ABCA1 and apoE protein expression by 22(R)-HC and 9CRA in THP-1 macrophages.

THP-1 macrophages were treated for 24h with 22(R)-HC (2μg/ml) and 9CRA (10μM) in the absence or the presence of Gö6976 at the indicated concentrations. The inhibitor was added 1h before the ligand (pre-treatment). Equal amount of protein (20-40μg) was subjected to SDS-PAGE and Western blot analysis using antibodies against ABCA1, apoE and the β-actin control as shown (panels A and B). The results in the histogram in panels C and D show relative expression (Rel Exp) (mean ±SD) normalised to the expression of β-actin. The relative expression in the presence of the ligand alone (Lig) has been arbitrarily assigned as 1 (from four independent experiments), as determined by densitometric analysis.

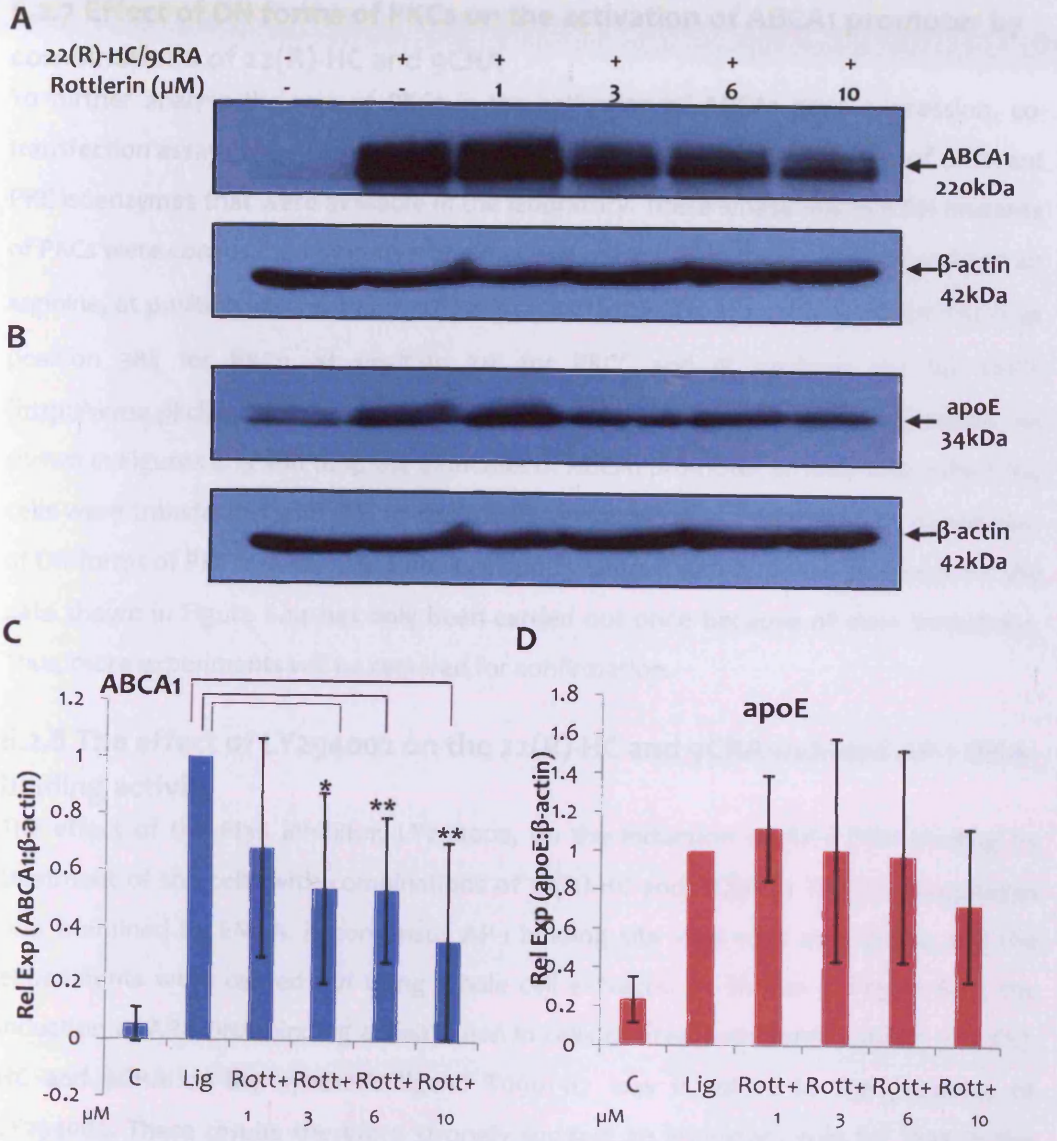


Figure 6.12 The effect of rottlerin on the induction of ABCA1 and apoE protein expression by 22(R)-HC and 9CRA in THP-1 macrophages.

Differentiated THP-1 macrophages were treated for 24h with 22(R)-HC (2μg/ml) and 9CRA (10μM) in the absence or the presence of rottlerin (Rott) at the indicated concentrations. The inhibitor was added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 20-40μg of whole cell extracts. Blotted membranes were incubated with antibodies against ABCA1, apoE or the β-actin control as shown. Antigen-antibody complexes were detected using the ECL detection system. The image shown in panels A and B is representative of five independent experiments. The histogram in panels C and D show the relative expression (Rel Exp) (mean ±SD) normalised to the expression of β-actin. The relative expression in the presence of the ligand alone (Lig) has been arbitrarily assigned as 1, as determined by densitometric analysis (*$P<0.05$, **$P<0.01$).

6.2.7 Effect of DN forms of PKCs on the activation of ABCA1 promoter by combinations of 22(R)-HC and 9CRA

To further analyse the role of PKCs in the activation of ABCA1 gene expression, co-transfection assays were carried out using plasmids specifying for DN forms of different PKC isoenzymes that were available in the laboratory. These kinase inactive DN mutants of PKCs were constructed by converting a critical lysine within the catalytic domain to an arginine, at position 368 for PKCα, at position 437 for PKCε, at position 376 for PKCδ, at position 384 for PKCη, at position 218 for PKCζ and at position 257 for PKCλ (http://www.pkclab.org/index.htm) (Soh et al., 1999; Soh and Weinstein, 2003). As shown in Figures 6.13 and 6.14, the induction of ABCA1 promoter activity seen when the cells were transfected with the control pcDNA3 plasmid was attenuated by expression of DN forms of PKC-α, δ and ε but not λ, η and ζ. Unfortunately, the experiment for the data shown in Figure 6.14 has only been carried out once because of time limitations. Thus, more experiments will be required for confirmation.

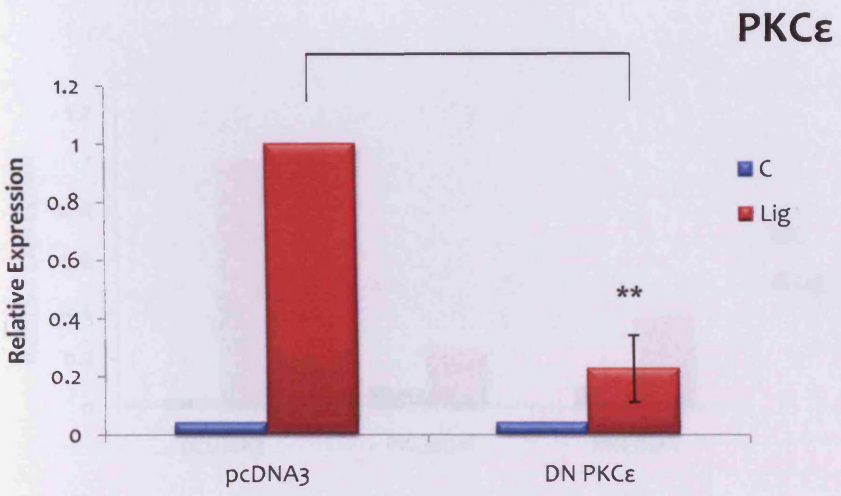
6.2.8 The effect of LY294002 on the 22(R)-HC and 9CRA-induced AP-1 DNA-binding activity

The effect of the PI3K inhibitor, LY294002, on the induction of AP-1 DNA binding by treatment of the cells with combinations of 22(R)-HC and 9CRA in THP-1 macrophages was examined by EMSA. A consensus AP-1 binding site was used as a probe and the experiments were carried out using whole cell extracts. As shown in Figure 6.15, the induction of AP-1 DNA binding activity seen in cells treated with combinations of 22(R)-HC and 9CRA or the synthetic ligand T0901317 was inhibited in the presence of LY294002. These results therefore strongly suggest an important role for PI3K in the induction of AP-1 DNA binding activity by the ligands.

6.2.9 The effect of PKC inhibitors on AP-1 DNA-binding activity

To explore the action of the PKC inhibitors on the induction of AP-1 DNA binding activity by combinations of 22(R)-HC and 9CRA, EMSA was carried out using a consensus AP-1 binding site probe. As shown in Figure 6.16, the AP-1 DNA binding activity seen when cells were treated with combinations of 22(R)-HC and 9CRA was inhibited in the presence of BIM, Gö6976 or rottlerin. These results therefore further substantiate a potentially important role for PKC in the activation of AP-1 binding by the ligands.

A



B

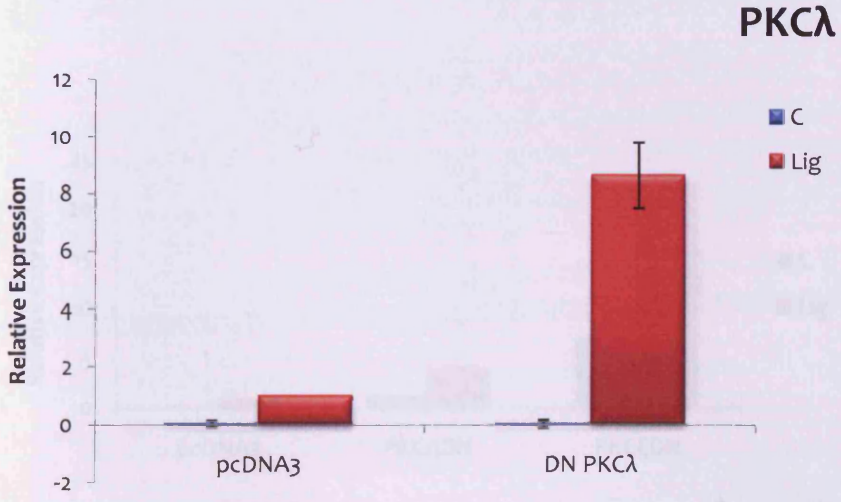


Figure 6.13 Effect of DN constructs of PKC-ε and -λ on the 22(R)-HC and 9CRA induced ABCA1 promoter activity.

U937 cells were transfected with the human ABCA1 promoter construct and DN constructs for PKC-ε (A) and -λ (B). Transfected cells were treated with combinations of 22(R)-HC (2μg/ml) and 9CRA (10μM) (Lig) or DMSO as a vehicle control (C) for 18h. The cells were then harvested and luciferase activity and protein assays carried out as described in Materials and Methods. Relative counts were normalised to protein concentration and values are expressed as relative expression (mean ±SD). The relative expression in cells transfected with the pcDNA3 vector and treated with the ligands has been arbitrarily assigned as 1. The results show the outcome of three independent experiments carried out in triplicate. The data was analysed by student's t-test, (**P<0.01).

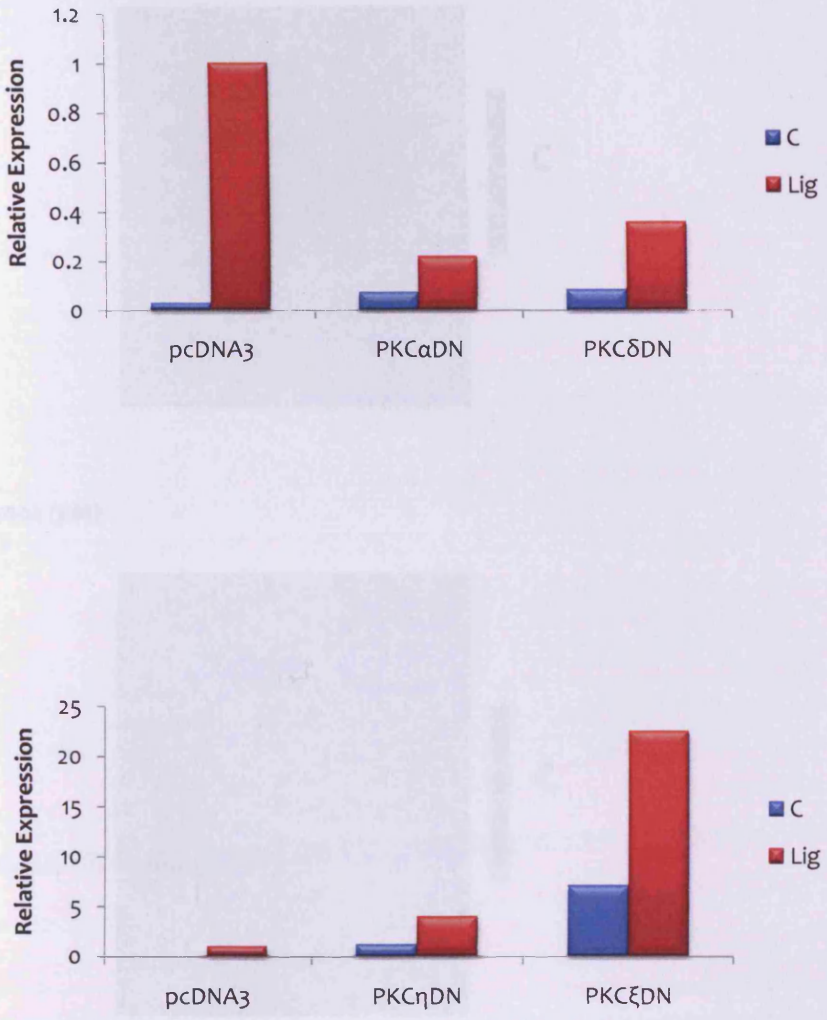


Figure 6.14 The effect of DN constructs for PKC isoforms on the 22(R)-HC and 9CRA-mediated induction of ABCA1 promoter activity.

U937 cells were co-transfected with the human ABCA1 promoter construct and the expression plasmids for DN PKC-α, -δ, -η or -ξ. Cells transfected with the control pcDNA3 plasmid were included for comparison. The cells were then treated with either DMSO as a vehicle control or combinations of 22(R)-HC (2μg/ml) and 9CRA (10μM) for 18h. The transfected cells were then harvested and the luciferase reporter activity was determined. Relative counts were normalised to protein concentration and values are expressed as relative expression. The relative expression in cells transfected with the pcDNA3 plasmid and treated with the ligands has been arbitrarily assigned as 1.

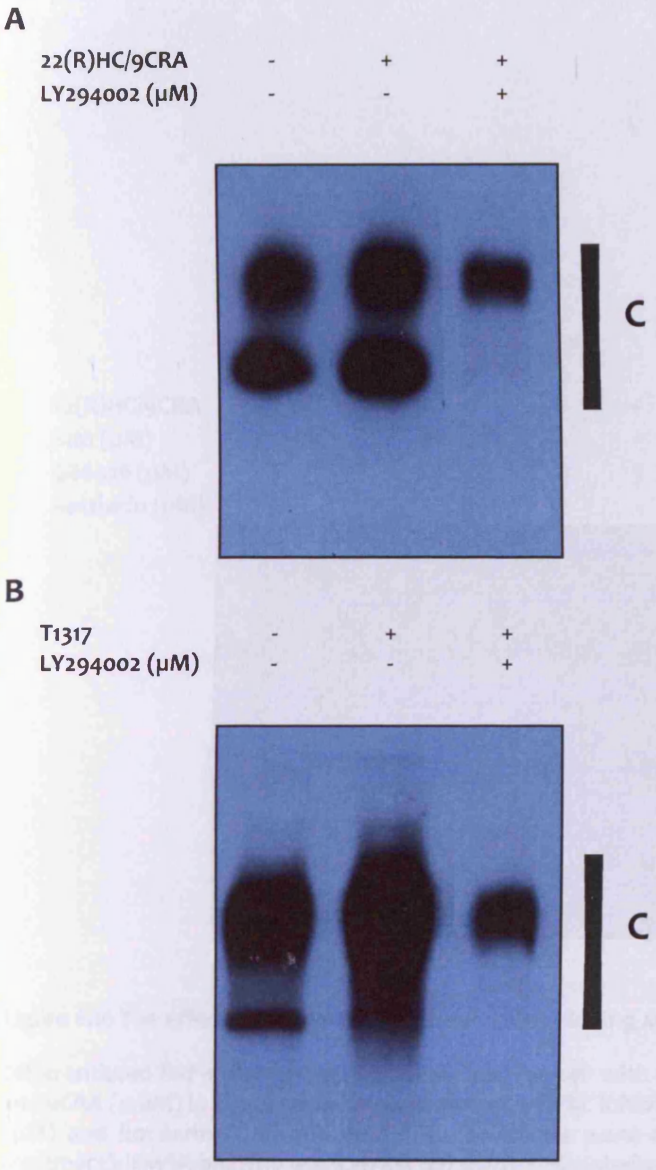


Figure 6.15 The effect of LY294002 on the LXR agonist-induced binding of AP-1.

Differentiated THP-1 macrophages were treated for 24h with 22(R)-HC (2μg/ml) and 9CRA (10μM) or T0901317 (10μM) or DMSO as a vehicle control in the absence or the presence of 100μM LY 294002. The inhibitor was added 1h before the ligand (pre-treatment). EMSA analysis was carried out using radiolabelled AP-1 consensus sequence probe. DNA protein complexes are shown by a vertical line labelled C. The free probe has migrated off the gel.

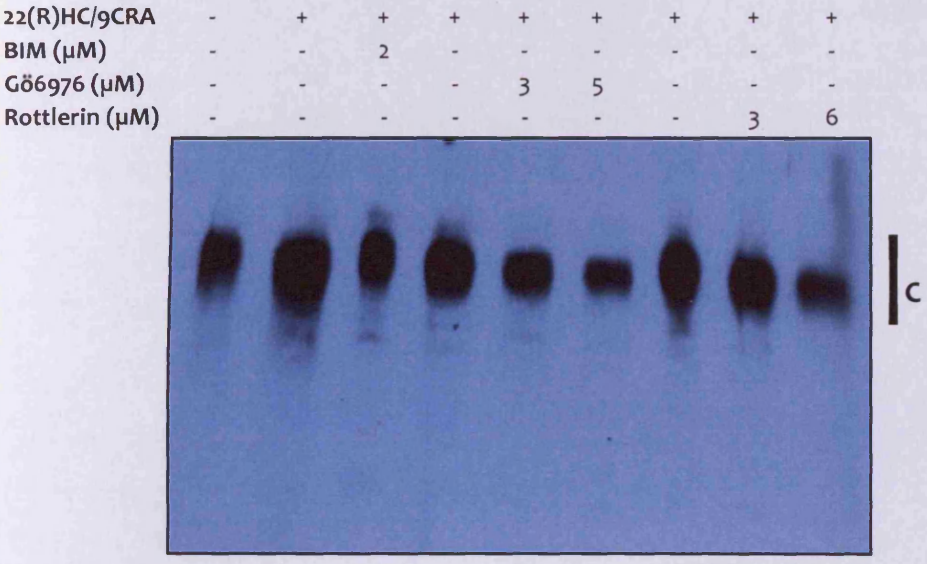


Figure 6.16 The effect of PKC inhibitors on AP-1 DNA-binding activity.

Differentiated THP-1 macrophages were treated for 24h with combinations of 22(R)-HC (2μg/ml) and 9CRA (10μM) in the absence or the presence of PKC inhibitors BIM (2μM), Gö6976 (3μM and 5μM) and Rottlerin (3μM and 6μM). The inhibitors were added 1h before the ligand (pre-treatment). EMSA analysis was carried out using radiolabelled AP-1 consensus sequence probe. DNA-protein complexes are shown by a vertical line labelled C. The free probe has migrated off the gel. The image shown is representative of two independent experiments.

6.3 Discussion

Studies presented in chapter 3 showed a potential role for the PI3K pathway in the regulation of gene expression by LXR/RXR agonists, as identified by the use of the inhibitor LY294002 in murine J774.2 macrophages and by co-transfection of a DN form of PKB and p110 with the LXR promoter in Hep3B. Studies presented in chapter 4 further showed a role of PI3K in the regulation of ABCA1 and apoE protein expression by LXR agonists in THP-1 macrophages through the use of LY294002. Together these results implicate an important role for the PI3K pathway in the LXR-mediated regulation of gene expression in murine and human macrophages. The aim of the studies presented in this chapter was to confirm the activation of this pathway in THP-1 cells in response to treatment with 22(R)-HC/9CRA and analyse its potential role in the responses mediated by these ligands. Several lines of evidence lend further support to PI3K/PKB playing an important role in the induction of ABCA1 and apoE expression by LXR agonists in THP-1 macrophages. For example, the phosphorylation of PKB at Ser 473 and its kinase activity were induced in THP-1 macrophages following treatment of the cells with 22(R)-HC plus 9CRA (Figures 6.2-6.5). Such an activation of PKB was inhibited by pre-treatment of the cells with LY294002 (Figures 6.4-6.6). In addition, the induction of ABCA1 promoter by 22(R)-HC/9CRA was inhibited in U937 cells transfected with a DN construct specifying for a mutant form of PKB compared to transfection with the empty vector, pcDNA3 (Figure 6.7).

Having concluded that treatment of THP-1 macrophages with LXR agonists results in an increase in PI3K phosphorylation and kinase activity, experiments were carried out to investigate whether any downstream targets of this pathway were involved in LXR-mediated regulation of ABCA1 and apoE expression. Several downstream targets of PKB, including GSK-3, mTOR, eNOS and FKHR, have been shown to be involved in NR signalling (Campbell et al., 2001; Gianni et al., 2002b; Lin et al., 2001; Rogatsky et al., 1998a). For example, recent work has shown that T0901317 and insulin down-regulate the transcription of the APOA5 gene in human and primary rat hepatocytes through the PI3K/ P70S6 kinase signalling pathways (Jakel et al., 2004; Nowak et al., 2005). However, further studies on the downstream targets for PKB actions showed that inhibition of neither GSK-3 nor mTOR affected the induction of ABCA1 and apoE expression by combinations of 22(R)-HC and 9CRA (Figure 6.9). Indeed, previous studies in the laboratory had shown that 22(R)-HC does not affect the phosphorylation-mediated

activation/deactivation of GSK-3 and FKHR (Greenow, K., 2004). In contrast to these findings, the use of both inhibitors and DN constructs showed a key role for PKC in the response (Figures 6.8, 6.10 - 6.14).

Several lines of evidence point to a direct role of the PKC signalling pathway in the regulation of transcriptional activity of some members of the NR family. For example, receptors for TH, vitamin D and RARα appear to be targets for PKCs (Delmotte et al., 1999; Goldberg et al., 1988; Hsieh et al., 1991; Tahayato et al., 1993). It has been shown that PKC phosphorylates the DNA binding domain of several NRs (e.g. RARα, RXRα, VDR and THRβ) and that phosphomimetic mutants of the Ser/Thr phosphorylation sites results in the cytoplasmic localization of RXRα and PPARα (Hsieh et al., 1991; Hsieh et al., 1993; Pailler-Rodde et al., 1999; Sun et al., 2007). In addition, it has been demonstrated that vitamin D3, estrogens and retinoids can modulate the expression of PKC isoforms in certain cells (Berry et al., 1996; Lissoos et al., 1993; Marino et al., 2002; Simboli-Campbell et al., 1994). Research by Delmotte et al. (1999) characterized PKC isoforms for their ability to phosphorylate hRARα and identified that PKCα can directly regulate its transcriptional activity by altering its ability to dimerize with RXRs (Delmotte et al., 1999). Recently, it has been shown that PKCs also control the transcriptional activity of PPARα in rat and human hepatocytes (Blanquart et al., 2004; Yaacob et al., 2001). However, so far, direct regulation of LXRs by the PKC pathway has not been reported. In our present study, we demonstrate for the first time, by using PKC inhibitors and by transfecting DN PKC plasmids, that they are required for the induction of LXR target genes by combinations of 22(R)-HC and 9CRA.

The regulation of AP-1 activity is complex and occurs at several levels, including c-Jun and c-Fos gene transcription along with its mRNA and protein turnover. (De Bosscher et al., 2003; Hazzalin and Mahadevan, 2002; Karin, 1995; Karin et al., 1997; Thomson et al., 1999; Wisdom, 1999). Reddy et al. (1997) have presented evidence that IL-1 not only causes a rapid and dramatic increase in PI3K activity but also induces the physical interaction of its receptor with the regulatory subunit of PI3K, and that overexpression of PI3K may be sufficient to induce AP-1 activity and increase c-Fos protein levels. In addition, Bian et al. (2004) recently linked the activation of AP-1 (c-Fos) to MCP-1 gene expression in human retinal pigment epithelial cells through the PI3K/PKB pathway, which was independent of ERK, p38 and JNK/SAPK pathways. Indeed, several previous studies have associated

PKCα and PKCε to the activation of AP-1 in a variety of cells (Hirai et al., 1994; Soh et al., 1999; Soh and Weinstein, 2003; Ueda et al., 1996; Vuong et al., 2000). For example, Genot et al. (1995) showed that PKCε and, to a lesser extent PKCα, but not PKCζ, can regulate AP-1 transcriptional activity. In addition, Akimoto et al. (1996) have implicated PI3K in the epidermal growth factor-induced AP-1 activation and reported that PKCλ was involved in such activation. In addition, another study demonstrated that transcriptional activation of both c-Fos and c-Jun by PKCα/δ enhances AP-1 activity (Soh and Weinstein, 2003). Moreover, a recent study by Song et al. (2005) indicated that bile acids induce mucin expression by activation of AP-1 via PKC (Song et al., 2005). In the light of these previous studies, the effect of LY294002 and PKC inhibitors on the LXR agonist-induced binding of AP-1 was investigated. EMSA revealed that the LXR ligands induced binding of AP-1 to its recognition sequence, and this was almost completely inhibited by LY294002 (Figure 6.15) and by inhibitors of PKC-α and -δ (Figure 6.16). It is worth noting that AP-1 has already been shown to be essential for the induction of ABCA1 expression by LXR agonists through the AP-1 binding site in its promoter region (See chapter 7).

The observed rapid effects of LXR ligands, 22(R)-HC/9CRA, to induce PKB activity after only 1h suggests the existence of an alternative mechanism in the regulation of ABCA1 and apoE expression by LXRs, in which LXR activators elicit a non-genomic effect. This time period (1h) is far too rapid to account for the activation of RNA and protein synthesis, which starts at 3h and peaks at 24h (Figure 4.6). Non-genomic actions are a common property of steroid hormones and other NRs and are frequently associated with the activation of various protein-kinase cascades (Losel and Wehling, 2003). Indeed, the non-genomic activation of the PI3K pathway has been observed for steroid hormone receptors (Björnström and Sjöberg, 2004; Hafezi-Moghadam et al., 2002; Simoncini et al., 2000). For example, it has been reported that direct interaction between ER-α and the p85 regulatory subunit of PI3K results in the phosphorylation of PKB at Ser 473 and the subsequent regulation of eNOS expression in endothelial cells (Gronemeyer et al., 2004; Pozo-Guisado et al., 2004; Simoncini et al., 2000). This concept of non-genomic effect has been further substantiated by studies on the actions of androgens (Baron et al., 2004; Heinlein and Chang, 2002). The androgen receptor interacts directly with the p85 regulatory subunit of PI3K and promotes the accumulation of PI3K generated lipid products, which increase PKB activity. Furthermore, the effect of the GR ligand

dexamethasone on the activation of eNOS expression in human endothelial cells is very rapid, which indicates a potential non-genomic nature of regulation (Hafezi-Moghadam et al., 2002). This effect was found to be mediated by GR-induced activation of PI3K which, through an increase in PtdIns(3,4,5)P_3 levels, activated downstream pathways involving PKB (Stellato, 2004). Recently, Moeller et al. (2006) have reported a new model for the action of TH that is very rapid and independent of protein synthesis, which is typical of non-genomic actions, and involves the PI3K pathway. In this new mechanism of TH action, the ligand bound-TR-β interacts directly with the regulatory subunit of PI3K (p85) in the cytosol (Cao et al., 2005). This leads to the activation of PI3K, and sequential phosphorylation and activation of PKB, leading to rapid activation of mTOR and its substrate p70S6K, with detectable phosphorylation within 10min after ligand treatment (Moeller et al., 2006). Two aspects distinguish this mechanism of TH action from most other non-genomic effects of the hormone. Firstly, it requires ligand binding to TH receptors. Secondly, its ultimate effect is genomic with the expression of specific genes being induced by this mechanism (Moeller et al., 2006). This novel model of regulating transcription via non-genomic-to-genomic signalling was also found to be applicable in a model for the action of ERs, whereby signal transduction pathways connect the non-genomic actions of estrogens to genomic responses (Björnström and Sjöberg, 2004, , 2005). Such a mechanism could potentially be applied to the LXR regulation of ABCA1 and apoE expression through activation of the PI3K pathway.

It is at present unclear how ligands could potentially activate downstream signalling pathways. Previous studies in vascular SMCs have indicated that the non-genomic activation of the PI3K and ERK pathways by PPARγ ligands is mediated via novel membrane receptors (Patel et al., 2005; Takeda et al., 2001). Therefore, it may be possible that a membrane bound LXR exists, however further investigation of this aspect is required. On the basis of published literature and the results presented, a hypothetical scheme can be proposed to explain, at least partially, the mechanisms by which LXR agonists (i.e. 22(R)-HC/9CRA) act through membrane bound LXR, which have not yet been identified, to induce ABCA1 and apoE expression in THP-1 cells by stimulating LXR activity via the PI3K pathway (Figure 6.17).

consensus sites for proline-dependent kinases, which include MAPKs such as JNK/SAPK and ERK1/2 (Chang and Karin, 2001; Morgan, 1997). Hence, such kinases, together with kinases that are activated by other signals (e.g. PKB and PKC) could cooperate with the NR ligands to enhance transcriptional activation (Germain et al., 2006). Li et al. 2000b have demonstrated a molecular link between the activation of PKC-ε and the transcription factors NF-κB and AP-1 in cardiac myocytes. Furthermore, it was demonstrated that both ERK1/2 and JNK/SAPK signalling pathways were essential mediators in the activation of these two factors (Li et al., 2000b). In the light of two signalling pathways, JNK/SAPK and PI3K, being involved in the upregulation of ABCA1 expression by LXR agonists, further studies are therefore required to clarify whether there is any cross-talk between these pathways. This aspect forms the focus of studies presented in the next chapter.

In conclusion, the work presented in this chapter has demonstrated that the PI3K pathway is activated in response to treatment of THP-1 macrophages with LXR agonists. In addition, our results also show that this pathway is activated rapidly, suggesting once again that combinations of 22(R)-HC and 9CRA may have a potential non-genomic action. These results are novel and may also be extended to other LXR target genes. Ultimately, further knowledge about the molecular mechanisms by which LXR regulates transcription of target genes should provide new potential targets for therapeutic intervention of atherosclerosis.

CHAPTER SEVEN:

THE INTERPLAY BETWEEN THE JNK/SAPK AND THE PI3K SIGNALLING PATHWAYS IN THE REGULATION OF ABCA1 GENE EXPRESSION BY LXR AGONISTS

Chapter 7: The interplay between the JNK/SAPK and the PI3K signalling pathways in the regulation of ABCA1 gene expression by LXR agonists

7.1 Introduction

The studies presented in chapters 5 and 6 demonstrated an important role for the JNK/SAPK and PI3K signalling pathways in the 22(R)-HC and 9CRA-induced expression of ABCA1 and apoE in THP-1 macrophages. Increasing number of recent studies suggest that activation of target gene expression by NRs is more complicated than initially thought, and may involve the regulation of receptor function through a potential cross-talk with other transcription factors and intracellular signalling pathways. There are several studies that indicate that NRs are also substrates for a multitude of kinases activated by a variety of signals, some of which are independent of the ligands, and such phosphorylation is important for ligand-dependent and –independent *trans*-activation (Cenni and Picard, 1999; Mani, 2001; Weigel and Zhang, 1998). Individual phosphorylation changes can act to either enhance or inhibit the *trans*-activation potential of the receptor (Table 7.1) (Rochette-Egly, 2003; Shao and Lazar, 1999). For example, ERs are phosphorylated at specific serine or threonine residues by MAPK in cells treated with epidermal growth factor (EGF) and insulin-like growth factor (IGF) *in vivo* and these phosphorylations enhance the transcriptional activity of ER (Kato et al., 1995). A specific tyrosine phosphorylation site located at the C-terminal region of the ER, which is a target for MAPK signalling pathway, is involved in ligand-independent transcriptional activity (Bunone et al., 1996; White et al., 1997). Furthermore, the action of a strong AF-1 domain in PPARα is modulated by phosphorylation by MAPK and this phosphorylation enhances its transcriptional activity (Juge-Aubry et al., 1999). However, phosphorylation of the A/B domain of PPARγ by the same kinase negatively regulates its transcriptional function. Interestingly, this modification reduces the binding of the ligand to the receptor, thereby showing that binding can be regulated by intermolecular communication between the modulatory domain and the C-terminal LBD (Shao et al., 1998). MAPK-dependent phosphorylation of RXR can also alter the biological actions of a partner receptor engaged in heterodimerisation (Solomon et al., 1999).

Table 7.1: Phosphorylation of NRs

NR	Kinase	Effect of phosphorylation	Reference
AR	PKA	Promotes ligand-dependent and – independent transcriptional activation	Nazareth and Weigel, 1996
ER-α	PKA	Inhibits dimerization and DNA binding	Chen et al., 1999
ER-α	MAPK	Promotes ligand-dependent and – independent *trans*-activation	Kato et al., 1998
ERβ	MAPK	Promotes ligand-dependent and – independent *trans*-activation	Tremblay et al., 1999
GR	MAPK	Inhibits ligand-dependent *trans*-activation	Krstic et al., 1997
GR	GSK-3	Inhibits ligand-dependent *trans*-activation	Rogatsky et al., 1998b
PR	CK2	Regulates hormone-dependent *trans*-activation	Zhang et al., 1994
TR-α	CK2	Inhibits monomer DNA binding	Katz et al., 1995
TR-α	PKA	Inhibits monomer DNA binding	Tzagarakis-Foster and Privalsky, 1998
TR-β	PKA	Promotes RXR heterodimerization	Bhat et al., 1994
RAR	PKA	RA-dependent *trans*-activation	Taneja et al., 1997
RXR	MAPK	Inhibits RXR and VDR ligand-dependent *trans*-activation	Dowhan and Muscat, 1996
RXR	PKA	RA-dependent *trans*-activation in muscle cells	Solomon et al., 1999
PPAR-γ 2	MAPK	Decreases ligand-independent *trans*-activation	Adams et al., 1997
PPAR-γ 2	MAPK	Decreases ligand-binding affinity	Shao et al., 1998
PPAR-γ 1	JNK/SAPK	Decreases ligand-dependent *trans*-activation	Camp et al., 1999
HNF4	PKA	Promotes DNA binding	Jiang et al., 1997

Abbreviations: AR, androgen receptor; CK2, casein kinase2; ER-α/β, estrogen receptor-α/β; GR, glucocorticoid receptor; GSK-3, glycogen synthase kinase-3; HNF4, hepatocytes nuclear factor-4; JNK/SAPK, c-Jun N-terminal kinase/ Stress activated protein kinase; MAPK, mitogen activated protein kinase; PKA, protein kinase A; PPAR-γ, peroxisome proliferator-activated receptor-γ; PR, progesterone receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; TR-α/β, thyroid hormone receptor-α/β.

Although cross-talk between NRs and signal transduction pathways primarily involves the phosphorylation of NRs, cell signalling pathways can also regulate the action of such receptors through direct modification of the coregulator proteins, including coactivators and corepressors (Table 7.2) (Lonard and O'Malley, 2007; Rochette-Egly, 2003). For most NRs (e.g. ERα/β, PPARα and AR), the phosphorylation of the N-terminal A/B region by MAPKs or PKB helps the recruitment of specific coactivators (Barger et al., 2001; Driggers et al., 2001; Lin et al., 2001; Mckenna and O'Malley, 2002b; Watanabe et al.,

2001; Yeh et al., 1999). Indeed, coactivators, such as SRC-1, PGC-1 and P300/CBP, are themselves targets for a variety of kinases, including PKA and MAPKs, which enhance their ligand-dependent binding to NRs, thereby facilitating the recruitment of chromatin remodelers and modifiers such as SWI/SNF and TRAP/DRIP, which decompact the repressive chromatin (Huang et al., 2003; Rochette-Egly, 2003).

Table 7.2: Post-translational modifications of selected coregulators

Coregulator	Posttranslational modification	Regulatory effect	Reference
PGC-1α	Phosphorylation	Increased transcriptional activity	Puigserver et al., 2001
SRC-1	Phosphorylation	Increased transcriptional activity	Rowan et al., 2000
SRC-2	Phosphorylation	Increased transcriptional activity	Lopez et al., 2001
SRC-3	Phosphorylation	Increased transcriptional activity	Wu et al., 2002; Wu et al., 2004b
N-CoR	Phosphorylation	Nuclear export	Hermanson et al., 2002
SMRT	Phosphorylation	Nuclear export	Jonas and Privalsky, 2004
CBP-1	Phosphorylation	Loss of repressor activity	Barnes et al., 2003
PCBP-1	Phosphorylation	Conversion to a transcriptional coactivator	Meng et al., 2007

Abbreviations: CBP, CREB binding protein; N-CoR, nuclear receptor corepressor; PCBP-1, poly(rC) binding protein-1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1α; SMRT, Silencing mediator for retinoid and thyroid hormone receptors; SRC, steroid receptor coactivator.

These interactions between chromatin remodelling complexes and coactivators increase the efficiency of recruitment of components of the RNA polymerase II transcriptional machinery and modulate positively the expression of target genes in response to a particular ligand (Figure 7.1) (Rochette-Egly, 2003). In contrast, phosphorylation of corepressors, such as NCoR and SMRT subsequent to the activation of MAPKs, has been shown to induce their redistribution from the nucleus to the cytoplasm and this correlates with an inhibition of their interaction with NRs (Germain et al., 2006; Hong and Privalsky, 2000).

It is also possible that phosphorylation can contribute to the attenuation and/or termination of the ligand response. However, the mechanisms by which post-translational modification facilities such an inhibition of NR action remains unknown. Phosphorylation may potentially inhibit receptor signalling at several levels, including

exclusion of NRs from the transcription complex, reduction in the affinity of ligand binding or induction of NR degradation by the ubiquitin-proteasome pathway (Aranda and Pascual, 2001; Bastien and Rochette-Egly, 2004; Gronemeyer et al., 2004). Purpose of such negative effects would be to ensure the activation of the right gene by the right activator at the right time for a defined period (Rochette-Egly, 2003).

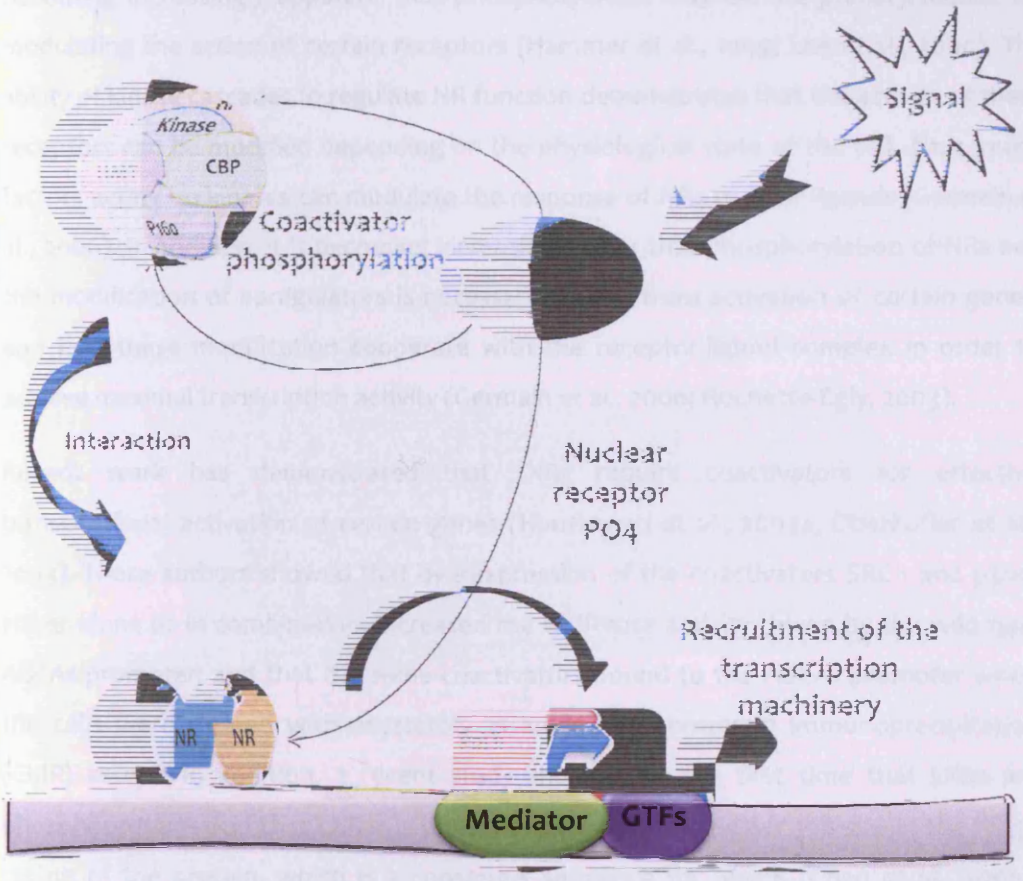


Figure 7.1 Positive regulation of nuclear receptor *trans*-activation through phosphorylation.

NR phosphorylation by kinases, activated in response to a variety of signals (MAPKs or PKB), aids the recruitment of coactivators, thereby facilitating the recruitment of histone remodelling complexes, which decondense the repressive chromatin. In addition, NR phosphorylation synergizes with the concomitant phosphorylation of the associcated coactivators, thereby affecting the *trans*-activation potential of the NRs. Phosphorylation of the NR also participates in the recruitment of components of the RNA polymerase II transcriptional machinery. CBP, p300/CREB-binding protein; HAT, histone acetyltransferase; HMT, histone methyltransferase; p160 family coactivators proteins (SRC-1,-2,-3 and GRIP1). Figure modified from Rochette-Egly, 2003.

Overall, therefore, phosphorylation has been shown to modulate the activity of many NRs, with a range of functions being affected, such as dimerisation, DNA binding and interactions with cofactors, all of which are likely to affect the *trans*-activation function of these transcription factors (Table 7.1 and 7.2) (Chen et al., 1999; Hammer et al., 1999). Although phosphorylation is usually secondary to ligand activation for most NRs, it is becoming increasingly apparent that phosphorylation may be the primary means for modulating the action of certain receptors (Hammer et al., 1999; Lee et al., 2000). The ability of kinase cascades to regulate NR function demonstrates that the activity of these receptors can be modified depending on the physiological state of the cell. Thus, many factors acting on kinases can modulate the response of NRs to their ligands (Germain et al., 2006). In addition, it is becoming increasingly clear that phosphorylation of NRs and the modification of coregulators is necessary for the *trans*-activation of certain genes, and that these modification cooperate with the receptor-ligand complex in order to achieve maximal transcription activity (Germain et al., 2006; Rochette-Egly, 2003).

Recent work has demonstrated that LXRs require coactivators for effective transcriptional activation of certain genes (Huuskonen et al., 2004a; Oberkofler et al., 2003). These authors showed that overexpression of the coactivators SRC-1 and p300, either alone or in combination, increased the luciferase activity driven by the wild-type ABCA1 promoter, and that the same coactivators bound to the ABCA1 promoter when the cells were treated with oxysterols as judged by chromatin immunoprecipitation (ChIP) assays. In addition, a recent study showed for the first time that LXRs are phosphoproteins and that LXR-α is phosphorylated at a single Ser 198 site in the hinge region of the protein, which is a consensus sequence for MAPK (Chen et al., 2006). Furthermore, it has been shown that direct phosphorylation of LXR-α by PKA results in a decrease in its DNA binding and recruitment of coactivators by LXRs (Yamamoto et al., 2007). Moreover, its dimerization partner RXR has been shown to be affected by several kinases, including MAPKs and PKA (Mann et al., 2005; Solomon et al., 1999). In the light of these studies and the outcome of previous research on this project (Chapters 3-6), the aim of the studies presented in this chapter was to investigate the potential interplay between the two identified cell signalling pathways in the LXR-mediated activation of ABCA1 and apoE expression in macrophages.

The experiments in this chapter were carried out with the aim of determining whether there was a cross-talk between JNK/SAPK and PI3K/PKB pathways in the regulation of ABCA1 and apoE gene expression by 22(R)-HC and 9CRA. For these investigations, the effect of SP600125 on the activation of PKB by 22(R)-HC and 9CRA in THP-1 macrophages was studied first. Secondly, the effect of LY294002 on the phosphorylation of JNK/SAPK was examined. Thirdly, the effect of a PAN PKC inhibitor BIM on the phosphorylation of PKB, JNK/SAPK, SEK1/MKK4 and c-Jun was analysed. All these aims were achieved through the continuing use of pharmacological inhibitors and antibodies specifically reacting with phosphorylated epitopes. Further studies on the potential role of the AP1 family in the activation of the ABCA1 promoter, and the potential interactions between the JNK/SAPK and PI3K/PKB pathways on this transcription factor were carried out by EMSA. Figure 7.2 illustrates the overall experimental strategy for the work presented in this chapter.

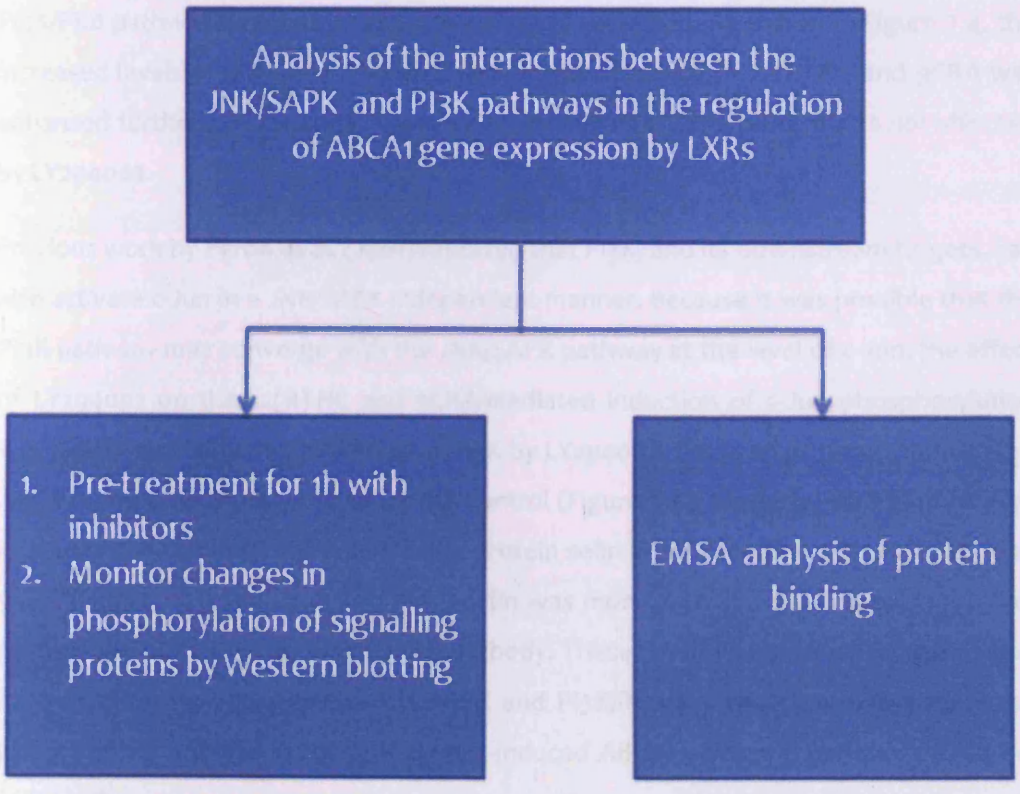


Figure 7.2 Summary of the experimental strategy used to delineate the cross-talk between signal transduction pathways underlying LXR agonist-mediated activation of ABCA1 gene expression in THP-1 macrophages.

7.2 Results

7.2.1 Effect of SP600126 on the phosphorylation of PKB induced by 22(R)-HC and 9CRA

In order to determine any potential cross-talk between the PI3K/PKB and JNK/SAPK signalling pathways, the effect of JNK/SAPK inhibitor SP600125 on the phosphorylation status of PKB in THP-1 macrophages by 22(R)-HC/9CRA was assessed. As shown in Figure 7.3, pre-treatment of THP-1 cells with 50μM and 100μM SP600125 inhibited the 22(R)-HC/9CRA-stimulated phosphorylation of PKB. On the other hand, the levels of total PKB were not affected. These results therefore suggest the existence of a potential cross-talk between the PI3K/PKB and JNK/SAPK signalling pathways.

7.2.2 Effect of the PI3K inhibitor LY294002 on the 22(R)-HC and 9CRA-mediated phosphorylation of JNK/SAPK in THP-1 macrophages

The phosphorylation status of JNK/SAPK by 22(R)-HC and 9CRA in the presence of the PI3K/PKB pathway inhibitor, LY294002, was next evaluated. As shown in Figure 7.4, the increased levels of phospho-JNK/SAPK seen in the presence of 22(R)-HC and 9CRA was enhanced further by LY294002. The amount of total JNK/SAPK protein was not affected by LY294002.

Previous work by Peron et al. (2001) reported that PI3K, and its downstream targets, can also activate c-Jun in a JNK/SAPK independent manner. Because it was possible that the PI3K pathway may converge with the JNK/SAPK pathway at the level of c-Jun, the effect of LY294002 on the 22(R)-HC and 9CRA-mediated induction of c-Jun phosphorylation was examined. Following inhibition of PI3K by LY294002, the level of phosphorylated c-Jun was not changed compared to the control (Figure 7.4). Similarly, inhibition of PI3K increased the total cell content of c-Jun protein seen in cells treated with 22(R)-HC and 9CRA (Figure 7.4). The expression of β-actin was monitored as a loading control by re-probing the blots with an anti-β-actin antibody. These results suggest once again some form of interplay between the JNK/SAPK and PI3K/PKB signalling pathways that may play an important role in the LXR agonist-induced ABCA1 and apoE gene expression in THP-1 cells.

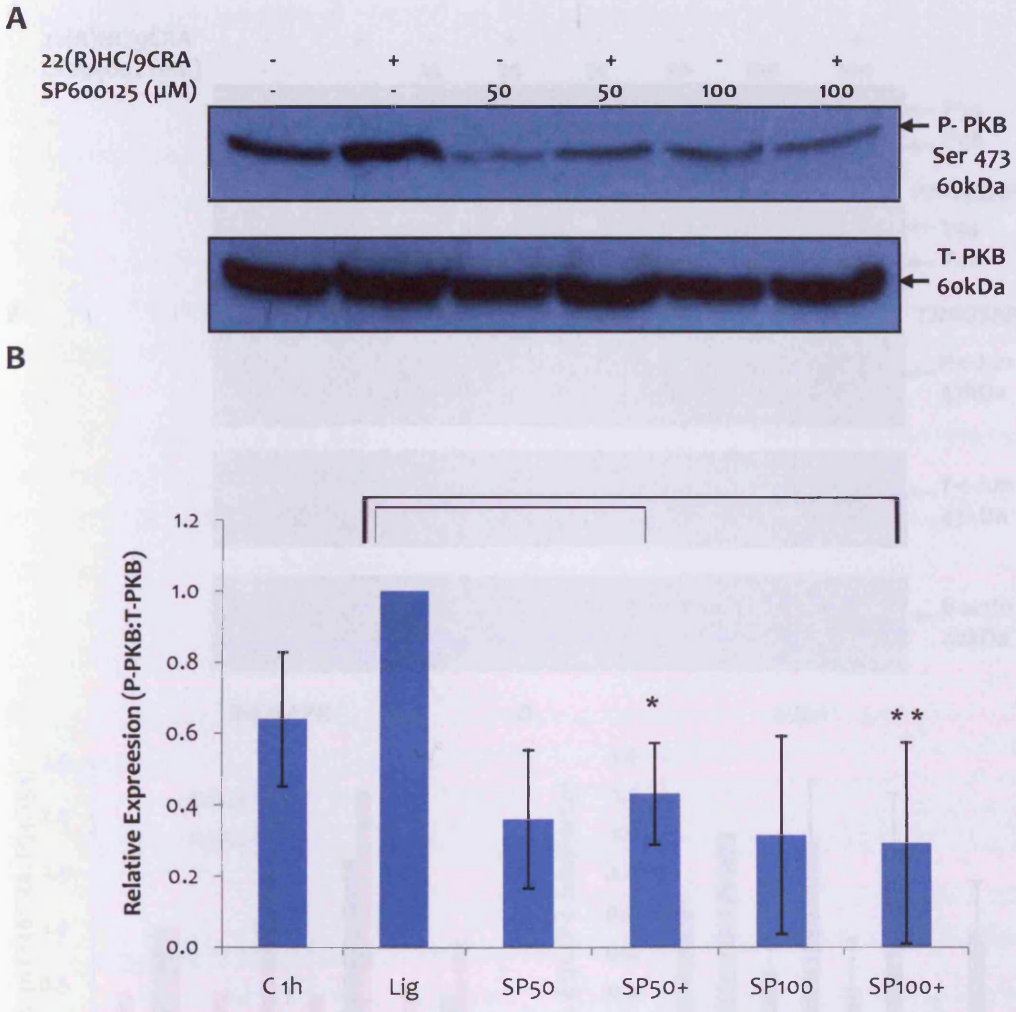


Figure 7.3 Effect of SP600125 on the 22(R)-HC and 9CRA-mediated posphorylation of PKB at Ser 473 in THP-1 macrophages.

Differentiated THP-1 macrophages were incubated for 1h in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (2μg/ml) and 9CRA (10μM) (Lig) in the absence or the presence of SP600125 at the indicated concentrations (50 and 100μM). In addition, cells were treated with DMSO as a vehicle control (C1h). The inhibitors were added 1h before the ligand (pre-treatment). Western blot analysis was carried out using equal volume of lysates (50μl). Blotted membranes were incubated with anti-phospho-PKB and anti-total-PKB primary antibodies. Antigen-antibody complexes were detected using the ECL detection system. The image shown in panel A is representative of four independent experiments. Densitometric analysis was carried out on the data and presented as relative expression (Rel Exp) (mean ±SD) normalised to the expression of T-PKB). The relative expression in the presence of the ligand alone (Lig) has been arbitrarily assigned as 1, as shown in panel B (*$P<0.05$, **$P<0.01$ compared to control).

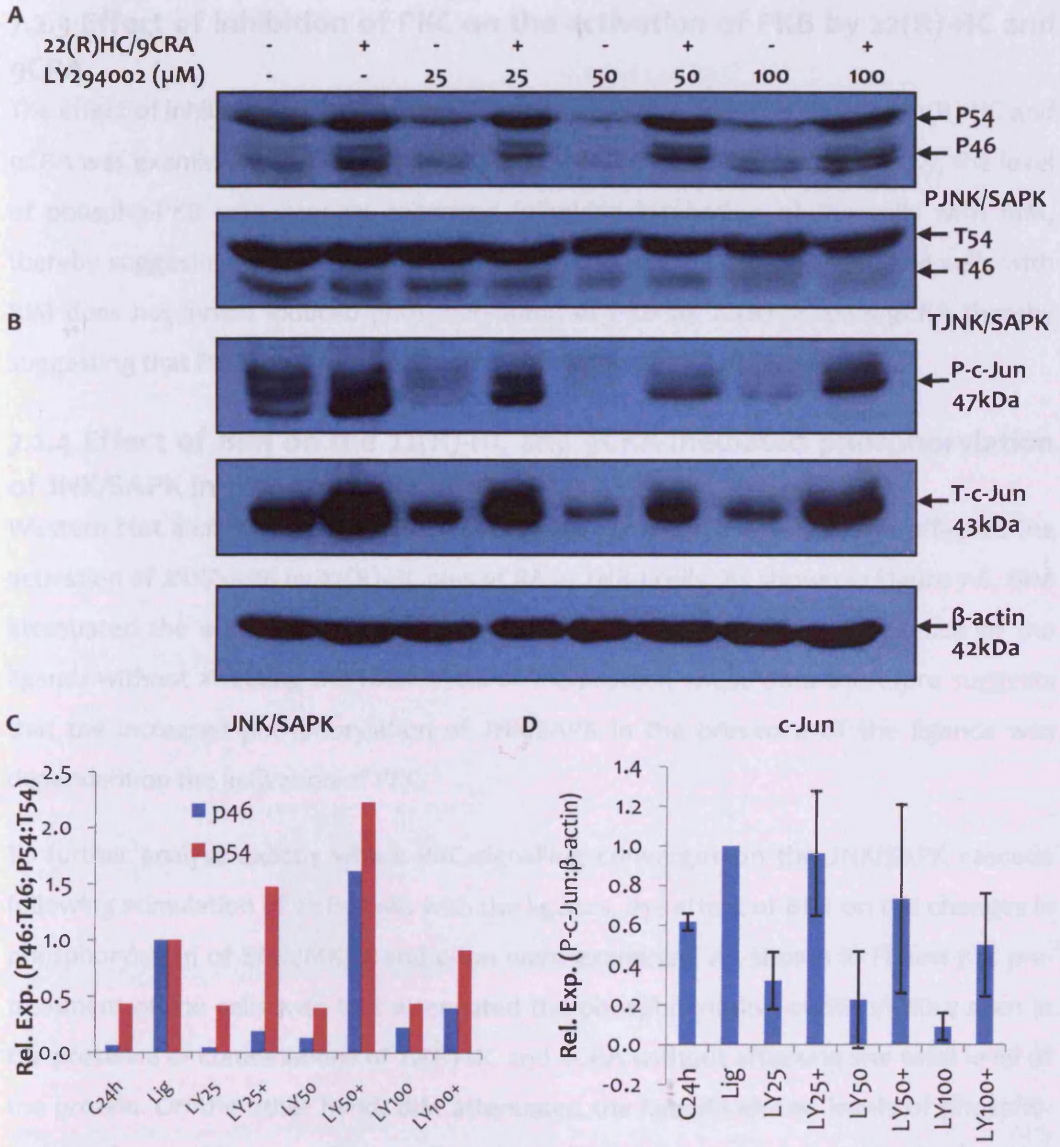


Figure 7.4 Effect of LY294002 on the phosphorylation of JNK/SAPK by 22(R)-HC and 9CRA in THP-1 macrophages.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (2μg/ml) and 9CRA (10μM) (Lig) in the absence or the presence of LY294002 at the indicated concentrations. In addition, cells were also treated with DMSO as a vehicle control (C24h). The inhibitors were added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 25-50μl of lysates. Blotted membranes were incubated with primary antibodies against phospho-JNK/SAPK (Thr183/Tyr185) and total-JNK/SAPK (A) or phospho-c-Jun and total-c-Jun (B). Antigen-antibody complexes were detected using the ECL detection system. Blotted membrane for c-Jun Western was re-probed with an anti-β-actin antibody to ensure equal loading of protein in each samples. The image shown in panels A and B is representative of two and three independent experiments respectively. Densitometric analysis was carried out on the data and presented as relative expression (Rel Exp) (average panel C) and (mean ±SD panel D) normalised to the expression of total JNK/SAPK (panel A) or β-actin (panel B). The relative expression in the presence of the ligand alone (Lig) has been arbitrarily assigned as 1.

7.2.3 Effect of inhibition of PKC on the activation of PKB by 22(R)-HC and 9CRA

The effect of inhibition of PKC on the activation of PKB by combinations of 22(R)-HC and 9CRA was examined using the PAN PKC inhibitor BIM. As shown in Figure 7.5, the level of phospho-PKB was strongly enhanced following incubation of the cells with BIM, thereby suggesting some sort of interaction. However, pre-treatment of the cells with BIM does not inhibit induced phosphorylation of PKB by 22(R)-HC plus 9CRA thereby suggesting that PKC is not required for the activation of PKB in THP-1 cells.

7.2.4 Effect of BIM on the 22(R)-HC and 9CRA-mediated phosphorylation of JNK/SAPK in THP-1 macrophages

Western blot analysis was carried out in order to investigate whether BIM affected the activation of JNK/SAPK by 22(R)-HC plus 9CRA in THP-1 cells. As shown in Figure 7.6, BIM attenuated the enhanced phosphorylation of JNK/SAPK seen in the presence of the ligands without affecting the total levels of the protein. These data therefore suggests that the increased phosphorylation of JNK/SAPK in the presence of the ligands was dependent on the activation of PKC.

To further analyse exactly where PKC signalling converges on the JNK/SAPK cascade following stimulation of THP-1 cells with the ligands, the effect of BIM on the changes in phosphorylation of SEK1/MKK4 and c-Jun were examined. As shown in Figure 7.7, pre-treatment of the cells with BIM attenuated the phosphorylation of SEK1/MKK4 seen in the presence of combinations of 22(R)-HC and 9CRA without affecting the total level of the protein. On the other hand, BIM attenuated the ligand-induced levels of phospho- and total-c-Jun (Figure 7.7). Overall, these results support the hypothesis that the activation of the SEK1/MKK4-JNK/SAPK-c-Jun signalling pathway in THP-1 macrophages by the ligands was PKC dependent. Thus, PKC was likely to be an upstream component in the JNK/SAPK pathway responsible for the regulation of ABCA1 and apoE expression by 22(R)-HC and 9CRA.

7.2.5 Effect of other PKC inhibitors on JNK/SAPK activation by 22(R)-HC and 9CRA

To investigate the potential role of various isoforms of PKC on 22(R)-HC plus 9CRA-mediated activation of JNK/SAPK in THP-1 macrophages, three isoform-specific inhibitors were used, rottlerin, Gö6976 and Gö6983. These inhibitors have been shown to

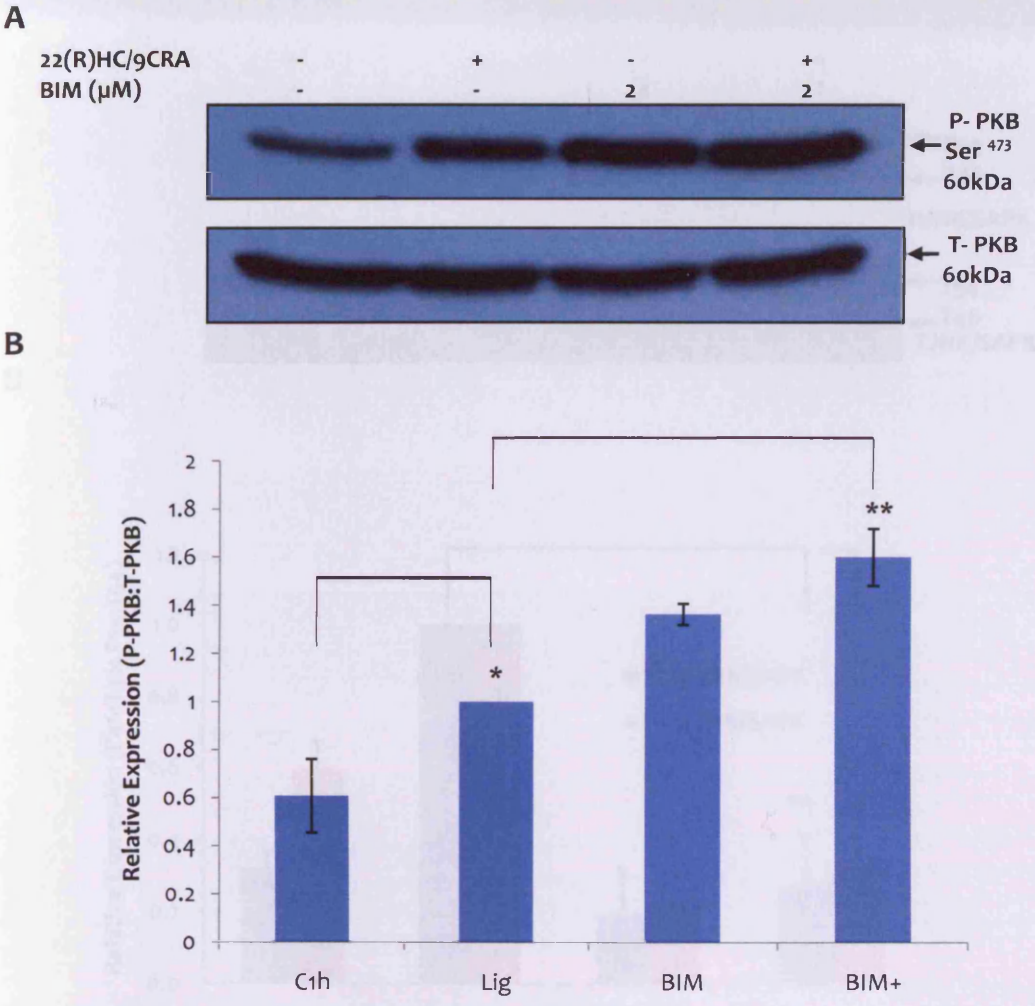


Figure 7.5 Effect of PKC inhibitor bisindoylmaleimide on the 22(R)-HC and 9CRA-mediated PKB posphorylation at Ser 473 in THP-1 macrophages.

Differentiated THP-1 macrophages were incubated for 1h in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (2μg/ml) and 9CRA (10μM) in the absence or the presence of 2μM of BIM. In addition, cells were treated with DMSO as a vehicle control (C1h). The inhibitors were added 1h before the ligand (pre-treatment). Western blot analysis was carried out using equal volume of lysates (50μl). Blotted membranes were incubated with anti-phospho-PKB and anti-total-PKB primary antibodies. Antigen-antibody complexes were detected using the ECL detection system. The image shown in panel A is representative of three independent experiments. Densitometric analysis was carried out on the data and presented as relative expression (mean ±SD, from three experiments). The relative expression in the presence of the ligand alone (Lig) has been arbitrarily assigned as 1, as shown in panel B; *$P<0.05$; **$P<0.01$.

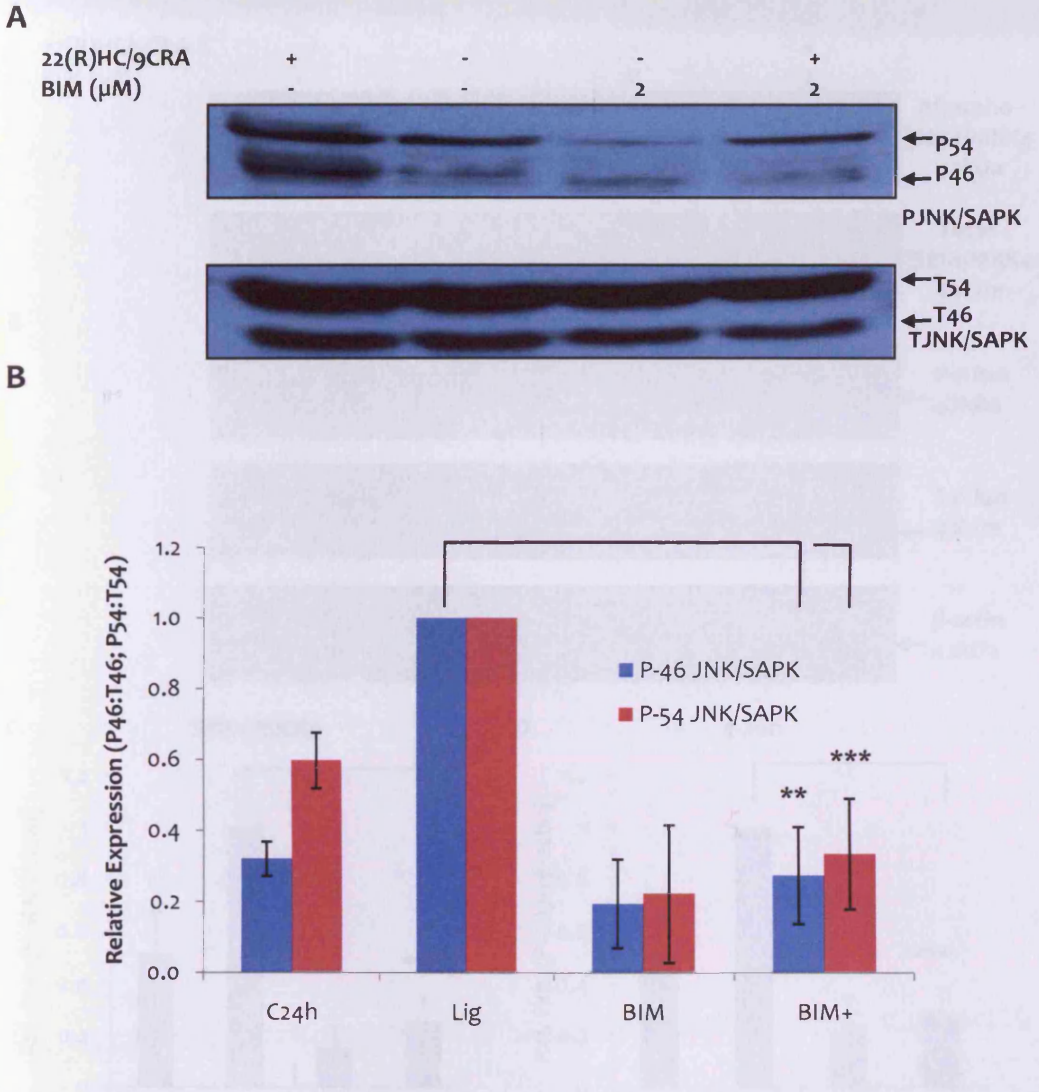


Figure 7.6 Effect of bisindoylmaleimide on JNK/SAPK phosphorylation by 22(R)-HC and 9CRA in THP-1 macrophages.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (2μg/ml) and 9CRA (10μM) in the absence or the presence of 2μM of BIM. In addition, cells were also treated with DMSO as a vehicle control (C24h). The inhibitors were added 1h before the ligand (pre-treatment). Western blot analysis was carried out using equal volume of lysates (50μl). Blotted membranes were incubated with anti-phospho-JNK/SAPK (Thr183/Tyr185) or anti-total-JNK/SAPK primary antibodies. Antigen-antibody complexes were detected using the ECL detection system. The image shown in panel A is representative of three independent experiments. Densitometric analysis was carried out on the data and is presented as relative expression (mean ±SD, from three experiments). The relative expression in the presence of the ligand alone (Lig) has been arbitrarily assigned as 1, as shown in panel (B) (**P<0.01; ***P<0.001).

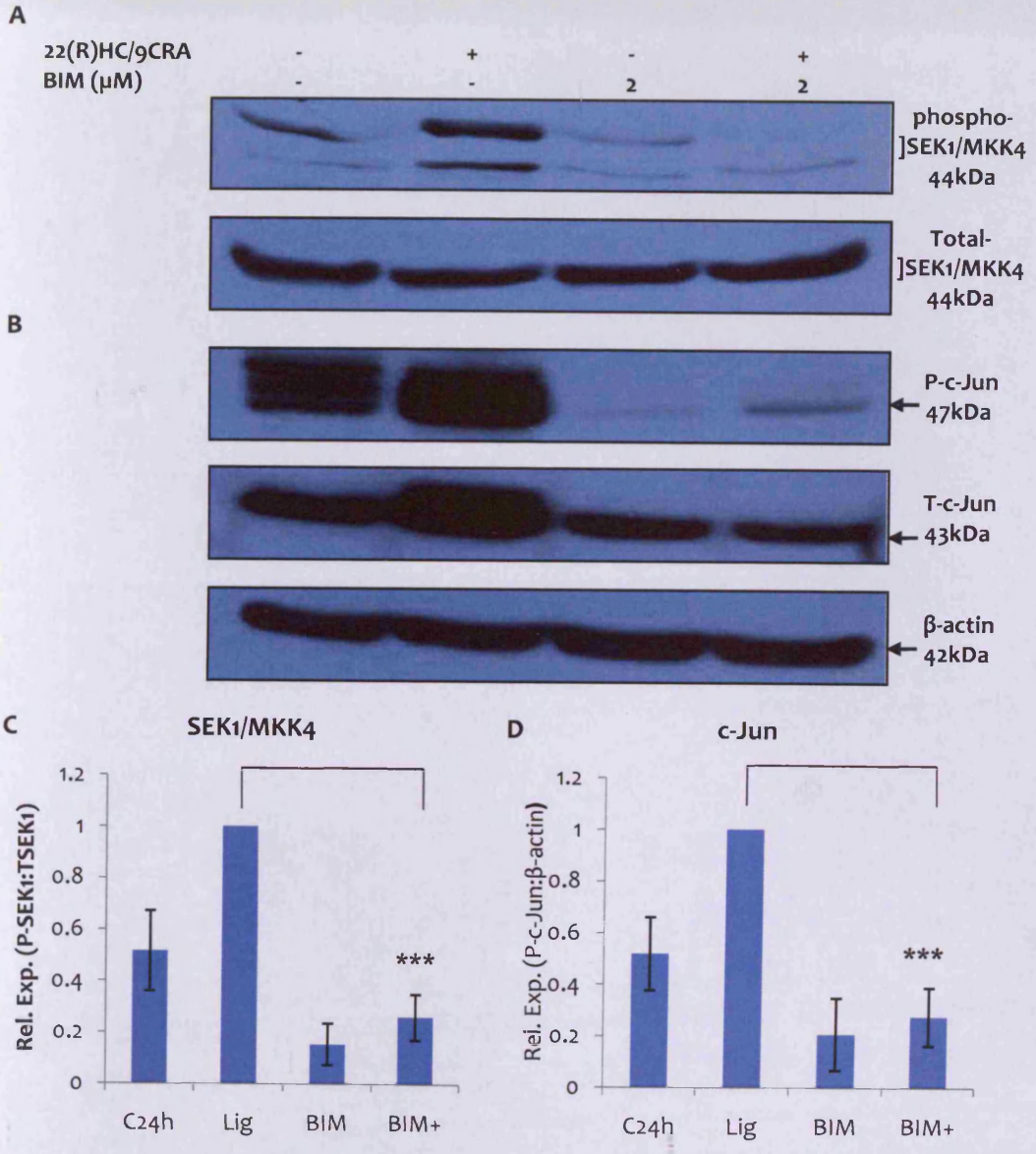


Figure 7.7 The effect of bisindoylmaleimide on the 22(R)-HC and 9CRA-mediated SEK1/MKK4 and c-Jun phosphorylation in THP-1 macrophages.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (2μg/ml) plus 9CRA (10μM) in the absence or the presence of 2μM of BIM. In addition, cells were treated with DMSO as a vehicle control (C24h). The inhibitors were added 1h before the ligand (pre-treatment). Western blot analysis was carried out using equal volume of the lysates (25-50μl). Blotted membranes were incubated with primary antibodies against phospho-SEK1/MKK4 or total-SEK1/MKK4 (A) and phospho-c-Jun or total-c-Jun (B). Antigen-antibody complexes were detected using the ECL detection system. The image shown in panels A and B is representative of three independent experiments. Densitometric analysis was carried out on the data and presented as relative expression (Rel Exp) (mean ±SD, from four experiments) normalised to the expression of total-SEK1/MKK4 (panel A) or β-actin (panel B). The relative expression in the presence of the ligand alone (Lig) has been arbitrarily assigned as 1, as shown in panels C and D (***$P<0.001$).

selectively inhibit PKC isoforms δ, α and ζ respectively (Gschwendt et al., 1994; Martiny-Baron et al., 1993; Wang et al., 1998).

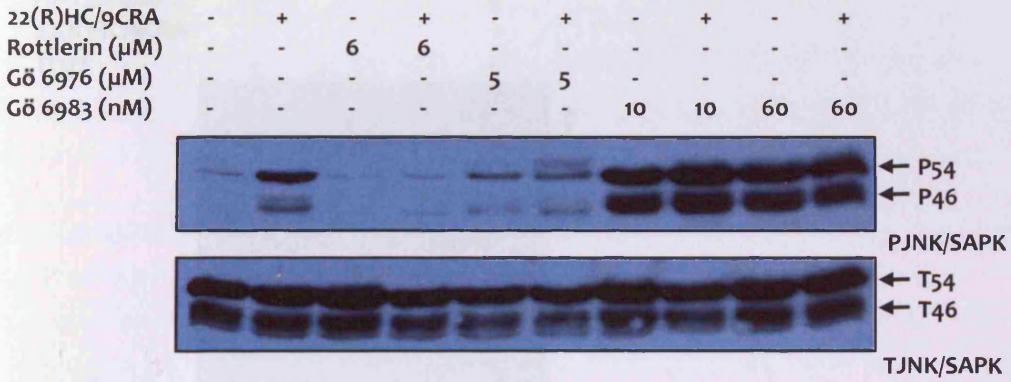
The increased phosphorylation of JNK/SAPK on Thr183/Tyr185 seen in cells treated with combinations of 22(R)-HC and 9CRA was attenuated by pre-treatment of the cells with Gö6976 or Rottlerin (Figure 7.8). The amount of total JNK/SAPK protein levels did not vary with ligand treatment. On the other hand, treatment of the cells with Gö6983, a selective inhibitor of PKCζ, had no effect on the levels of phospho- and total-JNK/SAPK proteins (Figure 7.8). Overall, these results with pharmacological inhibition of PKCα (Gö6976) and PKCδ (Rottlerin) strongly suggest that these kinases are likely to regulate the phosphorylation of JNK/SAPK.

7.2.6 EMSA analysis of protein binding to the ABCA1 promoter

Our studies into the regulation of ABCA1 expression in response to LXR agonists have suggested a potentially important role for the PI3K and JNK/SAPK pathways. Oxysterols and other NR agonists have been shown to affect AP-1 DNA binding activity in several cell lines and a recent study demonstrated that oxysterols induce the binding of AP-1 proteins via EMSA, and an increase in the expression of an AP-1 reporter as a result of activation of LXRs during keratinocyte differentiation (Schmuth et al., 2004). Analysis of the proximal promoter region of the human ABCA1 gene has revealed the presence of several putative regulatory elements for a number of transcription factors, including AP-1, NF-κB and SP-1 (see Figure 1.6). Therefore, the putative AP-1 binding site in the human ABCA1 promoter was selected for EMSA to further determine the role of JNK/SAPK in the LXR-mediated upregulation of ABCA1 gene expression and to investigate the potential involvement of this transcription factor in the response.

Whole cell protein extracts were prepared from untreated THP-1 macrophages and those treated for 24h with LXR agonists. The EMSA probe, containing the AP-1 element of the human ABCA1 promoter, was radiolabeled and incubated in a protein binding reaction with whole cell protein extracts. As shown in Figure 7.9A, the AP-1 DNA-binding activity was increased when extracts were used from THP-1 cells stimulated with either 22(R)-HC/9CRA or T0901317. The specificity of DNA-protein interactions was demonstrated by the ability of excess unlabeled DNA probe to compete for complex

A



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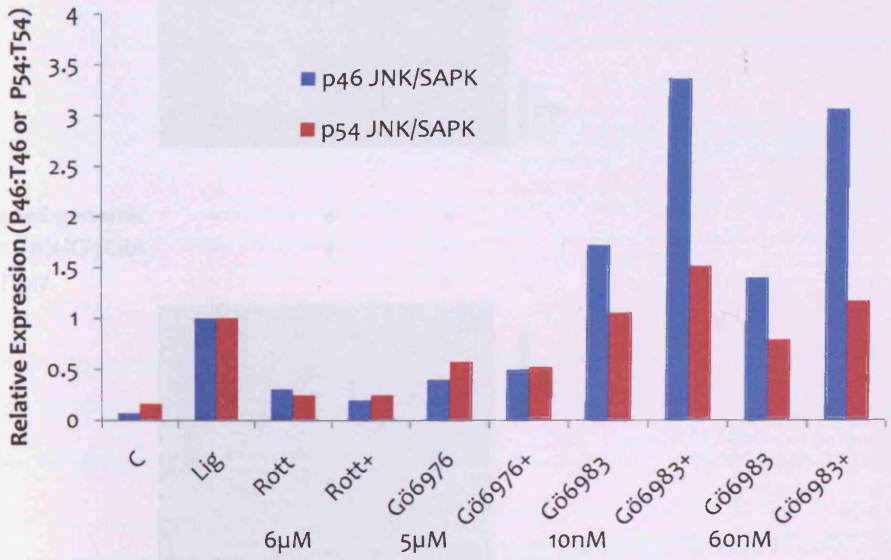

Figure 7.8 The effect of isoform-specific PKC inhibitors on JNK/SAPK phosphorylation by 22(R)-HC and 9CRA in THP-1 macrophages.

Differentiated THP-1 macrophages were incubated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with combination of 22(R)-HC (2μg/ml) and 9CRA (10μM) in the absence or the presence of the PKC isoform inhibitors Rottlerin (6μM), Gö6976 (5μM) and Gö6983 (10nM, 60nM). In addition, cells were treated with DMSO as a vehicle control (C). The inhibitors were added 1h before the ligand (pre-treatment). Western blot analysis was carried out using 50μl of cell lysates. Blotted membranes were incubated with primary antibodies against phospho-JNK/SAPK (Thr183/Tyr185) or total-JNK/SAPK. Antigen-antibody complexes were detected using the ECL detection system. The image shown in panel A is representative of two independent experiments. Densitometric analysis was carried out on the data and presented as relative expression (Rel Exp) (average). The relative expression in the presence of the ligand alone (Lig) has been arbitrarily assigned as 1, as shown in panel B.

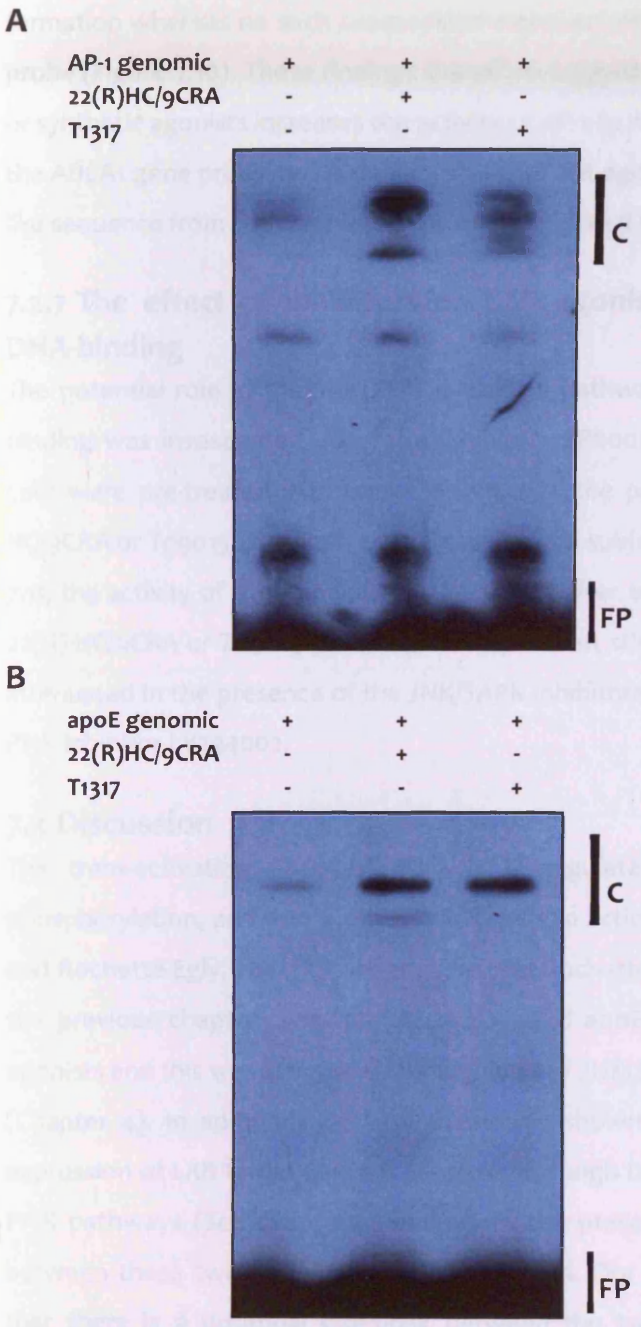

Figure 7.9 Effect of LXR agonists on binding of AP-1 to a putative recogination sequence in the ABCA1 and apoE gene promoters.

Differentiated THP-1 macrophages were cultured for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (2μg/ml) and 9CRA (10μM) or T1317 (10μM). In addition, cells were also treated with DMSO as a vehicle control. Whole cell protein extracts were prepared. EMSA analysis was carried out using the radiolabelled AP-1 genomic probe as indicated. DNA-protein complexes and free probe are shown by vertical lines labelled C and FP, respectively. The results shown are representative of three and two (panel A and B) independent experiments respectively.

formation whereas no such competition was seen with a nonspecific NF-κB binding site probe (Figure 7.10). These findings therefore suggest that activation of LXRs by natural or synthetic agonists increases the activity of AP-1 to its putative recognition sequence in the ABCA1 gene promoter. A similar action of LXR agonists was also seen with the AP-1–like sequence from the apoE gene promoter (Figure 7.9B).

7.2.7 The effect of inhibitors on LXR agonist-mediated changes in AP-1 DNA-binding

The potential role of the JNK/SAPK and PI3K pathways on the induction of AP-1 DNA binding was investigated using the inhibitors SP600125, curcumin and LY294002. The cells were pre-treated with these inhibitors in the presence or the absence of 22(R)-HC/9CRA or T0901317. Protein extracts were then subjected to EMSA. As shown in Figure 7.11, the activity of AP-1 binding was increased after treatment of the cells for 24h with 22(R)-HC/9CRA or T0901317. This ligand-dependent stimulation of AP-1 DNA binding was attenuated in the presence of the JNK/SAPK inhibitors, SP600125 and curcumin, and the PI3K inhibitor LY294002.

7.3 Discussion

The *trans*-activation potential of NRs is regulated by both ligand binding and phosphorylation, and they are substrates for the actions of a variety of kinases (Bastien and Rochette-Egly, 2004; Gianni et al., 2002a; Rochette-Egly, 2003). Studies presented in the previous chapters showed that ABCA1 and apoE expression was induced by LXR agonists and this was attenuated by inhibitors of JNK/SAPK and PI3K signalling pathways (Chapter 4). In addition, we have previously shown that the 22(R)-HC/9CRA-induced expression of LXR target genes is mediated through the activation of the JNK/SAPK and PI3K pathways (See chapters 5 and 6). In the present study, the potential cross-talk between these two pathways was investigated. Our results indicate for the first time that there is a potential cross-talk between the two signal transduction pathways, JNK/SAPK and PI3K, activated by LXR agonists in THP-1 macrophages. The 22(R)-HC/9CRA-dependent phosphorylation of both JNK/SAPK and PKB was attenuated by treatment of the cells with the JNK/SAPK inhibitor SP600125 (Figure 7.3). On the other hand, inhibition of the PI3K pathway with LY294002 amplified the observed 22(R)-HC/9CRA-induced increase in JNK/SAPK and c-Jun phosphorylation (Figure 7.4). In addition, the cell content of the c-Jun protein was increased by the inhibitor in the

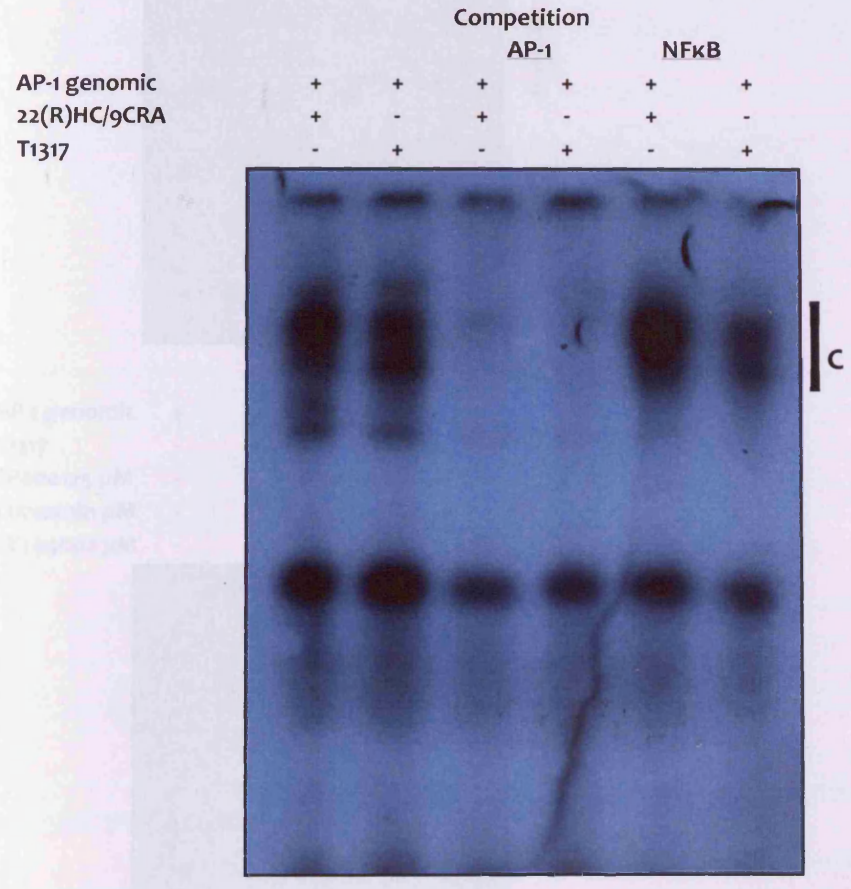

Figure 7.10 Competition EMSA on binding of AP-1 to a putative recogination sequence in the ABCA1 promoter.

Differentiated THP-1 macrophages were cultured for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (2μg/ml) and 9CRA (10μM) or T1317 (10μM). In addition, cells were treated with DMSO as a vehicle control. Whole cell protein extracts were prepared. EMSA analysis was carried out using radiolabelled AP-1 genomic sequence probe. Competition assays were carried out in the presence 200-fold molar excess of unlabeled specific (AP-1) or nonspecific (NF-κB) competitor oligonucleotides. The major AP-1: DNA- protein complex is shown by a vertical line labelled C. The free probe has migrated off the gel. The result shown is representative of two independent experiments.

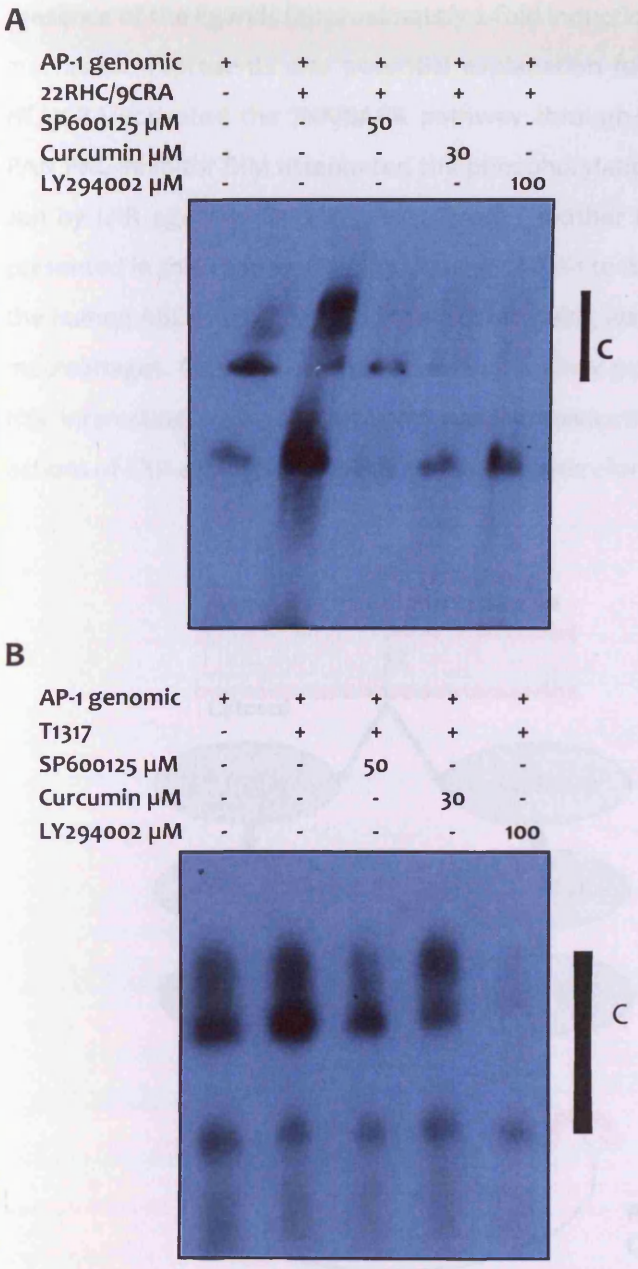

Figure 7.11 The effect of inhibitors on the LXR ligands-induced binding to AP-1 genomic sequence.

Differentiated THP-1 macrophages were treated for 24h in RPMI medium 1640 containing 10% (v/v) LPDS with 22(R)-HC (2µg/ml) and 9CRA (10µM) or T1317 (10µM) in the absence or the presence of the inhibitors SP600125, curcumin and LY294002 at the indicated concentrations. In addition, cells were also treated with DMSO as a vehicle control. The inhibitors were added 1h before the ligand (pre-treatment). Whole cell protein extracts were prepared and EMSA analysis was carried out using radiolabelled AP-1 genomic probe. The major AP-1: DNA-protein complexes are shown by a vertical line labelled C. The free probe has migrated off the gel. The results shown are representative of two independent experiments.

presence of the ligands (approximately 2-fold induction) (Figure 7.4). Feedback response mechanism represents one potential explanation for these results. In addition, 22(R)-HC/9CRA activated the JNK/SAPK pathway through a PKC-dependent pathway as the PAN PKC inhibitor BIM attenuated the phosphorylation of JNK/SAPK, SEK1/MKK4 and c-Jun by LXR agonists in THP-1 cells. Finally, another important result from the studies presented in this chapter was the binding of AP-1 to its putative recognition sequence in the human ABCA1 promoter and that such binding was induced by LXR agonists in THP-1 macrophages. Overall, therefore, our results allow postulation of a potential model for this interesting cross-talk between signal transduction pathways (Figure 7.12) in the actions of LXR agonists. Further studies could therefore focus on confirming this model.

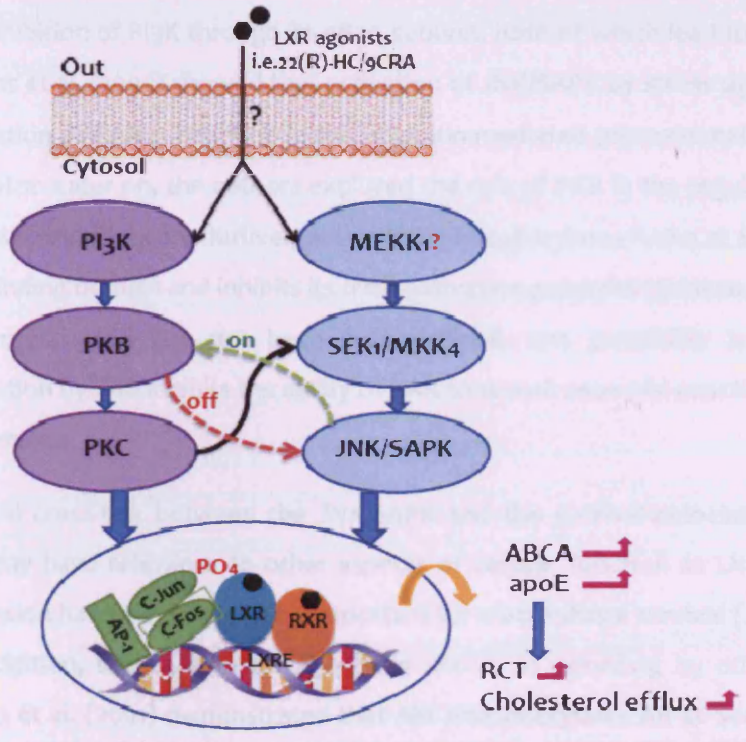

Figure 7.12 Potential model for LXR agonist-induced JNK/SAPK- and PI3K-dependent activation of ABCA1 and apoE gene expression in THP-1 macrohages.

LXR agonists stimulate the phosphorylation and activation of SEK1/MKK4, which then induces c-Jun-mediated AP-1 activation in a JNK/SAPK dependent manner. In addition, 22(R)-HC/9CRA also activates PKB by phosphorylation on Ser473 residue leading to PKC activation. PKC then causes cross-talk between PI3K/PKB/PKC and SEK1/MKK4/JNK/SAPK/c-Jun pathways. These pathways then regulate AP-1-dependent activation of ABCA1 and apoE gene expression, and hence cholesterol efflux. Whether the activation of these pathways requires direct binding of the LXR agonists to its receptor or if this is a non-genomic effect remains to be determined.

Phosphorylation of NRs provides an important link between signalling pathways and the regulation of expression of downstream genes. Such cross-talk has previously been described for several NRs. For example, recent reports have demonstrated that PKB negatively regulates retinoic acid (RA)-induced p38 activation (Gianni et al., 2002b; Kim et al., 2001; Park et al., 2002). Interestingly, Gianni et al. (2002b) also demonstrated that the RA-induced downregulation of the PI3K/PKB pathway targets the phosphorylation of RARγ2 through the activation of the p38 kinase. Thus, RARγ2 phosphorylation, RARγ2 turnover and RARγ2-mediated transcription of target genes are interrelated events resulting from the RA-induced downregulation of the PI3K/PKB pathway. The mechanism by which RA inhibits the PI3K/PKB pathway has been elucidated by Gianni et al. (2002b). They showed that RA acts at two levels, phosphorylation of the phosphotase PTEN and inhibition of PI3K through its p85α subunit, both of which lead to inhibition of PKB. Srinivas et al. (2005) showed that activation of JNK/SAPK by stress signals leads to phosphorylation of RARα, resulting in the ubiquitin-mediated proteasomal degradation of the receptor. Later on, the authors explored the role of PKB in the regulation of RAR function and found that constitutively active PKB phosphorylates RARα at Ser96 residue in its DNA binding domain and inhibits its *trans*-activation potential (Srinivas et al., 2006). While the mechanism has not been fully defined, one possibility is that RARα phosphorylation by PKB inhibits the ability of RAR to recruit essential coactivators to the receptor complex.

The potential cross-talk between the JNK/SAPK and the survival-associated PI3K/PKB pathways may have relevance to other aspects of cellular function as LXR-dependent gene expression has been found to be important for macrophage survival (Joseph et al., 2004). In addition, such a role has also been shown in signalling by other NRs. For example, Lin et al. (2001) demonstrated that Akt phosphorylates AR at Ser-210, inhibits AR *trans*-activation and blocks AR-induced apoptosis. Analysis of the literature suggests that PI3K is a key anti-apoptotic effector in the growth factor signalling pathway. PKB, beyond its role in regulating NR function, is also the product of a proto-oncogene and phosphorylates a number of substrates that are important regulators of cell survival via anti-apoptotic effects (Srinivas et al., 2006; Vivanco and Sawyers, 2002). Such an action serves a key role in mediating the anti-apoptotic actions of growth factors on cells (Datta et al., 1997). JNK/SAPK is a pathway well known for mediating stress-related responses and is important in inducing apoptosis. Indeed, several studies have shown

that the activation of MAPKs and, particularly JNK/SAPKs, might be positively or negatively regulated by the activation of PI3K. Some investigators have reported that inhibitors of PI3K block activation of MAPKs and JNK/SAPK, suggesting a positive regulatory role for the PI3K pathway in the MAPK cascade (Klippel et al., 1996; Logan et al., 1997; Lopez-Ilasaca et al., 1997). However, an increase in MAPK activity in some cell types in response to PI3K inhibitors implies a potential negative regulatory effect of PI3K/PKB on the MAPK pathway (Hui et al., 2005; Kwon et al., 2000; Levresse et al., 2000; Madge and Pober, 2000; Murakami, 2005; Park et al., 2002). For example, Murakami et al. (2005) showed that angiopoietin-1-dependent phosphorylation of PKB through PI3K leads to the inhibition of SEK1/MKK4-JNK/SAPK phosphorylation in human endothelial cells. In this case, inhibiting SEK1/MKK4 activation by angiopoietin-1, which occurs via phosphorylation of PKB at Ser 80, leads to the suppression of JNK/SAPK signalling pathway (Murakami et al., 2005; Park et al., 2002).

Recently, several mechanisms of cross-talk between JNK/SAPK and PKB pathways have been reported where the activation of PKB, as a potent survival factor, inhibits JNK/SAPK phosphorylation in many cell types. For example, PKB decreases JNK/SAPK signalling by phosphorylating apoptosis signal regulating kinase 1 (ASK1) at residue Ser 83 (Kim et al., 2001). It has also been shown that PKB interacts with JNK-associated scaffolding protein (JIP1) and decreases the ability of JIP1 to enhance JNK/SAPK activation (Kim et al., 2002). Barthwal et al. (2003) reported that phosphorylated PKB (Ser 473) could also phosphorylate Mixed lineage kinase3 (MLK3) on Ser 674, which then inhibits MLK3-mediated JNK/SAPK activation. Other studies indicate that PKB can regulate protein kinases upstream of JNK/SAPK, thereby inhibiting the phosphorylation of JNK/SAPK and protecting against JNK/SAPK-dependent apoptosis in susceptible cells (Brazil et al., 2004; Shahabi et al., 2006).

Cross-talk between different signalling pathways is relatively common and the involvement of PKC in NR regulation of target gene expression has been discussed in the previous chapter. Although studies in chapter 6 had shown that inhibitors of PKCs were able to inhibit the 22(R)-HC/9CRA-mediated induction of ABCA1 and apoE expression, it was necessary to investigate any potential cross-talk between PKC and JNK/SAPK and PI3K/PKB. Our results show that inhibition of PKC causes PKB activation by 22(R)-HC/9CRA to increase slightly (approximately 2-fold) (Figure 7.5). Accordingly, it would be

reasonable to speculate that PI3K-PKB modulates PKC activity and PKC could potentially negatively regulate PKB activity. Previous studies suggested that PI3K activity, through the generation of 3-phosphorylated lipids, could act as a second messenger for the regulation of most PKC isoforms (Akimoto et al., 1996; Moriya et al., 1996; Zhang et al., 1995). In addition, Konishi et al. (1996) have shown that PKB is able to phosphorylate PKC-δ, with which it associates via the PH domain.

Our results also suggest that PKC is potentially an upstream effector of the JNK/SAPK pathway activated by LXR agonists as inhibition of phosphorylation of JNK/SAPK cascade via PAN PKC inhibitor BIM was seen in THP-1 cells treated with 22(R)-HC/9CRA (Figures 7.6-7.7). Accordingly, it would be reasonable to speculate that PKB-nPKCε/δ-dependent JNK/SAPK activation by 22(R)-HC/9CRA mediates ABCA1 and apoE gene expression in THP-1 cells. In addition, the stimulatory effect of the ligand-mediated phosphorylation of JNK/SAPK was inhibited in THP-1 cells by inhibitors of PKCα/δ (Figure 7.8). Furthermore, the results also showed that the PKCζ inhibitor Gö6983 had no effect on the activation of JNK/SAPK by the ligands (Figure 7.8). However, PKCζ inhibitor Gö6983 attenuated the expression of ABCA1-induced by 22(R)-HC and 9CRA (Figure 6.10). Indeed, regulation of several other NRs (e.g. GR, PPAR and ER) through the activation of the MAPK cascade has also been documented and several studies suggests that PKC, as an activator of the MAPK pathway, might participate indirectly in the control of the transcriptional activity of these NRs (Hu et al., 1996; Kato et al., 1995; Schonwasser et al., 1998; Ueda et al., 1996). For example, vitamin D3 has been shown to activate the MAPK signalling cascade, including JNK/SAPK, through the activation of PKC (Beno et al., 1995). In this context, down regulation of PKC has been reported to block completely the activation of JNK/SAPK by phorbol esters in HeLa cells (Werlen et al., 1998).

Accordingly, our results suggest that novel PKC isoforms could be a link connecting PI3K to JNK/SAPK signalling pathways, and also suggests a link between PKC and JNK/SAPK since the PKC inhibitor BIM completely blocked the phosphorylation of the JNK/SAPK cascade. On the other hand, inhibition of PKC had a stimulatory effect on PKB phosphorylation, thereby indicating that PKB stimulation could be upstream of PKC in the PI3K pathway. However, further studies such as monitoring the effect of the pharmacological inhibitor LY294002 on PKC activity are required to confirm these

finding. Several studies demonstrate a cross-talk between the JNK/SAPK and PI3K signalling pathways (Greco et al., 2006; Hui et al., 2005; Lee et al., 2006a; Shahabi et al., 2006). Numerous studies also implicate PKC in signalling pathways leading to the activation of various MAPKs (Berra et al., 1995; Chang et al., 1998; Ghaffari-Tabrizi et al., 1999; Vincent et al., 2006). For example, PKCs have been shown to function as upstream components of the JNK/SAPK pathway. In addition, PKCε has been shown to induce the phosphorylation and activation of ERK1/2 and JNK/SAPK in rabbit cardiomyocytes (Ping et al., 1999a; Ping et al., 1999b). Moreover, work by Li et al. (2000) has established that MAPKs function as critical intermediate signalling molecules that transduce signals from PKC-ε to produce the activation of AP-1 and NF-κB in cardiomyocytes (Li et al., 2000b). It has also been shown that bile acids activate PKC, which then initiate a MAPK signal transduction pathway to phosphorylate JNK/SAPK, and thereby suppress the expression of the CYP7A1 gene (Chiang John, 2002). Recently, Greco et al. (2006) have shown that direct activation of PKC occurs through phosphorylation of PKB on Ser473, suggesting that PKB is upstream to PKC-δ/ε. The authors also showed that this activation was sufficient to activate the JNK/SAPK cascade. Taken together, our novel results, along with those from other laboratories detailed above, suggest that a potential interaction between the activation of PI3K/PKB/PKC and JNK/SAPK signalling cascade is mediated by 22(R)-HC and 9CRA, which work together in the upregulation of ABCA1 expression in human THP-1 macrophages.

Because of the essential role played by ABCA1 gene expression induced by LXR agonists in macrophages in relation to RCT, and its identification as a protector against the risk of CVD (Schmitz and Langmann, 2005), further studies were carried out on the regulation of ABCA1 expression by LXR agonists. Figure 1.6 illustrates the presence of several putative regulatory elements for known transcription factors in the ABCA1 promoter, including AP-1, SP-1 and NF-κB. It has been shown earlier in chapter 5 that AP-1 activity is increased by LXR ligands. In addition, AP-1 activity induced by LXR agonists was inhibited by inhibitors of PI3K and PKCs (See chapter 6). Consistent with these observations, AP-1 has been found to bind to its putative recognition sequence in the human ABCA1 promoter, with the binding induced in response to treatment of THP-1 macrophages to LXR ligands (Figure 7.9). This binding was competed by an excess of specific unlabelled probe but not by a consensus sequence for NF-κB (Figure 7.10). This binding of AP-1 to the proximal promoter of the human ABCA1 gene was inhibited in the presence of

specific pharmacological inhibitors of both the JNK/SAPK and PI3K pathways in THP-1 cells (Figure 7.11). In addition, the activation of AP-1 binding to a putative recognition site in the apoE gene promoter by 22(R)-HC/9CRA and T0901317 was also seen (Figure 7.9). This AP-1 element has already been shown to be essential for the induction of apoE expression during macrophage differentiation (Basheeruddin et al., 1994) and may additionally play a role in the LXR-mediated activation of apoE expression. Such a requirement for the AP-1 element in the LXR-mediated induction of apoE expression may be due to the low affinity of the LXR-REs present in the proximal promoter of the apoE gene for this NR (Laffitte et al., 2001b). Therefore, AP-1 may be required for the full stimulatory response of the apoE promoter to LXR agonists.

In summary, the results presented in this chapter have shown that a potential cross-talk between PI3K/PKB/PKC and JNK/SAPK signalling cascade exists in the action of 22 (R)-HC/9CRA in THP-1 macrophages. We have also shown that novel PKC isoforms can act as a link between the PI3K/PKB and JNK/SAPK pathways, indicating that PI3K/novel PKCε, δ-dependent JNK/SAPK activation may be essential for the ligand-induced expression of ABCA1 and apoE. This cross-talk between two identified pathways suggests a novel regulatory mechanism in LXR signalling and could offer new direction for therapeutic intervention of atherosclerosis.

CHAPTER EIGHT:

GENERAL DISCUSSION

Chapter 8: General Discussion

8.1 Overview of the results presented in this thesis

An initiating step in atherosclerosis is the formation of macrophage foam cells, which occurs when macrophages in the arterial wall become overloaded with excess cholesterol. Unlike other cells, macrophages take up cholesterol via scavenger receptors and therefore are highly dependent on RCT to reduce their cellular cholesterol content. The strong inverse relationship between circulating levels of HDL-cholesterol and the incidence of atherosclerosis is clearly evident (Cuchel and Rader, 2006). The LXRs are members of the NR superfamily, which are activated by oxysterols and play an important role in maintaining macrophage cholesterol homeostasis. Previous studies have identified the LXRs as important regulators of HDL metabolism because of their ability to control the expression of genes involved in RCT. For example, LXRs control the cholesterol efflux pathway in macrophages through the regulation of target genes implicated in the process, including ABCA1 and apoE (Laffitte et al., 2001b; Venkateswaran et al., 2000a; Repa and Mangelsdorf, 2000; Chawla et al., 2001b). The synthetic LXR ligand, GW3965, reduces the development of atherosclerosis in LDLR-/- and apoE-/- mice (Joseph et al., 2002b). Conversely, LXRα/β -/- mice exhibit accumulation of foam cells in multiple tissues (Joseph et al., 2002b; Schuster et al., 2002; Tangirala et al., 2002). Transplantation of LXRαβ-deficient bone marrow into LDLR-/- and apoE-/- mice results in increased atherosclerosis, thereby demonstrating the relevance of macrophage LXR activity in the prevention of atherosclerosis (Tangirala et al., 2002). The importance of ABCA1 in RCT is also exemplified by studies on the ABCA1 knockout mice. These mice have almost no circulating HDL and show signs of cholesterol accumulation in macrophages that is similar to that in patients with TD (Mcneish et al., 2000). Additionally, cells overexpressing human ABCA1 show increased cholesterol efflux activity (Lawn et al., 1999). Moreover, study by Wagner et al. (2003) have demonstrated that LXRs not only induce RCT when cholesterol levels are high but also mediate active repression of the process in the unliganded state, thereby also linking transcriptional repression to regulation of cholesterol homeostasis (Wagner et al., 2003). Under normal resting conditions, ABCA1 levels are low or absent (Langmann et al., 1999) and require specific stimuli to increase its expression (see Table 1.1). The combined ability to repress

or activate the expression of ABCA1 allows the existence of a tightly regulated and responsive system to handle sudden and pronounced changes in cellular cholesterol levels (Wagner et al., 2003). ApoE represents another LXR target gene that also contributes to the anti-atherogenic effects of the LXRs (Laffitte et al., 2001b). Macrophage apoE expression can significantly reduce atherosclerotic development as demonstrated by decreased lesion area in apoE-deficient mice that have received a bone marrow transplant from apoE+/+ donors (Boisvert et al., 1995; Linton et al., 1995). In summary, therefore, LXRs play a key role in the control of RCT and suggest that pharmaceuticals that increase their action, and thereby expression of their downstream targets such as ABCA1 and apoE, may be useful in the prevention of, or a decrease in, the incidence of CVD, and as a treatment for atherosclerosis.

Recent studies in the laboratory had demonstrated a novel role for PI3K and JNK/SAPK signalling pathways in the 22(R)-HC-mediated induction of apoE expression in THP-1 macrophages (Greenow, K., 2004). However, the potential role of these pathways in the action of other LXR agonists, such as T0901317, or in the regulation of expression of other downstream targets, such as ABCA1, had not been investigated. Such studies could identify potentially novel targets for therapy against atherosclerosis. The major focus of the studies presented in this thesis was therefore to delineate fully the signal transduction pathways underlying LXR agonist-mediated upregulation of ABCA1 and apoE expression in macrophages.

Studies presented in chapter 3 investigated the potential mechanisms underlying the regulation of ABCA1 gene expression by LXR agonists using J774.2 macrophages as a model system. It was found that LXR activators induce the expression of ABCA1 in these cells and that an additive activation was obtained when combinations of 22(R)-HC and 9CRA were used. We next investigate the potential signalling pathways that might be involved in the induction of ABCA1 mRNA expression by these ligands. This activation of ABCA1 expression by LXR/RXR was attenuated by LY294002, thereby extending the previous finding on apoE (Greenow, K., 2004) to ABCA1 and combinations of 22-(R)-HC and 9CRA. However, definitive conclusions on the role of the JNK/SAPK pathway could not be made as SP600125 had no effect and curcumin was found to be toxic to J774.2 macrophages. In the light of these results along with the various limitations associated with the J774.2 cell line (e.g. problems of efficient transfection with exogenous DNA), it

was decided to use human THP-1 macrophages for subsequent studies. These cells are human in origins and therefore more directly relevant to atherosclerosis, given that it is in essence a human disease.

The work presented in chapter 4 aimed to examine the effects of natural and synthetic LXR agonists on ABCA1 and apoE gene expression in differentiated THP-1 macrophages. Given that the function of apoE and ABCA1 in the control of cholesterol homeostasis is carried out by proteins, Western blot analysis was used to examine the changes in their expression rather than RT-PCR, as used for the studies presented in chapter 3. Transient transfection assays using the ABCA1 gene promoter were also used for further confirmation. The studies showed that natural and synthetic LXR agonists induce ABCA1 and apoE expression in THP-1 macrophages, with the overall induction levels being dependent on the ligands. Combinations of 22(R)-HC/9CRA produced the most activation followed by T0901317 and GW3965. Transfection assays in U937 cells also confirmed the induction of ABCA1 promoter activity by LXR agonists. The use of an ABCA1 promoter construct containing mutations in LXR-RE along with a DN form of LXR-α showed that LXRs play a crucial role in the induced expression of ABCA1. In addition, we assessed the involvement of the JNK/SAPK and PI3K pathways through the use of commercially available inhibitors. We showed that inhibitors of both these pathways attenuated the activation of ABCA1 and apoE gene expression by LXR agonists. Such an inhibition of the responses by the JNK/SAPK and PI3K inhibitors was also seen in HMDMs.

Further investigation of the ability of combinations of LXR/RXR to activate the JNK/SAPK pathway was then carried out (Chapter 5). The phosphorylation of JNK/SAPK and its enzyme activity was also induced in response to treatment of THP-1 macrophages with LXR agonists. Further studies also demonstrated that SEK1/MKK4 and c-Jun, upstream and downstream targets respectively for JNK/SAPK actions, become phosphorylated in response to treatment of THP-1 macrophages with combinations of 22(R)-HC and 9CRA. Experiments with the use of DN mutants against key components of the JNK/SAPK pathway showed that DN SEK1/MKK4 and DN c-Jun attenuated the induction of ABCA1 promoter activity by combinations of 22(R)-HC/9CRA. Although, the expression of DN JNK/SAPK had no effect on the induction of ABCA1 promoter activity by 22(R)-HC/9CRA, the potential role of JNK/SAPK in the induction of apoE expression by

the ligands was confirmed by siRNA-based knockdown. Another novel observation was that EMSA showed an increase in protein binding to an AP-1 recognition sequence in THP-1 macrophages treated with LXR ligands. Such activation of AP-1 DNA binding activity was inhibited by pre-treatment the cells with the inhibitors SP600126 and curcumin.

Studies presented in chapter 6 investigated the role of the PI3K pathway in detail. The results showed that phosphorylation of PKB at serine 473 was induced in THP-1 macrophages treated with combinations of 22(R)-HC and 9CRA. Such an activation of PKB was inhibited by pre-treatment of the cells with LY294002. The role of PI3K/PKB was substantiated further through the use of PKB kinase assays and a DN PKB plasmid in transfection assays with the ABCA1 promoter. Thus, the key role of the PI3K pathway in the LXR agonist-mediated induction of ABCA1 promoter activity was conserved in both human THP-1 and mouse J774.2 macrophages. In contrast to GSK-3 and mTOR, PKC was found to play an important role in the LXR-mediated regulation of ABCA1 and apoE expression in THP-1 macrophages. Inhibition of PKC action by the use of selective inhibitors effectively attenuated the LXR agonist-induced expression of ABCA1 and apoE in THP-1 cells. The role of PKC was also supported further by experiments using DN constructs. Thus, a DN form PKC-ε almost completely prevented the activation of the ABCA1 promoter in response to combinations of 22(R)-HC and 9CRA, and DN PKC-α and -δ did this to a slightly lesser extent. In contrast, PKCλ, ξ and η DN constructs did not inhibit the ABCA1 response. EMSA indicated that the LXR ligands-induced binding of AP-1 to its recognition sequence was almost completely inhibited by LY294002 and inhibitors of PKC-α and –δ.

The results so far demonstrated that the JNK/SAPK and PI3K pathways play a potentially important role in the LXR agonist-mediated regulation of ABCA1 and apoE expression in THP-1 macrophages. These findings therefore provided a base for further investigation of the cross-talk between these two pathways (Chapter 7). Initial observations indicated a potential cross-talk between JNK/SAPK and PI3K, a novel finding. Thus, activation of both JNK/SAPK and PKB by combinations of 22-(R)-HC and 9CRA was inhibited by pre-treatment of the cells with SP600125. On the other hand, treatment of the cells with LY294002 failed to inhibit the activation of JNK/SAPK and its downstream target c-Jun but instead slightly potentiated the response, thereby suggesting the possibility of the

existence of a negative regulatory loop between PI3K/PKB and the JNK/SAPK pathway. In addition, our results showed that 22(R)-HC/9CRA activated the JNK/SAPK pathway in a PKC dependent manner as the PAN PKC inhibitor BIM attenuated the phosphorylation-mediated activation of the JNK/SAPK cascade by LXR ligands in THP-1 macrophages. Finally, EMSA showed that the binding of AP-1 to its putative recognition sequence in the human ABCA1 and apoE gene promoters was induced by LXR agonists in THP-1 macrophages, and the response was attenuated by inhibition of JNK/SAPK and PI3K/PKB.

In summary, the work presented in this thesis have been successful in enhancing our understanding of the mechanisms underlying the 22(R)-HC/9CRA-mediated activation of ABCA1 and apoE expression in THP-1 macrophages. The major important findings are as follows:

1. Induction of ABCA1 and apoE protein expression by natural and synthetic LXR agonists in THP-1 macrophages.

2. Identification of a novel role for the JNK/SAPK and PI3K/PKC pathways in the 22(R)-HC/9CRA-mediated induction of ABCA1 and apoE gene expression in THP-1 macrophages.

3. The binding of AP-1 to its consensus sequence and a putative recognition element in the human ABCA1 and apoE promoters.

4. Convergence of the JNK/SAPK and PI3K pathways on AP-1, thereby suggesting that the full inducibility of ABCA1 and apoE gene transcription by LXR agonists may require AP-1 binding to the promoter region in addition to that of LXR/RXR to LXR-REs.

5. The existence of a potential cross-talk between both pathways, and the potential requirement of PKC in the activation of the JNK/SAPK pathway.

The maximal activation of ABCA1 and apoE expression by LXR agonists occurred at 24h. In contrast, the activation of the JNK/SAPK and PI3K signalling pathways was rapid within an increase in phosphorylation following treatment of the cells with the ligands occurring after 30min for JNK/SAPK and 1h for PI3K. These kinetics are clearly more rapid than that for the induction of ABCA1 and apoE expression by the ligands. Such rapid activation of the signalling pathways is unlikely to be due to gene transcription, and it is therefore possible that 22(R)-HC/9CRA may be able to exert a non-genomic effect in addition to its action on gene transcription. A putatitive model can be derived from all these studies as shown in Figure 8.1. A major focus of future studies should therefore be to investigate the genomic and non-genomic actions of LXRs and the potential interactions between them (See section 8.3 for details on future work).

Overall, the findings presented in this thesis can be approached with a good degree of confidence given that we have used a range of complementary techniques, performed appropriate positive controls, and accurately reproduced key findings in human monocyte-derived macrophages. Consequently, the next two sections will attempt to discuss the greater significance of the findings and the future studies that could be performed in order to advance the studies towards the ultimate goal of therapeutic treatment of atherosclerosis.

8.2 Wider perspectives of the novel findings in this thesis

Recent studies have demonstrated the selective targeting of protein kinases as a therapeutic approach, and it is a common procedure to use small inhibitor molecules (Buschbeck, 2006; Daub et al., 2004; Hannon and Rossi, 2004; Sawyer et al., 2005). Indeed, potential therapeutic strategies have been investigated in relation to the treatment of CVD that involves the inhibition of JNK/SAPK- or PI3K-dependent signalling (Andrés, 2004; Koh, 2007; Monaco and Paleolog, 2004; Verdeguer et al., 2007). The identification of a potential involvement of cell signalling pathways in the regulation of NR action provides another potential mechanism for therapeutic intervention. Further research into these pathways could lead to the development of specific therapeutics without undesired side effect.

An excellent therapeutic strategy against the development of atherosclerosis would be to enhance RCT by modulating ABCA1 gene transcription. ABCA1 mediates the initial step

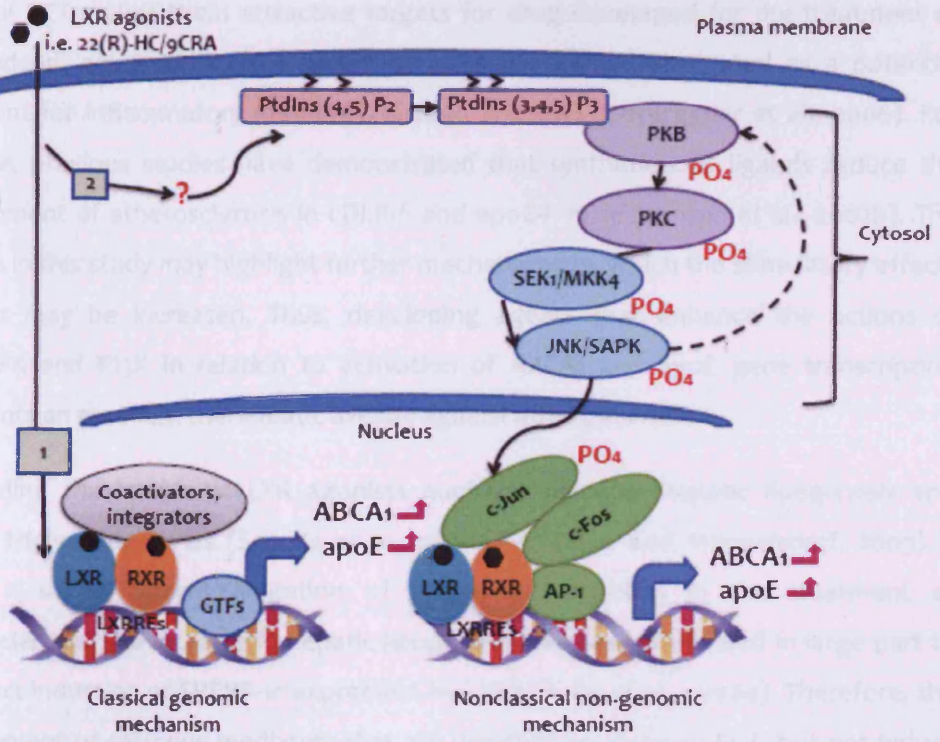

Figure 8.1 A putative model for the genomic and non-genomic actions of LXR agonists.

The LXR agonists 22(R)-HC and 9CRA could potentially exert their actions through a genomic and a non-genomic pathway. In the genomic pathway, 22(R)-HC/9CRA enters the target cells and binds to nuclear LXRs, thereby leading to its heterodimerization with RXR. The LXR/RXR heterodimer then induces target gene transcription by interacting with LXR-REs in their regulatory region (1). In the non-genomic pathway, LXR agonists activate PKB/PKC, possibly through interaction with a putative membrane bound or cytosolic receptor(s) (2), as found for other NRs, thereby leading to activation of JNK/SAPK and c-Jun. The subsequent increase in AP-1 binding might be required for the full inducibility of target genes.

of RCT, the efflux of cholesterol from cells to HDL particles (Oram and Vaughan, 2000). ABCA1 transcription is regulated by LXR (Costet et al., 2000; Repa et al., 2000b). Similarly the potent anti-atherogenic actions of apoE, its key role in RCT and its inducibility by LXRs is well documented (Greenow et al., 2005; Joseph et al., 2002b; Laffitte et al., 2001b; Repa and Mangelsdorf, 2002; Singh and Ramji, 2006). The ability of LXRs to promote RCT makes them attractive targets for drug developed for the treatment of CVD. Indeed, activation of the action of LXRs has been investigated as a potential treatment for inflammatory metabolic disease and CVD (Geyeregger et al., 2006). For example, previous studies have demonstrated that synthetic LXR ligands reduce the development of atherosclerosis in LDLR-/- and apoE-/- mice (Joseph et al., 2002b). The findings in this study may highlight further mechanisms by which the stimulatory effects of LXRs may be increased. Thus, developing agents that enhance the actions of JNK/SAPK and PI3K in relation to activation of ABCA1 and apoE gene transcription represents an excellent therapeutic avenue against atherosclerosis.

The finding that synthetic LXR agonists markedly increase hepatic lipogenesis and plasma triglyceride levels (Schultz et al., 2000; Tontonoz and Mangelsdorf, 2003) is clearly a major current limitation of using LXR agonists in the treatment of atherosclerosis. The increase in hepatic lipogenesis has been attributed in large part to the direct induction of SREBP-1c expression by LXRs (Repa et al., 2000a). Therefore, the development of selective mediators that are designed to increase RCT, but not induce hepatic SREBP-1c expression, would be a better therapeutic approach. Additionally, a better understanding of potential gene-specific differences in the action of LXRs would be useful, such as signalling pathways that are involved in the activation of individual genes. The development of partial or gene-specific agonists of NRs (e.g. selective ER modulators (SERMs)) have provided a framework on how such ligands could be identified (Bian et al., 2001; Gustafsson, 1998). Indeed, a newly developed LXR agonist, DMHCA, has been reported to have such a selective activity (Muscat et al., 2002; Quinet et al., 2004). Both *In vitro* and *in vivo* studies have shown that DMHCA mediates potent transcriptional activation of genes implicated in the control of RCT while exhibiting minimal effects on SREBP-1c expression (Quinet et al., 2004). In addition, it should also be taken into consideration that the effects of LXRs on lipogenesis may be tissue specific since it has been observed that in skeletal muscles, LXR ligands increase cholesterol efflux without affecting lipid deposition (Muscat et al., 2002).

In addition to gene- or tissue-specific agonists, an alternative approach to overcome the undesirable effects of LXR agonists on hepatic lipogenesis would be to develop isoform-specific LXR ligands. The development of LXRβ selective agonists could be a potential solution to dissociate LXR activation of RCT from the hepatic side effects. Indeed, in contrast to LXRβ-/- mice, LXRα-/- mice have reduced plasma triglyceride levels and decreased hepatic mRNA levels for multiple enzymes involved in fatty acid synthesis, thereby suggesting that LXRα is the isoform that controls the transcription of hepatic SREBP-1c (Alberti et al., 2001; Peet et al., 1998). Alberti and co-workers have therefore proposed that LXRβ-specific ligands may induce the desired RCT pathway but circumvent the hepatic complications that are attributed to LXRα (Alberti et al., 2001). On the other hand, macrophages from LXRβ-/- mice, but not LXRα-/- mice, have increased basal ABCA1 mRNA expression, indicating that LXRβ is the isoform responsible for controlling basal transcription of the ABCA1 gene in these cells (Laffitte et al., 2001b). In contrast, LXR agonist treatment of macrophages from LXRα-/- or LXRβ-/- mice show a comparable increase in expression of ABCA1 mRNA compared to wildtype mice (Repa et al., 2000b), thereby indicating that both LXRα/β are equally important for inducing ABCA1 transcription. Recently, Molteni and co-workers have identified a novel LXR agonist, N-acylthiadiazolines that activates the LXRβ subtype with selectivity over LXRα (Molteni et al., 2007). This selectivity of LXRβ was confirmed using macrophages derived from LXRα or β knockout mice (Molteni et al., 2007). Treatment of LXRα-/-apoE-/- mice with this ligand has also been found to ameliorate the cholesterol overload phenotype and reduce atherosclerosis (Bradley et al., 2007). Collectively, these observations provide *in vivo* support for drug development strategies on the development of agonists specific for LXRβ.

An additional alternative approach for the undesirable effects of LXR ligands on hepatic lipogenesis would be to target potential differences in coregulators requirements for different target genes (e.g. ABCA1/ apoE v/s SREBP-1c). This would require more research to be initially carried out on this aspect.

8.3 Future work

From the work presented in this thesis, we have gained significant new knowledge about LXR signalling in macrophages. Prior to these studies, relatively few investigations on LXR signalling had been reported. As a direct result of the studies presented herein,

several avenues for further investigations become apparent. For example, the widespread role of the JNK/SAPK and PI3K signalling could be identified by profiling expression in macrophages of known target genes (e.g. ABCG1, SREBP-1c) or at the whole genome level using either pharmacological inhibitors or expression of siRNA or DN constructs. The outcome of these investigation would reveal about potential gene-specific actions of these signalling pathways.

Although the potential involvement of the JNK/SAPK and PI3K pathways and their major upstream and downstream components have been identified, their exact roles in the LXR-mediated induction of ABCA1 and apoE expression should be extended to a detailed understanding of the potential genomic or non genomic effects along with the mechanisms in operation. Firstly, the role of LXRs on the ligand-mediated activation of JNK/SAPK and PI3K/PKB should be investigated. This can be achieved by transfection of cells with DN constructs for LXR-α and -β, which lack AF2 domain, or siRNA against LXR-α and/or LXR-β. The ligand–mediated activation of JNK/SAPK and PI3K/PKB should then be followed. The use of macrophages from LXR knockout mice represents another approach. The results of these investigations would verify whether LXRs are required for the activation of these signalling cascades.

Investigation of potential LXR phosphorylation by the ligands would also be important. For this, cells could be metabolically labelled with ^{32}P orthophosphate in the absence or the presence of the ligands. Equal amount of cellular extracts could be subjected to immunoprecipitation using LXR isoform-specific antibodies followed by SDS-PAGE and autoradiography. It is likely that subtle phosphorylations may remain undetected using this method, thereby possibly requiring 2D gel electrophoresis, which examines changes in protein isoelectric point due to phosphorylation. The role of the JNK/SAPK or PI3K pathways in any identified changes in phosphorylation of LXRs could then be analysed by repeating the analysis with cells incubated with pharmacological inhibitors or in which the expression of key kinases has been knocked down by siRNA. In the case of any problems with detection due to low expression of NRs in cells, the analysis could be carried out on cells transfected with plasmids specifying for epitope-tagged LXRs (antibodies against the epitope is used for immunoprecipitation). In the long-term, it would be useful to map the site(s) of phosphorylation, if any, and recent advances in mass spectrometry could be exploited.

Experiments could also be carried out to investigate whether any changes in phosphorylation affect the cellular localization of LXRs, and to delineate if any membrane-bound LXR exists, like other NRs, and if ligand-binding by such a receptor causes activation of upstream components of the JNK/SAPK and PI3K signalling pathways. This could be achieved by Western blot analysis of membrane fractions with antibodies against LXRs. The potential role of phosphorylation could be identified by immunocytochemistry on cells transfected with expression plasmids specifying for wildtype or mutant forms of hLXRα or β (e.g. mutations in the phosphorylation sites) using fluorescent antibodies.

ChIP is an elegant technique to study the mode of activation of NR (e.g. LXR) target genes. Importantly, this technique allows determination of which coactivators and corepressors are involved in the transcriptional regulation of a certain gene (e.g. ABCA1). Indeed, several new perspectives on NR function have emerged from the use of this technique (Auwerx et al., 2003; Mahajan and Samuels, 2005; Pellegrini et al., 2004; Yamamoto et al., 2007). In this technique, cells are first treated with vehicle or the LXR ligands and the DNA-bound proteins are then cross-linked to the coregulators by treatment with formaldehyde. Following this fixation step, nuclei are isolated and the chromatin fragmented to 500-1000bp size by sonication. Then, protein-DNA complexes are immunoprecipitated using an antibody specific for the coregulator protein of interest. The cross-links are then reversed and DNA from the immunoprecipitated protein/DNA fraction is purified. The identity and the amount of DNA fragments isolated from the complex with the coregulator protein of interest can then be determined by PCR using primers specific for the LXR-RE in the regulatory regions of target genes (e.g. ABCA1 or apoE). The results of these investigations would verify whether these sequences bind to coregulator proteins under investigation and reveal any potential gene-specific differences. The effect of blockage of JNK/SAPK and PI3K pathways on the recruitment of such coregulators could also be investigated. The role of a particular coregulator in governing expression of two different isoforms of LXR target genes could be carried out using macrophages in which the expression of individual isoform has been knocked down by siRNA or knocked out. In addition, potential phosphorylation of coregulators could be explored essentially as described above for LXRs.

It would be of interest to further analyse the involvement of PKCs in LXR action. This could include analysis of the phosphorylation or the activity of individual PKC isoforms in response to LXR agonists. For this, THP-1 macrophages are treated with LXR ligands in the absence or the presence of the isoform-specific PKC inhibitor and the activation of individual isoform followed by Western blot analysis using phospho-specific antibodies or by *in vitro* kinase activity, as used for JNK/SAPK and PKB in the studies presented in this thesis. The potential role of individual members could be confirmed by siRNA-mediated knockdown or use of macrophages from knockout mice.

It is also important that further investigations are carried out in relation to the potential cross-talk between the JNK/SAPK and PI3K/PKC pathways. Such studies could involve analysis of the activation of a particular pathway (e.g. JNK/SAPK) in cells where the action of individual signalling proteins, has been inhibited either by the use of pharmacological agents, siRNA-mediated knockdown or use of macrophages from knockout mice. Also, the potential involvement of several other key pathways that have been implicated in NR signalling, such as cdks and PKA, should be investigated. In this regard, it is of interest that PKA suppresses SREBP-1c expression via phosphorylation of LXR in the liver (Yamamoto et al., 2007).

Finally, the roles of JNK/SAPK and PI3K/PKB/PKC signalling in cellular changes, such as macrophage cholesterol efflux, should be investigated. This can be achieved by converting macrophages into foam cells using acetylated LDL in the presence of ^{14}C cholesterol before treatment of the cells with the vehicle or the LXR ligands, in the presence or the absence of specific inhibitors for JNK/SAPK, PI3K or PKC. The cholesterol efflux to HDL_3 acceptor could then be determined. The analysis could be extended to the use of cells following siRNA-mediated knockdown of key component or the use of macrophages from knockout mice.

Finally, it would be of great interest to perform at least some of the studies in apoE- or LDLR-deficient mice that have been fed an atherosclerotic diet, a system that has previously been used to elucidate the roles of numerous genes in the pathogenesis of atherosclerosis (Boucher and Gotthardt, 2004; Chow et al., 2007; Joseph et al., 2002b; Repa and Mangelsdorf, 2002; Shi et al., 2000; Su et al., 2006).

8.4 Concluding remarks

In summary, the studies presented in this thesis have identified the signalling pathways underlying the LXR agonist-mediated regulation of two key genes implicated in the control of macrophage cholesterol efflux. More specifically, the LXR agonist-inducible ABCA1 expression was mainly mediated by the PI3K/PKB-PKC-dependent-JNK/SAPK signal transduction pathway. Our studies, therefore, provide important new insights into LXR signalling, and reveal potential new avenues for therapeutic intervention. The use of specific LXR isoform- or tissue-specific agonists could be pharmaceutically attractive as far as treatment of CVD is concerned. It is therefore crucial to gain as complete an understanding as possible on the cellular actions of LXRs. The studies presented in this thesis are an important step towards this goal, and it is hoped that more significant progress will be made by pursuing the future studies suggested above.

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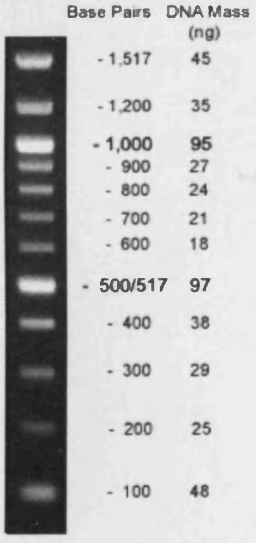
APPENDICES

Appendix I

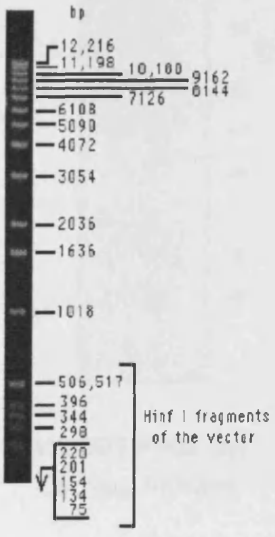
DNA Molecular weight Marker

NEB 100bp ladder



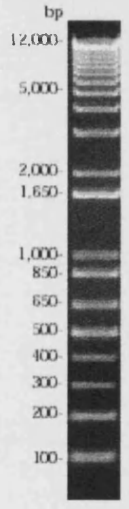
1.3% agarose gel
0.5µg/lane
Cat No. N3231S/N3231L

Invitrogen 1kb ladder



0.9% agarose gel
0.5µg/lane
Cat No. 15615-016

Gibco 1kb plus ladder

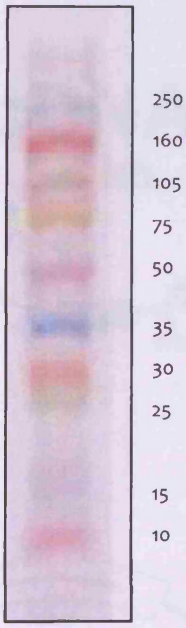


0.9% agarose gel
0.9µg/lane
Cat No. 10787-018

Appendix II

Protein Molecular weight Marker

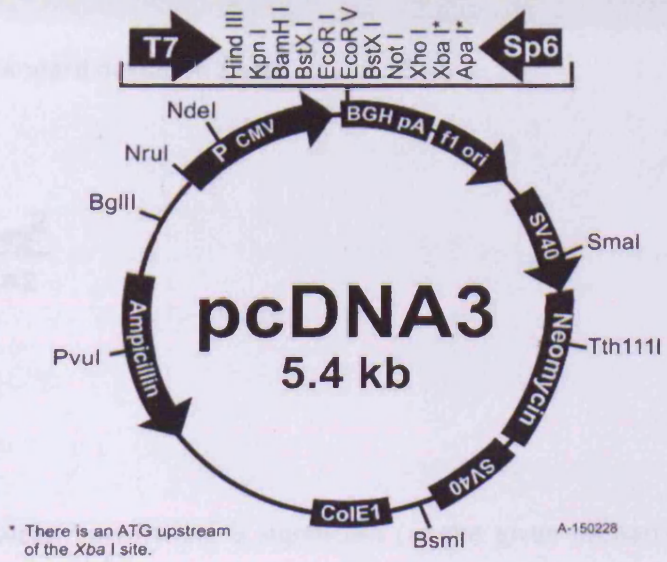
Amersham Full-Range Rainbow™
Molecular Weight Markers

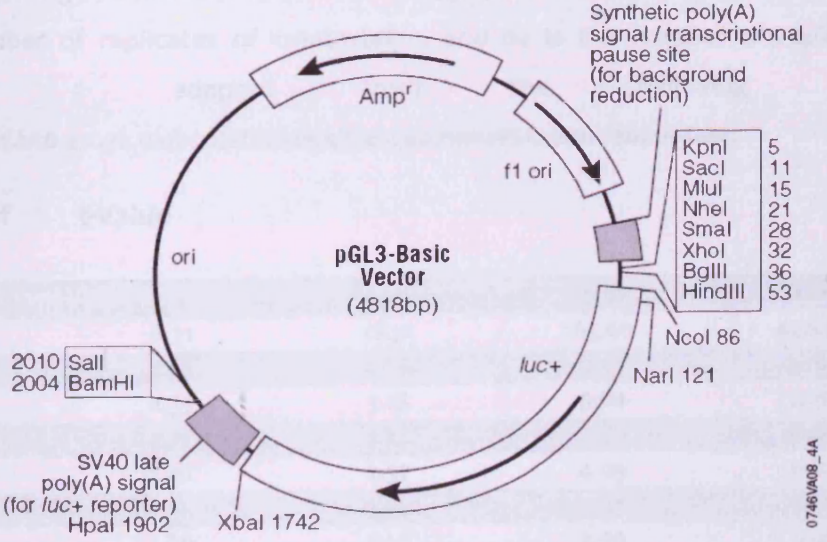


12% SDS-PAGE gel
Cat. No. RPN800

Appendix III

Plasmid vectors





Appendix IV

t-Test

Mean 1 = $\bar{x}1$ Standard deviation 1 = σ 1

Mean 2 = $\bar{x}2$ Standard deviation 2 = σ2

Variance = $\sigma d2$

$$\sigma_d^2 = \frac{\sigma_1^2}{n1} + \frac{\sigma_2^2}{n2}$$

$$t = \frac{\bar{x}1 - \bar{x}2}{\sigma_d}$$

t-Table

A difference between two means is significant (at the given probability level) if the calculated t value is greater than the value given in this table. A probability of p = 0.05 (95% probability of making a correct statement) is usually acceptable for biological work.

When comparing two means, the number of degrees of freedom is (n1 + n2)-2, where n1 is the number of replicates of treatment 1, and n2 is the number of replicates of treatment 2 adapted from the following website: http://helios.bto.ed.ac.uk/bto/statistics/tress4a.html#Student's%20t-test.

Degrees of Freedom	t-Value			
P-Value	0.1	0.05	0.01	0.001
1	6.31	12.71	63.66	636.62
2	2.92	4.30	9.93	31.60
3	2.35	3.18	5.84	12.92
4	2.13	2.78	4.60	8.61
5	2.02	2.57	4.03	6.87
6	1.94	2.45	3.71	5.96
7	1.89	2.37	3.50	5.41
8	1.86	2.31	3.36	5.04
9	1.83	2.26	3.25	4.78
10	1.81	2.23	3.17	4.59

Appendix V

Figure 3.2C	Exp 1	Exp 2	Exp 3	Exp 4					
samples	Fold Ind	Fold Ind	Fold Ind	Fold Ind	Mean	SD	t-test	P-value	
C12h	1	1	1	1	1				
22(R)-HC	3.05104	4.64194	6.60303	2.28940	4.14635	1.908652	3.30	0.05	*
22(S)-HC	0.33667	1.71921	1.83198	1.08560	1.243362	0.687964			
C24h	1	1	1	1	1				
22(R)-HC	1.28365	1.29190	2.00312	1.64062	1.554823	0.34205	3.24	0.05	*
22(S)-HC	1.15323	1.07864	2.11492	0.48162	1.207104	0.675736			

Figure 3.3C	Exp 1	Exp 2	Exp 3					
samples	Fol Ind	Fol Ind	Fol Ind	Mean	SD	t-test	P-value	
C12h	1	1	1	1				
9CRA	3.21859	2.49567	2.37856	2.697606	0.454967	6.46	0.01	**
22(R)HC	3.02976	2.29624	2.48080	2.602265	0.381545	7.27	0.01	**
9CRA/22(R)HC	5.50164	3.84876	5.27228	4.874228	0.895459	7.49	0.01	**

Figure 3.4	Exp 1	Exp 2	Exp 3					
samples	Fold Ind	Fold Ind	Fold Ind	Mean	SD	t-test	P-value	
C	1	1	1	1				
Lig	4.05387	5.21588	3.39316	4.220968	0.922777	6.05	0.01	**

Figure 3.5C	Exp 1	Exp 2	Exp 3	Exp 4					
samples	Rel Exp	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C 12h	0.15081	0.23523	0.09044	0.09221	0.142172	0.068084			
Lig	1	1	1	1	1				
LY	0.06084	0.13302	0.26857	0.19087	0.163324	0.088048			
LY+	0.54608	0.38957	0.42829	0.33140	0.423836	0.090703	12.70	0.001	***

Figure 3.6A/B	Exp 1	Exp 2	Exp 3	Exp 4					
samples	Fold Ind	Fold Ind	Fold Ind	Fold Ind	Mean	SD	t-test	P-value	
LXR+pcDNA									
UT	1	1	1	1	1				
T	3.28738	4.66884	4.83810	3.85363	4.16199	0.724353	8.73	0.001	***
LXR+DN p110									
UT	1	1	1	1	1				
T	3.43168	2.35903	3.07654	2.73497	2.90056	0.459613	2.94	0.05	*
LXR+ DN PKB									
UT	1	1		1	1				
T	2.50704	3.30260	2.30840	3.07670	2.79869	0.467852	3.16	0.05	*

Figure 3.7C	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average
Control	0.15701	0.22177	0.18939
Lig	1	1	1
SB216	0.06846	0.19634	0.132401
SB216+	0.42474	0.77154	0.598142
SB216+	0.66379	0.97101	0.817400
SB415	0.06189	0.16591	0.113901
SB415+	0.75014	0.87069	0.810417
SB415+	0.70177	0.82371	0.762737

Figure 3.8B	MCP-1	Bactin		
Samples	Raw value	Raw value	Ratio	Rel Exp
Control	31495.24	4977.49	6.32753	0.416809
IFNγ	72173.05	4754.20	15.18089	1
SB216	570.15	4755.46	0.11989	0.007898
SB216+IFNγ	24612.05	2814.26	8.74548	0.576085
SB415	1672.41	3554.50	0.47050	0.030993
SB415+IFNγ	414.91	3095.95	0.13402	0.008828

Figure 3.9C	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average
Control	0.15701	0.13948	0.14824
Lig	1	1	1
Rapa	0.21230	0.11334	0.16282
Rapa50+	0.99406	1.04405	1.01905
Rapa100+	1.59856	1.27453	1.43654
Rapa150+	1.03577	0.98197	1.00887

Figure 3.11C	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
Control	0.19323	0.17847	0.14917	0.173622	0.022425			
Lig	1	1	1	1		63.83	0.001	***
SP50	0.31161	0.50223	0.36966	0.394502	0.097711			
SP50+	1.19841	1.02086	1.16034	1.126536	0.093479			
SP100+	1.16693	0.82137	0.90293	0.963744	0.180629			

Figure 3.12C	apoE	28SrRNA		
samples	Raw value	Raw value	Ratio	Rel Exp
Control	4454.15	2609.08	1.70717	0.254551
TGFβ	7063.11	1053.16	6.70660	1
SP	2651.11	5806.51	0.45658	0.068078
SP+TGFβ	6126.22	2308.35	2.65394	0.395721

Figure 3.13	Exp 1	Exp 2	Exp 3					
samples	Fold Ind	Fold Ind	Fold Ind	Mean	SD	t-test	P-value	
LXR+pcDNA								
UT	1	1	1	1				
T	3.28738	4.56884	4.63810	4.164772	0.760636			
LXR+ DN JNK								
UT	1	1	1	1				
T	8.60983	7.55648	5.71978	7.295366	1.462613	3.29	0.05	*

Figure 4.2C	Exp 1	Exp 2	Exp 3	Exp 4					
samples	Fold Ind	Fold Ind	Fold Ind	Fold Ind	Mean	SD	t-test	P-value	
C	1	1	1	1	1				
9CRA	4.64449	5.14204			4.89326				
22(R)-HC	4.00123	6.48568	6.92959	6.75884	6.04384	1.3739562	7.34	0.01	**
22(R)-HC+9CRA	11.20951	7.66147	8.56996	15.08661	10.63189	3.3293209	5.79	0.01	**
22(S)-HC	1.14262	1.18435	1.71635	1.302714	1.33651	0.262152			

Figure 4.2D	Exp 1	Exp 2	Exp 3					
samples	Fold Ind	Fold Ind	Fold Ind	Mean	SD	t-test	P-value	
C	1	1	1	1				
9CRA	2.40450	3.06618		2.73534				
22(R)-HC	7.80943	7.43717	6.59001	7.27887	0.624936	17.40	0.001	***
22(R)-HC+9CRA	4.27482	3.28825	4.46498	4.00935	0.631685	8.25	0.01	**
22(S)-HC	1.33116	1.21541	1.06876	1.20511	0.131503			

Figure 4.3C	Exp 1	Exp 2	Exp 3					
samples	Fold Ind	Fold Ind	Fold Ind	Mean	SD	t-test	P-value	
DMSO	1	1	1	1				
GW3965 (1μM)	3.35321	3.42592	2.71814	3.165754	0.389345	9.63	0.01	**
GW3965 (5μM)	2.51479	2.31333		2.414063	0.142455	14.04	0.01	**
22(S)-HC	0.69478	1.22659	0.28355	0.734973	0.472803			

Figure 4.3D	Exp 1	Exp 2	Exp 3					
samples	Fold Ind	Fold Ind	Fold Ind	Mean	SD	t-test	P-value	
DMSO	1	1	1	1				
GW3965 (1μM)	2.31333	1.65903	2.13547	2.035943	0.338318	5.30	0.05	*
GW3965 (5μM)	1.88903	1.76891		1.828971	0.084934	13.80	0.001	***
22(S)-HC	1.21633	0.60531	1.17437	0.998672	0.341302			

Figure 4.4C	Exp 1	Exp 2	
samples	Fold Ind	Fold Ind	Average
DMSO	1	1	1
T1317 (1μM)	21.05673	19.32215	20.18944
T1317 (10μM)	27.56209	24.36016	25.96112
22(S)-HC	1.75786	1.30271	1.53029

Figure 4.4D	Exp 1	Exp 2	
samples	Fold Ind	Fold Ind	Average
DMSO	1	1	1
T1317(1μM)	3.51148	3.18047	3.345975
T1317(10μM)	4.92417	5.67440	5.299286
22(S)-HC	1.34818	1.18435	1.266264

Figure 4.5C	ABCA1	B-actin		
samples	Raw value	Raw value	Ratio	Fold Ind.
C	9912.18	190544.96	0.05202	1
0.5μM	174487.45	222145.94	0.78546	15.0992
1μM	162946.10	162112.66	1.00514	19.3221
5μM	166227.46	159139.19	1.04454	20.0795
7.5μM	190703.20	163872.45	1.16373	22.3707
10μM	187147.78	147683.80	1.26722	24.3602

Figure 4.5D	apoE	B-actin		
samples	Raw value	Raw value	Ratio	Fold Ind.
C	31413.07	190544.96	0.16486	1
0.5μM	141234.64	222145.94	0.63577	3.8565
1μM	85000.56	162112.66	0.52433	3.1805
5μM	130093.34	159139.19	0.81748	4.9587
7.5μM	155012.44	163872.45	0.94593	5.7378
10μM	138154.75	147683.80	0.93548	5.6744

Figure 4.6C	ABCA1	B-actin		
samples	Raw value	Raw value	Ratio	Fold Ind.
C	6737.20	139549.53	0.04828	1
0.5h	9190.58	132352.88	0.06944	1.43833
1h	11242.96	113355.15	0.09918	2.05442
3h	136305.17	117339.31	1.16163	24.06121
6h	312152.60	145712.30	2.14225	44.37306
12h	333882.65	132545.51	2.51900	52.17681
24h	552523.58	122139.58	4.52371	93.70075

Figure 4.6D	apoE	B-actin		
samples	Raw value	Raw value	Ratio	Fold Ind.
C	17469.83	139549.53	0.12519	1
0.5h	15938.83	132352.88	0.12043	0.96197
1h	26225.55	113355.15	0.23136	1.84809
3h	79349.57	117339.31	0.67624	5.40183
6h	162565.64	145712.30	1.11566	8.91194
12h	184029.51	132545.51	1.38843	11.09078
24h	316659.38	122139.58	2.59260	20.70978

Figure 4.7C	ABCA1	B-actin		
samples	Raw value	Raw value	Ratio	Fold Ind.
C	5753.10	142236.18	0.04045	1
0.5h	3544.36	135680.04	0.02612	0.64585
1h	4600.20	129794.74	0.03544	0.87625
3h	23959.17	118072.11	0.20292	5.01687
6h	106432.73	113179.77	0.94039	23.2495
12h	159614.30	132781.75	1.20208	29.7195
24h	411953.39	163004.11	2.52726	62.4824

Figure 4.7D	apoE	B-actin		
samples	Raw value	Raw value	Ratio	Fold Ind.
C	21732.25	142236.18	0.15279	1
0.5h	33107.81	135680.04	0.24401	1.59706
1h	28483.87	129794.74	0.21945	1.43631
3h	60499.78	118072.11	0.51240	3.35361
6h	98971.88	113179.77	0.87447	5.72333
12h	214083.51	132781.75	1.61230	10.5524
24h	332626.50	163004.11	2.04060	13.3556

Figure 4.8C	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C	0.106475	0.154186	0.144309	0.134990	0.025183			
22(R)-HC	1	1	1	1		59.49	0.001	***
22S	0.155529	0.151596	0.247685	0.184937	0.054377			
SP-	0.128655	0.060922	0.079707	0.089761	0.034968			
SP+	0.124190	0.260891	0.306929	0.230670	0.095044	14.02	0.001	***
Curc-	0.105374	0.030404	0.075694	0.070491	0.037755			
Curc+	0.021895	0.013595	0.082478	0.039323	0.037603	44.25	0.001	***

Figure 4.8D	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C	0.21221	0.13446	0.45340	0.266688	0.166301			
22(R)-HC	1	1	1	1		7.64	0.01	**
22S	0.25792	0.18797	0.39390	0.279933	0.104715			
SP-	0.31819	0.04390	0.12908	0.163722	0.140389			
SP+	0.23846	0.26263	0.30541	0.268834	0.033903	37.35	0.001	***
Curc-	0.14976	0.22454	0.10991	0.161405	0.058197			
Curc+	0.18183	0.21087	0.13981	0.177506	0.035725	39.88	0.001	***

Figure 4.9C	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C	0.13052	0.14069	0.01376	0.09499	0.07053			
9CRA+22R	1	1	1	1		22.22	0.001	***
22S	0.15459	0.07801	0.01593	0.08284	0.06946			
SP-	0.31298	0.06845	0.01869	0.13337	0.15752			
SP+	0.24995	0.47096	0.37594	0.36562	0.11087	9.91	0.01	**
Curc-	0.11753	0.01356	0.01808	0.04972	0.05876			
Curc+	0.10275	0.50907	0.01531	0.20904	0.26349	5.20	0.05	*

Figure 4.9D	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C	0.32614	0.38002	0.22397	0.310042	0.079264			
9CRA+22R	1	1	1	1		15.08	0.001	***
22S	0.34856	0.18803	0.42339	0.319991	0.120254			
SP-	0.33623	0.18317	0.21958	0.246326	0.079958			
SP+	0.10203	0.57781	0.42690	0.368917	0.243132	4.50	0.05	*
Curc-	0.08265	0.42128	0.22839	0.244107	0.169863			
Curc+	0.10848	0.66114	0.38750	0.385707	0.276337	3.85	0.05	*

Figure 4.10C	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C	0.36790	0.04040	0.29325	0.233851	0.171640			
GW3965	1	1	1	1		7.73	0.01	**
22S	0.10432	0.08556	0.21558	0.135149	0.070280			
SP-	0.38752	0.01389	0.08515	0.162187	0.198371			
SP+	0.34261	0.22851	0.14976	0.240295	0.096964	13.57	0.001	***
Curc-	0.26664	0.00135	0.07387	0.113953	0.137116			
Curc+	0.03222	0.02299	0.16923	0.074814	0.081897	19.57	0.001	***

Figure 4.10D	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C	0.60276	0.36917	0.38179	0.45124	0.131375			
GW3965	1	1	1	1		7.23	0.01	**
22S	0.34225	0.51183	0.34202	0.39870	0.097974			
SP-	0.39973	0.21057	0.27986	0.29672	0.095702			
SP+	0.38066	0.42219	0.28017	0.36101	0.073022	15.16	0.001	***
Curc-	0.25406	0.38819	0.22903	0.29043	0.085585			
Curc+	0.19979	0.32679	0.15660	0.22773	0.088464	15.12	0.001	***

Figure 4.11C	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average
C	0.15162	0.31612	0.23387
T1317	1	1	1
22S	0.16651	0.25775	0.212133
SP-	0.01500	0.04570	0.030351
SP+	0.23953	0.28318	0.261352
Curc-	0.10030	0.14419	0.122246
Curc+	0.08229	0.02023	0.051261

Figure 4.11D	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average
C	0.43963	0.34036	0.389995
Lig	1	1	1
22S	0.45648	0.54870	0.502587
SP	0.36578	0.32251	0.344146
SP+	0.49023	0.42502	0.457625
Curc	0.21611	0.12841	0.17226
Curc+	0.41946	0.26124	0.340352

Figure 4.12C	ABCA1	B-actin		
samples	Raw value	Raw value	Ratio	Rel Exp
C	5529.31	146504.69	0.03774	0.009972
Lig	512234.02	135347.85	3.78457	1
+SP25	265591.08	137666.91	1.92923	0.509761
+SP50	271864.45	142040.30	1.91400	0.505736
+SP75	42579.35	117087.03	0.36366	0.096089
+SP100	57874.18	163114.39	0.35481	0.093751

Figure 4.12D	apoE	B-actin		
samples	Raw value	Raw value	Ratio	Rel Exp
C	40445.70	146504.69	0.27607	0.329933
Lig	113252.00	135347.85	0.83675	1
+SP15	198272.81	137666.91	1.44024	1.721231
+SP25	144399.03	137666.91	1.04890	1.253546
+SP50	83699.44	142040.30	0.58927	0.704233
+SP75	44354.74	117087.03	0.37882	0.452727
+SP100	24913.65	163114.39	0.15274	0.182537

Figure 4.13C	ABCA1	B-actin		
samples	Raw value	Raw value	Ratio	Rel Exp
C	38668.515	148149.24	0.26101	0.107598
Lig	321929.16	132710.81	2.42579	1
+SP15	227140.03	128404.42	1.76894	0.729222
+SP25	153098.12	149896.91	1.02136	0.421040
+SP50	116287.07	142030.76	0.81875	0.337517
+SP75	124029.30	120956.26	1.02541	0.422709
+SP100	96016.510	167467.38	0.57334	0.236353

Figure 4.13D	apoE	B-actin		
samples	Raw value	Raw value	Ratio	Rel Exp
C	14564.61	148149.24	0.09831	0.060310
Lig	216331.23	132710.81	1.63009	1
+SP15	231344.43	128404.42	1.80169	1.105264
+SP25	187383.07	149896.91	1.25008	0.766875
+SP50	100345.18	142030.76	0.70650	0.433412
+SP75	37662.07	120956.26	0.31137	0.191013
+SP100	28091.96	167467.38	0.16775	0.102906

Figure 4.14C	ABCA1	B-actin		
samples	Raw value	Raw value	Ratio	Rel Exp
C	6964.29	146504.69	0.04754	0.011848
Lig	543031.24	135347.85	4.01212	1
+Curc15	194566.36	137666.91	1.41331	0.352261
+Curc25	48562.90	142040.30	0.34190	0.085216
+Curc35	4356.58	117087.03	0.03721	0.009274
+Curc50	2624.35	163114.39	0.01609	0.004010

Figure 4.14D	apoE	B-actin		
samples	Raw value	Raw value	Ratio	Rel Exp
C	5891.39	146504.69	0.04021	0.055314
Lig	98396.75	135347.85	0.72699	1
+Curc15	20856.19	137666.91	0.15150	0.208390
+Curc25	22633.71	142040.30	0.15935	0.219187
+Curc35	9712.83	117087.03	0.08295	0.114106
+Curc50	5814.38	163114.39	0.03565	0.049032

Figure 4.15C	ABCA1	B-actin		
samples	Raw value	Raw value	Ratio	Rel exp
C	51668.84	124119.10	0.41628	0.176815
Lig	247934.42	105309.16	2.35435	1
+Curc15	220917.26	103186.80	2.14094	0.909358
+Curc25	172179.18	105364.29	1.63413	0.694091
+Curc35	35561.85	130135.72	0.27327	0.116069
+Curc50	2622.47	122180.55	0.02146	0.009117

Figure 4.15D	apoE	B-actin		
samples	Raw value	Raw value	Ratio	Rel exp
C	83153.71	124562.91	0.66756	0.280697
Lig	253413.89	106555.36	2.37824	1
+Curc15	183658.99	94092.45	1.95190	0.820734
+Curc25	101908.16	96400.10	1.05714	0.444505
+Curc35	93749.17	127562.43	0.73493	0.309022
+Curc50	56200.98	112780.71	0.49832	0.209534

Figure 4.16C	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C	0.10648	0.14795	0.07008	0.108169	0.038965			
22(R)-HC	1	1	1	1		39.64	0.001	***
22S	0.15553	0.22118	0.58194	0.319551	0.229598			
LY-	0.26894	0.00553	0.32422	0.199562	0.170293			
LY+	0.23680	0.15700	0.25036	0.214720	0.050444	26.96	0.001	***

Figure 4.16D	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C	0.21221	0.24897	0.46693	0.30937	0.137685			
22(R)-HC	1	1	1	1		8.69	0.01	**
22S	0.25792	0.45468	0.46205	0.391551	0.115785			
LY-	0.17342	0.06450	0.22184	0.153255	0.080585			
LY+	0.23848	0.15388	0.40156	0.26464	0.125897	10.12	0.01	**

Figure 4.17C	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C	0.13052	0.11669	0.06617	0.104458	0.033877			
9CRA+22R	1	1	1	1		45.79	0.001	***
22S	0.15459	0.16902	0.05108	0.12490	0.064334			
LY-	0.22029	0.07557	0.09339	0.12975	0.078912			
LY+	0.14375	0.33688	0.07984	0.186821	0.133822	10.52	0.01	**

Figure 4.17D	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C	0.32614	0.40621	0.30411	0.345488	0.053731			
9CRA+22R	1	1	1	1		21.10	0.001	***
22S	0.34856	0.51135	0.34762	0.402512	0.094257			
LY-	0.09024	0.36128	0.11554	0.189019	0.149715			
LY+	0.24815	0.52963	0.20335	0.327044	0.176869	6.59	0.01	**

Figure 4.18C	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C	0.36790	0.02317	0.29189	0.2276539	0.181120			
GW3965	1	1	1	1		7.39	0.01	**
22S	0.10432	0.09202	0.20123	0.1325196	0.059818			
LY-	0.33292	0.05660	0.09169	0.1604022	0.150434			
LY+	0.28208	0.10710	0.37632	0.255168	0.1366152	9.44	0.010	**

Figure 4.18D	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
control	0.60276	0.38426	0.46828	0.485101	0.11022			
GW3965	1	1	1	1		8.09	0.01	**
22S	0.36486	0.42535	0.54993	0.446714	0.094368			
LY-	0.31154	0.12995	0.38092	0.274137	0.129597			
LY+	0.16695	0.44356	0.45247	0.354327	0.162335	6.89	0.01	**

Figure 4.19C	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average
C	0.24143	0.14217	0.1918
T1317	1	1	1
22S	0.27483	0.10653	0.1907
LY-	0.09469	0.07960	0.0871
LY+	0.14970	0.08807	0.1189

Figure 4.19D	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average
C	0.35173	0.27063	0.311184
T1317	1	1	1
22S	0.47225	0.55350	0.512873
LY-	0.26185	0.29552	0.278685
LY+	0.35887	0.44607	0.402470

Figure 4.20C	ABCA1	B-actin		
samples	Raw value	Raw value	Ratio	Rel Exp
C	3766.57	149351.25	0.02522	0.004877
Lig	547251.83	105832.95	5.17090	1
+LY25	315150.41	115384.09	2.73132	0.528209
+LY50	320800.48	105832.10	3.03122	0.586207
+LY75	265737.46	115380.11	2.30315	0.445405
+LY100	263158.31	105834.68	2.48650	0.480865

Figure 4.20D	apoE	B-actin		
samples	Raw value	Raw value	Ratio	Rel exp
C	53823.47	149351.25	0.36038	0.169338
Lig	225231.60	105832.95	2.12818	1
+LY25	95128.40	115384.09	0.82445	0.387397
+LY50	61949.01	105832.10	0.58535	0.275048
+LY75	54537.14	115380.11	0.47267	0.222102
+LY100	33480.92	105834.68	0.31635	0.148649

Figure 4.21C	ABCA1	B-actin		
samples	Raw value	Raw value	Ratio	Rel Exp
C	21716.07	130626.16	0.16625	0.081981
Lig	188938.19	93171.53	2.02785	1
+LY25	93299.72	108547.53	0.85953	0.423861
+LY50	55420.25	120656.33	0.45932	0.226507
+LY75	49575.06	124308.48	0.39881	0.196664
+LY100	48832.84	143904.15	0.33934	0.167341

Figure 4.21D	apoE	B-actin		
samples	Raw value	Raw value	Ratio	Rel exp
C	141300.51	130626.16	1.08172	0.405420
Lig	248594.28	93171.53	2.66814	1
+LY25	138435.46	108547.53	1.27534	0.477991
+LY50	71302.31	120656.33	0.59095	0.221486
+LY75	60161.45	124308.48	0.48397	0.181388
+LY100	37566.32	143904.15	0.26105	0.097840

Figure 4.22C	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average
C	1	1	1
22(R)-HC	2.42825	3.25992	2.84408
9CRA		2.14505	2.14505
22(R)-HC+9CRA	5.85906	8.24860	7.05383
GW3965	4.63100	5.77934	5.20517
T1317	7.33582	5.87168	6.60375
22S	1.20030	1.36268	1.28149

Figure 4.22D	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average
C	1	1	1
22(R)-HC	3.45038	3.01094	3.23066
9CRA		2.33783	2.33783
22(R)-HC+9CRA	3.13300	3.28009	3.20654
GW3965	2.65336	2.89375	2.77355
T1317	3.50923	2.64703	3.07813
22S	1.35401	0.85213	1.10307

Figure 4.23C	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average
C	0.27364	0.19087	0.2323
9CRA+22R	1	1	1
SP	0.41372	0.30384	0.35878
SP+	0.40556	0.18761	0.29658
Curc	0.03302	0.01706	0.02504
Curc+	0.04838	0.01243	0.03040
LY	0.55480	0.07355	0.31418
LY+	0.05897	0.21275	0.13586

Figure 4.23D	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average
C	0.33526	0.28025	0.30776
9CRA+22R	1	1	1
SP	0.22925	0.27013	0.24969
SP+	0.35077	0.15849	0.25463
Curc	0.40463	0.35593	0.38028
Curc+	0.34614	0.23256	0.28935
LY	0.27079	0.09709	0.18394
LY+	0.17122	0.01661	0.09392

Figure 4.24A	Exp 1	Exp 2	
samples	Fold Ind	Fold Ind	Average
WT ABCA1			
UT	1	1	1
T	5.68490	6.45706	6.070981
Mut ABCA1			
UT	1	1	1
T	0.53968	0.01221	0.275949

Figure 4.24B	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5					
samples	Fold Ind	Fold Ind	Fold Ind	Fold Ind	Fold Ind	Mean	SD	t-test	P-value	
9CRA/22RHC										
UT	1	1	1	1	1	1				
T	35.64191	37.62522	55.72278	17.76852	45.53798	38.45928	14.00783	5.98	0.01	**
T1317						Average				
UT	1	1				1				
T	8.96322	13.53161				11.24742				

Figure 4.25	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
pcDNA3								
UT	1	1	1	1				
T	14.88867	9.40993	11.12610	11.80823	2.802341			
DN LXR								
UT	1	1	1	1				
T	4.19291	4.74135	2.47496	3.803076	1.182417	4.56	0.05	*
pcDNA3								
UT	1	1	1	1				
T	9.88867	7.40993	6.12610	7.80823	1.912645	3.70	0.05	*
DN LXR								
UT	1	1	1	1				
T	3.71940	3.95684	2.49744	3.391229	0.783096	5.01	0.05	*

	P-46		
Figure 5.2B	Exp 1	Exp 2	
samples	FoI Ind	FoI Ind	Average p-46
0h	1	1	1
15min	2.50037	2.61396	2.557165
30min	1.76100	1.52208	1.641539
45min	1.53369	1.32906	1.431378
60min	0.65980	0.48357	0.571685
90min	0.45135	0.32730	0.389323
180min	0.45032	0.12404	0.287176

	P-54		
Figure 5.2B	Exp 1	Exp 2	
samples	FoI Ind	FoI Ind	Average p-54
0h	1	1	1
15min	1.76080	1.92520	1.843002
30min	1.59756	1.83948	1.718522
45min	1.94990	2.20575	2.077826
60min	1.52645	1.76625	1.646350
90min	1.72206	2.02927	1.875663
180min	1.73343	1.84294	1.788186

Figure 5.3B	Exp 1	Exp 2	
samples	FoI Ind	FoI Ind	Average
0h	1	1	1
15min	1.96633	1.46761	1.716972
30min	1.75957	1.47038	1.614975
45min	0.93234	0.69982	0.816079
60min	1.79182	1.13932	1.465572
90min	2.36069	1.21987	1.790283
180min	0.84640	0.62378	0.735090

	P-46		
Figure 5.4B	Exp 1	Exp 2	
samples	FoI Ind	FoI Ind	Average p-46
0h	1	1	1
1/2h	1.30871	1.28997	1.299342
1h	0.84841	0.90321	0.875806
3h	0.95616	1.06192	1.009039
6h	0.59608	0.71804	0.657059
12h	0.11049	0.16277	0.136628
24h	0.41467	0.58104	0.497854

Figure 5.4B	P-54 Exp 1	P-54 Exp 2	
samples	Fol Ind	Fol Ind	Average p-54
0h	1	1	1
1/2h	1.45659	1.48671	1.47165
1h	0.93225	0.99082	0.96154
3h	0.47171	0.52694	0.49932
6h	0.59520	0.60134	0.59827
12h	0.76356	1.10575	0.93465
24h	1.22914	1.55213	1.39063

Figure 5.5B	Exp 1	Exp 2	
samples	Fold Ind	Fold Ind	Average
0h	1	1	1
1/2h	1.84249	1.59569	1.719091
1h	2.97560	3.58901	3.282304
3h	2.94785	2.67468	2.811268
6h	1.92745	2.76973	2.348592
12h	1.42085	2.96261	2.191727
24h	4.31343	6.41282	5.363125

Figure 5.6B	Exp 1	Exp 2	
samples	Fold Ind	Fold Ind	Average
0h	1	1	1
1/2h	1.94917	1.15580	1.552488
1h	1.12902	1.10351	1.116264
3h	0.24334	0.66457	0.453953
6h	0.64038	0.28851	0.464444
12h	0.78179	0.39823	0.59001
24h	1.23191	0.72060	0.976253

Figure 5.7D	P-46 Exp 1	P-46 Exp 2	
samples	Rel Exp	Rel Exp	Average p-46
C	0.24378	0.41623	0.33000
Lig	1	1	1
SP50	0.33750	0.41790	0.37770
SP50+	0.07922	0.30719	0.19321
SP100	0.32024	0.24003	0.28013
SP100+	0.39358	0.32505	0.35932

	P-54		
Figure 5.7D	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average p-54
C	0.41544	0.58051	0.49797
Lig	1	1	1
SP50	0.15016	0.23817	0.19416
SP50+	0.10149	0.27217	0.18683
SP100	0.18546	0.53977	0.36262
SP100+	0.33571	0.57442	0.45507

Figure 5.7E	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C	0.60108	0.69690	0.62082	0.63960	0.050598			
Lig	1	1	1	1		10.07	0.01	**
SP50	0.45171	0.49697	0.67989	0.54285	0.120811			
SP+50	0.33350	0.36514	0.48432	0.39432	0.079529	10.77	0.01	**
SP100	0.41802	0.35013	0.33639	0.36818	0.043706			
SP100+	0.09630	0.33449	0.26649	0.23242	0.122695	8.85	0.01	**

Figure 5.7F	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average
C	0.33914	0.49925	0.41920
Lig	1	1	1
SP50	0.18084	0.16902	0.17493
SP50+	0.34034	0.16215	0.25124
SP100	0.13706	0.17658	0.15682
SP100+	0.08317	0.11248	0.09782

	P-46							
Figure 5.8D	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean p-46	SD	t-test	P-value	
C	0.63433	0.53211	0.16295	0.4431291	0.247966			
Lig	1	1	1	1		3.89	0.05	*
Curc	4.93215	5.54175	4.40385	4.9592488	0.569432			
Curc+	3.79002	3.46771	2.83494	3.3642275	0.485877			

	P-54							
Figure 5.8D	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean p-54	SD	t-test	P-value	
C	0.69558	0.42931	0.444463	0.5231179	0.149547			
Lig	1	1	1	1		5.52	0.05	*
Curc	1.71711	1.64163	1.350103	1.5696158	0.193814			
Curc+	1.66709	1.43324	1.305258	1.4685321	0.183481			

Figure 5.8E	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average
C	0.59625	0.50530	0.55078
Lig	1	1	1
Curc	0.95516	0.59843	0.77680
Curc+	2.69693	2.84259	2.76976

Figure 5.8F	Exp 1	Exp 2	Exp 3	Exp 4					
samples	Rel Exp	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C	0.73036	0.65897	0.88048	0.78593	0.76394	0.093474			
Lig	1	1	1	1	1		5.05	0.01	**
Curc	1.15387	1.01335	2.02633	1.17667	1.34255	0.399703			
Curc+	0.84792	0.97152	2.20120	1.15994	1.29514	0.534780			

Figure 5.9B	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average
C	0.41987	0.39690	0.40838
Lig	1	1	1
Curc	0.04367	0.32166	0.18267
Curc+	0.13292	0.24401	0.18847

Figure 5.10	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
pcDNA3								
UT	1	1	1	1				
T	15.88867	8.40993	12.12610	12.14157	3.739392	5.16	0.05	*
DN JNK/SAPK								
UT	1	1	1	1				
T	12.00598	19.08112	13.80307	14.96339	3.67752			
DN SEK-1								
UT	1	1	1	1				
T	4.01527	4.71168	5.60488	4.777275	0.796832	3.33617	0.05	*
DN c-Jun								
UT	1	1	1	1				
T	2.96661	2.04218	3.93621	2.981666	0.947106	4.11291	0.05	*

	pcDNA3			
Figure 5.11B	apoE	Bactin		
samples	Raw value	Raw value	Ratio	Fold Ind
C	46273.24	230471.47	0.20078	1
Lig	108043.61	177208.30	0.60970	3.036703
22(S)-HC	35813.30	184628.91	0.19397	0.966122
	DN JNK			
	apoE	Bactin		
samples	Raw value	Raw value	Ratio	Fold Ind
C	29105.06	168591.23	1	1
Lig	30566.97	106900.87	0.634083	0.634083
22(S)-HC	60354.86	153556.41	0.910821	0.910821

Figure 5.12B	Exp 1	Exp 2	
samples	Fold Ind	Fold Ind	Average
C	1	1	1
Lig	2.15450	1.80754	1.98102
C	0.58405	1.08590	0.83498
Lig	1.11867	1.12770	1.12318

Figure 6.2B	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
0h	1	1	1	1				
15min	1.84168	1.87657	1.53563	1.75129	0.187579	6.94	0.01	**
30min	1.83407	2.74168	2.22832	2.26803	0.455107	4.83	0.05	*
45min	1.00726	1.76657	2.13552	1.636452	0.575272			
60min	2.61325	3.26806	2.30081	2.727372	0.493618	6.06	0.01	**
90min	1.69135	1.95346	2.26652	1.970443	0.287959	5.84	0.01	**
180min	0.48005	0.50855	0.71004	0.566217	0.125371	5.99	0.01	**

Figure 6.3B	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
Control	1	1	1	1				
Lig	2.95064	1.98808	2.13958	2.359436	0.517573	4.55	o.o5	

Figure 6.4B	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
Control	0.17800	0.50493	0.46738	0.383436	0.178902			
Lig	1	1	1	1		5.97	0.01	**
LY-	0.36130	0.10943	0.06190	0.17754	0.160904			
LY+	0.33879	0.12095	0.10968	0.189807	0.129149	10.87	0.01	**

Figure 6.5B	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
control	1	1	1	1				
Lig	2.28330	2.73446	2.59290	2.536885	0.230736	11.54	0.01	**

Figure 6.6B	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
control	0.60286	0.36570	0.30750	0.425356	0.156455			
Lig	1	1	1	1		6.36	0.01	**
LY	0.25812	0.32407	0.64962	0.410604	0.2096			
LY+	0.20253	0.33829	0.52142	0.354082	0.160026	6.99	0.01	**

Figure 6.7	Exp 1	Exp 2	Exp 3					
samples	Fold Ind	Fold Ind	Fold Ind	Mean	SD	t-test	P-value	
pcDNA3								
UT	1	1	1	1	0			
T	15.88867	8.40993	12.12610	12.14157	3.739392			
DN PKB				Mean				
UT	1	1	1	1	0			
T	6.02230	4.01379	5.01627	5.017453	1.004253	3.19	0.05	*

Figure 6.8C	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C	0.01107	0.02430	0.02958	0.021649	0.009538			
Lig	1	1	1	1				
BIM	-0.00016	-0.00007	-0.00014	-0.00012	0.009538			
BIM+	0.11092	0.30900	0.40207	0.273996	0.148695	8.46	0.01	**
BIM	-0.00016	-0.00006	-0.00011	-0.00011	0.009538			
BIM+	0.05278	0.01964	0.27787	0.116764	0.140502	10.89	0.01	**

Figure 6.8D	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C	0.16079	0.23696	0.18563	0.194459	0.0388457			
Lig	1	1	1	1		35.92	0.001	***
BIM	0.04816	0.06919	0.02568	0.047675	0.021760			
BIM+	0.28131	0.42305	0.59994	0.434769	0.1596382	6.13	0.01	**
BIM	0.03944	0.07868	0.02028	0.046135	0.0297722			
BIM+	0.19728	0.21137	0.52582	0.311489	0.1857473	6.42	0.01	**

Figure 6.9C	ABCA1	B-actin		
samples	Raw value	Raw value	Ratio	Rel Exp
C	8876.96	142254.35	0.06240	0.03566
Lig	233245.10	133302.20	1.74975	1
Rapa500	-68.41	150852.96	-0.00045	-0.00026
Rapa500+	176633.64	140508.13	1.25711	0.71845
Rapa150	128.37	127457.02	0.00101	0.00058
Rapa150+	195204.75	123774.99	1.57709	0.90133

Figure 6.9D	apoE	B-actin		
samples	Raw value	Raw value	Ratio	Rel Exp
C	76315.39	142254.35	0.53647	0.430383
Lig	166160.73	133302.20	1.24650	1
Rapa500	27317.26	150852.96	0.18109	0.145275
Rapa500+	123605.64	140508.13	0.87970	0.705742
Rapa150	31928.81	127457.02	0.25051	0.200968
Rapa150+	124870.07	123774.99	1.00885	0.809346

Figure 6.10C	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average
C	0.08491	0.01906	0.051989
Lig	1	1	1
Gö6983	-0.00011	-0.00021	-0.000158
Gö6983+	0.73395	0.81050	0.772222
Gö6983	-0.00008	-0.00021	-0.000144
Gö6983+	0.53445	0.41398	0.474218

Figure 6.10D	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average
C	0.39028	0.19095	0.2906159
Lig	1	1	1
Gö6983	0.15616	0.10555	0.1308526
Gö6983+	0.64875	0.77230	0.7105235
Gö6983	0.12777	0.08625	0.1070105
Gö6983+	0.45669	0.52947	0.493081

Figure 6.11C	Exp 1	Exp 2	Exp 3	Exp 4		
samples	Rel Exp	Rel Exp	Rel Exp	Rel Exp	Mean	SD
C	0.01922	0.03413	0.07207	0.06201	0.046859	0.024434
Lig	1	1	1	1	1	
Gö6976+	0.78740	1.60225	0.36832	0.92477	0.920685	0.512323
Gö6976+	0.63653	1.59738	0.44883	0.73526	0.854499	0.509305
Gö6976+	0.56797	1.96383	0.34461	0.95617	0.958147	0.716489
Gö6976+	0.54797	1.74120	0.34842	0.89050	0.882021	0.614974

Figure 6.11D	Exp 1	Exp 2	Exp 3	Exp 4		
samples	Rel Exp	Rel Exp	Rel Exp	Rel Exp	Mean	SD
C	0.34631	0.10630	0.28859	0.33846	0.269916	0.112033
Lig	1	1	1	1	1	
Gö6976+	1.32577	1.66951	0.82094	1.00223	1.204616	0.373708
Gö6976+	1.12735	1.59670	1.02566	0.94727	1.174245	0.291125
Gö6976+	1.54450	1.43906	1.05470	1.17348	1.302934	0.227498
Gö6976+	1.25374	1.10153	0.96947	0.92549	1.062557	0.147787

Figure 6.12C	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5					
samples	Rel Exp	Rel Exp	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C	0.06717	0.00223	0.01118	0.14994	0.03394	0.052892	0.059769			
Lig	1	1	1	1	1	1				
Rott+	0.27136	0.26355	0.88757	0.86048	1.10435	0.677462	0.386042	1.87		
Rott+	0.37912	0.18559	0.91098	0.88246	0.32272	0.536174	0.336724	3.08	0.05	*
Rott+	0.57689	0.13841	0.73665	0.76624	0.44065	0.531768	0.255943	4.09	0.01	**
Rott+	0.05183	0.10169	0.38082	0.92356	0.30824	0.353227	0.347194	4.17	0.01	**

Figure 6.12D	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5		
samples	Rel Exp	Rel Exp	Rel Exp	Rel Exp	Rel Exp	Mean	SD
C	0.29038	0.06317	0.16539	0.31468	0.33801	0.234328	0.116614
Lig	1	1	1	1	1	1	
Rott+	0.64897	1.21814	1.15004	1.33630	1.23788	1.118266	0.270677
Rott+	0.39568	1.75432	1.09932	1.30775	0.47125	1.005662	0.574064
Rott+	0.37776	1.80430	1.06921	1.03164	0.60015	0.976611	0.547042
Rott+	0.30938	1.31716	0.72343	0.83916	0.43292	0.724412	0.394252

Figure 6.13A	Exp 1	Exp 2	Exp 3					
samples	Fold Ind	Fold Ind	Fold Ind	Mean	SD	t-test	P-value	
pCDNA3	0.04471	0.29326	0.07618	0.138053	0.135332			
DN PKCε	0.04471	0.29326	0.07618	0.1380526	0.135332			
pCDNA3+Lig	1	1	1	1				
DN PKCε+Lig	0.11021	0.23084	0.33845	0.2264995	0.11418	11.73	0.01	**

Figure 6.13B	Exp 1	Exp 2	Exp 3		
samples	Fold Ind	Fold Ind	Fold Ind	Mean	SD
pCDNA3	0.04471	0.29326	0.07618	0.138053	0.135332
DN PKCλ	0.04471	0.29326	0.07618	0.1380526	0.135332
pCDNA3+Lig	1	1	1	1	
DN PKCλ +Lig	8.51495	10.70830	7.71972	8.980991	1.547834

Figure 6.14	pcDNA3	PKCαDN	PKCδDN	PKCηDN	PKCξDN
samples	Rel Exp	Rel Exp	Rel Exp	Rel Exp	Rel Exp
UT	0.02908	0.07350	0.08311	1.218490	7.020931
T	1	0.21446	0.35552	3.9047216	22.42197

Figure 7.3B	Exp 1	Exp 2	Exp 3	Exp 4					
samples	Rel Exp	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C 1h	0.47501	0.77388	0.83039	0.48291	0.64055	0.188033			
Lig	1	1	1	1	1		3.82	0.05	*
SP50			0.49674	0.22418	0.36046	0.192725			
SP50+			0.53194	0.33121	0.43157	0.141939	5.66	0.05	*
SP100	0.15044	0.01637	0.61039	0.48243	0.31491	0.277818			
SP100+	0.14647	0.01630	0.66485	0.34421	0.29296	0.282212	5.01	0.01	**

	P-46		
Figure 7.4C	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average p-46
C24h	0.09775	0.02874	0.063244
Lig	1	1	1
LY25		0.04842	0.04842
LY25+		0.19102	0.19102
LY50	0.22219	0.04312	0.13265
LY50+	1.83360	1.40366	1.61863
LY100	0.34027	0.11474	0.22751
LY100+	0.51191	0.29008	0.40099

	P-54		
Figure 7.4C	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average p-54
C24h	0.424124	0.439093	0.431609
Lig	1	1	1
LY25		0.417133	0.417133
LY25+		1.473149	1.473149
LY50	0.592707	0.204468	0.398587
LY50+	2.413024	2.044029	2.228527
LY100	0.447441	0.426464	0.436952
LY100+	0.499990	1.321354	0.910672

Figure 7.4D	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C24h	0.66561	0.58488	0.60086	0.61712	0.042749			
Lig	1	1	1	1		15.51	0.001	***
LY25		0.42333	0.22020	0.32176	0.143637			
LY25+		1.18421	0.73875	0.96148	0.314984			
LY50		0.39648	0.05344	0.22496	0.242564			
LY50+		1.06888	0.39689	0.73289	0.475169			
LY100	0.04154		0.13828	0.08991	0.068406			
LY100+	0.31762		0.68472	0.50117	0.25958			

Figure 7.5B	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C1h	0.76596	0.59996	0.45983	0.608583	0.15325			
Lig	1	1	1	1		4.42	0.05	*
BIM	1.31379	1.37393	1.39864	1.362118	0.043643			
BIM+	1.53547	1.73738	1.52848	1.600444	0.118639	8.77	0.01	**

	P-46							
Figure 7.6B	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean p-46	SD	t-test	P-value	
C24h	0.33117	0.26811	0.36268	0.32065	0.0481576			
Lig	1	1	1	1		24.43	0.001	***
BIM	0.33798	0.13361	0.11228	0.19462	0.1246091			
BIM+	0.37492	0.11841	0.32738	0.27357	0.136459	9.22	0.01	**
	P-54							
Figure 7.6B	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean p-54	SD	t-test	P-value	
C24h	0.51560	0.67169	0.60505	0.597448	0.078322			
Lig	1	1	1	1		8.90	0.01	**
BIM	0.44041	0.15111	0.07439	0.221970	0.193023			
BIM+	0.50235	0.30552	0.19545	0.334438	0.155482	7.41	0.001	***

Figure 7.7C	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C24h	0.51797	0.67166	0.35999	0.51654	0.155843			
Lig	1	1	1	1		5.37	0.05	*
BIM	0.13937	0.08323	0.24205	0.154885	0.08054			
BIM+	0.30350	0.32150	0.15862	0.261205	0.089297	14.33	0.001	***
Figure 7.7D	Exp 1	Exp 2	Exp 3					
samples	Rel Exp	Rel Exp	Rel Exp	Mean	SD	t-test	P-value	
C24h	0.36593	0.64706	0.54263	0.518541	0.142105			
Lig	1	1	1	1		5.87	0.01	**
BIM	0.20344	0.35023	0.07052	0.208063	0.139913			
BIM+	0.27052	0.39188	0.16584	0.276083	0.113123	11.08	0.01	**

	P-46		
Figure 7.8B	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average p-46
C	0.10194	0.03487	0.06841
Lig	1	1	1
Rott	0.06249	0.54665	0.30457
Rott+	0.21732	0.17224	0.19478
Gö6976	0.47556	0.31863	0.39710
Gö6976+	0.58004	0.40408	0.49206
Gö6983	2.65076	0.78106	1.71591
Gö6983+	3.51683	3.20008	3.35846
Gö6983	2.14176	0.65590	1.39883
Gö6983+	1.78738	4.33632	3.06185

	P-54		
Figure 7.8B	Exp 1	Exp 2	
samples	Rel Exp	Rel Exp	Average p-54
C	0.10670	0.21036	0.15853
Lig	1	1	1
Rott	0.04267	0.43681	0.23974
Rott+	0.09349	0.39098	0.24224
Gö6976	0.55261	0.58240	0.56750
Gö6976+	0.44242	0.58335	0.51288
Gö6983	1.27904	0.82342	1.05123
Gö6983+	1.74465	1.27348	1.50906
Gö6983	1.04126	0.52655	0.78390
Gö6983+	0.97184	1.36497	1.16840

Publications

Huwait, E., Greenow, K., Singh, N. N. and Ramji, D. P. The liver-X-receptor-mediated activation of apolipoprotein E and ATP-binding casette transporter A1 expression in macrophages requires the c-Jun N-terminal kinase/stress-activated protein kinase and phosphoinositide-3-kinase signalling pathways. In preparation for submission to the Journal of Biological Chemistry.

Huwait, E., Singh, N. N. and Ramji, D. P. A potentially key role for protein kinase C in signalling by liver-X-receptors. In preparation for submission to Arteriosclerosis, Thrombosis, and Vascular Biology.

Singh, N. N., Foka, P., Harvey, E. J., **Huwait, E.,** Ali, S., Li, N. and Salter, R. (2007) Signalling pathways underlying cytokine regulated expression of key genes in macrophages implicated in atherosclerosis. Atherosclerosis 8:4.

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Ramji, D. P., Irvine, S. A., Foka, P., Singh, N. N., Rogers, S., Evans, S., Harvey, E., Ali,S. and **Huwait, E.** (2005) Molecular mechanisms involved in the cytokine-regulated expression of genes in macrophages implicated in foam cell formation and atherosclerosis. Atherosclerosis 6:76.

Irvine, S. A., Singh, N. N., Foka, P., Greenow, K., Hughes, T. R., Evans, S., Rogers, S.,Harvey, E., Ali, S., **Huwait, E.** and Ramji, D. P. (2004) Molecular mechanisms involved in thecytokine-mediated regulation of genes implicated in lipid homeostasis and atherosclerosis. The 2004 HDL Workshop, Heraklion, Crete, Greece.