STUDIES ON THE REGULATION OF APOLIPOPROTEIN E GENE EXPRESSION IN MACROPHAGES

BY

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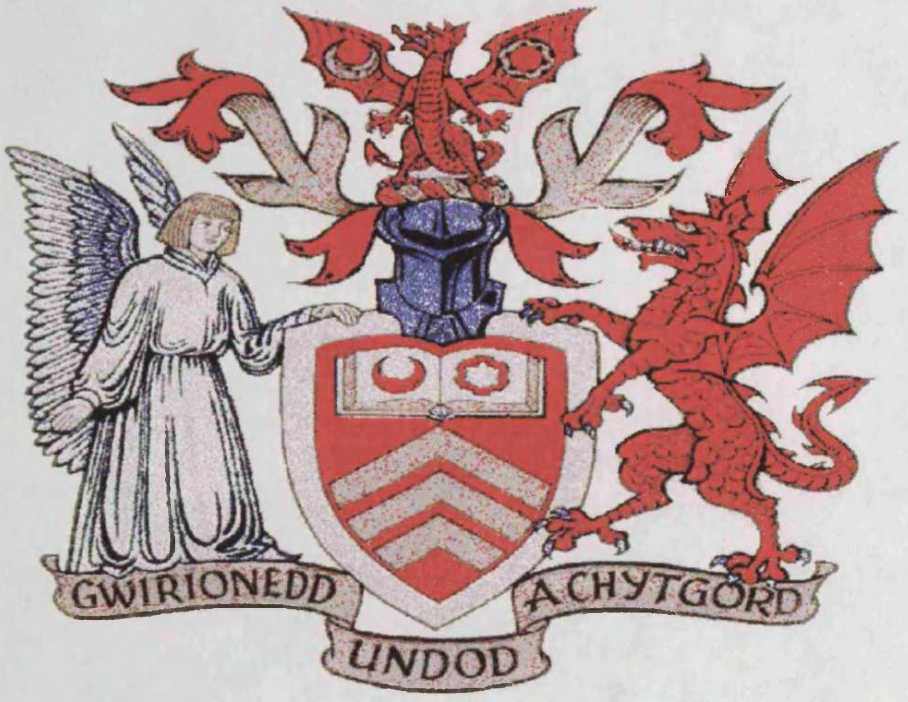

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ABSTRACT

Apolipoprotein E (apoE) plays a vital role in lipid metabolism and reverse cholesterol transport, and is highly expressed in the atherosclerotic plaque, where it is mainly associated with macrophage-derived foam cells. Macrophage apoE has several anti-atherogenic functions including key roles in the maintenance of macrophage lipid homeostasis and cellular cholesterol efflux. ApoE may therefore be considered as a potential therapeutic target in the prevention or treatment of atherosclerosis and it is therefore essential that a detailed understanding is obtained of the regulation of apoE in macrophages.

The initial aim of the project was to determine, in detail, the mechanism(s) involved in the induction of apoE expression during macrophage differentiation and to identify the *cis*-acting elements in the apoE promoter that were responsible for this upregulation. These aims required the optimisation of a macrophage-based transfection system, which allowed the analysis of several DNA constructs containing regions of the apoE promoter and the enhancer regions, ME1 and ME2. However, whilst this system was able to demonstrate the previously reported TNF-α induction of the apoE promoter, no such induction was observed during PMA-induced differentiation, indicating that the expression of apoE during macrophage differentiation may require other sequences in addition to the promoter and enhancer sequences.

Consequently, it was decided to investigate the regulation of apoE by the LXR subfamily of nuclear receptors. LXRs have atheroprotective properties due to their ability to upregulate the expression of genes involved in the reverse cholesterol transport process. ApoE is a direct target of these ligand-mediated transcription factors and a clearer understanding of the LXR-mediated transcription of apoE would aid the development of potential therapeutic agents for atherosclerosis. Therefore, the involvement of signal transduction pathways in the LXR-mediated regulation of apoE in macrophages was investigated.

Through the use of commercially available inhibitors, we firstly identified a novel role for the JNK/SAPK MAPK, PI3K and CK2 pathways in the LXR-mediated induction of apoE mRNA, protein and secretion in human THP-1 macrophages. This inhibition of apoE induction was also shown to occur in human primary monocyte-derived macrophages and further investigations demonstrated the potential role of these cell signalling pathways in the LXR-mediated regulation of ABCA1, ABCG1 and LXRα. It was also found, for the first time, that treatment of THP-1 macrophages with the oxysterol LXR ligand, 22(R)-hydroxycholesterol, induced JNK phosphorylation and kinase activity, and the subsequent phosphorylation of the c-jun transcription factor. Treatment of THP-1 macrophages with the LXR ligand also resulted in the phosphorylation and activation of Akt, a downstream component of PI3K signalling. In addition CK2 activity was found to be increased in THP-1 macrophages treated with 22(R)-hydroxycholesterol.

In conclusion, the studies presented in this thesis demonstrated, for the first time, an important role for the JNK/SAPK MAPK, PI3K and CK2 pathways in the activation of macrophage apoE gene expression by the LXR subfamily of nuclear receptors. A potential role for these cell signalling pathways was also implicated in the LXR-mediated regulation of ABCA1, ABCG1 and LXRα.

ABBREVIATIONS

ABC	ATP binding cassette
ACAT-1	acyl-coA: cholesterol acyltransferase-1
AF-2	activation function-2
AP-1	activator protein-1
apigenin	4', 5, 7-trihydroxyflavone
apo	apolipoprotein
APS	ammonium persulphate
AR	androgen receptor
ASC-2	activating signal cointegrator-2
ATF-2	activating transcription factor-2
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
cdk	cyclin dependent kinase
cDNA	complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
CETP	cholesteryl ester transfer protein
Ci	Curie(s)
CK2	Casein kinase 2
CMV	cytomegalovirus
COX-2	cyclooxygenase
CREB	cAMP response element binding protein
C-terminal	carboxyl terminal
curcumin	1, 7-bis(4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione
CYP7A1	cholesterol 7α-hydroxylase
Da	Daltons
DAG	diacylglycerol
dATP	deoxyadenosine triphosphate
DBD	DNA binding domain
dCTP	deoxycytidine triphosphate
DEAE	diethylaminoethyl
dGTP	deoxyguanosine triphosphate
DIPE	di-isopropyl ether
DMSO	dimethyl sulphoxide
DN	dominant negative
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DRIP	vitamin D3 receptor interacting proteins
DTT	dithiothreitol
ECL	enhanced chemi-luminescence
EDTA	ethylenediaminetetracetic acid
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FCS	foetal calf serum

FGF	fibroblast growth factor
g	grams
g	gravity
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte/macrophage-colony stimulating factor
GR	glucocorticoid receptor
GRIP1	glucocorticoid receptor-interacting protein-1
GSK-3	glycogen synthase kinase-3
h	hour(s)
HAT	histone acetyltransferase
HCR	hepatic control region
HDL	high density lipoprotein
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HI-FCS	heat-inactivated foetal calf serum
HL	hepatic lipase
HRP	horse radish peroxidase
HSPG	heparin sulphate proteoglycans
ICAM	intracellular adhesion molecule
IDL	intermediate density lipoprotein
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
JNK	c-jun N-terminal kinase
Kb	kilobase(s)
kDa	kilo Dalton(s)
LB	Luria Bertani
LBD	ligand binding domain
LCAT	Lecithin: cholesterol acyltransferase
LDL	low density lipoprotein
LDL-R	low density lipoprotein receptor
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LRP	low density lipoprotein receptor-related protein
LXR	liver X receptors
LXRE	LXR response element
LY294002	2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
MAPK	mitogen-activated kinase
MCP	monocyte chemoattractant protein
M-CSF	macrophage-colony stimulating factor
ME	multienhancer
min	minutes
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MMLV	Maloney Murine Leukemia Virus
MOPS	3-(N-morpholino)-propanesulphonic acid
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NCoA	nuclear receptor co-activator
NCoR	nuclear receptor co-repressor
NF-κB	nuclear factor for kappa light chain in B cells
NO	nitric oxide
N-terminal	amino-terminal

OD	optical density
ONPG	o-nitrophenyl β-D-galactopyranoside
oxLDL	oxidised LDL
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
P/CAF	p300/CBP-associated factor
PCR	polymerase chain reaction
PD98059	2'-amino-3'-methoxyflavone
PDGF	platelet-derived growth factor
PEG	polethyleneglycol
PI3K	phosphoinositide-3-kinase
PIP	phosphatidylinositol phosphate
PK	protein kinase
PLTP	phospholipids transfer protein
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulphonyl fluoride
PPAR	peroxisome proliferator-activated receptor
PR	progesterone receptor
PVDF	polyvinylidene fluoride
RAR	retinoic acid receptor
RNA	ribonucleic acid
RNase	ribonuclease
RSV	Rous sarcoma virus
RT	reverse transcriptase
RXR	retinoid X receptor
SAPK	stress-activated protein kinase
SB202190	4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)1H-imidazole
SD	standard deviation
SDS	sodium dodecyl sulphate
sec	second(s)
SEK	SAPK/ERK kinase
SMC	smooth muscle cell(s)
SMRT	*silencing mediator for RXR and TR*
SP600125	anthra[1,9-cd]pyrazol-6(2H)-one
SR	scavenger receptor
SRC	steroid receptor co-activator
SREBP	sterol response element binding protein
SWI/SNF	switch/sucrose non-fermenting
TAF	TBP associated factor
TBE	Tris-borate-ethylenediaminetetracetic acid
TBP	TATA-binding protein
TE	Tris-ethylenediaminetetracetic acid
TEMED	N, N, N, N-tetramethylethylenediamine
TG	triglycerides
TGF	transforming growth factor
TGRP	triglyceride-rich particles
TNF	tumour necrosis factor
TR	thyroid receptor
TRAP	thyroid hormone receptor associated protein
U	unit
V	volts

v/v	volume to volume
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VLDL	very low density lipoprotein
WCE	whole cell extract
w/v	weight to volume

CHAPTER ONE – GENERAL INTRODUCTION

1.1. ATHEROSCLEROSIS

Atherosclerosis is a slow progressive disease of the arteries and is the major underlying cause of heart attack and stroke, which accounts for 50% of all deaths in Western societies. The disease is characterised by the accumulation of lipids, fibrous elements and blood constituents in the intima of large arteries. Epidemiological studies have revealed several important environmental and genetic risk factors related to the increased incidence of this disease, including smoking, hypertension, diabetes mellitus, stress, physical inactivity and hypercholesterolaemia. The development of atherosclerotic lesions involves a complicated sequence of events in which various cell types, including monocyte-derived macrophages, T cells and the normal cellular elements of the arterial wall, such as smooth muscle cells and collagen, interact to form a complicated atherosclerotic plaque (Ross, 1999).

Atherosclerosis can be considered to be a form of chronic inflammation caused by a primarily protective mechanism that is triggered after injury to the endothelial lining of the arterial wall. Injury to the endothelium may be induced by several possible risk factors, including hypertension and raised plasma lipid levels, and results in changes in the permeability of the arterial wall, the increased expression of several cell surface adhesion molecules, and the production of cytokines (Ross, 1999; Glass and Witzum, 2001). These changes in endothelial function result in an increased migration of monocytes and T-lymphocytes from the circulation into the intima of the arterial wall. Once monocytes enter the intima, they differentiate into macrophages and begin to accumulate cell debris, lipoproteins and oxidatively modified lipoproteins, resulting in the development of lipid-laden foam cells (Lusis, 2000; Ross, 1999). These activated foam cells then amplify their cellular invasion by secreting their own growth factors and cytokines. This early atherosclerotic lesion consisting of cholesterol-engorged macrophages in the intima of the arterial wall is termed the fatty streak. The fatty streak progresses to an intermediate fibrofatty lesion, which consists of several layers of foam cells and smooth muscle cells (SMCs) with T-lymphocytes, surrounded by a relatively poorly developed matrix of connective tissue (Ross, 1999). The transition from the fatty streak to the more complex lesion is characterised by the migration of smooth muscle cells from the medial layer of the artery wall into the intima. Intimal smooth muscle cells proliferate and take up modified lipopoproteins, contributing to foam cell formation, and synthesise extracellular matrix proteins that lead to the development of the fibrous cap (Ross, 1999). Further increase of the lesion severity subsequently results in the formation of an advanced fibrous lesion that is characterised by a dense fibrous cap, which protrudes into the arterial lumen impeding the flow of blood, and covers a core of macrophages, smooth muscle cells, T-lymphocytes, extracellular lipid and necrotic material (Ross, 1999; Lusis, 2000). Instability of the

atherosclerotic lesion may then lead to rupture of the plaque and the induction of secondary haemorrhage and thrombosis, which may ultimately lead to occlusion of the artery.

1.1.1. LIPOPROTEINS AND ATHEROSCLEROSIS

Among the various genetic and environmental risk factors that have been identified, elevated levels of cholesterol in the blood seems to be unique due to its ability to drive the development of atherosclerosis in humans and experimental animals, even in the absence of other known risk factors (Glass and Witzum, 2001). Serum cholesterol is carried by several lipoprotein particles, which are responsible for the transport of dietary and endogenously produced lipids. Lipoproteins are spherical macromolecular particles that comprise of a core of hydrophobic cholesteryl esters and triglycerides surrounded by a shell of phospholipids and unesterified free cholesterol. The phospholipids in the lipoprotein shell are polarized and the charged head groups face the hydrophilic blood compartment, while the hydrophobic fatty acid chains are in contact with the core lipids. In addition to the phospholipids and the unesterified free cholesterol, the lipoprotein shell also contains a number of specific proteins, called apolipoproteins, that play a major role in the regulation of lipoprotein metabolism and the stabilisation of the lipoprotein particles.

Five major classes of lipoproteins can be distinguished, including chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). These different classes of lipoproteins differ in properties such as their size, electrophoretic mobility, composition of lipids and apolipoproteins, all of which effect their interaction with different tissues and their metabolism. Each class also vary in their function; chylomicrons are primarily responsible for the transport of dietary lipids, while VLDL, LDL and HDL function to transport endogenous lipids.

VLDL particles are synthesised by the liver from cholesterol and triglycerides derived from either *de novo* synthesis or lipoprotein uptake (Gibbons, 1990). VLDL particles contain apolipoprotein B-100 (apoB-100), apolipoprotein CI, II and III, and apolipoprotein E (apoE). These particles are responsible for the transport of fatty acids to peripheral tissues such as adipose tissue and muscle. In the plasma, the triglycerides in VLDL are subject to lipolysis by lipoprotein lipase (LPL), which is bound to the capillary endothelium of skeletal muscle, cardiac muscle and adipose tissue (Goldberg, 1996). The free fatty acids are taken up, mainly by adipocytes where they are stored in triglyceride droplets. Progressive hydrolysis of core triglycerides of VLDL, is also accompanied by transfer of phospholipids by phospholipid transfer protein (PLTP) and apolipoproteins to HDL. After triglyceride removal in the peripheral tissues, a proportion of the remaining VLDL remnants are rapidly removed from the circulation by the high-affinity binding sites on parenchymal liver cells, where the cholesterol is either used in membrane or lipoprotein

biosynthesis or excreted as free cholesterol or bile acids. VLDL remnant particles that are not endocytosed by the liver are further processed by the enzyme hepatic lipase and converted into LDL particles. The conversion of VLDL remnants into LDL is accompanied by further removal of core triglycerides and dissociation of all apolipoproteins apart from apoB-100.

Although 70% of the formed LDL is taken up by hepatic LDL receptors, it is mainly LDL particles in humans that are responsible for the delivery of cholesterol to the peripheral tissues. Therefore, high LDL cholesterol levels are associated with increased risk for cardiovascular disease. The uptake of LDL by cells occurs via LDL receptors that recognise an N-terminal domain of apoB-100, its sole lipoprotein. The circulating level of LDL is determined in large by its rate of uptake through the hepatic LDL receptor pathway, as evidenced by the fact that lack of functional LDL receptors is responsible for the massive accumulation of LDL in patients with homozygous familial hypercholesterolaemia (Goldstein and Brown, 1977). Control of LDL receptor levels through transcription of the LDL receptor gene is regulated by a family of membrane-bound transcription factors called sterol regulatory element binding proteins (SREBPs), which are in turn regulated by the cholesterol content of the cell. Low levels of intracellular cholesterol lead to activation of SREBP-2, which stimulates the transcription of the LDL receptor gene and other genes involved in cholesterol biosynthesis (Brown and Goldstein, 1999).

The recruitment of macrophages to the intima of the arterial wall and their subsequent uptake of LDL-derived cholesterol are the major cellular events contributing to fatty streak formation (Figure 1.1). Native LDL is not taken up by macrophages rapidly enough to generate foam cells and several studies have suggested that oxidative modification of the lipid and apoB-100 component of LDL may drive the initial formation of fatty streaks (Navab *et al.*, 1996). Oxidation of LDL by free radicals and lipoxygenases occurs in the arterial wall and becomes prevalant when levels of circulating LDL are raised. LDL diffuses passively through endothelial cell junctions to the subendothelial matrix, and its retention in the vessel wall involves the interaction of the apoB component of LDL with matrix proteoglycans (Schwenke and Carew, 1989; Boren *et al.*, 1998). Studies have demonstrated that mice lacking 12/15-lipoxygenase have considerably diminished atherosclerosis, suggesting that this enzyme may be an important source of reactive oxygen species in LDL oxidation (Cyrus *et al.*, 1999). Also evidence that inducible nitric oxide synthase (iNOS) contributes to LDL oxidation *in vivo* has recently been provided by studies demonstrating that inhibitors of iNOS decrease atherosclerosis in rabbits (Behr-Roussel *et al.*, 2000; Detmers *et al.*, 2000). The specific properties of oxidized LDL, depends on the extent of modification, which can range from minimal to extensive. Minimal modification (mmLDL), allows the LDL particle to be recognised by the LDL receptor as normal (Navab *et al.*, 1996), whereas extensive modification of LDL (oxLDL) results in the particle being bound by scavenger receptors expressed on the surface

of macrophages and smooth muscle cells. The inability of the extensively modified LDL particle to bind to the LDL receptor is due to the apoB-100 component of LDL being fragmented and the lysine residues of the particle becoming covalently modified with reactive breakdown products of oxidized lipids (Steinberg *et al.*, 1989).

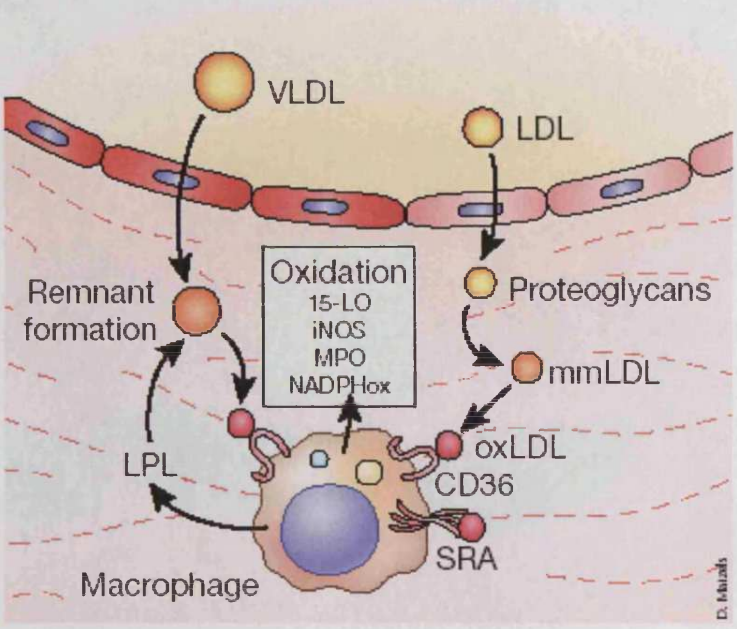


FIGURE 1. 1. MECHANISM CONTRIBUTING TO FOAM-CELL FORMATION.

LDL penetrates into the artery wall where it binds to proteoglycans. These interactions are thought to trap the LDL particles and increase their susceptibility to oxidation. Enzymes contributing to LDL oxidation include lipoxygenases, myeloperoxidase and iNOS. VLDL particles are subject to modification by LPL and the resulting remnants are subject to trapping by the proteoglycans, oxidative modification and uptake by macrophages. Macrophage uptake of modified lipoproteins occurs via the scavenger receptors CD36 and SR-A. VLDL, very low density lipoproteins; LDL, low density lipoproteins; mmLDL, minimally modified LDL; oxLDL, oxidised LDL; 15-LO, 15-lipoxygenase; iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; NADPHox, NADPH oxidase; LPL, lipoprotein lipase; SRA, scavenger receptor A. See text for further explanation. Figure taken from Li and Glass (2002).

LDL also undergoes other types of modifications such as non-enzymatic glycation, enzymatic degradation and aggregation, all of which, consequently, generates a wide range of oxidation-specific neo-epitopes, which renders the modified LDL immunogenic and leads not only to a cellular immune response but a humoral response also (Horkko *et al.*, 2000). Also lipoprotein accumulation in the artery wall is a strong stimulus to the activation of several inflammatory pathways and VLDL and other triglyceride-containing particles are able to activate the transcription factor, nuclear factor-κB (NFκB) [Dichtl *et al.*, 1999], which increases the expression of several inflammatory genes, including cyclooxygenase-2 (COX-2) [Belt *et al.*, 1999].

1.1.2. MACROPHAGES AND ATHEROSCLEROSIS

Monocytes and macrophages are present at all stages of atherosclerotic lesion development, from the fatty streak to the more complicated atherosclerotic plaque. The turnover of macrophages in the atherosclerotic plaque is the net effect of monocyte infiltration, local proliferation, efflux, and cell death. Monocytes are derived from pluripotent haematopoietic stem cells in the bone marrow and migrate into the blood circulation 24h after production (van Furth, 1989). Once they enter the blood circulation they either remain in the blood stream or migrate into tissues and body cavities where they differentiate into macrophages. Macrophages play an important role in protecting the vascular wall from injury and in ordinary inflammatory reactions macrophages scavenge debris, such as cytotoxic and proinflammatory oxLDL particles or apoptotic cells and subsequently remove the debris by migrating away from the site of injury into the blood circulation. Although the recruitment of monocytes to the arterial wall and their subsequent differentiation into macrophages, initially serves a protective function, the progressive accumulation of macrophages and their uptake of oxLDL, ultimately results in a prolonged inflammatory response and an increase in the severity of the atherosclerotic lesion. The importance of macrophages in the development of atherosclerosis has been illustrated by the osteopetrotic mouse, which carries a naturally occurring mutation in the gene encoding macrophage colony stimulating factor (M-CSF), an important factor involved in the proliferation and survival of monocytes. These mice exhibit a near complete absence of macrophages, and when bred to apoE-deficient mice they are extremely resistant to the development of atherosclerosis, despite an increase in circulating cholesterol levels (Smith *et al.*, 1995a; Qiao *et al.*, 1997).

The development of atherosclerosis is initiated by the adhesion of monocytes to the endothelial cell layer of the vessel wall. The recruitment of monocytes to lesion-prone sites of large arteries is regulated by several cell adhesion molecules that are expressed on the surface of the endothelial cells in response to inflammatory stimuli. Initial adhesion involves selectins, which mediates a rolling interaction, which is followed by a firmer attachment by means of integrins (Figure 1.2). Vascular cell adhesion molecule-1 (VCAM-1) was the first cell adhesion molecule to be implicated in the initial recruitment of monocytes, due to its increased expression on endothelial cells over lesion-prone areas in response to cholesterol feeding and also its pattern of regulation by pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) [Cybulsky and Gimbrone, 1991]. Although targeted deletion of VCAM-1 in mice causes embryonic lethality, studies where hypomorphic variants of VCAM-1 have been introduced into atherosclerosis-susceptible mice (apoe-/-) have demonstrated a reduction in atherosclerotic lesion formation (Cybulsky *et al.*, 2001). In addition to VCAM-1, both E-selectin and P-selectin appear to play quantitative roles in monocyte adhesion, as apoE-deficient mice lacking both genes show a

40-60% decrease in atherosclerosis (Dong *et al.*, 1998). Similarly, a reduction in monocyte recruitment to atherosclerotic lesions can be seen in apoE-deficient mice with a deletion of the gene encoding intracellular adhesion molecule-1 (ICAM-1) [Collins *et al.*, 2000].

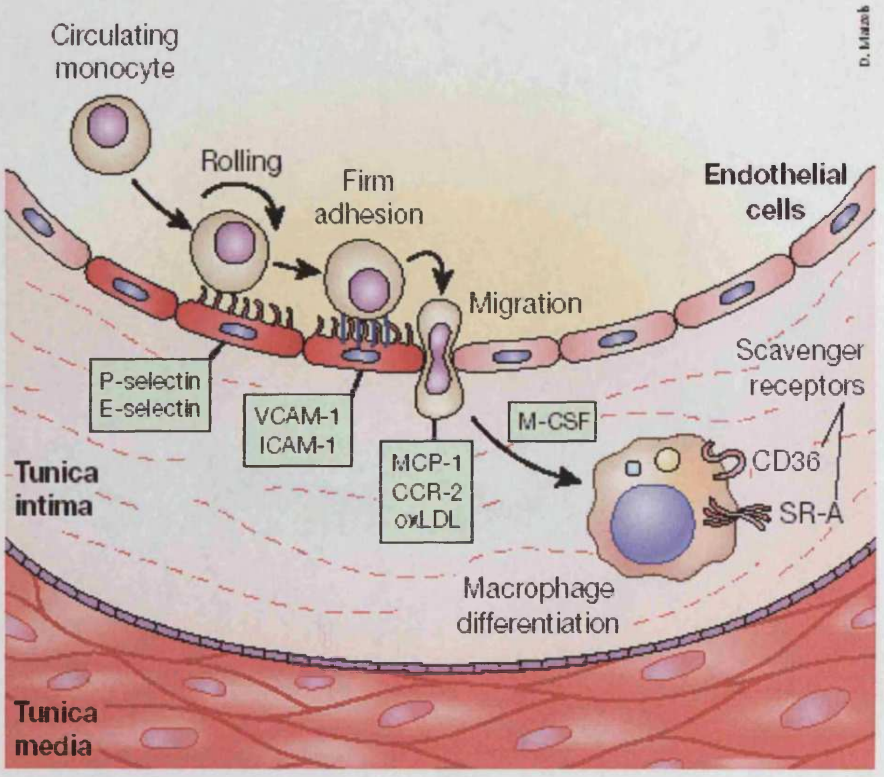


FIGURE 1.2. MONOCYTE RECRUITMENT AND DIFFERENTIATION.

Circulating monocytes attach to endothelial cells by cell adhesion molecules that are induced in response to inflammatory signals. Selectins mediate low-affinity interactions that permit rolling, whereas integrins mediate firm adhesion. Monocytes migrate through the endothelial layer into the intima, where they differentiate into macrophages in response to locally produced factors such as M-CSF. This process of differentiation is accompanied by the upregulation of scavenger receptors. VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemotactic protein-1; CCR-2, MCP-1 receptor; oxLDL, oxidised LDL; M-CSF, macrophage colony stimulating factor; SR-A, scavenger receptor A. See text for further explanation. Figure taken from Li and Glass (2002).

Adherent monocytes then migrate through endothelial cell junctions into the sub-endothelial space under the influence of chemoattractant molecules. The migration of monocytes into the intima of the arterial wall is likely to be stimulated in part by oxLDL, which is able to directly attract monocytes (Steinberg *et al.*, 1989) and induce the expression of chemotactic molecules by endothelial cells, such as monocyte chemotactic protein-1 (MCP-1) [Navab *et al.*, 1996]. Recent work using compound mutant mice lacking MCP-1 or its receptor CCR2, which are susceptible to atherosclerosis (apoE-/- or LDLR-/- mice), have shown striking decreases in monocyte accumulation and local lipid levels (Gu *et al.*, 1998; Boring *et al.*, 1998). IL-8, which is present in

atherosclerotic lesions, may also play a role in the migration of cells into the intima, as mice lacking CXCR2, a high affinity receptor for IL-8 results in significantly reduced risk of atherosclerosis (Boisvert *et al.*, 1998).

Once monocytes have entered the intima, they differentiate into macrophages and start to accumulate massive amounts of cholesteryl esters resulting in the development of cholesterol-filled foam cells. This accumulation of cholesterol by macrophages is thought to be mediated primarily by the uptake of modified forms of LDL via scavenger receptors (Yamada *et al.*, 1998). Two proteins shown to significantly contribute to this process, are scavenger receptor A (SR-A) and CD36 as apoE-deficient mice lacking SR-A or CD36 develop significantly less atherosclerosis than control apoE-deficient mice (Suzuki *et al.*, 1997). OxLDL taken up by macrophage scavenger receptors is delivered to lysosomes, where its cholesteryl ester content is hydrolysed to free cholesterol and fatty acids. This free cholesterol has a number of potential metabolic fates, including esterification and storage in the lipid droplets that characterise foam cells. The cholesteryl ester stores of macrophages have been shown to undergo a continuous cycle of hydrolysis (to free cholesterol) and re-esterification (Brown *et al.*, 1980). The hydrolytic step of this cycle is mediated by a neutral cytoplasmic cholesteryl ester hydrolase, which is responsive to cyclic-AMP (Khoo *et al.*, 1981) and the re-esterification step is mediated by acyl CoA: cholesterol acyltransferase-1 (ACAT-1) [Brown *et al.*, 1980]. As intracellular cholesterol levels increase, the proteolytic activation of SREBP transcription factors required for cholesterol biosynthesis and LDL receptor expression (Brown and Goldstein, 1999) are inhibited. Although this prevents the further accumulation of cholesterol via these pathways, cholesterol is still taken up via scavenger receptors or phagocytic mechanisms and cholesterol homeostasis can not be maintained. As mammalian cells do not have the ability to degrade the sterol ring the majority of the excess sterols can only be eliminated from the body by biliary excretion. Macrophages and other cell types must export cholesterol to extracellular acceptors for transport to the liver. Therefore, mechanisms mediating cholesterol efflux are critical for the maintenance of cholesterol homeostasis in the macrophage.

The macrophage has two potential mechanisms for disposing of excess cholesterol: enzymatic modification to the more soluble forms and efflux via membrane transporters. The enzyme 27-hydroxylase is expressed in macrophages at relatively high levels and may play a role in cholesterol excretion by converting it to the more soluble 27-oxygenated steroids that can be readily accepted by albumin (Babiker *et al.*, 1997). The major mechanism for cholesterol efflux however, is reverse cholesterol transport, where HDL serves as the primary extracellular acceptor. There is also evidence that macrophages may contribute directly to the availability of extracellular cholesterol levels through the secretion of apolipoprotein E (apoE) [Linton *et al.*, 1995], which contributes to the formation of HDL particles.

During lesion development, significant cross-talk occurs among the cellular elements of the plaque. Macrophages, endothelial cells, T cells and smooth muscle cells become active during atherogenesis and secrete a variety of proteins. A wide range of the activated macrophage products are involved in the initiation and progression of the lesion (Table 1.1) and although all of the macrophage secretory products indicate the importance of macrophages in atherogenesis, discussion of all these products is beyond the scope of this thesis, therefore the remainder of this chapter will discuss in detail the role of apolipoprotein E in the development of atherosclerosis.

TABLE 1.1. MONOCYTE/MACROPHAGE PRODUCTS AND THEIR POTENTIAL ROLE IN ATHEROGENESIS

PRODUCT	FUNCTION IN ATHEROGENESIS
SECRETED PROTEINS	
Apolipoprotein E	Lipoprotein metabolism and cholesterol efflux
Interleukin-1	▲ Adhesion molecules and SMC migration and proliferation
Interleukin-6	Activation of coagulation
Interleukin-8	Recruitment of lymphocytes and endothelial cells
M-CSF	Monocyte proliferation, survival, and modulation of gene expression
TNF-α	▲ Adhesion molecules, monocyte attraction, SMC proliferation
MCP-1	Monocyte chemoattraction
ENZYMES AND INHIBITORS	
Collagenase	Matrix degradation, cell migration and plaque rupture
Complement C3b	Prothrombotic
Factor XIIIa	Prothrombotic
Gelatinases (MMP-2/MMP-9)	Matrix degradation, cell migration and plaque rupture
Lipoprotein lipase	Alteration of lipoprotein metabolism
15-Lipoxygenase	Lipid oxidation and foam cell formation
Metalloproteinases	Matrix degradation and plaque rupture
Myeloperoxidase	Formation of cytotoxins and reactive oxygen species
CELL-ASSOCIATED PROTEINS	
VLA-4	Monocyte/macrophage activation and retention
LDL-receptor-related protein	Lipoprotein uptake and protease clearance
LDL receptor	Lipoprotein uptake
VLDL receptor	Lipoprotein uptake
Scavenger receptors	Lipoprotein uptake, monocyte recruitment and adhesion
Thrombin receptor	Monocyte/macrophage activation
Tissue factor	Coagulation and thrombosis

M-CSF, macrophage colony stimulating factor; TNF-α, tumour necrosis factor-α; MCP-1; monocytic chemotactic protein-1; MMP, matrix metalloproteinase; VLA-4, alpha 4 beta 1 integrin; LDL, low density lipoprotein; VLDL, very low density lipoprotein; ▲, increase. Table derived from van Eck (1999).

1.2. APOLIPOPROTEIN E

Apolipoprotein E (apoE) is a 34kDa protein with a mature form comprising of 299 amino acids (Rall *et al.*, 1982). Initially apoE was termed the "arginine-rich apoprotein" and was first identified in 1973 by Shore and Shore as a lipoprotein constituent of triglyceride-rich VLDL (Shore and Shore, 1973). ApoE has since been found to be a major protein constituent of several plasma lipoproteins that carry dietary- and liver-derived cholesterol, including very low density lipoproteins (VLDL), chylomicrons and a sub-population of high density lipoproteins (HDL) (Mahley *et al.*, 1984). Most of the apoE found circulating in the body is synthesised by the liver, although 20-40% is derived from extra-hepatic sources (Newman *et al.*, 1985), such as macrophages. One important role of apoE is in lipoprotein metabolism, where it is the primary determinant for the receptor-mediated catabolism of all triglyceride-rich lipoproteins, apart from LDL. ApoE mediates the uptake and degradation of lipoproteins via its high affinity binding to the low-density lipoprotein receptor (LDL-R) on parenchymal liver cells. ApoE is an extremely efficient ligand for the receptor, as it has a 25-fold greater affinity for the receptor than LDL itself (Innerarity and Mahley, 1978), in which apoB-100 is the ligand. The importance of the interaction of apoE with the LDL receptor is underscored by the massive accumulation of lipoproteins and lipoprotein remnants in the plasma of patients with type III hyperlipoproteinaemia, where an apoE variant is expressed, which is defective in LDL receptor binding (Weisgraber *et al.*, 1982).

ApoE also binds to a second hepatic receptor involved in lipoprotein metabolism, the low density lipoprotein receptor-related protein (LRP) which has been implicated in the clearance of chylomicron remnants (Herz and Willnow, 1995), a process which has been shown to be dependant on apoE (Linton *et al.*, 1998). Evidence for the existence of the remnant receptor comes from the ability of individuals, with familial hypercholesterolaemia, that have defective or absent LDL receptors to clear chylomicron remnants from their plasma (Mahley, 1988). The clearance of chylomicron remnants by apoE binding to the LRP, involves the ability of apoE to bind to heparan sulfate proteoglycans (HSPG). This binding of apoE to HSPGs serves a secretion-capture role in lipoprotein remnant metabolism as apoE-enriched remnants appear to bind to HSPG before it interacts with LRP on the hepatic surface (Ji *et al.*, 1994a). The requirement for HSPG binding by apoE has been confirmed in studies using heparinase treated cells and the infusion of heparinase in mice (Ji *et al.*, 1993; Ji *et al.*, 1994b; Ji *et al.*, 1995). In addition, lipoproteins are required to be artificially enriched in apoE in order to be active *in vitro* (Kowal *et al.*, 1989).

The HSPG–LRP pathway involves a three-step process, involving sequestration, processing, and uptake (Mahley and Ji, 1999). Initially, lipoprotein particles are sequestered in the hepatic space of Disse, which is rich in apoE; there the particles become enriched with apoE, which interacts with the HSPG molecules anchored on the hepatic surface (Ji *et al.*, 1994a). The

retained lipoproteins then undergo lipolytic events involving either lipoprotein-bound LPL or cell-surface bound hepatic lipase (Ji *et al.*, 1994c; Ji *et al.*, 1997). Finally, the "secretion-capture" role of apoE facilitates the interaction of lipoproteins with the LDL receptor, the LRP receptor, or HSPG acting as a receptor. It appears that lipoproteins containing apoE are either transferred from HSPG to the LRP and then taken up as a ligand-receptor complex by endocytosis or are taken up as an HSPG-LRP-lipoprotein complex together by the hepatocytes (Ji *et al.*, 1997). The HSPG-LRP system has been shown to function *in vivo* by several studies (Ji *et al.*, 1995; Willnow *et al.*, 1994; Hussain *et al.*, 1991) and although the LDL receptor-mediated pathway is sufficient for clearing lipoprotein remnants during fasting, the LRP/HSPG-mediated pathway is thought to be required for efficient clearance of post-prandial lipoprotein remnants (Mahley and Ji, 1999).

1.2.1. REVERSE CHOLESTEROL TRANSPORT

In addition to the role of apoE in the metabolism of lipoproteins, apoE plays a vital role in cholesterol efflux. The elimination of cholesterol from the body occurs mainly through the liver via bile formation. Therefore, it is essential that excess cholesterol in peripheral tissues is transported back to the liver for excretion, via a process involving HDL, called "reverse cholesterol transport" (Glomset, 1968). Nascent preβ-HDL, a discoidal apoA-I phospholipid particle, which is secreted by the intestine or the liver, is a potent acceptor of excess cholesterol from peripheral tissues. Once HDL has taken up free cholesterol from peripheral cells, the cholesterol becomes esterified to cholesteryl esters by the enzyme lecithin-cholesterol acyltransfrease (LCAT) and nascent HDL is converted to small, spherical HDL_3 (Figure 1.3). Subsequently HDL_3 is converted into HDL_2 by acquirement of phospholipids and apolipoproteins that are released into the interstitial fluid during the lipolysis of triglycerides in chylomicrons or VLDL by LPL. ApoE, which is present in the interstitial fluid due to secretion by various cell types, has avidity for HDL as it becomes cholesterol-enriched and studies have also shown that the presence of apoE actually facilitates HDL uptake of cholesterol. For example, macrophages derived from apoE-/- mice exhibit a decreased capacity to efflux cholesterol and other lipids to HDL_3, lipid-free apoAI or lipid-free apoE3 compared with those from wild-type mice (Langer *et al.*, 2000; Hayek et al., 1994).

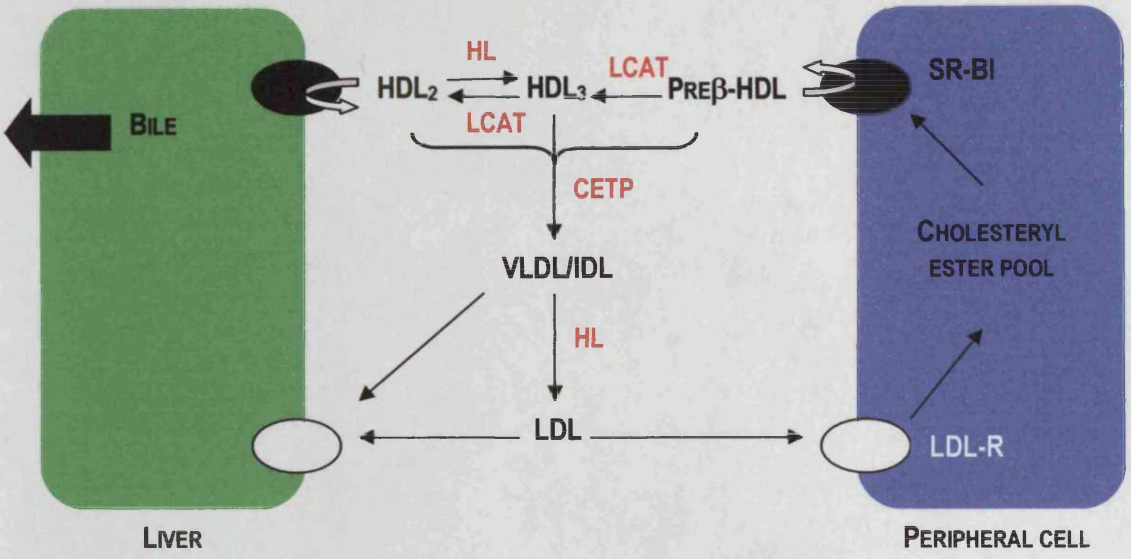


FIGURE 1.3. SCHEMATIC ILLUSTRATION OF REVERSE CHOLESTEROL TRANSPORT.
HDL, high density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HL, hepatic lipase; LCAT, lecithin-cholesterol acyltransfrease; CETP, cholesteryl ester transfer protein; SR-BI, scavenger receptor BI; LDL-R, low density lipoprotein receptor. See text for further details.

The return of peripheral cholesterol to the liver can occur via three different pathways. The first is by the direct uptake of the whole HDL particle by the LDL receptors of the liver, via apoE binding. The second pathway is indirect but appears to be more important in humans than the direct ligand role of apoE in HDL (Tall, 1993). This pathway occurs via the transfer of the cholesteryl esters in HDL to other lipoproteins, such as VLDL, IDL or LDL by the cholesteryl ester transfer protein (CETP), these lipoproteins are then subsequently taken up by the liver, where apoE behaves as a ligand. The anti-atherogenic properties of this CETP/HDL pathway is confirmed by recent data that demonstrate that CETP-deficiency is associated with increased atherosclerosis in humans, despite increased HDL levels (Bruce *et al.*, 1998). Also over-expression of CETP in mice that normally lack CETP activity is anti-atherogenic (Bruce *et al.*, 1998). The third pathway of reverse cholesterol transport is the selective delivery of cholesteryl esters in HDL to the liver via scavenger receptor class B1 (SR-B1) [Acton *et al.*, 1996]. SR-B1, which is expressed mainly in the liver and steroidogenic tissues, binds HDL with great affinity, and mediates the selective delivery of cholesteryl esters to the liver without internalisation and degradation of the HDL particle. The direct uptake of cholesteryl ester from HDL is facilitated by apoE-mediated binding of apoE-containing HDL to cell surface HSPG (Arai *et al.*, 1999). Subsequently, this apoE-HSPG interaction enhances the ability of the cell surface protein SR-BI to mediate the delivery of HDL-cholesteryl esters to cells. A direct interaction of cell surface apoE with these receptors may not be required; only the close proximity of apoE as a sterol acceptor may be needed although

recent studies have shown that apoE contains an SR-BI binding sequence (Li *et al.*, 2002a). The importance of SR-BI in the reverse cholesterol transport process has been demonstrated by the inactivation of this gene in mice, which was associated with a dramatic increase in serum cholesterol levels and the size of HDL, without influencing the HDL protein concentration (Rigotti *et al.*, 1997). Also liver-specific overexpression of this protein in mice resulted in reduced HDL levels and an increase in reverse cholesterol transport (Wang *et al.*, 1998).

The process of cholesterol efflux is particularly important in maintaining cholesterol homeostasis in cells that are incapable of limiting their uptake of lipids, such as macrophages. This removal of free cholesterol from macrophages by specific cholesterol acceptors is vital in the prevention of foam cell formation. Reverse cholesterol transport is an important pathway in maintaining the cholesterol balance of macrophages and the antiatherogenic properties of apoE are largely attributed to its ability to deplete foam cell macrophages of cholesterol. Various studies have confirmed the involvement of apoE in macrophage cholesterol efflux. It has been extensively reported that secretion of apoE and cholesterol from macrophages occurs via independent but concurrent mechanisms and it was thought that apoE facilitates cholesterol efflux by improving the cholesterol accepting properties of HDL (Mazzone, 1996; Basu *et al.*, 1983; Dory, 1989; Mazzone and Reardon, 1994). Work by Zhu *et al.* (1998) has determined that reduced atherosclerosis in human apoE transgenic mice, compared to apoE deficient mice was due to the ability of the transgenic apoE to associate with α-migrating HDL, increasing its capacity to accept cholesterol. Also the capacity of apoE-depleted HDL from humans or mice to promote cholesterol efflux from mouse peritoneal macrophages is decreased and can be restored to normal by the addition of exogenous apoE (Basu *et al.*, 1982; Hayek *et al.*, 1994). The expression of endogenous apoE also enhances cholesterol efflux in macrophages (Zhang *et al.*, 1996; Mazzone and Reardon, 1994; Smith *et al.*, 1996; Kruth *et al.* 1994), and the experimental characteristics of cholesterol efflux in such cases can be distinguished from that produced by the exogenous addition of lipid free apoE (Lin *et al.*, 1998). The precise mechanisms by which endogenous apoE stimulates cholesterol efflux currently remains unclear although its retention at the cell surface via association with proteoglycans makes a contribution (Lin *et al.*, 2001). ApoE arrives at the cell surface in a relatively lipid-poor state and conformation changes attained during lipidation may cause its release (Lin *et al.*, 2001). ApoE held in proximity to the plasma membrane by proteoglycan binding could facilitate passive desorption of lipid from the plasma membrane by a process that may involve SR-BI or ATP-binding cassette transporter A1 (ABCA1) (Krieger, 1999; Orso *et al.*, 2000). Indeed, it has previously been shown that apoE is an acceptor for free cholesterol and phospholipid released from macrophages by ABCA1 (Smith *et al.*, 1996) and work by Krimbou *et al.*, (2004) has demonstrated that lipid-free apoE is able to interact with ABCA1 *in vitro*. It may be possible that the

interactions of apoE with ABCA1 leads to the lipidation of apoE through a membrane microsolubilisation process whereby there is simultaneous release of phospholipids and free cholesterol to apoE, as recently suggested for the lipidation of apoAI (Liu *et al.*, 2003). This is in agreement with the idea that the transfer of phospholipids and cholesterol from the active site of ABCA1 transporter to apoE molecule weakens the interaction of apoE/ABCA1 and causes disocciation of the lipidated apoE product. However, it is worth noting that apoE may be the primary driver of cholesterol efflux in macrophages through a pathway independent of ABCA1 expression or activity (Huang *et al.*, 2001).

In addition to the ability of apoE to facilitate HDL cholesterol efflux, apoE is able to directly stimulate hepatic VLDL and triglyceride production as demonstrated by studies using apoE-/- mouse hepatocytes that have impaired secretion of VLDL triglycerides (Kuipers *et al.*, 1997). Also hepatocytes stably transfected with human apoE showed a two-fold increase in VLDL triglyceride secretion with increasing levels of human apoE (Huang *et al.*, 1998) and apoE transgenic mice lacking murine apoE with inhibited lipolysis showed increased VLDL production of 50% compared with that of non-transgenic controls (Huang *et al.*, 1998). Plasma apoE concentration is also an important determinant for VLDL metabolism which indirectly affects HDL levels. Studies using apoE-expressing transgenic mice have shown that the apoE:apoCII ratio is a major factor in determining whether VLDL is a good substrate for lipoprotein lipase (Huang *et al.*, 1998) and increased levels of apoE may increase triglycerides by displacing the lipoprotein lipase cofactor apoCII and inhibiting lipolysis. This increase in apoE and triglyceride levels reduces HDL levels, which is probably due to the inhibition of VLDL lipolysis, which reduces the availability of surface components for HDL production.

Another role of apoE in reverse cholesterol transport, separate to its role as a high-affinity ligand for the various lipoproteins, is in the activation of enzymes involved in lipoprotein metabolism such as HL, CETP and LCAT. The hydrolysis of phospholipid monolayers by HL in apoE-enriched HDL was found to be enhanced as compared to apoE-poor HDL, indicating that apoE activates HL (Thuren *et al.*, 1992). ApoE also enhances the cholesteryl and triglyceride transfer between VLDL and HDL via CETP by enhancing the affinity of CETP for VLDL (Kinoshita *et al.*, 1993), and the C-terminal domain of apoE has been reported to play a role in the activation of LCAT (De Pauw *et al.*, 1995). ApoE is also thought to indirectly facilitate LPL-mediated hydrolysis of triglycerides in VLDL as apoE-mediates binding to the VLDL receptor, which co-localizes with LPL (Takahashi *et al.*, 1995; Niemeier *et al.*, 1996).

1.2.2. APOLIPOPROTEIN E AND ATHEROSCLEROSIS.

Interest in apoE has mainly arisen from the observation that apoE is an atheroprotective protein. Depressed expression of apoE in humans leads to a pro-atherogenic lipoprotein profile associated with increased risk for atherosclerosis (Schaefer *et al.*, 1986; Ghiselli *et al.*, 1981). In addition, apoE knockout (apoE-/-) mice are severely hypercholesterolemic compared to the wild-type counterparts (500mg/dl compared with 60-90 mg/dl), and the increase in cholesterol is largely distributed in the lower density lipoproteins due to their impaired clearance (Plump *et al.*, 1992; Zhang *et al.*, 1992 and 1994a; Nakashima *et al.*, 1994). ApoE deficient mice also loose their normal resistance to cholesterol feeding and their cholesterol levels can rise to greater than 2000mg/dl (Plump *et al.*, 1992; Zhang *et al.*, 1992 and 1994a; Nakashima *et al.*, 1994). This plasma accumulation of remnant lipoproteins results in the development of complex atherosclerotic lesions, which develop even when mice are fed a low fat diet (Plump *et al.*, 1992; Zhang *et al.*, 1992 and 1994a; Nakashima *et al.*, 1994; Reddick *et al.*, 1994; Piedrahita *et al.*, 1992). In addition, atherosclerosis in apoE-null mice has been shown to be prevented by increasing circulating apoE levels through recombinant adenovirus-mediated apoE gene transfer to the liver (Kashyap *et al.*, 1995). The decrease in atherosclerosis seen in this model was accompanied by decreased total cholesterol and VLDL/IDL levels (Kashyap *et al.*, 1995) suggesting that apoE prevents atherosclerosis by lowering plasma cholesterol levels.

ApoE expression is absent in normal vessels, but during atherosclerosis apoE is expressed in the atherosclerotic plaque and although it can enter the artery wall from the periphery, it is mainly thought to be synthesised locally by resident macrophages (Rosenfeld *et al.*, 1993; O'Brien *et al.*, 1994). Macrophage apoE plays a direct role in preventing or delaying foam cell formation, and lack of macrophage apoE leads to induction and progression of atherosclerosis despite the abundance of apoE in plasma and on lipoproteins (Fazio *et al.*, 1997). Studies by Boisvert and Curtiss (1999) confirm that the majority of apoE in the lesion is synthesised locally by lesion macrophages and established that the expression of this macrophage-derived apoE in the lesion can retard the development of atherosclerosis in male mice fed a high fat diet. Several other *in vivo* studies have demonstrated that the secretion of apoE by macrophages exerts an important protective effect. Selective expression of apoE in macrophages of apoE-/- mice through bone marrow transplantation or transgenic expression decreases hypercholesterolaemia and reduces lesion development (Linton *et al.*, 1995). Conversely, the transplantation of apoE-/- bone marrow into apoE +/+ mice confers increased susceptibility to atherosclerosis (Fazio *et al.*, 1997).

Several studies have shown that macrophage-derived apoE exerts anti-atherogenic properties largely independent of its effects on plasma lipid levels (Fazio *et al.*, 1997; Boisvert and Curtiss, 1999; Van Eck *et al.*, 2000). For example, work by Zhang *et al.* (1994a) demonstrated that

atherosclerosis was found to be more severe in cholesterol-fed apoE-heterozygous(+/-) mice than in cholesterol-fed apoE(+/+) mice, despite the relative similar plasma cholesterol level in the two groups. It has also been shown that the lack of macrophage apoE in C57BL/6 mice leads to a 10-fold increase in diet-induced atherosclerosis in the absence of any changes in serum cholesterol levels or lipoprotein profiles (Fazio *et al.*, 1997; Boisvert and Curtiss, 1999). Moreover, transgenic mice expressing human apoE in the vessel wall show reduced atherosclerotic lesions in the absence of any changes in plasma cholesterol and lipoprotein profile (Shimano *et al.*, 1995). In addition, the expression of low levels of apoE in the artery wall of apoE-deficient mice using a retroviral transduction system leads to reduced early foam cell lesion formation (Hasty *et al.*, 1999). Therefore, although the ability of apoE to influence plasma cholesterol homeostasis and facilitate cellular cholesterol efflux are central to its atheroprotective role, apoE is also thought to protect from the development of atherosclerosis by other independent mechanisms, which will be discussed in more detail below.

1.2.3. OTHER ANTI-ATHEROGENIC FUNCTIONS OF APOLIPOPROTEIN E

As described above, a number of studies have shown that low expression of apoE in the vessel wall is able to limit the development of atherosclerosis without having any effect on plasma cholesterol levels or lipoprotein profile (Fazio *et al.*, 1997; Boisvert and Curtiss, 1999; Van Eck *et al.*, 2000; Shimano *et al.*, 1995; Bellosta *et al.*, 1995; Hasty *et al.*, 1999; Gough and Raines, 2003). This suggests a potent anti-atherogenic role of apoE that is independent of its action on cholesterol metabolism and transport. Further evidence in support of an alternative anti-atherogenic role for apoE comes from recent work by Thorngate *et al.* (2003) who demonstrated that low-level apoE expression, is able to inhibit atherogenesis using a mechanism separate to its ability to enhance cholesterol efflux via the ABCA1 pathway or by facilitating reverse cholesterol transport via apoAI or apoAI-containing HDL. They also demonstrated that other HDL-dependent cholesterol efflux pathways do not play a role in mediating the atheroprotective effects of low level apoE. Therefore it must be considered that apoE may block atherogenesis by mechanisms that are independent of its ability to normalise plasma cholesterol concentrations.

ApoE has been shown to exhibit local cytokine- and hormonal-like effects on the surrounding cells of the vessel wall, which also contributes to its atheroprotective role. It was originally thought that HDL had anti-platelet activities (Aviram and Brook, 1983) but further research revealed that the anti-aggregatory action of HDL was due to a minor subset of HDL which contained apoE (Riddell *et al.*, 1996). For example, apoE inhibits agonist-induced platelet aggregation, which has been proposed to occur through apoE binding to specific receptors on the platelet surface, which in turn initiates cell signalling events that results in the stimulation of nitric

oxide-synthase (Riddell *et al.*, 1999). The cytoplasmic tail of this cell surface protein, apoE receptor 2 (apoER2) is thought to contain domains important in cellular signalling, including potential targets for cyclic-GMP- and cyclic-AMP-dependant protein kinases and consensus sequences for Src Homology 3 (SH3) recognition. The binding of apoE to apoER2 stimulates endothelial nitric oxide release in an isoform-dependant manner, and it has been proposed that tyrosine phosphorylation of apoER2 initiates phosphoinositide-3-kinase (PI3K) signalling and activation of nitric oxide synthase (Sacre *et al.*, 2003). Nitric oxide (NO) is a potent inhibitor of platelet activation and aggregation and acts through the activation of platelet soluble guanylate cyclase to increase levels of cGMP (Lincoln *et al.*, 1997).

ApoE can also directly modify the T lymphocyte- and smooth muscle cell-mediated inflammatory responses of atherosclerosis. T-lymphocytes have been shown to accumulate locally in complicated human atherosclerotic plaques (Jonasson *et al.*, 1986), and apoE-deficient mice crossed with mice defective in both T and B lymphocyte function show a 42% reduction in atherosclerosis when fed a chow diet (Dansky *et al.*, 1997). However when these same mice are fed cholesterol, lesion formation was unaffected, which indicates that atherosclerosis-prone mice display a prominent immune system component within their lesions and that once early lesions develop, immune responses modulate lesion progression (Dansky *et al.*, 1997). Various studies have shown that apoE can suppress the activation and proliferation of peripheral blood mononuclear cells as well as purified T cells stimulated *in vitro* with polyclonal mitogens and antigens (Avila *et al.*, 1982; Pepe and Curtiss, 1986; Okano *et al.*, 1985; Cardin *et al.*, 1988; Hui and Harmony, 1980a; Dyer *et al.*, 1991). This antiproliferative effect of apoE is thought to be due to inhibition of critical events such as intracellular calcium accumulation and phosphatidylinositol turnover (Hui and Harmony, 1980a and 1980b), which results in the reduction of IL-2 expression (Kelly *et al.*, 1994). Also apoE was shown to be capable of inhibiting the high affinity IL-2 receptor (IL-2R) on primary IL-2 dependant T cells, which consequently arrests lymphocytes at the G1A/G1B boundary in the cell cycle (Mistry *et al.*, 1995).

Smooth muscle cells (SMCs) also participate in inflammatory responses within the intima, and it was recently reported that apoE inhibits SMC migration and proliferation induced by the inflammatory agonists platelet-derived growth factor (PDGF) and oxLDL (Ishigami *et al.*, 2000). This inhibition of SMC proliferation is thought to be mediated by apoE inhibition of mitogen-activated protein (MAP) kinase activity and a corresponding decrease in cyclin D1 expression (Ishigami *et al.*, 1998). Recent work has also demonstrated that the inhibition of PDGF-stimulated cell migration is mediated via suppression of signal transduction through the LRP. Tyrosine phosphorylation of the cytoplasmic tail of LRP is stimulated by PDGF and this activity is inhibited by apoE (Boucher *et al.*, 2002). There is also evidence that this inhibition of SMC proliferation is partly

due to an apoE-dependant increase in heparan sulfate proteoglycan synthesis (inhibitors of smooth muscle cell proliferation) from endothelial cells within the lesion (Paka *et al.*, 1988).

Another anti-atherogenic property of apoE is its ability to inhibit lipid oxidation (Miyata *et al.*, 1996), this antioxidative property of apoE may influence early events of atherogenesis by preventing the accumulation of oxLDL. Evidence of enhanced oxidative stress has been found in apoE-deficient mice and LDL oxidation-specific epitopes have been identified in aortic lesions of apoE-null mice by immunohistochemical techniques (Palinski *et al.*, 1994). According to Hayek *et al.* (1994) circulating lipoproteins in apoE-deficient mice are more oxidized and more susceptible to oxidation *in vitro* than lipoproteins from the wild-type animals. Although the mechanism is not completely understood, it has been shown that apoE can bind metal ions such as copper and iron, possibly sequestering them and preventing their participation in the oxidation process (Miyata and Smith, 1996).

A fraction of macrophage-secreted apoE is sequestered within the pericellular proteoglycan matrix, where apoE, in addition to influencing the uptake and retention of atherogenic lipoproteins into the vessel wall can modulate the availability of cytokines and growth factors retained within it. ApoE is an effective inhibitor of endothelial cell proliferation and is able to inhibit the chemotactic response of endothelial cells to basic fibroblast growth factor (bFGF) [Vogel *et al.*, 1994], which was partly due to the competition of apoE with growth factors for interactions with cell surface HSPG. ApoE has also been shown to inhibit VCAM-1 expression by endothelial cells (Stannard *et al.*, 2001). Another potential anti-atherogenic role of apoE is its ability to increase triglyceride-rich particle (TGRP) accumulation in the endothelial layer, these particles become bound by the arterial wall matrix which seems to effectively block deep penetration of TGRPs into the arterial wall (Mullick *et al.*, 2002).

Therefore, the above properties of apoE demonstrate how this protein may potentially inhibit atherogenesis without affecting plasma cholesterol levels. Also these characteristics of apoE may explain how low levels of apoE can protect from atherosclerosis. Work by Thorngate *et al.* (2000) using apoE-deficient mice that express only adrenal apoE have revealed that low levels of apoE (< 1% to 2% of wild-type plasma apoE) can effect the progression of atherosclerosis without affecting plasma cholesterol levels. These studies also demonstrated that apoE plasma levels of up to 3% of wild-type levels can be achieved by adrenal expression and that this level is sufficient to normalise total plasma cholesterol levels, indicating that although in wild-type mice the liver is the major source of plasma apoE, apoE protein from any source, when present in the plasma compartment, can contribute to the hepatic uptake of the cholesterol-containing lipoproteins. Also heterozygous apoE+/- mice do not have elevated plasma cholesterol (Zhang *et al.*, 1992) and plasma apoE concentrations of only 40ug/dL generated solely by bone marrow-transplanted

macrophages can effectively lower plasma cholesterol levels (Hasty *et al.*, 1999). Conversely, over-expression of human apoE in apoE-/- mice at levels of more than 30mg/dl leads to hypertriglyceridaemia (Huang *et al.*, 1998) which is due to both an apoE-dependant increase in hepatic VLDL triglyceride production (Mensenkamp *et al.*, 1999) and interference with apoC-II-dependant VLDL lipolysis by LPL (Huang *et al.*, 1998), indicating that an optimal range of apoE exists, in which it can prevent atherogenesis.

1.2.4. BIOCHEMICAL CHARACTERISATION OF APOLIPOPROTEIN E

1.2.4.1. THE APOLIPOPROTEIN E GENE

The apoE gene consists of 3.7 kb of DNA and has been mapped to chromosome 19 where it is located at the 5' end of a 50 kb gene-cluster with apoCI, an apoCI pseudogene, apoCII and apoCIV (Allan *et al.*, 1995). The mRNA consists of 1163 bases and the coding region contains four exons separated by three introns. ApoE is produced in most organs and significant quantities of apoE mRNA have been detected in the liver, brain, spleen, lung, ovary, adrenal gland, kidney and muscle, in several different species (Driscoll and Getz, 1984; Elshourbagy *et al.*, 1985). Tissue specific expression of apoE is regulated by multiple independent positive and negative regulatory elements, many of which are located between the apoE and apoC-I gene (Smith *et al.*, 1988; Simonet *et al.*, 1991). The highest expression of apoE is found in the parenchymal liver cells, where its expression is directed by two distal regulatory elements, the hepatic control regions, located downstream of the apoE promoter (Simonet *et al.*, 1993). ApoE mRNA expression has also been demonstrated in the spleen and lungs, where macrophages are most likely to be responsible for its synthesis (Srivastava *et al.*, 1996). The expression of apoE in macrophages is thought to be controlled by two homologous regulatory regions, designated multi-enhancer 1 and 2, both of which are located downstream of the apoE promoter (Shih *et al.*, 2000).

1.2.4.2. TRANSCRIPTIONAL REGULATION OF APOLIPOPROTEIN E

ApoE gene expression is extremely complex due to interactions from a number of proteins that bind to the proximal promoter region as well as downstream elements (Paik *et al.*, 1988; Smith *et al.*, 1988; Simonet *et al.*, 1991,1993; Berg *et al.*, 1996). Expression of the apoE gene varies between different cell types and several potential regulatory regions have been identified that may be involved in basal and tissue-specific control of the gene, although much less is known about the transcription factors that regulate expression.

1.2.4.2.i. PROXIMAL APOE REGULATORY ELEMENTS AND FACTORS

Little is known about the regulatory elements controlling apoE gene expression in macrophages, although one study by Basheeruddin *et al.* (1994), showed that the –623 to –447 promoter region was necessary for transcriptional activation during 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced differentiation of human THP-1 monocytes into macrophages. This study suggests that AP-1-like proteins were involved, possibly due to the phorbol ester activating the protein kinase C (PKC) pathway which in turn modulates the binding activity of AP-1 transcription factors. A study by Andreani-Mangeney *et al.* (1996) demonstrated that cyclic-AMP (cAMP) inhibits apoE transcription in HepG2 cells, which is mediated by elements located between –614 to –804 in the apoE promoter region. Interestingly, this study also provided evidence of a minimal promoter region, –200/-1, which contains elements that respond positively to cAMP, thereby indicating a more complex regulation by this factor. Indeed, work by Garcia *et al.* (1996) has identified the transcription factor AP-2, which interacts with the –48 to –74 and –107 to –135 regions of the human apoE promoter, as a mediator of synergistic activation by cAMP and retinoic acid in astrocytic cells. It was also suggested that protein kinase A (PKA)-mediated phosphorylation of AP-2 may be important in this regulation.

Promoter deletions and footprinting analysis of the apoE promoter have also identified several other proximal promoter regulatory elements, many of which are thought to be recognised by Sp1. A strong non-specific enhancer, termed upstream regulatory element (URE) 1, has been identified at nucleotides –193 to –124 upstream of the first exon (Paik *et al.*, 1988; Smith *et al.*, 1988). Also another non-specific enhancer was identified at nucleotides –366 to –246, and a GC-box motif that binds the Sp1 transcription factor was identified at nucleotides –59 to –45, both of which promote apoE gene expression (Paik *et al.*, 1995; Paik *et al.*, 1988; Smith *et al.*, 1988; Chang *et al.*, 1990). Also by using nuclear extracts from various cell lines, a DNase I footprint was identified at nucleotides -103 to –87, termed URE3 (Jo *et al.*, 1995). This positive transcriptional element may have a dual role in apoE gene expression depending on the cell type, as a part of the URE3 region has been shown to be a binding site for the nuclear repressor factor BEF-1, which becomes phosphorylated for activity via a PKC pathway in HepG2 cells (Berg *et al.*, 1995; Berg *et al.*, 1996)

In addition to the promoter elements described above, several consensus sequences for various other transcription factors that interact with the proximal promoter region have been identified through the use of various techniques, including transfection assays, DNase-I and *in vivo* footprinting, cell free transcription assays and the yeast one-hybrid system. A summary of these transcription factors are shown in Table 1.2.

TABLE 1.2. TRANSCRIPTION FACTOR CONSENSUS SEQUENCES IDENTIFIED AS AFFECTING APOE GENE EXPRESSION

CONSENSUS SEQUENCE	CELL TYPE	REFERENCES
SP-1	HepG2, mouse liver nuclei	Paik *et al.*, 1995; Smith *et al.*, 1988; Dang and Taylor, 1996
AP-2	U87	Garcia *et al.*, 1996
AP-1	THP-1	Basheeruddin *et al.*, 1994
HNF3	Mouse liver nuclei	Dang and Taylor, 1996
HNF4	Mouse liver nuclei	Dang and Taylor, 1996
TF-LF2	Mouse liver nuclei	Dang and Taylor, 1996
GATA-1	Mouse liver nuclei	Dang and Taylor, 1996
C/EBP	Mouse liver nuclei	Dang and Taylor, 1996
BEF-1	Hep G2	Berg *et al.*, 1995
Aa	Hep G2	Smith *et al.*, 1988
B1b	Hep G2	Smith *et al.*, 1988
B2b	Hep G2	Smith *et al.*, 1988
Zic1, Zic2	U87	Salero *et al.*,2001

1.2.4.2.II. DISTAL REGULATORY ELEMENTS AND FACTORS

Although various promoter elements have been identified, studies have shown that promoters lack the ability to direct apoE gene transcription *in vivo*, in any cell in the absence of distal enhancers (Simonet *et al.*, 1991). Various such distal regulatory elements that control the transcription of the apoE gene in liver and other tissues have been determined using transgenic mice and it has been demonstrated that a number of sequences in the intergenic region between apoE and apoCI are required for the expression of both these genes in a variety of tissues including liver, testis, spleen, skin, submaxillary gland, kidney, brain, small intestine, heart, stomach, and the pancreas (Simonet *et al.*, 1993). In addition, two hepatocyte-specific enhancers, hepatic control regions (HCR) 1 and 2, have been reported and have been found to be essential for the hepatic expression of apoE/apoCI gene locus (Simonet *et al.*, 1991). Both of these enhancers are located 15kb (HCR1) and 26kb (HCR2) 3' to the apoE gene and share 87% sequence identity, which is thought to have arisen as a result of an evolutionary gene duplication that yielded the apoCI and apoCI' genes (Allan *et al.*, 1995).

Two enhancer regions which direct macrophage- and adipose-specific expression in transgenic mice were also identified recently (Shih *et al.*, 2000). These two homologous enhancers,

designated multi-enhancer 1 and 2 (ME1 and ME2) are located 3.3kb and 15.9kb downstream of the apoE gene and contain 620 and 619 nucleotides, respectively. Several binding motifs for the glucocorticoid receptor (GR) and C/EBP-α and C/EBP-β have been located in these enhancer regions although the precise mechanism of activation is unknown. Another recent study has demonstrated that the nuclear liver-X-receptor (LXR) -α and -β regulate apoE expression in macrophages and adipocytes. Laffitte *et al.* (2001b) identified that both the promoter and the ME1 and ME2 enhancers of the apoE gene contain a conserved LXR-responsive element (LXRE) that activated gene transcription in response to oxysterol ligands in macrophages, but not monocytes. Also work by Galetto *et al.* (2001) has demonstrated a peroxisome proliferator-activated receptor (PPAR)-γ response element within the apoE/apoCI intergenic sequence, which may play a vital role in apoE up-regulation during monocyte differentiation.

Although recent studies are uncovering binding motifs important in apoE expression, further research is essential in order to determine precisely what regions and what transcription factors are important for the regulation of macrophage-derived apoE expression. In addition, the signalling pathways associated with inducible apoE expression are poorly understood and need to be elucidated in detail.

1.2.4.3. POLYMORPHISMS OF APOLIPOPROTEIN E

Three common isoforms of apoE exist in humans, E2, E3 and E4 and are products of three alleles ε2, ε3, and ε4 at a single gene locus (Zannis and Breslow, 1981). The three apoE isoforms can be distinguished from each other by isoelectric focusing due to charge differences, which are the result of cysteine-arginine amino acid substitutions at residues 112 and 158. Although the isoforms differ from one another only by a single amino acid substitution, these changes have profound functional consequences at the cellular and molecular level. The most common phenotype in humans is E3/3 (60%) and seems to be the normal isoform in all known functions; therefore apoE3 (Cys^{112}, Arg^{158}) is considered to be the parent form of the protein, and apoE4 (Arg^{112}, Arg^{158}) and E2 (Cys^{112}, Cys^{158}) are variants (Rall *et al.*, 1982). The frequency of the different alleles in a normolipidemic population is 8%, 77%, and 15% for apoE2, apoE3 and apoE4 respectively (Rall *et al.*, 1982).

Polymorphism of apoE is one of the major determinants of the initiation and progression of atherosclerosis (Davignon *et al.*, 1988; De Andrade *et al.*, 1995) and each of the three isoforms differ with respect to their binding affinity for the LDL receptor, lipoprotein preference and their affinity for heparan sulfate proteoglycans (Davignon *et al.*, 1988). *In vitro* LDL receptor binding studies have demonstrated that the binding affinity of the apoE2 phenotype for the LDL receptor is less than 2% of the affinity of apoE3 and apoE4 (Weisgraber *et al.*, 1982). Due to this impaired

receptor binding, the E2 phenotype is associated with the development of type III hyperlipidemia, a disorder caused by impaired metabolism of apoE containing lipoproteins, which results in a higher proportion of VLDL and IDL in the plasma, a risk factor in atherosclerosis (Gregg *et al.*, 1981). Patients with this form of hyperlipidemia are homozygous for the apoE2 allele, however, other genetic or environmental factors must influence the phenotype because only 1% to 2% of E2 homozygotes have symptomatic hyperlipidemia (Mahley *et al.*, 1999). In fact, most E2 homozygotes have either normal or low cholesterol levels (Rall and Weisgraber, 1982; Davignon *et al.*, 1988). Although the apoE4 phenotype displays normal receptor binding it is still associated with an increased risk for atherosclerosis due to elevated levels of plasma LDL cholesterol (Davignon *et al.*, 1988). ApoE4 is also associated with increased risk for Alzheimer's disease, impaired cognitive function, and reduced neurite outgrowth; isoform-specific differences in cellular signaling events may also exist (Huang *et al.*, 2004; Mahley and Huang, 1999).

The apoE phenotype is also responsible for the preference of apoE variants for the different lipoproteins in the circulation. Human apoE4 preferentially associates with VLDL, whereas apoE3 and apoE2 preferentially associate with HDL (Dong and Weisgraber, 1996). In addition, apoE2 has an even greater affinity for HDL as compared to apoE3 (Luc *et al.*, 1997). Therefore the apoE genotype has a profound effect on the efficiency of cholesterol efflux (Zhang *et al.*, 1996; Cullen *et al.*, 1998; Huang *et al.*, 1998).

Several other naturally-occuring rare variants of apoE have been described, most of which are characterised by substitution of a single amino acid within the LDL receptor binding domain, thereby directly reducing interactions with the LDL receptor. However, one apoE variant with a defective LDL-receptor binding capability, apoE3-Leiden, consists of a 7-amino acid tandem repeat of residues 121-127, which is located outside the LDL receptor binding domain of apoE (Wardell *et al.*, 1989).

A secondary form of apoE polymorphism is explained by posttranslational glycosylation (Mahley, 1988). Several glycosylated isoforms arise from either the presence or the absence of a mono- or disialyted carbohydrate moiety at a single site in apoE, O-linked glycosylation of threonine 194 (Wernette-Hammond *et al.*, 1989).

1.2.4.4. PROTEIN SYNTHESIS AND SECRETION.

ApoE is a 299-amino acid secretory protein, which follows the classical pathway for secretory protein synthesis and release. The primary translation product is 317 amino acids and contains 18 amino acid extension at the N-terminus, which acts as a signal peptide, directing the nascent chain to the endoplasmic reticulum (Zannis *et al.*, 1984). The protein is transported to the Golgi prior to secretion and the protein is glycosylated with carbohydrate chains containing sialic acid (Mazzone

et al., 1986). The glycosylation is O-linked and newly synthesised apoE differ by –2, -4 and –6 charges from the corresponding asialo form that is detected in plasma. Sialation is thought to be a requirement for apoE secretion as 92% of secreted apoE is sialyted in comparison to 42% of intracellular apoE and 24% of plasma apoE (Zannis *et al.*, 1984).

The secretion of apoE is a complex process and a significant proportion of newly synthesised apoE undergoes proteasomal degradation prior to secretion (Mazzone *et al.*, 1992; Duan *et al.*, 1997), as has been shown for apoAI and apoB in liver (White *et al.*, 1999; Yeung *et al.*, 1996). For example, the rate of apoE secretion from mouse peritoneal macrophages is only 44% of the measured rate of apoE synthesis (Dory, 1991). In addition, newly secreted apoE may remain tightly bound to proteoglycans on the cell surface (Lucas and Mazzone, 1996), and this source of the protein can serve as a precursor for the secreted apoE. ApoE arrives at the cell surface in a relatively lipid-poor state and whilst retained at the cell surface by proteoglycans, acquires lipid, this lipidation of the protein results in changes in apoE conformation and subsequent release from the cell surface (Zhao and Mazzone, 2000). This efflux mechanism may explain why endogenous apoE expression is more efficient at producing cholesterol efflux from macrophages compared with exogenous apoE (Lin *et al.*, 1999). Endogenously synthesised apoE is more efficiently sequestered at the cell surface due to an increased exposure to low affinity-binding sites at the cell surface during passage through the pericellular space.

ApoE retained at the cell surface may also undergo re-uptake and then be degraded in a lysosomal compartment (Deng *et al.*, 1995), or recycled back to the cell surface/extracellular medium (Lucas and Mazzone, 1996: Zhao and Mazzone, 2000). This recycling of proteoglycan-bound apoE through intracellular membrane compartments including the Golgi could facilitate lipid acquisition by apoE (Zhao and Mazzone, 2000) and also enable cells to maintain their apoE levels at an optimal level. Work by Heeren *et al.* (1999) showed by the use of radiolabeled triglyceride-rich lipoproteins, that 60% of the labelled apoE was released back into the medium, where it again became part of lipoproteins. In addition to hepatocytes, evidence for apoE recycling has been provided in macrophages where a substantial amount of the internalised lipoprotein-associated apoE is retained inside the cell and then reappears in the secretion medium (Takahashi and Smith, 1999). This recent discovery that internalised apoE may escape the lysosomal degradation pathway (Fazio *et al.*, 1999) suggests the presence of an active cellular mechanism which dissociates apoE from the lipoprotein particle and carries apoE back to the secretory apparatus.

1.2.4.5. STRUCTURE OF APOLIPOPROTEIN E

The predicted secondary structure of apoE is thought to consist of α-helices (62%), β-sheet structures (9%) and β-turns (11%) and the remaining 18% having a random structure (Mahley, 1988). Within the apoE structure two distinct independantly folded structural domains are thought to exist and are joined by a randomly arranged protease-susceptible hinge region (Mahley, 1988). Digestion of apoE with thrombin, that cleaves at residues 191 and 215, generates a 22kDa N-terminal fragment (residues 1-191) and a 10kDa C-terminal fragment (residues 216-299) [Aggerbeck *et al.*, 1988]. The molecule is predicted to have a highly ordered structure throughout the amino-terminal half of the molecule. Then, beginning at residue 165, a long stretch of predicted random structure extends for about 35 residues. This random structure is then followed by a highly ordered structure representing approximately the carboxyl-terminal third of the molecule.

1.2.4.5.i. N-TERMINAL DOMAIN OF APOE

The structure of the N-terminal domain of apoE has been revealed by crystallization and X-ray diffraction analysis. The domain is a compact structure consisting of 5 helices, in which 4 of the 5 helices are arranged in an antiparallel helix bundle (Wilson *et al.*, 1991). This region of apoE has been implicated as the LDL receptor and LRP binding domain and a basic arginine- and lysine-rich segment of helix 4 (residues 136-158) is thought to be essential for receptor binding. *In vitro* studies using apoE that has been chemically modified at the lysine or arginine residues within this LDL receptor binding domain, have demonstrated the complete inhibition of receptor binding (Mahley *et al.*, 1977). ApoE variants associated with type III hyperlipoproteinaemia show a reduced binding activity, the most common variant is apoE2 (Arg^{158} to Cys), where the normally occurring arginine at position 158 is replaced with cysteine. This reduced binding capability of the apoE2 variant is due to the absence of a positive charge at position 158, which dramatically changes salt bridges within helix 4 and between helices 3 and 4 (Innerarity *et al.*, 1984). This absence of salt bridges alters the conformation of the LDL receptor-binding region and greatly lowers the positive ion potential of this region (Dong *et al.*, 1996; Wilson *et al.*, 1994). Also, a region spanning residues 171-183 contains critical elements necessary for the stabilisation or the alignment of the LDL receptor-binding domain (Lalazar and Mahley, 1989). In addition, lipid association of the N-terminal domain is thought to be essential for high-affinity binding to the LDL receptor, as the four helix bundle undergoes a structural reorganization upon lipid binding, where the helical segments of the bundle realign, presenting a convex receptor-active surface (Raussens *et al.*, 1998).

A segment of helix 4 (residues 142-147) also contains a high-affinity HSPG binding site that is highly complementary to heparan sulfate rich in N- and O-sulfo groups such as that found in the liver and brain (Libeu *et al.*, 2001). This binding of apoE to proteoglycans involves an

interaction between the positively charged arginine- and lysine-rich region of helix 4 with the negatively charged carboxylate and sulfate groups of proteoglycans and is the initial step of the HSPG-LRP pathway. Rare variants of apoE with substitutions for the normally occurring basic amino acids in this region are moderately defective in binding to the LDL receptor but tend to be severly defective in binding to HSPG (Ji *et al.*, 1994b).

Recent work has also suggested that the N-terminal domain of apoE contains an SR-BI binding sequence (Li *et al.*, 2002a). However, work by Thuahnai *et al.* (2003) has also demonstrated that the C-terminal region of apoE is also required for SR-BI binding in the lipid-free state.

1.2.4.5.II. C-TERMINAL DOMAIN OF APOE

The C-terminal region of apoE contains 3 amphipathic helices spanning the regions 203-222, 225-266, and 268-289 (Nolte and Atkinson, 1992), all of which are thought to be important for lipid binding. In the absence of lipids, apoE self-associates as a tetramer due to protein-protein interactions of the C-terminal domains (Aggerbeck *et al.*, 1988). In the presence of lipid, no self-association occurs as the C-terminal domain is required for the association of apoE with lipoproteins. Studies with synthetic peptides from the carboxyl terminus (Sparrow *et al.*, 1992) and apoE3 C-terminus truncation studies (Westerlund and Weisgraber, 1993) revealed that residues 263-286 are critical for apoE tetramerization and lipoprotein association. The C-terminal domain of apoE also contains a glycosaminoglycan binding site that is located between residues 243-272 (Weisgraber *et al.*, 1986). It has also been proposed that the C-terminal region of apoE plays a role in the activation of the enzyme LCAT, which is the enzyme responsible for transforming cholesterol into cholesteryl esters (De Pauw *et al.*, 1995).

The structure of apoE is also responsible for the lipoprotein preferences of the apoE isoforms as domain interaction between the amino- and carboxyl- segments of apoE distinguish the lipoprotein preferences of apoE4 for VLDL versus apoE3 and apoE2 for HDL. This domain interaction in apoE4 is due to an arginine residue replacing a cysteine at position 112, which causes a reorientation in the sidechain of arginine 61 (Dong and Weisgraber, 1996). This reorientation allows arginine 61 to adopt a different exposed position on helix 2, which leads to the formation of a new salt bridge between glutamic acid 109 and residue 112 in helix 3, and more importantly causes the side chain of arg 61 to interact with the carboxyl terminus causing an alteration in conformation which distinguishes it from apoE3 and apoE2 (Dong and Weisgraber, 1996).

1.2.4.6. MODULATION OF APOLIPOPROTEIN E EXPRESSION

The synthesis and secretion of macrophage apoE is controlled at multiple loci, and is thought to be regulated via transcriptional, posttranscriptional and posttranslational mechanisms. Altering the functional state and metabolism of macrophages such that they mimic situations in atherogenesis results in altered apoE expression. For example, cholesterol loading of macrophages is accompanied by a 10-fold induction of apoE gene transcription (Mazzone *et al.*, 1989; Mazzone *et al.*, 1987). In addition to cholesterol loading, macrophage apoE production can be altered by extracellular cholesterol acceptors and cytokines and also the very act of monocyte differentiation induces apoE expression (Werb and Chin, 1983; Auwerx *et al.*, 1988; Menju *et al.*, 1989; Tajima *et al.*, 1985). For example, bone marrow cells cultured in the presence of colony stimulating factor-1 for 9 days mature into macrophages and secrete apoE (Werb and Chin, 1983). Also, studies in THP-1 cells, which require activation with phorbol ester in order to acquire the macrophage phenotype have revealed that differentiation is associated with increased apoE expression mediated at the level of mRNA, cellular protein and secreted protein (Auwerx *et al.*, 1988; Menju *et al.*, 1989; Tajima *et al.*, 1985). Basheeruddin *et al.* (1992) postulated that this induction in apoE was due to apoE mRNA being more slowly degraded in macrophages compared to monocytes due to inhibition of a specific nuclease, eventually leading to increased levels of apoE. Another mode of apoE regulation is degradation, as a substantial amount of newly synthesised apoE in macrophages is degraded prior to secretion due to either direct targeting of apoE to lysosomal pathways (Deng *et al.*, 1995) or ubiquitinated-mediated proteasomal degradation (Duan *et al.*, 1997; Wenner *et al.*, 2001), or association of secreted apoE to an extracellular matrix that is then subject to rapid degradation (Lucas and Mazzone, 1996).

1.2.4.6.I. ROLE OF CYTOKINES ON MACROPHAGE APOE

Inflammatory cytokines are considered to be important in the pathogenesis of atherosclerosis and other vascular diseases. Various cytokines are produced by multiple cell types during atherogenesis and many of these cytokines regulate apoE expression in macrophage foam cells. For example, the inflammatory agent, bacterial lipopolysaccharide (LPS) decreases apoE secretion from mouse macrophages when added exogenously or injected *in vivo* (Werb and Chin, 1983), which may be due to LPS stimulation of TNF-α secretion (Zuckerman and O'Neal, 1994; Zuckerman *et al.*, 1992; Starck *et al.*, 2000). This indirect form of apoE regulation, through stimulation of TNF-α may also be the case for granulocyte-macrophage colony-stimulating factor (GM-CSF) down-regulation of apoE (Zuckerman and O'Neal, 1994). ApoE production by macrophages is also inhibited by interferon-γ (IFN-γ), which is due to increased intracellular degradation of newly synthesised apoE (Brand *et al.*, 1993). Interleukin-1 (IL-1) also affects apoE

expression in HepG2 cells through phosphorylation of the trans-repressor BEF-1, which, in turn inhibits apoE gene expression (Berg *et al.*, 1996). Conversely, the anti-inflammatory agent TGF-β has been associated with an increase in apoE mRNA expression and secretion (Zuckerman *et al.*, 1992; Duan *et al.*, 1995). Various other cellular factors also modulate apoE expression, including the thyroid hormone (Vandenbrouck *et al.*, 1994), insulin (Ogbonna *et al.*, 1993), oestrogen (Srivastava *et al.*, 1996), and cAMP (Andreani-Mangeney, 1996).

1.2.4.6.II. EFFECT OF STEROL LOADING ON MACROPHAGE APOE

A number of studies have investigated the effects of cholesterol loading on apoE expression as cholesterol loaded macrophages are prominent within the atherosclerotic lesion. Although these studies have shown an enhancement of apoE secretion and synthesis, and stimulation of apoE mRNA expression due to increased gene transcription, the mechanisms of regulation have not yet been elucidated (Dory, 1989; Rees *et al.*, 1999; Cader *et al.*, 1997; Mazzone and Basheeruddin, 1991). Work by Paik *et al.* (1988) demonstrated that the sterol-mediated upregulation of apoE required a positive element for apoE gene transcription (PET) [-169/-140], a core sequence of the URE1 region of the human apoE gene. This sequence has previously been shown to have a high homology with the SRE42 of the human LDL receptor gene (Paik *et al.*, 1988) and has been renamed as SRE4 based on its new role as a sterol sensor.

The segregation of cell-surface apoE between secretion and degradation is modulated by the presence of extracellular lipid, such as phospholipid vesicles or lipoproteins (Mazzone *et al.*, 1992). Work by Duan *et al.* (1997) has demonstrated that pre-incubation of macrophages with sterol increases secretion of apoE, which is due to the suppression of apoE intracellular degradation. This effect of sterol on apoE degradation requires the presence of the C-terminal domain of apoE (Duan et al., 2000). This domain is thought to to be necessary for apoE to interact with membrane lipids, which results in an alteration in apoE conformation, which leads to a decrease in susceptibility to proteolysis (Duan *et al.*, 2000). This reduction in apoE degradation may also be due to sequestration of apoE away from proteases (Duan et al., 2000).

In human monocyte-derived macrophages (HMDM) the release of cholesterol and apoE are kinetically parallel, although there is substantial evidence that the two processes are independent of each other (Dory, 1991; Rees *et al.*, 1999; Basu *et al.*, 1983). Work by Rees *et al.* (1999) revealed that upon cholesterol loading there was greater spontaneous release of cholesterol from apoE secreting macrophages from normal mice than those of apoE knockout mice. However there was no difference in cholesterol efflux to apoAI between the two cell sources. Also, when cholesterol-loaded HMDM were incubated with β-cyclodextrins, which are known to promote

cholesterol efflux without associated phospholipid release, cholesterol efflux was observed without increased apoE secretion (Rees et al., 1999).

Oxidised forms of cholesterol, such as those present in foam cells, also modulate apoE expression. Incubation of mouse peritoneal macrophages with 25-hydroxycholesterol increased synthesis (Mazzone *et al.*, 1987) and also increased apoE mRNA levels in differentiated THP-1 cells (Mazzone and Basheeruddin, 1991). However the relevance of these studies to the *in vivo* situation is limited as 25-hydroxycholesterol is a minor constituent in the atherosclerotic plaque. When differentiated THP-1 cells were incubated with copper oxidised LDL, which has 7-ketocholesterol as its major oxysterol [the most abundant oxysterol in the atherosclerotic plaque (Brown and Jessup, 1999)] an increase in apoE mRNA abundance and protein was detected (Cader *et al.*, 1997), which was due to an increase in gene transcription. The action of oxysterols is mediated through the nuclear receptors LXRα and LXRβ. A recent study by Laffitte *et al.* (2001b) has demonstrated that, in addition to controlling the tissue-specific expression of apoE in macrophages and adipocytes, the ME1 and ME2 enhancers are also likely to facilitate gene induction in response to cellular lipid loading. Recent work in knockout mice has also shown that synthetic LXR ligands are able to inhibit the development of atherosclerosis in mice (Joseph *et al.*, 2002b) and various studies have established a role for these nuclear receptors in dietary cholesterol absorption and in the control of cholesterol efflux in peripheral cells (Repa and Mangelsdorf, 2000; Venkateswaran *et al.*, 2000a). In addition, a recent study by Perez *et al.* (2003) demonstrated the sequential and co-ordinate regulation of apoE and nuclear receptor transcription, providing further evidence for the roles of nuclear receptors in apoE induction.

1.2.4.6.III. REGULATION OF APOE BY CHOLESTEROL ACCEPTORS

ApoE expression can also be regulated by HDL and apoAI, the protein constituent of HDL, both of which are extracellular acceptors of the reverse cholesterol transport pathway (Rees *et al.*, 1999; Bielicki *et al.*, 1999). Studies show that incubation of apoAI with cholesterol loaded macrophages from human monocyte derived macrophages, mouse peritoneal macrophages and THP-1 macrophages results in enhanced cholesterol efflux as well as enhanced apoE secretion (Rees *et al.*, 1999; Bielicki *et al.*, 1999). This regulation is likely to occur at the post-transcriptional level as incubation of apoAI treated cholesterol-loaded human monocyte-derived macrophages with actinomycin D, an inhibitor of transcription did not block apoE secretion (Rees *et al.*, 1999). This regulation of apoE by apoAI is of physiological relevance as this protein is small and can easily penetrate the arterial intima and access foam cells within the lesion. Also, the incubation of cholesterol-loaded mouse peritoneal macrophages (MPM) with HDL did not alter apoE mRNA levels or rates of protein synthesis although an increase in apoE secretion was observed (Dory,

1991). Work by Lin *et al.* (1998) has demonstrated that the binding affinity of HDL to macrophages increases in cells expressing apoE. Treatment with heparinase significantly reduced this HDL binding, suggesting that apoE secreted was from a cell surface pool, although cycloheximide decreased apoE secretion induced by apoAI, indicating that ongoing protein synthesis is required for apoE secretion in addition to a preformed pool (Rees *et al.*, 1999). A possible explanation for post-translational regulation of apoE is re-direction from a degradatory pathway to a secretory pathway, which may occur via a receptor-mediated mechanism, whereby apoAI or HDL are internalised and come into contact with vesicles containing new apoE.

The ATP binding cassette transporter ABCA1, and to a lesser extent ABCG1, both of which are important regulators of cholesterol efflux (Oram and Vaughn, 2000) have also recently been shown to modulate apoE secretion from human monocyte-derived macrophages (von Eckardstein *et al.*, 2001). Cholesterol loading and cAMP treatment, both of which increase apoE expression, also up-regulate ABCA1 expression in macrophages (Bortnick *et al.*, 2000; Langmann *et al.*, 1999; Takahashi *et al.*, 2000; Oram *et al.*, 2000). ABCA1 may play a role in the intracellular trafficking of apoE as inhibition of ABCA1 leads to the disappearance of apoE containing granular structures from the plasma membrane (von Eckardstein *et al.*, 2001). It is also possible that ABCA1 may serve either as a regulator of vesicular transport between the trans-Golgi network and the plasma membrane (Orso *et al.*, 2000) or as a channel protein within the plasma membrane (Orso *et al.*, 2000; Wang *et al.*, 2000; Oram and Vaughn, 2000), both of which would allow a role of ABCA1 in re-direction of apoE from a degradatory pathway.

Although the previous section has given a brief overview of the various factors that are able to modulate apoE expression in macrophages, the major focus of this thesis is the regulation of apoE by LXRs. Therefore, the next section will discuss these members of the nuclear receptor family in more detail.

1.3. LIVER X RECEPTORS

The liver X receptors (LXRs) are a family of transcription factors that were first identified as orphan members of the nuclear receptor superfamily. Nuclear receptors, includes both non-steroidal and steroid receptors, which regulate every major metabolic and developmental process by regulating gene transcription in response to ligand binding. Identified ligands for these receptors are small, lipophilic molecules such as steroids, retinoids, thyroid hormone, and vitamin D. In addition, >30 different orphan nuclear members of this receptor family have been identified for which ligands and/or biological functions are yet to be determined (Mangelsdorf and Evans, 1995).

The LXRs were originally isolated in 1995 using low-stringency hybridization screening of liver cDNA libraries (Willy *et al.*, 1995). Two members of the LXR family have been identified. LXRα (NR1H3, also known as RLD-1) and LXRβ (NR1H2, also known as UR, NER, OR-1 and RIP15) and sequence analysis shows that human LXRα and β share 77% amino acid identity in both their DNA- and ligand-binding domains (Teboul *et al.*, 1995). The expression patterns of the two LXR receptors differ significantly, with LXRα expression being restricted predominantly to tissues involved in lipid metabolism, with highest levels being found in the liver and also in the kidney, small intestine, spleen, adipose tissue, macrophages, pituitary and adrenals (Apfel *et al.*, 1994; Willy *et al.*, 1995; Auboeuf *et al.*, 1997). In contrast the expression of LXRβ is much more widespread and it is found in almost every tissue examined, including liver and brain (Song *et al.*, 1995; Kainu *et al.*, 1996). Recent work has shown that the LXRs are important mediators of the effects of oxLDL on macrophage gene expression, and are also central components of the cellular response to lipid loading. To date more than a dozen LXR target genes have been identified and the majority of which 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.

1.3.1. LXR AGONISTS

The identification of a specific class of oxidised derivatives of cholesterol as ligands for the LXRs has been crucial to helping understand the function of these receptors *in vivo* and first suggested their role in the regulation of lipid metabolism. Cholesterol neither activates nor binds LXR, although the introduction of an epoxide, hydroxyl, or keto group on the side chain renders this compound biologically active (Janowski *et al.*, 1996; Lehmann *et al.*, 1997). The original identification of oxysterols as natural ligands for LXR was reported almost simultaneously by three laboratories and initial ligand screening experiments employing organic extracts isolated from several tissues identified the first activators as a unique class of meiosis-activating sterols (Forman *et al.*, 1997; Janowski *et al.*, 1996; Lehmann *et al.*, 1997). Subsequent screening of numerous

compounds in the cholesterol metabolic pathway identified a specific group of mono-oxidised derivatives of cholesterol as the most potent ligands (Janowski *et al.*, 1996; Lehmann *et al.*, 1997; Forman *et al.*, 1997). These studies also demonstrated a structure-activity relationship for these compounds, and a requirement for the 3β-hydroxyl group of cholesterol and a single, stereoselective oxygen on the sterol side chain, which functions as a hydrogen bond acceptor.

The natural ligands of LXRs are oxysterols and include: 24(S), 25-epoxycholesterol, which is formed from squalene in a shunt pathway of cholesterol biosynthesis and is abundant in the liver; 22(R)-hydroxycholesterol, which is a transient intermediate in steroid hormone synthesis and abundant in the adrenal gland; 24(S)-hydroxycholesterol, which is abundant in the central nervous system; 20(S)-hydroxycholesterol; 6α-hydroxy bile acids; and mevalonate, which is an intermediate of cholesterol biosynthesis. Importantly, these oxysterols elicit an LXR response at concentrations consistent with those found in tissue extracts (Lehmann *et al.*, 1997; Janowski *et al.*, 1999) and the naturally occurring enantiomers are consistently more active than the synthetic enantiomers. Studies have shown that these compounds bind directly to the LXRs with K_d values ranging from 0.1 to 0. 4μM (Janowski *et al.*, 1999; Spencer *et al.*, 2001). Recently, it was found that 27-hydroxycholesterol, formed via the action of the enzyme 27-hydroxylase, is also an endogenous ligand for LXRs in cholesterol-loaded human macrophage cells, with K_d values of 85 and 71nM for LXRα and LXRβ respectively (Fu *et al.*, 2001). However it is not known whether 27-hydroxycholesterol binds to LXR or whether a metabolite is produced, which subsequently binds and activates the nuclear receptor, although recent work has demonstrated that cholestenoic acid, a metabolite of 27-hydroxycholesterol is able to activate LXRα at physiological concentrations (Song *et al.*, 2000).

No significant differences in the ability of LXRα or LXRβ to respond to oxysterols have been reported, although recently the oxysterol 5,6-24(S), 25-diepoxycholesterol has been shown to be a relatively selective LXRα ligand, suggesting the possibility of developing subtype-selective LXR ligands for pharmacological applications (Janowski *et al.*, 1999). Also, natural and synthetic 6α-hydroxy bile acids appear to be more selective activators of LXRα than LXRβ (Song *et al.*, 2000) and cholestenoic acid, a metabolite of 27-hydroxycholesterol, appears to be a naturally occurring ligand for LXRα, but not for LXRβ (EC_{50} for LXRα= 200nM, EC_{50} for LXRβ ≥ 5μM).

A number of synthetic pharmacological LXR ligands have also been developed, which are structurally unrelated to oxysterols. These compounds are high-affinity LXR-specific agonists and their increased potency compared to physiological ligands makes them useful tools for the study of LXR function. The first such ligands were the compound T0314407 and its derivative T0901317, which showed EC_{50} values for LXRα of 100nM and 20nM, respectively, compared to 300nM for

24(S), 25-epoxycholesterol (Schultz *et al.*, 2000). Another compound, acetyl-podocarpic (APD) was shown to be 1000-fold more potent than 22(R)-hydroxycholesterol for the activation of LXRα and LXRβ, and the maximal stimulation achieved was more than 6-fold higher than for the endogenous ligand (Sparrow *et al.*, 2002). More importantly, APD was approximately 1000-fold more potent than endogenous ligands for the induction of LXR target genes (Sparrow *et al.*, 2002). Most recently, a tertiary amine, GW683965, has been identified as an orally active LXR agonist (Collins *et al.*, 2002).

1.3.2. LXR ANTAGONISTS

Antagonists are compounds that compete with agonists for binding and prevent activation of receptors. Although it was initially assumed that the binding sites for agonists and antagonists were the same, there is now evidence that they can be different, although overlapping is required. It is thought that the conformation of the antagonist-bound receptor blocks productive interaction with one or more proteins required for transcription, or prevents efficient DNA binding, thereby reducing the ability of the receptor to activate target genes.

In contrast to the oxysterols, another product of the cholesterol metabolic pathway, geranylgeranyl-PP, negatively regulates LXRα and LXRβ transcriptional activity by inhibiting interaction of the LXRs with nuclear coactivators (Forman *et al.*, 1997; Gan *et al.*, 2001). Unsaturated fatty acids also antagonise LXR activity by acting as competitive inhibitors of LXR ligands (Ou *et al.*, 2001; Yoshikawa *et al.*, 2002). Additionally, human plasma contains natural antagonists for LXRα and LXRβ (Song *et al.*, 2001). These antagonists are specific 3-sulfate derivatives of oxysterols, and may be formed by the oxidation of cholesterol 3-sulfate, which is present in elevated plasma concentrations in hypercholesterolaemia (Tamasawa *et al.*, 1993).

Interestingly, the bile acid-responsive farnesol X receptor (FXR) dramatically represses LXRα activation in the absence of LXR ligands (Wang *et al.*, 1999). Since FXR and LXRα show overlapping expression patterns (liver, intestine, kidney), it may be hypothesised that their co-regulation by oxysterol and bile acid derivatives of cholesterol enables the organism to coordinately regulate cholesterol homeostatic pathways in several tissues.

1.3.3. LXR PROTEIN STRUCTURE

The LXRs, like other transcriptional regulators, exhibit a modular structure with different regions of the protein corresponding to autonomous functional domains that can be interchanged between related proteins without loss of function. The modular structure of nuclear receptors is similar throughout, despite the wide variation in ligand sensitivity between the receptor sub-types. LXRs, like all the nuclear receptors, contains a variable NH_2-terminal domain, also called the A/B region,

which contains a ligand-independent transcriptional activation function (AF-1) [Figure 1.4]. The A/B domain also shows promoter- and cell-specific activity, which suggests that it may contribute to the specificity of action among receptor isoforms and may interact with cell type-specific factors (Aranda and Pascual, 2001). This domain also contains several phosphorylation sites that may be the target of various different signaling pathways (Shao and Lazar, 1999). Although no phosphorylation of LXR has yet been shown, several studies, using other members of the nuclear receptor family have shown that this phosphorylation is able to affect transcriptional activity (Rochette-Egly *et al.*, 1997; Kato *et al.*, 1995; Patrone *et al.*, 1996; Juge-Aubry *et al.*, 1999).

The DNA-binding domain (DBD) is the most conserved domain of nuclear receptors and contains two highly conserved zinc finger motifs that target the receptor to specific DNA sequences (Luisi *et al.*, 1991; Schwabe *et al.*, 1993). These zinc finger motifs are located within two α-helices that cross at right angles to form the core of the DBD that recognises a hemi-site of the response element. Between the DBD and the ligand-binding domain (LBD) is a hinge region (D domain) [Figure 1.4] that allows protein flexibility and rotation of the DBD for simultaneous receptor dimerisation and DNA binding. This D domain in many nuclear receptors contains the nuclear localisation signals and contains residues whose mutation abolishes interaction with nuclear receptor corepressors (Aranda and Pascual, 2001).

The ligand-binding domain (LBD) is a large multifunctional domain located at the COOH-terminal of the receptor (Figure 1.4). This domain encompasses the ligand-binding domain, dimerisation interface, and contains a ligand-dependant activation function (AF-2) [Wurtz *et al.*, 1996]. The LBD is formed by twelve α-helices, with a conserved β-turn situated between helix 5 and 6. The helices are folded into a three-layered, antiparallel helical sandwich that contains a cavity, the ligand-binding pocket, which accomodates the ligand (Aranda and Pascual, 2001). This cavity is mainly hydrophobic and is buried within the bottom half of the LBD.

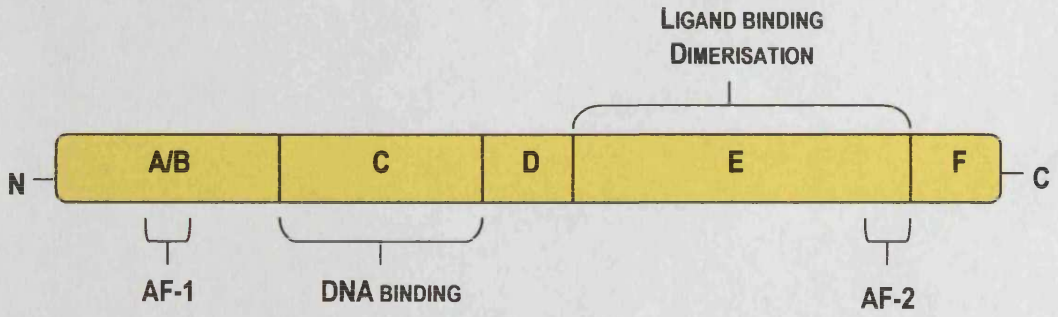


FIGURE 1.4. SCHEMATIC REPRESENTATION OF A NUCLEAR RECEPTOR
A typical nuclear receptor is composed of several functional domains. See text for further detail. Figure adapted from Aranda and Pascual, 2001.

The structure of ligand-bound receptors is much more compact than the unliganded receptor, demonstrating that upon ligand binding the receptor undergoes a conformational change (Aranda and Pascual, 2001). This conformational change is thought to enable transcriptional activation by the receptor as it co-ordinately dissociates corepressors and facilitates recruitment of coactivator proteins, necessary for ligand activation (Svensson *et al.*, 2003). Recruitment of coactivators appears to depend on specific interactions between the receptor and coactivator, and involves a common, highly conserved, LXXLL interaction domain found in the nuclear receptor interaction domain of many coactivators (McInerney *et al.*, 1998). Ligand-dependent recruitment of coactivators is dependent on AF-2, which consists of a short conserved helical sequence within the LBD (Bourguet *et al.*, 2000), although a distinct set of coactivators can also associate with the AF-1 domain. Point mutations in this AF-2 region had little or no effect on the binding of regulatory ligands, dimerisation or DNA-binding, but did abolish transcriptional activation (Svensson *et al.*, 2003). This region is therefore predicted to serve as an adapter surface for interactions with other molecules necessary for transcriptional activation (Danielian *et al.*, 1992; Barettino *et al.*, 1994).

1.3.4. LXR AND RXR

LXRs belong to the nonsteroidal subclass of nuclear receptors, which include the farnesol X receptor (FXR), peroxisome-proliferator activated receptors (PPAR), and the vitamin D receptor (VDR). As with other non-steroidal nuclear receptors, the majority of LXR proteins are localized in the nucleus and form obligate heterodimers with the retinoid X receptor (RXR) for function (Willy *et al.*, 1995). Target genes of this heterodimer are characterised by the presence of specific *cis* elements in their proximal promoter, distal enhancers or introns. The heterodimers bind to an LXR response element (LXRE) that contains a direct repeat of two similar hexanucleotide half-sites (consensus-AGGTCA) separated by 4 nucleotides, also known as a direct repeat-4 (DR-4) element (Edwards *et al.*, 2002; Mangelsdorf and Evans, 1995; Zhang *et al.*, 2001). Analysis of the functional LXREs that have been characterised in the known LXR target genes indicates that the consensus LXRE contains a number of invariant nucleotides in each half-site, and that the nucleotides at other positions can vary considerably. Although LXRα and LXRβ both bind with similar affinities to consensus LXR response elements, it is likely that these two nuclear receptors have differential effects on certain target genes.

The LXR/RXR heterodimer is a so-called permissive heterodimer, which is characterised by the ability to be activated independently by either the RXR ligand (i.e. 9-cis retinoic acid), the LXR ligand, or synergistically by both receptors' ligands together. This mechanism of dual-ligand activation contrasts with that exhibited by the second, well-characterised subclass of RXR heterodimers that include the vitamin D receptor (VDR), thyroid hormone receptor (TR), and

retinoic acid receptor (RAR) as partners. In these heterodimers, the RXR ligand cannot activate the heterodimer complex (as in the case of RXR/VDR and RXR/TR) or can do so only after the partner's ligand has first bound the receptor (RXR/RAR). One particularly novel aspect of the RXR/LXR heterodimer is that it only requires the carboxy-terminal activation function (the AF-2 domain) of LXR to become transcriptionally active (Willy and Mangelsdorf, 1997; Wiebel and Gustaffson, 1997), demonstrating that binding of the RXR ligand results in a conformational change in LXR that leads to transcriptional activation.

1.3.5. LXR AND COREGULATORS

Transcriptional regulation by the LXR/RXR heterodimer is achieved when the LXRE-bound dimer is activated by its ligand. As with other transcriptional regulatory proteins, the primary mechanism by which LXR heterodimers affect the rate of RNA polymerase II-directed transcription likely involves the interaction of receptors with components of the transcription preinitiation complex. This interaction may be direct or it may occur indirectly through the action of coregulators, which act as bridging factors. Evidence for the recruitment by activated receptors of coregulators came initially from yeast experiments based on transcriptional interference (Gill and Ptashne, 1988) and subsequently from squelching noted between cotransfected receptors in mammalian cell-based reporter assays (Meyer *et al.*, 1989). In general, coregulators play one or more of the following roles in nuclear receptor transcriptional activity: (1) they function as bridging factors to recruit cofactors to DNA-bound nuclear receptors, e.g. the steroid receptor coactivator (SRC) proteins, which can recruit p300/CREB-binding protein (CBP) to DNA-bound receptors; (2) they acetylate nucleosomal histones and various transcription factors at the promoters of hormone target genes, e.g. p300/CBP and p300/CBP-associated factor (P/CAF), which have potent nucleosomal histone acetyltransferase (HAT) and factor acetyltransferase activities; and (3) they function as bridging factors between the DNA-bound receptors and the basal transcriptional machinery e.g. the human mediator-like thyroid hormone receptor associated protein (TRAP)/vitamin D3 receptor interacting proteins (DRIP) multipolypeptide complex. Although the activation mechanisms and coregulators used by LXR have so far been unclear, recent work has identified a potential coregulator model for the activation of LXR transcription.

In this model, the non-induced transcriptional state of LXR is thought to occur when corepressors, such as silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor co-repressor (N-CoR), are bound to LXR/RXR in the absence of ligand (Wagner *et al.*, 2003; Hu *et al.*, 2003). Upon ligand binding, a basal rate of transcription occurs when the corepressors start to dissociate from the LXR/RXR heterodimer and transcription of the target gene becomes fully activated when coactivators are recruited to the ligand-activated

LXR/RXR heterodimer. This recruitment of co-activator proteins occurs via a two-step mechanism. Firstly, corepressors dissociate and the p160 coactivators, steroid receptor coactivator-1 (SRC-1) or glucocorticoid receptor-interacting protein 1 (GRIP1), appear to be directly recruited to the liganded receptors (Huuskonen *et al.*, 2004). These coactivators then serve as a platform to recruit p300 or CBP (Huuskonen *et al.*, 2004). The next step in this activation process is currently unclear for the LXR heterodimer. Other nuclear receptors appear to recruit associated proteins such as P/CAF, and by using their HAT activities, they remodel the nucleosomal structures so that the mediator complex TRAP/DRIP can replace SRC-1/CBP and bind the liganded receptors. Subsequent recruitment of RNA polymerase II complex to TRAP/DRIP completes the second step in nuclear receptor transactivation. Unfortunately, how the activation signal is transmitted from LXR-p160 coactivator p300/CBP to the basal transcription machinery is currently unknown and recent work has ruled out a role for P/CAF in LXR transcriptional activation (Huuskonen *et al.*, 2004). It is possible that p300/CBP directly interacts with the basal transcription machinery (Felzien *et al.*, 1999) or that p300/CBP can acetylate histones and recruit SWI/SNF chromatin remodelling complexes to the nuclear receptors thereby facilitating the interaction with basal machinery (Huang *et al.*, 2003). Therefore, these interactions among LXR, chromatin remodeling complexes and coactivators at oxysterol-activated promoters lead to the stimulation of gene transcription.

LXR transactivation can also be activated by at least two other mechanisms. Activating signal co-integrator 2 (ASC-2) has been shown to interact specifically with LXR and activate transcription (Kim *et al.*, 2003). The precise mechanism isn't known although ASC-2 exists in a complex containing methyltransferases. Futhermore, LXR can interact with multi-protein bridging family 1 (MBF-1) [Brendel *et al.*, 2002], which directly interacts with transcription factor IID (TFIID), making it a possible bridging molecule between LXR and basal transcription machinery.

In addition to the ability of the unliganded LXR/RXR heterodimers to actively repress target genes by recruiting corepressors (NcoR and SMRT) [Wagner *et al.*, 2003], LXR is capable of inhibiting the activities of other transcription factors, such as AP-1 and NFκB, in a ligand dependant manner. This effect does not require DNA binding by the nuclear receptor and is termed transrepression and is thought to be the primary mechanism by which nuclear receptors inhibit pro-inflammatory genes in macrophages (Joseph *et al.*, 2003; Schule *et al.*, 1991; Ray and Prefontaine, 1994; Ricote *et al.*, 1998).

Another function of nuclear receptors that does not require DNA binding is a recently characterised mechanism, termed non-genomic or extranuclear signalling, whereby nuclear receptor agonists also appear to participate in rapid signalling. Although no such action has yet been described for the LXRs, it is becoming increasingly obvious that non-genomic signalling of nuclear receptor agonists is an important aspect of nuclear receptor-regulated activation of genes.

Whilst the traditional mechanism of target gene transactivation by nuclear receptors and their agonists is characterised by the delayed onset of action, and its sensitivity to blockers of transcription and protein synthesis, the non-genomic action of nuclear receptors and their agonists is rapid, and inhibitors of transcription and protein synthesis have no effect (Schmidt *et al.*, 2000). This non-genomic function is thought to be the mechanism responsible for the recently observed effects of nuclear receptor ligands on intracellular signalling pathways and whilst it is still not clear the exact mode of mechanism, recent studies have suggested the existence of a sub-population of nuclear receptors that exist in the cytoplasm and at the plasma membrane, to which agonists bind and activate cell signalling cascades (Schmidt et al., 2000; Boonyaratanakornkit and Edwards, 2004).

1.3.6. LXR TARGET GENES

The characterisation of LXR target genes in the macrophage, as well as in liver and intestine, and the function of these target genes in reverse cholesterol transport and atherosclerosis are briefly described in the following section. The use of LXR null mice and LXR-selective agonists have been crucial in the identification of these genes and in establishing the important role of LXRs as sterol sensors that govern the absorption, transport, and catabolism of cholesterol.

TABLE 1.3. LXR REGULATION OF GENES INVOLVED IN LIPID METABOLISM.

TARGET GENE	TARGET TISSUES	FUNCTION	LXRE	REFERENCES
↑*CYP7A1*	Liver	Bile acid synthesis	TGGTCActcaAGTTCA rat TGGTCAcccaAGTTCA mouse TGGTCAtccaAGTTCA hamster TTGTCaccaAGCTCA human	Repa and Mangelsdorf, 2000; Lehmann *et al.*, 1997; Peet *et al.*, 1998.
↑*ABCA1*	Macrophage Intestine	Cholesterol efflux	AGGTTActatCGGTCA human GGATCAcctgAGGTCA human AGATCActtgAGGTCA human AGGTTActgaAGGCCA human	Repa *et al.*, 2000b; Costet *et al.*, 2000; Schwartz *et al.*, 2000; Singaraja *et al.*, 2001.
↑*ABCG5/G8*	Liver Intestine	Sterol transport	Not identified	Berge *et al.*, 2000; Repa *et al.*, 2002.
↑*ABCG1*	Macrophage	Cholesterol efflux	TGGTCActcaAGTTCA human AGTTTAtaatAGTTCA human	Venkateswaran *et al.*, 2000b; Kennedy *et al.*, 2001.

↑*ApoE*	Macrophage Adipocyte	Cholesterol efflux	GGGTCActggCGGTCA human GAATCActtaAGGTCA mouse	Laffitte *et al.*, 2001b.
↑*LPL*	Liver Macrophage	Triglyceride hydrolysis	A/T?GGTCAccacCGGTCA mouse AGGTGActgcAGGTCA human	Zhang *et al.*, 2001.
↑*CETP*	-	Cholesteryl ester transfer	GGGTCAttgtCGGGCA human	Luo and Tall, 2000.
↑*SREBP-1c*	Liver Intestine	Fatty acid synthesis	GGGTTActggCGGTCA mouse GGGTTActagCGGGCG human	Repa *et al.*, 2000a; Schultz *et al.*, 2000; Yoshikawa *et al.*, 2001.
↑ *FAS*	Liver	Fatty acid synthesis	GGGTTActgcCGGTCA human GGGTTActacCGGTCA rat	Joseph *et al.*, 2002a.
↑*LXRα*	Macrophage	Cholesterol sensor	AGGTTActgcTGGTCA human	Laffitte *et al.*, 2001a; Whitney *et al.*, 2001.

CYP7A1, cholesterol 7α-hydroxylase; ABCA1, ATP-binding cassette A1; ApoE, apolipoprotein E; LPL, lipoprotein lipase; CETP, cholesteryl ester transfer protein; SREBP-1c, sterol response element-binding protein-1; FAS, fatty acid synthase; LXR, liver X receptor. Table derived from Zhang and Mangelsdorf, 2002.

1.3.6.1. LXRs AND THE CONTROL OF CHOLESTEROL HOMEOSTASIS

Cholesterol homeostasis is maintained via dietary intake, intestinal absorption and the regulated pathways of *de novo* synthesis and catabolism. Cholesterol biosynthesis is controlled by the accumulation of sterols in the liver, which feedback and inhibit further synthesis through a protease-sensitive mechanism involving the SREBP family of transcription factors (Brown and Goldstein, 1997). Excess cholesterol also activates a feed-forward pathway that leads to the catabolic elimination of cholesterol as bile acids. Bile acid production from cholesterol and the solubilization of cholesterol by bile acids are the final steps in reverse cholesterol transport, facilitating cholesterol excretion from the body. The LXRs are important in the regulation of each of these processes and the following section will discuss the role of these ligand-mediated transcription factors and the function of its target gene in more detail.

1.3.6.1.i. CHOLESTEROL SYNTHESIS

A role for the LXRs in the negative control of cholesterol synthesis was first suggested by the observation that the expression of several important cholesterogenic genes is altered in the liver of LXRα-/- mice (Peet *et al.*, 1998). The expression of the cholesterol synthesis transcriptional regulator SREBP-2, as well as the cholesterogenic enzymes 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and synthase, farnesyl diphosphate synthase (FPP synthase), and squalene synthase (SQS) were significantly increased in these mice compared to wild-type mice (Peet *et al.*, 1998). This action of LXRs on cholesterol synthesis has since been confirmed by the ability of oxysterols to suppress the nuclear levels of SREBP-2 (Yoshikawa *et al.*, 2001; DeBose-Boyd *et al.*, 2001). The regulation of cholesterol synthesis is thought to differ between the two LXR isoforms as only minor increases in the hepatic expression of HMG-CoA reductase, FPP synthase, and SQS were observed in LXRβ-/- mice (Alberti *et al.*, 2001), and SREBP-2 expression was unchanged, indicating that LXRα is more important as a regulator of cholesterol synthesis than LXRβ. This role of LXRs in cholesterol synthesis has been further supported by studies demonstrating that the liver expression of SQS and HMG-CoA synthase is markedly reduced following the administration of the LXR agonist T0901317 to wild-type mice (Schultz *et al.*, 2000).

1.3.6.1.ii. DIETARY CHOLESTEROL ABSORPTION

Dietary and secreted biliary cholesterol enters the intestinal lumen and is absorbed by proximal enterocytes. In the small intestine, at least three ABC transporters are transcriptionally regulated by LXRs (Repa et al, 2000b; Berge *et al.*, 2000) and limit cholesterol absorption by mediating free cholesterol export from inside the enterocyte into the intestinal lumen. ABCA1-/- mice show a marked increase in cholesterol absorption, confirming the importance of ABCA1 in this process (McNeish *et al.*, 2000). Ligands of LXR and RXR increase ABCA1 expression and decrease cholesterol absorption in the distal duodenum and jejenum; however these responses are absent in LXRαβ-/- mice (Repa *et al.*, 2000b). Recently it has been suggested that ABCA1 may reside in the abluminal membrane of the enterocyte, and its role may be to redirect absorbed cholesterol into a secretion pathway involving HDL-like particles, rather than the typical chylomicrons that enter the lymph (Murthy *et al.*, 2002; Wellington *et al.*, 2002).

Two additional targets for LXR regulation are ABCG5 and ABCG8. ABCG5 and ABCG8 are half-transporters, each composed of a single transmembrane domain and ATP-binding cassette (Berge *et al.*, 2000) and are co-expressed exclusively in the liver and intestine. Mutations in either gene cause a rare autosomal recessive disorder called sitosterolemia, which is characterised by increased absorption of cholesterol and toxic plant sterols (sitosterols), and decreased biliary sterol secretion, resulting in the development of premature cardiovascular

disease (Lee *et al.*, 2001; Berge *et al.*, 2000). *In vivo* and *in vitro* experiments indicate that ABCG5 and ABCG8 are direct targets of LXRs (Berge *et al.*, 2000; Repa *et al.*, 2002). In mice treated with LXR agonists, ABCG5 and ABCG8 expression is enhanced (Repa *et al.*, 2002), biliary cholesterol is increased and cholesterol absorption is decreased (Repa *et al.*, 2000b; Plosch *et al.*, 2002). Similar changes occur in transgenic mice overexpressing the human ABCG5 and ABCG8 genes (Plosch *et al.*, 2002; Yu *et al.*, 2002b). Additional evidence that ABCG5 and ABCG8 are important in this system is the finding that LXR activity still affects biliary secretion and cholesterol absorption in the ABCA1-/- mouse (Plosch *et al.*, 2002).

1.3.6.III. REVERSE CHOLESTEROL TRANSPORT

The elimination of cholesterol through bile acid production occurs exclusively in the liver, so that excess cholesterol from peripheral tissues must be returned to the liver, in a process termed reverse cholesterol transport for catabolic elimination. As described previously in this chapter, this process is particularly important in macrophages. LXRs are implicated as key sensors of sterol metabolism, maintaining normal cholesterol balance by promoting sterol efflux from peripheral cells, increasing circulating HDL-cholesterol, and ultimately increasing hepatic sterol catabolism and excretion. LXR regulation of reverse cholesterol transport also suggests a role for LXRs in protecting against atherogenesis, a hypothesis that has been confirmed by studies of apoE-/- mice in which activation of LXR reduced atherosclerosis (Claudel *et al.*, 2001; Joseph et al., 2002b). Recent studies suggest that LXRs control lipid efflux from macrophages through the coordinate regulation of a set of genes involved in cholesterol efflux, including ABCA1 and apoE (Laffitte *et al.*, 2001b; Claudel *et al.*, 2001; Repa *et al.*, 2000b; Venkateswaran *et al.*, 2000a).

THE ABC TRANSPORTERS

ABCA1 facilitates the transport of cholesterol and phospholipids to apoAI, apoE or HDL, the first step in reverse cholesterol transport. The role of LXR in the regulation of ABCA1 expression and cholesterol efflux has been supported by multiple studies (Repa *et al.*, 2000b; Costet *et al.*, 2000; Schwartz *et al.*, 2000; Venkateswaran *et al.*, 2000a). Venkateswaran *et al.* (2000a) and Schwartz *et al.* (2000) have demonstrated that ABCA1 mRNA expression is induced in macrophages in response to lipid loading and that expression and activation of LXR stimulates apoAI mediated efflux of cholesterol. This ability of LXR ligands to stimulate cholesterol efflux is lost in fibroblasts from Tangier patients, whose disease is characterised by mutations in the ABCA1 gene, therefore, confirming the importance of LXR induction of ABCA1 in this process (Venkateswaran *et al.*, 2000a). The ability of LXR ligands to raise HDL-cholesterol levels in mice is also consistent with the established function of ABCA1 in cholesterol efflux (Schultz *et al.*, 2000). Also LXR$\alpha\beta$-/- mice

develop splenomegaly and accumulate foam cells in multiple peripheral tissues, a phenotype remarkably similar to that of the ABCA1-/- mice (Schuster *et al.*, 2002; Tangirala *et al.*, 2002). Furthermore, oxysterol-induced ABCA1 expression can be attenuated in cells expressing a dominant-negative form of LXRα (Venkateswaran *et al.*, 2000a). Work by Costet *et al.* (2000) has identified a DR-4 element in a region of the ABCA1 promoter that is responsible for the sterol-mediated activation of ABCA1 promoter. In addition, a study addressing the role of ABCA1 in endothelial cells showed that native LDL acts via LXR activation to up-regulate ABCA1 expression and cholesterol efflux, indicating that ABCA1 may additionally play an important role in cholesterol homeostasis in the vascular endothelium (Liao *et al.*, 2002).

In accordance with the above evidence confirming the role of LXRs in ABCA1 expression, the synthetic LXR agonist APD is able to stimulate macrophage ABCA1 expression and cholesterol efflux, this induction is also much greater in comparison to the induction observed in response to cholesterol loading (Sparrow *et al.*, 2002). Also, the LXR agonist GW3965 was shown to induce the expression of ABCA1 and ABCG1 in the aortas of hyperlipidemic mice, and to concurrently cause a dramatic 50% reduction in lesion development in two mouse models of atherosclerosis (Joseph *et al.*, 2002b).

Similar to ABCA1, expression of ABCG1 is also induced in macrophages in response to cholesterol loading and specific oxysterol LXR ligands (Venkateswaran *et al.*, 2000b) and the gene also contains two functional LXREs (Kennedy *et al.*, 2001). The function of ABCG1 is currently unknown, but it has been proposed to play a role in cholesterol efflux, perhaps by working in concert with ABCA1 (Klucken *et al.*, 2000). Peritoneal macrophages isolated from mice deficient for both LXRα and LXRβ failed to show an increase in ABCG1 expression in response to oxysterol treatment (Venkateswaran *et al.*, 2000b). A novel ABC half-transporter, ABCG4, has also been characterised (Costet *et al.*, 2000; Dean *et al.*, 2001; Engel *et al.*, 2001) and it shares high sequence identity with ABCG1. ABCG4 expression is also believed to be upregulated in response to oxysterols and retinoids in human macrophages, suggesting a possible role for this transporter in macrophage lipid homeostasis (Engel *et al.*, 2001).

APOLIPOPROTEIN E

As described earlier in this chapter, another protein involved in the cholesterol efflux pathway is apoE. In macrophages, apoE transcription is increased by cholesterol loading, indicating the existence of a molecular sensor that regulates apoE expression in response to lipid. The LXRs represent this molecular sensor, since LXR ligands increase apoE expression in human macrophages, an effect mediated via two LXREs present in the apoE enhancer regions (Laffitte *et al.*, 2001). ApoE was the first gene shown to be regulated by LXR/RXR heterodimers in a tissue-

specific manner as LXR mediates lipid-inducible expression of the apoE gene in adipose tissue and macrophages but not in liver (Laffitte *et al.*, 2001b). This induction of apoE expression by LXR ligand is significantly reduced in macrophages from either LXRα-/- or LXRβ-/- mice, even in the presence of high concentrations of ligand (Laffitte *et al.*, 2001b).

ApoE is present in a gene cluster that contains apoCI, apoCII, and apoCIV, and recent work has demonstrated that these apolipoprotein genes are also LXR responsive, in both human and murine macrophages (Mak *et al.*, 2002a). Like apoE the other members of this gene cluster have been shown to serve as acceptors in ABCA1-mediated cholesterol efflux (Bortnick *et al.*, 2000) and the induction of these acceptors in macrophages within the arterial wall would be expected to promote cholesterol efflux and reverse cholesterol transport. The induction of the members of this gene cluster by LXR ligand was reduced in LXRαβ-/- mice, but was generally unchanged in LXRα-/- or LXRβ-/- mice, indicating that both LXR isoforms are functional regulators of lipid-inducible apolipoprotein expression (Mak *et al.*, 2002a).

LIPOPROTEIN REMODELING ENZYMES

LXR has also been shown to influence the expression of several enzymes that act on lipoproteins, including lipoprotein lipase (LPL), cholesteryl ester transfer protein (CETP) and the phospholipid transfer protein (PLTP) (Cao *et al.*, 2002; Laffitte *et al.*, 2003; Luo *et al.*, 2000; Mak *et al.*, 2002b). Both CETP and PLTP are lipoprotein-remodelling enzymes that transfer lipids between lipoproteins. CETP is secreted by the liver and circulates in plasma principally bound to HDL (Barter, 2000). CETP mediates the transfer of HDL cholesteryl esters to apoB-containing lipoproteins for return to the liver, and in exchange HDL receives triglycerides (Oliveira *et al.*, 1997). This modification of HDL by CETP makes HDL more susceptible to hydrolysis by hepatic lipase at the hepatocyte surface, which is an important component of the regeneration of small pre-β-HDL-like particles and free apoAI that can re-circulate in the reverse cholesterol transport pathway. The contribution of CETP activity to atherosclerosis is unclear, although the generation of pre-β-HDL-like particles, suggests an antiatherogenic role. However, over-expression of human CETP in atherosclerotic mice (apoE-/- or LDLR-/-) lowers plasma HDL levels and increases lesion formation (Plump *et al.*, 1999). The LXR/RXR heterodimer acts via a DR-4 element in the promoter of the human CETP gene to mediate sterol induction of the expression of CETP in response to a high fat diet (Luo and Tall, 2000). In transient transfection experiments, the CETP gene could be transactivated by both LXRα and LXRβ, suggesting that both LXR isoforms play a role in the control of CETP expression (Luo and Tall, 2000).

PLTP has been identified as a key modulator of HDL metabolism, and may also be involved in reverse cholesterol transport (van Tol, 2002). In addition, PLTP has recently been shown to regulate VLDL secretion from the liver as PLTP-/- mice exhibit decreased levels of VLDL and LDL in an apoE-deficient or apoB-transgenic background (Jiang *et al.*, 1999). During the lipolysis of serum VLDL by LPL, surface remnants containing phospholipids and apolipoproteins are transferred by PLTP and contribute to pre-β-HDL and large α-HDL production. PLTP can also mediate lipid transfer between HDL particles to produce a small pre-β-HDL and a large α-HDL and the expression of a human PLTP transgene in mice increases production of pre-β-HDL and enhances hepatic uptake and clearance of cholesteryl ester. Recent work has demonstrated that LXR ligands induce the expression of PLTP in cultured HepG2 cells and mouse liver *in vivo* and also in macrophages (Laffitte *et al.*, 2003; Cao *et al.*, 2002; Mak *et al.*, 2002b). Also PLTP activity is increased in mice treated with the LXR ligands and this ability of synthetic and oxysterol ligands to regulate PLTP mRNA was lost in LXRαβ-/- mice (Laffitte *et al.*, 2003). These studies also demonstrated that the PLTP promoter contains a high-affinity LXRE that is bound by LXR/RXR heterodimers *in vitro* and is activated by LXR/RXR in transient-transfection studies (Mak *et al.*, 2002; Laffitte *et al.*, 2003). Finally, immunohistochemistry studies reveal that PLTP is highly expressed by macrophages within human atherosclerotic lesions, suggesting a potential role for this enzyme in lipid-loaded macrophages (Laffitte *et al.*, 2003).

LPL catalyses the hydrolysis of lipoprotein triglycerides and is highly expressed in adipose tissue and muscle, and is also produced by macrophages (Mead *et al.*, 2002). The contribution of LPL expression to atherogenesis is complex and seems to be context dependent. On the anti-atherogenic side, non-macrophage LPL is able to aid the clearance of serum cholesterol-rich lipopoproteins by remodelling triglyceride-rich chylomicrons and VLDL into chylomicron remnants and LDL for uptake and this hydrolysis of lipoproteins also results in the concomitant generation of material for HDL formation. Indeed systemic overproduction of human LPL in atherogenic animal models appears to protect against atherosclerosis and diet-induced atherogenesis (Shimada *et al.*, 1996; Yagyu *et al.*, 1999). In addition to its enzymatic activity, LPL can also facilitate HDL-associated cholesterol uptake into liver cells by acting as a bridging factor (Merkel *et al.*, 2002; Rinninger *et al.*, 1998). Hepatic LPL expression also promotes the maturation of HDL in mice (Strauss *et al.*, 2001) and liver-specific expression of human LPL by adenovirus-mediated gene delivery in apoE-/- and LDLR-/- mice improves the hyperlipidemic serum profile seen in these mice (Zsigmond *et al.*, 1997). On the other hand, evidence suggests that macrophage expression of LPL in the artery wall may be pro-atherogenic, as studies in which macrophage-specific overproduction of LPL in apoE-/- and LDLR-/- mice was shown to accelerate atherosclerosis (Babaev *et al.*, 2000; Wilson *et al.*, 2001). Also a study showing that macrophage-selective elimination of LPL led to a

decrease in lesion formation in LDLR-/- mice (Babaev *et al.*, 2000). LPL would promote the conversion, and perhaps retention, of triglyceride rich lipoproteins to cholesterol-rich lipoproteins such as LDL, thereby creating an environment conductive to foam cell development (Pentikainen *et al.*, 2002). In addition, recent evidence indicates that LPL may serve as a bridging factor that links lipoprotein particles to the arterial extracellular matrix, thus increasing their residence time in the arterial wall and potentiating their conversion into more atherogenic forms (Saxena *et al.*, 1992; Eisenberg *et al.*, 1992).

Similar to apoE, the regulation of LPL gene expression by LXR is tissue specific as LXR agonists induce expression of LPL in liver and macrophages but not in adipose tissue (Zhang *et al.*, 2001). Liver LPL expression is greatly increased in wild-type mice fed a high cholesterol diet or treated with a synthetic LXR agonist, while no change in LPL expression occurs in LXRαβ-/- mice (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 DR-4-like LXRE that has been identified within the murine LPL gene and conserved in the human LPL gene (Zhang *et al.*, 2001).

1.3.6.1.IV. BILE ACID SYNTHESIS

Conversion of cholesterol to bile acids in the liver is a quantitatively important pathway for elimination of cholesterol from the body. Bile acids are physiological agents important for the absorption and transport of lipid-soluble vitamins, steroids and xenobiotics. Bile acid synthesis occurs exclusively in the liver and is initiated by cholesterol 7α-hydroxylase (CYP7A1), a cytochrome P450 isozyme of the CYP7A family (Russell and Setchell, 1992; Chiang, 1998). CYP7A1 is the rate-limiting enzyme in the classic bile acid synthesis pathway and transcription of this gene is inhibited by bile acid feedback and is stimulated by dietary factors such as cholesterol. This regulation of the CYP7A1 gene by cholesterol appears to be mediated by LXR, and a functional LXRE has been identified in the proximal promoter region of the rat CYP7A1 gene, and has been shown to be activated by RXR/LXR heterodimers in an oxysterol and retinoid-dependant manner (Lehmann *et al.*, 1997; Peet *et al.*, 1998). Evidence supporting LXR regulation of CYP7A1 came from work demonstrating that on a high cholesterol diet, LXRα-/- mice fail to up-regulate CYP7A1 expression and bile acid production, resulting in increased plasma LDL cholesterol, decreased HDL cholesterol levels and massive cholesterol accumulation in the liver (Peet *et al.*, 1998). This study also observed that the knockout of the LXRα gene did not effect the basal activity of CYP7A1, indicating that LXRs may not play a significant role in CYP7A1 expression in hepatocytes under normal dietary conditions. Despite the strong evidence of a role for LXRα in the control of CYP7A1 expression in rats and mice, it appears that this function of LXRα is not present

in humans as co-transfection with LXRα and RXR expression plasmids strongly stimulates the activity of the rat CYP7A1 promoter but has no significant effect on the human CYP7A1 promoter (Chiang *et al.*, 2001). This difference is due to the LXR element not being conserved in the promoter of the human CYP7A1 gene (Agellon *et al.*, 2002).

The function of LXRβ in CYP7A1 regulation is still unclear. Allthough LXRβ is also expressed in the liver and appears to respond to the same ligands as LXRα, it is unable to rescue the altered bile acid phenotype in LXRα-/- mice. This suggests that the two LXRs may subserve distinct biological roles. Accordingly, LXRβ-/- mice maintain normal hepatic cholesterol metabolism and are thus resistant to dietary cholesterol (Alberti *et al.*, 2001); however LXRαβ-/- mice have an exacerbated liver phenotype (Zhang and Mangelsdorf, 2002). Co-transfection assays have also shown that the CYP7A1 LXRE is a much stronger response element for LXRα than LXRβ (Lehmann *et al.*, 1997; Peet *et al.*, 1998), which may explain the difference between the regulation of CYP7A1 by the two LXR isoforms.

1.3.6.2. LXRs AND THE CONTROL OF FATTY ACID SYNTHESIS

Although all of the target genes described so far demonstrate that LXRs have the ability to play an antiatherogenic role, some of the earliest studies on LXR pointed to an important role for these receptors in the control of fatty acid synthesis. Mice carrying a targeted disruption in the LXRα gene were noted to be deficient in the hepatic expression of SREBP-1c, FAS, steroyl coenzyme A desaturase 1 (SCD-1), acyl coenzyme A carboxylase (ACC) [Peet *et al.*, 1998]. Consistent with these observations, oral administration of synthetic ligands to mice triggers induction of the lipogenic pathway and elevates plasma and hepatic triglyceride levels (Schultz *et al.*, 2000; Joseph *et al.*, 2002a). These effects were also confirmed by the finding that the treatment of LXRαβ-/- mice with the synthetic ligand, T0901317, induced only a minor increase in plasma triglyceride levels, and had no effect on liver expression of SREBP-1, SCD-1, FAS or ACC (Schultz *et al.*, 2000). The primary mechanism through which LXR agonists stimulate lipogenesis appears to be through direct activation of the SREBP-1c promoter (Repa *et al.*, 2000a; Yoshikawa *et al.*, 2001). SREBP-1c is a membrane-bound transcription factor that governs the expression of several enzymes in the biosynthesis and esterification of unsaturated fatty acids (Shimano *et al.*, 1999; Kim and Spiegelman, 1996; Horton and Shimomura, 1999). Such SREBP-1c-activated genes include acetyl-CoA synthetase, acetyl-CoA carboxylase, FAS, SCD-1, glycerol phosphate acyltransferase and CTP:phosphocholine cytidylyltransferase. Dietary cholesterol or the administration of LXR or RXR agonists specifically induces the expression of the SREBP-1c isoform in the tissues of wild-type, but not LXRαβ-/- mice (Repa *et al.*, 2000a). Interestingly it has been shown that the basal

expression of SREBP-1c in a rat hepatoma cell line is dependant on the activation of LXR by an endogenous oxysterol (DeBose-Boyd *et al.*, 2001). *In vitro* studies have shown that LXR and RXR agonists, as well as LXRα or LXRβ over-expression, induced increased transcriptional activity of the SREBP-1c proximal promoter via two inverted LXREs of the DR-4 type, thus demonstrating that SREBP-1c is a direct target gene of the oxysterol-activated LXR/RXR heterodimer (Repa *et al.*, 2000a; Yoshikawa *et al.*, 2001). Differential regulation by the two LXR isoforms has also been demonstrated for SREBP-1c as LXRα appears to be a more potent regulator of SREBP-1c expression in the liver than LXRβ, since LXRβ was unable to compensate for the absence of LXRα in LXRα-/- mice (Peet *et al.*, 1998). An additional mechanism for LXR-mediated up-regulation of fatty acid synthesis was identified with the finding that the LXRs can up-regulate FAS expression directly through interaction with an LXRE in the FAS promoter, as well as indirectly via SREBP-1c (Joseph *et al.*, 2002a). This LXR/RXR-mediated upregulation of SREBP-1c and FAS expression has been shown to occur in the human hepatoma cell line HepG2, indicating that LXR activation may increase fatty acid synthesis in humans (Yoshikawa *et al.*, 2001).

Although the ability of LXRs to regulate fatty acid synthesis is widely considered as a negative aspect of LXR activation, it is also important to note that this regulation of lipogenesis by the LXRs may also contribute to its role in cholesterol homeostasis. Regulation of lipogenesis may function to coordinate the levels of fatty acids and phospholipids, both of which are components essential for the mobilisation of free cholesterol. For example, oleoyl-CoA, a product of SCD-1 catalysis, is the preferred 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 cholesterol accumulation. In addition, phospholipids facilitate bile acid flow, lipoprotein transport of excess cholesterol, and help maintain the appropriate ratio of cholesterol to other lipids in plasma membranes (Repa *et al.*, 2000a; Yoshikawa *et al.*, 2001). Therefore, it is possible to appreciate why a transcription factor that upregulates cholesterol efflux and downregulates cholesterol synthesis, would also upregulate fatty acid synthesis. Unfortunately, pharmacological activation of LXRs increases lipogenesis to potentially harmful levels, causing hypertriglyceridaemia (Yoshikawa *et al.*, 2001).

1.3.7. REGULATION OF LXR EXPRESSION

Although more and more mediators that effect the gene expression of the LXRs are being identified (Tobin *et al.*, 2000; Tobin *et al.*, 2002; Lundholm *et al.*, 2004), the mechanisms that control expression of these genes are not well understood. In mice LXRα is expressed primarily in the liver, intestine, adipose tissue and macrophages, whereas LXRβ is ubiquitously expressed,

indicating that distinct *trans*-acting factors must be involved in the regulation of these genes as expression of the LXRs is tissue specific and regulated in response to certain metabolic signals. Recent studies attempting to characterise the promoter region of the mouse LXRα gene have identified a role for the transcription factors Zta, Ets, and Hes1 in LXR gene regulation (Steffensen *et al.*, 2003). These factors have been linked to cell cycle and differentiation processes (Sinclair, 2003; Oikawa and Yamada, 2003; Kageyama *et al.*, 2000) suggesting that expression of LXRα might be under control of signalling mechanisms regulating cell proliferation. Also several putative binding sites of the glucocorticoid receptor (GR) were also identified in the LXRα promoter, and transient co-transfections of the GR and LXRα promoter constructs induced reporter gene activity, suggesting cross-talk between GR and LXR signalling (Steffensen *et al.*, 2003). Furthermore, members of the nuclear factor 1 (NF1) and CCAAT/enhancer binding protein (C/EBP) families have also been shown to affect the transcriptional potential of the mouse LXRα promoter (Steffensen *et al.*, 2001 and 2002).

Although information on the transcriptional control of the LXR genes is limited, studies have demonstrated that macrophages have established mechanisms whereby LXR gene expression can be amplified during cholesterol loading.

1.3.7.1. HUMAN LXRα AUTOREGULATION

Several studies (Whitney *et al.*, 2001; Laffitte *et al.*, 2001a; Li *et al.*, 2002b) have demonstrated the existence of an auto-regulatory loop controlling the expression of LXRα, but not LXRβ. Studies have shown that natural and synthetic LXR ligands induce the expression of LXRα in multiple human cell types, including primary human macrophages. This induction of LXRα occurs via three functional LXREs identified in the human LXRα promoter (Whitney *et al.*, 2001; Laffitte *et al.*, 2001a; Li *et al.*, 2002b). One of these LXREs in the distal portion of the LXRα promoter was strongly activated by both LXRα and LXRβ, while the other two LXREs were selectively but more weakly activated by LXRα (Li *et al.*, 2002). Although this region is conserved in both the mouse and human promoter, autoregulation is only observed in human cells, suggesting that humans may be more responsive to LXRα agonists than mice. As a result, the induction of LXR target genes, by LXR ligands may be significantly greater in human than in murine macrophages, allowing amplification of the cholesterol catabolic cascade and maximise the effects of LXR (Laffitte *et al.*, 2001a; Whitney *et al.*, 2001).

1.3.7.2. LXRα REGULATION BY PPARS

In addition to autoregulation the macrophage has evolved an additional mechanism to further amplify the LXR signalling pathway. Certain lipid components of oxLDL can activate another nuclear receptor subfamily expressed in human macrophages, the peroxisome proliferator-activated receptors (PPARs) [Delerive *et al.*, 2000; Han *et al.*, 2000]. This activation of the PPAR family in turn causes a marked increase in LXRα expression (Shiffman *et al.*, 2000). Two subtypes of the PPARs, PPARα and PPARγ, are particularly interesting as they are well characterised and are involved in lipid metabolism and have also been found in macrophages that are associated with atherosclerotic plaques. Work in rat hepatocytes has demonstrated that unsaturated fatty acids are able to increase the mRNA and protein expression of LXRα, but caused no change in the expression of LXRβ (Tobin *et al.*, 2000). This upregulated expression of LXRα was mainly due to an increase in transcription, which was due to the binding of PPARα to a PPAR response element (PPRE) in the promoter region of the murine LXRα gene (Tobin *et al.*, 2000). Subsequently, a PPRE was also identified in the human LXRα promoter region (Laffitte *et al.*, 2001a) and was found to be responsible for the induction of the LXRα gene by PPARγ agonists (Chawla *et al.*, 2001a). Notably, in this study only ligands for the PPARγ isoform were able to induce LXRα gene expression, whilst ligands for the other isoforms had no effect. PPARγ agonists have also been shown to induce LXRα expression in human primary macrophages (Chinetti *et al.*, 2001). Interestingly, no regulation of LXRβ has been observed thus far in any studies, implicating distinct regulation of LXRα and LXRβ genes.

This transcriptional regulation of LXRα by the PPAR family suggests an overlap between LXR and PPAR signalling pathways, which allows the PPARs to affect LXR target genes involved in cholesterol efflux. Exposure of macrophages to PPARγ ligands increases the expression of LXR target genes such as ABCA1 and ABCG1 and promotes cholesterol efflux in human macrophages through a transcriptional cascade mediated by LXRα (Chawla *et al.*, 2001b; Chinetti *et al.*, 2001). In addition to the ABC transporters it has been determined that the apoE gene is regulated by the PPARγ-LXR axis, and the macrophage specific deletion of PPARγ reduces levels of apoE and drastically decreases basal cholesterol efflux (Akiyama *et al.*, 2002).

Another way in which the LXR and PPAR pathways may overlap is through direct interaction of the receptors. It has been suggested that LXRα can bind directly to PPARα, as well as to the common heterodimerization partner RXRα (Miyata *et al.*, 1996). Although LXRα did not form a DNA binding complex with PPARα on naturally occurring PPREs or LXREs, LXRα was able to inhibit the binding of PPARα/RXRα heterodimers to PPREs, and coexpression of LXRα in

mammalian cells antagonized peroxisome proliferator signalling mediated by PPARα/RXRα *in vivo*. These findings suggested that LXRα plays a role in modulating PPAR-signalling pathways in the cell (Miyata *et al.*, 1996). Further evidence for LXR-PPAR overlap has come from recent studies identifying a role for the LXRs in adipocyte differentiation (Seo *et al.*, 2004). In adipocytes, LXR activation with T0901317 primarily enhanced the expression of lipogenic genes such as the SREBP-1c and FAS genes and substantially increased the expression of PPARγ. This study showed that the PPARγ promoter contains a conserved LXRE and was transactivated by the expression of LXRα (Seo *et al.*, 2004). Furthermore, the suppression of LXRα by small interfering RNA attenuated adipocyte differentiation. Taken together, these results suggest that LXR plays a role in the execution of adipocyte differentiation by regulation of lipogenesis and adipocyte-specific gene expression.

1.3.8. LXRs AND ATHEROSCLEROSIS

As described previously in this chapter, oxidised LDL uptake in macrophages of the arterial wall is central to the pathogenesis of atherosclerosis. In addition to serving as a source of lipids to the macrophage and other cells of the atherosclerotic plaque during lesion development, oxLDL regulates gene expression in macrophages, smooth muscle cells and endothelial cells. Recent data suggests that LXRs may mediate many of the effects of oxLDL on gene expression in vascular cells. LXRs are expressed in one or more of the cell types present in the atherosclerotic lesion. LXRα is expressed at a low level in circulating monocytes and at a much higher level following differentiation into macrophages (Laffitte *et al.*, 2001a; Nagy *et al.*, 1998) and the exposure of macrophages to oxLDL also induces the expression of LXRα mRNA (Laffitte *et al.*, 2001a). The uptake of oxLDL by macrophages during atherogenesis, supplies the cells with ligand for the activation of LXR. Therefore oxLDL induces both the expression and the transcriptional activity of LXRs in macrophages. Oxysterol ligands of LXR can also be derived intracellularly through enzymatic modification of cholesterol. Consequently, loading macrophages with acetylated LDL, which does not contain oxysterols, also activates LXR (Laffitte *et al.*, 2001a; Laffitte *et al.*, 2001b; Venkateswaran *et al.*, 2000b). Therefore LXRs are likely to be transcriptionally active within atherosclerotic lesions. As described earlier, the activation of these receptors and their target genes stimulates cholesterol efflux in macrophages, promotes bile acid synthesis in liver, and inhibits intestinal cholesterol absorption, actions that would collectively be expected to reduce atherosclerotic risk.

Recent studies have directly addressed the role of the LXR signalling pathway in atherosclerosis using mouse models. Tangirala *et al.* (2002) were able to address the importance

of macrophage LXR signalling using bone marrow transplantation studies into apoE-/- and LDLR-/- mice. They showed that selective elimination of macrophage LXRs by lethal irradiation, and transplant with LXRαβ-/- bone marrow, led to a significant increase in atherosclerotic lesion formation in both mouse models. Also, foam cell accumulation occurred in several other tissues including lung, spleen and brain (Joseph *et al.*, 2002b; Tangirala *et al.*, 2002; Schuster *et al.*, 2002). Additionally, macrophage-specific rescue of LXRs significantly decreased atherosclerosis development in these mice, despite no significant changes in circulating lipid levels (Tangirala *et al.*, 2002). Direct evidence for the potential use of LXR activators in atherosclerosis has also come from studies in murine models. The LXR agonist GW3965 was shown to decrease lesion area approximately 50% in both apoE-/- and LDLR-/- mice (Joseph *et al.*, 2002b; Terasaka *et al.*, 2003). This reduction in atherosclerosis was thought to be due to increased ABCA1 and ABCG1 expression, as demonstrated in atherosclerotic aortas of apoE-/- mice treated with LXR agonists, which probably caused an increase in cholesterol efflux, causing net lipid loss from macrophage foam cells, and antagonism of fatty streak formation (Joseph *et al.*, 2002b; Claudel *et al.*, 2001). Chronic ligand administration only moderately affected the lipoprotein profile of these mice, confirming that the antiatherogenic ability of LXR ligands is probably through a direct effect on the cells of the artery wall. A similar reduction in atherosclerosis was observed with the RXR agonist, LG268 (Claudel *et al.*, 2001). Therefore, despite the undesirable effects of LXR agonists on lipogenesis, the net effect of whole body LXR activation can be considered to be anti-atherogenic.

Two other nuclear receptors, RXR and PPARγ, have also been associated with the pathogenesis of atherosclerosis and treatment of atherogenic mouse models with RXR or PPARγ agonists results in significant decreases in lesion development (Claudel *et al.*, 2001; Li *et al.*, 2000). Interestingly both RXR and PPARγ impact LXR-regulated pathways and LXRα may be a particularly important downstream target with regards to the antiatherogenic effects of PPARγ ligands. The LXR/RXR heterodimers are permissive heterodimers that respond to agonists for both receptors, therefore it is not surprising that RXR agonists mimic many of the effects of LXR activators including induction of ABCA1 and reverse cholesterol transport in macrophages (Repa *et al.*, 2000b; Claudel *et al.*, 2001). Also studies have demonstrated strong and synergistic induction of ABCA1, ABCG1 and apoE by LXR and RXR agonists in LXRαβ+/+ but not LXRαβ-/- macrophages indicating that any effects of RXR agonists on these genes are indeed mediated by LXR (Tangirala *et al.*, 2002). As mentioned in the above section, the expression of the LXRα gene is responsive to PPARγ and as a result of this crossregulation, PPARγ and LXR agonists have additive effects on ABCA1 expression and reverse cholesterol transport in macrophages (Chawla *et al.*, 2001a; Laffitte *et al.*, 2001b; Chinetti *et al.*, 2001). Transplantation of PPARγ-/- bone marrow

into LDLR -/- mice also increases atherosclerosis (Chawla *et al.*, 2001a), suggesting that PPARγ-LXR cross-talk may be important for atherosclerosis susceptibility. Consistent with this, PPARγ deficiency results in a marked reduction of LXRα expression and macrophage-cholesterol efflux (Chawla *et al.*, 2001a; Moore *et al.*, 2001; Akiyama *et al.*, 2002). Also PPARγ agonists have been reported to reduce atherosclerosis in mouse models (Li *et al.*, 2000; Claudel *et al.*, 2001) and clinical trials (Minamikawa *et al.*, 1998) which is most likely a consequence of indirect LXR-mediated effects. In combination, this *in vivo* data highlights the potential usefulness of LXR, PPAR or RXR agonists in the management of atherosclerosis.

1.3.9. LXRs AND INFLAMMATION

Although the role of LXRs in the regulation of lipid metabolism is well established and it is clear from mouse model studies that LXRs exert an important atheroprotective role in macrophages, recent work has been aimed at identifying other potentially atheroprotective roles for these receptors in macrophages, distinct from reverse cholesterol transport. Atherosclerosis has long been recognised as a chronic inflammatory disease as well as a disorder of lipid metabolism and recent observations have identified LXRs as regulators of immune functions in macrophages. LXR agonists have been shown to inhibit the macrophage response to bacterial pathogens and prevented the induction of several pro-inflammatory genes, such as IL-1β, IL-6, MMP-9, iNOS, COX-2, MCP-1 and MCP-3, macrophage inflammatory protein (MIP)-1β and IP-10 (Joseph *et al.*, 2003; Castrillo *et al.*, 2003a). Consistent with these *in vitro* effects, LXR null mice exhibit enhanced responses to inflammatory stimuli and LXR ligands reduce inflammation in a murine model of contact dermatitis (Joseph *et al.*, 2003).

Work by Castrillo *et al.* (2003a) demonstrated that LXRα and LXRβ inhibit the basal and inducible expression of MMP-9. MMP-9 is highly expressed by macrophages and smooth muscle cells in atherosclerotic plaques and participates in the degradation of ECM components, which leads to plaque instability and ultimately plaque rupture (Mason *et al.*, 1999; Prescott *et al.*, 1999; Brown *et al.*, 1995). Therefore mechanisms by which excessive MMP production can be limited are of therapeutic interest. Treatment of murine peritoneal macrophages with synthetic LXR ligands reduces MMP-9 mRNA expression and decreased its induction by pro-inflammatory stimuli including LPS, IL-1β and TNFα. This regulation was strictly receptor-dependant and absent in macrophages from LXRαβ-/- mice. In addition, treatment of apoE-/- mice with LXR agonists reduced the expression of the inflammatory mediator MMP-9 in atherosclerotic aortas (Joseph *et al.*, 2003). These observations suggest that LXR agonists may exert their antiatherogenic effects not only by promoting cholesterol efflux, but also by acting to limit the production of inflammatory

mediators in the artery wall. Both studies demonstrated that this regulation was LXR-dependant and involved repression of NF-κB signalling.

Conversely, inflammatory pathways might alter the LXR-mediated metabolism of cholesterol. Recent work has demonstrated that viral and bacterial pathogens antagonise LXR transcriptional activity and cholesterol efflux. Some viral and bacterial pathogens activate Toll-like receptor 3 (TLR3) and TLR4 in macrophages, which triggers a rapid antimicrobial response through stimulation of proinflammatory pathways. This in turn inhibits LXR-induced expression of ABCA1 and apoE, and therefore decreases macrophage cholesterol efflux (Castrillo *et al.*, 2003b). This effect is NF-κB-independent and appears to involve IRF3, a transcriptional regulator of TLR signalling that inhibits the transcriptional activity of LXR on its target promoters. This crosstalk between signaling pathways controlling innate immunity and macrophage cholesterol metabolism may contribute to the ability of bacterial and viral pathogens to modulate cardiovascular disease.

Unfortunately, LXR has also been shown to play a role in the production of the pro-inflammatory cytokine tumor necrosis factor-α (TNFα) [Landis *et al.*, 2002]. Co-administration of 22(R)-hydroxycholesterol and the RXR-specific ligand 9-cis-retinoic acid to human monocytes resulted in a dramatic increase in TNFα production. LXR activation induced TNFα gene transcription and translation via an LXRE in the TNFα promoter, while RXR activation induced the release of TNFα protein from cells co-stimulated with an LXR activator (Landis *et al.*, 2002). It may be possible that specific LXR-mediated stimulation of TNFα expression may reduce atherosclerotic lesion size by the induction of apoptosis (Landis *et al.*, 2002). LXR also induces vascular endothelial growth factor (VEGF), a cytokine produced by macrophages and is a primary inducer of angiogenesis and neovascularization. VEGF mRNA was induced in response to synthetic LXR agonists in murine and human primary macrophages as well as in murine adipose tissue *in vivo* and LXR heterodimers bound directly to a conserved LXRE in the promoter of the murine and human VEGF genes (Walczak *et al.*, 2004).

1.4. AIMS OF THE STUDY

The evidence presented in this chapter clearly demonstrates a protective role for apoE in the development of atherosclerosis. ApoE is expressed at high levels in the atherosclerotic lesions, where it is mainly associated with macrophage-derived foam cells (Rosenfeld *et al.*, 1993; O'Brien *et al.*, 1994). This macrophage-specific expression of apoE is potentially of great importance and several studies have demonstrated that macrophage-derived apoE has potent anti-atherogenic properties (Linton *et al.*, 1995; Boisvert and Curtiss, 1999; Fazio *et al.*, 1997). Therefore macrophage apoE can be considered as an excellent target for therapy against atherosclerosis and studies investigating the regulation of this protein would be of great therapeutic potential.

Regulation of apoE expression is complex and involves multiple levels of control, including gene transcription, mRNA stability, synthesis, degradation and secretion (Duan *et al.*, 1995; Zuckerman *et al.*, 1992; Menju *et al.*, 1989; Basheeruddin *et al.*, 1992; Mazzone *et al.*, 1989). Although it has been well established through numerous studies that differentiation of monocytes into macrophages is associated with increased apoE expression (Werb and Chin, 1983; Auwerx *et al.*, 1988; Menju *et al.*, 1989; Tajima *et al.*, 1985), relatively little is known about the mechanisms involved in this activation of apoE. Currently, investigations into the differentiation-dependent activation of apoE in macrophages, is currently restricted to only a single study, which was rather limited and partly inconclusive (Basheeruddin *et al.*, 1994). Therefore the initial aims of this project were:

(i) To delineate fully the regulatory elements in the apoE promoter responsible for apoE activation during macrophage differentiation, and;

(ii) Identify the nuclear factors involved in this differentiation-dependent activation of apoE.

Also, during this project, studies in transgenic mice suggested that two distal enhancers, ME1 and ME2 were important in the macrophage- and adipocyte-specific expression of the apoE gene (Shih *et al.*, 2000; Laffitte *et al.*, 2001b). However, the precise regulatory sequences had not been identified, although multiple C/EBP sites were suggested as potential targets. Therefore, as an extension to the initial aims of the study, the role of ME1 and ME2 in apoE expression during macrophage differentiation was investigated.

The outcome of these initial aims are presented in Chapter 3 and show that, similar to the case in hepatocytes, the regulation of macrophage apoE expression is extremely complex. More specifically, in contrast to the endogenous gene, neither the promoter nor the enhancer were activated during macrophage differentiation. Therefore, as a result of this outcome, the overall aims of the project were modified slightly to understanding the regulation of apoE transcription by the Liver X receptor (LXR) family of nuclear receptors.

As described previously in this chapter, the activation of LXRs *in vivo* by ligands leads to a number of favourable changes in lipid metabolism, including promotion of reverse cholesterol transport, elevation of plasma HDL cholesterol, inhibition of cholesterol absorption, and the antagonism of inflammatory signalling. The relevance of these effects for the development of cardiovascular disease is clear from studies showing that synthetic ligands inhibit atherosclerosis in animal models (Joseph *et al.*, 2002b). Unfortunately, at the same time, the potent lipogenic activity of the current generation of LXR agonists is a significant limitation. From a theapeutic standpoint,

the most desirable LXR agonist would be one that was a strong inducer of genes involved in cholesterol efflux, such as ABCA1, apoE, ABCG1, and a strong suppressor of inflammatory gene expression yet lacked activity on the SREBP-1c and FAS promoters. This requirement to dissociate fatty acid synthesis from the beneficial effects on cholesterol metabolism requires a much clearer understanding of LXR-regulated transcription.

LXR-mediated regulation of target gene transcription has recently been shown to be more complicated than first anticipated. The requirement of coregulators for ligand activation of target genes has revealed that LXR transcription is regulated by mechanisms, other than just the availability of the ligand. In addition, recent studies have demonstrated an essential role for cell signalling pathways in nuclear receptor signalling (Rochette-Egly, 2003). These pathways may be responsible for the nuclear receptor recruitment of coregulators by affecting the availability and activity of these proteins and therefore affecting transcription. Also, recent studies have demonstrated that nuclear receptors are phosphoproteins, whose transcriptional activation may be modified directly through phosphorylation (Shao and Lazar, 1999). As very little work has so far been done on the effect of cell signalling on the ligand-induced activation of LXR target genes, the revised aims for this project were:

(i) Investigate the mechanism of oxysterol action on the expression of apoE

(ii) Identify signalling pathways that may be involved in this regulation of apoE

This has led to the identification of a novel involvement of cell signalling pathways such as the c-jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK), casein kinase 2 (CK2), and phosphoinositide-3-kinase (PI3K) pathways in the ligand-mediated induction of apoE in macrophages (see Chapters 5 and 6).

CHAPTER TWO - MATERIALS AND METHODS

2.1. MATERIALS

The chemical reagents and materials required for general use in this study are listed alongside their suppliers in Table 2.1.

TABLE 2.1 MATERIALS/ CHEMICAL REAGENTS AND THEIR SUPPLIERS

MATERIALS	SUPPLIER
Acrylamide:bisacrylamide (37.5:1)	Anachem, Luton, UK
[γ-^{32}P]-ATP, X-OMAT Kodak X-ray film, ECL-Western blotting detection reagent, Rainbow protein size markers, Streptavidin-HRP complex	Amersham Int., Buckinghamshire, UK
MicroAmp® 96-well reaction tubes, TaqMan® Universal PCR mastermix, TaqMan® primers, TaqMan® probes	Applied Biosystems, Warrington, UK
RPMI-1640 with stabilix	Autogen Bioclear, Wiltshire, UK
Goat polyclonal ApoE antibody	Biogenesis Ltd, Poole, UK
Curcumin, LY294002, PD98059, SB202190, SP600125	Calbiochem, Nottingham, UK
Agarose, BIOTAQ DNA polymerase, Magnesium Chloride, 10x NH_4 Buffer	BioLine, London, UK
Cell lines: THP-1 and U937	European Collection of Animal Cell Culture, Salisbury, UK
Butan-1-ol, EDTA, ethanol, formaldehyde, formamide, hydrochloric acid, isopropanol, methanol, SDS, sodium chloride, sodium fluoride, sodium hydroxide, sodium orthovanadate, sodium phosphate, sodium pyrophosphate, sulphuric acid, tri-sodium citrate, Tris-buffer and other general chemicals	Fisher, Loughborough, UK

Saran Wrap	Genetic Research Instrumentation, Essex, UK
Concert™ plasmid purification kit, FCS, 1Kb DNA marker, Trypsin/EDTA	Gibco BRL Life Technologies Ltd, Paisley, UK
Tissue culture plastics, 96-well plates, 6-well plates, Falcon 15ml and 50ml polypropylene tubes	Greiner, Gloucestershire, UK
Human serum apoE, SB biotinylated secondary antibody, goat serum	GlaxoSmithKline, Stevenage, UK
Goat polyclonal ATAB atlantic ApoE antibody	Inc Star, Minnesota, USA
Immobilin™-P^{SQ} PVDF membrane	Millipore Corporation, Massachusetts, USA
10x TBE	National Diagnostics, Atlanta, USA
Akt antibody, Phospho-Akt (Ser473) antibody, Phospho-Akt (Thr308) antibody, Akt kinase assay kit, Phospho ATF-2 (Thr71) antibody, DNA markers, Phospho-FKHR (Ser256) antibody, Phospho-GSK-3β (Ser9) antibody, JNK kinase assay kit, JNK total antibody, Phospho-JNK (Thr183/Tyr185) antibody, c-jun antibody, Phospho c-jun (Ser63) antibody, Phospho-PDK1 (Ser241) antibody, Phospho-PTEN (Ser 380) antibody, Restriction endonucleases and buffers	New England Biolabs, Herts, UK
Lymphoprep™	Nycomed Pharma, Oslo, Norway
TGF-β, TNF-α	PeproTech EC Ltd, London, UK
Immunopure®TMB substrate kit	Perbio Science UK Ltd, Cheshire, UK

Deoxyribonucleoside triphosphate, Random hexanucleotides	Pharmacia, Hertfordshire, UK
Micro BCA™ protein assay kit	Pierce, Chester, UK
Purified CK2 enzyme, 5x Passive Lysis Buffer, Wizard® *Plus* SV miniprep kit, MMLV reverse transcriptase, 5x reverse transcriptase buffer, Firefly Luciferase assay reagent, RNasin® Ribonuclease inhibitor	Promega, Southampton, UK
Effectene™ transfection reagent, RNeasy Total RNA isolation kit, Superfect™ transfection reagent	Qiagen Ltd, West Sussex, UK
Casein kinase 2 (α-subunit) antibody, Protein A/G agarose	Santa Cruz Biotechnology Inc., California, USA
Accuspin™ tubes, β-Actin antibody, Alkaline Protease, Ampicillin, Apigenin, Aprotonin, APS, Benzamidine, Bromophenol blue, BSA, β-Casein, DTT, DMSO, Ethidium Bromide, Gamma-globulin, Glycerol, HEPES, 22-(R) hydroxycholesterol, 22-(S) hydroxycholesterol, LB-Agar capsules, LB-Medium, Leupeptin, β-mercaptoethanol, Mineral oil, MOPS, ONPG, Penicillin/Streptomycin, Pepstatin A, PMA, PMSF, Ponceau S solution, TEMED, Tissue culture water, Triton X-100, Trypan blue solution, TWEEN-20, TWEEN-40, zinc chloride	Sigma-Aldrich Company Ltd, Dorset, UK
RT-PCR primers	Sigma-Genosys, Cambridgeshire, UK
Mammalian transfection kit (DEAE-dextran)	Stratagene, California, USA
Buffy coat, human serum	Welsh blood service, Talbot Green, UK

2.1.1. PLASMIDS

During this study several DNA constructs were used to analyse promoter activity and to investigate various signalling pathways. All constructs were kind gifts from various laboratories and the details of the cloning process for each construct can be found in the appropriate reference (Table 2.2).

TABLE 2.2. THE VARIOUS DNA CONSTRUCTS USED IN THIS STUDY

PLASMID	REFERENCE
ApoE –3000/+86 pGL3	Basheeruddin *et al.*, 1994
pGL-apoE-890	Laffitte *et al.*, 2001b
pGL-apoE-890-ME.1.	Laffitte *et al.*, 2001b
pGL-apoE-890-ME.2.	Laffitte *et al.*, 2001b
LPL-1824/+188	Gimble *et al.*, 1989
pSG-alpha (K68A)	Heriche *et al.*, 1997
SAPKα-VPF	Moriguchi *et al.*, 1995
SEK1D/N	Sanchez *et al.*, 1994

2.1.2. BACTERIAL CULTURE MEDIA

Both the LB-medium and LB-agar were supplied in capsule form and made up according to the manufacturer's instructions.

2.1.3. STOCK SOLUTIONS

All stock solutions used in the procedures described in this chapter are shown in Tables 2.3, 2.4, 2.5, 2.6, 2.7, 2.8 and 2.9. The solutions were prepared using double distilled water and were of molecular biology grade unless otherwise stated.

TABLE 2.3. SOLUTIONS USED FOR THE ISOLATION OF DNA

SOLUTIONS	COMPOSITION
Miniprep Resuspension solution	50mM Tris-HCl (pH 7.5), 0.1mM EDTA, 100μg/ml RNase A
Miniprep Lysis solution	0.2M sodium hydroxide, 1% (w/v) SDS
Miniprep Neutralisation solution	2.55M potassium acetate (pH 4.8)
Miniprep Column Wash solution	200mM NaCl, 20mM Tris-HCl (pH 7.5), 5mM EDTA
Miniprep Resin	1.5% (w/v) celite resin in 7M guanidine hydrochloride (pH 5.5)
Maxiprep Cell Suspension buffer	50mM Tris-HCl (pH 8.0), 10mM EDTA, 0.2mg/ml RNase A
Maxiprep Cell Lysis solution	200mM NaOH, 1% (w/v) SDS
Maxiprep Neutralization buffer	3.1M potassium acetate (pH 5.5)
Maxiprep Equilibration buffer	600mM NaCl, 100mM sodium acetate (pH 5.0), 0.15% (v/v) Triton® X-100
Maxiprep Wash buffer	800mM NaCl, 100mM sodium acetate (pH 5.0)
Maxiprep Elution buffer	1.25M NaCl, 100mM Tris-HCl (pH 8.5)
TE buffer	10mM Tris-HCl (pH 8.0), 0.1mM EDTA

TABLE 2.4. SOLUTIONS FOR ELECTROPHORESIS OF DNA AND RNA

SOLUTION	COMPOSITION
10x TBE	890mM Tris-HCl, 890mM boric acid, 20mM EDTA
10x DNA loading dye	1xTBE, 50% (v/v) glycerol, 0.25% (w/v) bromophenol blue
RNA loading dye stock	50% (v/v) glycerol, 1M EDTA, 0.25% (w/v) bromophenol blue, pH 8.0
2x RNA loading buffer	RNA loading dye stock: 10x MOPS: formaldehyde: formamide [4:4:7:20(v/v)]
20x MOPS	400mM MOPS, 100mM sodium acetate, 100mM EDTA, pH 7.0

TABLE 2.5. SOLUTIONS FOR CELL TRANSFECTIONS

SOLUTION	COMPOSITION
2x β-galactosidase assay buffer	200mM sodium phosphate buffer (120mM Na_2HPO_4, 80mM NaH_2PO_4) [pH 7.3], 2mM $MgCl_2$, 100mM β-mercaptoethanol, 1.33mg/ml ONPG
2x HBS	10g/l Hepes, 16g/l NaCl, pH 7.1
100x PO_4 buffer	70mM Na_2HPO_4, 70mM NaH_2PO_4 in a 1:1 ratio

TABLE 2.6. SOLUTIONS FOR THE PREPARATION OF CELL EXTRACTS

SOLUTION	COMPOSITION
PBS	137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4
Whole Cell Extraction buffer	10mM HEPES (pH 7.9), 400mM NaCl, 0.5mM DTT, 2μg/ml Aprotinin, 0.5mM PMSF, 5% (v/v) glycerol, 2mM 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 tetra-sodium pyrophosphate ($Na_4P_2O_7$), 5μM $ZnCl_2$, 100μM sodium orthovanadate (Na_3VO_4), 1mM DTT, 2.8μg/ml aprotinin, 2.5μg/ml leupeptin, 2.5μg/ml pepstatin, 0.5mM benzamidine, 0.5mM PMSF

TABLE 2.7. SOLUTIONS FOR WESTERN BLOT ANALYSIS OF PROTEINS

SOLUTION	COMPOSITION
SDS-PAGE upper buffer	1M Tris-HCl (pH 6.8), 10% (w/v) SDS
SDS-PAGE lower buffer	1.5M Tris-HCl (pH 8.8), 10% (w/v) SDS
SDS-PAGE 10x running buffer	25mM Tris-HCl (pH 8.3), 250mM glycine, 0.1% (w/v) SDS
SDS-PAGE gel loading buffer	50mM Tris-HCl (pH 6.8), 20% (w/v) sucrose, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, 5%(v/v)β-mercaptoethanol
Western blot transfer buffer	25mM Tris-HCl (pH 8.3), 192mM glycine, 20% (v/v) methanol
1x TBS	10mM Tris-HCl, 200mM NaCl, pH 7.4

TABLE 2.8. SOLUTIONS FOR ELISA

SOLUTION	COMPOSITION
ELISA assay buffer	0.5% (w/v) BSA, 0.05% (w/v) Gamma-globulin, 50mM Tris-HCl, 150mM NaCl, 0.01% (v/v) TWEEN-40, pH 7.4
Blocking Buffer	1% BSA (w/v), 50mM Tris-HCl, 150mM NaCl, 1mM $MgCl_2$, pH 7.4
Washing Buffer	10mM Tris-HCl, 150mM NaCl, 0.05% (v/v) TWEEN-20, pH7.4

TABLE 2.9. SOLUTIONS FOR PROTEIN KINASE ASSAYS

SOLUTION	COMPOSITION
CK2 kinase buffer	100mM Tris-HCl (pH 8.0), 100mM NaCl, 20mM $MgCl_2$, 50mM KCl, 100µM Na_3VO_4, 5mg/ml β-casein
1x Akt kinase buffer	25mM Tris-HCl (pH 7.5), 5mM β-glycerophosphate, 2mM DTT, 0.1mM Na_3VO_4, 10mM $MgCl_2$
1x JNK kinase buffer	25mM Tris-HCl (pH 7.5), 5mM β-glycerophosphate, 2mM DTT, 0.1mM Na_3VO_4, 10mM $MgCl_2$

2.1.4. PREPARATION OF CERAMICS, PLASTICS AND GLASSWARE

All glassware, ceramics and plastics (including pipette tips and Eppendorf tubes) were autoclaved for 20min at 121°C and at a pressure of 975 kPa.

2.2. ISOLATION AND MANIPULATION OF DNA

2.2.1. PREPARATION OF COMPETENT CELLS

For the preparation of competent bacterial cells, 10ml of LB-medium was inoculated with a single bacterial colony of the DH5α *E.coli* strain [*supE44, Δ lacU169, (Φ80 lac ZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1*] and incubated overnight at 37°C, with moderate shaking. Following incubation, 100μl of the culture was used to inoculate 100ml of fresh LB-medium and incubated with moderate shaking at 37°C, until an OD_{550} of between 0.5 and 0.6 was reached. The cells were then pelleted by centrifugation at 3,000g at 4°C for 5min and resuspended in 50ml of ice-cold $CaCl_2$ solution (50mM). The suspension was left on ice for 25min and the cells were again pelleted by centrifugation (3,000g for 5min at 4°C). The cells were then resuspended in 10ml of ice-cold $CaCl_2$ solution (50mM) and mixed with 40% (v/v) sterile glycerol. The suspension was aliquoted into chilled Eppendorf tubes and stored at -70°C.

2.2.2. TRANSFORMATION OF COMPETENT CELLS

For each transformation, 10ng of recombinant plasmid DNA was added to 200μl of competent cells (Section 2.2.1) and subsequently incubated on ice for 40min. The cells were then heat-shocked for 90sec at 42°C. Following heat shock treatment, the cells were placed on ice for 2min and 800μl of pre-warmed (37°C) LB-medium was added. The mixture was then left to incubate for 1h at 37°C with moderate shaking. After incubation, 200μl aliquots were spread on agar plates containing ampicillin (100μg/ml) for selection of transformed bacteria and left to incubate overnight at 37°C. An ampicillin plate spread with untransformed cells was used as a control for each transformation.

2.2.3. SMALL-SCALE DNA PREPARATION (MINIPREP PROTOCOL)

Small-scale preparation of recombinant plasmid DNA was carried out using the Wizard® *Plus* SV miniprep kit (Promega) according to the manufacturer's protocol. 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. Cells were pelleted by centrifugation at 10,000g, at room temperature for 5min and resuspended in 250μl Cell Resuspension solution (Table 2.3). The suspension was then transferred to a microcentrifuge tube and 250μl of Cell Lysis solution was added (Table 2.3). The suspension was mixed by inversion and clearing was observed confirming that lysis was complete. Alkaline Protease (10μl) was added to the solution and after inversion the mixture was incubated at room temperature for 5min. Once the incubation period was over, 350μl of Neutralisation solution (Table 2.3) was added, mixed by inversion and centrifuged at 14,000g for 10min. The clear supernatant was transferred to a spin column inserted into a collection tube and

subjected to centrifugation at 14,000g for 1min. The flow through was discarded and the spin column was washed twice with Column Wash solution (Table 2.3). The column was then dried by centrifugation at 14,000g for 2min. The DNA was eluted from the column by adding 50µl of Nuclease-Free water and centrifuging at 14,000g for 1min.

2.2.4. LARGE-SCALE DNA PREPARATION (MAXIPREP PROTOCOL)

Large-scale preparation of plasmid DNA was carried out using the Concert™ plasmid purification kit (Gibco) according to the manufacturer's protocol. A single colony of transformed bacteria was used to inoculate 5ml of LB-medium containing 100µg/ml of ampicillin and incubated for 4-6h at 37°C with moderate shaking. This mini-culture was then added to 200ml of LB-ampicillin medium and left to grow overnight with shaking at 37°C. The cells were then pelleted by centrifugation at 6,000g for 20min, at a temperature of 4°C. All medium was thoroughly removed and the resulting pellet was resuspended, using 10ml of Cell Suspension buffer (Table 2.3). The mixture was lysed using 10ml of Cell Lysis solution (Table 2.3) and mixed gently by inversion. Following incubation at room temperature for 5min, 10ml of Neutralization solution (Table 2.3) was added. The solution was mixed by inversion and centrifuged at 15,000g at room temperature for 10min. The supernatant was then transferred to a column, which had previously been equilibrated by allowing 30ml of Equilibration buffer (Table 2.3) to drain through the column by gravity flow. The resulting flow-through was discarded and the column was washed with 60ml of Wash buffer (Table 2.3). The DNA was then eluted by adding 15ml of Elution buffer (Table 2.3) 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 a temperature of 4°C. The pellet was washed with 5ml of 70% (v/v) ethanol by centrifugation at 15,000g for 5min at 4°C. The ethanol was removed and the DNA pellet was left to air-dry for 10min and then dissolved in 500µl of TE buffer (Table 2.3).

2.2.5. QUANTIFICATION OF DNA

The concentration and purity of recombinant plasmid DNA was determined by measuring the ratio of absorbance at 260nm and 280nm, percentage purity and yield in µg/µl of each sample using the GeneQuant spectrophotometer (Pharmacia).

2.2.6. RESTRICTION ENDONUCLEASE DIGESTION OF DNA

Restriction endonuclease digestion of DNA was carried out using buffers and conditions recommended by the supplier (New England Biolabs). The incubation times varied depending on the enzymes with the majority of the reactions being incubated for at least 2h at 37°C. The digests

were typically carried out using 10 Units of each enzyme. The only exception was in the case of digests involving KpnI where 20 Units were used due to the enzyme's 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 each digest performed in this study, the reaction mixture was supplemented with 100μg/ml BSA. All reactions were stopped by incubation at 65°C for 10min.

2.2.7. GEL ELECTROPHORESIS OF DNA

Size-fractionation of DNA was carried out using agarose gel electrophoresis. The gels used in the present study were 0.7% to 1.5% (w/v) and the sizes of the various fragments were determined by comparison of their migration with standard molecular weight markers (Appendix I). Both the electrophoresis buffer and gel contained ethidium bromide in 0.25x TBE at a final concentration of 0.5μg/ml. Before loading, all samples were mixed with 10x DNA loading buffer (Table 2.4) where bromophenol blue was used as the tracking dye. Electrophoresis was routinely performed in a horizontal gel apparatus (GNA-100 and GNA-200, Pharmacia) at 100V for 1.5-2h. Following electrophoresis, the DNA was visualised using an ultraviolet transilluminator and the gel photographed using Genetools (Syngene).

2.3. TISSUE CULTURE AND RELATED TECHNIQUES

2.3.1. MAINTENANCE OF CELL LINES IN CULTURE

The human monocyte THP-1 and U937 cell lines were both grown in RPMI-1640 with Stabilix supplemented with 10% (v/v) heat-inactivated (30min, 56°C) foetal calf serum (HI-FCS) in the presence of penicillin (100U/ml) and streptomycin (100ug/ml). Before addition to the media, all solutions were filter-sterilised by passing them through a 0.2μm sterile filter. The cells were maintained at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. The cell culture medium for each cell line was changed every three days.

2.3.2. SUB-CULTURING OF CELLS

Both monocyte cell lines (THP-1 and U937) were sub-cultured when they reached approximately 70% confluency ($1x10^6$ cells/ml). The U937 cell line was centrifuged at 1,000g for 5min and the pellet resuspended in medium containing 10% (v/v) HI-FCS. The cells were then plated into fresh tissue culture flasks at a ratio of 1:4 (i.e. cells from 1 tissue culture flask was sub-cultured into 4 new flasks) and incubated as above. The THP-1 cell line was also sub-cultured at a ratio of 1:4, which was carried out by dilution of the appropriate amount of cells into fresh medium containing 10% (v/v) HI-FCS.

2.3.3. HUMAN PRIMARY MONOCYTE-DERIVED MACROPHAGE CELL CULTURE

2.3.3.1. ISOLATION OF PRIMARY MONOCYTE-DERIVED MACROPHAGES

The isolation of human monocytes was carried out using Accuspin™ centrifuge tubes (Sigma-Aldrich) and the separation medium used was Lymphoprep™ (Nycomed Pharma). To each Accuspin™ centrifuge tube, 15ml of Lymphoprep™ (warmed to room temperature), was layered on top of the polyethylene barrier ("frit") and centrifuged at 800g for 30sec in order to place the Lymphoprep™ in the chamber below the "frit". After this brief centrifugation step, 30ml of buffy coat (Welsh blood service) was layered over the "frit" and centrifuged at 800g for 30min at room temperature. The resultant mononuclear band was collected and transferred to a new centrifuge tube, to which 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, after which a 0.2% saline solution (10ml) was added to the pellet (on ice) for 30sec followed by a 1.6% saline solution (10ml), and centrifuged immediately at 800g for 5min at 4°C. The resultant pellet was then washed 6-8 times with ice-cold PBS-0.4% (w/v) tri-sodium citrate to remove contaminating platelets. Monocytes were plated out in 75cm^2 flasks (8x10^{6}cells/10ml/flask) in RPMI-1640 with Stabilix containing antibiotics (100U/ml penicillin and 100µg/ml streptomycin) and supplemented with 5% (v/v) human serum. The cells were allowed to adhere for 3h in an incubator at 37°C, 5% (v/v) CO_2 and then the medium was replaced with fresh culture medium. The monocytes were then left to differentiate for 7-10 days before use in experiments.

2.3.3.2. CULTURE ROUTINE OF HUMAN PRIMARY MONOCYTE-DERIVED MACROPHAGES

Every day, the medium in which the cells were growing was removed and replaced with fresh culture medium pre-warmed to 37°C, containing the appropriate antibiotics (100U/ml penicillin and 100µg/ml streptomycin) and 5% (v/v) human serum.

2.3.4. DELIPIDATION OF HI-FCS

Lipids were removed from HI-FCS according to the method of Cham and Knowles (1976) with minor modification. To 10ml of HI-FCS, 20ml of 2:3 (v/v) butanol:di-isopropyl ether (DIPE) was added and mixed with rotation for 30min at room temperature. The phases were then separated by centrifugation at 2,000g for 2min and the serum removed. In order to remove any residual solvent, nitrogen was bubbled through the serum for 2-3h.

2.3.5. TRANSIENT TRANSFECTION OF THE THP-1 AND U937 CELL LINES

Prior to each transfection (about 24h), the cells were split 1:2 and put into fresh flasks. Approximately $1x10^6$ cells were seeded into each well of a 6-well plate (Greiner) four hours prior to transfection. All cells used for transfection experiments were between passages 3 and 10.

2.3.5.1. TRANSIENT TRANSFECTION OF CELLS USING SUPERFECT™

Transient transfection with Superfect™ was carried out as described in the manufacturer's protocol (Qiagen). For each transfection, 2μg of the appropriate reporter DNA construct and 0.5μg of the internal control plasmid were made up to 100μl with serum-free and antibiotic-free culture medium. Each transfection DNA mix was then mixed by vortexing with the Superfect™ transfection reagent (a charge ratio of 1:4 was used for all transfections carried out in this study i.e. 4μl of Superfect™ was used per 1μg of DNA). The mixture was then left to incubate at room temperature for 10min in order to allow the formation of the DNA:Superfect™ complexes. Complete medium (containing HI-FCS and penicillin/streptomycin) was then added to each transfection mixture (400μl) and the transfection complexes were added drop-wise onto the cells. The 6-well plates were swirled to allow even distribution of the complexes and then left to incubate (37°C, 5% (v/v) CO_2) for the appropriate length of time for gene expression.

2.3.5.2. TRANSIENT TRANSFECTION OF CELLS USING EFFECTENE™

Transient transfection with Effectene™ was carried out as described in the manufacturer's protocol (Qiagen). For each transfection, 0.4μg of the appropriate plasmid construct and 0.2μg of the internal control plasmid were made up to 100μl with buffer EC (Qiagen), along with 3.2μl of the Enhancer reagent (Qiagen). The mixture was mixed by vortexing and incubated at room temperature for 5min. The DNA-Enhancer solution was then centrifuged for a few seconds and 10μl of the Effectene™ reagent was added and mixed by pipetting. The solution was left to incubate at room temperature for 10min and 600μl of complete cell culture medium was added to the transfection mixture. The transfection complexes were then added drop-wise to the cells and the plates were swirled to ensure uniform distribution of the complexes. The cells were incubated at 37°C with 5% (v/v) CO_2 for the appropriate length of time.

2.3.5.3. TRANSIENT TRANSFECTION OF CELLS USING THE DEAE-DEXTRAN MAMMALIAN TRANSFECTION KIT

Transient transfection with the DEAE-dextran mammalian transfection kit was carried out as described in the manufacturer's protocol (Stratagene) with only minor modification. In order for suspension cells to be transfected using this system it was necessary to bulk transfect the cells in

15ml polypropylene tubes (Falcon). Therefore, cells at a concentration of $1x10^6$cells/ml were washed twice with PBS and re-suspended in serum-free, antibiotic-free medium to the same concentration. Before transfection, the transfection solutions-A (2μg of DNA made up to 170μl with PBS) and -B (85μl of solution no.3 [2mg/ml DEAE-dextran in 0.9% NaCl] with 85μl PBS) were prepared. The solutions (A and B) were combined and added drop-wise to the cells. The transfection mix was left at room temperature for 15min and then removed from the cells. The cells were washed twice with PBS and resupended in complete medium. The cells were then aliquoted into 6-well plates and the final volume for each well was made up to 2ml with fresh medium. The plates were then left to incubate for 36h at 37°C with 5% (v/v) CO_2.

2.3.6. DIFFERENTIATION OF MONOCYTIC CELL LINES

Differentiation of monocytes was routinely carried out by the addition of an appropriate concentration (depending on the experimental conditions) of the differentiation agent PMA. Prior to each experiment (about 24h), the cells were split 1:2 and put into fresh flasks. Before each experiment, the cells were plated out at the appropriate density and left to incubate for 4h, following which, PMA was added to the cells. For all transfection experiments, PMA was added once the transfection of cells was complete. After the addition of PMA, the cells were left to incubate as normal (Section 2.3.3.1) for the requisite time period.

2.3.7. TREATMENT OF CELLS WITH EXTRACELLULAR MEDIATORS

For certain transfection experiments it was necessary to treat the transfected cells with the extracellular mediator TNF-α, in order to monitor the effect of this cytokine on promoter activity. Therefore, where necessary, the transient transfection protocol (Sections 2.3.5.1, 2, 3 and 4) was modified so that 16h after transfection, TNF-α was added to provide a final concentration of 1000U/ml. The cells were then left to incubate for a further 36h at 37°C with 5% (v/v) CO_2.

2.3.8. TREATMENT OF CELLS WITH OXYSTEROL LIGANDS

For certain experiments the addition of the LXR ligands 22(R)- and 22(S)-hydroxycholesterol was necessary. The ligands were added 24h after PMA treatment of cells, at a concentration of 2μg/ml. The cells were then left to incubate as normal (Section 2.3.3.1) for the requisite time period.

2.3.9. TREATMENT OF CELLS WITH INHIBITORS

Investigation of the signalling pathways involved in LXR regulation of apoE required the addition of various inhibitors 1h prior to the addition of the above ligands. Once the inhibitors were added in

their appropriate concentrations (depending on the experimental conditions) the cells were left to incubate as normal (Section 2.3.3.1) for the requisite incubation period of the study.

2.3.10. PREPARATION OF CELL EXTRACTS FOR THE DETERMINATION OF REPORTER GENE ACTIVITY

Following the completion of transient transfection (Sections 2.3.5.1, 2, 3), cells were detached from the plate using a cell scraper and the mixture was transferred to a microcentrifuge tube. The sample was centrifuged for 1min at 12,000g and the pellet was washed twice with PBS. The pellet was then re-suspended in 200μl of 1x Passive Lysis buffer (Promega) and left for 10min at room temperature. The cell lysate was centrifuged for 2min at 15,000g and the supernatant was retained and used for the determination of the reporter gene activity.

2.3.11. MEASUREMENT OF LUCIFERASE ACTIVITY

During this study, the apoE promoter and enhancer activity was monitored using the luciferase gene as the reporter. The assay was carried out essentially as described in the manufacturer's protocol (Promega). For each assay, 40μl of cell extract (Section 2.3.9) was mixed with 100μl of the Luciferase Assay Reagent (Promega) in a luminometer tube, and the light output for each sample was determined in duplicate using a Turner Designs 50/50 Luminometer. The luminometer was set at a sensitivity value of 70% with a 2sec-delay period and 20sec integrate period.

2.3.12. MEASUREMENT OF β-GALACTOSIDASE ACTIVITY

In some of the transfections carried out, β-galactosidase was used as an internal control for transfection efficiency and the luciferase activity was normalised to this value. The assay for β-galactosidase was carried out in 96-well plates (Greiner). In each well, 50μl of cell extract (Section 2.3.10) was mixed with 50μl of 2x β-galactosidase assay buffer (Table 2.5). The assay mixture was then incubated at 37°C for 1h. The reaction was terminated by the addition of 100μl of 1M sodium carbonate and the absorbance of the samples were measured at 405nm, using a Titertek Multiscan MCC/340 microplate reader.

For certain transfection assays it was necessary to perform a protein assay in order to normalise the luciferase activity of the cell extracts, as described in Section 2.5.2.

2.3.13. TRYPAN BLUE EXCLUSION ASSAY

Cell viability was assessed using trypan blue. Briefly, cells were adjusted to a density of 10^6/ml and 20μl of this suspension was mixed with an equal volume of 0.4% (v/v) trypan blue and left for 5min. The cells were then placed on a haemocytometer and viewed using phase contrast microscopy. At

least 200 cells were counted and the percentage of dead cells (i.e. the cells that had absorbed the stain) was estimated.

2.4. RNA-RELATED TECHNIQUES

2.4.1. RNA ISOLATION

Total cellular RNA was purified from cells using the RNeasy Total RNA isolation kit (Qiagen) according to the manufacturer's protocol. The cells used for RNA extraction in this study were the THP-1 cell line, therefore the method used was that for cells grown in suspension. The cells (8x 10^6 per RNA extraction) and their media were transferred to a 15ml polypropylene tube (Falcon) and centrifuged at 1,000g for 5min. The resulting supernatant was discarded and the cell pellet was lysed by the addition of 600µl of Buffer RLT (Qiagen) [containing 10µl/ml of β-mercaptoethanol]. The solution was mixed by pipetting to remove all clumps and then homogenized by passing the lysate through a 0.9mm needle attached to a 1ml syringe for at least 10 times. Once the homogenization step was complete 600µl of 70% (v/v) ethanol was added and the lysate was mixed well by pipetting. The sample was transferred 700µl at a time to an RNeasy spin column inserted into a 2ml collection tube and centrifuged at 8,000g for 15sec. The flow-through was discarded and 700µl of buffer RW1 (Qiagen) was used to wash the column by centrifuging the sample for 15sec at 8,000g. Once again the flow-through was discarded and the RNeasy column was transferred to a new collection tube. The column was washed again by adding 500µl of buffer RPE (Qiagen) and centrifuging the column for 15sec at 8,000g. The final wash was carried out using 500µl of buffer RPE (Qiagen) and centrifuging the column for 2min at 10,000g. The RNA was eluted from the column by placing the column into a new 1.5ml collection tube and adding 50µl of RNase-free water onto the column. The column was then centrifuged for 1min at 8,000g and the purified RNA was stored at –70°C.

2.4.2. ANALYSIS OF RNA ON DENATURING AGAROSE GELS

Size-fractionation and determination of RNA quality was carried out using denaturing formaldehyde agarose gels. The gels routinely used in this study were 1% (w/v) agarose gels, and were prepared using sterile double distilled water. For a 100ml gel, 1g of agarose was dissolved in 77ml of water to which were then added 5ml of 20x MOPS (Table 2.4) and 18ml of 37% (v/v) formaldehyde. Each RNA sample (5µl) was prepared by adding an equal volume of RNA loading buffer (Table 2.4) and 1µl of 1mg/ml ethidium bromide. The mixture was heated at 65°C for 10min and then cooled on ice before loading. Electrophoresis was performed for approximately 2h at 75V in a GNA-100 horizontal gel apparatus (Pharmacia) using 1x MOPS as the "running" buffer. The

ethidium bromide stained RNA was then visualised and photographed as described previously (Section 2.2.7).

2.4.3. QUANTIFICATION OF RNA

All isolated RNA was quantified using a GeneQuant spectrophotometer (Pharmacia) as described previously for DNA (Section 2.2.5).

2.4.4. REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

RT-PCR was carried out in two stages; cDNA synthesis using reverse transcriptase and the subsequent amplification of the synthesised cDNA using the Polymerase Chain Reaction.

2.4.4.1. COMPLEMENTARY DNA (cDNA) SYNTHESIS FROM TOTAL RNA

For each RNA sample, 1μg of total RNA was used as a template to generate random primed cDNA. To 1μg of total RNA in 12.5μl of RNase-free water, 200pmole of random hexamer primers were added. The mixture was incubated at 70°C for 5min and then cooled on ice. Then, 4μl of 5x reverse transcriptase NH_4 buffer (Bioline), 1μl of dNTP mix (10mM each), 0.5μl of 40U/μl RNase inhibitor and 1μl of 200U/μl Molony murine Leukemia virus (MMLV) reverse transcriptase were added to the mixture and incubated at 42°C for 1h. The reaction was then terminated by heating the mixture to 94°C for 2min and the cDNA was diluted 5-fold by adding 80μl of sterile double distilled water. The cDNA was either used immediately for Polymerase Chain Reaction (Section 2.4.4.2.) or stored at –70°C.

2.4.4.2. POLYMERASE CHAIN REACTION

During this study all PCR reactions were carried out in a final volume of 50ul. Each standard PCR reaction contained Taq polymerase (1.25Units), 10x PCR buffer (Bioline) [50mM KCl, 10mM Tris-HCl (pH 9.0), 1% (v/v) Triton® X-100], 0.1mM of each dNTP, 50pmoles of both the 3' and 5' primers. The concentration of $MgCl_2$ was varied according to the template/primer set used, and in some cases DMSO (5%, v/v) was added to the reaction to increase amplification specificity. Each reaction mixture was overlaid with 50ul of mineral oil and the cycle programmes used were optimised for each template/primer set. All PCR reactions were carried out using a Biometra TRIO Thermoblock. The PCR amplification products were analysed by agarose gel electrophoresis using a 1.5% (w/v) agarose gel as described in Section 2.2.6. The sequences of primers used are shown in Table 2.10.

TABLE 2.10. SEQUENCES OF PRIMERS USED IN PCR ANALYSIS

TARGET MRNA	FORWARD PRIMER	REVERSE PRIMER
Human ApoE	5'-*TTCCTGGCAGGATGCCAGGC*-3'	5'-*GGTCAGTTGTTCCTCCAGTTC*-3'
Human ABCA1	5'-*GTTGGAAAGATTCTCTATACACCTGA*-3'	5'-*CGTCAGCACTCTGATGATGGCCTG*-3'
Human LXRα	5'-*ATGGTGGATGGAGACGTAG*-3'	5'-*ATGGACACCTACATGCGTC*-3'
Human GAPDH	5'-*CCCTTCATTGACCTCAACTACATG*-3'	5'-*AGTCTTCTGGGTGGCAGTGATGG*-3'

The primer sequences were obtained from the following references: Human ApoE (Wang et al., 1989); human ABCA1 (Klucken et al., 2000); human LXRα (Kohro et al., 2000).

TABLE 2.11. OPTIMISED PCR TEMPERATURE AND CYCLING CONDITIONS FOR EACH PRIMER SET[1]

TARGET MRNA	PCR PROGRAMME
Human ApoE	*Initial treatment of 96°C for 5min, 24 cycles of [60°C(1min), 72°C(2min), 93°C(30sec)], and 72°C for 10min at end*
Human ABCA1	*Initial treatment of 92.5°C for 2min, 31 cycles of [60°C(44sec), 60°C(40sec), 71°C(46sec)], and 72°C for 10min at end*
Human LXRα	*Initial treatment of 96°C for 5min, 32 cycles of [60°C(1min), 72°C(2min), 93°C(30sec)], and 72°C for 10min at end*
Human GAPDH	*Initial treatment of 96°C for 5min, 18 cycles of [60°C(1min), 72°C(2min), 93°C(30sec)], and 72°C for 10min at end*

[1]*The optimised conditions for each primer set were within the exponential phase of amplification and, therefore, provided a direct correlation between the amount of amplification products and RNA template abundance in the sample.*

2.4.5. TAQMAN REAL-TIME QUANTITATIVE RT-PCR

The cDNA synthesis step was carried out as described in section 2.4.4.1. The PCR reactions were carried out in 96 sample tubes in a final volume of 29μl. Each standard PCR reaction contained 2x Taqman Master mix (Applied Biosystems), 300nM of both the 3' and 5' primers and 300nM of the probe, and 25ng of cDNA. The mixture was then overlaid with 12μl of mineral oil. Reactions were cycled on an ABI PRISM 7700 Sequence detector (Applied Biosystems) using the following default programme: a 50°C incubation for 2min, 95°C for 10min, followed by 40 cycles of 95°C for 15sec for denaturation and 60°C for annealing and extension. Each set of reactions also contained a standard curve, where human liver cDNA was diluted to give a range of concentrations (60ng/μl, 30ng/μl, 10ng/μl, 3.34ng/μl, 1.11ng/μl, 0.38ng/μl, 0.124ng/μl, 0.05ng/μl, 0.014ng/μl, 0.0046ng/μl, 0.00152ng/μl, 0.0005ng/μl). Reactions were monitored in real time mode using Sequence Detection Systems, version 1.7 software. Samples were normalised using the β-microtubulin Taqman probe. The sequences of the primers and probes used are shown in Table 2.12.

2.5. PROTEIN ANALYSIS

2.5.1. PREPARATION OF WHOLE CELL EXTRACTS

The cells used for the isolation of whole cell extracts in this study were the THP-1 cell line. The cells (8x 10^6 per isolation) and their media were transferred to a 15ml Polypropylene tube (Falcon) and centrifuged at 1000g for 5min. The pellet was then washed three times in cold PBS by centrifugation for 1min at 10,000g. The resulting pellet was placed on dry ice for 5min and then resuspended in 5 volumes of whole cell extract buffer (Table 2.6). The cells were lysed by pipetting and the mixture was then centrifuged at 10,000g for 5min at 4°C. The resulting lysate was transferred to a fresh Eppendorf tube and stored at –70°C.

For certain experiments the maintenance of proteins in their phosphorylated state was vital, and it was therefore necessary to slightly modify the method for whole cell extract isolation. In these instances, whole cell extracts were made in a buffer containing phosphatase inhibitors as well as protease inhibitors, as described by Hipskind *et al.* (1994). The cells (8x10^6 per isolation) and their media were centrifuged at 1000g for 5min and the resulting pellet was washed twice with ice-cold PBS containing 10mM NaF and 100μM sodium orthovanadate (Na_3VO_4). The pellet was then resuspended in 500μl of phosphatase-free whole cell extraction buffer (Table 2.6) and vortexed vigorously for 45sec at 4°C. The lysate was cleared by centrifugation at 10,000g for 10min at 4°C and stored at –70°C.

TABLE 2.12. SEQUENCE OF PRIMERS AND PROBES USED IN TAQMAN REAL-TIME RT-PCR

TARGET MRNA	FORWARD PRIMER	REVERSE PRIMER	PROBE
Human ApoE	5'-*CGTTGCTGGTCACATTCCTG*-3'	5'-*GCTCTGTCTCCACCGCTTG*-3'	5'-*CAGGATGCCAGGCCAAGGTGGA*-3'
Human ABCA1	5'-*TCCAGGCCAGTACGGAATTC*-3'	5'-*TTCCTCGCCAAACCAGTAGG*-3'	5'-*AGGCCCTGGTATTTTCCTTGCACCAA*-3'
Human ABCG1	5'-*ATGGAAGGCTGCCACAGC*-3'	5'-*AGGTCCTCTTGAAGAGGATGCA*-3'	5'-*TCTCTGCCAGCTGCCTCACGCA*-3'
Human LXRα	5'-*AGCCCTGCATGCCTACGTC*-3'	5'-*GCATCCGTGGGAACATCAG*-3'	5'-*CCATCCACCATCCCCATGACCG*-3'
Huamn PPARγ	5'-*CACAGGCCGAGAAGGAGAA*-3'	5'-*TCGATATCACTGGAGATCTCCGCCAAC*-3'	5'-*TCAGCGGACTCGGATTCAG*-3'
Human β-microtubulin	5'-*CGCTCCGTGGCCTTAGC*-3'	5'-*GAGTACGCTGGATAGCCTCCA*-3'	5'-*TGCTCGCGCTACTCTCTCTTTCTGGC*-3'

2.5.2. DETERMINATION OF PROTEIN CONCENTRATION

The concentration of total protein in the cell extracts was determined using the BCA™ protein assay kit, according to the manufacturer's protocol (Pierce). A standard curve was produced for each assay using suitable dilutions of a 2mg/ml bovine serum albumin solution (BSA) in order to give concentrations of 5μg/ml, 10μg/ml, 15μg/ml, 20μg/ml and 25μg/ml. The standards along with 100μl of diluted cell extract were placed into the wells of a 96-well plate. Both the standards and the test samples were diluted in PBS. To each well, a 100μl of the BCA working reagent was added, and after mixing the plate, was left to incubate at 37°C for 2h. The plate was then left to cool to room temperature and the absorbance of each sample was read at 595nm using a Titertek Multiscan MCC/340 microplate reader. The protein concentration of each sample was then calculated from the standard curve.

2.5.3. IMMUNOPRECIPITATION OF PROTEINS

Immunoprecipitation of proteins was carried out using Protein A/G agarose (Santa Cruz Biotechnology). Whole cell extracts, prepared using the phosphatase-free extraction buffer (Section 2.5.1), were left to incubate overnight at 4°C with gentle rolling with an antibody raised against the desired protein (2μg/ml). The resulting protein-antibody complex was then captured by the addition of Protein A/G agarose (20μl) with gentle rolling for 2h at 4°C. The beads, containing the captured immunocomplex, were collected by centrifugation at 13,000g, for 3min at 4°C and washed twice with phosphatase-free extraction buffer (without Triton X-100). At this point, the protocol differed according to the desired use of the immunoprecipitated protein. For Western blot analysis, the bead/immunocomplex pellet were resuspended in 50μl of 0.1M Glycine, pH 2.5, vortexed, and incubated with rolling for 10min at 4°C. The sample was then centrifuged at 9,000g (4°C) for 2min and the supernatant kept. To each sample 5μl of 1M Tris (pH 8.0) was added along with 30μl of SDS-PAGE gel loading buffer. The samples were boiled for 5min and used immediately for analysis by SDS-PAGE (Section 2.5.4). For the CK2 kinase assay (Section 2.6.1), the bead/immunocomplex pellet, after the extraction buffer washes was immediately incubated with the substrate mix.

2.5.4. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

During this study, SDS-PAGE was carried out using separating gels and stacking gels containing 10% (w/v) and 5% (w/v) acrylamide, respectively. For a 10% separating gel (10ml), 2.5ml of acrylamide:bisacrylamide (37.5:1) was added to 2.5ml of lower buffer (Table 2.7), whilst the 5% stacking gel (10ml) consisted of 1.25ml of acrylamide:bisacrylamide (37.5:1) in 2.5ml of upper

buffer (Table 2.7). Both gels also contained 100µl of 10% (w/v) APS and 10µl of TEMED and the volume of each was made up to 10ml with double distilled water.

Electrophoresis was carried out using a Mini-PROTEAN II slab electrophoresis Cell (Bio-Rad Laboratories) and was assembled according to the manufacturer's instructions. The separating gel was poured first, allowing a space of 1.5cm at the top for the stacking gel. Butan-1-ol was layered on top of the gel and the gel was left to polymerise for 30-40min. The butan-1-ol was then washed away using distilled water and the stacking gel was poured on top of the separating gel. The gel comb was inserted into the gel and the stacking gel was left to polymerise. After polymerisation of the gel, the comb was removed and the wells were washed with distilled water. The gel was then placed in the tank and the inner and outer chambers were filled with 1x running buffer (Table 2.7).

Prior to loading of the gel, 10-80µg of each whole cell extract sample was mixed with an equal volume of gel loading buffer (Table 2.7) and heated to 100°C for 2min. Once cooled the samples were loaded onto the gel. Onto the first lane of each gel, 10µl of the Amersham Rainbow protein size markers (Appendix II) were loaded to allow size estimation of the detected protein. Electrophoresis was carried out at 200V for 30-45min and the gel was then subjected to Western blotting (Section 2.5.4).

2.5.5. *WESTERN BLOTTING*

The electrophoretic transfer of proteins from the gel to a Immobilin™-P^{SQ} PVDF membrane was performed using a Bio-Rad Trans-Blot Electrophoretic Transfer Cell. Following SDS-PAGE (Section 2.5.3), the gel was removed and the stacking gel was carefully cut away. The gel was then left to soak for 15min in transfer buffer (Table 2.7). In addition to the gel, the PVDF membrane (which had been activated by soaking in methanol), Whatman 3MM paper (both pre-cut to the gel size) and the sponge pads of the transfer apparatus were also pre-soaked in transfer buffer. The PVDF membrane was then placed on the gel and sandwiched between the Whatman paper and the sponge pads, before being placed into the blotting cassette. The cassette was then subjected to electro-blotting in a tank containing transfer buffer (Table 2.7) at a temperature of 4°C. The gel was left to blot at a constant voltage of 100V for 1h or over-night at a constant voltage of 15V.

2.5.6. *IMMUNODETECTION OF PROTEINS*

Electrophoretic transfer of proteins was carried out as described in Section 2.5.4. Once the electro-blotting process was complete, the Rainbow markers were cut from the PVDF membrane and stored in aluminium foil. The membrane was removed from the blotting cassette and incubated in a blocking solution [TBS (Table 2.5); 0.1% (v/v) TWEEN-20, containing 5% or 10% (w/v) non-fat

dried milk] for 1h. The membrane was then washed three times, for 5min each, in TBS (Table 2.7) containing 0.1% (v/v) TWEEN-20 and left to incubate in blocking solution containing an appropriate dilution of the primary antibody for 1h. Certain primary antibodies required overnight incubation at 4°C, where the 5% (w/v) non-fat dried milk in the blocking solution was replaced by 5% (w/v) BSA. The membrane was then washed in TBS containing 0.1% (v/v) TWEEN-20 as before, and left to incubate in blocking solution containing the appropriate dilution of the second antibody-Horse Radish Peroxidase (HRP) conjugate. The membrane was then washed with TBS containing 0.1% (v/v) TWEEN-20 and detection of the membrane bound antigen-antibody complexes was carried out using Enhanced Chemi-luminescence (ECL) as specified by the ECL manufacturer's instructions (Amersham). The membrane was exposed to Kodak X-ray film in a light-proof X-ray cassette (Genetic Research Instrumentation) and developed using an automated developer (Gevamatic-60, AGFA). The expected protein size was then verified by aligning the Rainbow marker with the developed image.

2.5.7. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The cells used for the ELISA experiments in this study were the THP-1 cell line. Approximately $1x10^6$ cells were seeded into each well of a 6-well plate (Greiner) and differentiated with PMA. After 24h, the cells were treated with the appropriate ligand and inhibitors and left for a further 24h. To each well, 200µl of ELISA assay buffer (Table 2.8) was added to the medium and left to incubate, with swirling, for 10min at room temperature. An aliquot of medium (500µl) was then removed from each well and analysed by ELISA.

Each 96 well plate was coated with primary antibody, goat polyclonal ATAB atlantic anti-apoE (Inc Star) at a concentration of 250ng/100µl PBS/well and left to incubate over-night at 4°C. Unbound antibody was shaken away and 250µl of Blocking solution (Table 2.8) was added per well and left at 37°C for 1h, without shaking. The ELISA plate was washed three times with 300µl washing buffer (Table 2.8) and 50µl of SB biotinylated secondary antibody (GSK), prepared with 1% goat serum in assay buffer, was added to each well, followed by 50µl of apoE standards or samples. The standards used were human apoE serum (GSK) diluted in assay buffer to give a range of concentrations (160ng/ml, 80ng/ml, 40ng/ml, 20ng/ml, 10ng/ml, 5ng/ml). The plates were left at room temperature for 2h, with shaking and then washed four times with 300µl of washing buffer. To each well, 100µl of streptavidin-HRP complex (Amersham), prepared in assay buffer (1/2000) was added and left for 30min at room temperature with shaking. Each plate was washed three times with 300µl washing buffer and equal volumes of the Immunopure® TMB concentrate substrate solution (Pierce) and hydrogen peroxide solution were mixed and 100µl was added to

each well. The reaction was left for 30min at room temperature, with shaking and stopped with 100µl of 2M sulphuric acid. The absorbance was read at 450nm using a Titertek Multiscan MCC/340 microplate reader and the protein concentration of each sample was then calculated from the standard curve.

2.6. PROTEIN KINASE ACTIVITY ASSAYS

2.6.1. CASEIN KINASE 2 ASSAY

This method was derived from those used by Sung *et al.* (2001) and Lodie *et al.* (1997) with minor modifications. Whole cell extracts were isolated as described in Section 2.5.1 using the phosphatase-free whole cell extraction buffer and immunoprecipitation was carried out as described in Section 2.5.3. using the CK2 (α-subunit) antibody. The immunoprecipitates were recovered by centrifugation and the resulting pellet was washed twice using phosphatase-free whole cell extraction buffer, without the Triton X-100. The pellet was then resuspended in 25µl of kinase buffer (Table 2.9) to which 1µCi [γ- ^{32}P]-ATP was added. The kinase reaction was incubated for 15 min at 37°C and stopped by the addition of 10µl SDS-PAGE sample buffer. Samples were immediately subjected to SDS-PAGE using 15% (w/v) acrylamide gels. After electrophoresis, gels were fixed for 20 min in a solution containing 40% (v/v) methanol and 10% (v/v) acetic acid. These were washed with distilled water before being dried under vacuum and visualised by autoradiography.

2.6.2. AKT KINASE ASSAY

The Akt kinase assays were carried out using the Akt non-radioactive kinase assay kit (New England Biolabs) with only minor modification to the manufacturer's protocol. Whole cell extracts were isolated as described in Section 2.5.1 using the phosphatase-free whole cell extraction buffer. To 250µg of whole cell extract, 20µl of immobilised Akt antibody slurry was added and was left to incubate with gentle rocking for 3h, at 4°C. Following incubation, the mix was centrifuged for 30sec at 10,000g, and the resulting pellet was washed twice with 300µl of phosphatase-free whole cell extraction buffer, without Triton X-100 (Table 2.7) and twice with 1xAkt kinase buffer (Table 2.9). After the final wash, the pellet was resuspended in 40µl of 1xAkt kinase buffer supplemented with 200µM ATP and 1µg of GSK-3 fusion protein. This kinase mix was then incubated at 30°C for 30min. The kinase reaction was then terminated with 20µl SDS-PAGE sample buffer. The sample was vortexed and centrifuged at 10,000g for 2min. The supernatant was then boiled for 5min and loaded onto a SDS-PAGE gel. SDS-PAGE electrophoresis and Western Blotting were carried out

as described in sections 2.5.5 and 2.5.6, respectively and the immunodetection of proteins (Section 2.5.7) was carried out using the phospho GSK-3α/β (Ser21/9) antibody.

2.6.3. JNK KINASE ASSAY

The JNK kinase assays were carried out using the JNK non-radioactive kinase assay kit (New England Biolabs) with only minor modification to the manufacturer's protocol. Whole cell extracts were isolated as described in Section 2.5.1 using the phosphatase-free whole cell extraction buffer. To 250μg of whole cell extract, 20μl of c-jun fusion protein beads was added and was left to incubate overnight with gentle rocking, at 4°C. Following incubation, the mix was centrifuged for 30sec at 10,000g, and the resulting pellet was washed twice with 300μl of phosphatase-free whole cell extraction buffer, without Triton X-100 (Table 2.7) and twice with 1xJNK kinase buffer (Table 2.9). After the final wash the pellet was resuspended in 50μl 1xJNK kinase buffer supplemented with 100μM ATP. This kinase mix was then incubated at 30°C for 30min. The kinase reaction was then terminated with 25μl SDS-PAGE sample buffer and the supernatant was boiled for 5min and loaded onto a SDS-PAGE gel. SDS-PAGE electrophoresis and Western Blotting were carried out as described in sections 2.5.5 and 2.5.6, respectively and the immunodetection of proteins (Section 2.5.7) was carried out using the phospho c-jun (ser63) antibody.

CHAPTER THREE - STUDIES ON APOLIPOPROTEIN E PROMOTER ACTIVITY IN RESPONSE TO PMA

3.1. INTRODUCTION

As described previously in Chapter one, apoE is found to be expressed at high levels in the atherosclerotic plaque, where it is mainly associated with macrophage-derived foam cells (Rosenfeld et al., 1993; O'Brien et al., 1994). Macrophage apoE has several anti-atherogenic functions including key roles in the maintenance of macrophage lipid homeostasis, cellular cholesterol efflux and reverse cholesterol transport. Therefore, macrophage apoE represents an excellent target for therapy against atherosclerosis. Although it is known that the expression of apoE is induced during differentiation of monocytes into macrophages (Werb and Chin, 1983; Auwerx *et al.*, 1988; Menju *et al.*, 1989; Tajima *et al.*, 1995), the regulation of this process is poorly understood. A detailed understanding of how macrophage apoE expression is regulated would provide further information about factors involved in lesion development.

In order to investigate the regulation of apoE gene expression during the differentiation of monocytes into macrophages the human monocytic leukemia cell lines THP-1 (Tsuchiya *et al.*, 1980) and U937 (Sundstrom and Nilsson, 1976) were used as the model systems during this study. Both cell lines have previously been widely used as *in vitro* models for studying the mechanisms involved in macrophage differentiation and atherogenesis (Olsson *et al.*, 1983; Auwerx, 1991; Liu and Wu, 1992; Rival *et al.*, 2004; Fu and Borensztajn *et al.*, 2002) and several studies have shown similarities in the response of these cells with those observed in primary cultures of human monocyte-derived macrophages (Li and Hui, 1997; Frostegard *et al.*, 1990). Both cell lines can be considered as monoblasts as they represent a relatively immature cell of the monocyte-macrophage cell lineage (Abrink *et al.*, 1994). Although both these cell lines are blocked at certain steps in the differentiation process they can be induced to differentiate along the monocytic pathway by treatment with phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) [Tsuchiya *et al.*, 1982; Koren *et al.*, 1979; Abrink *et al.*, 1994; Auwerx, 1991; Ways *et al.*, 1987]. Phorbol esters mimic the physiological activator diacylglycerol and are agonists of the α-, δ-, ε-, ξ-isoforms of protein kinase C (PKC) (Schwende *et al.*, 1996), a family of 12 related serine/threonine kinases implicated in the regulation of cell proliferation, differentiation and other cellular functions (Parker *et al.*, 1989; Ono *et al.*, 1988). After PMA treatment, both cell lines differentiate into a macrophage-like cell, which resembles native monocyte-derived macrophages with regard to their morphological characteristics (Tsuchiya *et al.*, 1982; Auwerx, 1991; Larrick *et al.*, 1980), their membrane expression of antigens and receptors (Auwerx, 1991; Auwerx *et al.*, 1992; Larrick *et al.*, 1980), and their production of several secretory products, including apoE (Auwerx, 1991; Auwerx *et al.*, 1988; Tajima *et al.*, 1985).

ApoE expression can be regulated at a number of levels, with transcriptional control being responsible, at least in part, for the induction seen during macrophage differentiation (Auwerx *et al.*, 1988), in response to oxysterol ligands (Laffitte *et al.*, 2001b) and cholesterol loading (Mazzone *et al.*, 1989). An understanding of the mechanisms involved in the transcriptional regulation of apoE during differentiation is lacking and there has only been a single, rather limited and partly inconclusive study (Basheeruddin *et al.*, 1994) that aimed to identify the sequence elements that are involved in transcriptional activation during macrophage differentiation. Basheeruddin *et al.* (1994), demonstrated that the –623 to –447 region of the apoE promoter was necessary for the induction of apoE transcription during PMA-induced differentiation of monocytes into macrophages. This –623 to –447 region contained a consensus sequence for the AP-1 family of transcription factors. The binding of AP-1 members to this sequence was induced in response to exposure of THP-1 cells to PMA and mutation of this site produced a significant reduction in mediator-induced gene activity. Unfortunately, there are several limitations associated with this study; firstly the analysis of the regulatory elements was restricted solely to the AP-1 site with no experiments for sites of other transcription factors that may be present in this region. This point is particularly important as mutation of the AP-1 site produced only a slightly reduced response in apoE expression, suggesting that in the native gene, the AP-1 element functions in co-operation with other regulatory elements to induce the endogenous gene during PMA-induced differentiation. Secondly, the analysis of the interaction of nuclear proteins with the AP-1 response element was restricted to competition gel retardation experiments, with no information on the precise nature of the interacting factors (i.e. supershift experiments) and the mechanisms that are involved in their regulation. Also, gel retardation assays indicated that mutation of the AP-1 site caused the binding of an additional factor, the abundance of which was increased in differentiated cells, indicating that this additional DNA-protein complex, which could not be competed out by an AP-1 site oligonucleotide, was responsible for the reduced response of the mutated AP-1 construct. Another problem of this study was that all the experiments were carried out on THP-1 cells, therefore any results obtained may be a peculiar property of this cell line.

In light of the limitations of the study by Basheeruddin *et al.*, (1994), the aim of the work presented in this chapter was to systematically optimise a transient transfection method in order to re-evaluate the regulatory sequences in the apoE promoter that are required for increased apoE expression during macrophage differentiation. Also, whilst these studies were being carried out, work in other laboratories using transgenic mice showed that two distal enhancers, ME1 and ME2 were important in the macrophage- and adipocyte-specific expression of the apoE gene (Shih *et al.*, 2000). Therefore, further extension studies investigated the importance of these enhancer regions in macrophage differentiation.

3.2. RESULTS

3.2.1. MORPHOLOGICAL ANALYSIS OF THP-1 CELLS DURING PMA-INDUCED DIFFERENTIATION INTO MACROPHAGES

Previous studies have shown that apoE mRNA and protein expression are induced during PMA-induced differentiation of THP-1 monocytes into macrophages (Auwerx *et al.*, 1988; Tajima *et al.*, 1985; Menju *et al.*, 1989). It was therefore first decided to investigate whether this was also the case for the THP-1 cells present in our laboratory. Before investigating the apoE mRNA expression profile, the morphology of the cells was investigated during PMA-induced differentiation. PMA was used at a concentration of 0.16μM as this concentration has previously been shown to be the lowest concentration required to induce the complete differentiation of THP-1 cells (Auwerx *et al.*, 1988). As shown in Figure 3.1, after treatment with PMA, the THP-1 cells began to differentiate and after 48h, 95% of all cells present were adherent to the tissue culture plastic. In addition to cell adhesion and the arrest of proliferation, differentiation was accompanied by marked changes in the morphology of the cells as the cells become irregular and flattened in shape, and demonstrated a 'stellate' phenotype.

FIGURE 3.1. EFFECT OF PMA ON THE MORPHOLOGY OF THP-1 CELLS

THP-1 cells were grown in 10% (v/v) HI-FCS and treated with PMA (0.16μM). The cells were observed using phase contrast microscopy and photographed at the times indicated.

3.2.2. ANALYSIS OF APOE MRNA AND PROTEIN EXPRESSION DURING PMA-INDUCED DIFFERENTIATION OF THP-1 MONOCYTES INTO MACROPHAGES

Analysis of apoE mRNA expression was carried out in order to reproduce the previously noted mRNA induction during differentiation (Auwerx *et al.*, 1988; Menju *et al.*, 1989). Previous studies demonstrated that the increase in apoE mRNA was linear with time after the addition of PMA, reaching a maximum at 48h. RT-PCR was employed in this study to examine the expression of apoE mRNA during PMA-induced differentiation of THP-1 monocytes into macrophages. For this, THP-1 cells were grown in 10% (v/v) HI-FCS and treated for 24 and 48h with 0.16μM PMA. RNA was then isolated and the generation of cDNA from total cellular RNA was performed as described in Section 2.4.4. For comparative purposes, in addition to differentiated THP-1 cells (denoted 24h+ and 48h+), RNA was also isolated from monocytes (denoted 24h- and 48h-) at each time-point along with monocytes from the start of the experiment (0h). The quality of RNA was assessed by agarose gel electrophoresis (Figure 3.2a) and with all RNA used in this study the expected ratio of 2:1(28s:18s) was obtained, confirming that the RNA had not been degraded. PCR was carried out using conditions (Table 2.11) and primers (Table 2.10) that were based on previous studies in the laboratory. PCR using primers against GAPDH (Table 2.10) were also included in parallel in order to provide a control for the amount of RNA. The PCR conditions for GAPDH (Table 2.11) had also been established previously in the laboratory, where it was also shown that its mRNA expression remains constant through macrophage differentiation. As shown in Figure 3.2b, apoE mRNA expression was increased dramatically after PMA-induced differentiation, with the greatest induction occurring at 48h. This marked induction of apoE is consistent with previous studies (Auwerx *et al.*, 1988; Menju *et al.*, 1989).

Western blot analysis was carried out to evaluate whether the induction of apoE mRNA expression during differentiation of THP-1 cells was also shown at the level of protein. Previous studies have demonstrated that the time course of protein synthesis showed a similar profile to that of mRNA, with maximal induction occurring at 48h (Menju *et al.*, 1989). For this analysis, whole cell extracts were isolated from THP-1 cells treated with 0.16μM PMA and untreated cells as described in Section 2.5.1. Of each sample 20μg of total protein was subjected to SDS-PAGE (Section 2.5.4) and then transferred to a PVDF membrane (Section 2.5.5). The membrane was then incubated with an anti-apoE primary antibody (Biogenesis) and immunodetection was carried out using the appropriate secondary antibody and the ECL detection system (Amersham) as described in Section 2.5.6. The results of this experiment are presented in Figure 3.2c, and it can be seen that the level of apoE protein was also induced dramatically during macrophage differentiation.

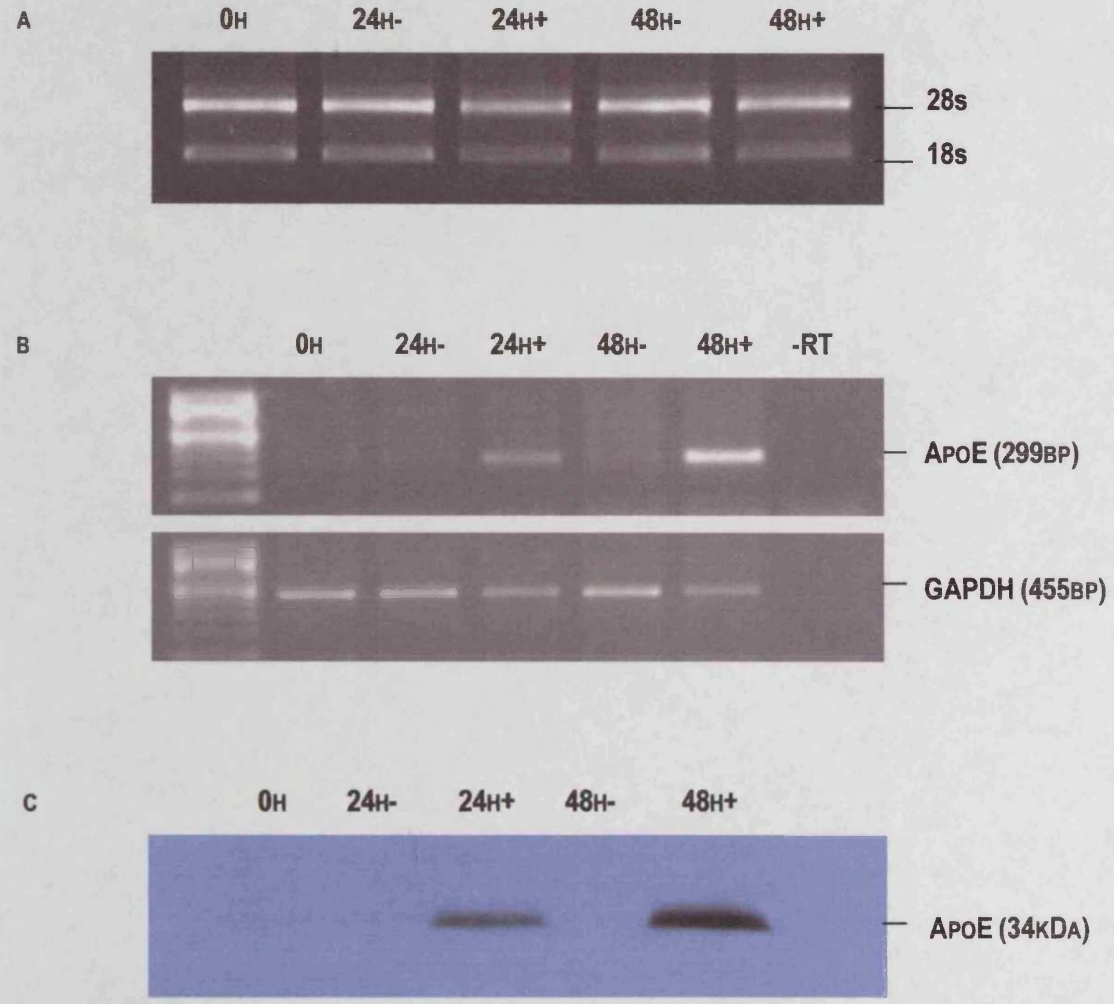


FIGURE 3.2. EFFECT OF PMA-INDUCED DIFFERENTIATION ON APOE MRNA AND PROTEIN LEVELS IN THP-1 CELLS

THP-1 cells grown in 10% (v/v) HI-FCS were exposed to PMA (0.16μM) for the indicated times. For comparative purposes, sample isolation was carried out from both monocytes (denoted 24h- and 48h-) and differentiated macrophages (denoted 24h+ and 48h+) at each time-point along with monocytes at the start of the experiment (0h). ***a*** *Total RNA was extracted and the integrity was assessed by agarose gel electrophoresis (1% [w/v]).* ***b*** *RT-PCR was carried out using apoE- and GAPDH-specific primers. Each set of RT-PCR reactions included a negative control, where a reverse transcriptase reaction contained all the components except for the reverse transcriptase enzyme (denoted –RT). Following amplification, the products were subjected to electrophoresis using a 1.5% (w/v) agarose gel and product size was confirmed using the 100bp molecular weight marker (Appendix I).* ***c*** *Western blot analysis of whole cell extracts (20μg) isolated from THP-1 cells. Blotted membranes were incubated with anti-apoE antibody (goat polyclonal IgG) and detected using an anti-goat horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. All results are representative of three separate experiments.*

3.2.3. TRANSFECTION ANALYSIS OF APOE PROMOTER ACTIVITY DURING PMA-INDUCED DIFFERENTIATION OF MONOCYTES INTO MACROPHAGES

Having established that both the apoE mRNA levels and protein content are induced during macrophage differentiation, the promoter activity of apoE was investigated using transient transfection assays. For this study, a DNA construct, containing the –3000/+86 promoter fragment of apoE linked to the luciferase gene in the pGL3 vector (a kind gift from Dr. T. Mazzone, of Rush Medical College, Chicago [Figure 3.3]), was transfected into THP-1 and U937 cell lines, both of which have been used extensively to investigate the regulation of genes during macrophage differentiation (Olsson *et al.*, 1983; Auwerx, 1991; Liu and Wu, 1992; Rival *et al.*, 2004; Fu and Borensztajn et al., 2002). Transfection experiments were carried out in both THP-1 and U937 cells to ensure that any results obtained were not a peculiar property of the cell line used.

FIGURE 3.3. SCHEMATIC REPRESENTATION OF THE APOE-3000/+86 PGL3 PROMOTER CONSTRUCT

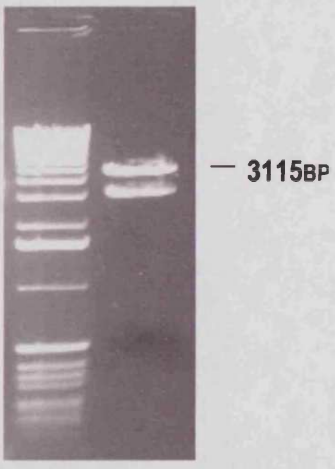


FIGURE 3.4. ANALYTICAL DIGEST OF THE APOE-3000/+86-PGL3 PLASMID.

The apoE-3000/+86 pGL3 promoter construct was digested with KpnI and Hind III restriction enzymes, which released the 3115bp apoE promoter fragment from pGL3 vector. The digest products were size-fractionated by electrophoresis on a 1% (w/v) agarose gel along with the 1kb molecular weight marker (Appendix I).

The activity of the apoE promoter was monitored by transient transfection of monocytes using the polycation transfection reagent, Superfect™ (Qiagen). The initial analysis of promoter activity was assessed using the human monocytic cell line, THP-1. The apoE-3000/+86 pGL3 promoter construct (2μg) was added to the cells, along with the internal transfection efficiency control plasmid CMVβ-galactosidase (0.5μg) [Appendix III]. RSV-luciferase (De wet et al, 1987) was used as a positive control and pGL3-Basic vector (Appendix IV) was used as the negative control. All DNA used for transfection experiments was purified using the Concert™ plasmid purification kit (Gibco) and the purity and quality of the DNA preparation was checked by analytical digests using the appropriate restriction enzymes and subjected to fractionation using agarose gel electrophoresis (Figure 3.4). Following transfection, the cells were treated with 1μM PMA. This higher concentration of PMA was used in the initial transfection experiments as this concentration had been used in previous studies in the laboratory, involved in the detailed dissection of the LPL promoter in U937 cells (Hughes *et al.*, 2002). After PMA treatment, cells were harvested at the indicated time-points (Figure 3.5) and the luciferase and β-galactosidase activity was determined as described in Section 2.3.11 and 2.3.12, respectively. Each transfection was carried out in triplicate and each time-point had a control where the cells were untreated.

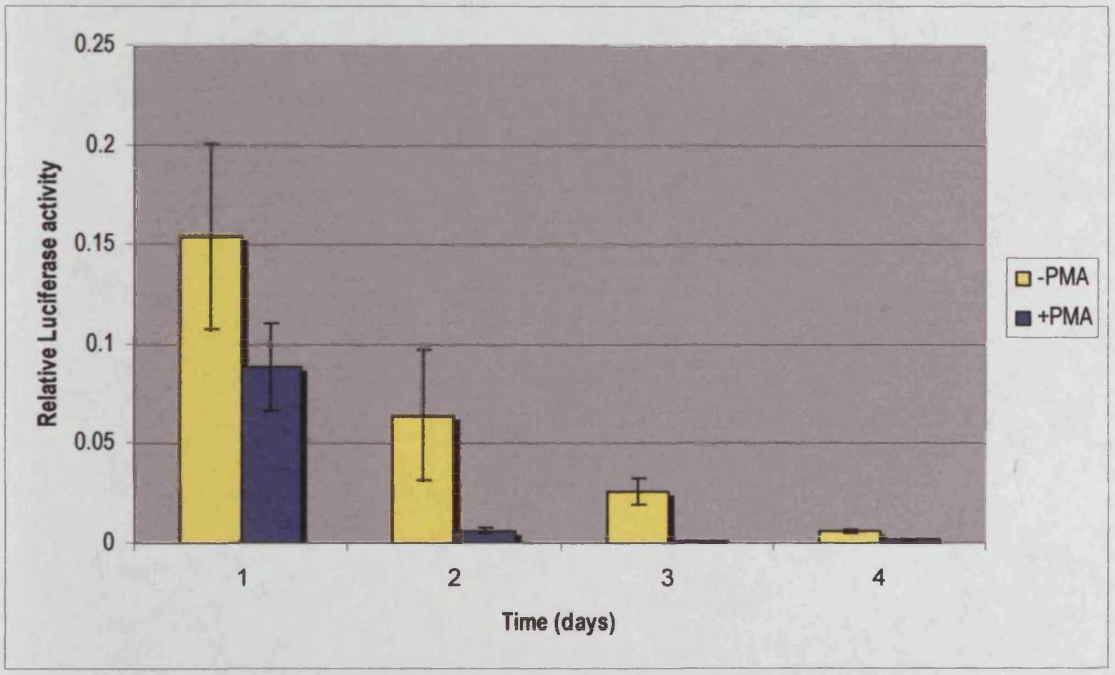


FIGURE 3.5. ANALYSIS OF APOE PROMOTER ACTIVITY DURING MACROPHAGE DIFFERENTIATION.

Transfection was performed in THP-1 cells as described in Section 2.3.5.1. The cells were transfected with the apoE-3000/+86 reporter construct in medium containing 3% (v/v) HI-FCS, and incubated with 1μM PMA. The luciferase activity was normalised to the β-galactosidase value and expressed as relative luciferase activity. Each experiment was carried out in triplicate and contained a control (untreated cells) for each time-point.

As shown in Figure 3.5, the relative luciferase activity was greater in the untreated cells at each time-point, indicating that apoE promoter activity did not increase during the differentiation process. These results were therefore contradictory to those obtained by Basheeruddin *et al.* (1994), which demonstrated that the apoE promoter activity increased with PMA treatment. One explanation for the higher apoE promoter activity in untreated THP-1 cells may be due to the use of the transfection reagent, Superfect™. Therefore, the transfection procedure was repeated using Effectene™ (Qiagen), a lipid-based reagent that has also been extensively used in transfection-based studies in macrophages (Shanmugam *et al.*, 2003; Kikuchi *et al.*, 2002; Schwarz and Murphy, 2001; Nishiguchi *et al.*, 2001). All other transfection conditions were kept constant, with the exception of the amount of DNA used per experiment. According to the procedures supplied by the manufacturer, transfection using the Effectene™ reagent (Section 2.3.5.2) only required 0.4μg of the apoE-3000/+86-pGL3 construct and 0.2μg of the CMVβ-galactosidase control plasmid. The cells were treated with 1μM PMA after transfection and all assays were carried out as specified in Section 2.3.11 and 2.3.12.

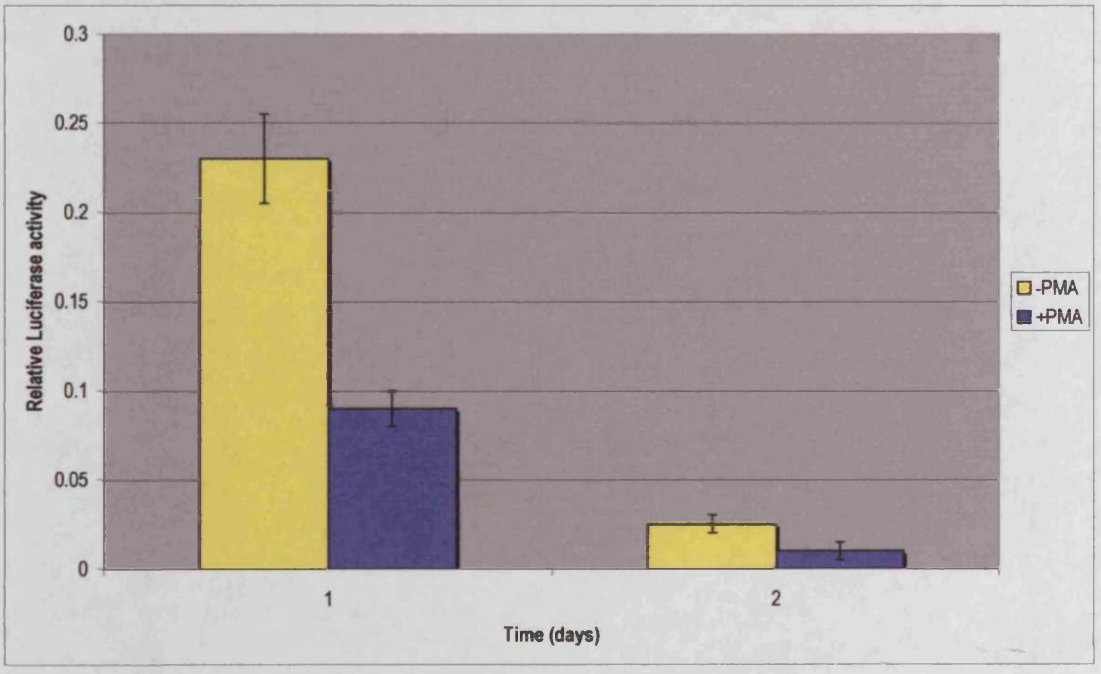


FIGURE 3.6. ANALYSIS OF APOE PROMOTER ACTIVITY DURING MACROPHAGE DIFFERENTIATION.

Transfection was performed in THP-1 cells as described in Section 2.3.5.2. The cells were transfected with the apoE-3000/+86 reporter construct in medium containing 3% (v/v) HI-FCS, and incubated with 1μM PMA. The luciferase activity was normalised to the β-galactosidase value and expressed as relative luciferase activity. Each experiment was carried out in triplicate and contained a control (untreated cells) for each time-point.

The results obtained demonstrated that the transfection reagent was not responsible for the higher apoE promoter activity in monocytes compared to macrophages as transfection with the Effectene™ reagent gave the same results as those obtained with the Superfect™ (Figure 3.6). The transfection was then carried out using the U937 cell line to ensure that the results obtained were not due to the THP-1 cell line. The transfection of the U937 cell line was carried out using Superfect™ as this reagent had been used successfully to dissect regulatory regions in the LPL gene promoter in this cell line (Hughes et al., 2002). All transfection conditions were identical to that of the THP-1 cell line, and each experiment was carried out in triplicate. The results (Figure 3.7) were consistent with those using THP-1 cells, as the relative luciferase activity was much greater in the control (untreated) cells than that in PMA-treated, differentiated cells.

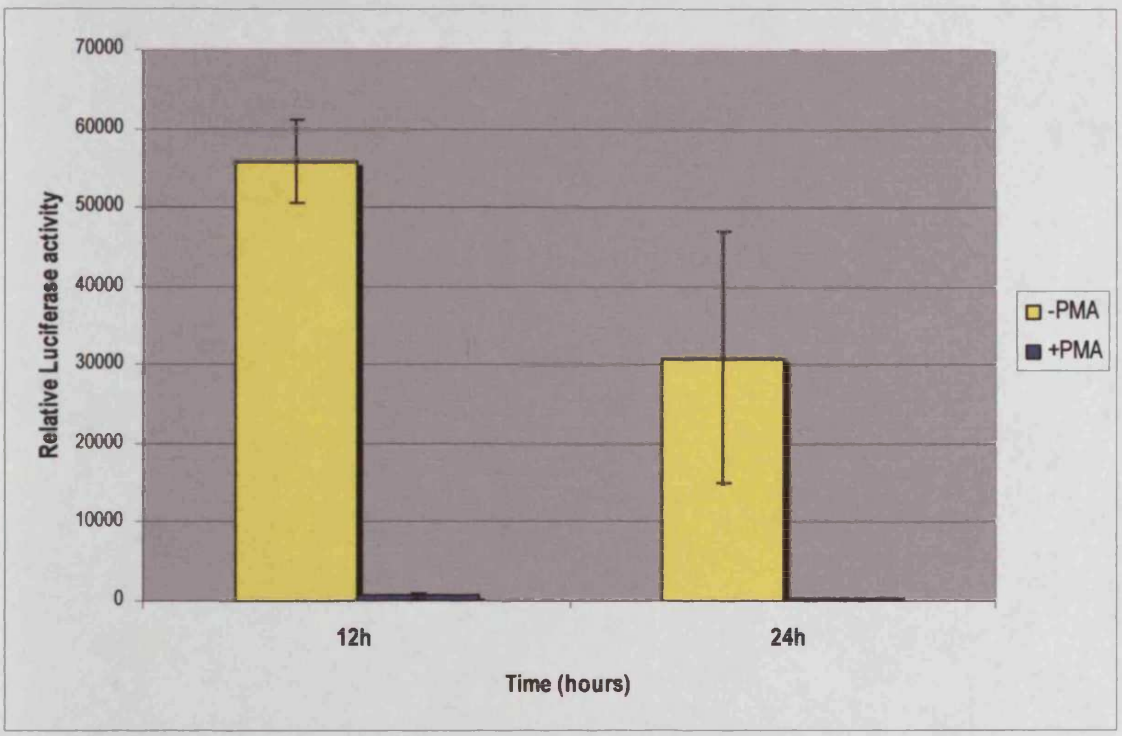


FIGURE 3.7. ANALYSIS OF APOE PROMOTER ACTIVITY DURING MACROPHAGE DIFFERENTIATION.

Transfection of U937 cells was carried out as specified in Section 2.3.5.1. The cells were transfected with the apoE-3000/+86 reporter construct in medium containing 3% (v/v) HI-FCS, and incubated with 1μM PMA. The luciferase activity was normalised to the β-galactosidase value and expressed as relative luciferase activity. Each experiment was carried out in triplicate and contained a control (untreated cells) for each time-point.

In order to ensure that the transfection results were not due to the concentration of PMA used, U937 cells transfected with the apoE-3000/+86 promoter construct were treated with 0, 0.5 and 1μM PMA for 24h. The cells were transfected using Superfect™, as specified above, and harvested as normal. The results demonstrated that varying the PMA concentration used to differentiate the cells had no effect on the apoE promoter activity, as the untreated cells still

showed a greater activity (Figure 3.8). Although the result of this experiment suggested that the observed results from the transfections were independent of the concentration of PMA used, all subsequent experiments were carried out using a PMA concentration of 0.32μM, as used by Basheeruddin *et al.* (1994), as this lower concentration of PMA was found to be sufficient to induce macrophage differentiation.

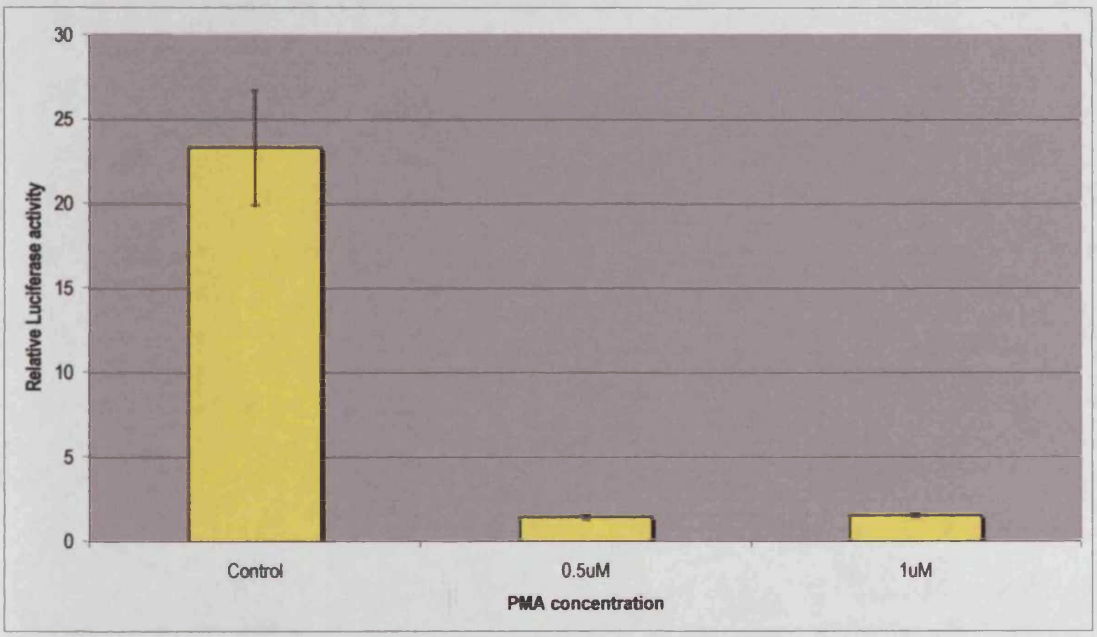


FIGURE 3.8. EFFECT OF PMA CONCENTRATION ON APOE PROMOTER ACTIVITY IN U937 CELLS

Transfection of U937 cells was carried out as specified in Section 2.3.5.1. The cells were transfected with the apoE-3000/+86 reporter construct in medium containing 3% (v/v) HI-FCS, and incubated with various concentrations of PMA. The luciferase activity was normalised to protein concentration and expressed as relative luciferase activity. Each experiment was carried out in triplicate and the control refers to untreated cells.

The concentration of serum in which transfection assays are carried out, 3% (v/v) HI-FCS, was based on previous extensive optimisation experiments in the laboratory (T.R. Hughes, personal communication). Factors present in serum are known to modulate the activity of several promoters, therefore, in order to ensure that the concentration of serum used was not the cause of the results obtained, THP-1 cells were transfected in medium containing 10% (v/v) and 3% (v/v) HI-FCS, respectively. As shown in Figure 3.9, the promoter activity was regulated marginally by serum concentration, with that from cells incubated in 3% (v/v) HI-FCS being approximately two-fold higher than that in 10% (v/v) HI-FCS. However, the previously noted higher apoE activity in undifferentiated cells was independent of serum concentration, being seen at both 3% (v/v) FCS and 10% (v/v) FCS. All subsequent transfections were carried out in medium containing 10% FCS

in order to limit the variables that may be affecting the activity of the apoE promoter during macrophage differentiation.

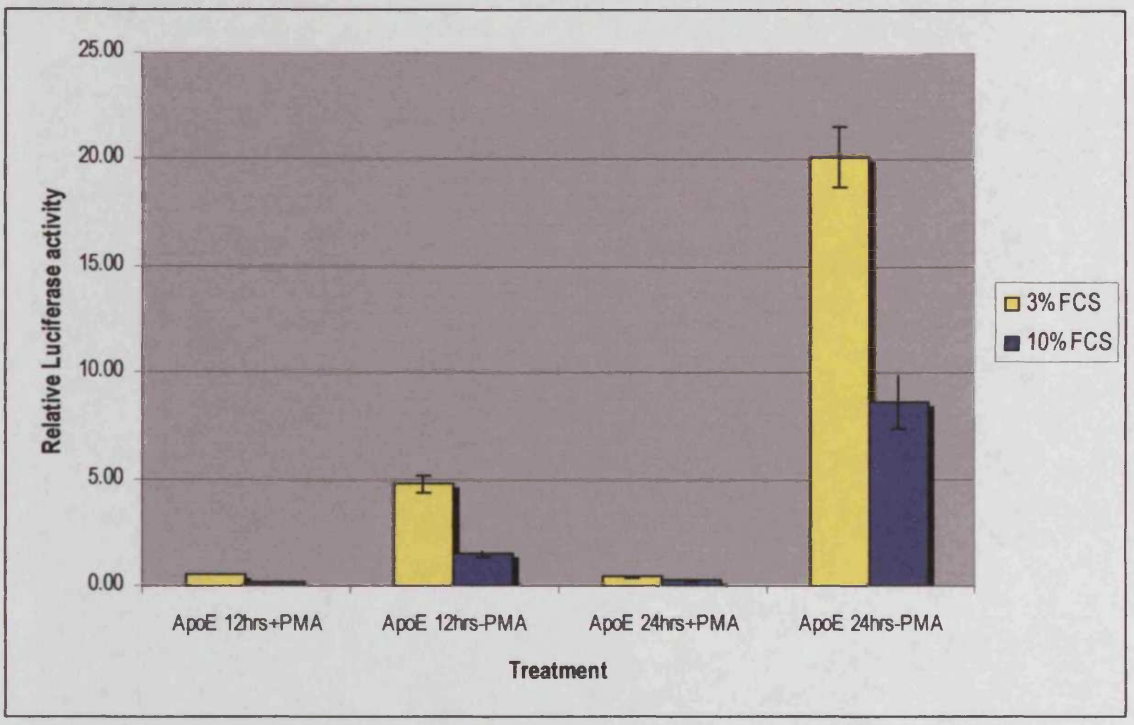


FIGURE 3.9. EFFECT OF FCS ON THE PROMOTER ACTIVITY OF APOE DURING MACROPHAGE DIFFERENTIATION.

THP-1 cells were transfected with apoE-3000/+86-pGL3 as described in Section 2.3.3.2. The cells were transfected in medium containing either 3% (v/v) or 10% (v/v) HI-FCS. The luciferase activity was normalised to protein concentration, and the values are expressed as relative luciferase activity. Each experiment was carried out in triplicate and contained a control (untreated cells) for each time-point.

Initially the aim of this project was the analysis of the regulatory sequences in the apoE promoter that are required for activation during macrophage differentiation. The constant higher activity in undifferentiated cells in all of the transfection experiments in this project raised further doubts on the previously published study by Basheeruddin *et al.* (1994), on the promoter region of apoE. In addition, these results have raised questions about the ability of the apoE gene promoter to be activated during macrophage differentiation. Recent studies in transgenic mice have suggested that the apoE promoter lacks sufficient information for transcription during differentiation, and that the key regulatory elements for expression in macrophages in transgenic mice lie in the two enhancer regions, ME1 and ME2 (Laffitte *et al.*, 2001b). In order to compare the ability of the apoE-3000/+86 promoter construct to activate apoE expression with that of the ME1 and ME2 enhancers, three other apoE-luciferase DNA constructs were therefore analysed. In addition to the apoE-3000/+86 promoter construct, the THP-1 cell line was transfected with a reporter construct containing only the –890 to +93 promoter region (pGL-890) and constructs containing the ME1 or

ME2 upstream of this apoE promoter sequence (pGL-890-ME1 and pGL-890-ME2). All three DNA constructs (Figure 3.10) were kind gifts from Dr B. Laffitte, of University of California, Los Angeles and were transfected into the cells using Effectene™ (Qiagen). After transfection, the cells were treated with 0.32μM PMA and left to incubate for 12 and 24 hours. Cell extracts were prepared and assays were carried out as specified in Section 2.3.11 and 2.3.12. As shown in Figure 3.11, the activity of the apoE-3000/+86 construct was markedly less than that of pGL-890-ME1 and –ME2. However, surprisingly, the shorter, pGL-890 promoter construct which was transfected for comparative purposes showed similar activity to that of the enhancer constructs, which further raises the question of whether the higher activity of the pGL-890-ME1 and –ME2 constructs is actually due to the enhancer sequences or the promoter fragment present. Although these results demonstrate the difference in the activation levels of the different constructs, the previously noted increased activity in untreated cells remained consistent, especially at the 24h time-point. It is interesting to note that the level of apoE promoter activity in untreated and PMA-treated cells was similar at 12h.

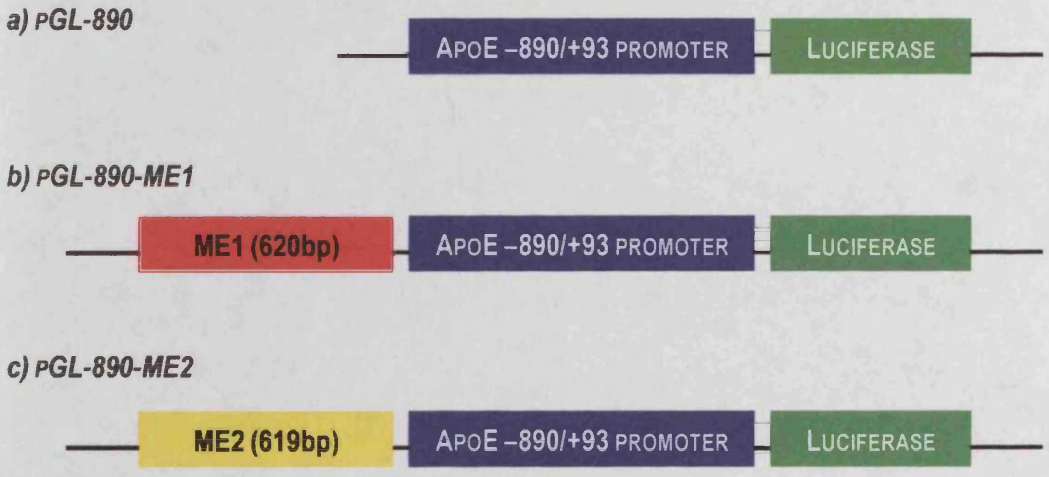


FIGURE 3.10. SCHEMATIC REPRESENTATION OF THE APOE PROMOTER AND ENHANCER DNA CONSTRUCTS USED FOR THE TRANSFECTION OF THP-1 CELLS.

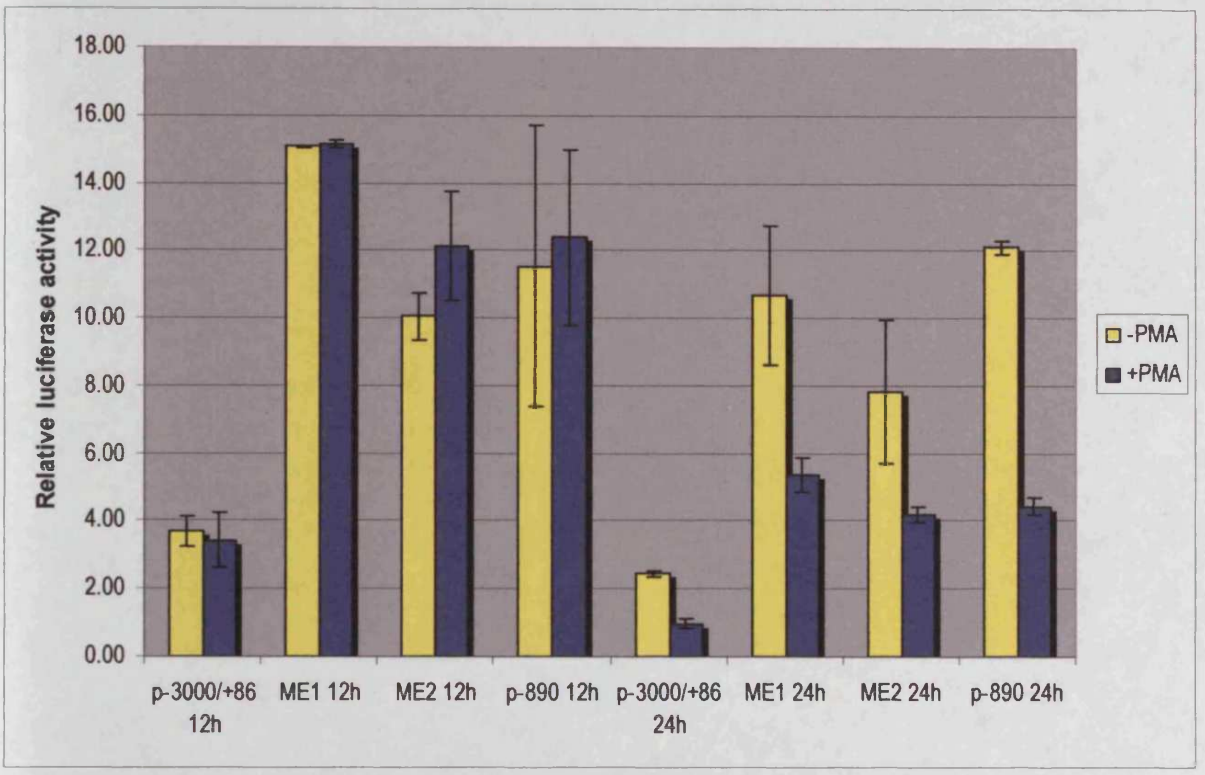


FIGURE 3.11. COMPARISON OF THE ACTIVITY OF THE DIFFERENT APOE PROMOTER AND ENHANCER SEQUENCES DURING MACROPHAGE DIFFERENTIATION

THP-1 cells were transfected as described in Section 2.3.5.2 and treated with 0.32μM PMA. The cells were transfected in 10% (v/v) HI-FCS and harvested at the indicated time-points. The luciferase activity was normalised with protein concentration, and values are expressed as relative luciferase activity. Each experiment was carried out in triplicate and contained a control (untreated cells) for each time-point.

In order to ensure that the increased activity of the apoE promoter in untreated monocytes was specific to this gene and not due to the cellular system, a promoter construct for the LPL gene, -1824/+188 LPL (Figure 3.12) [Gimble *et al.*, 1989], whose activity is known to be induced during differentiation was transfected into the U937 cell line. In addition to the LPL construct, the apoE-3000/+86-pGL3 and pGL-890-ME2 constructs, were transfected. Transfection was carried out using the Superfect™ transfection reagent and the cells were treated with 0.32μM PMA. The transfection was carried out in 3% (v/v) FCS because previous studies had shown this to be optimal for the LPL promoter (T.R. Hughes, personal communication).

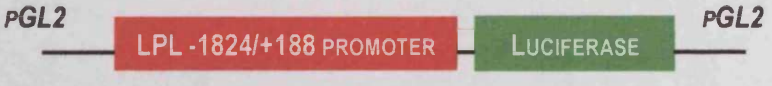
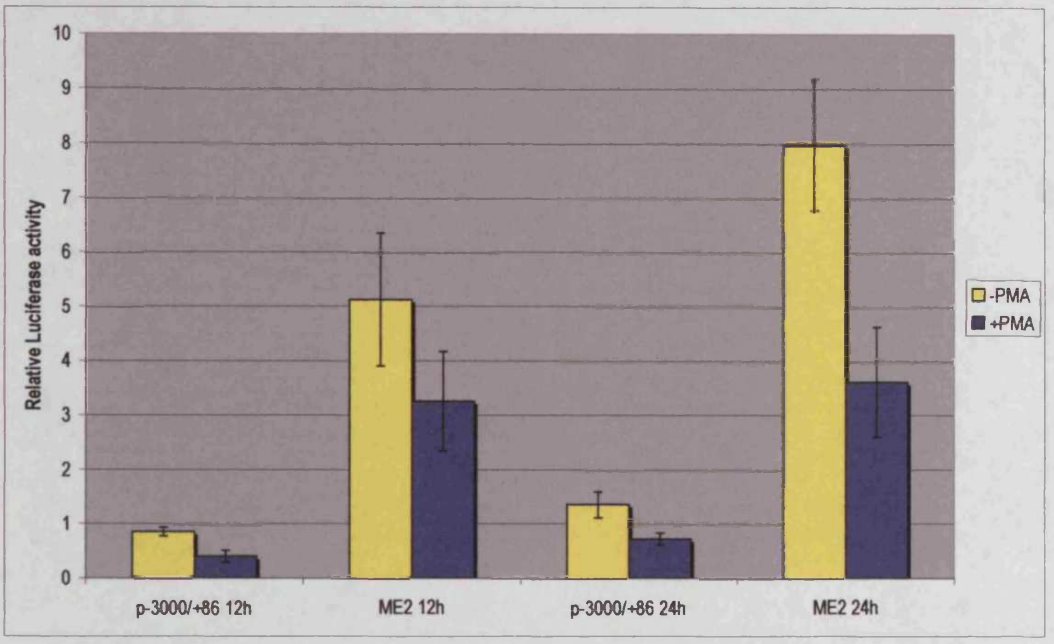


FIGURE 3.12. SCHEMATIC REPRESENTATION OF THE LPL -1824/+188 DNA PROMOTER CONSTRUCT USED FOR THE TRANSFECTION OF THP-1 CELLS.

A



B

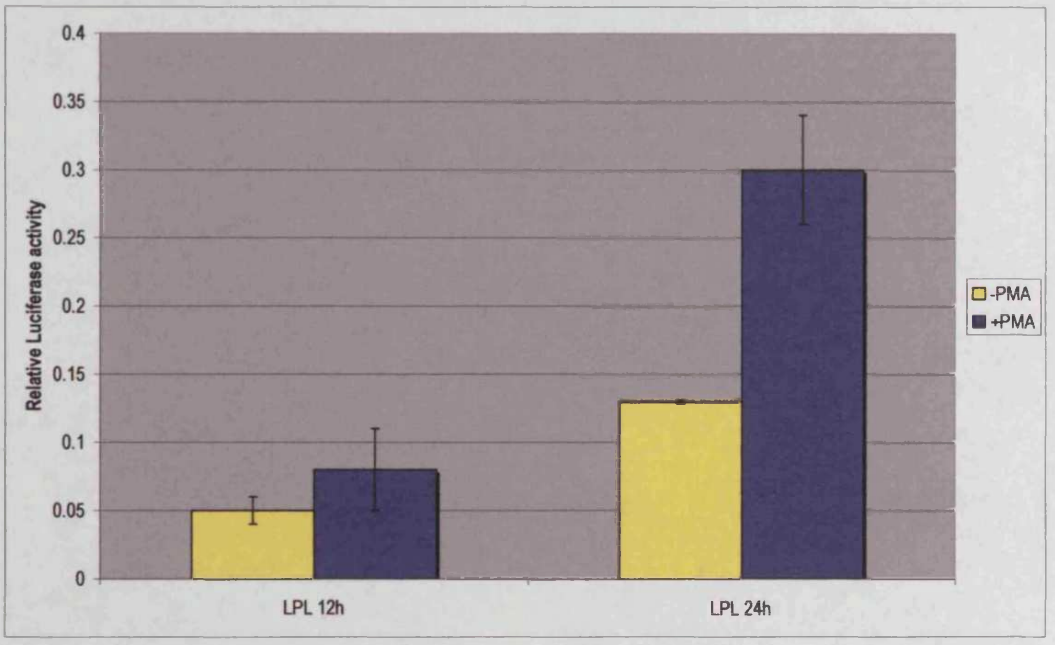


FIGURE 3.13. COMPARISON OF THE ACTIVITY OF THE LPL -1824/+188 PROMOTER CONSTRUCT WITH THE APOE PROMOTER AND ENHANCER SEQUENCES DURING MACROPHAGE DIFFERENTIATION

U937 cells were transfected as described in Section 2.3.5.1 and treated with 0.32μM PMA. The cells were transfected in 3% (v/v) HI-FCS and harvested at the indicated time-points. The luciferase activity was normalised with protein concentration, and values are expressed as relative luciferase activity. Each experiment was carried out in triplicate and contained a control (untreated cells) for each time-point.

The results of the transfection showed that the cellular system was working, as the activity of the LPL promoter was higher in cells treated with PMA (Figure 3.13b), although the apoE promoter activity was still greater in the untreated cells at the 24h time-point (Figure 3.13a). Once again, at the 12h time-point, the relative luciferase activity for the apoE constructs showed little or no difference between the treated and untreated cells. This similarity in apoE expression at 12 hours raised the question of whether transcriptional activation of apoE was an earlier event, as a previous study by Basheeruddin *et al.* (1992) demonstrated that transcriptional induction of apoE could be seen at 6h. Therefore a time-course experiment was carried out. The THP-1 cell line was transfected with the pGL-890 and pGL-890-ME1 constructs using Effectene™ (Qiagen) and harvested after 1, 3, 6, and 12 hours. The cells were transfected in 10% HI-FCS and treated with 0.32μM PMA. The results at 1 and 3 hours demonstrated that no apoE promoter activity was detectable (data not shown). After 6 hours there was a slight activity, with the highest activity seen at 12 hours (Figure 3.13), where the activity in the untreated cells was marginally higher than the differentiated cells.

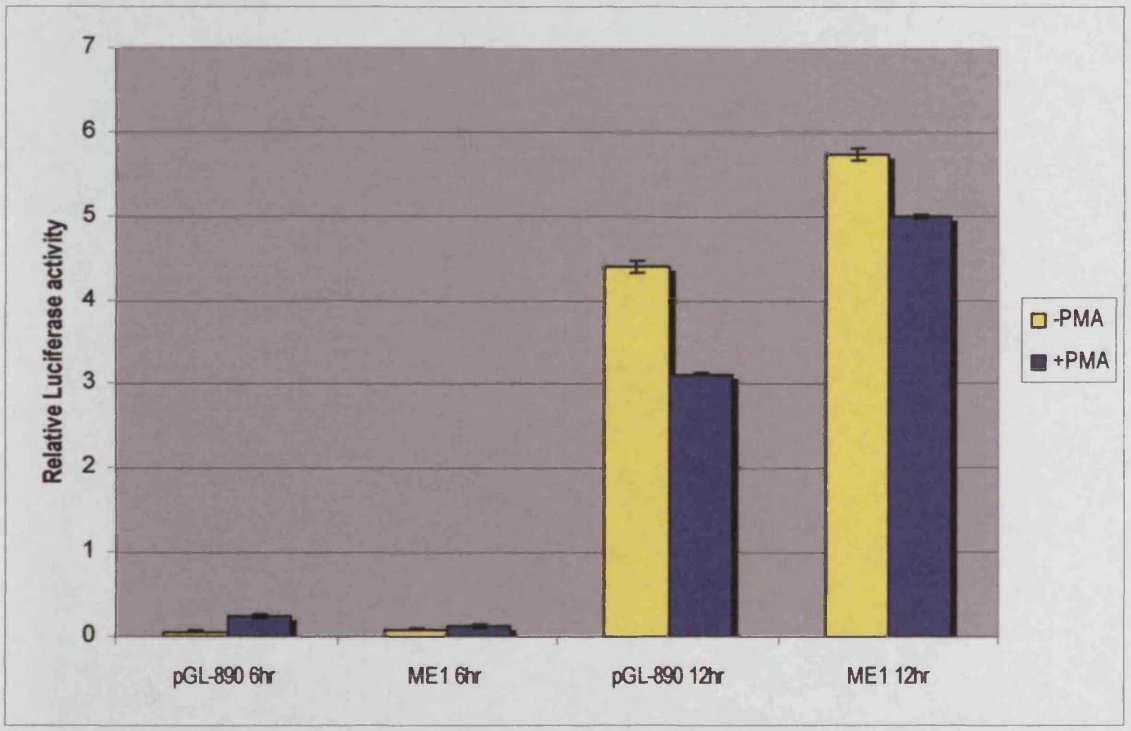


FIGURE 3.13. TIME-COURSE RESPONSE OF APOE PROMOTER ACTIVITY DURING DIFFERENTIATION

THP-1 cells were transfected as described in Section 2.3.5.2 and treated with 0.32μM PMA. The cells were transfected in 10% (v/v) HI-FCS and harvested at the indicated time-points. The luciferase activity was normalised with protein concentration and values are expressed as relative luciferase activity. Each experiment was carried out in triplicate and contained a control (untreated cells) for each time-point.

The final two transfections were carried out using the THP-1 cell line. The first transfection was carried out using a slightly modified transfection protocol. The cells were transfected using Effectene™ as described in Section 2.3.3.2 and then incubated for 16h with the DNA before the addition of 0.32μM PMA. This alteration in the protocol was to ensure that PMA was not affecting the uptake of DNA by the cells. Although the results demonstrated a decrease in the difference of apoE promoter activity between treated and untreated cells (data not shown), the increase in apoE promoter activity seen by Basheeruddin *et al.* (1994) and by mRNA expression studies was not apparent.

The final transfection in the monocytic cell line THP-1, was carried out using the DEAE-dextran method as described in Section 2.3.5.3. DEAE-dextran was employed as a transfection method as it has previously been used extensively for the transfection of macrophages (Wu *et al.*, 1990; Rupprecht and Coleman, 1991; Mack *et al.*, 1998). The cells were transfected with the apoE-3000/+86-pGL construct and treated with 0.32μM PMA. The action of TNFα (1000U/ml) on the apoE promoter was also investigated for comparative purposes as apoE mRNA expression has previously been shown to be induced by this cytokine (Duan *et al.*, 1995), therefore this experiment would also demonstrate whether other aspects of promoter regulation are functioning as normal. Altering the method of transfection made no difference to the activity of apoE in PMA-treated cells, as it was markedly less than that seen in the control (untreated) cells (Figure 3.14). As expected, the activity of apoE in cells treated with TNFα was increased nearly two-fold, which is similar to that shown previously by Duan *et al.* (1995), thereby indicating that the cellular system was suitable for the analysis of certain aspects of apoE promoter activity.

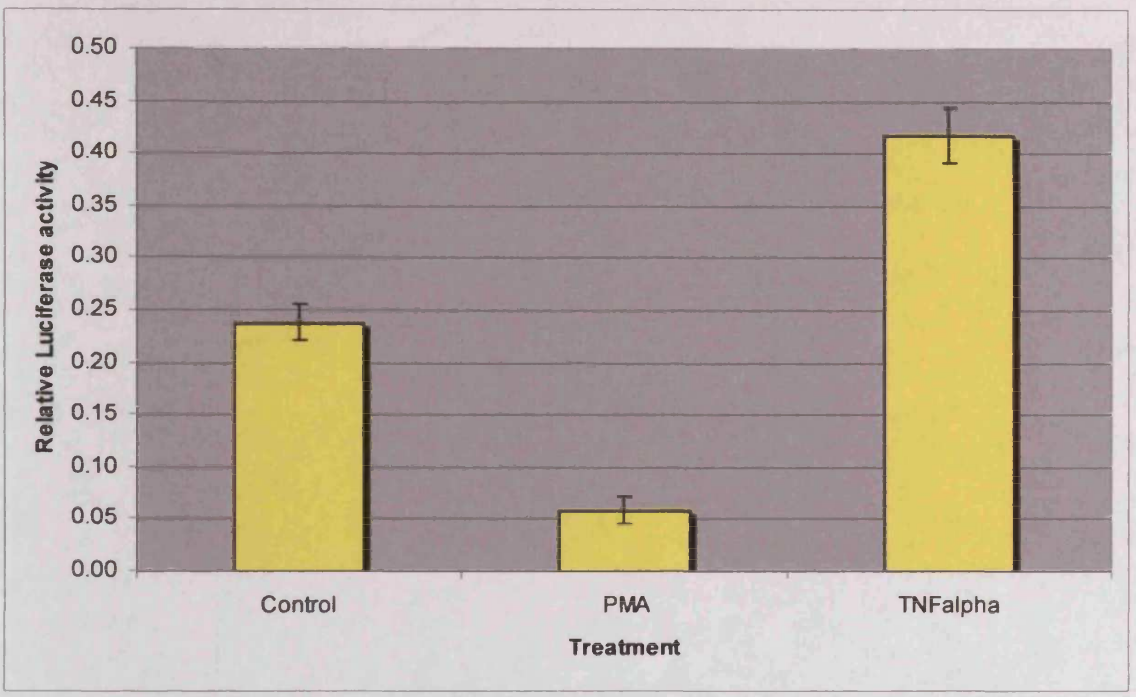


FIGURE 3.14. EFFECT OF DIFFERENT MEDIATORS ON THE ACTIVITY OF THE APOE PROMOTER

THP-1 cells were transfected with 2μg of the apoE-3000/+86 DNA construct using DEAE-dextran (Section 2.3.5.3). After 16h the cells were treated with either 0.32μM PMA or 1000U/ml TNFα. After 36h, the cells were harvested and the assays were carried out as specified in Section 2.3.8. and 2.3.9. Luciferase activity was normalised to protein concentration and values are expressed as relative luciferase activity.

3.3. DISCUSSION

The initial aim of the studies described in this chapter was to analyse the regulatory sequences in the apoE promoter that are required for activation during macrophage differentiation. Both RT-PCR and Western blot analysis (Figure 3.2) confirmed the previously noted induction of apoE mRNA and protein expression during macrophage differentiation. However, the apoE promoter activity was not increased during differentiation despite extensive transfection experiments in which a number of parameters were varied. Although the previously reported induction of apoE promoter activity by TNF-α (Duan *et al.*, 1995) was reproduced in the system, the results raise further doubts on the findings of Basheeruddin *et al.* (1994) regarding macrophage differentiation. As described in section 3.1, there were several problems associated with the studies described in this paper and these have now been confirmed by the results of the experiments carried out in this chapter. The results of this chapter also show that the recently identified ME1 and ME2 apoE enhancers are not sufficient for the activation of the gene seen during macrophage differentiation.

Several possibilities could account for the transfection results obtained in this chapter. Firstly, similar to the complexity seen for hepatocyte-specific expression, activation of apoE during macrophage differentiation may require other sequences in addition to the promoter and

enhancers. With regards to the liver-specific expression of apoE, several studies have demonstrated that the proximal regulatory elements are not sufficient to direct this expression of the apoE gene *in vivo* (Smith *et al.*, 1988; Simonet *et al.*, 1991), and various regions in the intergenic sequence between apoE and apoCI have been shown to be required for the expression of these genes in a variety of tissues including liver, testis, spleen, skin, submaxillary gland, kidney, brain, small intestine, heart, stomach and pancreas (Simonet *et al.*, 1993). Moreover, a region in the intergenic sequence between apoCI and the apoCI pseudogene has been shown to contain an element, termed the hepatic control region 1 (HCR1), which is vital for the hepatic expression of the human apoE and apoCI genes (Simonet *et al.*, 1993; Taylor *et al.*, 1987; Simonet *et al.*, 1991). Also, a second hepatic control region designated HCR2 has been identified and is located in the intergenic sequence between the apoCI pseudogene and apoCIV gene and has also been shown to be essential in the liver-specific expression of apoE (Allan *et al.*, 1995a; 1995b). Previous studies have also demonstrated that expression of the apoE gene is controlled by a complex interaction of several independent tissue-specific transcriptional enhancers and silencers and that the absence of more dominant regulatory sequences may result in cryptic elements exerting their silencing effects (Simonet *et al.*, 1993). Therefore, it is a possibility that the important regulatory elements for the control of apoE expression during differentiation may reside outside the sequences contained in the constructs used for these studies, and a thorough understanding of the mechanisms controlling apoE expression during macrophage differentiation would be facilitated through an analysis of DNA constructs containing different combinations of regulatory elements. Indeed, recent work in macrophages has identified a sequence located in the apoE/apoCI intergenic region, separate to the ME1 enhancer element, which regulates apoE expression. This sequence, a PPARγ response element was shown through transfection based studies using THP-1 cells to be the only sequence of the apoE/CI intergenic region that responds to the inducers of macrophage and adipocyte differentiation (Galetto *et al.*, 2001).

An alternative explanation for the transfection results obtained in this chapter may be that the induction of apoE mRNA expression during macrophage differentiation is mediated predominately at the level of mRNA stability with transcriptional control making only a minor contribution. Indeed, the activation of the apoE promoter during macrophage differentiation seen in the studies by Basheeruddin *et al.* (1994) was approximately 3-fold. However, the activation of mRNA and protein expression seen in this study (Figure 3.3 and 3.4) along with those of other studies (Auwerx *et al.*, 1988; Basheeruddin et al., 1992; Werb and Chin, 1983; Menju *et al.*, 1989; Tajima *et al.*, 1985) is at least 10-15-fold. Evidence supporting this theory has previously been demonstrated by Basheeruddin *et al.* (1992), who demonstrated that an increase of up to 10-fold in steady state levels of apoE mRNA during differentiation was not accompanied by a corresponding

increase in the rate of transcription initiation or elongation. They also demonstrated that this disparity between mRNA levels and transcription could be attributed to mRNA degradation, as a more rapid level of degradation of mature mRNA occurred in undifferentiated cells compared to differentiated (Basheeruddin *et al.*, 1992). Although no studies have elucidated the precise mechanism for this increase in mRNA stability, work by Wager and Assosian (1990) has demonstrated that PMA treatment of U937 cells leads to increased levels of TGF-β mRNA, which is due to a PMA-mediated inhibition of the activity of a specific nuclease. This induction of mRNA stability in response to PMA treatment has also been shown for several other genes in macrophages (Kwon and Kim, 2003; Park *et al.*, 2001; Doyle *et al.*, 1997; Ohh *et al.*, 1994), indicating that increased mRNA stability may be a common mechanism for gene up-regulation during differentiation. Therefore, it is possible to speculate that the increase in apoE mRNA observed in PMA-treated cells is due to an increase in mRNA stability as a result of the inhibition of a nuclease responsible for degradation, and that transcriptional regulation makes only a minor contribution to this mode of apoE regulation during differentiation.

In conclusion, this Chapter has demonstrated that the regulation of apoE expression during macrophage differentiation is much more complex than first anticipated. Although apoE expression is induced by PMA at the level of mRNA and protein we were unfortunately unable to identify how transcriptional regulation relates to this induction, as our transfection system was not suitable for the analysis of the apoE promoter during differentiation. Therefore, in the light of these findings and the recent developments in the apoE field, it was decided to slightly modify the aims of the project, which are described in the next chapter.

CHAPTER FOUR- EFFECT OF PHARMOLOGICAL INHIBITORS ON THE LXR-MEDIATED INDUCTION OF APOE

4.1. INTRODUCTION

The work presented in Chapter 3 has shown that the regulation of apoE during macrophage differentiation is a much more complex story than first anticipated. The results of the previous chapter have demonstrated that additional studies are required to further elucidate the factors which control apoE expression. As a result of this outcome and in light of the recent developments in the apoE field, the overall aims of the project were modified slightly to understanding the regulation of apoE transcription by the Liver X receptor (LXR) family of nuclear receptors. ApoE regulation by the LXRs occurs in response to lipid loading, whereby these receptors act as a molecular sensor, increasing apoE expression (Laffitte *et al.*, 2001b). Although it is known that LXRs mediate their effects through binding to two LXREs in the apoE enhancer regions and that this regulation is tissue-specific (Laffitte *et al.*, 2001b), very little else is known about LXR regulation of this gene, especially with regards to the involvement of cell signalling pathways. Therefore this chapter will address the role of cell signalling in nuclear receptor activation and identify the potential involvement of these pathways in LXR regulation of apoE.

More and more studies reveal that nuclear receptor activation of target genes is much more complicated than initially thought, and may involve the regulation of receptor function through cross-talk with other transcription factors and intracellular signalling pathways. One way in which cell signalling pathways affect nuclear receptor activity is through the modification of coregulator proteins (Figure 4.1) [Hermanson *et al.*, 2002; McKenna and O'Malley, 2002]. Coactivator recruitment by DNA-bound nuclear receptors occurs in response to ligand binding. The binding of ligand leads to a conformational change, which alters the affinity of the nuclear receptors for coregulator proteins, which results in the dissociation of corepressors, and the recruitment of coactivators. Although ligand binding is primarily responsible for coregulator recruitment by nuclear receptors, cell signalling pathways can regulate this function through direct modification of the coactivator and corepressor proteins. Post-translational modification of coregulators exerts considerable control over their availability and their recruitment to transcriptional complexes and also their enzymatic activity (Rowan *et al.*, 2000; Hong and Privalsky, 2000; Chawla *et al.*, 1998).

Cell signalling pathways can also regulate coregulator recruitment and various other receptor functions, through direct phosphorylation of the nuclear receptor (Figure 4.1). Both the ligand-binding domain and the DNA-binding domain, in addition to the N-terminal domain of nuclear receptors contain several, potential phosphorylation sites (Rochette-Egly, 2003; Shao and Lazar, 1999). Phosphorylation of the nuclear receptors by signalling pathways may occur in response to ligand or in the absence of ligand and individual phosphorylations can act either to enhance or to inhibit the transactivation potential of the receptor.

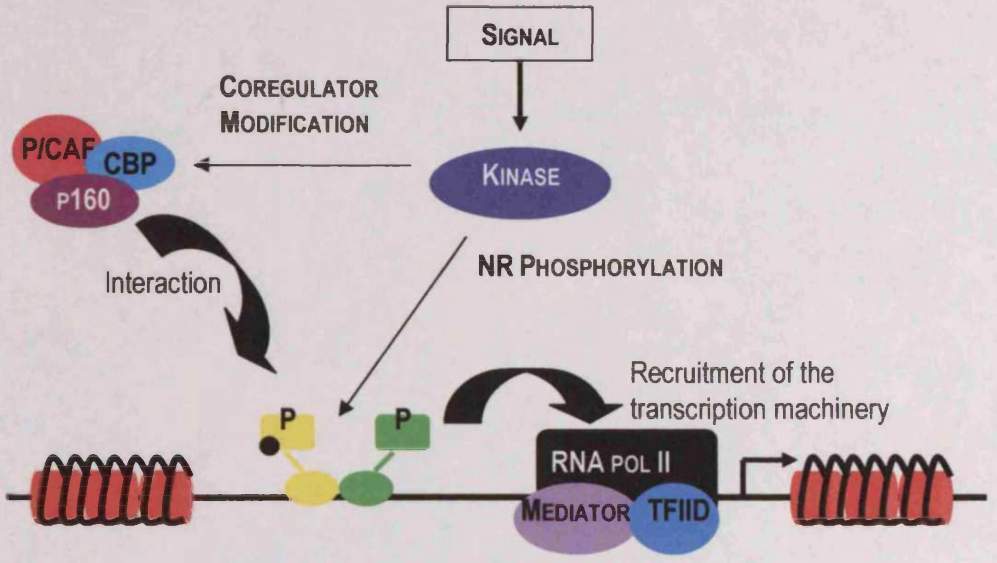


FIGURE 4.1. POSITIVE REGULATION OF NUCLEAR RECEPTOR TRANSACTIVATION THROUGH PHOSPHORYLATION

Nuclear receptor phosphorylation by kinases activated in response to a variety of signals, aids coactivator recruitment, thereby facilitating the recruitment of histone remodeling complexes, which decondense chromatin and promote a transcriptionally permissive environment at the promoter. Phosphorylation of the nuclear receptor also participates in the recruitment of components of the RNA polymerase II transcriptional machinery. In addition, coactivator modification also effects coregulator recruitment and enzymatic function thereby indirectly effecting nuclear receptors transactivation potential. P/CAF, p300/CBP-associated factor; CBP, p300/CREB-binding protein; P, phosphate residue; NR, nuclear receptor.

Phosphorylation has been shown to modulate the activity of many nuclear receptors, with a range of functions being affected, such as dimerisation, DNA binding and interactions with cofactors, all of which affect the transactivation function of these transcription factors (Chen *et al.*, 1999; Auricchio *et al.*, 1996; Hammer *et al.*, 1999). Although phosphorylation is usually secondary to ligand activation for most nuclear receptors, it is becoming apparent that phosphorylation may be the primary method for modulating certain receptors (Hammer *et al.*, 1999; Tremblay *et al.*, 1999; Arnold *et al.*, 1995; Lee *et al.*, 2000). The ability of kinase cascades to regulate nuclear receptor functions demonstrates that the activity of these receptors can be modified depending on the physiological state of the cell. Also, it is becoming increasingly clear that the phosphorylation of nuclear receptors and the modification of coregulators is necessary for the transactivation of certain genes, and that these modifications cooperate with the receptor ligand in order to achieve maximal transcriptional activity.

Although recent work has demonstrated that the LXRs require coactivators for effective transcriptional activation of certain genes (Huuskonen *et al.*, 2004; Oberkofler *et al.*, 2003) no involvement of any cell signalling pathways in the LXR-mediated regulation of genes have so far

been demonstrated, although its dimerisation partner RXR has been shown to be affected by several kinases, including the mitogen-activated protein kinases (MAPKs) and protein kinase A (PKA) [Solomon *et al.*, 1999; Dowhan and Muscat, 1996]

Therefore the aim of this chapter, was to determine whether cell signalling pathways play a role in the LXR-mediated activation of apoE transcription in macrophages through the use of commercially-available inhibitors against various cell signalling pathways. For these studies, it was decided to use several different inhibitors, as no previous work on the involvement of cell signalling pathways in the LXR-mediated regulation of genes has been published. All inhibitors chosen for this study have been used extensively in research examining gene regulation in macrophages (Garcia-Garcia *et al.*, 2002; Ma *et al.*, 2001; Zhao *et al.*, 2002; Abe *et al.*, 1999; Liang *et al.*, 1999; Wang *et al.*, 2002) and have been chosen to target pathways previously shown to be involved in nuclear receptor signalling. Below is a brief summary of the pathways to be investigated and their involvement so far in nuclear receptor signalling:

MITOGEN-ACTIVATED PROTEIN KINASES (MAPKS)- MAPK signalling pathways play an important role in regulating gene expression in eukaryotic cells and have been shown to participate in a variety of processes, such as cell differentiation, movement, division and cell death. Five distinctly regulated MAPK subgroups have been identified so far, all of which can signal independently from each other. These include the extracellular signal-regulated kinase 1 and 2 (ERK1/2) cascade, which preferentially regulates cell growth and differentiation, as well as the c-jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) and p38 MAPK cascades, which function mainly in stress responses like inflammation and apoptosis.

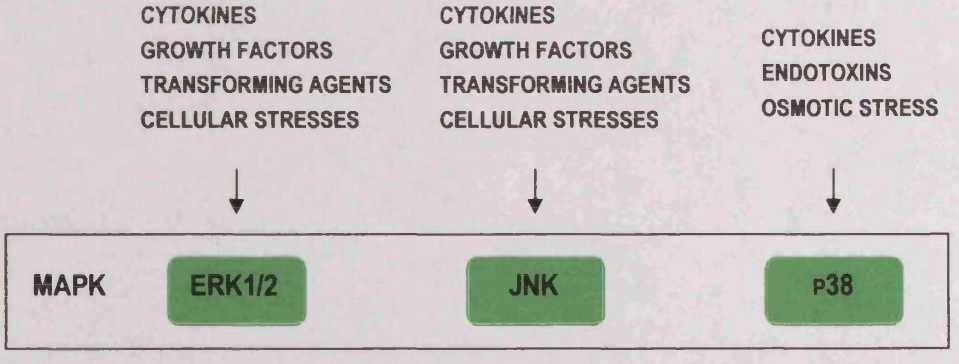


FIGURE 4.2. SCHEMATIC SUMMARY OF THE THREE MAIN MAPK PATHWAYS

Each of these three branches of the MAPK pathway has been shown to affect nuclear receptor activity through phosphorylation of the nuclear receptor itself or through phosphorylation of coregulator proteins. The AF-1 domain of several nuclear receptors have been reported to be

substrates for the ERK or p38 MAPKs, including the progesterone receptor (PR) [Lange *et al.*, 2000; Shen *et al.*, 2001], estrogen receptor-α (ERα) [Bunone *et al.*, 1996; Kato *et al.*, 1995], ERβ (Tremblay *et al.*,1999; Driggers *et al.*, 2001), androgen receptor (AR) [Yeh *et al.*, 1999], PPARs (Hu *et al.*, 1996; Juge-Aubry *et al.*, 1999), and RARγ (Gianni *et al.*, 2002). Whilst the JNK/SAPK subfamily has been shown to target the AF-1 domain of RXRα (Adam-Stitah *et al.*, 1999; Lee *et al.*, 2000). The JNK/SAPK pathway is also thought to potentiate the activation function of ER through phosphorylation of ER-associated proteins, CBP and GRIP1 (Feng *et al.*, 2001). All three MAPK pathways are also able to modulate the activities of transcription coregulators, such as p300 and SMRT (Chen *et al.*, 2003).

PHOSPHOINOSITIDE 3' KINASE (PI3K)- PI3Ks modulate several physiological processes including metabolism, cell growth, proliferation and cell migration (Wymann *et al.*, 2003). PI3Ks phosphorylate inositol lipids at the 3' position of the inositol ring to generate the 3-phosphoinositides PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3, which recruit 3-phosphoinositide-binding proteins to the plasma membrane and activate downstream signalling pathways (Leevers *et al.*, 1999). One downstream target of PI3K is Akt/PKB, a serine/threonine kinase that regulates a variety of downstream kinases (Datta *et al.*, 1999). Upon activation and subsequent translocation into the nucleus, Akt has been shown to phosphorylate certain nuclear receptors such as ERα (Campbell *et al.*, 2001) and AR (Lin *et al.*, 2001b) in their N-terminal region. The PI3K/Akt pathway also plays an important role in RAR signalling (Gianni *et al.*, 2002) and a downstream target of Akt, glycogen synthase kinase-3 (GSK-3), has also been shown to regulate the transcriptional activation function of the glucocorticoid receptor (GR) [Rogatsky *et al.*, 1998].

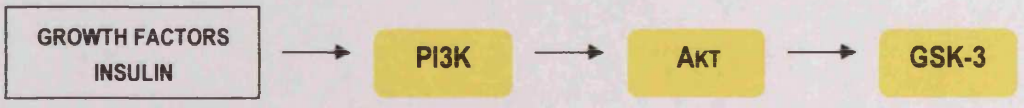


FIGURE 4.3. SCHEMATIC REPRESENTATION OF THE PI3K/AKT/GSK-3 PATHWAY

CASEIN KINASE 2 (CK2)- CK2 is a ubiquitous, constitutively active serine/threonine protein kinase and is typically found in tetrameric complexes consisting of two catalytic (α and/or α') subunits and two regulatory β subunits. CK2 appears to reside in a variety of cellular compartments and participates in the phosphorylation and regulation of a broad array of substrates (Meggio and Pinna, 2003). CK2 has been shown to modulate the transactivation functions of several nuclear receptors through phosphorylation. Phosphorylation of the human Vitamin D receptor (VDR) at

Ser208 by CK2, specifically modulates its transcriptional capacity, as phosphorylation alters the conformation of VDR and potentiates its interaction with the basal transcription machinery (Jurutka *et al.*, 1996). Other targets of CK2 phosphorylation include PRβ (Zhang *et al.*, 1994b), TRα2 (Katz *et al.*, 1995), ER (Castano *et al.*, 1997) and the corepressor SMRT (Zhou *et al.*, 2001). SMRT phosphorylation occurs in the C-terminal domain of the corepressor, and stabilizes the ability of the SMRT protein to interact with nuclear hormone receptors (Zhou *et al.*, 2001).

4.2. RESULTS

4.2.1. EFFECT OF LXR LIGAND 22(R)-HYDROXYCHOLESTEROL ON APOE MRNA EXPRESSION IN THP-1 MACROPHAGES

Before addressing the overall aim of this chapter on the involvement of cell signalling pathways in LXR-mediated transactivation of apoE in macrophages, it was necessary to ensure that the earlier data produced by Laffitte *et al.* (2001b), which demonstrated that the LXR ligand 22(R)-hydroxycholesterol induced apoE expression, could be reproduced. It was therefore decided to examine the ability of 22(R)-hydroxycholesterol to increase apoE mRNA expression in macrophages. For this experiment THP-1 monocytes were differentiated for 24h with 0.16μM PMA. After the 24h differentiation period 22(R)-hydroxycholesterol (2μg/ml) was added to the appropriate flasks and left to incubate for 12h (Figure 4.4). In addition to 22(R)-hydroxycholesterol, as a control for this experiment, THP-1 cells were treated with the ligand 22(S)-hydroxycholesterol (2μg/ml), an inactive enantiomer of 22(R)-hydroxycholesterol which binds but does not activate the LXR receptor. As both ligands were solubilised in DMSO, it was ensured that the final concentration was less than 0.1% (v/v) to ensure that it had no effect on cell viability. Also, as an additional control, all control samples for these experiments were treated with an equal volume of DMSO to ensure that any observed result was due to the ligand and not the DMSO. Total RNA was then extracted (Section 2.4.1) and RT-PCR was carried out (Section 2.4.4) using the conditions (Table 2.11) and primers for apoE and GAPDH (Table 2.10).

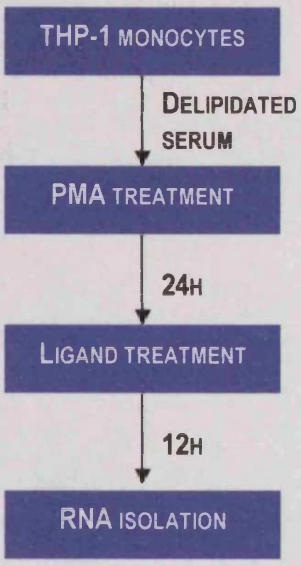
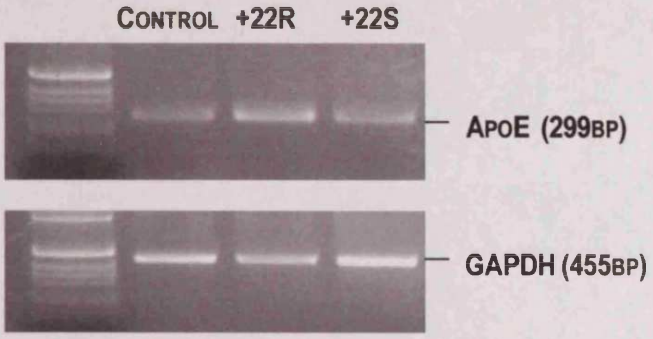


FIGURE 4.4. SCHEMATIC REPRESENTATION OF THE EXPERIMENTAL STRATEGY USED TO INVESTIGATE THE EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON APOE MRNA EXPRESSION IN THP-1 MACROPHAGES.

For all experiments investigating the ability of oxysterol ligands to induce apoE gene expression it was necessary to use 10% (v/v) delipidated serum, in order to ensure that no other external source of sterols would effect the LXR-mediated induction of apoE. Delipidation of serum was carried out as described in Section 2.3.4 and was substituted for "normal" HI-FCS at the beginning of each experiment (Figure 4.4). This substitution of FCS had no effect on cell viability or rate of growth and did not affect PMA-induced adhesion of cells (data not shown). In addition to the use of delipidated serum all experiments investigating the LXR-mediated induction of apoE required a differentiation period of only 24h. Also, all PCR-based experiments required only a12h incubation with the LXR ligand. The reason for this was that the effect of the ligand on apoE expression in macrophages becomes hidden after this time-point by the increasing stimulation of apoE due to PMA.

From Figure 4.5 it can be seen that treatment of differentiated THP-1 cells with the oxysterol ligand 22(R)-hydroxycholesterol results in an increase in apoE mRNA expression as demonstrated previously by Laffitte *et al.* (2001b). This induction of apoE by 22(R)-hydroxycholesterol was not achieved in monocytes (data not shown), which also confirms the previous finding by Laffitte et al. (2001b) that LXR-mediated induction of apoE is differentiation dependent. THP-1 cells treated with the inactive ligand, 22(S)-hydroxycholesterol showed no change in apoE levels (Figure 4.5), which demonstrates that the ligand-mediated induction of apoE requires LXR activation.



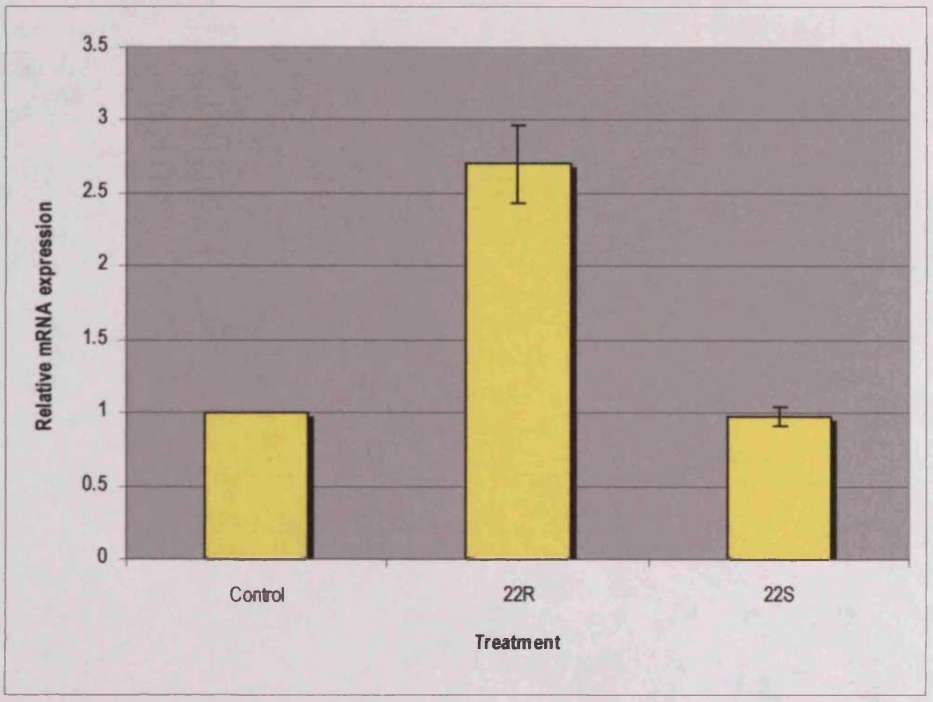


FIGURE 4.5. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON APOE MRNA EXPRESSION IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligands, 22R-hydroxycholesterol (denoted 22R) [2μg/ml] and 22(S)-hydroxycholesterol (denoted 22S) [2μg/ml] for 12h. RT-PCR was carried out using apoE- and GAPDH-specific primers. Although not shown on this Figure, each set of RT-PCR reactions included a negative control, where no reverse transcriptase had been added during the cDNA synthesis step of the control sample. No signal was obtained from these samples, indicating that there was no genomic DNA contamination in the PCR. Following amplification, the products were subjected to electrophoresis using a 1.5% (w/v) agarose gel and product size was confirmed using the 100bp molecular weight marker (Appendix II). The apoE and GAPDH signals for each sample were determined by densitometric analysis and plotted on a bar chart. The apoE:GAPDH ratio in macrophages treated with DMSO only (denoted control) has been assigned as 1, with the ratio for the remaining samples being represented relative to this control. Results are representative of three separate experiments and the data shown in the histogram is the mean value ±SD.

4.2.2. EFFECT OF LXR LIGAND 22(R)-HYDROXYCHOLESTEROL ON APOE PROTEIN EXPRESSION IN THP-1 MACROPHAGES

Although the study by Laffitte *et al.*, (2001b) demonstrated an induction in apoE mRNA by the LXR ligand, 22(R)-hydroxycholesterol, the effect of this ligand on apoE protein levels was not shown. Therefore it was decided that the effect 22(R)-hydroxycholesterol on apoE protein levels should be investigated. Whole cell protein extracts were prepared (see Section 2.5.1) from differentiated THP-1 cells treated with 2μg/ml of 22(R)-hydroxycholesterol or 22(S)-hydroxycholesterol for 24h. Of each sample 20μg of total protein was subjected to SDS-PAGE (Section 2.5.4) and then transferred to a PVDF membrane (Section 2.5.5). The membrane was then incubated with an anti-apoE primary antibody (Biogenesis) and immunodetection was carried out using the appropriate secondary antibody and the ECL detection system (Amersham) as described in Section 2.5.6. As shown in Figure 4.6, 22(R)-hydroxycholesterol treatment of differentiated THP-1 cells increases apoE protein levels, and this induction was shown to be time-dependent, with maximal induction occurring at 24h (Figure 4.7). Also, as shown at the mRNA level (Figure 4.5) the inactive enantiomer, 22(S)-hydroxycholesterol had no effect on apoE expression levels.

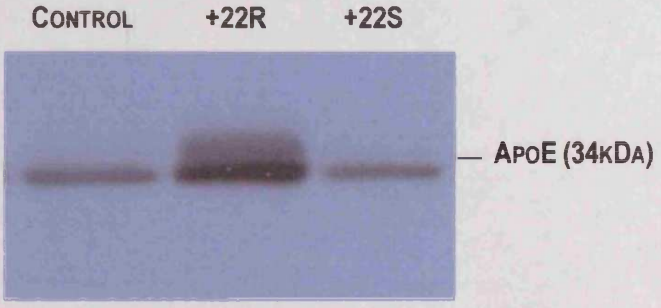


FIGURE 4.6. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON APOE PROTEIN EXPRESSION IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligands, 22R-hydroxycholesterol (denoted 22R) [2μg/ml] and 22(S)-hydroxycholesterol (denoted 22S) [2μg/ml] for 24h. In addition, differentiated cells were treated with vehicle (DMSO), in the place of ligand (denoted control). Western blot analysis was carried out using 20μg of whole cell extracts. Blotted membranes were incubated with anti-apoE primary antibody (goat polyclonal IgG) and detected using an anti-goat horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of three separate experiments.

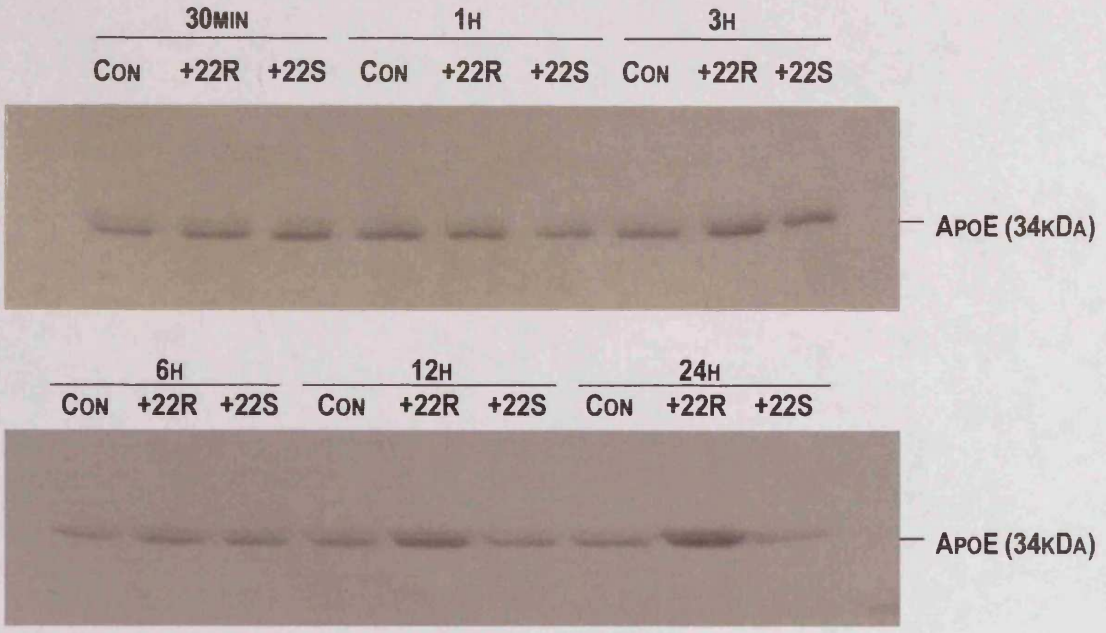


FIGURE 4.7. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON APOE PROTEIN EXPRESSION IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligands, 22R-hydroxycholesterol (denoted 22R) [2μg/ml] and 22(S)-hydroxycholesterol (denoted 22S) [2μg/ml] for various time-points. In addition, for each time-point, differentiated cells were treated with vehicle (DMSO) in the place of ligand (denoted control). Western blot analysis was carried out using 20μg of whole cell extracts. Blotted membranes were incubated with anti-apoE primary antibody (goat polyclonal IgG) and detected using an anti-goat horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of three separate experiments.

4.2.3. EFFECT OF LXR LIGAND 22(R)-HYDROXYCHOLESTEROL ON APOE SECRETION IN THP-1 MACROPHAGES

As apoE is a secretory protein and its secretion by macrophages in the atherosclerotic plaque is largely responsible for its atheroprotective role, the effect of 22(R)-hydroxycholesterol on apoE secretion in macrophages was investigated. THP-1 monocytes were differentiated for 24h with 0.16μM PMA and then treated with 2μg/ml of 22(R)-hydroxycholesterol or the control ligand, 22(S)-hydroxycholesterol for 24h. After treatment, the apoE content of the cell culture medium was determined by ELISA as described in Section 2.5.7. As shown in Figure 4.8, the level of apoE secretion increases with 22(R)-hydroxycholesterol treatment and remains unchanged in cells treated with 22(S)-hydroxycholesterol.

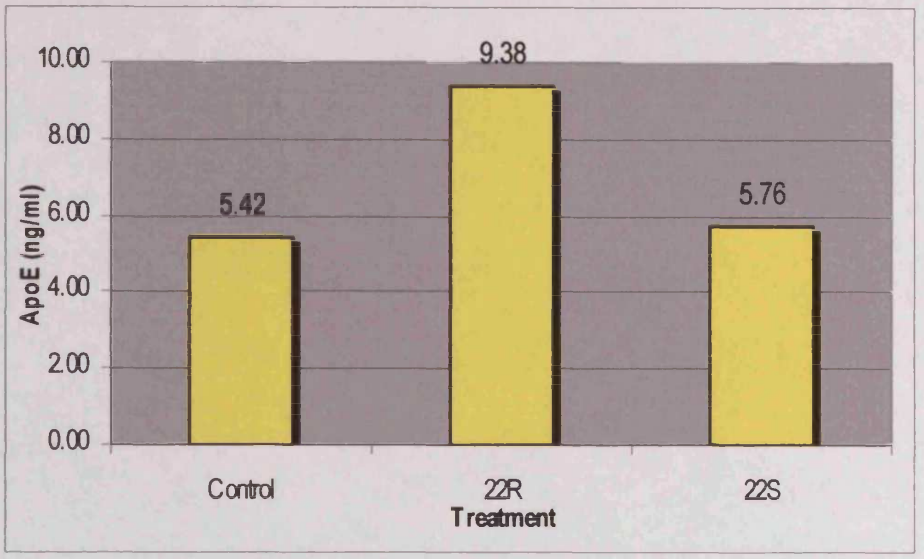


FIGURE 4.8. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON APOE SECRETION IN THP-1 MACROPHAGES
THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligands, 22R-hydroxycholesterol (denoted 22R) [2μg/ml] and 22(S)-hydroxycholesterol (denoted 22S) [2μg/ml] for 24h. In addition, differentiated cells were treated with vehicle (DMSO) in the place of ligand (denoted control). Each ELISA was carried out as described in Section 2.5.7. The level of apoE protein is shown as a value above each bar and results are representative of three separate experiments.

4.2.4. EFFECT OF INHIBITORS ON THE 22(R)-HYDROXYCHOLESTEROL-MEDIATED INDUCTION OF APOE MRNA EXPRESSION IN THP-1 MACROPHAGES

The results from the previous sections have shown clearly that the oxysterol ligand 22(R)-hydroxycholesterol increases apoE expression at both the mRNA and protein level, giving a strong basis from which we can confidently approach the primary aim of this chapter, which is to determine, through the use of inhibitors, whether cell signalling pathways play a role in the LXR-mediated activation of apoE transcription in macrophages. For this study, THP-1 cells were differentiated in 10% (v/v) delipidated HI-FCS with PMA (0.16μM) for 24h. After the 24h-differentiation period, inhibitors were added for 1h before treatment with LXR ligand (Figure 4.9), in order to ensure cellular uptake and inhibition of the target enzyme. The ligand 22(R)-hydroxycholesterol (2μg/ml) was then added to the appropriate flasks and left to incubate for a further 12h. After incubation with the inhibitors and ligand, total RNA was extracted (Section 2.4.1). As all the inhibitors used in this study were solubilised in DMSO, it was ensured that the final concentration was less than 0.1% (v/v) to ensure that that it had no effect on cell viability, and as an additional control, all control samples for these experiments were treated with an equal volume of DMSO to ensure that the observed effect was due to the inhibitor and not the DMSO. In addition,

trypan blue exclusion assays were carried out to ensure that the inhibition was not due to any cytotoxic effect of the compound.

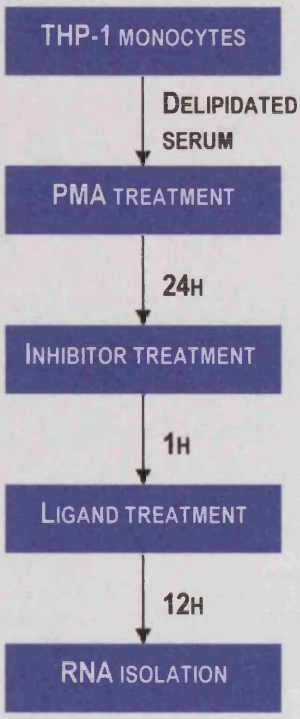


FIGURE 4.9. SCHEMATIC REPRESENTATION OF THE EXPERIMENTAL STRATEGY USED TO INVESTIGATE THE EFFECT OF CELL SIGNALLING INHIBITORS ON THE LIGAND MEDIATED INDUCTION OF APOE MRNA EXPRESSION IN THP-1 MACROPHAGES

As the induction of apoE expression at the mRNA level is modest (2-2.5x), a more quantitative method of measuring mRNA expression was employed for these inhibitor studies. Taqman Real-Time quantitative RT-PCR was carried out using apoE-specific primers (Table 2.12) as described in Section 2.4.5 and reactions were monitored in real time mode using Sequence Detection Systems, version 1.7 software. In addition, Taqman reactions were carried out using primers specific for β-microtubulin (Table 2.12), which was used as the constitutive control as none of the inhibitors used in these experiments affected the expression of this gene. The concentration of the inhibitors used in the following experiments were based on previous work in our laboratory, where these inhibitors were used to elucidate key signalling pathways in macrophages and also previously published studies whereby these inhibitors were employed to investigate the regulation of macrophage gene expression (Liu et *al.*, 2001; Salomonsson *et al.*, 2002; Katsuyama *et al.*, 2001; Abe *et al.*, 1999; Liang *et al.*, 1999; Wang *et al.*, 2002). The inhibitors used and the pathways affected by them are shown in Table 4.1.

TABLE 4.1. INHIBITORS USED TO ELUCIDATE THE CELL SIGNALLING PATHWAYS INVOLVED IN THE LXR-MEDIATED INDUCTION OF APOE EXPRESSION

INHIBITOR	PATHWAY	MECHANISM	REFERENCE
APIGENIN	CK2	Reversible ATP/GTP-competitive inhibitor of CKII	Critchfield *et al.*, 1997
CURCUMIN	JNK/SAPK MAPK	Inhibits an upstream kinase of the JNK pathway, thought to be MEKK1	Chen and Tan, 1998; Jobin *et al.*, 1999
LY294002	PI3K	Reversible ATP-competitive inhibitor of PI3K	Vlahos *et al.*, 1994
PD98059	ERK MAPK	Inhibits MEK1 phosphorylation and activation of ERK	Dudley *et al.*, 1995
SB202190	p38 MAPK	Bind the inactive form of p38 and inhibit its activation	Lee *et al.*, 1994; Frantz *et al.*, 1998
SP600125	JNK/SAPK MAPK	Reversible ATP-competitive inhibitor of JNK1, -2 and –3	Bennett *et al.*, 2001

The results of this experiment are presented in Figure 4.10 and it can be seen that inhibitors against three of the pathways investigated, had a significant effect upon the 22(R)-hydroxycholesetrol-mediated induction of apoE mRNA. Both the inhibitors, curcumin and SP600125, which target the JNK branch of the MAPK pathway, completely eliminated the 22(R)-hydroxycholesterol induced expression of apoE mRNA, indicating that the JNK/SAPK pathway is likely to play a key role in the LXR-mediated regulation of apoE transcription. The PI3K inhibitor, LY294002, also completely blocked this induction of apoE mRNA by 22(R)-hydroxycholesterol, as did the CK2 inhibitor, apigenin. The inhibitors against the ERK (PD98059) and p38 (SB202190) branches of the MAPK cascade did not show any significant inhibition of this apoE induction, indicating that these pathways are not involved in the LXR-mediated regulation of apoE.

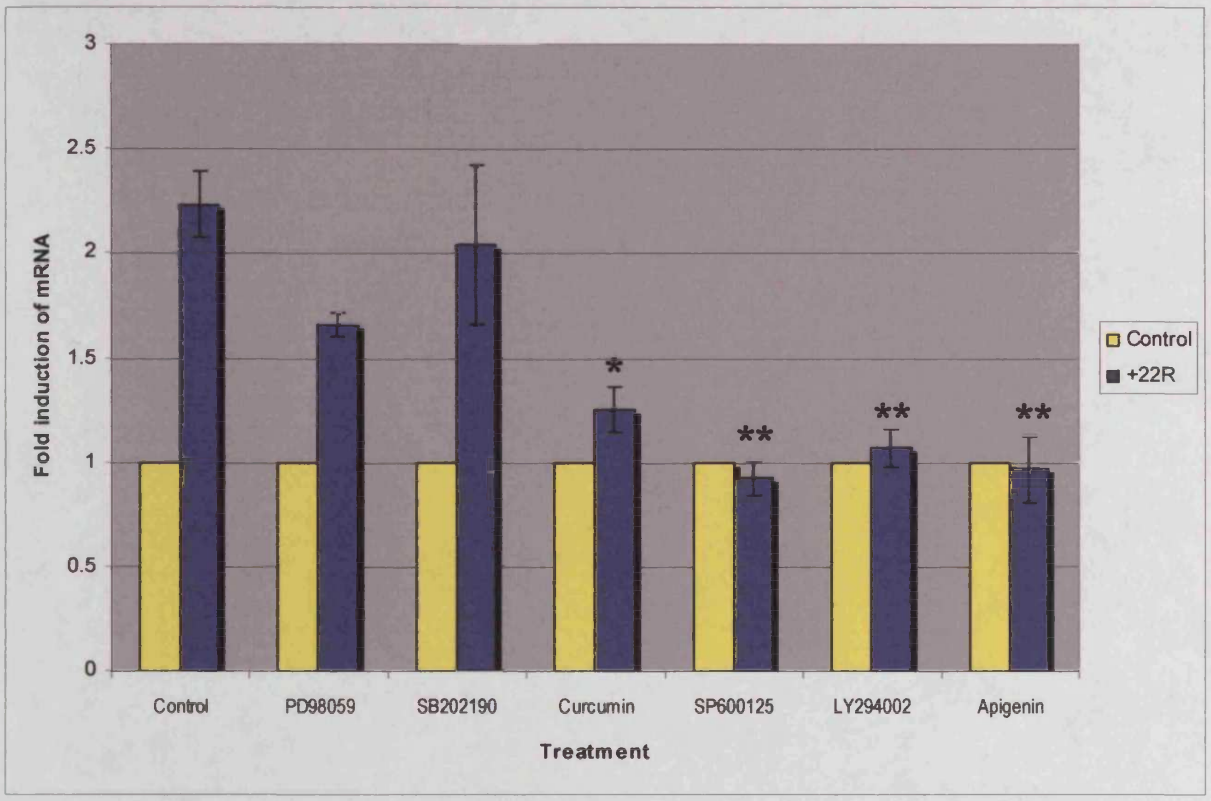


FIGURE 4.10. EFFECT OF CELL SIGNALLING INHIBITORS ON THE 22(R)-HYDROXYCHOLESTEROL-MEDIATED INDUCTION OF APOE MRNA EXPRESSION

*THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22R-hydroxycholesterol (denoted 22R) [2μg/ml] for 12h. In addition, differentiated cells were treated with vehicle (DMSO) in the place of ligand (denoted control). Inhibitors were added 1h before the ligand at various concentrations: PD98059 (50μM), SB202190 (10μM), Curcumin (25μM), SP600125 (50μM), LY294002 (100μM), Apigenin (40μM). Taqman Real-Time quantitative RT-PCR was carried out using apoE- and β-microtubulin-specific primers and reactions were monitored in real time mode using Sequence Detection Systems, version 1.7 software. The apoE:β-microtubulin ratio in macrophages treated with vehicle (DMSO) only has been assigned as 1, with the ratio for the remaining samples being represented relative to this control. The data shown is the mean ±SD from three independent experiments. The data was analysed by Student's t-test. * Represents the significant difference compared to control cells treated with 22(R)-hydroxycholesterol (*p<0.05; **p<0.005).*

4.2.5. EFFECT OF INHIBITORS ON THE 22(R)-HYDROXYCHOLESTEROL-MEDIATED INDUCTION OF APOE PROTEIN EXPRESSION IN THP-1 MACROPHAGES

The effect of these inhibitors on apoE expression was also confirmed at the protein level. Whole cell extracts were prepared from differentiated THP-1 cells, which were subsequently treated with inhibitors for 1hr before 22(R)-hydroxycholesterol (2μg/ml) addition for 24h. Western blot analysis was carried out as described previously in this chapter (Section 4.2.2). As shown in Figure 4.11, the effect of the inhibitors at the protein level is consistent with that seen at the mRNA level, with inhibition of the ligand–mediated response occurring in cells pre-treated with curcumin (JNK/SAPK

inhibitor), LY294002 (PI3K inhibitor) and apigenin (CK2). Also, as was the case for apoE mRNA, no inhibition of the apoE induction by the ligand occurred in cells pre-treated with the ERK MAPK inhibitor, PD98059 or the p38 MAPK inhibitor SB202190.

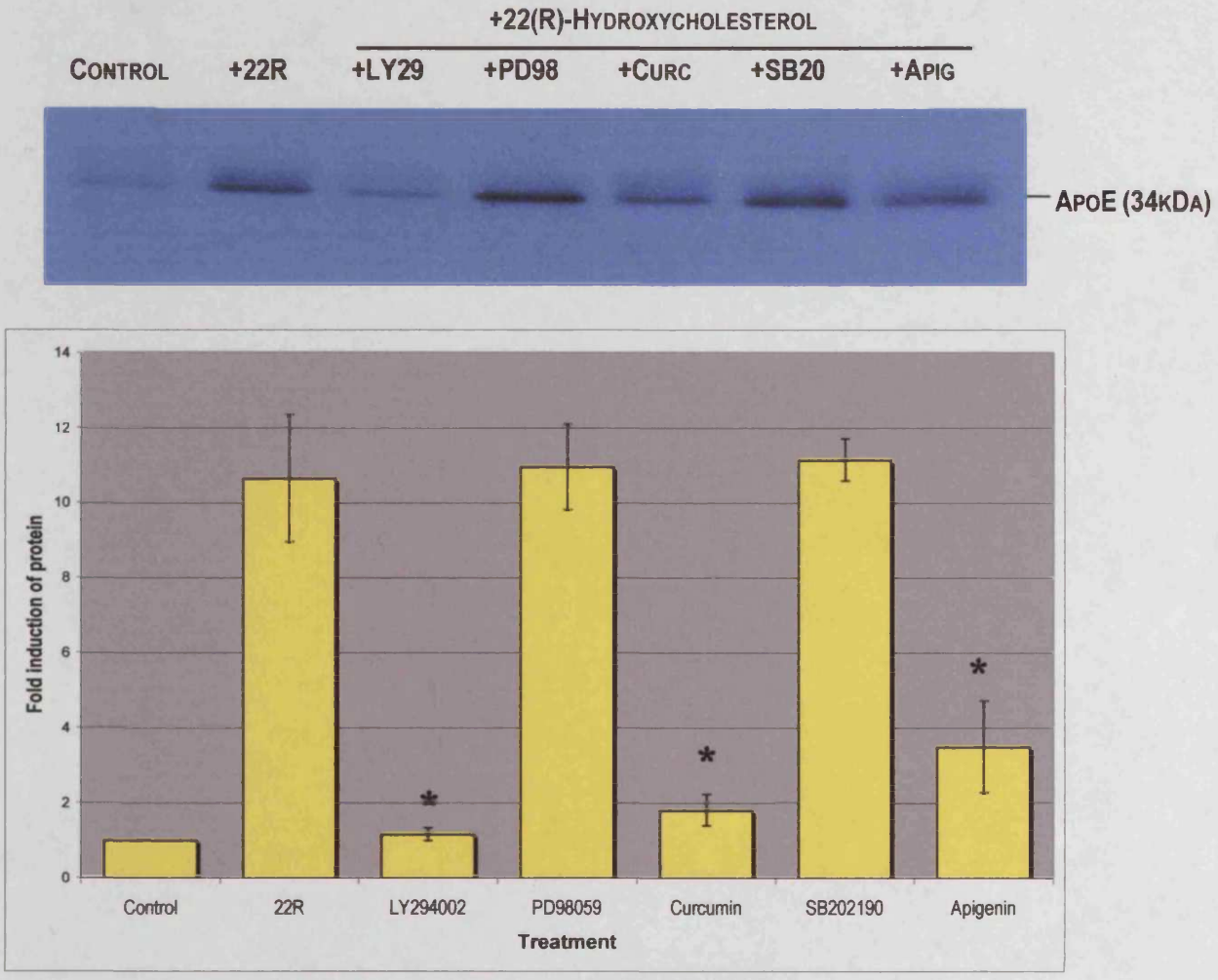


FIGURE 4.11. EFFECT OF CELL SIGNALLING INHIBITORS ON THE 22(R)-HYDROXYCHOLESTEROL-MEDIATED INDUCTION OF APOE PROTEIN EXPRESSION

*THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22R-hydroxycholesterol (denoted 22R) [2μg/ml] for 24h. In addition, differentiated cells were treated with vehicle (DMSO) in the place of ligand (denoted control). Inhibitors were added 1h before the ligand at various concentrations: LY294002 (100μM), PD98059 (50μM), Curcumin (25μM), SB202190 (10μM), Apigenin (40μM). Western blot analysis was carried out using 20μg of whole cell extracts. Blotted membranes were incubated with anti-apoE primary antibody (goat polyclonal IgG) and detected using an anti-goat horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. The apoE protein level for each sample was determined by densitometric analysis and plotted on a bar chart. The control represents macrophages treated with vehicle (DMSO) only has been assigned as 1, with the apoE protein level for the remaining samples being represented relative to this control. The data shown is the mean ±SD from three independent experiments. The data was analysed by Student's t-test. * Represents the significant difference compared to control cells treated with 22(R)-hydroxycholesterol (*p<0.05).*

The inhibition observed by pre-treatment with the JNK/SAPK, PI3K and CK2 inhibitors was also shown to be concentration-dependent (see Figure 4.12), as we next performed dose-response experiments with each inhibitor. Whole cell extracts were prepared from differentiated THP-1 cells, which were subsequently treated with varying concentration of inhibitors for 1hr before 22(R)-hydroxycholesterol (2μg/ml) addition for 24h.

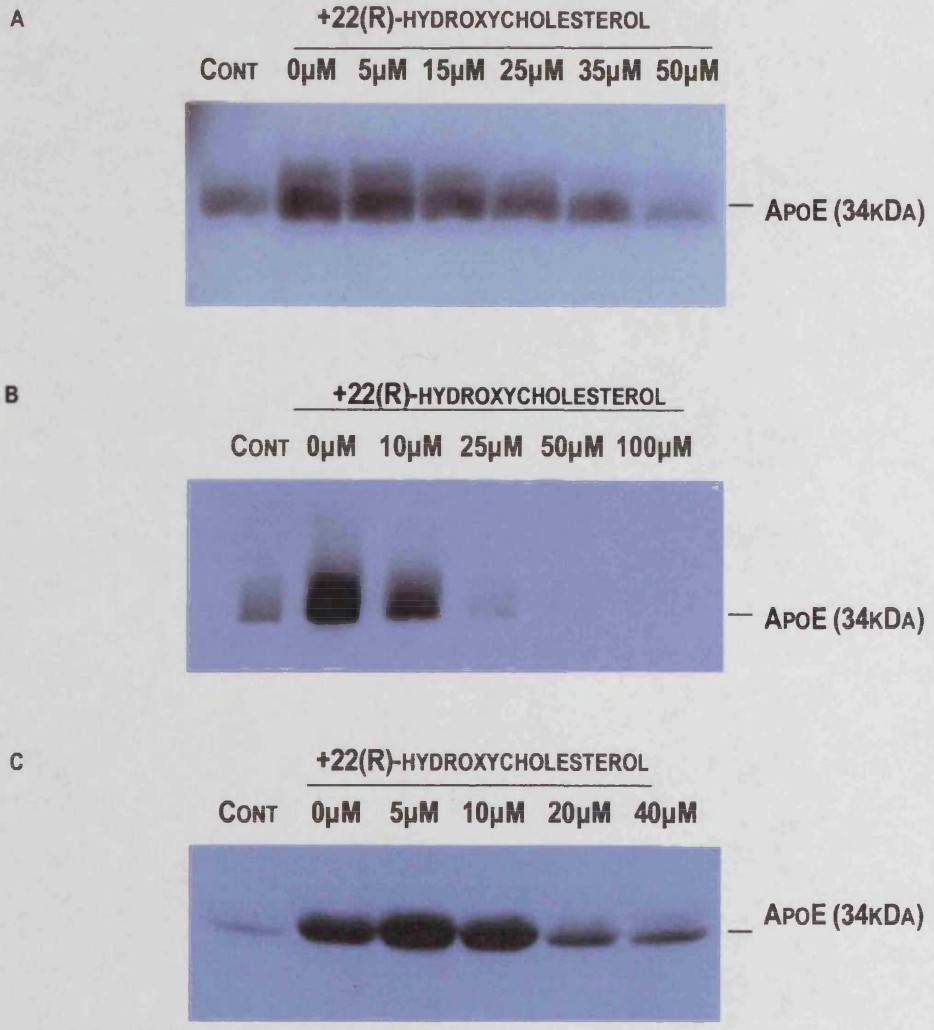


FIGURE 4.12. EFFECT OF INHIBITOR CONCENTRATION ON THE 22(R)-HYDROXYCHOLESTEROL-MEDIATED INDUCTION OF APOE PROTEIN EXPRESSION

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22R-hydroxycholesterol (denoted 22R) [2μg/ml] for 24h. Inhibitors: (A) curcumin; (B) LY294002 and; (C) apigenin were added 1h before the ligand at the indicated concentrations. In addition, differentiated cells were treated with vehicle (DMSO) in the place of ligand (denoted control). Western blot analysis was carried out using 20μg of whole cell extracts. Blotted membranes were incubated with anti-apoE primary antibody (goat polyclonal IgG) and detected using an anti-goat horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of three separate experiments.

4.2.6. EFFECT OF INHIBITORS ON 22R-HYDROXYCHOLESTEROL-MEDIATED INDUCTION OF APOE SECRETION

The effect of these inhibitors on the LXR-mediated induction of apoE was extended to apoE protein secretion. THP-1 monocytes were differentiated for 24h with 0.16μM PMA and subsequently treated with inhibitors for 1h before 22(R)-hydroxycholesterol (2μg/ml) addition for 24h. After incubation with the inhibitors and ligand, the apoE content of the cell culture medium was determined by ELISA as described in Section 2.5.7. Figure 4.13 shows that the inhibitors against the JNK/SAPK, PI3K and CK2 cell signalling pathways inhibit the induction seen in apoE secretion by ligand treatment. This result confirms further that these pathways are involved in the LXR-mediated induction of apoE. Also from Figure 4.13 it can be seen that the result for the PD98059 (ERK MAPK) inhibitor is also consistent with that seen for the mRNA and protein levels, as it had no inhibitory effect. However, these experiments did show a conflicting result for SB202190 (p38 MAPK), as this inhibitor reduced apoE secretion by nearly 50% (Figure 4.13). As this inhibition only occurs at the level of protein secretion, it may be that the p38 MAPK pathway plays a role in the post-translational regulation of apoE. Also curcumin reduced the level of apoE to below that of the control, indicating that this pathway may also be involved in the basal regulation of apoE secretion.

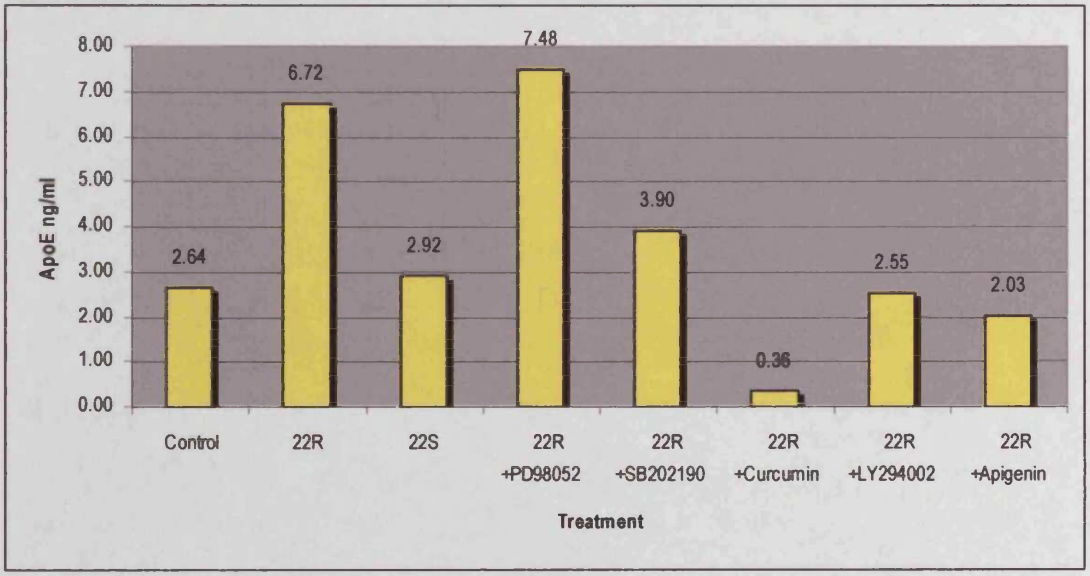


FIGURE 4.13. EFFECT OF INHIBITORS ON THE 22(R)-HYDROXYCHOLESTEROL-MEDIATED INDUCTION OF APOE SECRETION

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22R-hydroxycholesterol (denoted 22R) [2μg/ml] for 24h. In addition, differentiated cells were treated with vehicle (DMSO) in the place of ligand (denoted control). Inhibitors were added 1h before the ligand at various concentrations: LY294002 (100μM), PD98059 (50μM), Curcumin (25μM), SB202190 (10μM), Apigenin (40μM). As a control, differentiated cells were treated with vehicle (DMSO) in the place of ligand. Each ELISA was carried out as described in Section 2.5.7. The level of apoE protein is shown as a value above each bar and results are representative of three separate experiments.

4.2.7. EFFECT OF 22(R)-HYDROXYCHOLESTEROL AND CELL SIGNALLING INHIBITORS ON APOE PROTEIN EXPRESSION IN HUMAN PRIMARY MONOCYTE-DERIVED MACROPHAGES

Although the results from the previous sections have demonstrated clearly that the LXR-ligand 22(R)-hydroxycholesterol induces apoE expression in human THP-1 macrophages and that this inhibition can be abolished by pre-treatment with inhibitors against the JNK/SAPK, PI3K and CK2 pathways. In order for these results to be fully relevant to human atherosclerosis, they must be reproduced in primary cultures of human monocyte-derived macrophages as there are many instances where there are differences in response to stimuli between primary monocytes/macrophages and cell lines. Therefore, the effect of 22(R)-hydroxycholesterol on apoE induction was tested in human primary monocyte-derived macrophages, as was the effect of the inhibitors. Isolation of primary cells was carried out as described in Section 2.3.3 and the monocytes were left to differentiate for 7 days before use in these experiments. Once the cells had differentiated they were treated with inhibitors for 1h before treatment with 22(R)-hydroxycholesterol for 24h. For all experiments using primary cultures it is important to note that the concentration of ligand added to the cells was increased to 3μg/ml, as the concentration of cells per sample was much greater than that used in experiments using the THP-1 cell line. Whole cell extracts were prepared as described previously (Section 2.5.1) and western blot analysis was carried out as described in Section 4.2.2 of this chapter.

From Figure 4.14 it can be seen that 22(R)-hydroxycholesterol induced a 3-4-fold induction of apoE protein expression in these cells and this induction was significantly blocked by inhibitors against the JNK/SAPK and PI3K pathways, whilst pre-treatment with the CK2 inhibitor, apigenin had no significant inhibitory effect in these cells. Also as previously shown in the THP-1 cell line, the ERK and p38 MAPK inhibitors had no effect on the ligand-mediated induction of apoE.

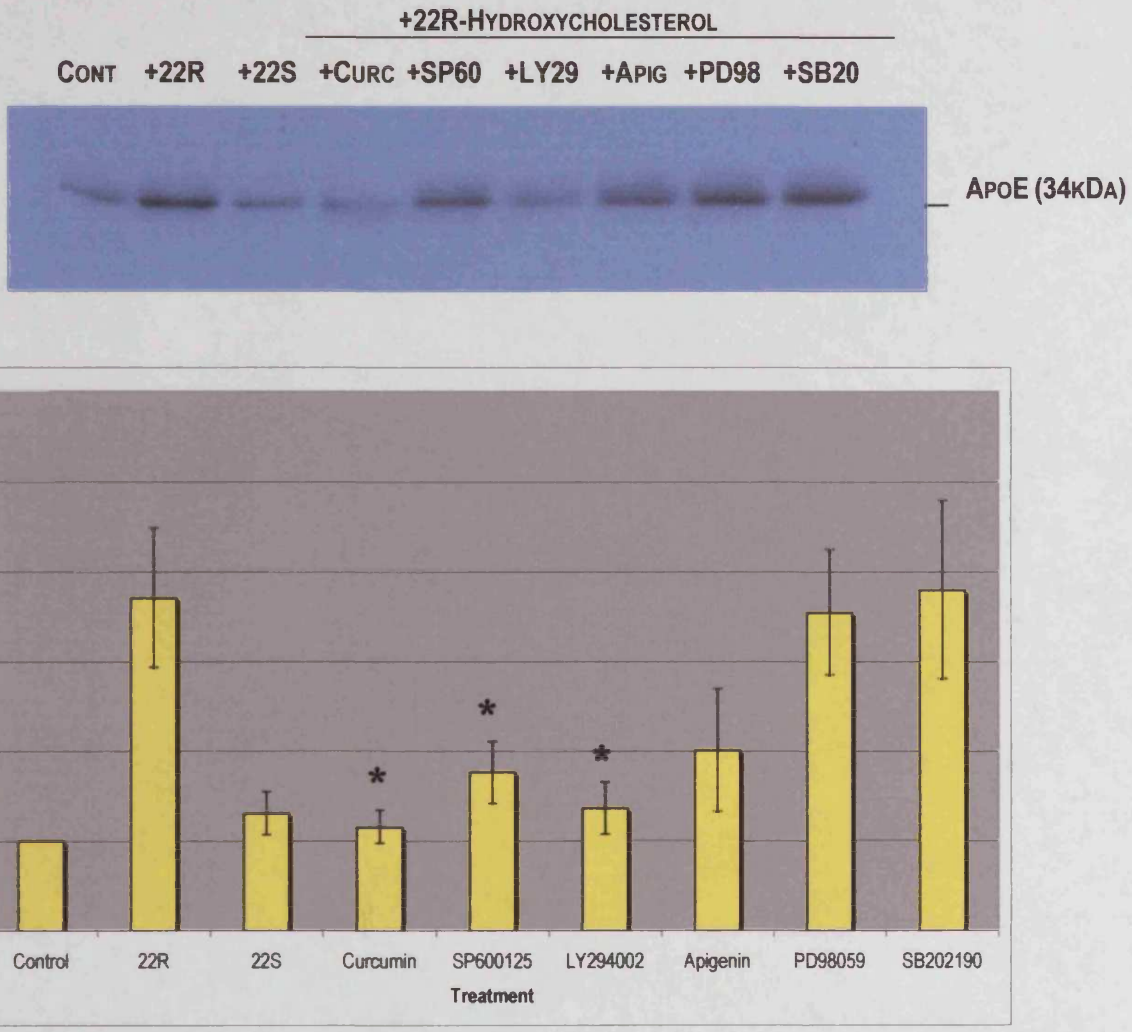


FIGURE 4.14. EFFECT OF 22R-HYDROXYCHOLESTEROL AND INHIBITORS ON APOE PROTEIN EXPRESSION IN HUMAN PRIMARY MONOCYTE-DERIVED MACROPHAGES

*Human monocytes were isolated and cultured for 7days in RPMI supplemented with 5% (v/v) human serum, after-which they were treated with 3μg/ml of 22(R)-hydroxycholesterol (denoted 22R) or 22(S)-hydroxycholesterol (denoted 22S) for 24h. In addition, differentiated cells were treated with vehicle (DMSO) in the place of ligand. Inhibitors were added 1h before the ligand at various concentrations: Curcumin (35μM), SP600125 (50μM), LY294002 (100μM), Apigenin (40μM), PD98059 (50μM), SB202190 (10μM). Cells were also treated with 3μg/ml of the control ligand 22(S)-hydroxycholesterol (denoted 22S). Western blot analysis was carried out using 20μg of whole cell extracts. Blotted membranes were incubated with anti-apoE primary antibody (goat polyclonal IgG) and detected using an anti-goat horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. The apoE protein level for each sample was determined by densitometric analysis and plotted on a bar chart. The control represents macrophages treated with vehicle (DMSO) only has been assigned as 1, with the apoE protein level for the remaining samples being represented relative to this control. The data shown is the mean ±SD from three independent experiments. The data was analysed by Student's t-test. * Represents the significant difference compared to control cells treated with 22(R)-hydroxycholesterol (*p<0.05).*

4.2.8. EFFECT OF 22(R)-HYDROXYCHOLESTEROL AND CELL SIGNALLING INHIBITORS ON ABCA1 AND ABCG1 MRNA EXPRESSION IN THP-1 MACROPHAGES

Given the involvement of the JNK/SAPK, PI3K and CK2 signalling pathways in the regulation of apoE, it was decided to investigate whether these pathways are also involved in the activation of other LXR target genes, such as ABCA1 and ABCG1. To analyse this, THP-1 cells were differentiated in 10% (v/v) delipidated HI-FCS with PMA (0.16μM) for 24h. After the 24h-differentiation period, inhibitors were added for 1h before ligand treatment, and 22(R)-hydroxycholesterol (2μg/ml) was then added to the appropriate flasks and left to incubate for a further 12h. Total RNA was then extracted (Section 2.4.1) and Taqman Real-Time quantitative RT-PCR was carried out using ABCA1-, ABCG1- and β-microtubulin-specific primers (Table 2.12) as described in Section 2.4.5 and reactions were monitored in real time mode using Sequence Detection Systems, version 1.7 software.

As shown in Figure 4.15 and Figure 4.16, 22(R)-hydroxycholesterol induced both ABCA1 and ABCG1 mRNA, to that of levels previously shown in several studies (Costet *et al.*, 2000; Whitney *et al.*, 2001; Venkateswaran *et al.*, 2000a; Venkateswaran *et al.*, 2000b). The results from these experiments also demonstrated that pre-treatment with the p38 MAPK inhibitor, SB202190 had no significant effect on ABCA1 or ABCG1 mRNA induction. In contrast, similar to their effects on apoE mRNA, the JNK/SAPK inhibitors, SP600125 and curcumin, both significantly reduced the ligand-mediated induction for both ABCA1 and ABCG1, as did apigenin (CK2 inhibitor). However, the PI3K inhibitor, LY294002 only had an inhibitory effect on the ligand-mediated induction of ABCG1 mRNA and had no effect on ABCA1 mRNA levels. In addition, similar to the result seen for LY294002, the ERK MAPK inhibitor, PD98059, only reduced the mRNA induction of ABCG1 and demonstrated no effect on ABCA1 mRNA levels.

4.2.9. EFFECT OF 22(R)-HYDROXYCHOLESTEROL AND CELL SIGNALLING INHIBITORS ON LXRα MRNA EXPRESSION IN THP-1 MACROPHAGES

The LXRα promoter is another important target of the LXRs and it was therefore decided to investigate the effect of these same inhibitors on the 22(R)-hydroxycholesterol-induction of LXRα. Total RNA was extracted from differentiated THP-1 cells treated with ligand and inhibitors for 12h and Taqman Real-Time quantitative RT-PCR was carried out using LXRα- and β-microtubulin-specific primers (Table 2.12) as described in Section 2.4.5.

As shown in Figure 4.17, 22(R)-hydroxycholesterol treatment results in a 5-fold induction in LXRα mRNA expression, which is attenuated by pre-treatment with inhibitors against the p38

MAPK, JNK MAPK, PI3K and CK2 cell signalling pathways. The only inhibitor that had no significant effect in this case was the ERK MAPK inhibitor, PD98059.

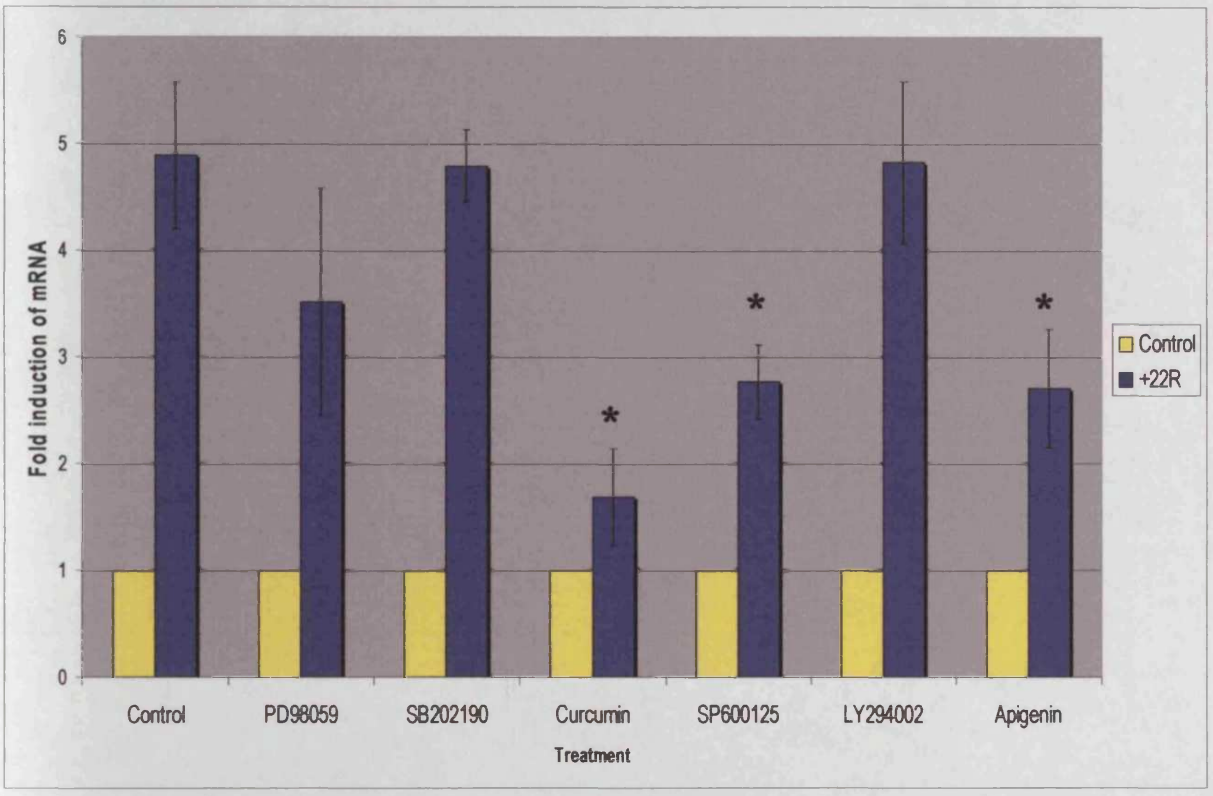


FIGURE 4.15. EFFECT OF CELL SIGNALLING INHIBITORS ON THE 22(R)-HYDROXYCHOLESTEROL-MEDIATED INDUCTION OF *ABCA1* mRNA EXPRESSION

*THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22R-hydroxycholesterol (denoted 22R) [2μg/ml] for 12h. In addition, differentiated cells were treated with vehicle (DMSO) in the place of ligand (denoted control). Inhibitors were added 1h before the ligand at various concentrations: PD98059 (50μM), SB202190 (10μM), Curcumin (25μM), SP600125 (50μM), LY294002 (100μM), Apigenin (40μM). Taqman Real-Time quantitative RT-PCR was carried out using ABCA1- and β-microtubulin-specific primers and reactions were monitored in real time mode using Sequence Detection Systems, version 1.7 software. The ABCA1:β-microtubulin ratio in macrophages treated with vehicle only has been assigned as 1, with the ratio for the remaining samples being represented relative to this control. The data shown is the mean ±SD from three independent experiments. The data was analysed by Student's t-test. * Represents the significant difference compared to control cells treated with 22(R)-hydroxycholesterol (*p<0.05).*

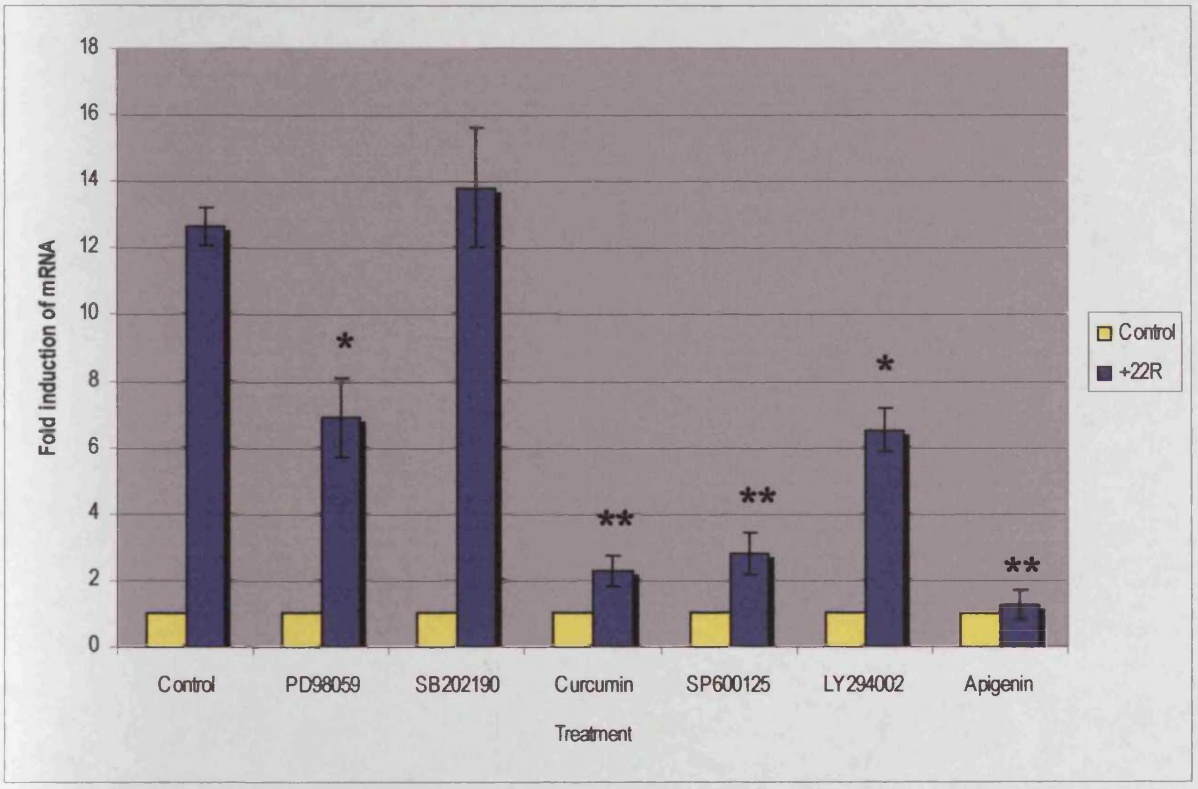


FIGURE 4.16. EFFECT OF CELL SIGNALLING INHIBITORS ON THE 22(R)-HYDROXYCHOLESTEROL-MEDIATED INDUCTION OF ABCG1 MRNA EXPRESSION

*THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22R-hydroxycholesterol (denoted 22R) [2μg/ml] for 12h. In addition, differentiated cells were treated with vehicle (DMSO) in the place of ligand (denoted control). Inhibitors were added 1h before the ligand at various concentrations: PD98059 (50μM), SB202190 (10μM), Curcumin (25μM), SP600125 (50μM), LY294002 (100μM), Apigenin (40μM). Taqman Real-Time quantitative RT-PCR was carried out using ABCG1- and β-microtubulin-specific primers and reactions were monitored in real time mode using Sequence Detection Systems, version 1.7 software. The ABCG1:β-microtubulin ratio in macrophages treated with vehicle only has been assigned as 1, with the ratio for the remaining samples being represented relative to this control. The data shown is the mean ±SD from three independent experiments. The data was analysed by Student's t-test. * Represents the significant difference compared to control cells treated with 22(R)-hydroxycholesterol (*p<0.05; **p<0.005).*

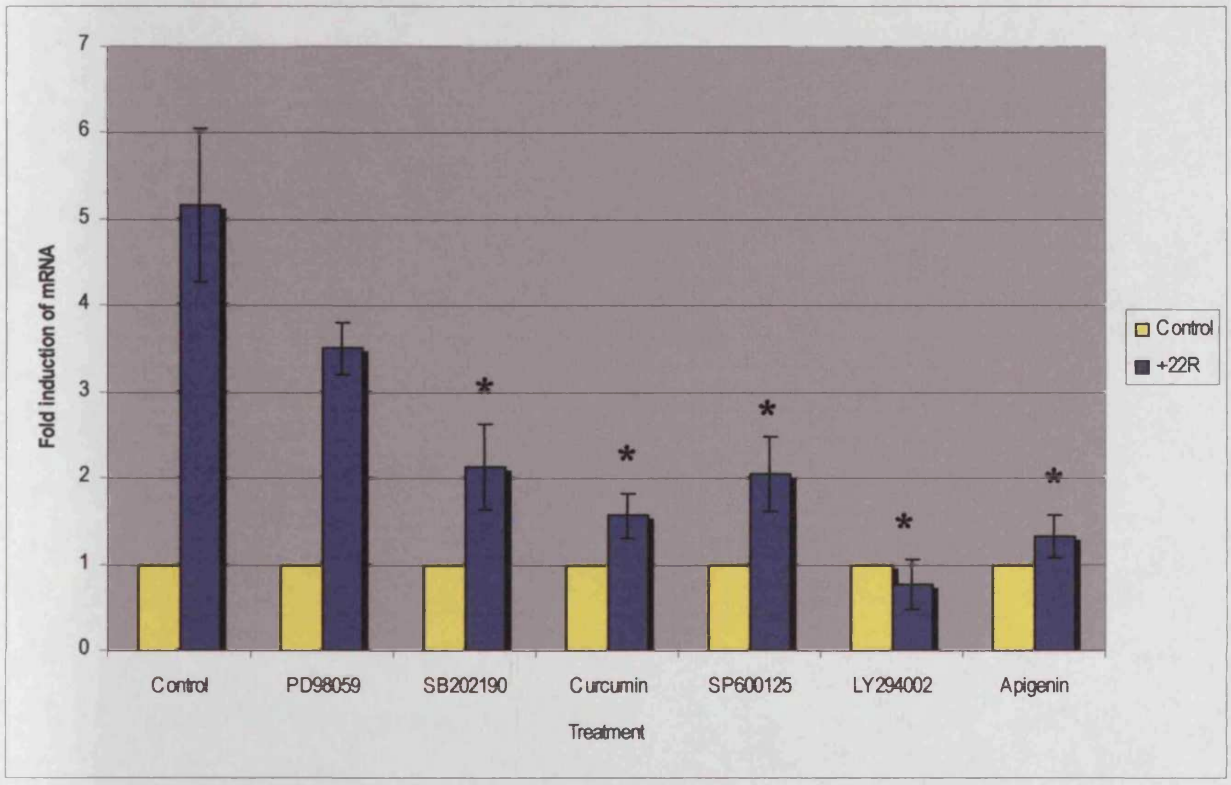


FIGURE 4.17. EFFECT OF CELL SIGNALLING INHIBITORS ON THE 22(R)-HYDROXYCHOLESTEROL-MEDIATED INDUCTION OF LXRα MRNA EXPRESSION

*THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22R-hydroxycholesterol (denoted 22R) [2μg/ml] for 12h. In addition, differentiated cells were treated with vehicle (DMSO) in the place of ligand (denoted control). Inhibitors were added 1h before the ligand at various concentrations: PD98059 (50μM), SB202190 (10μM), Curcumin (25μM), SP600125 (50μM), LY294002 (100μM), Apigenin (40μM). Taqman Real-Time quantitative RT-PCR was carried out using LXRα- and β-microtubulin-specific primers and reactions were monitored in real time mode using Sequence Detection Systems, version 1.7 software. The LXRα:β-microtubulin ratio in macrophages treated with vehicle only has been assigned as 1, with the ratio for the remaining samples being represented relative to this control. The data shown is the mean ±SD from three independent experiments. The data was analysed by Student's t-test. * Represents the significant difference compared to control cells treated with 22(R)-hydroxycholesterol (*p<0.05).*

4.3. DISCUSSION

Cell signalling pathways have been implicated in the transactivation function of several nuclear receptors either through direct phosphorylation of the nuclear receptor itself or through modification of coregulator proteins necessary for active transcription. This modification of nuclear receptor function by kinase cascades allows an additional mechanism for regulating the transactivation potential and the specificity of these transcription factors, and allows cross-talk to occur between nuclear receptors and cell signalling pathways. For example, there are several reports indicating that retinoic acid receptors (RARs) and other receptors can be phosphorylated by cyclin-dependant kinases (cdks) and that this phosphorylation is important for ligand-dependant transactivation (Rochette-Egly *et al.*, 1997; Rochette-Egly *et al.*, 1992; Taneja *et al.*, 1997). Furthermore, other nuclear receptors such as the estrogen receptors (ERs) are phosphorylated at serine or threonine residues by the mitogen-activated protein kinase (MAPK) *in vitro*, and in cells treated with growth factors that stimulate the Ras-MAPK cascade, and this phosphorylation enhances transcriptional activity (Kato *et al.*, 1995; Patrone *et al.*, 1996). PPARα is also modulated by phosphorylation by MAPK, and this phosphorylation enhances transcriptional activity (Juge-Aubry *et al.*, 1999). However, phosphorylation of the AF-1 domain of PPARγ by the same kinase negatively regulates its transcriptional functions. Interestingly, this modification reduces ligand binding to the receptor, showing that binding can be regulated by intramolecular communication between the modulatory domain and the C-terminal LBD (Shao *et al.*, 1998). In addition, MAPK-dependent phosphorylation of the RXR can also alter biological actions of a partner receptor (Solomon *et al.*, 1999). Therefore, as no studies have previously shown that cell signalling pathways are involved in the LXR-mediated regulation of genes, the overall aim of this chapter was to determine whether any pathways were involved with respect to the LXR-mediated induction of apoE expression.

Work by Laffitte *et al.* (2001b) demonstrated that the nuclear receptors LXRα and LXRβ and their oxysterol ligands were key regulators of lipid-inducible expression of apoE in both macrophages and adipose tissue. They also showed that the LXR/RXR heterodimer regulated apoE transcription directly, through interaction with a LXRE present in both ME1 and ME2. As this newly revised project was based on the ability of oxysterol ligands, to induce apoE transcription, it was necessary to ensure that the earlier data produced by Laffitte *et al.* (2001b), could be reproduced. It was therefore decided to examine the ability of the LXR ligand, 22(R)-hydroxycholesterol to increase apoE mRNA expression in macrophages (Figure 4.5). In addition to being extensively used in previous LXR studies (Laffitte *et al.*, 2001; Venkateswaran *et al.*, 2000b; Costet *et al.*, 2000; DeBose-Boyd *et al.*, 2001; Chiang *et al.*, 2001), 22(R)-hydroxycholesterol was used in this study due to its ability to specifically activate LXRs without crossreactivity with other known nuclear receptors (Janowski *et al.*, 1996). As shown in Figure 4.5, 22(R)-hydroxycholesterol

induced apoE mRNA expression, and this induction was also observed at the protein level (Figure 4.6). This induction of apoE protein levels by 22(R)-hydroxycholesterol was also shown to be time-dependent (Figure 4.7) with a maximum induction occurring at 24h. Ligand treatment of THP-1 macrophages also increased the level of secreted apoE (Figure 4.8), which is of potential therapeutic value as apoE secretion by macrophages is thought to be atheroprotective, not only through its role in the reverse cholesterol transport process but also due to its local anti-inflammatory and anti-proliferative effects on the surrounding cells of the vessel wall. Although the study by Laffitte *et al.* (2001b) did not investigate whether 22(R)-hydroxycholesterol induced apoE secretion, a study by Perez *et al.* (2003) has since shown, in agreement with this result, that the oxysterol ligand 22(R)-hydroxycholesterol increases apoE secretion in THP-1 macrophages. In addition to treatment with the LXR activator 22(R)-hydroxycholesterol, THP-1 cells were treated with S enantiomer of this ligand, 22(S)-hydroxycholesterol. Though this ligand binds to both subtypes of LXR competitively and with a high affinity *in vitro*, in cells it is completely inactive (Janowski *et al.*, 1996). As can be seen from Figure 4.5, 4.6 and 4.8, 22(S)-hydroxycholesterol had no effect at all on apoE, confirming that the induction of apoE expression seen with 22(R)-hydroxycholesterol, is due to LXR activation.

Confident that the ligand induction of apoE expression was well established, we investigated the action of inhibitors against key cell signalling pathways on this LXR-mediated induction of apoE expression. Several inhibitors were used, and the inhibitors against the JNK/SAPK MAPK, PI3K and CK2 signalling pathways, successfully blocked the ligand-mediated induction of apoE at the mRNA, protein and secreted protein level (Figure 4.10, 4.11, and 4.13). This inhibition was not due to any cytotoxic affect of the compound, as determined by trypan blue exclusion assays, and this inhibition with each compound could be seen to occur dose-dependently (Figure 4.12). Therefore, it can be concluded from these experiments that apoE induction in macrophages by the LXR family of transcription factors occurs through a JNK/CK2/PI3K-dependent manner. This is clearly a novel observation and requires further investigation.

Inhibitors against the ERK MAPK and p38 MAPK were also used, and were shown to have no affect on the induction of apoE expression at the mRNA and protein level, although an affect on apoE secretion (50% reduction) was seen by SB202190 inhibitor (Figure 4.13). This reduction in apoE secretion, could indicate that this LXR-mediated regulation of apoE secretion may have a post-translational component that is affected by the p38 MAPK pathway, although further work would be required to confirm this. The lack of inhibition by these inhibitors at the mRNA and protein level was not due the inhibitor themselves being inactive as the positive action of the SB202190 and PD98059 inhibitors used in this study have been confirmed by other studies in the laboratory. PD98059 (ERK inhibitor) was shown to successfully inhibit the PMA-induced induction of LPL

expression in THP-1 monocytes (Mead, 2002; Singh, 2003), whereas SB202190 (p38 inhibitor) has been shown to prevent the TGF-β-mediated induction of apoE mRNA in THP-1 monocytes (Singh, 2003). Thus, in contrast to the effect of inhibitors of the JNK, CK2 and PI3K pathways on the ligand-mediated induction of apoE expression, inhibitors of the ERK MAPK and p38 MAPK pathways had no effect, which implies that these pathways are not involved in the LXR-mediated regulation of apoE.

An important result of this chapter is the ability of the ligand 22(R)-hydroxycholesterol to induce apoE protein expression in primary human macrophages (Figure 4.14). This has not been shown previously and confirms that the LXR regulation of apoE may be of potential therapeutic value in the treatment of human atherosclerosis. In addition, the effect of the cell signalling inhibitors on this ligand-mediated induction of apoE was investigated in primary human macrophages. This confirmation of the results seen in the THP-1 cell line was necessary as there are many instances where there are differences in response to stimuli between primary monocytes/macrophages and cell lines. For example, with respect to the MAPK signalling pathways, LPS stimulation has been shown to activate all three branches of the MAPK cascade in primary human macrophages, yet only the JNK/SAPK branch is activated in the THP-1 cell line (Rao, 2001). Similar differences between cell lines and primary cultures are found in other kinase cascade systems, such as the PI3K pathway (Rao, 2001). Our results demonstrate that a similar mechanism of regulation may be involved in the LXR-mediated regulation of apoE in primary cells as the inhibitors against the JNK/SAPK and PI3K pathways blocked apoE induction by 22(R)-hydroxycholesterol in these cells also. Although it is important to note that a difference exists between primary cells and THP-1 macrophages with regards to CK2, as apigenin had no significant affect in primary human cells (Figure 4.14). Whilst this lack of inhibition by apigenin does imply that CK2 does not play an important role in the LXR-mediated induction in human primary monocyte-derived macrophages, further studies are essential to confirm this as an extensive dose-response experiment was not carried out with this inhibitor in primary cells due to limited amounts of material. Overall the results of these experiments indicates that there are similarities between the LXR-mediated regulation of apoE in human primary cells and the THP-1 cell line and indicates that the THP-1 cell line is a suitable model for further investigation into the LXR-mediated regulation of apoE and that the results obtained in this cell line are applicable to primary cell investigations.

A primary aim of this project is to gain a better understanding of LXR-regulated transcription. Although the focus gene of the project is apoE, LXR regulates several other genes of the reverse cholesterol transport pathway. Therefore we decided to investigate the effects of inhibitors on the 22(R)-hydroxycholesterol induction of ABCA1 (Figure 4.15) and ABCG1 (Figure 4.16), in order to see whether certain LXR target genes share a common mode of regulation

through LXR. From Figure 4.15, it can be seen that both the JNK and CK2 pathways are involved in the ligand induction of ABCA1, although the PI3K pathway does not seem to play a role. ABCG1 regulation by LXR seems to be very similar to that of apoE as all three pathways appear to be involved, however the ERK MAPK inhibitor PD98059 also appears to have a significant effect. In addition to ABCA1 and ABCG1, the effect of these inhibitors was investigated on the autoregulation function of LXRα. The LXRα induction, was successfully blocked by the inhibitors against the p38 MAPK, JNK/SAPK MAPK, PI3K and CK2 cell signalling pathways (Figure 4.17). The only inhibitor that had no effect in this case was the ERK MAPK inhibitor, PD98059. These results indicate that a common mode of regulation for these LXR target genes through cell signalling pathways may exist, although a few subtle differences have been identified. These minor differences between the LXR-mediated regulation of the various target genes may be of value in devising therapeutic approaches that could exploit these differences, allowing selective up-regulation of atheroprotective target genes without the unfortunate side-effect of triglyceride synthesis. In addition, these findings are consistent with other studies that have demonstrated that key LXR target genes have differential requirements with regards to coregulator recruitment (Wagner *et al.*, 2003).

In conclusion, the results in this chapter have successfully identified three signal transduction pathways (JNK/SAPK MAPK, PI3K and CK2) that may be involved in the LXR-mediated induction of apoE. This is novel data and provides a basis for the further investigation of these pathways in the regulation of apoE by the nuclear receptor LXR. The following chapters will look at each of these pathways in more detail.

CHAPTER FIVE – ROLE OF JNK/SAPK MAP KINASE SIGNALLING IN THE LXR-MEDIATED REGULATION OF APOE

5.1. INTRODUCTION

The results of the previous chapter have shown that the JNK/SAPK branch of the MAPK signalling cascade is involved in the LXR-mediated activation of apoE gene expression. As the JNK/SAPK pathway has not been described previously in this thesis, a brief description of the major components of this pathway is given below.

As mentioned earlier in Section 4.1, the JNK/SAPK subfamily belongs to the mitogen-activated protein kinase (MAPK) group. MAPKs are a large superfamily of evolutionary conserved enzymes connecting cell-surface receptors to critical regulatory targets within the cell. MAPK signalling pathways have been demonstrated to modulate gene expression through the specific phosphorylation and activation of a number of transcription factors. All MAPKs are "proline-directed" and phosphorylate Ser/Thr residues only if followed immediately by proline (Roux and Blenis, 2004).

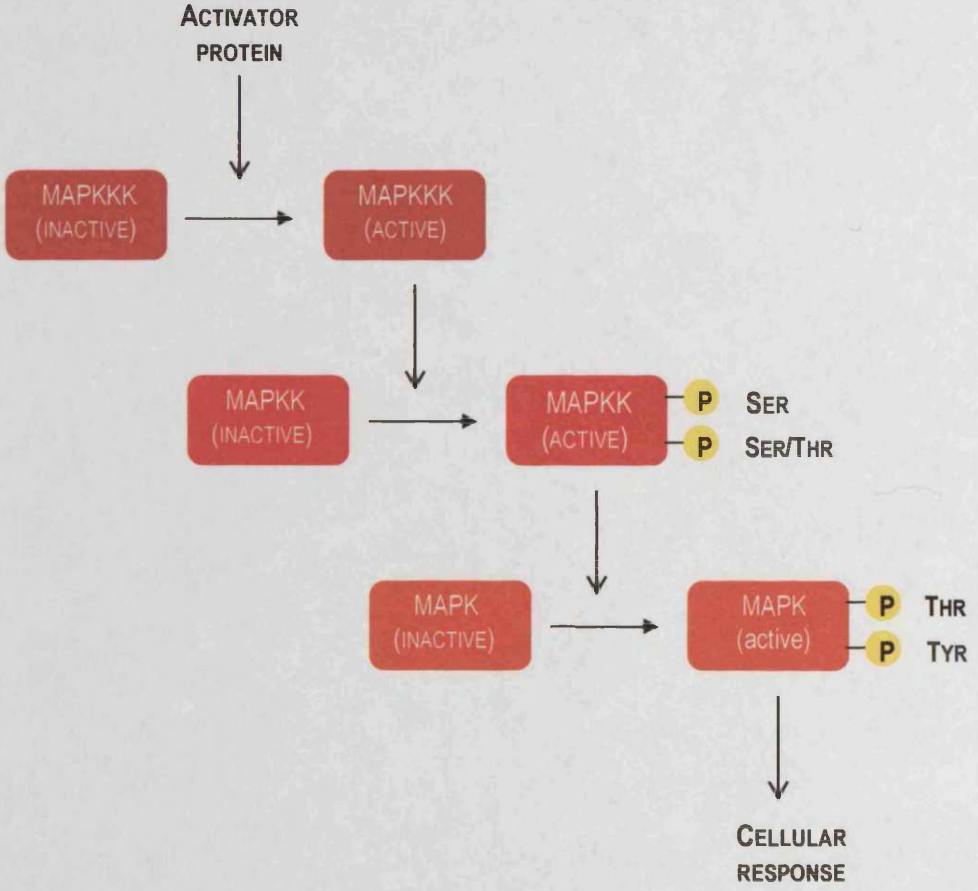


FIGURE 5.1. THE MAP KINASE CORE SIGNALLING MODULE

Various extracellular signals feed into the MAPK kinase kinase (MAPKKK)→ MAPK kinase→ MAPK core 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. The downstream targets of this signalling module then mediate the appropriate cellular response. Figure adapted from Kyriakis and Avruch, 2001.

All MAPK pathways are organised into core three-tiered signalling modules (Figure 5.1). Each MAPK is activated by dual phosphorylation of a tripeptide motif (Thr-Xaa-Tyr) located in the activation loop (T-loop) [Kyriakis and Avruch, 2001]. This phosphorylation is catalysed by a family of dual specificity kinases, MAPK kinases (MAPKKs), which are, in turn, regulated by Ser/Thr phosphorylation, catalysed by any of several protein kinase families collectively referred to as MAPK-kinase-kinases (MAPKKK). Upstream of MAPKKKs, MAPK core signalling modules are themselves regulated by a wide variety of upstream activators and inhibitors (Herskowitz, 1995; Kyriakis and Avruch, 2001; Marshall, 1995). In mammals, several MAPK subfamilies have been identified (Schaeffer and Weber, 1999) and each of these MAPK groups are respectively integrated into different signal transduction pathways and show differences in substrate specificity. The ERK and p38 groups of MAPK are related to enzymes found in budding yeast and contain the dual phosphorylation motifs Thr-Glu-Tyr and Thr-Gly-Tyr, respectively, whilst the c-jun N-terminal kinases (JNK), also known as the stress-activated protein kinases (SAPKs), represent a third group of MAPKs and contains the dual phosphorylation motif Thr-Pro-Tyr.

The JNK/SAPKs were initially identified by their ability to bind and phosphorylate the amino-terminal domain of the c-jun transcription factor in response to UV stimulation, and have since been shown to be activated by proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), and by environmental stress (Hibi *et al.*, 1993; Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994; Minden *et al.*, 1994). Phosphorylation of the JNK/SAPKs is mediated by the MAPKKs, MKK4/SEK1 and MKK7, both of which are present in the cytoplasm and also the nucleus (Tournier *et al.*, 1999). The upstream components of MAPKK appear to be cell type and stimuli dependent. Several MAPKKKs have been reported to activate the JNK/SAPK signalling pathway. These include members of the MEKK group (MEKK1-4), the mixed-lineage protein kinase group (MLK1, MLK2, MLK3, DLK and LZK), the apoptosis signal-regulating kinase group (ASK1 and ASK2), TGF-β-activated kinase (TAK) and TPL-2 (Figure 5.2). In most cases *in vitro* experimental data has demonstrated that these MAPKKKs phosphorylate and activate MKK4 and/or MKK7, although, it has not been established whether these protein kinases are physiological regulators of the JNK/SAPK pathway *in vivo,* and it is also unclear which MAPKKKs are relevant to specific physiological stimuli. Further upstream are the Ste-20-like MAPKKKKs such as the p21-activated kinases (PAKs), germinal centre kinase (GCK) and histidine protein kinase (HPK). Just how these kinases are regulated remains to be fully elucidated, but roles have been suggested for adaptor proteins (e.g. Nck, Crk, Shc or TNF-receptor-associated factors) and/or small GTP-binding proteins of the Rho and Rac families (Kyriakis, 1999).

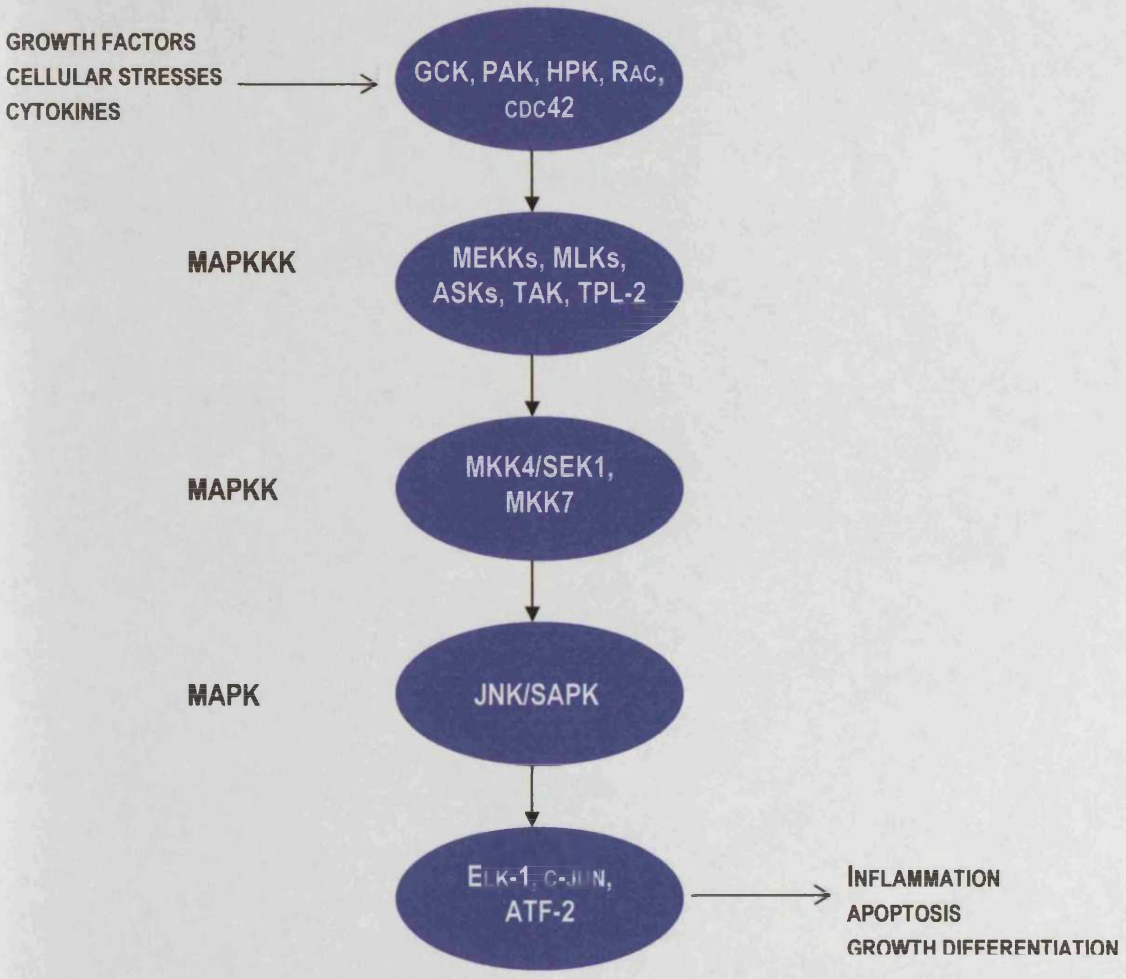


FIGURE 5.2. SCHEMATIC REPRESENTATION OF THE JNK/SAPK MAPK PATHWAY

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.

JNK/SAPK has several other downstream targets in addition to c-jun, many of which, like c-jun are components of the AP-1 complex. AP-1 is a heterodimer comprised of bZIP transcription factors, typically c-jun and JunD, along with members of the fos (usually c-fos) and ATF (usually ATF-2) families. All bZIP transcription factors contain leucine zippers that enable homo- and heterodimerisation, and AP-1 components are organised into jun-jun, jun-fos, or jun-ATF dimers (Karin *et al.*, 1997). Activation of AP-1 involves both, the direct phosphorylation or dephosphorylation of AP-1 components, as well as the phosphorylation and activation of transcription factors that induce elevated expression of c-jun or c-fos. Phosphorylation of c-jun or ATF-2 within their N-terminal transactivation domains by JNK/SAPK correlates with enhanced transactivating activity (Derijard *et al.*, 1994; Gupta *et al.*, 1995; Kyriakis *et al.*, 1994; Pulverer *et al.*, 1991). The Elk-1 transcription factor is another direct JNK/SAPK target (Cavigelli *et al.*, 1995),

which is involved in the induction of the c-fos gene, thereby indirectly effecting AP-1 activity (Treisman, 1995).

The involvement of cell signalling pathways in the nuclear receptor regulation of target genes has been discussed in the previous chapter. Although we have shown that inhibitors of the JNK/SAPK pathway are able to inhibit the ligand-mediated induction of apoE and other LXR target genes (Chapter 4), it is necessary to investigate how this pathway may potentially effect LXR signalling. The possibility exists that any one of the components of the JNK/SAPK pathway may be affecting the LXR transcription complex. The most straightforward and likely hypothesis behind the involvement of the JNK/SAPK pathway in LXR transcription would be the phosphorylation of the LXR protein or one of the coregulator components of the transcription complex, which would result in an increase in transcriptional activation of the target genes. This mechanism of involvement of cell signalling pathways in nuclear receptor signalling has been described extensively in the previous chapter and the JNK/SAPK pathway has been shown to directly phosphorylate several nuclear receptors including PPARγ (Adams *et al.*, 1997; Hu *et al.*, 1996; Camp *et al.*, 1999), GR (Rogatsky *et al.*, 1998) and RXR (Lee *et al.*, 2000).

Another hypothesis for the involvement of the JNK/SAPK pathway in LXR transcription would be through the activation of one of the various downstream transcription factors, such as the AP-1 complex. Recent work in keratinocytes has demonstrated that LXR activators induce a general increase in expression of AP-1-regulated genes (Schmuth *et al.*, 2004). In addition several other nuclear hormone receptors have been shown to alter transcription in various other cell types through the AP-1 response element (Uht *et al.*, 1997). This involvement of the AP-1 complex may occur through several mechanisms including interaction with the nuclear receptors or coregulators directly, binding to AP-1 elements at the promoters of coregulators or target genes, or even through the binding to AP-1 elements at the promoters of the nuclear receptors. Whether any of these mechanisms are involved in the regulation of apoE transcription by the LXRs remains to be determined and will be discussed further in Section 5.3.

Therefore, the aim of this chapter was to confirm whether the LXR ligand 22(R)-hydroxycholesterol activates the JNK/SAPK pathway in THP-1 macrophages and to elucidate which components of the pathway are involved in this LXR-mediated regulation of apoE.

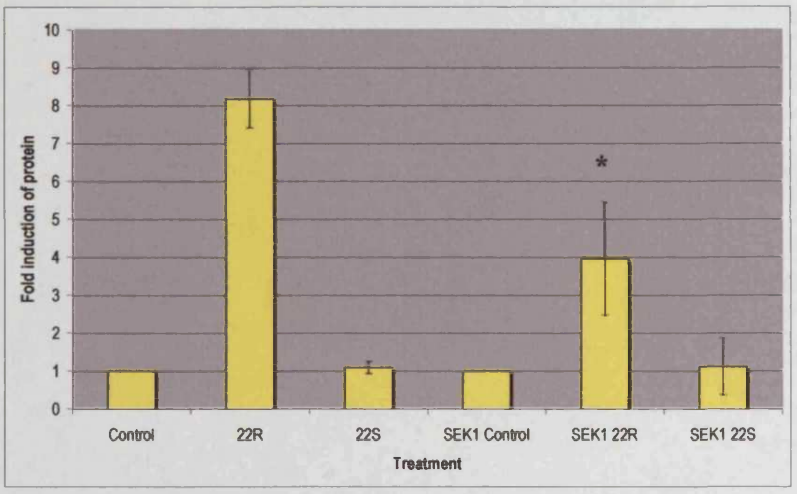
5.2. RESULTS

5.2.1. EFFECT OF DOMINANT NEGATIVE EXPRESSION PLASMIDS ON THE LXR-MEDIATED INDUCTION OF APOE IN THP-1 MACROPHAGES

In order to provide further, independent, confirmation of the results obtained from the previous chapter, which demonstrated that the JNK/SAPK pathway was involved in the LXR regulation of apoE, it was decided to use dominant negative expression plasmids for the MKK4/SEK1 MAPKK and the JNK/SAPK MAPK. The cDNA of the enzymatically inactive form of SEK1 was kindly provided by J.R. Woodgett (Ontario Cancer Institute), whilst the dominant negative JNK/SAPK plasmid, SAPKα-VPF, in which the phosphorylation sites Thr-Pro-Tyr were changed to Val-Pro-Phe, was a gift from E.Nishida (Kyoto university).

Transfections were carried out in THP-1 monocytes and the DNA constructs were transfected using the Effectene™ transfection reagent (Qiagen), as described in Section 2.3.5.2. PMA (0.16μM) was added immediately after the transfection of cells was complete and the cells were left to differentiate for 24h. After the 24h incubation, cells were treated with 2μg/ml of 22(R)-hydroxycholesterol or 22(S)-hydroxycholesterol for a further 24h, after which whole cell protein extracts were prepared (see Section 2.5.1). Of each sample, 20μg of total protein was subjected to SDS-PAGE (Section 2.5.4) and then transferred to a PVDF membrane (Section 2.5.5). The membrane was then incubated with an anti-apoE primary antibody (Biogenesis) and immunodetection was carried out using the appropriate secondary antibody and the ECL detection system (Amersham) as described in Section 2.5.6. In addition to the dominant negative constructs, as a control, cells were transfected with the empty $pcDNA_3$ vector (Appendix IV) to ensure that the results obtained were due to the expression of the dominant negative and not the plasmid or the transfection reagent.

From Figure 5.3, it can be seen that both the SEK1 and the JNK/SAPK dominant negative plasmids result in a 50% decrease in the induction of apoE by 22(R)-hydroxycholesterol. This reduction can be attributed to the presence of the dominant negative construct as the induction in the apoE protein levels in the control cells transfected with the empty vector remains similar to that seen in previous experiments (Chapter 4). Therefore, this result is consistent with the data obtained with curcumin and SP600125 in the previous chapter and clearly confirms a role for the JNK/SAPK pathway in the LXR-mediated regulation of apoE. Also the reduction of the apoE induction by the SEK1 dominant negative construct, allows us to speculate that this kinase is an upstream component involved in the JNK/SAPK regulation of the LXR- mediated induction of apoE.



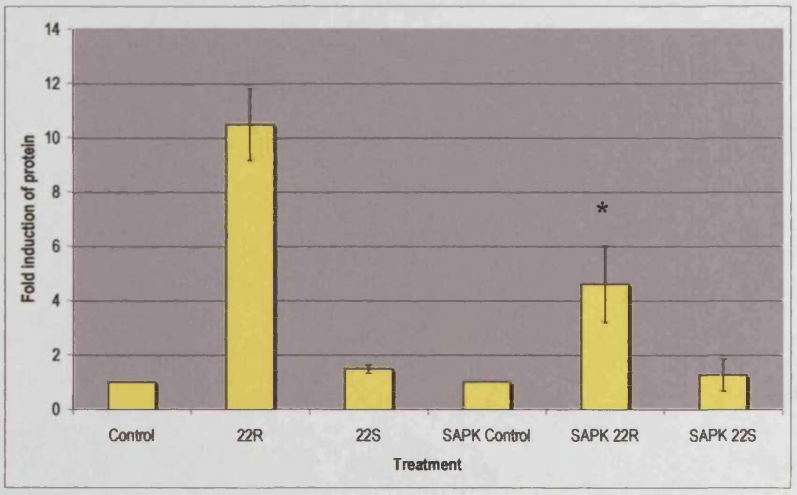


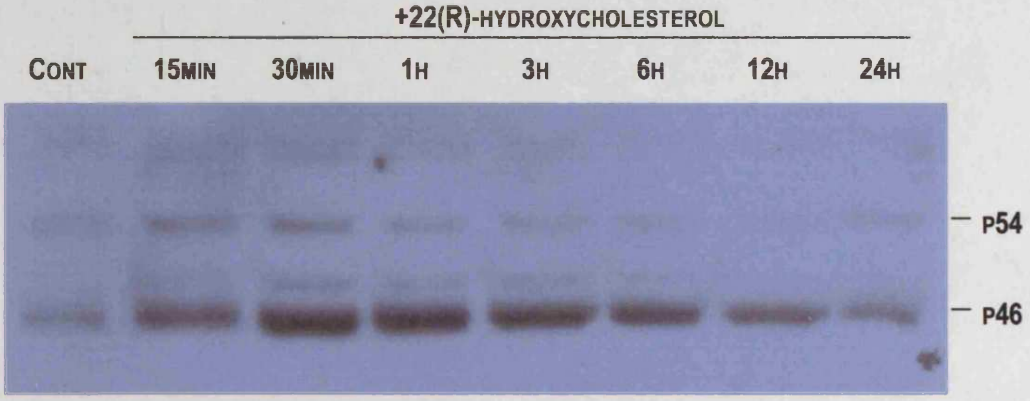
FIGURE 5.3. EFFECT OF DOMINANT NEGATIVE EXPRESSION PLASMIDS ON THE LXR-MEDIATED INDUCTION OF APOE IN THP-1 MACROPHAGES

*THP-1 monocytes were transfected (see section 2.3.5.2) with the indicated expression plasmids or the control vector and differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h. The LXR ligands, 22(R)-hydroxycholesterol (denoted 22R) [2μg/ml] or 22(S)-hydroxycholesterol (denoted 22S) [2μg/ml] were then added to the cells for a further 24h. Western blot analysis was carried out using 20μg of whole cell extracts. Blotted membranes were incubated with anti-apoE primary antibody (goat polyclonal IgG) and detected using an anti-goat horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. The apoE protein level for each sample was determined by densitometric analysis and plotted on a bar chart. The control represents macrophages treated with vehicle (DMSO) in the place of ligand and has been assigned as 1 in each case, with the apoE protein level for the remaining samples being represented relative to this control. The data shown is the mean ±SD from three independent experiments. The data was analysed by Student's t-test. * Represents the significant inhibition compared to pcDNA3 transfected cells treated with 22(R)-hydroxycholesterol (*p<0.05).*

5.2.2. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON JNK/SAPK PHOSPHORYLATION IN THP-1 MACROPHAGES

In order to fully confirm a role for the JNK/SAPK pathway in the regulation of apoE by LXR, it was necessary to investigate whether the ligand, 22(R)-hydroxycholesterol activates this pathway. The JNK/SAPK pathway is a phosphorylation cascade and the various components of the pathway become active once phosphorylated, therefore it was decided to ensure that the main component of this pathway, JNK/SAPK itself is in fact phosphorylated in response to ligand treatment. JNK/SAPK phosphorylation by upstream MAPKKs, such as SEK1 occurs on the dual phosphorylation motif, Thr183-Pro-Tyr185 and recognition of this phosphorylated form of JNK/SAPK can be accomplished through western blot analysis. Antibodies that specifically recognise this phosphorylated form of JNK/SAPK are commercially available and have been extensively used to investigate the role of JNK/SAPK signalling in macrophages (Waetzig *et al.*, 2002; Wang *et al.*, 2002; Dreskin *et al.*, 2001).

Whole cell extracts from differentiated THP-1 cells treated with 22(R)-hydroxycholesterol for various time-points were isolated using the phosphatase-free isolation buffer as described in Section 2.5.1, and western blot analysis (Section 2.5.4, 2.5.5 and 2.5.6) was carried out using the anti-phospho-JNK/SAPK antibody with overnight incubation. As shown in Figure 5.4, 22(R)-hydroxycholesterol appears to induce JNK/SAPK phosphorylation. This induction was dependent on ligand treatment and occurred in a time-dependent manner. Ligand-mediated phosphorylation of the p46 isoform peaked with a 4.5-fold induction at 30min and returned to baseline levels after 24h. The following studies presented in this chapter will also demonstrate that this induction was not due to *de novo* protein synthesis as no change in total protein occurred with ligand treatment, and that this induction was dependent on LXR activation as the inactive enantiomer of 22(R)-hydroxycholesterol, 22(S)-hydroxycholesterol did not induce JNK/SAPK activation (Section 5.2.5).



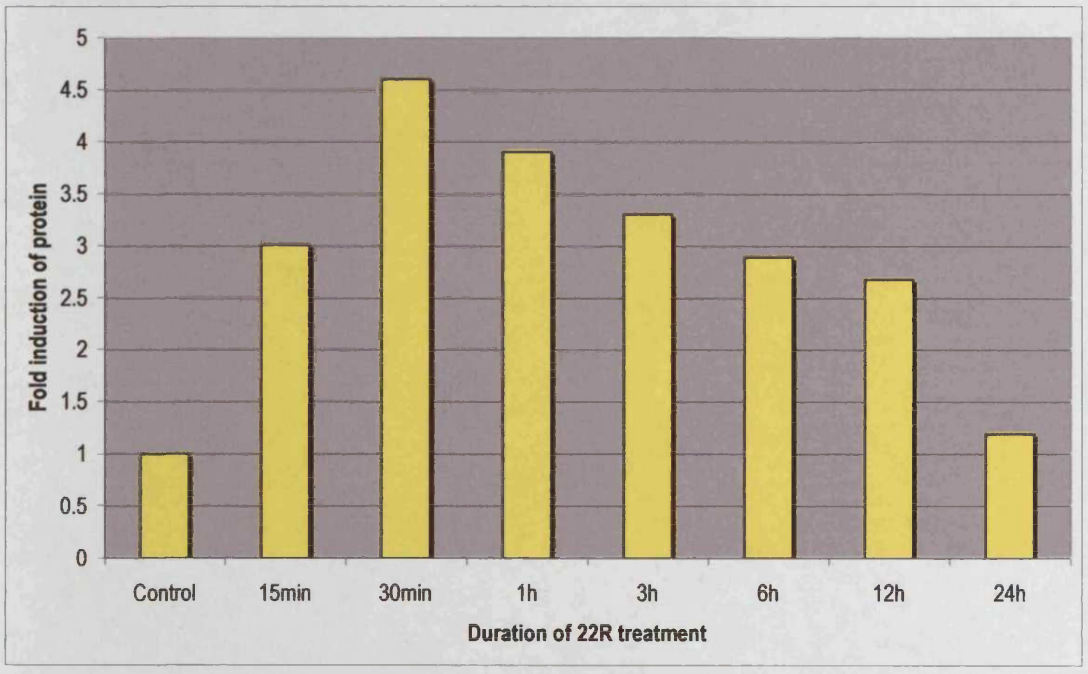


FIGURE 5.4. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON JNK/SAPK PHOSPHORYLATION IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22(R)-hydroxycholesterol (denoted 22R) [2μg/ml] for the indicated time-points. Western blot analysis was carried out using 80μg of whole cell extracts. Blotted membranes were incubated with phospho-JNK/SAPK (Thr183/Tyr185) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. The control sample represents differentiated THP-1 cells treated with vehicle (DMSO) only, in the place of ligand and was taken at the 15min time-point in this experiment. The p46 phosphorylated protein level for each sample was determined by densitometric analysis and plotted on a bar chart. The control (DMSO-treated cells) has been assigned as 1, with the phosphorylated level for the remaining samples being represented relative to this control. Results are representative of three separate experiments.

5.2.3. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON JNK/SAPK ACTIVITY IN THP-1 MACROPHAGES

Although protein phosphorylation is an excellent marker of activity, another method of measuring JNK/SAPK activity is to determine the kinase activity directly from cell extracts. JNK/SAPK activation can be routinely measured by protein kinase activity towards the transcription factor substrate c-jun. In this study, a non-radioactive method of measuring JNK/SAPK activity was employed based on the JNK/SAPK Assay Kit (NEB). Differentiated THP-1 cells were treated with 22(R)-hydroxycholesterol or DMSO for 30min and whole cell extracts were isolated using the phosphatase-free isolation buffer (Section 2.5.1). The kinase assay was carried out as described in Section 2.6.3, whereby a c-jun fusion protein linked to agarose beads is used to "pull down" the JNK/SAPK enzyme from whole cell extracts. The isolated JNK/SAPK enzyme, upon addition of a kinase buffer and ATP, phosphorylates the c-jun substrate and this reaction mix was then subjected to SDS-PAGE, whereby the phospho-c-jun (Ser63) antibody was used to measure JNK/SAPK activity. The phospho- c-jun (Ser63) antibody used detects c-jun only when catalytically activated by phosphorylation at Ser63.

From Figure 5.5, it can be seen that JNK/SAPK isolated from 22(R)-hydroxycholesterol treated THP-1 macrophages phosphorylates c-jun to a greater extent (2-3-fold) than JNK/SAPK obtained from control cells treated with DMSO. These results are consistent with that seen previously for JNK/SAPK phosphorylation by the ligand and clearly demonstrates the ability of 22(R)-hydroxycholesterol to activate the JNK/SAPK pathway.

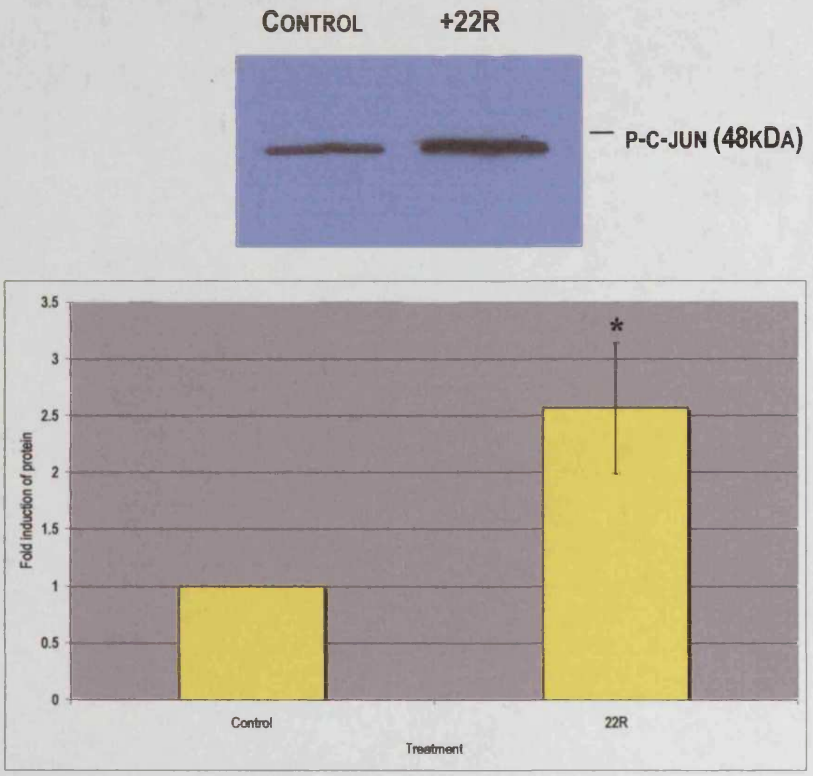


FIGURE 5.5. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON JNK/SAPK ACTIVITY IN THP-1 MACROPHAGES

*THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22(R)-hydroxycholesterol (denoted 22R) [2μg/ml] for 30min. The kinase assay was carried out using 250μg of whole cell extracts as described in Section 2.6.3 and blotted membranes were incubated with phospho-c-jun (Ser63) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. The c-jun phospho-protein level for each sample was determined by densitometric analysis and plotted on a bar chart. The control represents macrophages treated with vehicle (DMSO) in the place of ligand and has been assigned as 1, with the phospho-protein level for the ligand-treated sample being represented relative to this control. The data shown is the mean ±SD from three independent experiments. The data was analysed by Student's t-test. * Represents the significant difference compared to control cells treated with DMSO (*p<0.05).*

5.2.4. EFFECT OF CURCUMIN AND SP600125 ON THE LXR-MEDIATED INDUCTION OF JNK/SAPK ACTIVITY IN THP-1 MACROPHAGES

In order to confirm that the inhibition of the 22(R)-hydroxycholesterol-mediated induction of apoE by the JNK/SAPK inhibitors was due to an inhibition in JNK/SAPK activity, we next investigated the effect of curcumin and SP600125 on the ligand-mediated induction of JNK/SAPK kinase activity.

Whole cell extracts were isolated using the phosphatase-free isolation buffer (Section 2.5.1) from differentiated THP-1 cells that were pre-treated with inhibitors for 1h before the addition of 22(R)-hydroxycholesterol for 30min. The JNK/SAPK kinase assay was then carried out as described in Section 2.6.3. From Figure 5.6a, it can be seen that both curcumin and SP600125

inhibited the induction in JNK/SAPK activity caused by ligand treatment. This inhibition by the JNK/SAPK inhibitors is consistent with the previously observed result, whereby curcumin and SP600125 prevent the ligand-mediated induction of apoE and therefore confirms that activation of JNK pathway is essential in the LXR-mediated regulation of apoE. Also included in this experiment was the inactive ligand, 22(S)-hydroxycholesterol, which did not effect JNK/SAPK activity, indicating that 22(R)-hydroxycholesterol induction of the JNK/SAPK pathway requires LXR activation.

Although unlikely, there is a possibility that the increased activity of JNK/SAPK in 22(R)-hydroxycholesterol-treated cells may be the result of *de novo* protein synthesis. Therefore, to rule out this possibility the whole cell lysates used for the kinase assays were subjected to western blot analysis using an antibody which detects the endogenous levels of total JNK/SAPK protein. As can be seen from Figure 5.6b, both the control and 22(R)-hydroxycholesterol-treated cells showed similar steady-state protein levels. Also from these results it can be seen that the effect of the inhibitors was mainly due to an inhibition of JNK/SAPK activity and not due to an effect on the endogenous levels of JNK/SAPK, although a slight reduction in the total protein of the p46 JNK/SAPK isoform was observed in cells pre-treated with curcumin.

5.2.5. EFFECT OF LY294002 AND APIGENIN ON THE LXR-MEDIATED INDUCTION OF JNK/SAPK ACTIVITY IN THP-1 MACROPHAGES

Given that the inhibitors against the PI3K and CK2 pathways also inhibit the apoE induction by 22(R)-hydroxycholesterol, it was decided to investigate whether these inhibitors affected apoE levels indirectly through the JNK/SAPK pathway. Both PI3K and CK2 have been shown to function as upstream components for the JNK/SAPK pathway and inhibitors which target these pathways may, therefore, inhibit JNK/SAPK activity (Go *et al.*, 1998; Assefa *et al.*, 1999; Eom *et al.*, 2001; Fleming *et al.*, 2000).

Whole cell extracts were isolated using the phosphatase-free isolation buffer (Section 2.5.1) from differentiated THP-1 cells that were pre-treated with LY294002 or apigenin for 1h before the addition of ligand for 30min. The JNK/SAPK kinase assay was then carried out as described in Section 2.6.3.

Figure 5.7a shows that 22(R)-hydroxycholesterol induced the JNK/SAPK activity as shown previously and demonstrates that neither LY294002 nor apigenin had a significant effect on this induction of JNK/SAPK activity or on the endogenous JNK/SAPK protein levels (Figure 5.7b). This result clearly indicates that neither the PI3K, nor the CK2 enzymes are acting upstream of JNK/SAPK and that the JNK/SAPK pathway is independent to these pathways in the LXR-mediated regulation of apoE.

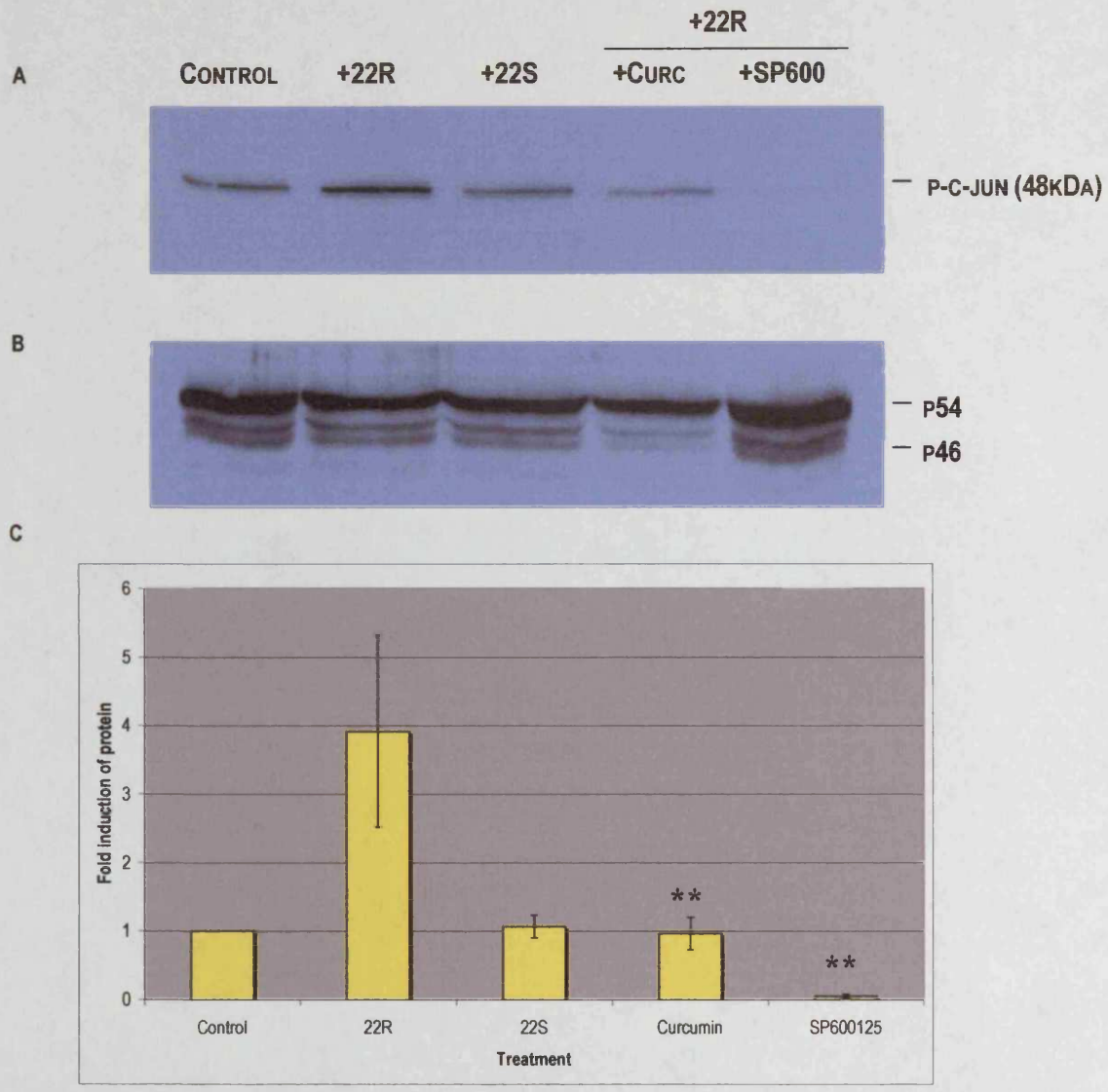


FIGURE 5.6. EFFECT OF CURCUMIN AND SP600125 ON THE LXR-MEDIATED INDUCTION OF JNK/SAPK ACTIVITY IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligands, 22(R)-hydroxycholesterol (denoted 22R){2μg/ml] or 22(S)-hydroxycholesterol (denoted 22S) [2μg/ml] for 30min. In addition, cells were treated with the vehicle, DMSO in the place of ligand (denoted control). Inhibitors were added 1h before the ligand at various concentrations: Curcumin (25μM), SP600125 (50μM). **A** *The kinase assay was carried out using 250μg of whole cell extracts as described in Section 2.6.3 and blotted membranes were incubated with phospho-c-jun (Ser63) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody.* **B** *Western blot analysis was carried out using 80μg of whole cell extracts. Blotted membranes were incubated with total-JNK/SAPK primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system.*
C *The c-jun phospho-protein and total JNK level for each sample was determined by densitometric analysis and the c-jun:total protein for each sample has been represented relative to the control, which has been assigned as 1 and plotted on a bar chart. The data shown is the mean ±SD from three independent experiments. The data was analysed by Student's t-test. ** Represents the significant inhibition compared to control cells treated with 22(R)-hydroxycholesterol (**p<0.005).*

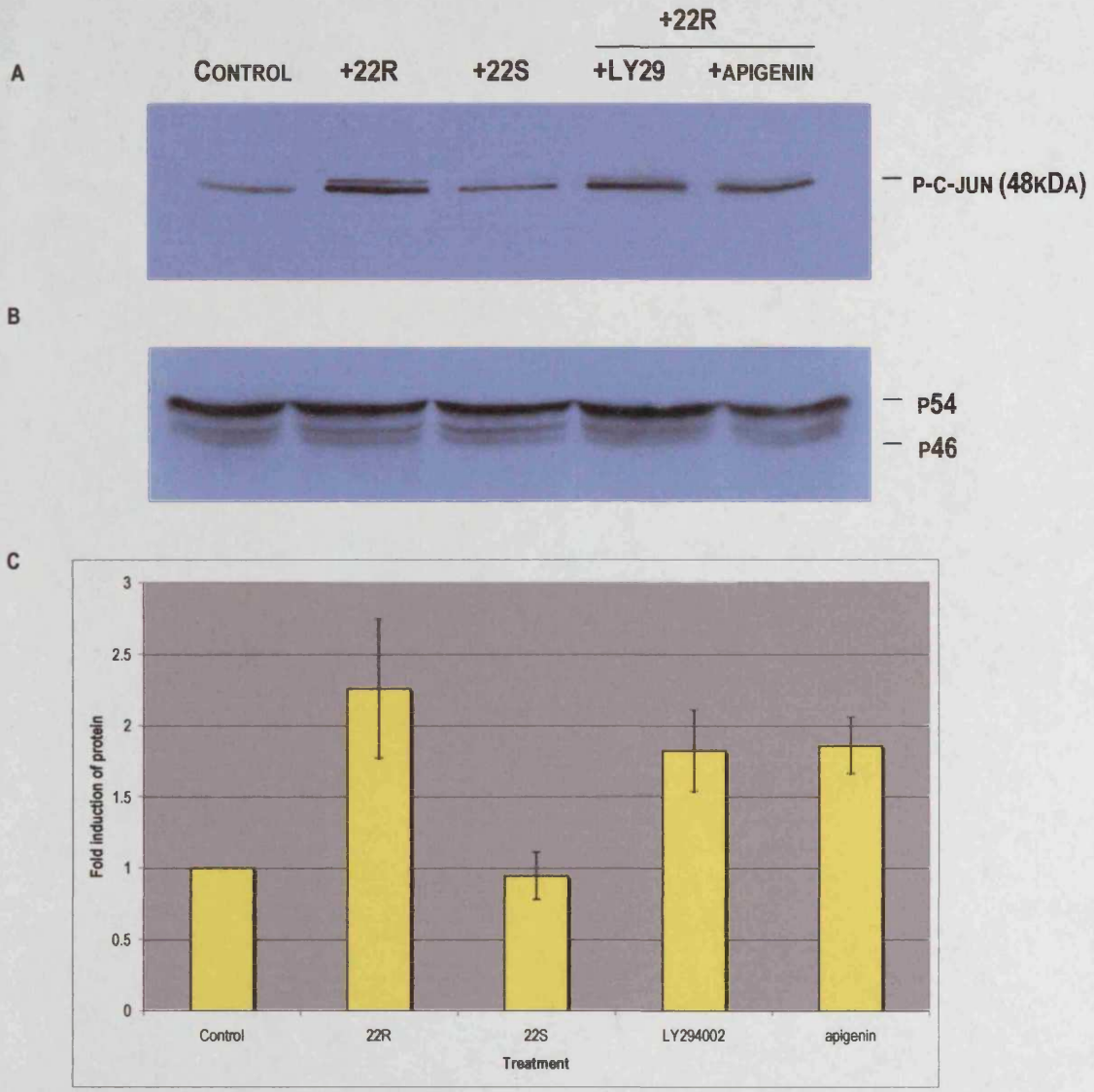


FIGURE 5.7. EFFECT OF LY294002 AND APIGENIN ON THE LXR-MEDIATED INDUCTION OF JNK/SAPK ACTIVITY IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligands, 22(R)-hydroxycholesterol (denoted 22R){2μg/ml] and 22(S)-hydroxycholesterol (denoted 22S) [2μg/ml] for 30min. In addition, cells were treated with the vehicle, DMSO in the place of ligand (denoted control). Inhibitors were added 1h before the ligand at various concentrations: LY294002 (25μM), apigenin (40μM). A *The kinase assay was carried out using 250μg of whole cell extracts as described in Section 2.6.3 and blotted membranes were incubated with phospho-c-jun (Ser63) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody.* B *Western blot analysis was carried out using 80μg of whole cell extracts. Blotted membranes were incubated with total-JNK/SAPK primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of three separate experiments.* C *The c-jun phospho-protein and total JNK level for each sample was determined by densitometric analysis and the c-jun:total protein for each sample has been represented relative to the control, which has been assigned as 1 and plotted on a bar chart. The data shown is the mean ±SD from three independent experiments. The data was analysed by student's t-test.*

5.2.6. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON C-JUN PHOSPHORYLATION IN THP-1 MACROPHAGES

The results from the previous sections have clearly demonstrated that the LXR-mediated transcription of apoE requires the activity of JNK/SAPK. Exactly how this cell signalling pathway is involved in the LXR-mediated regulation of apoE is unclear. As described previously cell signalling pathways may effect nuclear receptors regulation of target genes through various methods. Therefore, in order to gain a better understanding of the role of the JNK/SAPK pathway in LXR signalling it was decided to investigate which downstream target, if any was being activated by JNK/SAPK.

One of the best-known substrates of the JNK/SAPK MAPKs is c-jun, the major component of the AP-1 complex (Bohmann *et al.* 1987; Angel *et al.* 1988). c-jun is phosphorylated by the JNK/SAPKs on Ser63 and Ser73 and this phosphorylation enhances its transcriptional activity (Pulverer *et al.* 1991; Smeal *et al.* 1991). Antibodies that specifically recognise the phosphorylated form of c-jun are commercially available therefore c-jun phosphorylation can be investigated by western blot analysis.

For these experiments whole cell extracts were isolated using the phosphatase-free isolation buffer (Section 2.5.1) from differentiated THP-1 cells that were treated with 22(R)-hydroxycholesterol for 30min. Total c-jun proteins were immunoprecipitated from whole cell lysates (Section 2.5.3) and were subjected to western blot analysis (Section 2.5.4, 2.5.5 and 2.5.6) using the anti-phospho-c-jun (Ser63) antibody with overnight incubation. As shown in Figure 5.8, 22(R)-hydroxycholesterol induced c-jun phosphorylation after only 30min. This induction was ligand specific as the following studies presented in this chapter demonstrate that the inactive enantiomer of 22(R)-hydroxycholesterol, 22(S)-hydroxycholesterol did not induce c-jun phosphorylation (Section 5.2.8).

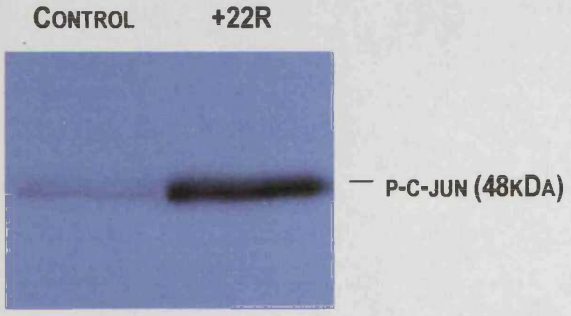


FIGURE 5.8. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON C-JUN PHOSPHORYLATION IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22R-hydroxycholesterol (denoted 22R) [2μg/ml] for 30min. In addition, cells were treated with the vehicle, DMSO in the place of ligand (denoted control). Immunoprecipitation of whole cell extracts (250μg) was carried out using a total-c-jun antibody as described in Section 2.5.3 and the isolated c-jun proteins were subjected to western blot analysis. Blotted membranes were incubated with a phospho-c-jun (Ser63) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of three separate experiments.

5.2.7. EFFECT OF CURCUMIN AND SP600125 ON THE LXR-MEDIATED PHOSPHORYLATION OF C-JUN IN THP-1 MACROPHAGES

In order to confirm that the inhibition of the 22(R)-hydroxycholesterol-mediated induction of apoE by the JNK/SAPK inhibitors was due to an inhibition in c-jun activity, we next investigated the effect of curcumin and SP600125 on the ligand-mediated phosphorylation of c-jun. Whole cell extracts were isolated using the phosphatase-free isolation buffer (Section 2.5.1) from differentiated THP-1 cells that were pre-treated with inhibitors for 1h before the addition of 22(R)-hydroxycholesterol for 30min. Total c-jun proteins were immunoprecipitated from whole cell lysates (Section 2.5.3) and were subjected to western blot analysis (Section 2.5.4, 2.5.5 and 2.5.6) using the anti-phospho-c-jun antibody with overnight incubation. The results in Figure 5.9, demonstrate, as shown in the previous section, that 22(R)-hydroxycholesterol treatment of THP-1 macrophages results in c-jun phosphorylation, and that both, curcumin and SP600125 are able to inhibit this induction. This inhibition by the JNK/SAPK inhibitors is consistent with the previously observed result, whereby curcumin and SP600125 prevent the ligand-mediated induction of apoE and JNK/SAPK activity and therefore confirms that activation of c-jun is essential in the LXR-mediated regulation of apoE. Also included in this experiment was the inactive ligand, 22(S)-hydroxycholesterol, which had no effect on c-jun phosphorylation, which indicates that this phosphorylation of c-jun is mediated through the activation of LXR.

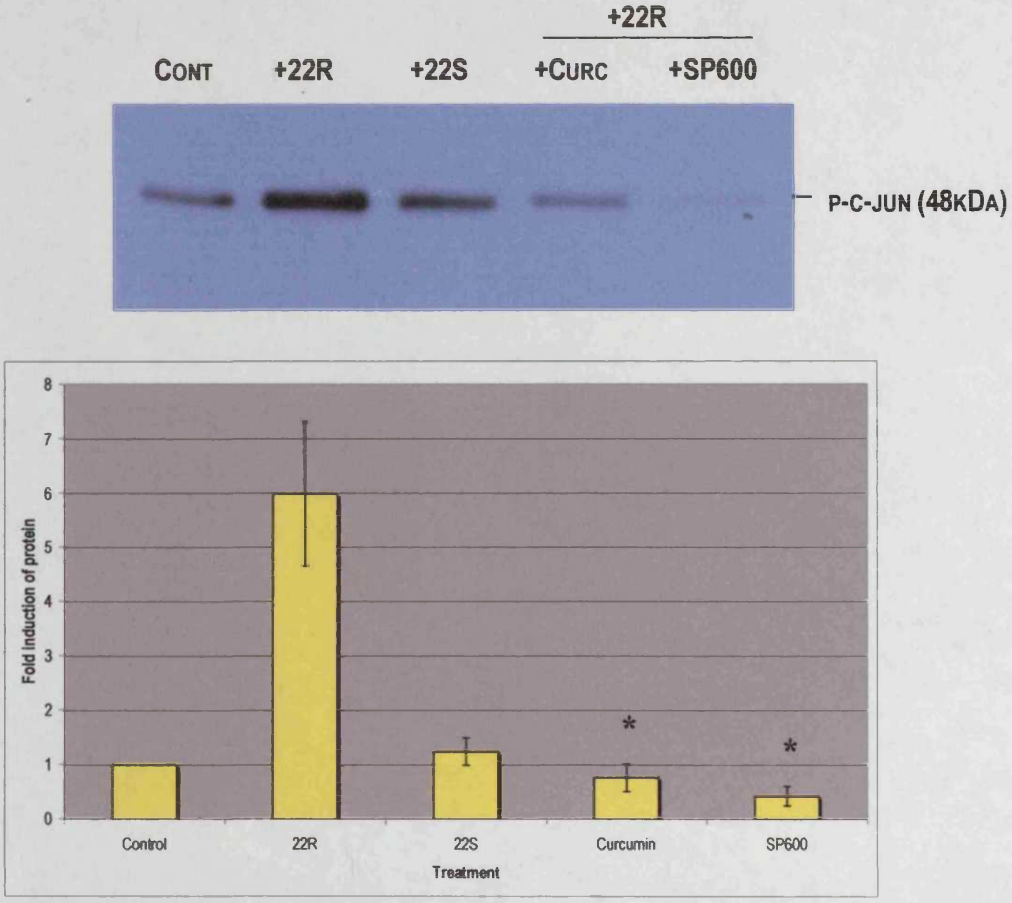


FIGURE 5.9. EFFECT OF CURCUMIN AND SP600125 ON THE LXR-MEDIATED PHOSPHORYLATION OF C-JUN IN THP-1 MACROPHAGES

*THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligands, 22(R)-hydroxycholesterol (denoted 22R) [2μg/ml] and 22(S)-hydroxycholesterol (denoted 22S) [2μg/ml] for 30min. Inhibitors were added 1h before the ligand at various concentrations: Curcumin (25μM), SP600125 (50μM). Immunoprecipitation of whole cell extracts (250μg) was carried out using a total-c-jun antibody as described in Section 2.5.3 and the isolated c-jun proteins were subjected to western blot analysis. Blotted membranes were incubated with a phospho-c-jun (Ser63) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. The c-jun phospho-protein level for each sample was determined by densitometric analysis and plotted on a bar chart. The control represents macrophages treated with vehicle (DMSO) in the place of ligand and has been assigned as 1, with the phospho-protein level for the ligand-treated sample being represented relative to this control. The data shown is the mean ±SD from three independent experiments. The data was analysed by Student's t-test. * Represents the significant inhibition compared to control cells treated with 22(R)-hydroxycholesterol (*p<0.05).*

5.2.8. EFFECT OF LY294002 AND APIGENIN ON THE LXR-MEDIATED PHOSPHORYLATION OF C-JUN IN THP-1 MACROPHAGES

Cross-talk between different signalling pathways is relatively common, and although the PI3K and CK2 pathway inhibitors did not affect the JNK/SAPK pathway upstream or at the level of JNK/SAPK, it is possible that the PI3K and CK2 pathways may converge with the JNK/SAPK pathway at the level of c-jun as both CK2 and downstream components of the PI3K pathway have been shown to activate c-jun in a JNK-independent manner (Pinna *et al.*, 1995; Lin *et al.*, 1992; Li *et al.*, 2004; Peron *et al.*, 2004). Therefore, in order to rule out the possibility that the effect of LY294002 and apigenin on the ligand-mediated induction of apoE was mediated through the inhibition of c-jun phophorylation, the effect of these inhibitors on c-jun phosphorylation was investigated.

Whole cell extracts were isolated using the phosphatase-free isolation buffer (Section 2.5.1) from differentiated THP-1 cells that were pre-treated with inhibitors for 1h before the addition of 22(R)-hydroxycholesterol for 30min. Total c-jun proteins were immunoprecipitated from whole cell lysates (Section 2.5.3) and were subjected to western blot analysis (Section 2.5.4, 2.5.5 and 2.5.6) using the anti-phospho-c-jun antibody with overnight incubation.

From Figure 5.10, it can be seen that neither LY294002 nor apigenin had an affect on c-jun phosphorylation, which is consistent with the results of previous experiments in this chapter, whereby these inhibitors had no affect on JNK activity (Figure 5.7). This lack of inhibition by LY294002 and apigenin at the level of c-jun phosphorylation confirms once again that the inhibition of the ligand-mediated induction of apoE seen by these inhibitors does not occur through the JNK/SAPK pathway and that this pathway, in this instance is independent from the PI3K and CK2 pathways in its involvement in the LXR regulation of apoE.

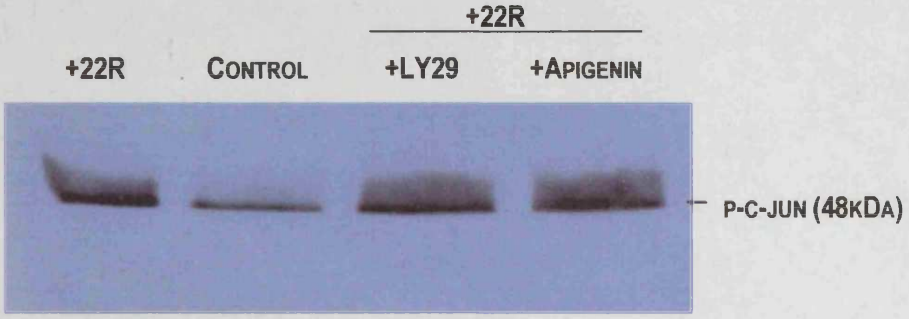


FIGURE 5.10. EFFECT OF LY294002 AND APIGENIN ON THE LXR-MEDIATED PHOSPHORYLATION OF C-JUN IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligands, 22(R)-hydroxycholesterol (denoted 22R) [2μg/ml] and 22(S)-hydroxycholesterol (denoted 22S) [2μg/ml] for 30min. In addition, cells were treated with the vehicle, DMSO in the place of ligand (denoted control). Inhibitors were added 1h before the ligand at various concentrations: LY294002 (25μM), apigenin (40μM). Immunoprecipitation of whole cell extracts (250μg) was carried out using a total-c-jun antibody as described in Section 2.5.3 and the isolated c-jun proteins were subjected to western blot analysis. Blotted membranes were incubated with a phospho-c-jun (Ser63) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of two separate experiments.

5.2.9. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON ATF-2 PHOSPHORYLATION IN THP-1 MACROPHAGES

JNK/SAPKs can also phosphorylate ATF-2 at Thr69 and Ser71, which activates the *trans*-activating activity of this protein (Gupta *et al.*, 1995). ATF-2 heterodimerises with c-jun, forming an AP-1 complex and also stimulates the expression of the c-jun gene (van Dam *et al.*, 1995). It was therefore decided to investigate whether 22(R)-hydroxycholesterol treatment also resulted in the phosphorylation of this member of the AP-1 family of transcription factors. Antibodies that specifically recognise the phosphorylated form of ATF-2 are commercially available therefore phosphorylation of this transcription factor can be investigated by western blot analysis.

Differentiated THP-1 cells were treated with the oxysterol ligand, 22(R)-hydroxycholesterol for various time-points and whole cell extracts were isolated using the phosphatase-free isolation buffer as described in Section 2.5.1. Western blot analysis (Section 2.5.4, 2.5.5 and 2.5.6) was carried out using the anti-phospho-ATF-2 antibody with overnight incubation. Treatment of THP-1 macrophages with 22(R)-hydroxycholesterol did not change the level of phosphorylation of ATF-2 (Figure 5.11). This result rules out a role for the ATF-2 transcription factor in the LXR-mediated regulation of apoE.

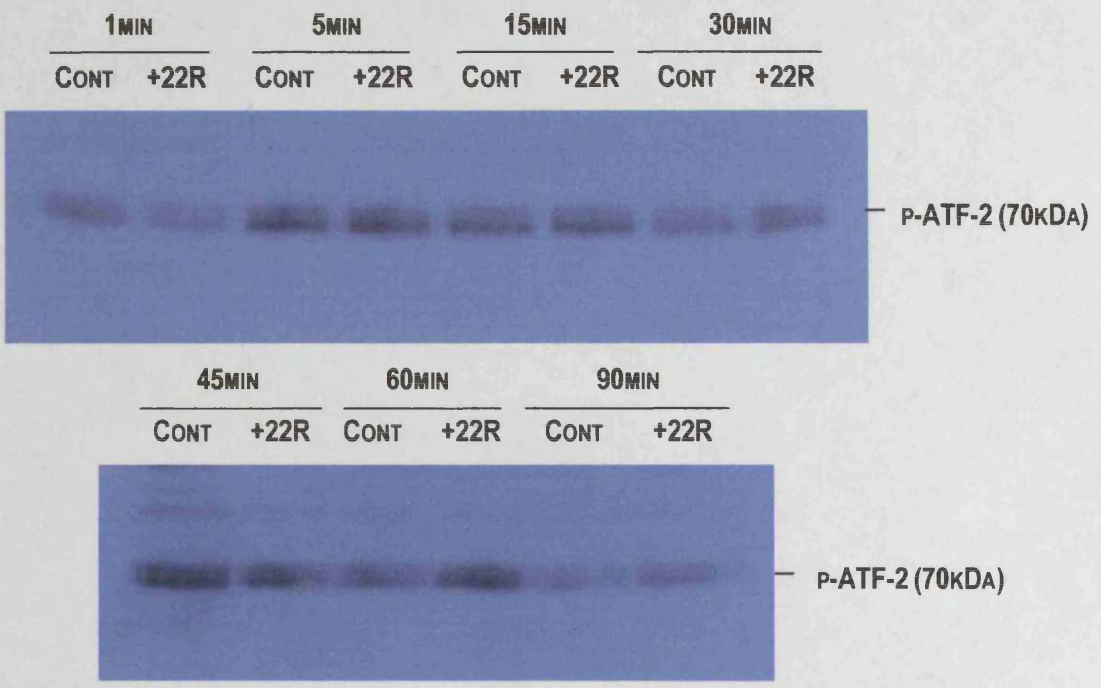


FIGURE 5.11. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON ATF-2 PHOSPHORYLATION IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22R-hydroxycholesterol (denoted 22R) [2μg/ml] for the indicated time-points. As a control at each time-point, cells were treated with the vehicle, DMSO in the place of ligand. Western blot analysis was carried out using 80μg of whole cell extracts. Blotted membranes were incubated with phospho-ATF-2 (Thr71) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of two separate experiments.

5.3. DISCUSSION

The results from the previous chapter indicated a role for the JNK/SAPK pathway in the LXR-mediated regulation of apoE. Therefore, the aim of this chapter was to investigate this pathway in more detail and confirm its involvement in apoE regulation. Data from Chapter 4 also demonstrated that the JNK/SAPK pathway was involved in the LXR-mediated induction of other target genes, such as ABCA1, ABCG1 and LXRα itself. Therefore, the data presented in this chapter may also have a wide-spread relevance with regards to LXR regulation of a range of genes.

The initial experiments of this chapter confirmed through the use of SEK1 and JNK/SAPK dominant negative constructs that the JNK/SAPK pathway is essential for the induction of apoE by the LXR agonist 22(R)-hydroxycholesterol (Figure 5.3). These experiments also identified that SEK1 MAPKK activity in addition to JNK/SAPK MAPK activity was essential in the oxysterol induction of apoE. The following experiments, whereby JNK/SAPK phosphorylation and activation were investigated, also confirmed the role of the JNK/SAPK pathway in the LXR regulation of apoE, and demonstrated that treatment of macrophages with the oxysterol ligand 22(R)-

hydroxycholesterol, induced both phosphorylation (Figure 5.4) and the activity (Figure 5.5) of this enzyme after only 30min. In addition the results from these experiments confirmed that the induction of the JNK/SAPK pathway was due to LXR activation as 22(S)-hydroxycholesterol, which binds but does not activate the receptor had no affect on JNK/SAPK activity (Figure 5.6a). These results have therefore demonstrated a novel mechanism, whereby 22(R)-hydroxycholesterol is able to rapidly activate JNK phosphorylation and kinase activity in a ligand-specific manner that requires LXR activation and does not involve *de novo* protein synthesis.

The rapid kinetics in which the JNK/SAPK pathway is activated by 22(R)-hydroxycholesterol in this study suggests that the activation of this pathway by LXR ligands is not dependent on gene activation. This ability of a nuclear receptor agonist to rapidly activate a cell signalling pathway independent of gene transcription is a recently characterised phenomenon, termed non-genomic or extranuclear signalling. These rapid actions of agonists have been described to generate intracellular second messengers, to activate or inhibit cell signalling pathways and modulate ion channels, and studies have indicated in part that this initiation of intracellular signalling mechanisms occurs through the nuclear receptor agonist binding to a receptor present at the plasma membrane or in the cytoplasm (Schmidt *et al.*, 2000; Boonyaratanakornkit and Edwards, 2004). It may be that a subpopulation of nuclear receptors mediates these actions by somehow associating with the cell membrane and/or signalling complexes in the cytoplasm (Schmidt *et al.*, 2000). Although studies have also suggested the potential existence of separate membrane receptors unrelated to the classical nuclear receptors which are responsible for this non-genomic action of nuclear receptor ligands (Boonyaratanakornkit and Edwards, 2004).

Although no such function has previously been described for the LXR subfamily of nuclear receptors, the non-genomic action for several other members of the nuclear receptor family has been well-characterised. For example, the human progesterone receptor (PR) has been shown to mediate rapid progestin activation of the Src/Ras/Raf/MAPK signalling pathway in mammalian cells, this is thought to occur through a direct interaction between a motif located in the N-terminal domain of the receptor and the Src homology 3 domain of Src tyrosine kinases (Migliaccio *et al.*, 1998; Boonyaratanakornkit *et al.*, 2001). Additionally, a novel membrane protein unrelated to nuclear PR was recently identified that has properties of a G-protein-coupled receptor for progesterone and has been shown to be involved in mediating the non-genomic signalling actions of progesterone that promotes oocyte maturation in fish (Boonyaratanakornkit and Edwards, 2004).

Another nuclear receptor agonist that has several rapid non-genomic signalling actions is estrogen. Estrogen induces an increase in the intracellular cGMP level and the release of nitric oxide within a few minutes of administration in endothelial cells (Caulin-Glaser *et al.*, 1997). Also,

estrogen activates the ERK MAPK pathway within 15min (Singh *et al.*, 1999). These actions appear to be mediated via a novel membrane-bound estrogen receptor. A protein termed modulator of non-genomic activity of ER (MNAR) has been identified that acts as an adaptor between ER and Src, and thus provides a mechanism for coupling of estrogen and ER with rapid-estrogen-induced activation of Src and the downstream MAPK signalling cascades. Consistent with these findings, bovine serum albumin-conjugated estrogen, which is impermeable to cell membranes, has been reported to activate the ERK pathway in endothelial cells (Russell *et al.*, 2000) and in a neuroblastoma cell line (Watters *et al.*, 1997). Recent work has also established a non-genomic mechanism of gene regulation for PPARγ, whereby its ligand, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-d-PGJ2) resulted in enhanced AP-1 binding activity in VSMCs (Takeda *et al.*, 2001). This induction of AP-1 activity occurred within 30min and was shown to be due to activation of the ERK pathway, which occurred in the presence of actinomycin D and was partially dependent on PI3K.

Therefore from the results presented here and from the recent advancements in the understanding of nuclear receptor signalling it is possible to conclude that the LXR-mediated regulation of apoE requires the non-genomic activation of the JNK/SAPK pathway by 22(R)-hydroxycholesterol in addition to the 22(R)-hydroxycholesterol-mediated activation of the LXR heterodimer at the apoE promoter and enhancer sequences (Laffitte *et al.*, 2001b). As no previous work has investigated a non-genomic role for LXR agonists, it is unclear how the ligand is activating the JNK/SAPK pathway. It may be possible that a membrane-bound LXR receptor exists or perhaps 22(R)-hydroxycholesterol is behaving independently of LXR as a signalling molecule, either suggestion requires further investigation, although a membrane-bound receptor is the most probable mechanism as our studies have demonstrated that JNK/SAPK activation is ligand-specific and requires LXR activation as 22(S)-hydroxycholesterol did not activate the JNK/SAPK pathway (Figure 5.6a).

Having concluded that oxysterol ligand treatment of THP-1 macrophages results in an increase in JNK/SAPK phosphorylation and kinase activity, experiments were carried out to investigate whether any downstream targets of this pathway are involved in LXR-mediated regulation of apoE. As described earlier in this chapter, c-jun is a major downstream target of the JNK/SAPK pathway and heterodimerises with other transcription factors, such as c-fos and ATF-2 to form the AP-1 complex. Oxysterols and other nuclear receptor agonists have been shown to affect AP-1 activity in several cell lines and a recent study demonstrated that oxysterols induce specific AP-1 proteins during keratinocyte differentiation, which is due to LXR activation (Schmuth *et al.*, 2004). Therefore, c-jun phosphorylation was investigated and shown to be induced by 22(R)-hydroxycholesterol treatment (Figure 5.8).

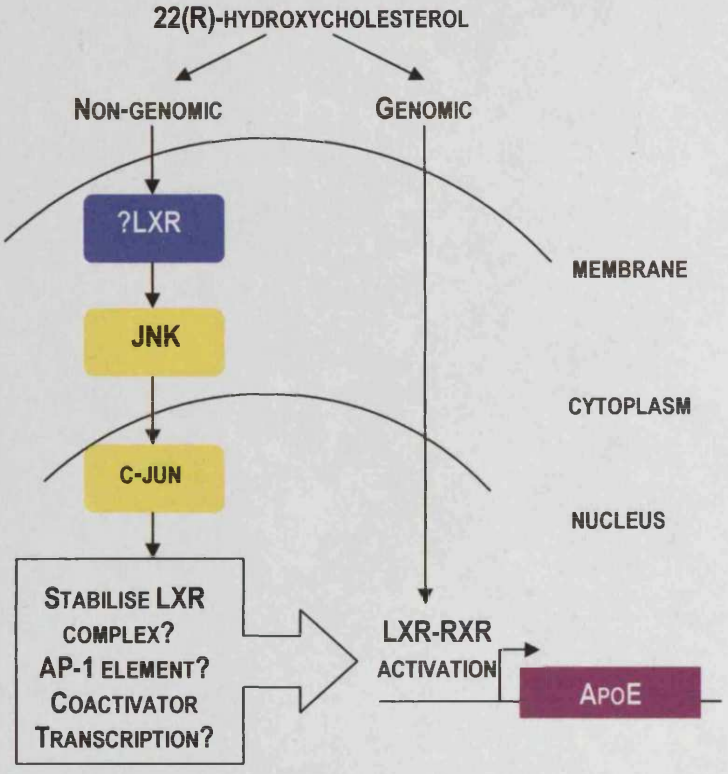


FIGURE 5.12. SCHEMATIC REPRESENTATION OF THE INVOLVEMENT OF THE JNK/SAPK PATHWAY IN THE LXR-MEDIATED INDUCTION OF APOE. *See text for further details*

Although the original hypothesis on the involvement of cell signalling pathways was that the LXRs or coregulators were being targeted for phosphorylation, the activation of a transcription factor by 22(R)-hydroxycholesterol suggests the existence of an alternative mechanism in the regulation of apoE by the LXRs (Figure 5.12). It is possible that c-jun may be interacting with the LXR transcriptional complex at the apoE promoter and this has been shown previously to occur in estrogen signalling, whereby ERα, c-jun, and the p160 coactivator GRIP1 form a multiprotein complex *in vitro* and in intact cells, and the ERα-c-Jun interaction is crucial for the stability of this complex (Teyssier *et al.*, 2001). Another potential mechanism for the role of c-jun in LXR signalling is that this transcription factor, as part of the AP-1 complex is activating apoE through the AP-1 element of the apoE promoter. 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 also play a role in the LXR activation of apoE. This requirement for the AP-1 element in the LXR-mediated induction of apoE may be due to the low-affinity of the LXRE present in the proximal promoter of apoE (Laffitte *et al.*, 2001b), and the AP-1 transcription factor may therefore be required for the full stimulatory response of the apoE promoter to oxysterol ligands. Indeed, such a mechanism has recently been demonstrated for the rat SREBP-1c promoter, which requires in the

addition to a LXRE, the binding sites for Sp1, nuclear factor Y (NF-Y) and SREBP itself for full activation of the SREBP-1c promoter by insulin (Cagen *et al.*, 2004).

Perhaps the most likely mechanism is that c-jun is upregulating the expression of genes encoding coactivator proteins necessary for the LXR activation of apoE. Recently a model for the farnesoid X receptor (FXR) regulation of the CYP7A1 gene has been proposed, which also involves the JNK/SAPK pathway (Gupta *et al.*, 2001). In this model, bile acids activate JNK/SAPK and c-jun, in addition to FXR. Both FXR and c-jun bind through different promoter elements, IR-1 and AP-1 respectively, and enhance the transcription of the SHP-1 corepressor, which in turn represses *CYP7A1* transcription. This model could potentially be applied to the LXR regulation of apoE, with the JNK/SAPK pathway inducing a coactivator protein. This mechanism may also explain the delay between the apoE induction at 24h (Chapter 4) and the induction of c-jun at 30min.

Therefore, in summary, the results of this chapter have shown conclusively that c-jun is being phosphorylated by the JNK/SAPK pathway in response to ligand treatment and that inhibition of this phosphorylation of c-jun, eliminates the induction of apoE gene expression by 22(R)-hydroxycholesterol. We have also shown that this induction of the JNK/SAPK pathway occurs after only 30min of treatment, which may be due to a non-genomic action of the ligand. Although this rapid induction of the c-jun transcription factor may have one of several roles in the LXR-mediated regulation of apoE (Figure 5.12), it is clear that the overall function of this pathway is to facilitate the LXR-mediated transcription of apoE.

CHAPTER SIX- ROLE OF PI3KINASE AND CK2 SIGNALLING IN THE LXR-MEDIATED REGULATION OF APOE

6.1. INTRODUCTION

In addition to the JNK pathway, the data presented in Chapter 4 clearly demonstrates a role for the PI3K and CK2 pathways in the LXR-mediated regulation of apoE. As with the JNK/SAPK pathway neither of these signalling pathways has been described previously in this thesis, therefore a brief description of the main components of these pathways is given below.

Phosphoinositide 3-kinases (PI3Ks) are a large family of intracellular signal transducers that catalyse the formation of specific inositol lipid mediators in response to external stimuli (Auger *et al.*, 1989; Rameh and Cantley, 1999). PI3Ks phosphorylate the 3'-OH position of the inositol ring in inositol phospholipids, generating 3'-phosphoinositides and *in vivo* PI3Ks produce three lipid products namely PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3, all of which are able to initiate signalling cascades (Toker, 2000). Through the initiation of signalling pathways PI3Ks play a central role in numerous cellular processes including cell growth, differentiation, motility, insulin action, and cell survival to name a few (Toker, 2000). Furthermore, PI3Ks control more specialised enzyme systems such as NADPH oxidase in leukocytes and nitric oxide synthase in endothelial cells (Wymann *et al.*, 2003). Thus, PI3Ks modulate several physiological processes and are involved in the pathology of various diseases including inflammatory and cardiovascular disease (Cantley, 2002).

Members of the PI3K family are divided into three classes according to their structure and *in vivo* substrate specificities and the predominant form of PI3K is a member of the class I subfamily of PI3K enzymes, and comprises of the p85α regulatory subunit, and the p110 catalytic subunit (Carpenter and Cantley, 1990). Class I PI3Ks can be activated by either receptor tyrosine kinase (RTK) or G-protein-coupled receptors (GPCR) and once activated these PI3Ks generate PtdIns(3,4,5)P_3 leading to the recruitment and activation of protein kinases that contain specialised lipid-binding domains such as the pleckstrin homology (PH) domain. Conversely, PI3K production of phosphoinositides is antagonised by the protein phosphatase and tensin homologue deleted on chromosome 10 (PTEN) tumour suppressor. PTEN possesses 3'-phosphoinositide phosphatase activity, and hydrolyses PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2 *in vitro* and *in vivo*, which results in down-regulation of PI3K signalling pathways (Maehama and Dixon, 1998; Myers *et al.*, 1998; Stambolic *et al.*, 1998; Leslie and Downes, 2002).

One of the best characterised targets of the PI3K lipid products is Akt, a 60kDa Ser/Thr kinase with a PH domain that preferentially binds PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 over other PIs (James *et al.*, 1996; Stephens *et al.*, 1998). In unstimulated cells Akt resides in the cytosol, in a low-activity conformation and upon PI3K stimulation, becomes activated through recruitment to the cellular membranes by PI3K generated lipid products and subsequent phosphorylation by the Ser/Thr kinase, PDK1 (Vanhaesebroeck and Alessi, 2000). Although the exact mechanism of Akt

activation is still not completely known, the widely accepted model is that the interaction of the PH domain of Akt with the PI3K generated lipids at the inner leaflet of the plasma membrane alters the conformation of this protein kinase allowing PDK1 access to previously hidden residues, which become phosphorylated, resulting in Akt activation (Vanhaesebroeck and Alessi, 2000). Once activated Akt translocates through the cytosol to the nucleus (Figure 6.1), where it is able to phosphorylate a range of downstream targets, the majority of which have been implicated in insulin signalling or in cell survival pathways, including glycogen synthase kinase-3β (GSK-3β), mammalian target of rapamycin (mTOR), the forkhead in rhabdomyosarcoma (FKHR) transcription factor, endothelial nitric oxide synthase (eNOS) and several anti-apoptotic effectors (Rameh and Cantley, 1999; Wurmser *et al.*, 1999; Ellson *et al.*, 2002).

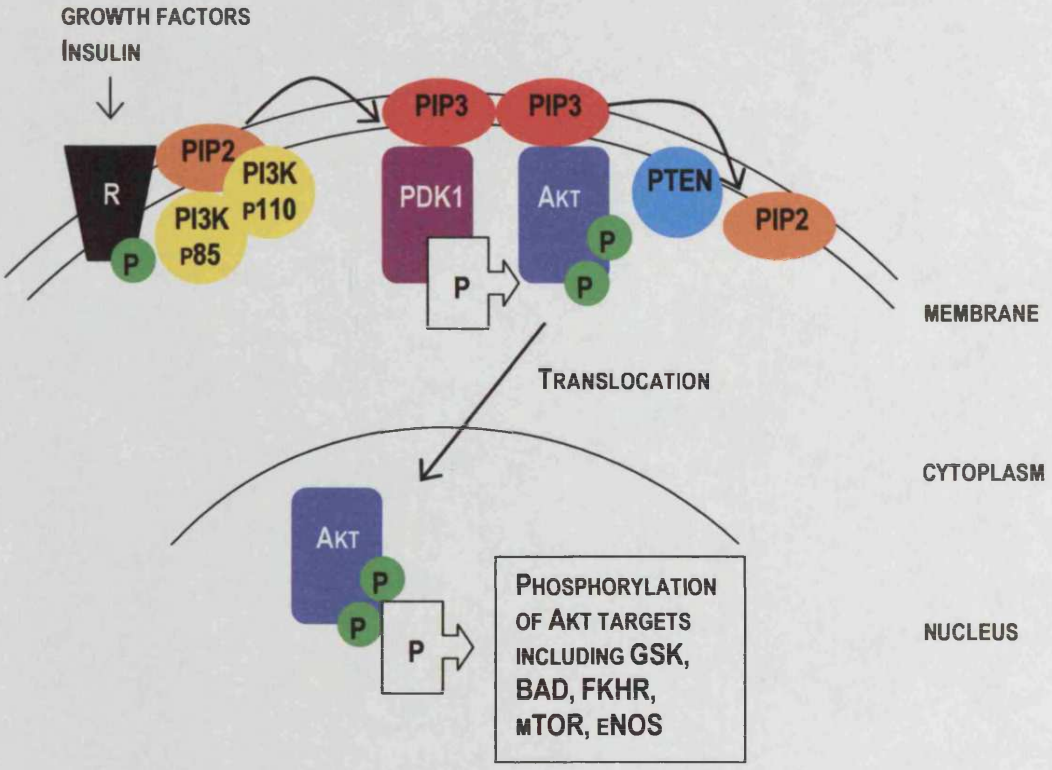


FIGURE 6.1. SCHEMATIC REPRESENTATION OF THE PI3K/AKT PATHWAY

P, phosphate residue; PIP2, phosphatidylinositol(4,5)P_2; PIP3, phosphatidylinositol(3,4,5)P_3; PI3K, phosphatidylinositol-3-kinase; PDK1, phosphoinositide dependent kinase-1; PTEN, protein phosphate and tensin homologue deleted on chromosome 10; GSK, glycogen synthase kinase; BAD, Bcl-2/BclL-antagonist, causing cell death; FKHR, forkhead in rhabdomyosarcoma; mTOR, mammalian target of rapamycin; eNOS, endothelial nitric oxide synthase; R, receptor. See text for further details.

Protein kinase CK2 is an extremely conserved pleiotropic Ser/Thr protein kinase that is ubiquitously distributed in eukaryotic organisms. CK2 has many functions and appears to be implicated in a wide variety of cellular functions such as control of metabolism, gene expression, protein synthesis, cell division and proliferation (Allende and Allende, 1995; Pinna and Meggio, 1997). CK2 usually exists in tetrameric complexes consisting of two catalytic subunits ($\alpha\alpha$, $\alpha'\alpha'$, or $\alpha\alpha'$), which are essential for cell viability (Padmanabha *et al.*, 1990), and a dimer of two non-catalytic β subunits that play an important regulatory role as they are responsible for increasing enzyme activity, determining substrate specificity and stabilising the holoenzyme (Faust and Montenarh, 2000; Lebrin *et al.*, 2001). In addition several studies have also demonstrated that CK2α may also exist free of CK2β as the β subunits can undergo reversible dissociation under physiological conditions (Litchfield *et al.*, 2003).

The mechanism of regulation of CK2 is poorly understood, although it has been postulated that this enzyme is constitutively active (Niefind *et al.*, 1998) and independent of either second messengers or phosphorylation events (Meggio and Pinna, 2003). However, several recent studies have indicated that this may not be the case as CK2 activity appears to be regulated by variety of signalling molecules (Solyakov *et al.*, 2004; Hilgard *et al.*, 2004; Cavin *et al.*, 2003). Some of the mechanisms that may contribute to the regulation of CK2 in cells include regulated expression and assembly, regulation by covalent modification, and regulatory interactions with protein and /or non-protein molecules (Litchfield *et al.*, 2003).

Although the upstream components of CK2 are still unclear, downstream, CK2 has a growing list of more than 300 substrates, all of which share typical phospho-acceptor sites specified by multiple acidic residues located mainly downstream of the phosphor-acceptor residue (Meggio and Pinna, 2003). These substrates are involved in a wide variety of cellular functions including the regulation of transcription, signal transduction and other nuclear functions (Meggio and Pinna, 2003).

Therefore in light of the results in Chapter 4 and the diverse array of signalling components of these pathways, exactly how these signalling molecules are involved in the LXR regulation of apoE forms the focus of this chapter. The aim is to confirm the activation of these pathways by ligand treatment and to attempt to delineate a potential mechanism for the involvement of these signalling cascades in the LXR-mediated regulation of apoE.

6.2. RESULTS

6.2.1. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON AKT SER473 PHOSPHORYLATION IN THP-1 MACROPHAGES

As mentioned earlier in this chapter the protein kinase Akt is a well-established target of PI3K activation. Akt plays an important role in cell survival, whereby growth factors and matrix-contact signals utilise the PI3K/Akt pathway to counteract apoptosis (Downward, 1998; Wymann *et al.*, 2003). In addition, Akt appears to be common to signalling pathways that mediate the metabolic effects of insulin in several physiologically important target tissues (Whiteman *et al.*, 2002).

Stimulation of PI3K by receptor tyrosine kinases results in Akt becoming activated through recruitment to the cellular membrane by PI3K generated lipid products and subsequent phosphorylation by PDK1 (Vanhaesbroeck and Alessi, 2000). Two specific sites, one in the kinase domain (Thr308) and the other in the C-terminal regulatory region (Ser473), need to be phosphorylated for full Akt activation (Alessi *et al.*, 1996) and once activated this protein kinase detaches from the membrane and translocates through the cytoplasm to the nucleus (Andjelkovic *et al.*, 1997; Meier *et al.*, 1997).

In order to confirm activation of the PI3K pathway by 22(R)-hydroxycholesterol, it was decided to investigate whether Akt becomes phosphorylated in response to ligand treatment. Akt phosphorylation is an effective marker for PI3K activity and several studies have used this approach in investigating the PI3K pathway (Qiu *et al.*, 2004; Jaiswal *et al.*, 2004; Hassan *et al.*, 2004). Whole cell extracts from differentiated THP-1 cells treated with 22(R)-hydroxycholesterol or DMSO for various time-points, were isolated using the phosphatase-free isolation buffer as described in Section 2.5.1, and western blot analysis (Section 2.5.4, 2.5.5 and 2.5.6) was carried out using an anti-phospho-Akt antibody that was specific for Akt phosphorylation at Ser473.

From Figure 6.2a, it can be seen that ligand treatment induced a two-fold induction of Akt phosphorylation at the Ser473 residue after 1h of treatment. This induction in Akt phosphorylation was not due to *de novo* protein synthesis as western blot analysis using an antibody that detects endogenous levels of total Akt protein showed similar steady-state protein levels (Figure 6.2b). Also this induction was specific to 22(R)-hydroxycholesterol as the following studies presented in this chapter will demonstrate that treatment with 22(S)-hydroxycholesterol showed no change in Akt phosphorylation (Section 6.2.2).

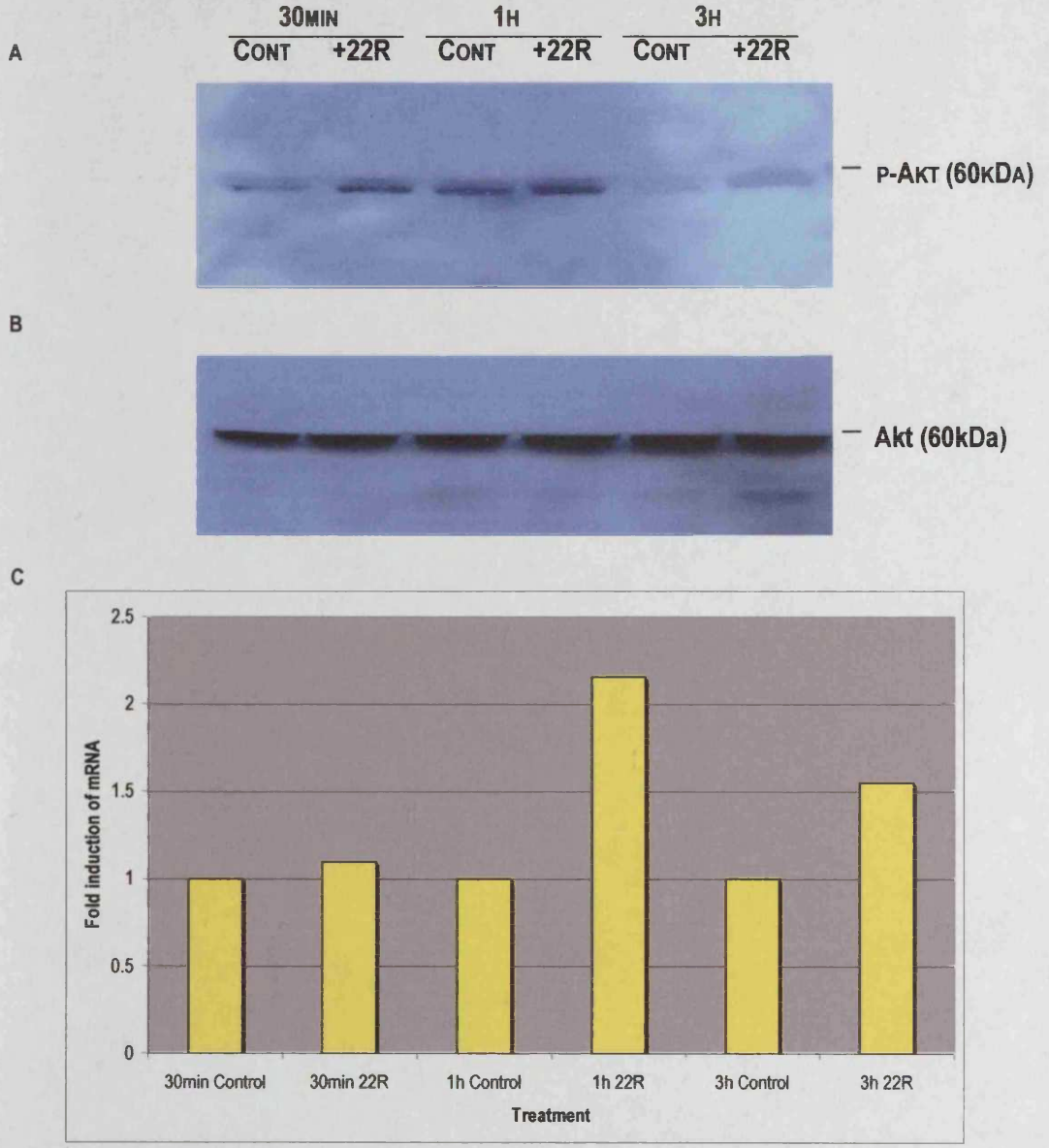


FIGURE 6.2. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON AKT PHOSPHORYLATION IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22(R)-hydroxycholesterol (denoted 22R) [2μg/ml] for the indicated time-points. As a control at each time-point, cells were treated with DMSO in the place of ligand. Western blot analysis was carried out using 80μg of whole cell extracts. **A** *Blotted membranes were incubated with phospho-Akt (Ser473) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody.* **B** *Blotted membranes were incubated with total-Akt primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system.* **C** *The Akt phospho-protein and total Akt level for each sample was determined by densitometric analysis and the Akt:total protein for each sample has been represented relative to its control, which has been assigned as 1. Results are representative of two separate experiments and the data presented in the form of a histogram is the mean value from these experiments.*

6.2.2. EFFECT OF LY294002 AND APIGENIN ON THE LXR-MEDIATED PHOSPHORYLATION OF AKT SER473 IN THP-1 MACROPHAGES

In order to confirm that the inhibition of the 22(R)-hydroxycholesterol-mediated induction of apoE by LY294002 was due to an inhibition of the PI3K pathway, we next investigated the effect of LY294002 on Akt phosphorylation. Whole cell extracts were isolated using the phosphatase-free isolation buffer (Section 2.5.1) from differentiated THP-1 cells that were pre-treated with inhibitors for 1h before the addition of 22(R)-hydroxycholesterol for 1h. Total Akt proteins were immunoprecipitated from whole cell lysates (Section 2.5.3) and were subjected to western blot analysis (Section 2.5.4, 2.5.5 and 2.5.6) using the anti-phospho-Akt (ser473) antibody with overnight incubation.

As shown in Figure 6.3, LY294002 inhibits the ligand-mediated induction of Akt Ser473 phosphorylation at 1h, which demonstrates that the activation of this pathway is necessary for the LXR-mediated regulation of apoE. Also, from this experiment it can be seen that the increase in Akt phosphorylation is mediated by LXR activation as the control ligand, 22(S)-hydroxycholesterol had no effect on Akt phosphorylation. In addition to LY294002, cells were pre-treated with the CK2 inhibitor apigenin. It was decided to include this inhibitor as previous data (see Chapter 4) has shown that the apoE induction caused by 22(R)-hydroxycholesterol can be prevented by pre-treatment with apigenin, and it is therefore important to establish whether this inhibition is independent of the PI3K pathway. In addition, several studies have demonstrated that apigenin can directly inhibit PI3K activity and Akt kinase activity in various cellular systems (Way *et al.*, 2004; Liorens *et al.*, 2002; Agullo *et al.*, 1997). From Figure 6.3, it is clear that apigenin is having an inhibitory effect on the ligand-mediated phosphorylation of Akt. Whether this inhibition is due apigenin directly affecting Akt, or whether CK2 converges with the PI3K pathway upstream of Akt and therefore apigenin is indirectly affecting Akt through CK2, is unclear from these studies and requires further investigation.

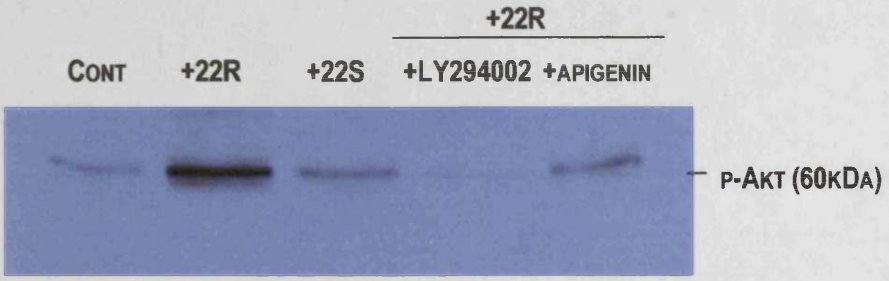


FIGURE 6.3. EFFECT OF LY294002 AND APIGENIN ON THE LXR-MEDIATED PHOSPHORYLATION OF AKT IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligands, 22(R)-hydroxycholesterol (denoted 22R) [2μg/ml] and 22(S)-hydroxycholesterol (denoted 22S) [2μg/ml] for 1h. In addition cells were treated with vehicle, DMSO in the place of ligand (denoted control). Inhibitors were added 1h before the ligand at various concentrations: LY294002 (25μM), apigenin (40μM). Immunoprecipitation of whole cell extracts (250μg) was carried out using a total-Akt antibody as described in Section 2.5.3 and the isolated Akt proteins were subjected to western blot analysis. Blotted membranes were incubated with a phospho-Akt (Ser473) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of three separate experiments.

6.2.3. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON AKT THR308 PHOSPHORYLATION IN THP-1 MACROPHAGES

In addition to Ser473 phosphorylation, full activation of Akt also requires the phosphorylation of a threonine residue located in the activation loop of Akt. This phosphorylation is thought to be necessary for substrate recognition and for the transfer of a phosphate group (Huse and Kuriyan, 2002). In order to fully investigate the mechanism whereby 22(R)-hydroxycholesterol activates the PI3K pathway, the levels of Akt phosphorylation at Thr308 were also assessed in response to ligand treatment.

Whole cell extracts from differentiated THP-1 cells treated with 22(R)-hydroxycholesterol for various time-points were isolated using the phosphatase-free isolation buffer as described in Section 2.5.1, and western blot analysis (Section 2.5.4, 2.5.5 and 2.5.6) was carried out using an anti-phospho-Akt (Thr308) antibody with overnight incubation. From Figure 6.4, it is clear that a basal level of phosphorylation of Akt at Thr308 exists in macrophages. However, no additional phosphorylation of Akt at this residue in response to 22(R)-hydroxycholesterol could be seen.

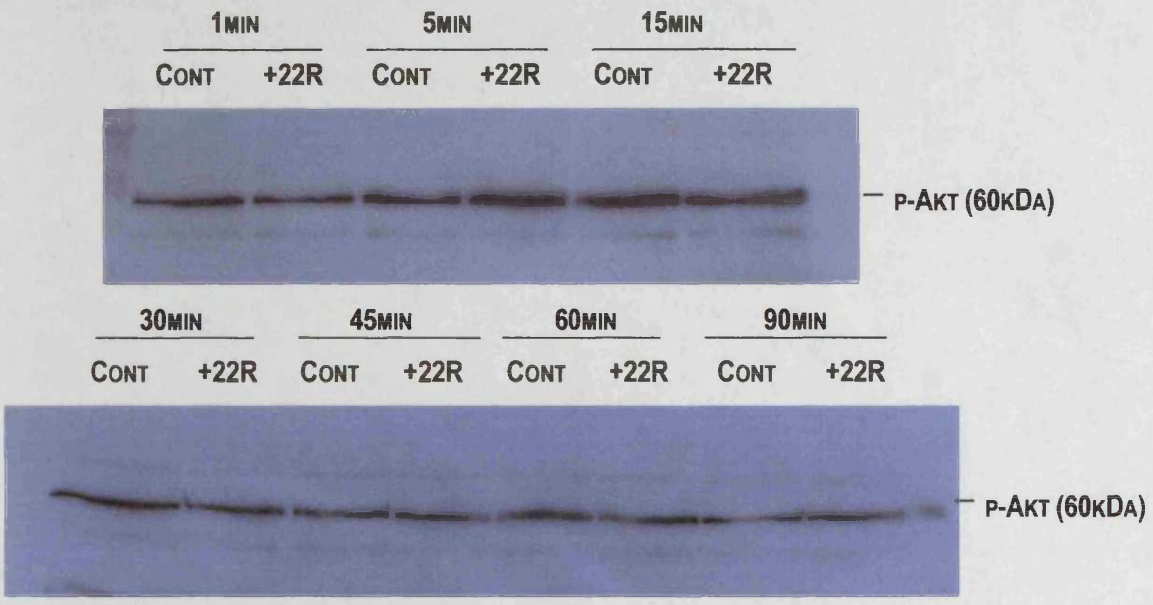


FIGURE 6.4. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON AKT THR308 PHOSPHORYLATION IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22(R)-hydroxycholesterol (denoted 22R) [2μg/ml] for the indicated time-points. As a control at each time-point, cells were also treated with DMSO in the place of ligand. Western blot analysis was carried out using 80μg of whole cell extracts. Blotted membranes were incubated with phospho-Akt (Thr308) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of two separate experiments.

6.2.4. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON AKT ACTIVITY IN THP-1 MACROPHAGES

Athough we have shown that ligand treatment of THP-1 macrophages results in the phosphorylation of Akt Ser473 (Figure 6.2), the inability of 22(R)-hydroxycholesterol to increase Thr308 phosphorylation indicates that the kinase activity of Akt may not be activated by ligand treatment. Therefore, it was necessary to investigate the effect of 22(R)-hydroxycholesterol on Akt kinase activity. For these experiments, a non-radioactive method of measuring Akt activity was employed based on the Akt Assay Kit (NEB), whereby the ability of Akt to phosphorylate one of its many downstream targets was measured. Differentiated THP-1 cells were treated with 22(R)-hydroxycholesterol for 1h and whole cell extracts were isolated using the phosphatase-free isolation buffer (Section 2.5.1). The kinase assay was carried out as described in Section 2.6.2, whereby an immobilized Akt Monoclonal Antibody was used to immunoprecipitate Akt from whole cell extracts. Then, an *in vitro* kinase assay was performed using GSK-3 Fusion Protein as a substrate and phosphorylation of GSK-3 was measured by western blot analysis (Section 2.5.5 and 2.5.6) using a phospho-GSK-3α/β(Ser21/9) antibody. From Figure 6.5, it is clear that ligand treatment of differentiated THP-1 cells does in fact result in an increase in Akt kinase activity, which

demonstrates that although no induced phosphorylation occurred at Thr308, the ligand-mediated phosphorylation of Ser473 is sufficient for an increase in Akt activity in this LXR-mediated mechanism.

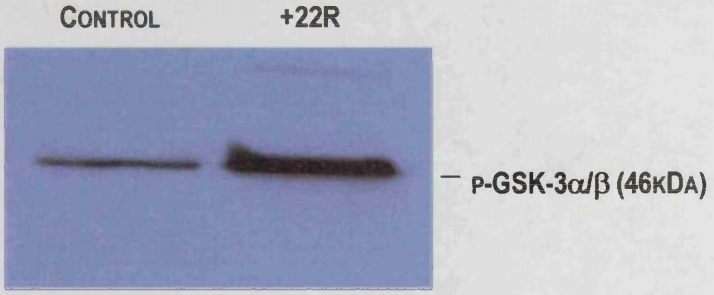


FIGURE 6.5. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON AKT ACTIVITY IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22(R)-hydroxycholesterol (denoted 22R) [2μg/ml] for 1h. In addition, cells were treated with vehicle, DMSO in the place of ligand (denoted control). The kinase assay was carried out using 250μg of whole cell extracts as described in Section 2.6.2 and blotted membranes were incubated with phospho-GSK3α/β (Ser21/9) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of three separate experiments.

6.2.5. EFFECT OF LY294002 AND APIGENIN ON THE LXR-MEDIATED INDUCTION OF AKT ACTIVITY IN THP-1 MACROPHAGES

The effect of the LY294002 and apigenin inhibitors on the ligand-mediated induction of Akt kinase activity was also investigated. Whole cell extracts were isolated using the phosphatase-free isolation buffer (Section 2.5.1) from differentiated THP-1 cells that were pre-treated with inhibitors for 1h, before the addition of 22(R)-hydroxycholesterol for 1h. The kinase assay was carried out as described in Section 2.6.2 and phosphorylation of GSK-3 was measured by western blot analysis (Section 2.5.5 and 2.5.6) using a phospho-GSK-3α/β(Ser21/9) antibody. As shown in Figure 6.6, both inhibitors eliminated the 22(R)-hydroxycholesterol-mediated induction of Akt kinase activity. This data is consistent with the previous result, whereby LY294002 and apigenin inhibited the ligand-induced phosphorylation of Akt Ser473 (Figure 6.3) and demonstrates that although no induced phosphorylation of Akt occurred at Thr308, the ligand-mediated phosphorylation of Ser473 is sufficient for an increase in Akt activity in this LXR-mediated mechanism and that inhibition of this Ser473 phosphorylation by LY294002 and apigenin results in the inhibition of Akt activity.

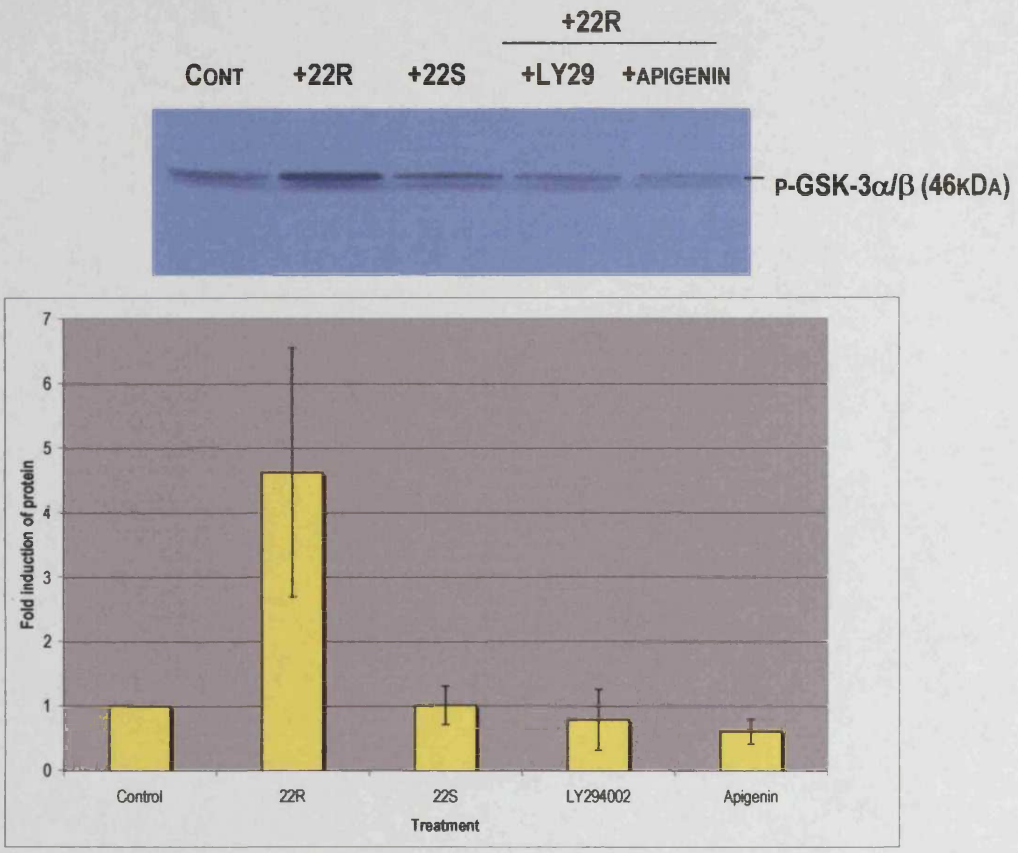


FIGURE 6.6. EFFECT OF LY294002 AND APIGENIN ON THE LXR-MEDIATED INDUCTION IN AKT ACTIVITY IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligands, 22(R)-hydroxycholesterol (denoted 22R) [2μg/ml] and 22(S)-hydroxycholesterol (denoted 22S) [2μg/ml] for 1h. In addition, cells were treated with vehicle, DMSO in the place of ligand (denoted control). Inhibitors were added 1h before the ligand at various concentrations: LY294002 (25μM), apigenin (40μM). The kinase assay was carried out using 250μg of whole cell extracts as described in Section 2.6.2 and blotted membranes were incubated with phospho-GSK-3α/β (Ser21/9) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. The GSK-3 phospho-protein level for each sample was determined by densitometric analysis and plotted on a bar chart. The control represents macrophages treated with vehicle (DMSO) in the place of ligand and has been assigned as 1, with the phospho-protein level for the ligand-treated sample being represented relative to this control. The data shown is the mean ±SD from three independent experiments.

6.2.6. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON PDK1 PHOSPHORYLATION IN THP-1 MACROPHAGES

Akt phosphorylation at Thr308 requires the protein kinase PDK1. PDK1, like Akt possesses a PH domain that can bind to $PI(3,4)P_2$ or $PI(3,4,5)P_3$ and requires translocation to the plasma membrane for full activation (Andjelkovic *et al.*, 1997; Anderson *et al.*, 1998). PDK1 exists in an active, phosphorylated configuration under basal conditions and appears to be susceptible to additional activation and phosphorylation upon cell stimulation with agonists which activate PI3K (Alessi *et al.*, 1997; Casamayor *et al.*, 1999). Recent work has also suggested that PDK1, in complex with other proteins, may also mediate the phosphorylation Akt on Ser473 (Balendran *et*

al., 1999). Although the main requirement for PDK1 activation is $PI(3,4,5)P_3$ binding, phosphorylation of this kinase is also equally critical. PDK-1 is phosphorylated at Ser241, which is present in the activation loop, and this phosphorylation occurs through autophosphorylation rather than by an upstream kinase (Casamayor *et al.*, 1999). Several other phosphorylation sites have been identified, although phosphorylation at Ser241 is necessary for PDK1 activity (Casamayor *et al.*, 1999).

Therefore, as PDK1 is the kinase responsible for Akt activation it was next decided to investigate whether this kinase becomes phosphorylated in response to ligand treatment. Whole cell extracts from differentiated THP-1 cells treated with 22(R)-hydroxycholesterol for various time-points were isolated using the phosphatase-free isolation buffer as described in Section 2.5.1, and western blot analysis (Section 2.5.4, 2.5.5 and 2.5.6) was carried out using an anti-phospho-PDK1 (Ser241) antibody with overnight incubation. From Figure 6.7 it can be seen that similar to the result seen for Akt Thr308 phosphorylation, a basal level of PDK-1 phosphorylation exists, but no additional phosphorylation of this protein kinase occurs in response to ligand treatment.

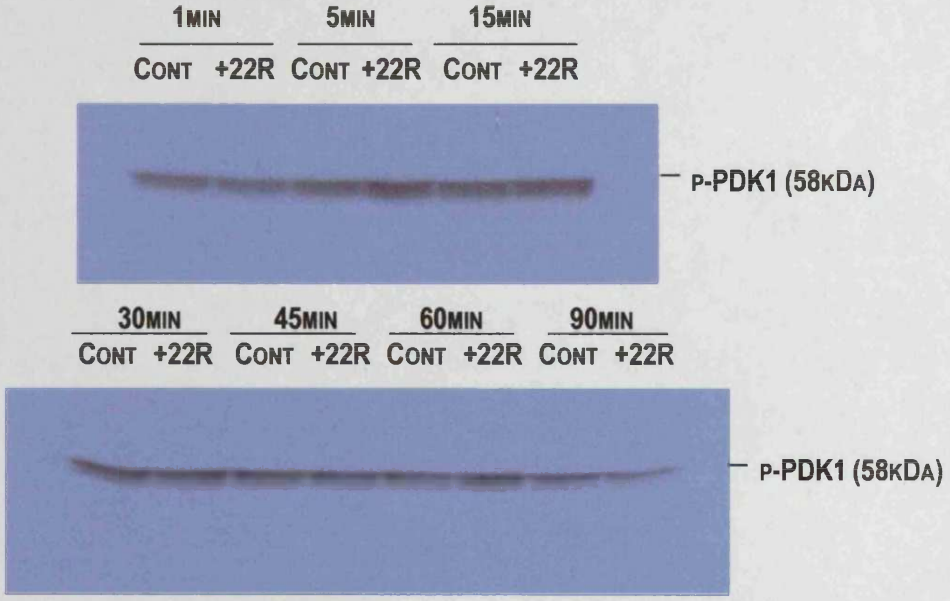


FIGURE 6.7. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON PDK1 PHOSPHORYLATION IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22(R)-hydroxycholesterol (denoted 22R) [2μg/ml] for the indicated time-points. As a control at each time-point, cells were also treated with DMSO in the place of ligand. Western blot analysis was carried out using 80μg of whole cell extracts. Blotted membranes were incubated with phospho-PDK1 (ser241) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of two separate experiments.

6.2.7. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON GSK-3 PHOSPHORYLATION IN THP-1 MACROPHAGES

The data presented in this chapter has so far demonstrated that the PI3K-Akt pathway is activated by the LXR ligand 22(R)-hydroxycholesterol and that the activation of this pathway is necessary for the LXR-mediated induction of apoE gene expression. In order to identify exactly what role this pathway may play in the apoE induction it is necessary to identify any downstream targets that may also be activated by the oxysterol ligand.

One of the first identified and major substrates of Akt is glycogen synthase kinase-3 (GSK-3) [Burgering and Coffer, 1995]. GSK-3 belongs to a family of conserved Ser/Thr kinases present in all eukaryotic organisms and one of its primary functions is to phosphorylate and inactivate glycogen synthase in response to insulin stimulation (Cross *et al.*, 1995). Two GSK-3 isoforms exist in humans (GSK3α and β) and both have a phosphorylation site in the amino-terminal region (Ser21 and Ser9, respectively), where they are phosphorylated and inactivated by Akt in a PI3K-dependent manner. GSK-3β is a ubiquitously expressed, constitutively active serine/threonine kinase that phosphorylates cellular substrates and negatively regulates downstream signalling mechanisms. Akt phosphorylation of GSK-3β results in the inactivation of this kinase and stimulates many cellular functions by removing the negative constraint imposed by GSK-3β.

GSK-3β has also been found to play a role in nuclear receptor signalling, whereby phosphorylation of nuclear receptors such as GR and AR by GSK-3β reduces the transcriptional activity of the receptor (Rogatsky *et al.*, 1998; Salas *et al.*, 2004; Liao *et al.*, 2004). Akt phosphorylation of GSk-3β therefore results in the activation of these nuclear receptors by inhibiting GSK-3β. It was therefore decided to investigate the phosphorylation state of this protein kinase as GSK-3β is a potential candidate that may link the PI3K pathway with LXR transcriptional regulation of genes.

For these experiments, THP-1 cells were differentiated and treated with 22(R)-hydroxycholesterol for various time-points. Whole cell extracts were isolated using the phosphatase-free isolation buffer (Section 2.5.1) and western blot analysis was carried out as described in Section 2.5.5 and 2.5.6, using an anti-phospho-GSK-3β (Ser9) antibody with overnight incubation. Figure 6.8 shows clearly that no phosphorylation of GSK-3β occurs in response to oxysterol treatment and rules out the involvement of this kinase in the LXR regulation of apoE.

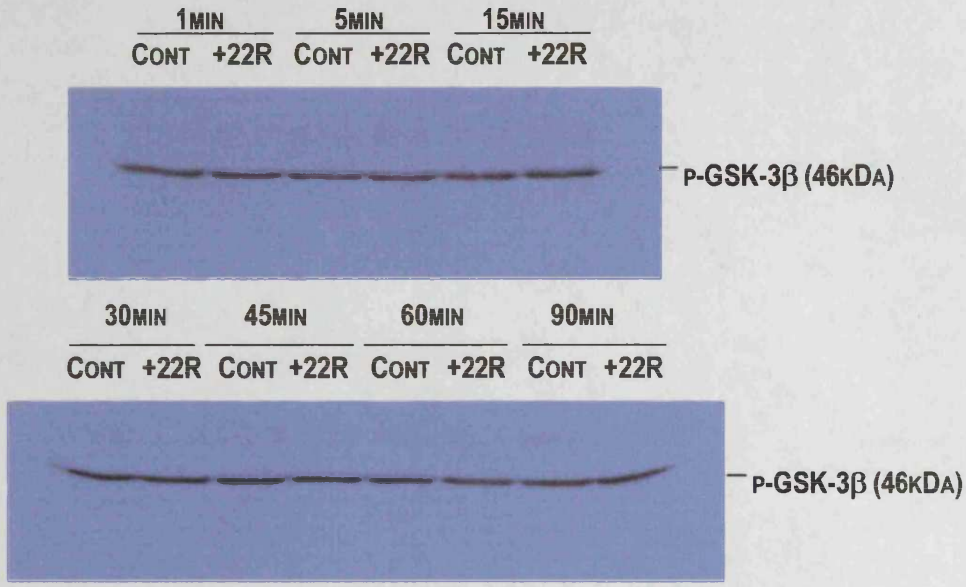


FIGURE 6.8. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON GSK-3β PHOSPHORYLATION IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22(R)-hydroxycholesterol (denoted 22R) [2μg/ml] for the indicated time-points. As a control at each time-point, cells were also treated with DMSO in the place of ligand. Western blot analysis was carried out using 80μg of whole cell extracts. Blotted membranes were incubated with phospho-GSK-3β (Ser9) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of two separate experiments.

6.2.8. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON FKHR PHOSPHORYLATION IN THP-1 MACROPHAGES

Another downstream target of the PI3K-Akt pathway is the FKHR transcription factor, which is responsible for the regulation of pro-apoptotic genes (Brunet *et al.*, 1999; Rena *et al.*, 1999). Akt phosphorylates FKHR at several sites and represses its transactivation function by promoting redistribution of the transcription factor from the nucleus to the cytoplasm (Brunet *et al.*, 1999; Medema *et al.*, 2000). In addition to its original function as a DNA binding transcription factor, several studies have demonstrated a role for FKHR as a nuclear receptor intermediary protein (Zhao *et al.*, 2001; Hirota *et al.*, 2003). FKHR has been shown to interact directly with several nuclear receptors, which enables this transcription factor to affect the transactivation function of these receptors (Zhao *et al.*, 2001; Schuur *et al.*, 2001; Li *et al.*, 2003; Dowell *et al.*, 2003). FKHR has also been shown to be phosphorylated in the non-genomic function of the AR receptor (Baron *et al.*, 2004). Therefore the phosphorylation state of this transcription factor was investigated in response to ligand treatment.

THP-1 cells were differentiated and treated with 22(R)-hydroxycholesterol for different time-points. Whole cell extracts were isolated using the phosphatase-free isolation buffer (Section 2.5.1) and western blot analysis was carried (Section 2.5.5 and 2.5.6) using an anti-phospho-FKHR

(Ser256) antibody with overnight incubation. Two separate experiments demonstrated that no phosphorylation of FKHR occurs in response to oxysterol treatment (Figure 6.9).

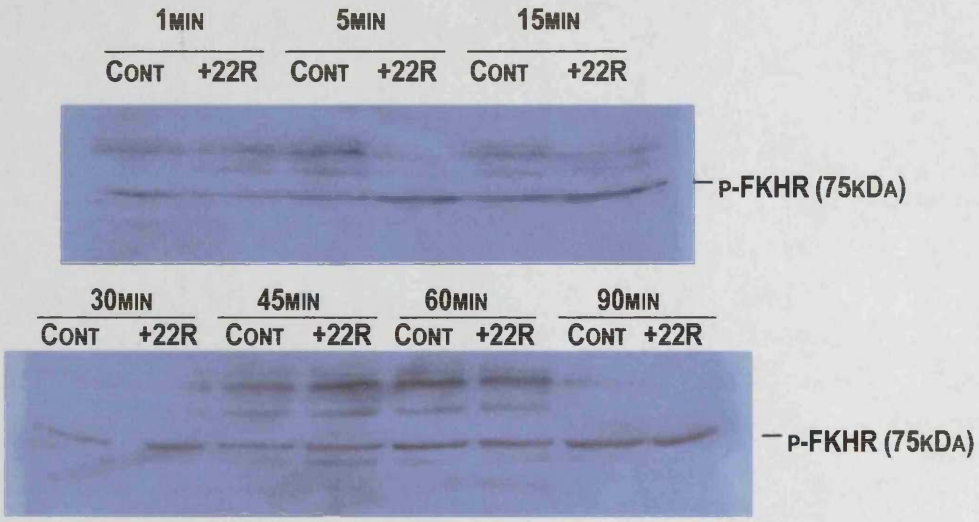


FIGURE 6.9. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON FKHR PHOSPHORYLATION IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22(R)-hydroxycholesterol (denoted 22R) [2μg/ml] for the indicated time-points. As a control at each time-point, cells were also treated with DMSO in the place of ligand. Western blot analysis was carried out using 80μg of whole cell extracts. Blotted membranes were incubated with phospho-FKHR (Ser256) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of two separate experiments.

6.2.9. EFFECT OF CK2 DOMINANT NEGATIVE EXPRESSION PLASMIDS ON THE LXR-MEDIATED INDUCTION OF APOE IN THP-1 MACROPHAGES

The results form Chapter 4 demonstrated that apigenin is able to repress the effect of the LXR ligand, 22(R)-hydroxycholesterol, on apoE gene expression. This effect was originally thought to occur through the inhibition of the CK2 enzyme as apigenin has been well-established as an inhibitor of this protein kinase and has extensively been used in CK2 studies (Critchfield *et al.*, 1997; Ford *et al.*, 2000; Tsai and Seto, 2002; Mead *et al.*, 2003). Unfortunately, the data in Section 6.2.2 demonstrates that apigenin is affecting the PI3K pathway, which indicates that CK2 may not be involved in the LXR-mediated induction of apoE. Therefore, in order to confirm the involvement of CK2, it was decided to use a dominant negative expression plasmid specific for the CK2 enzyme. The pSG-α (K68A) plasmid encoding the kinase-inactive mutant of CK2α catalytic subunit (with the substitution Lys68 to Ala) was a kind gift from Dr E. M. Chambaz and Dr C. Cochet (INSERM, Grenoble, France).

Transfections were carried out in THP-1 monocytes and the DNA constructs were transfected using the Effectene™ transfection reagent (Qiagen), as described in Section 2.3.5.2. PMA (0.16μM) was added immediately after the transfection of cells was complete and the cells were left to differentiate for 24h. After the 24h incubation, cells were treated with 2μg/ml of 22(R)-hydroxycholesterol or 22(S)-hydroxycholesterol for a further 24h, after which whole cell protein extracts were prepared (see Section 2.5.1). Of each sample 20μg of total protein was subjected to SDS-PAGE (Section 2.5.4) and then transferred to a nitrocellulose membrane (Section 2.5.5). The membrane was then incubated with an anti-apoE primary antibody (Biogenesis) and immunodetection was carried out using the appropriate secondary antibody and the ECL detection system (Amersham) as described in Section 2.5.6. In addition to the dominant negative construct, as a control, cells were transfected with the empty pSG5-α vector (Appendix IV) to ensure that response seen was due to the dominant negative CK2 and not the plasmid vector or the transfection reagent. From Figure 6.10, it can be seen that the CK2 dominant negative plasmid caused a 50% decrease in the induction of apoE by 22(R)-hydroxycholesterol, which confirms that CK2 is indeed involved in the LXR-mediated induction of apoE.

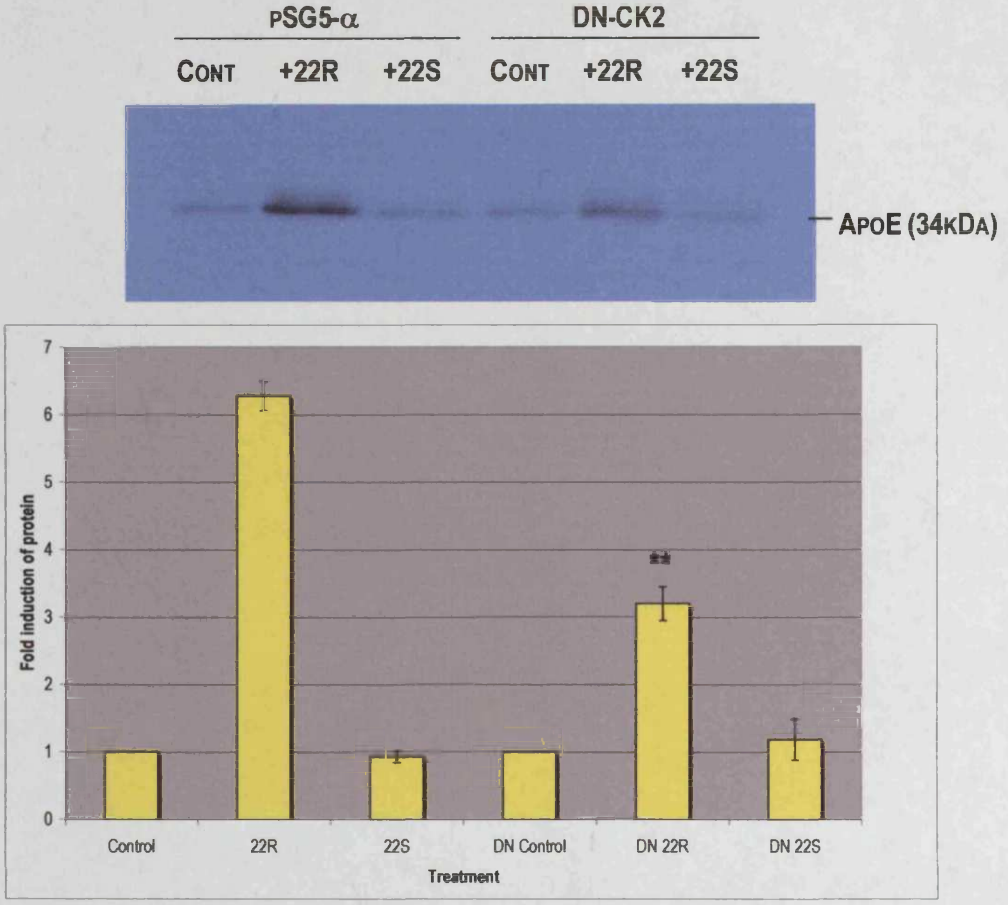


FIGURE 6.10. EFFECT OF DOMINANT NEGATIVE EXPRESSION PLASMIDS ON THE LXR-MEDIATED INDUCTION OF APOE IN THP-1 MACROPHAGES

*THP-1 monocytes were transfected (see section 2.3.5.2) with the indicated expression plasmids or the control vector and differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h. The LXR ligands, 22(R)-hydroxycholesterol (denoted 22R) [2μg/ml] and 22(S)-hydroxycholesterol (denoted 22S) [2μg/ml] were then added to the cells for a further 24h. Western blot analysis was carried out using 20μg of whole cell extracts. Blotted membranes were incubated with anti-apoE primary antibody (goat polyclonal IgG) and detected using an anti-goat horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of three separate experiments. The apoE protein level for each sample was determined by densitometric analysis and plotted on a bar chart. The control represents macrophages treated with vehicle (DMSO) in the place of ligand and has been assigned as 1 in each case, with the apoE protein level for the remaining samples being represented relative to this control. The data shown is the mean ±SD from three independent experiments. The data was analysed by Student's t-test. ** Represents the significant difference compared to pSG5α transfected cells treated with 22(R)-hydroxycholesterol (**p<0.005).*

6.2.10. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON CK2 ACTIVITY IN THP-1 MACROPHAGES

To our knowledge, no studies have been published with respect to the 22(R)-hydroxycholesterol ligand and increased CK2 activity. Therefore, it was important to prove that the two are linked, which was carried out by performing an *in vitro* assay for CK2 activity. For these experiments, THP-1 cells were differentiated for 24h with PMA and then treated with 22(R)-hydroxycholesterol for 30 min in the absence or presence of apigenin. This time-point was chosen as a sample time-point based on previous studies in our laboratory on CK2 activity. After preparation of whole cell extracts using the phosphatase-free isolation buffer (Section 2.5.1), CK2 was immunoprecipitated using an antibody against the α-subunit and subjected to an assay where its ability to phosphorylate β-casein was measured. This assay utilised γ-^{32}P-labelled ATP, which CK2 uses *in vivo* as a phosphate donor (although it may also use GTP), in the substrate mix. After allowing the reaction to proceed for 15 min at 37°C, the contents were sized-fractionated on a SDS-PAGE gel, and the phosphorylation of β-casein, a 24kDa protein, by the immunoprecipitated CK2 was assessed by autoradiography. The results of this experiment are shown in Figure 6.11.

From Figure 6.11a, it can be seen that β-casein was phosphorylated to a greater extent by CK2 immunoprecipitated from 22(R)-hydroxycholesterol-treated cells, than by CK2 isolated from control cells and that this activation was prevented by pre-treatment with the CK2 inhibitor, apigenin. These results, therefore demonstrates the ability of 22(R)-hydroxycholesterol to activate CK2 in THP-1 cells, and clearly indicates that CK2 activation is essential in the LXR-mediated regulation of apoE. The results in Figure 6.11b also show that the increase in CK2 activity is LXR mediated, as the inactive ligand 22(S)-hydroxycholesterol had no effect on CK2 β-casein phosphorylation. The LY294002 inhibitor was also used in these experiments to ensure that CK2 activation was independent of the PI3K pathway. This was confirmed as LY294002 had no effect of CK2 activity (Figure 6.11b). Also included in Figure 6.11a are positive and negative controls. The positive control reaction was performed using purified CK2 enzyme, and the negative control was carried out with substrate alone. The identical signals produced by the purified enzyme and immunoprecipitated CK2 confirm that the assay is specific for CK2, and the lack of any signal in the negative control shows that the signals obtained in experimental lanes are not spuriously created by ^{32}P-labelled ATP in the substrate mix.

The possibility existed that the increased activity of CK2 in 22(R)-hydroxycholesterol-treated cells was the result of *de novo* protein synthesis. This was discounted by the observation that western blot analysis with whole cell lysates obtained from control and ligand treated cells all showed similar steady-state protein levels (Figure 6.11c).

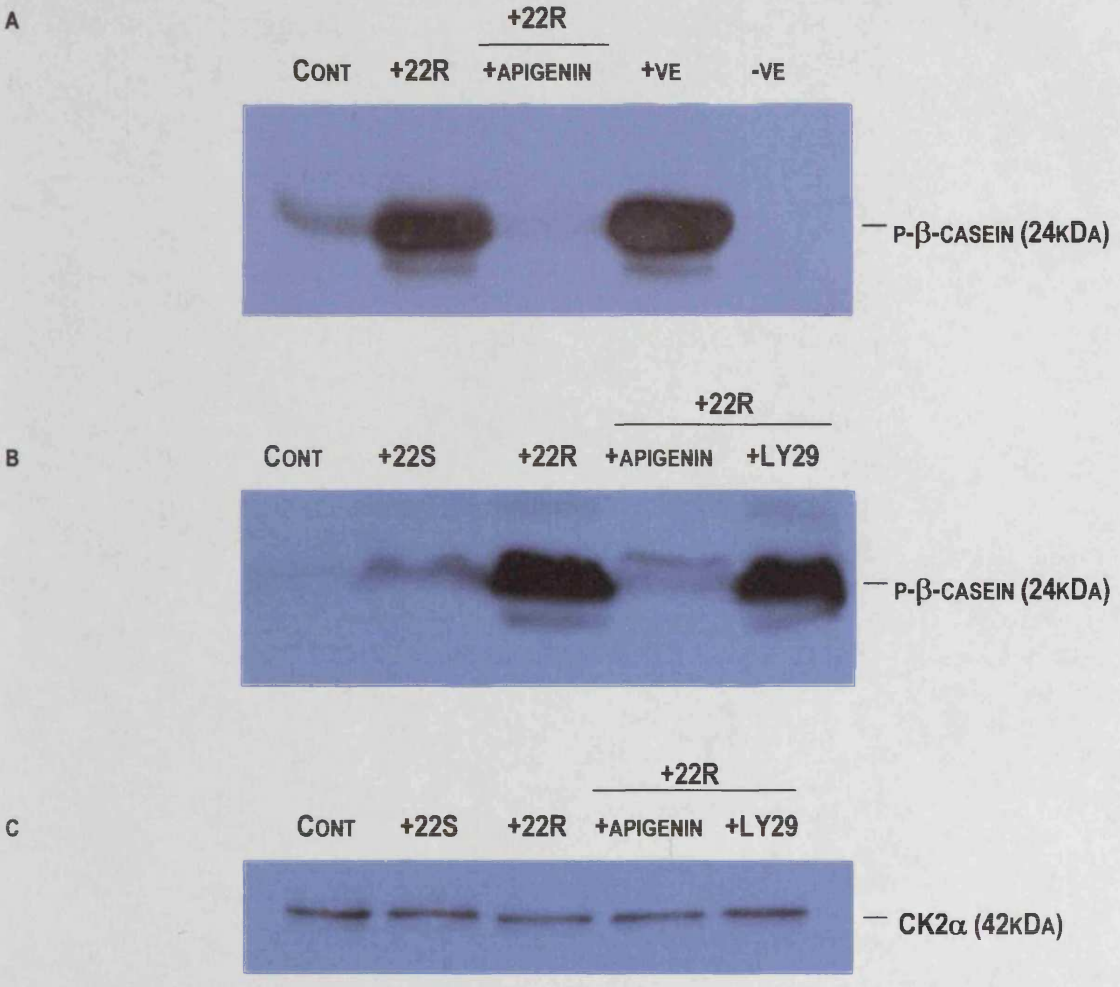


FIGURE 6.11. EFFECT OF 22(R)-HYDROXYCHOLESTEROL AND THE INHIBITORS APIGENIN AND LY294002 ON CK2 ACTIVITY AND PROTEIN EXPRESSION IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligands, 22(R)-hydroxycholesterol (denoted 22R){2μg/ml] and 22(S)-hydroxycholesterol (denoted 22S) [2μg/ml] for 30min. As a control, cells were also treated with DMSO in the place of ligand. Inhibitors were added 1h before the ligand at various concentrations: LY294002 (25μM), apigenin (40μM). **A** *and* **B** *The kinase assay was carried out using 200μg of whole cell extracts as described in Section 2.6.1. The contents of the kinase reaction were sized-fractionated on a SDS-PAGE gel [15% (v/v)], and the phosphorylation of β-casein, by the immunoprecipitated CK2 was assessed by autoradiography. Also shown are a positive control with purified CK2 enzyme (1U; Promega) and a negative control where only the substrate mix was size fractionated on the gel. The figure is representative of a gel that was fixed, dried and subsequently exposed to film for 1h.* **C** *Immunoprecipitation of whole cell extracts (250μg) was carried out using an anti-CK2α antibody (goat polyclonal IgG) as described in Section 2.5.3 and the isolated CK2α proteins were subjected to western blot analysis. Blotted membranes were incubated with the CK2α primary antibody and detected using an anti-goat horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of three separate experiments.*

6.2.11. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON PTENPHOSPHORYLATION IN THP-1 MACROPHAGES

The results from the previous sections confirm that CK2 is in fact being activated by 22(R)-hydroxycholesterol. Although the CK2 inhibitor, apigenin has been shown in other studies to affect the PI3K pathway and was shown to be inhibiting Akt phosphorylation in Figure 6.3, it may be that this inhibitor is affecting the PI3K pathway indirectly through CK2 inhibition. Several studies have shown that PTEN, a 3'-phosphoinositide phosphatase that antagonises PI3K signalling, is a substrate of CK2 (Miller *et al.*, 2002; Torres and Pulido, 2001). CK2 phosphorylation of PTEN is inhibitory, and therefore any inhibition of CK2 activity leads to a decrease in Akt activation (Miller *et al.*, 2002; Torres and Pulido, 2001). Therefore we next investigated whether CK2 activation by 22(R)-hydroxycholesterol resulted in the phosphorylation of PTEN.

Whole cell extracts from differentiated THP-1 cells treated with 22(R)-hydroxycholesterol for various time-points were isolated using the phosphatase-free isolation buffer as described in Section 2.5.1, and western blot analysis (Section 2.5.5 and 2.5.6) was carried out using an anti-phospho-PTEN (Ser380) antibody with overnight incubation. From Figure 6.12, it is clear that a basal level of PTEN phosphorylation exists in macrophages, but no additional phosphorylation occurs in response to ligand treatment, which indicates that CK2 is not affecting the PI3K pathway through PTEN phosphorylation.

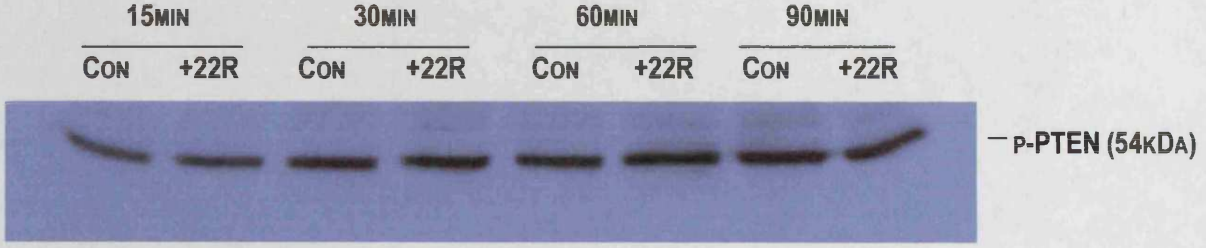


FIGURE 6.12. EFFECT OF 22(R)-HYDROXYCHOLESTEROL ON PTEN PHOSPHORYLATION IN THP-1 MACROPHAGES

THP-1 monocytes were differentiated with PMA (0.16μM) in 10% (v/v) delipidated HI-FCS for 24h and then exposed to the LXR ligand, 22R-hydroxycholesterol (denoted 22R) [2μg/ml] for the indicated time-points. As a control at each time-point, cells were also treated with DMSO in the place of ligand. Western blot analysis was carried out using 80μg of whole cell extracts. Blotted membranes were incubated with phospho-PTEN (Ser380) primary antibody (rabbit polyclonal IgG) and detected using an anti-rabbit horseradish peroxidase secondary antibody. Antigen-antibody complexes were detected using the ECL detection system. Results are representative of two separate experiments.

6.3. DISCUSSION

As no studies have been published regarding the involvement of PI3K and CK2 in LXR regulation of target genes, the initial aim of this chapter was to confirm the activation of these pathways in response to ligand treatment.

Firstly, we investigated PI3K activation by 22(R)-hydroxycholesterol. The PI3K pathway has been shown to play a vital role in nuclear receptor signalling with various components of the pathway being involved in regulating the transactivation functions of these transcription factors. The primary role for this pathway seems to be in the phosphorylation of nuclear receptors with receptors such as the androgen receptor (AR) and the estrogen receptor (ER) being phosphorylated in response to a variety of signals (Lin *et al.*, 2001b; Campbell *et al.*, 2001). In order to investigate the role of this pathway in the LXR-mediated regulation of apoE it was necessary to firstly identify whether ligand treatment increased PI3K activity in THP-1 macrophages. Therefore it was decided to measure phosphorylation of Akt, a well-established downstream signalling component of PI3K. From the results presented in this chapter it is clear that ligand treatment of THP-1 macrophages resulted in Akt phosphorylation at Ser473 (Figure 6.2) and kinase activation (Figure 6.5) after only 1h. This induction of Akt was ligand-specific and required LXR activation as 22(S)-hydroxycholesterol had no effect on Akt phosphorylation or activity. Also through the use of inhibitors we are able to conclude that Akt activity is essential for the LXR-mediated regulation of apoE as inhibition of this pathway by LY294002 is consistent with the inhibition of apoE with this inhibitor. We also demonstrated that the induction in Akt kinase activity did not require an increased level of Akt Thr308 phosphorylation (Figure 6.4) or an increase in PDK1 phopshorylation (Figure 6.7) although a basal level of phosphorylation of both these proteins does exist in THP-1 macrophages.

This ability of the LXR ligand, 22(R)-hydroxycholesterol to induce Akt activity after only 1h may be attributed to a non-genomic function of the ligand. As described in Chapter 5 nuclear receptors are able to activate cell signalling pathways through non-transcriptional mechanisms in addition to their ligand-inducible transcription factor function. These non-genomic or extranuclear actions of nuclear receptors are rapid and defy the time-course required for transcriptional activation of genes. From our results, which demonstrate that 22(R)-hydroxycholesterol is able to induce PI3K activity after 1h it is possible to conclude that PI3K activation, similar to the JNK/SAPK activation of the previous chapter occurs through a non-genomic action of the ligand, and this rapid activation of PI3K is necessary for the LXR-mediated transactivation of the apoE gene.

Although it is clear that 22(R)-hydroxycholesterol is activating the PI3K pathway through a non-genomic mechanism that does not require transcriptional activation, the precise mechanism of this action requires further investigation. One way in which cell signalling pathways have been

shown to be rapidly activated by nuclear receptors is through protein interactions. Indeed, several studies have previously shown that this is the case for the non-genomic activation of the PI3K pathway by the ER receptor. ERα has been shown to bind and activate the p85 regulatory subunit of PI3K which results in Akt Ser473 phosphorylation and kinase activation (Simoncini *et al.*, 2000; Pozo-Guisado *et al.*, 2004; Haynes *et al.*, 2000; Hisamoto *et al.*, 2001). This has also been shown to be the case for the non-genomic action of androgens, as the AR receptor interacts directly with the p85 regulatory subunit of PI3K and promotes the accumulation of PI3K generated lipid products, which in turn results in an increase in Akt activity (Baron *et al.*, 2004). This mechanism of activation of the PI3K pathway could potentially be applied to an LXR model, whereby a membrane-bound LXR receptor binds, in a ligand-dependent manner to PI3K, which would result in Akt recruitment and activation of this pathway, which ultimately increases the LXR-mediated transcription of apoE. In support of the potential mechanism, whereby LXR binds to a component of the PI3K pathway, resulting in Akt activation, a scan for motifs present in the amino acid sequence of the LXRs was carried out using the Scansite database (Obenauer *et al.*, 2003). The results of this scan indicated several PI3K pathway motifs, including PI3K p85 binding groups and PDK1 binding motifs (AppendixV). These results demonstrate that the necessary sequences for PI3K binding exist in the LXR proteins although further work is required to confirm this hypothesis.

Once Akt activation was established, it was decided to investigate whether any downstream targets of Akt were phosphorylated in response to 22(R)-hydroxycholesterol treatment. The Akt targets investigated were GSK-3β and FKHR, both of which are negatively regulated by phosphorylation by Akt and have been shown to be involved in nuclear receptor signalling (Rogatsky *et al.*, 1998; Salas *et al.*, 2004; Zhao *et al.*, 2001; Schuur *et al.*, 2001; Hirota *et al.*, 2003). Although neither one of these proteins showed any change in phosphorylation in response to ligand treatment (Figure 6.8 and 6.9), the possibility that the PI3K pathway is mediating its effects on the LXR regulation of apoE through a downstream component of Akt, can not be ruled out. More than 50 downstream targets have been identified so far for Akt and several others, in addition to GSK-3β and FKHR, have been shown to play a role in nuclear receptor signalling. For example MDM2, a ubiquitin ligase for p53 (Haupt *et al.*, 1997) has been shown to be a positive regulator of ERα in MCF-7 cells (Saji *et al.*, 2001). Also the TSC2 protein has been shown to bind and selectively modulate transcription mediated by GR, PPARα and VDR (Henry *et al.*, 1998). Therefore, although it is possible that Akt may be the last step in the PI3K pathway involved in the LXR regulation of apoE and may be directly phosphorylating LXR or one of the coregulator proteins required for transcriptional activation, it is impossible to rule out the activation of another downstream target at this stage. In support of this potential mechanism, whereby the PI3K pathway results in phosphorylation of LXR either through Akt, or a downstream target of Akt,

a scan for kinase motifs present in the amino acid sequence of these nuclear receptors was carried out using the Scansite database (Obenauer *et al.*, 2003). The results of this scan indicated several phosphorylation motifs, including Akt and GSK-3α phosphorylation sequences (AppendixV). The identification of these potential phosphorylation sites is of great interest and future work should aim to identify the importance of these residues in the involvement of the PI3K pathway in the LXR-mediated regulation of apoE.

In addition to the PI3K pathway, the aim of this chapter was to confirm and investigate the activation of CK2. Although the work presented in Chapter 4 demonstrated conclusively that apigenin inhibited the effect of the LXR ligand, 22(R)-hydroxycholesterol, on apoE gene expression, the data in Figure 6.3 indicated that this may be due to inhibition of the PI3K pathway rather than CK2. Therefore, the involvement of CK2 in the LXR-mediated regulation of apoE was confirmed through the use of dominant negative constructs (Figure 6.10). Also a CK2 kinase assay was carried out and clearly demonstrated that ligand treatment results in CK2 activation, which is blocked by pre-treatment with apigenin (Figure 6.11). Therefore, although apigenin may also affect the PI3K pathway, the results from this chapter prove conclusively that CK2 is activated by ligand treatment. It is also possible that apigenin is inhibiting the PI3K pathway indirectly through CK2 inhibition. This may be due to phosphorylation of PTEN by CK2. Although we have shown that no change in PTEN phosphorylation at Ser380 occurs in response to ligand treatment (Figure 6.12), it does not rule out CK2 phosphorylation of this phosphatase, as recent studies have demonstrated that CK2 phophorylates PTEN at several other critical residues (Torres and Pullido, 2001).

Although the exact role of CK2 in the LXR-mediated regulation of apoE has not been identified in this study, it is possible that CK2 may play one of several roles in LXR signalling as the list of CK2 substrates is extensive and ever-growing. CK2 may directly phosphorylate the LXR receptor, as has been shown for several other nuclear receptors (Jurutka *et al.*, 1996; Zhang *et al.*, 1994; Katz *et al.*, 1995). In fact, a Scansite search of the LXR protein sequences has demonstrated the existence of a CK2 phosphorylation motif in both LXRα and LXRβ (Appendix V). Other substrates of CK2 that may be involved in LXR signalling include coregulators (Zhou *et al.*, 2001; Meggio and Pinna, 2003), transcription factors (Armstrong *et al.*, 1997; Meggio and Pinna, 2003), and various members of the basal transcriptional machinery (Pinna, 1990; Maldonado and Allende, 1999; Ghavdel and Schultz, 1997).

Therefore, in conclusion the studies in this chapter have conclusively demonstrated that both the PI3K pathway and CK2 are activated in response to ligand treatment. These observations are novel and may also be extended to other LXR target genes. We have also shown that these pathways are activated rapidly, suggesting once again that 22(R)-hydroxycholesterol may have a non-genomic function which supports the LXR-mediated transcription of apoE.

CHAPTER SEVEN – GENERAL DISCUSSION

7.1 OVERVIEW OF THE RESULTS PRESENTED IN THIS THESIS

The role of apoE as an atheroprotective protein has been well established, with multiple studies demonstrating its ability to retard the development of atherosclerosis (Schaefer *et al.*, 1986; Ghiselli *et al.*, 1981; Plump *et al.*, 1992; Zhang *et al.*, 1992; Nakashima *et al.*, 1994; Reddick *et al.*, 1994; Piedrahita *et al.*, 1992). Macrophage-derived apoE is potentially of great importance due to its direct role in preventing or delaying foam cell formation (Fazio *et al.*, 1997). In addition to its role in the regulation of macrophage cholesterol homeostasis, macrophage apoE exerts its anti-atherogenic properties through various alternative mechanisms including its ability to modify T-lymphocyte- and smooth muscle cell-mediated inflammatory responses (Avila *et al.*, 1982; Hui and Harmony, 1980a; Ishigami *et al.*, 1998). Therefore, apoE may be considered as a potential therapeutic target that merits further investigation. Indeed, therapeutic approaches that would modulate macrophage apoE production may be useful in the prevention or treatment of atherosclerosis. It is therefore essential that a detailed understanding is obtained of the regulation of macrophage apoE expression, particularly the mechanisms that are responsible for increasing production.

Unfortunately, there is a general paucity of relevant information on the regulation of macrophage apoE gene expression and, additionally, several previous studies have produced incomplete and often conflicting results (Basheeruddin *et al.*, 1994; Duan *et al.*, 1995; Zuckerman *et al.*, 1992; Menju *et al.*, 1989). Therefore, the overall objective of this thesis was to gain a better understanding of the regulation of apoE expression in macrophages.

This objective was initially approached using transfection based studies, whereby the aim of the project was to elucidate, in detail, the mechanism(s) by which macrophage differentiation induced apoE expression, and to determine the *cis*-acting elements in the apoE promoter that were responsible for this upregulation. A macrophage-based transfection system was therefore systematically optimised to enable the analysis of several DNA constructs containing regions of the apoE promoter and the enhancer regions, ME1 and ME2 of the apoE gene. However, whilst this system was able to demonstrate the previously reported TNF-α induction of the apoE promoter and also the differentiation-dependent induction of the LPL promoter, no such induction was observed for the apoE promoter or enhancer constructs during PMA-induced differentiation, despite the variation of several parameters. There are several possible reasons for this, but the most likely is that the constructs used did not contain the necessary elements required to modulate the effect. To fully overcome this problem, the cloning of greater regions of the apoE promoter is likely to be required, also constructs containing additional sequences such as that of the intergenic region would be of great use.

It was therefore decided, in line with our original aims, to investigate the regulation of apoE by the LXR subfamily of nuclear receptors. As described in Chapter 1, members of the LXR subfamily regulate multiple aspects of lipid metabolism and recent work analysing the effect of a synthetic LXR ligand in murine models of atherosclerosis has provided direct evidence for the atheroprotective effect of LXR agonists (Joseph *et al.*, 2002b). Work by Laffitte *et al.* (2001b) has also demonstrated the direct regulation of apoE by LXRs. This ability of LXR agonists to upregulate apoE gene expression and to regulate other key genes of the cholesterol efflux pathway, such as ABCA1 and ABCG1, is of potential therapeutic value. A clearer understanding of LXR-regulated transcription of these genes would enable the development of promising agents for intervention in human cardiovascular disease. As cell signalling pathways appear to be essential in the transactivation function of several nuclear receptors it was decided to determine the signalling pathways involved in the LXR regulation of apoE in macrophages through the use of commercially available inhibitors.

We demonstrated that the oxysterol ligand, 22(R)-hydroxycholesterol, was able to induce apoE mRNA levels, protein levels and secretion and that pre-treatment with inhibitors against the JNK/SAPK, PI3K and CK2 pathways prevented this induction. This inhibition of apoE induction was also shown in human primary-monocyte-derived macrophages. Further investigation also demonstrated that these inhibitors were able to prevent the ligand-mediated induction of ABCG1 and LXRα mRNA. However, in the case of ABCA1, only the JNK and CK2 inhibitors had an affect on the ligand-mediated induction of this gene. Also, in addition to the JNK, PI3K and CK2 inhibitors, the ligand-mediated induction of LXRα was also shown to be eliminated by the p38 inhibitor, SB202190 and the ligand-mediated induction of ABCG1 was inhibited by the ERK MAPK inhibitor.

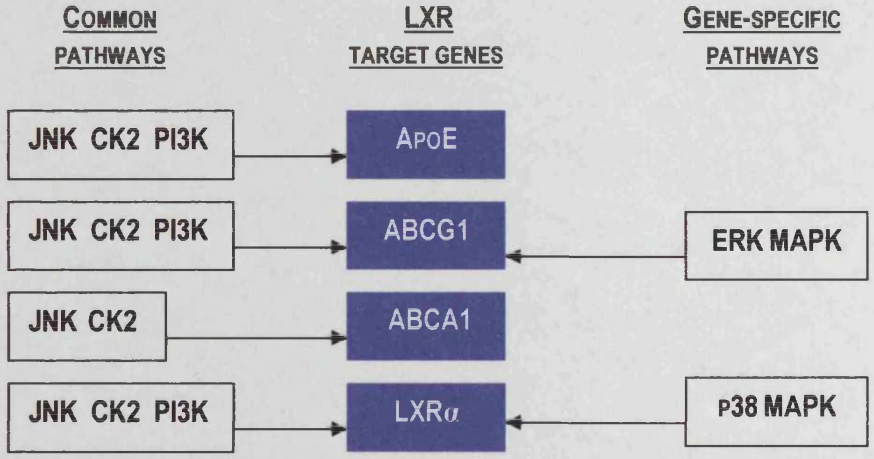


FIGURE 7.1. SCHEMATIC SUMMARY OF THE CELL SIGNALLING PATHWAYS IDENTIFIED THROUGH INHIBITOR STUDIES TO PARTICIPATE IN THE LXR-MEDIATED REGULATION OF TARGET GENES

The results of these experiments are novel findings, demonstrating that the LXR regulation of target genes is more complicated than initially thought. We have demonstrated that cell signalling pathways play a vital role in the LXR-regulation of apoE expression and several other LXR-regulated genes, and have shown that although a common mode of regulation may exist for these genes, there are some gene-specific differences (Figure 7.1). These results, therefore provided a basis for further investigation of the JNK/SAPK, PI3K and CK2 pathways in the LXR-mediated regulation of apoE.

We next investigated the ability of the LXR ligand, 22(R)-hydroxycholesterol to activate the JNK pathway. It was subsequently confirmed that the JNK/SAPK MAPK was indeed phosphorylated in response to ligand treatment and that the kinase activity of this enzyme was also induced. This induction of the JNK pathway was rapid with an increase in phosphorylation occurring after only 30min. The rapid kinetics in which this pathway was activated by 22(R)-hydroxycholesterol indicated that this activation was not dependent on gene transcription and that this ligand may be able to exert non-genomic or extranuclear actions in addition to its transcription-based function. This is a novel observation and suggests that similar to other nuclear receptor agonists such as estrogens and progestins, that have well-characterised non-genomic actions, the activation of these cell signaling pathways may be due to the existence of a receptor outside the nucleus. Further investigation also revealed that the c-jun transcription factor, a downstream target of JNK, becomes phosphorylated in response to ligand treatment, indicating, that similar to the LXR-mediated differentiation of keratinocytes, whereby oxysterols induce AP-1 proteins (Schmuth *et al.*, 2004), the AP-1 transcription complex is important in the LXR-mediated regulation of apoE.

In addition to the JNK pathway, the involvement of the PI3K and CK2 pathways in the LXR-mediated regulation of apoE was also investigated. These studies demonstrated that treatment of THP-1 macrophages with the LXR ligand, 22(R)-hydroxycholesterol resulted, in Akt, a downstream component of PI3K signalling, being phosphorylated and its kinase activity being induced. Akt activates several nuclear factors and we decided to investigate whether any of these factors, with known roles in nuclear receptor signalling were phosphorylated in response to ligand treatment. Both GSK-3β and FKHR were eliminated from involvement in this regulation of apoE, as neither demonstrated phosphorylation in response to ligand treatment.

CK2 involvement in the LXR-mediated regulation of apoE was also demonstrated through dominant negative studies and the kinase activity of this enzyme was investigated, and was shown to be activated in response to ligand treatment.

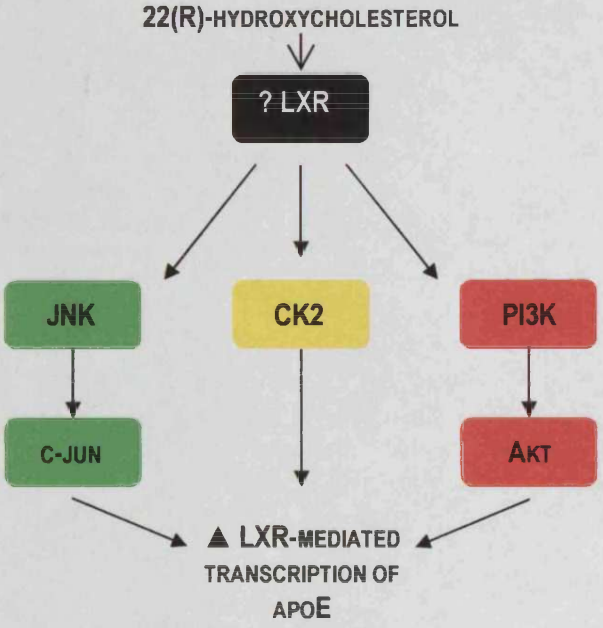


FIGURE 7.2. SCHEMATIC SUMMARY OF THE SIGNALLING PATHWAYS IDENTIFIED IN THE LXR-MEDIATED INDUCTION OF APOE IN THP-1 MACROPHAGES

Therefore, in summary, the work presented in this thesis has successfully addressed the role of cell signalling in the LXR-mediated regulation of apoE and has led to the following important findings:

- Induction of apoE mRNA, protein and secretion by the oxysterol ligand 22(R)-hydroxycholesterol.
- Identification of a novel role of JNK, PI3K and CK2 pathways in the LXR-mediated induction of apoE gene expression in THP-1 macrophages (Figure 7.2).
- A potential role of cell signalling pathways in ABCA1, ABCG1 and LXRα expression with some common components and potential gene-specific differences.

Furthermore, the data presented in this thesis can be approached with a good degree of confidence given that we have performed positive controls, accurately produced previously published work and verified novel findings through the use of several experimental techniques where possible. In addition, the key findings of this study were also confirmed in primary cultures, indicating that the results presented here may be relevant to human atherosclerosis. Consequently, the following sections will attempt to discuss the greater significance of these findings and the future studies that could be performed in order to advance towards the ultimate goal of a therapeutic treatment of atherosclerosis.

7.2. WIDER PERSPECTIVES OF THE NOVEL FINDINGS IN THIS THESIS

Nuclear receptors represent excellent molecular targets for drug therapy and some of the currently marketed drugs are well-established agonists of these transcription factors. Although several animal studies demonstrate support for the overall beneficial role of LXR activation in the control of atherosclerosis, it is still unclear whether or not the biological actions of LXRs make these receptors potential therapeutic targets. Indeed, recent findings have indicated that the treatment of db/db diabetic mice with the LXR ligand T-0901317 causes more severe lipogenic pathology due the LXR transactivation of fatty acid biosynthesis genes, demonstrating that special caution should be taken whilst evaluating LXR ligands as therapeutic agents to lower plasma cholesterol levels and to prevent atherosclerosis (Chisholm *et al.*, 2003). Therefore, the development of selective agonists that dissociate the antiatherosclerotic effects from the triglyceride raising effects seems to be a promising direction for future LXR study from a therapeutic perspective and an understanding of the gene-specific differences in signalling components may help in designing such therapy.

Recently, it has been reported that, in the skeletal muscle of mice and in a myogenic cell line, LXR agonists specifically induce cholesterol metabolism gene expression without affecting genes involved in fatty acid synthesis (Muscat *et al.*, 2002). Also, YT-32 [(22E)-ergost-22-ene-1α, 3β-diol], a synthetic derivative of phytosterol, has been demonstrated to act as a selective LXR agonist to specifically activate intestinal ABC transporters in mice without increasing plasma triglyceride levels (Kaneko *et al.*, 2003). Moreover, gene-selective modulation by the synthetic oxysterol ligand DMHCA (N, N-dimethyl-3β-hydroxycholenamide), whereby ABCA1 transcriptional activity is stimulated with minimal effects on SREBP-1c, has been shown to occur in human THP-1 macrophages (Quinet *et al.*, 2004). Also, the generation of selective LXR modulators that disrupt the binding of LXR to corepressors without leading to coactivator recruitment may have the potential to selectively increase cholesterol efflux genes and thus be used for anti-atherogenic purposes without having a side-effect on lipogenesis. Recent studies have demonstrated NCoR, one of the corepressors recruited by the unliganded LXR heterodimer, is not recruited to LXR target genes in LXR-/- macrophages, which is sufficient to allow increased expression of the ABCA1 gene and enhanced cholesterol efflux, but does not result in derepression of SREBP-1c or increased fatty acid biosynthesis (Hu *et al.*, 2003).

In addition, to selective agonists, the identification of cell signalling pathway involvement in the regulation of nuclear receptor signalling provides another potential mechanism for therapeutic intervention. Not only could research into these pathways enable the differentiation between various target genes of these receptors and provide a better understanding of the precise mechanisms involved in nuclear receptor-activated transcription, which would allow the desired therapeutic outcomes to be achieved without unwanted side-effects, but recent developments have

also demonstrated that the selective targeting of protein kinases is becoming a therapeutic reality (Heldin *et al.*, 2001) and the clinical use of small molecule inhibitors may perhaps become a common procedure.

7.3. FUTURE STUDIES

As a result of the work presented in this thesis, many avenues for further experimental studies have become apparent. Although potential mechanisms for the involvement of the JNK, PI3K and CK2 have been suggested, further work in elucidating the major upstream and downstream components of these pathways and their the exact roles in the LXR-mediated regulation of apoE expression must be undertaken. Firstly, it is important to determine how these signalling pathways are being activated by the LXR ligand, 22(R)-hydroxycholesterol. It may be, as has been shown for several other nuclear receptors, that a membrane- or cytoplasm-based LXR receptor exists and ligand-binding by the receptor may then result in LXR activation of various upstream components of these pathways. The identification of an LXR receptor outside the nucleus could possibly be undertaken through investigations using fluorescent microscopy to identify the location of LXR receptors within the cell or perhaps western blot analysis using extracts for the various cellular compartments and a specific LXR antibody. How this receptor is activating these pathways could then be analysed by co-immuniprecipitation studies using an antibody against LXR and the signalling components of this pathway such as JNK , CK2 or the p85 regulatory subunit of PI3K. The results of these investigations would identify whether LXR is binding to the signalling molecules and would provide a potential mechanism for the activation of cell signalling pathways by LXR. Unfortunately, these experiments were not carried out during this study due to time restraints and the lack of a specific, commercial LXR antibody, however, a monoclonal antibody against the human LXRα has recently been established (Watanabe *et al.*, 2003). In addition to identifying the mechanism behind the non-genomic activation of these pathways by 22(R)-hydroxycholesterol, these experiments would further confirm that the activation of the JNK, PI3K and CK2 signalling pathways was LXR-dependent.

With respect to the JNK pathway, additional experiments identifying how c-jun is involved in the LXR-mediated upregulation of apoE would be worthwhile. One suggested mechanism is that c-jun is activating the transcription of a coregulator protein necessary for the LXR activation of apoE. Therefore, before these experiments are carried out it may firstly be useful to identify potential coregulators involved in the LXR-mediated transcription of apoE. Transfection based analysis using expression plasmids for coregulator proteins represents one way in which we could carryout these studies, whilst chromatin immuniprecipitation (CHIP) assays would confirm the interaction *in vivo*, in a normal chromatin context. Identification of coregulators through CHIP

analysis would entail THP-1 cells being treated with ligand for the requisite time and then incubated with formaldehyde to cross-link the coregulators and DNA-binding proteins to the DNA *in vivo*. Cell nuclei are then prepared and, following lysis, the chromatin is fragmented by sonication and subjected to immunoprecipitation using antibodies specific to the coregulator of interest. The cross-links are then reversed and the precipitated DNA purified and subjected to PCR using primers against the LXRE sequences of the apoE promoter in order to identify whether these sequences were precipitated with the coregulator protein. Once potential coregulators have been identified it would then be possible to investigate whether c-jun is involved in the transcription of these proteins. It may also be possible that c-jun is affecting the apoE promoter directly through the AP-1 element (Basheeruddin *et al.*, 1994), which could be tested by EMSA analysis or chromatin immunoprecipitation. It would also be of great interest to identify whether the promoters of other LXR target genes such as ABCA1, ABCG1 and LXRα contain an AP-1 element, as the induction of both these genes was inhibited by pre-treatment with curcumin.

As detailed in Chapter 6, we have also demonstrated the involvement of the PI3K and CK2 pathways in the LXR-mediated induction of apoE. Although the studies presented in this thesis have demonstrated the activation of Akt, further studies are necessary to identify whether any Akt targets are activated by LXR treatment, this could be carried out through western blot analysis as was done for GSK-3β and FKHR in this study, or possibly through the use of inhibitors.

Akt has been shown to phosphorylate certain nuclear receptors such as ERα and AR (Campbell *et al.*, 2001; Lin *et al.*, 2001b) and we have also identified Akt phosphorylation sites in the LXR proteins, therefore investigation of LXR phosphorylation would be important to progress these studies. This could be undertaken by an *in vitro* phosphorylation assay, whereby a recombinant LXR protein could be used as a substrate for a radioactive Akt kinase assay. This assay would entail immunoprecipitating Akt from cell extracts, which would then be used in a kinase reaction using a specific kinase buffer, LXR as the substrate and γ-^{32}P-labelled ATP as the phosphate donor. Once the kinase reaction is complete the contents of this reaction could be size-fractionated on a SDS-PAGE gel and phosphorylation of LXR by Akt could be assessed by autoradiography. This kinase assay could also be used to investigate the ability of CK2 to phosphorylate LXR as we have also identified a potential CK2 phosphorylation motif in the LXR proteins.

With respect to the involvement of cell signalling pathways in the LXR regulation of genes, it would be of great interest to establish whether the activation of the pathways identified in this study are ligand-specific. The data in this thesis could be further extended to synthetic compounds as several synthetic LXR ligands have been shown to demonstrate atheroprotective properties in mouse models and a greater understanding of the mechanism and signalling pathways involved in

this LXR-mediated effect would be of great benefit. Also the potential involvement of other cell signalling pathways should be further investigated with regards to the ligand, 22(R)-hydroxycholesterol, in order to gain a better understanding of the proposed mechanisms. Cell signalling pathways very rarely act in isolation and although we have ruled out the ERK MAPK and p38 MAPK pathways in the LXR-mediated up-regulation of apoE, several other pathways which have been shown to be extensively involved in nuclear receptor signalling, such as protein kinase A (PKA) and the cyclin-dependent kinases (cdks) also require investigation.

It is also important, with regards to the wider implications of this work to determine the involvement of cell signalling pathways in the LXR-mediated regulation of genes involved in lipogenesis such as FAS and SREBP-1c. Again, these experiments could initially be carried out using inhibitors against key signalling pathways, as was carried out in this study. The results of these investigations would provide further information, which could aid the development of therapeutic strategies to selectively activate the expression of atheroprotective genes such as apoE and ABCA1, without the unwanted side-effects of lipogenesis.

7.4. CONCLUDING REMARKS

From the work presented in this thesis, it can be seen that the LXR-mediated regulation of the apoE gene in macrophages is dependent on several cell signalling pathways. The atheroprotective ability of apoE and the ability of LXR to regulate genes of the reverse cholesterol transport pathway and limit foam cell formation, demonstrates the importance of these proteins in the pathogenesis of atherosclerosis and also their potential as therapeutic targets. Therefore, it is crucial to gain as complete an understanding as possible of all aspects of their molecular mechanisms. By pursuing the future studies suggested above, it is hoped that further progress regarding this objective will be made.

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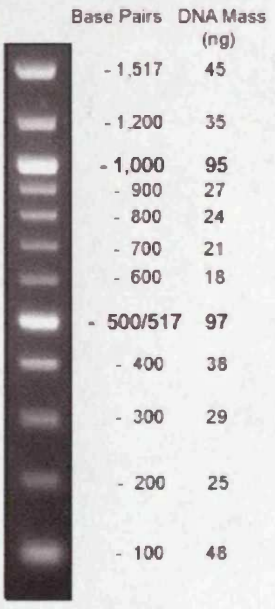
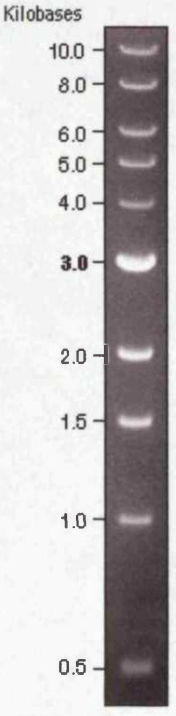
APPENDICES

APPENDIX I – MAPS OF DNA MOLECULAR WEIGHT MARKERS



100BP DNA MARKER (NEB)

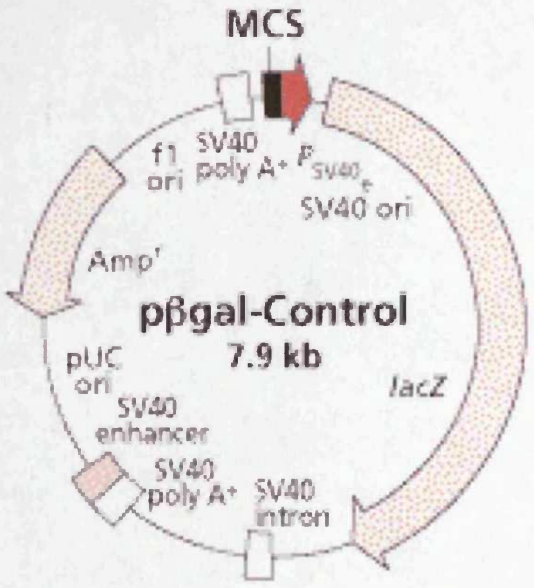


1KB DNA MARKER (GIBCO)

APPENDIX II – MAP OF THE RAINBOW PROTEIN SIZE MARKER

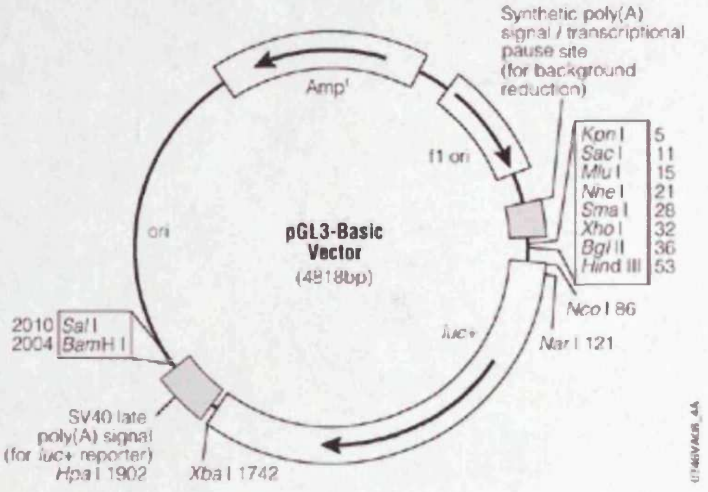
ADAPTED FROM THE AMERSHAM-PHARMACIA PRODUCT MANUAL

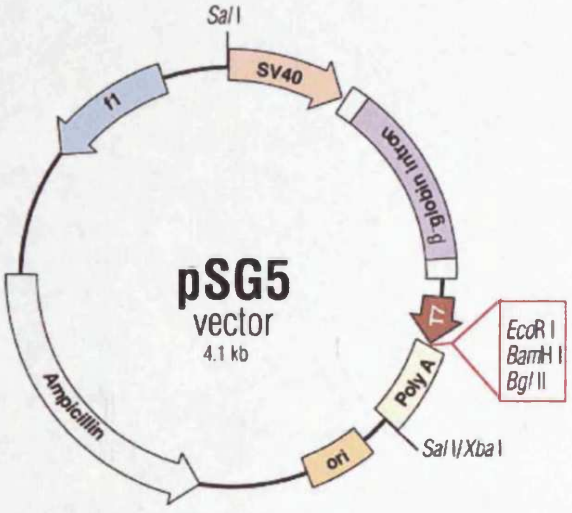
APPENDIX III – MAP OF THE PCMV-βGAL PLASMID

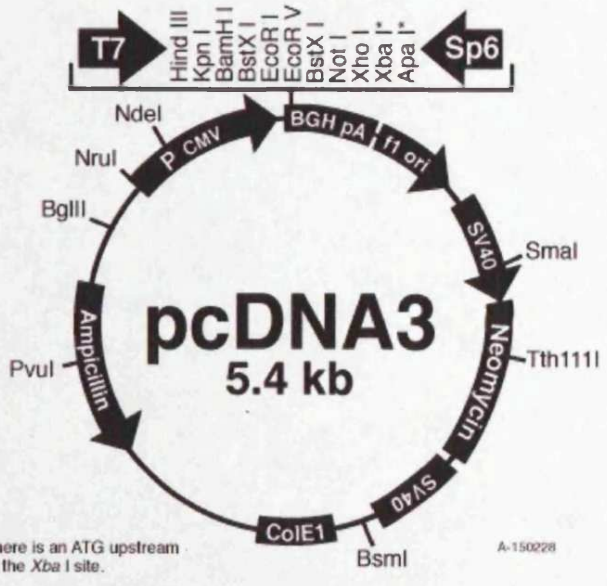


PCMV-βGAL (CLONETECH)- PLASMID USED TO EXPRESS β-GALACTOSIDASE IN MAMMALIAN CELLS FROM THE HUMAN CMV IMMEDIATE EARLY GENE PROMOTER

APPENDIX IV – MAPS FOR PLASMID VECTORS USED IN THIS STUDY







* There is an ATG upstream of the *Xba* I site.

APPENDIX V – SCANSITE MOTIF SCAN PROFILE FOR THE HUMAN LXRα AND LXRβ PROTEIN SEQUENCES

LXRα (ACCESSION NUMBER NP_005684) - SCANNED FOR ALL MOTIFS AT LOW STRIGENCY

Phosphoserine/threonine binding group (pST_bind)				
14-3-3 Mode 1			Gene Card YWHAZ	
Site	Score	Percentile	Sequence	SA
S244	0.5958	3.368 %	QCNKRSFSDQPKVTP	1.594
Tyrosine kinase group (Y_kin)				
Fgr Kinase			Gene Card FGR	
Site	Score	Percentile	Sequence	SA
Y370	0.5180	3.490 %	LGLDDAEYALLIAIN	0.432
Lck Kinase			Gene Card LCK	
Site	Score	Percentile	Sequence	SA
Y370	0.5871	3.768 %	LGLDDAEYALLIAIN	0.432
Src homology 2 group (SH2)				
PLCg N-terminal SH2			Gene Card PLCG1	
Site	Score	Percentile	Sequence	SA
Y399	0.4943	3.899 %	VEALQQPYVEALLSY	1.249
PLCg C-terminal SH2			Gene Card PLCG1	
Site	Score	Percentile	Sequence	SA
Y399	0.5265	3.505 %	VEALQQPYVEALLSY	1.249
PLCg C-terminal SH2			Gene Card PLCG1	
Site	Score	Percentile	Sequence	SA
Y370	0.5282	3.611 %	LGLDDAEYALLIAIN	0.432
Fyn SH2			Gene Card FYN	
Site	Score	Percentile	Sequence	SA
Y406	0.5297	2.154 %	YVEALLSYTRIKRPQ	0.786
Shc SH2			Gene Card SHC1	
Site	Score	Percentile	Sequence	SA
Y406	0.5810	4.373 %	YVEALLSYTRIKRPQ	0.786
Src homology 3 group (SH3)				
Grb2 SH3			Gene Card GRB2	
Site	Score	Percentile	Sequence	SA
P44	0.4986	0.447 %	PWPGGPDPDVPGTDE	1.754
Abl SH3			Gene Card ABL1	
Site	Score	Percentile	Sequence	SA
P44	0.5274	1.570 %	PWPGGPDPDVPGTDE	1.754
Grb2 SH3			Gene Card GRB2	
Site	Score	Percentile	Sequence	SA
P21	0.5348	0.954 %	PGNGPPQPGAPSSSP	1.467

Grb2 SH3			Gene Card GRB2	
Site	Score	Percentile	Sequence	SA
P14	0.5835	2.135 %	SSLDTPLPGNGPPQP	0.739
Cortactin SH3			Gene Card HCLS1	
Site	Score	Percentile	Sequence	SA
P21	0.5837	0.538 %	PGNGPPQPGAPSSSP	1.467
Grb2 SH3			Gene Card GRB2	
Site	Score	Percentile	Sequence	SA
P18	0.5945	2.586 %	TPLPGNGPPQPGAPS	2.425
Src SH3			Gene Card SRC	
Site	Score	Percentile	Sequence	SA
P44	0.6089	2.992 %	PWPGGPDPDVPGTDE	1.754
Crk SH3			Gene Card CRK	
Site	Score	Percentile	Sequence	SA
P21	0.6105	3.862 %	PGNGPPQPGAPSSSP	1.467
Nck 2nd SH3			Gene Card NCK1	
Site	Score	Percentile	Sequence	SA
P185	0.6114	2.665 %	SQSQSQSPVGPQGSS	0.934
Crk SH3			Gene Card CRK	
Site	Score	Percentile	Sequence	SA
P185	0.6277	4.822 %	SQSQSQSPVGPQGSS	0.934
Src SH3			Gene Card SRC	
Site	Score	Percentile	Sequence	SA
P21	0.6449	4.583 %	PGNGPPQPGAPSSSP	1.467
Nck 2nd SH3			Gene Card NCK1	
Site	Score	Percentile	Sequence	SA
P77	0.6461	4.481 %	ERKRKKGPAPKMLGH	2.191
PLCg SH3			Gene Card PLCG1	
Site	Score	Percentile	Sequence	SA
P251	0.6581	2.188 %	SDQPKVTPWPLGADP	0.509
PLCg SH3			Gene Card PLCG1	
Site	Score	Percentile	Sequence	SA
P24	0.6637	2.401 %	GPPQPGAPSSSPTVK	0.853
Cbl-Associated protein C-SH3			Gene Card N/A	
Site	Score	Percentile	Sequence	SA
P188	0.6907	0.451 %	QSQSPVGPQGSSSSA	0.598
PLCg SH3			Gene Card PLCG1	
Site	Score	Percentile	Sequence	SA
P188	0.6953	4.085 %	QSQSPVGPQGSSSSA	0.598
Itk SH3			Gene Card ITK	
Site	Score	Percentile	Sequence	SA
P77	0.7087	2.723 %	ERKRKKGPAPKMLGH	2.191

p85 SH3 mode2			Gene Card PIK3R1	
Site	Score	Percentile	Sequence	SA
P24	0.7260	3.704 %	GPPQPGAPSSSPTVK	0.853
Cbl-Associated protein C-SH3			Gene Card N/A	
Site	Score	Percentile	Sequence	SA
P21	0.7729	1.290 %	PGNGPPQPGAPSSSP	1.467
Src SH3			Gene Card SRC	
Site	Score	Percentile	Sequence	SA
P185	0.6449	4.583 %	SQSQSQSPVGPQGSS	0.934
Basophilic serine/threonine kinase group (Baso_ST_kin)				
PKC mu			Gene Card PRKCM	
Site	Score	Percentile	Sequence	SA
T430	0.4419	0.308 %	MKLVSLRTLSSVHSE	0.791
PKC epsilon			Gene Card PRKCE	
Site	Score	Percentile	Sequence	SA
T29	0.4830	1.979 %	GAPSSSPTVKEEGPE	1.762
PKC delta			Gene Card PRKCD	
Site	Score	Percentile	Sequence	SA
S114	0.4886	1.893 %	CKGFFRRSVVRGGAR	1.271
PKC delta			Gene Card PRKCD	
Site	Score	Percentile	Sequence	SA
T29	0.5023	2.571 %	GAPSSSPTVKEEGPE	1.762
PKC alpha/beta/gamma			Gene Card PRKCA	
Site	Score	Percentile	Sequence	SA
S427	0.5029	1.683 %	RMLMKLVSLRTLSSV	0.438
PKC mu			Gene Card PRKCM	
Site	Score	Percentile	Sequence	SA
T308	0.5074	1.410 %	IALLKASTIEIMLLE	0.381
PKC alpha/beta/gamma			Gene Card PRKCA	
Site	Score	Percentile	Sequence	SA
S114	0.5136	2.064 %	CKGFFRRSVVRGGAR	1.271
PKC epsilon			Gene Card PRKCE	
Site	Score	Percentile	Sequence	SA
T334	0.5223	4.789 %	ITFLKDFTYSKDDFH	2.008
Calmodulin dependent Kinase 2			Gene Card CAMK2G	
Site	Score	Percentile	Sequence	SA
T334	0.5364	1.251 %	ITFLKDFTYSKDDFH	2.008
Calmodulin dependent Kinase 2			Gene Card CAMK2G	
Site	Score	Percentile	Sequence	SA
S244	0.5380	1.289 %	QCNKRSFSDQPKVTP	1.594
Protein Kinase A			Gene Card PRKACG	
Site	Score	Percentile	Sequence	SA
S114	0.5547	2.140 %	CKGFFRRSVVRGGAR	1.271

PKC mu			Gene Card PRKCM	
Site	Score	Percentile	Sequence	SA
S436	0.5685	4.650 %	RTLSSVHSEQVFALR	0.690
Calmodulin dependent Kinase 2			Gene Card CAMK2G	
Site	Score	Percentile	Sequence	SA
S178	0.6050	4.109 %	RKQQQQESQSQSQSP	3.702
Calmodulin dependent Kinase 2			Gene Card CAMK2G	
Site	Score	Percentile	Sequence	SA
T308	0.6090	4.361 %	IALLKASTIEIMLLE	0.381
PKC zeta			Gene Card PRKCZ	
Site	Score	Percentile	Sequence	SA
S95	0.6148	4.338 %	RVCGDKASGFHYNVL	0.723
Protein Kinase A			Gene Card PRKACG	
Site	Score	Percentile	Sequence	SA
T334	0.6156	4.455 %	ITFLKDFTYSKDDFH	2.008
PKC zeta			Gene Card PRKCZ	
Site	Score	Percentile	Sequence	SA
S114	0.6198	4.643 %	CKGFFRRSVVRGGAR	1.271
PKC zeta			Gene Card PRKCZ	
Site	Score	Percentile	Sequence	SA
T328	0.6198	4.643 %	NHETECITFLKDFTY	0.177
Protein Kinase A			Gene Card PRKACG	
Site	Score	Percentile	Sequence	SA
T308	0.6204	4.699 %	IALLKASTIEIMLLE	0.381
Protein Kinase A			Gene Card PRKACG	
Site	Score	Percentile	Sequence	SA
S427	0.6254	4.952 %	RMLMKLVSLRTLSSV	0.438
Akt Kinase			Gene Card AKT1	
Site	Score	Percentile	Sequence	SA
S244	0.6469	1.853 %	QCNKRSFSDQPKVTP	1.594
DNA damage kinase group (DNA_dam_kin)				
DNA PK			Gene Card PRKDC	
Site	Score	Percentile	Sequence	SA
S8	0.5082	0.602 %	MSSPTTSSLDTPLPG	1.181
DNA PK			Gene Card PRKDC	
Site	Score	Percentile	Sequence	SA
S209	0.5227	0.758 %	PGGSEAGSQGSGEGE	0.705
ATM Kinase			Gene Card ATM	
Site	Score	Percentile	Sequence	SA
S178	0.5266	0.848 %	RKQQQQESQSQSQSP	3.702
DNA PK			Gene Card PRKDC	
Site	Score	Percentile	Sequence	SA
S178	0.5419	1.028 %	RKQQQQESQSQSQSP	3.702

DNA PK			Gene Card PRKDC	
Site	Score	Percentile	Sequence	SA
S180	0.5464	1.099 %	QQQQESQSQSQSPVG	2.865
DNA PK			Gene Card PRKDC	
Site	Score	Percentile	Sequence	SA
S182	0.5678	1.542 %	QQESQSQSQSPVGPQ	2.558
ATM Kinase			Gene Card ATM	
Site	Score	Percentile	Sequence	SA
S161	0.5696	1.769 %	MREQCVLSEEQIRKK	0.976
ATM Kinase			Gene Card ATM	
Site	Score	Percentile	Sequence	SA
S180	0.5901	2.482 %	QQQQESQSQSQSPVG	2.865
ATM Kinase			Gene Card ATM	
Site	Score	Percentile	Sequence	SA
T49	0.5970	2.761 %	PDPDVPGTDEASSAC	1.479
ATM Kinase			Gene Card ATM	
Site	Score	Percentile	Sequence	SA
S182	0.6097	3.368 %	QQESQSQSQSPVGPQ	2.558
ATM Kinase			Gene Card ATM	
Site	Score	Percentile	Sequence	SA
S336	0.6146	3.625 %	FLKDFTYSKDDFHRA	3.873
ATM Kinase			Gene Card ATM	
Site	Score	Percentile	Sequence	SA
S103	0.6245	4.213 %	GFHYNVLSCEGCKGF	0.173
Acidophilic serine/threonine kinase group (Acid_ST_kin)				
Casein Kinase 1			Gene Card CSNK1G2	
Site	Score	Percentile	Sequence	SA
S196	0.3823	0.219 %	QGSSSSASGPGASPG	0.630
Casein Kinase 1			Gene Card CSNK1G2	
Site	Score	Percentile	Sequence	SA
S8	0.4365	0.912 %	MSSPTTSSLDTPLPG	1.181
Casein Kinase 1			Gene Card CSNK1G2	
Site	Score	Percentile	Sequence	SA
S209	0.4431	1.077 %	PGGSEAGSQGSGEGE	0.705
Casein Kinase 1			Gene Card CSNK1G2	
Site	Score	Percentile	Sequence	SA
T29	0.4752	2.063 %	GAPSSSPTVKEEGPE	1.762
Casein Kinase 1			Gene Card CSNK1G2	
Site	Score	Percentile	Sequence	SA
S54	0.5268	4.939 %	PGTDEASSACSTDWV	0.302
GSK3 Kinase			Gene Card GSK3A	
Site	Score	Percentile	Sequence	SA
S25	0.5402	0.612 %	PPQPGAPSSSPTVKE	1.332

Casein Kinase 2			Gene Card CSNK2B	
Site	Score	Percentile	Sequence	SA
S53	0.5651	2.439 %	VPGTDEASSACSTDW	0.390
GSK3 Kinase			Gene Card GSK3A	
Site	Score	Percentile	Sequence	SA
S201	0.5843	1.343 %	SASGPGASPGGSEAG	0.465
Casein Kinase 2			Gene Card CSNK2B	
Site	Score	Percentile	Sequence	SA
T29	0.5877	3.449 %	GAPSSSPTVKEEGPE	1.762
GSK3 Kinase			Gene Card GSK3A	
Site	Score	Percentile	Sequence	SA
S205	0.5927	1.549 %	PGASPGGSEAGSQGS	0.521
Casein Kinase 2			Gene Card CSNK2B	
Site	Score	Percentile	Sequence	SA
T49	0.6051	4.553 %	PDPDVPGTDEASSAC	1.479
GSK3 Kinase			Gene Card GSK3A	
Site	Score	Percentile	Sequence	SA
S178	0.6169	2.329 %	RKQQQQESQSQSQSP	3.702
GSK3 Kinase			Gene Card GSK3A	
Site	Score	Percentile	Sequence	SA
S180	0.6335	2.990 %	QQQQESQSQSQSPVG	2.865
GSK3 Kinase			Gene Card GSK3A	
Site	Score	Percentile	Sequence	SA
S454	0.6491	3.876 %	KKLPPLLSEIWDVHE	0.267
Proline-dependent serine/threonine kinase group (Pro_ST_kin)				
p38 MAPK			Gene Card MAPK14	
Site	Score	Percentile	Sequence	SA
S184	0.4495	4.766 %	ESQSQSQSPVGPQGS	0.810
Cdk5 Kinase			Gene Card CDK5	
Site	Score	Percentile	Sequence	SA
S27	0.5119	1.058 %	QPGAPSSSPTVKEEG	0.914
Erk1 Kinase			Gene Card EPHB2	
Site	Score	Percentile	Sequence	SA
T11	0.5330	0.828 %	PTTSSLDTPLPGNGP	0.898
Cdc2 Kinase			Gene Card CDC2	
Site	Score	Percentile	Sequence	SA
S27	0.5379	0.927 %	QPGAPSSSPTVKEEG	0.914
Cdc2 Kinase			Gene Card CDC2	
Site	Score	Percentile	Sequence	SA
S201	0.5828	1.703 %	SASGPGASPGGSEAG	0.465
Erk1 Kinase			Gene Card EPHB2	
Site	Score	Percentile	Sequence	SA
S184	0.6028	2.144 %	ESQSQSQSPVGPQGS	0.810

Cdk5 Kinase			Gene Card CDK5	
Site	Score	Percentile	Sequence	SA
S201	0.6108	3.238 %	SASGPGASPGGSEAG	0.465
Erk1 Kinase			Gene Card EPHB2	
Site	Score	Percentile	Sequence	SA
T250	0.6193	2.649 %	FSDQPKVTPWPLGAD	1.234
Cdk5 Kinase			Gene Card CDK5	
Site	Score	Percentile	Sequence	SA
T11	0.6312	3.910 %	PTTSSLDTPLPGNGP	0.898
Cdk5 Kinase			Gene Card CDK5	
Site	Score	Percentile	Sequence	SA
S184	0.6369	4.108 %	ESQSQSQSPVGPQGS	0.810
Erk1 Kinase			Gene Card EPHB2	
Site	Score	Percentile	Sequence	SA
S27	0.6490	3.732 %	QPGAPSSSPTVKEEG	0.914
Erk1 Kinase			Gene Card EPHB2	
Site	Score	Percentile	Sequence	SA
S196	0.6536	3.908 %	QGSSSSASGPGASPG	0.630
Erk1 Kinase			Gene Card EPHB2	
Site	Score	Percentile	Sequence	SA
S201	0.6614	4.274 %	SASGPGASPGGSEAG	0.465
Cdc2 Kinase			Gene Card CDC2	
Site	Score	Percentile	Sequence	SA
T11	0.6650	4.049 %	PTTSSLDTPLPGNGP	0.898
Cdc2 Kinase			Gene Card CDC2	
Site	Score	Percentile	Sequence	SA
S184	0.6701	4.227 %	ESQSQSQSPVGPQGS	0.810
Cdc2 Kinase			Gene Card CDC2	
Site	Score	Percentile	Sequence	SA
T250	0.6829	4.701 %	FSDQPKVTPWPLGAD	1.234
Kinase binding site group (Kin_bind)				
PDK1 Binding			Gene Card PDPK1	
Site	Score	Percentile	Sequence	SA
T334	0.5942	0.602 %	ITFLKDFTYSKDDFH	2.008
PDK1 Binding			Gene Card PDPK1	
Site	Score	Percentile	Sequence	SA
D339	0.6249	0.982 %	DFTYSKDDFHRAGLQ	2.950
Erk D-domain			Gene Card MAPK1	
Site	Score	Percentile	Sequence	SA
V426	0.6299	0.416 %	PRMLMKLVSLRTLSS	0.607
PDK1 Binding			Gene Card PDPK1	
Site	Score	Percentile	Sequence	SA
T272	0.6374	1.139 %	QQRFAHFTELAIISV	0.562

PDK1 Binding			Gene Card PDPK1	
Site	Score	Percentile	Sequence	SA
E355	0.6566	1.309 %	EFINPIFEFSRAMRR	0.548
Erk D-domain			Gene Card MAPK1	
Site	Score	Percentile	Sequence	SA
I311	0.6568	0.634 %	LKASTIEIMLLETAR	0.131
Erk D-domain			Gene Card MAPK1	
Site	Score	Percentile	Sequence	SA
L274	0.6996	1.218 %	RFAHFTELAIISVQE	0.234
Erk D-domain			Gene Card MAPK1	
Site	Score	Percentile	Sequence	SA
L293	0.7171	1.580 %	AKQVPGFLQLGREDQ	0.229
Erk D-domain			Gene Card MAPK1	
Site	Score	Percentile	Sequence	SA
L449	0.7304	1.883 %	LRLQDKKLPPLLSEI	1.490
Erk D-domain			Gene Card MAPK1	
Site	Score	Percentile	Sequence	SA
L254	0.6461	0.533 %	PKVTPWPLGADPQSR	0.513

LXRβ (ACCESSION NUMBER NP_005684) - SCANNED FOR ALL MOTIFS AT LOW STRIGENCY

Phosphoserine/threonine binding group (pST_bind)				
14-3-3 Mode 1			Gene Card YWHAZ	
Site	Score	Percentile	Sequence	SA
S198	0.3941	0.371 %	SLPPRASSPPQILPQ	1.722
14-3-3 Mode 1			Gene Card YWHAZ	
Site	Score	Percentile	Sequence	SA
S191	0.5218	1.637 %	EEQAHATSLPPRASS	0.883
14-3-3 Mode 1			Gene Card YWHAZ	
Site	Score	Percentile	Sequence	SA
S230	0.5551	2.277 %	QCNRRSFSDRLRVTP	0.961
Src homology 2 group (SH2)				
p85 SH2			Gene Card PIK3R1	
Site	Score	Percentile	Sequence	SA
Y145	0.7008	4.296 %	GHCPMDTYMRRKCQE	3.285
Src homology 3 group (SH3)				
Grb2 SH3			Gene Card GRB2	
Site	Score	Percentile	Sequence	SA
P10	0.4948	0.416 %	LWLGAPVPDIPPDSA	0.736
Nck 2nd SH3			Gene Card NCK1	
Site	Score	Percentile	Sequence	SA
P88	0.5470	1.063 %	PQKRKKGPAPKMLGN	2.191

Crk SH3			Gene Card CRK	
Site	Score	Percentile	Sequence	SA
P10	0.5492	1.597 %	LWLGAPVPDIPPDSA	0.736
Src SH3			Gene Card SRC	
Site	Score	Percentile	Sequence	SA
P10	0.5493	1.385 %	LWLGAPVPDIPPDSA	0.736
Amphiphysin SH3			Gene Card AMPH	
Site	Score	Percentile	Sequence	SA
P239	0.5869	1.034 %	RLRVTPWPMAPDPHS	0.891
Abl SH3			Gene Card ABL1	
Site	Score	Percentile	Sequence	SA
P193	0.5963	3.923 %	QAHATSLPPRASSPP	1.198
Abl SH3			Gene Card ABL1	
Site	Score	Percentile	Sequence	SA
P10	0.5982	4.021 %	LWLGAPVPDIPPDSA	0.736
Intersectin SH3A			Gene Card ITSN	
Site	Score	Percentile	Sequence	SA
P13	0.6119	0.312 %	GAPVPDIPPDSAVEL	1.435
Src SH3			Gene Card SRC	
Site	Score	Percentile	Sequence	SA
P193	0.6243	3.619 %	QAHATSLPPRASSPP	1.198
Grb2 SH3			Gene Card GRB2	
Site	Score	Percentile	Sequence	SA
P193	0.6255	4.044 %	QAHATSLPPRASSPP	1.198
Nck 2nd SH3			Gene Card NCK1	
Site	Score	Percentile	Sequence	SA
P73	0.6434	4.325 %	LLTRAEPPSEPTEIR	3.405
PLCg SH3			Gene Card PLCG1	
Site	Score	Percentile	Sequence	SA
P242	0.6509	1.942 %	VTPWPMAPDPHSREA	1.245
Cortactin SH3			Gene Card HCLS1	
Site	Score	Percentile	Sequence	SA
P193	0.6658	2.001 %	QAHATSLPPRASSPP	1.198
Cbl-Associated protein C-SH3			Gene Card N/A	
Site	Score	Percentile	Sequence	SA
P193	0.6743	0.360 %	QAHATSLPPRASSPP	1.198
Itk SH3			Gene Card ITK	
Site	Score	Percentile	Sequence	SA
P88	0.6922	2.064 %	PQKRKKGPAPKMLGN	2.191
PLCg SH3			Gene Card PLCG1	
Site	Score	Percentile	Sequence	SA
P237	0.6963	4.160 %	SDRLRVTPWPMAPDP	0.611

p85 SH3 model			Gene Card PIK3R1	
Site	Score	Percentile	Sequence	SA
P10	0.7006	3.315 %	LWLGAPVPDIPPDSA	0.736
PLCg SH3			Gene Card PLCG1	
Site	Score	Percentile	Sequence	SA
P14	0.7036	4.659 %	APVPDIPPDSAVELW	0.868
Cortactin SH3			Gene Card HCLS1	
Site	Score	Percentile	Sequence	SA
P73	0.7084	3.711 %	LLTRAEPPSEPTEIR	3.405
Cortactin SH3			Gene Card HCLS1	
Site	Score	Percentile	Sequence	SA
P10	0.7213	4.437 %	LWLGAPVPDIPPDSA	0.736
Intersectin SH3A			Gene Card ITSN	
Site	Score	Percentile	Sequence	SA
P193	0.7245	1.230 %	QAHATSLPPRASSPP	1.198
p85 SH3 model			Gene Card PIK3R1	
Site	Score	Percentile	Sequence	SA
P239	0.7282	4.788 %	RLRVTPWPMAPDPHS	0.891
Cbl-Associated protein C-SH3			Gene Card N/A	
Site	Score	Percentile	Sequence	SA
P242	0.8671	3.697 %	VTPWPMAPDPHSREA	1.245
Cbl-Associated protein C-SH3			Gene Card N/A	
Site	Score	Percentile	Sequence	SA
P13	0.8708	3.828 %	GAPVPDIPPDSAVEL	1.435
Basophilic serine/threonine kinase group (Baso_ST_kin)				
PKC mu			Gene Card PRKCM	
Site	Score	Percentile	Sequence	SA
T416	0.4419	0.308 %	MKLVSLRTLSSVHSE	0.791
PKC alpha/beta/gamma			Gene Card PRKCA	
Site	Score	Percentile	Sequence	SA
S125	0.4732	0.927 %	CKGFFRRSVIKGAHY	1.226
PKC delta			Gene Card PRKCD	
Site	Score	Percentile	Sequence	SA
S363	0.4801	1.585 %	FALLIAISIFSADRP	0.177
PKC epsilon			Gene Card PRKCE	
Site	Score	Percentile	Sequence	SA
S320	0.4993	3.027 %	ITFLKDFSYNREDFA	2.276
PKC alpha/beta/gamma			Gene Card PRKCA	
Site	Score	Percentile	Sequence	SA
S413	0.5029	1.683 %	RMLMKLVSLRTLSSV	0.438
PKC epsilon			Gene Card PRKCE	
Site	Score	Percentile	Sequence	SA
S363	0.5097	3.645 %	FALLIAISIFSADRP	0.177

Protein Kinase A			Gene Card PRKACG	
Site	Score	Percentile	Sequence	SA
S125	0.5357	1.690 %	CKGFFRRSVIKGAHY	1.226
PKC alpha/beta/gamma			Gene Card PRKCA	
Site	Score	Percentile	Sequence	SA
S363	0.5525	4.273 %	FALLIAISIFSADRP	0.177
PKC zeta			Gene Card PRKCZ	
Site	Score	Percentile	Sequence	SA
S125	0.5595	1.449 %	CKGFFRRSVIKGAHY	1.226
PKC mu			Gene Card PRKCM	
Site	Score	Percentile	Sequence	SA
S293	0.5651	4.365 %	QIALLKTSAIEVMLL	1.087
PKC mu			Gene Card PRKCM	
Site	Score	Percentile	Sequence	SA
S422	0.5685	4.650 %	RTLSSVHSEQVFALR	0.690
Protein Kinase A			Gene Card PRKACG	
Site	Score	Percentile	Sequence	SA
S230	0.6096	4.163 %	QCNRRSFSDRLRVTP	0.961
Calmodulin dependent Kinase 2			Gene Card CAMK2G	
Site	Score	Percentile	Sequence	SA
S282	0.6115	4.530 %	LPGFLQLSREDQIAL	2.485
Calmodulin dependent Kinase 2			Gene Card CAMK2G	
Site	Score	Percentile	Sequence	SA
S230	0.6131	4.635 %	QCNRRSFSDRLRVTP	0.961
Protein Kinase A			Gene Card PRKACG	
Site	Score	Percentile	Sequence	SA
S228	0.6134	4.348 %	QQQCNRRSFSDRLRV	2.283
Protein Kinase A			Gene Card PRKACG	
Site	Score	Percentile	Sequence	SA
S320	0.6184	4.594 %	ITFLKDFSYNREDFA	2.276
Protein Kinase A			Gene Card PRKACG	
Site	Score	Percentile	Sequence	SA
S413	0.6254	4.952 %	RMLMKLVSLRTLSSV	0.438
Akt Kinase			Gene Card AKT1	
Site	Score	Percentile	Sequence	SA
S198	0.6784	2.682 %	SLPPRASSPPQILPQ	1.722
Akt Kinase			Gene Card AKT1	
Site	Score	Percentile	Sequence	SA
S230	0.6893	3.020 %	QCNRRSFSDRLRVTP	0.961
Clk2 Kinase			Gene Card CLK2	
Site	Score	Percentile	Sequence	SA
S230	0.7203	0.979 %	QCNRRSFSDRLRVTP	0.961

Clk2 Kinase			Gene Card CLK2	
Site	Score	Percentile	Sequence	SA
S418	0.7913	2.204 %	LVSLRTLSSVHSEQV	0.495
DNA damage kinase group (DNA_dam_kin)				
ATM Kinase			Gene Card ATM	
Site	Score	Percentile	Sequence	SA
S207	0.4605	0.247 %	PQILPQLSPEQLGMI	2.034
ATM Kinase			Gene Card ATM	
Site	Score	Percentile	Sequence	SA
S170	0.5552	1.404 %	MREECVLSEEQIRLK	0.976
ATM Kinase			Gene Card ATM	
Site	Score	Percentile	Sequence	SA
S282	0.5814	2.154 %	LPGFLQLSREDQIAL	2.485
ATM Kinase			Gene Card ATM	
Site	Score	Percentile	Sequence	SA
S114	0.6245	4.213 %	GFHYNVLSCEGCKGF	0.173
ATM Kinase			Gene Card ATM	
Site	Score	Percentile	Sequence	SA
S30	0.6345	4.876 %	PGAQDASSQAQGGSS	1.260
DNA PK			Gene Card PRKDC	
Site	Score	Percentile	Sequence	SA
S30	0.6467	4.798 %	PGAQDASSQAQGGSS	1.260
DNA PK			Gene Card PRKDC	
Site	Score	Percentile	Sequence	SA
S312	0.6467	4.798 %	RYNPGSESITFLKDF	0.624
Acidophilic serine/threonine kinase group (Acid_ST_kin)				
Casein Kinase 2			Gene Card CSNK2B	
Site	Score	Percentile	Sequence	SA
S74	0.5320	1.440 %	LTRAEPPSEPTEIRP	2.838
GSK3 Kinase			Gene Card GSK3A	
Site	Score	Percentile	Sequence	SA
T68	0.5673	1.004 %	AEPTALLTRAEPPSE	0.771
GSK3 Kinase			Gene Card GSK3A	
Site	Score	Percentile	Sequence	SA
S198	0.6103	2.087 %	SLPPRASSPPQILPQ	1.722
GSK3 Kinase			Gene Card GSK3A	
Site	Score	Percentile	Sequence	SA
S191	0.6321	2.927 %	EEQAHATSLPPRASS	0.883
GSK3 Kinase			Gene Card GSK3A	
Site	Score	Percentile	Sequence	SA
S74	0.6423	3.461 %	LTRAEPPSEPTEIRP	2.838

GSK3 Kinase			Gene Card GSK3A	
Site	Score	Percentile	Sequence	SA
S440	0.6491	3.876 %	KKLPPLLSEIWDVHE	0.267
Proline-dependent serine/threonine kinase group (Pro_ST_kin)				
Erk1 Kinase			Gene Card EPHB2	
Site	Score	Percentile	Sequence	SA
S198	0.5495	1.051 %	SLPPRASSPPQILPQ	1.722
Cdk5 Kinase			Gene Card CDK5	
Site	Score	Percentile	Sequence	SA
S198	0.6163	3.412 %	SLPPRASSPPQILPQ	1.722
Erk1 Kinase			Gene Card EPHB2	
Site	Score	Percentile	Sequence	SA
T236	0.6284	2.944 %	FSDRLRVTPWPMAPD	1.209
Cdc2 Kinase			Gene Card CDC2	
Site	Score	Percentile	Sequence	SA
S198	0.6514	3.592 %	SLPPRASSPPQILPQ	1.722
Cdk5 Kinase			Gene Card CDK5	
Site	Score	Percentile	Sequence	SA
T236	0.6555	4.789 %	FSDRLRVTPWPMAPD	1.209
Cdc2 Kinase			Gene Card CDC2	
Site	Score	Percentile	Sequence	SA
T236	0.6871	4.854 %	FSDRLRVTPWPMAPD	1.209
Kinase binding site group (Kin_bind)				
Erk D-domain			Gene Card MAPK1	
Site	Score	Percentile	Sequence	SA
L233	0.5212	0.068 %	RRSFSDRLRVTPWPM	1.297
PDK1 Binding			Gene Card PDPK1	
Site	Score	Percentile	Sequence	SA
E341	0.6206	0.929 %	EFINPIFEFSRAMNE	0.548
PDK1 Binding			Gene Card PDPK1	
Site	Score	Percentile	Sequence	SA
D325	0.6249	0.982 %	DFSYNREDFAKAGLQ	2.271
PDK1 Binding			Gene Card PDPK1	
Site	Score	Percentile	Sequence	SA
S320	0.6270	1.007 %	ITFLKDFSYNREDFA	2.276
Erk D-domain			Gene Card MAPK1	
Site	Score	Percentile	Sequence	SA
V412	0.6299	0.416 %	PRMLMKLVSLRTLSS	0.607
PDK1 Binding			Gene Card PDPK1	
Site	Score	Percentile	Sequence	SA
T258	0.6374	1.139 %	QQRFAHFTELAIVSV	0.562

Erk D-domain			Gene Card MAPK1	
Site	Score	Percentile	Sequence	SA
V297	0.6487	0.555 %	LKTSAIEVMLLETSR	0.139
Erk D-domain			Gene Card MAPK1	
Site	Score	Percentile	Sequence	SA
L260	0.6996	1.218 %	RFAHFTELAIVSVQE	0.248
Erk D-domain			Gene Card MAPK1	
Site	Score	Percentile	Sequence	SA
L279	0.7043	1.314 %	AKQLPGFLQLSREDQ	0.310
Erk D-domain			Gene Card MAPK1	
Site	Score	Percentile	Sequence	SA
I202	0.7223	1.678 %	RASSPPQILPQLSPE	0.950
Erk D-domain			Gene Card MAPK1	
Site	Score	Percentile	Sequence	SA
V386	0.7565	2.652 %	ERLQHTYVEALHAYV	0.555
Erk D-domain			Gene Card MAPK1	
Site	Score	Percentile	Sequence	SA
L435	0.7304	1.883 %	LRLQDKKLPPLLSEI	1.490
Lipid binding group (Lip_bind)				
PIP3-binding PH			Gene Card PIP3-E	
Site	Score	Percentile	Sequence	SA
F121	0.7529	1.070 %	SCEGCKGFFRRSVIK	1.305

PUBLICATIONS ARISING AS A RESULT OF THIS THESIS

GREENOW K., PEARCE N., RAMJI D.P., (2004). The key role of apolipoprotein E in atherosclerosis. *Journal of Molecular Medicine (in revision).*

GREENOW K., PEARCE N., RAMJI D.P., (2004). LXRs, from basic biochemistry to atherosclerosis. *(in preparation).*

GREENOW K., PEARCE N., RAMJI D.P., (2004). A critical role for the JNK/SAPK signal transduction pathway in the activation of apolipoprotein E gene expression in macrophages. *(in preparation).*

GREENOW K., PEARCE N., RAMJI D.P., (2004). A critical role for the phosphoinositide-3-kinase signal transduction pathway in the activation of apolipoprotein E gene expression. *Atherosclerosis (Supplements)* 5(1): 25.

RAMJI D.P., FOKA P., IRVINE S., HUGHES T., ROGERS S., SINGH N., GREENOW K., EVANS S., HARVEY E., ALI S., (2004). Signal transduction pathways and transcriptional control mechanisms involved in the cytokine-mediated regulation of key genes in macrophages implicated in foam cell formation and atherosclerosis. *Atherosclerosis (Supplements)* 5(1): 29.