GENERATION AND METABOLISM OF 12/15-LOX ESTERIFIED PRODUCTS

Alwena H Morgan-Davies

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789 East Eisenhower Parkway
P.O. Box 1346
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Some days you are the bug, some days you are the windshield

John's normal workplace banter doesn't go over as well in the supermarket.

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I Geraint, Mam a Dad

Diolch am bopeth

PUBLICATIONS

Full papers

Morgan AH, Hammond V, Morgan L, Evans T, Thomas CP, Tallman KA, Garcia-Diaz Y, Porter NA, Murphy RC & O'Donnell VB (2010). Quantitative assays for new families of esterified eicosanoids generated by immune cells. *Nature Protocols*. 5 (11); 1-13.

Morgan AH, Dioszeghy V, Maskrey BH, Thomas CP, Clark SC, Mathie SA, Lloyd CM, Kuhn H, Topley N, Coles BC, Taylor PR, Jones SA & O'Donnell VB. (2009) Phosphatidylethanolamine-esterified Eicosanoids in the Mouse. Tissue localization and inflammation- dependent formation inTh-2 disease. *Journal of Biological Chemistry*. 284 (32); 21185-21191.

Maskrey BH, Bermúdez-Fajardo A, Morgan AH, Stewart-Jones E, Dioszeghy V, Taylor GW, Baker PR, Coles B, Coffey MJ, Kühn H, O'Donnell VB (2007). Activated platelets and monocytes generate four hydroxyphosphatidylethanolamines via lipoxygenase. *Journal of Biological Chemistry*. 282(28):20151-63.

Presentations

Morgan AH, Maskrey, BH, Dioszeghy V, Thomas P, Topley N & O'Donnell V B (2008). In vivo and in vitro generation of hydroxy- and hydroperoxy-eicosatetraenoic acid-phosphatidylethanolamine products by murine macrophages and human monocytes. Symposium on Lipid Signalling, Frankfurt, Germany (Poster).

Morgan AH, Maskrey, BH, Dioszeghy V, Thomas P, Topley N & O'Donnell V B (2007). In vivo and in vitro generation of hydroxy- and hydroperoxy-eicosatetraenoic acid-phosphatidylethanolamine products by murine macrophages and human monocytes. Annual Postgraduate Research Day, Cardiff University(Poster).

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SUMMARY

12/15-LOX is suspected to be involved in inflammatory disorders such as atherosclerosis but the exact mechanism of its action is unclear. Novel esterified products called 12- and 15-H(p)ETE-PEs were recently identified in murine macrophages and human monocytes respectively, which may at least partially account for the effects of 12/15-LOX (Maskrey *et al*, 2007).

In this thesis the generation and metabolism of 12- and 15-H(p)ETE-PEs was characterised in murine macrophages and human monocytes. 12- and 15-H(p)ETE-PEs increased by 15 minutes following cell activation and were metabolised by three hours. However, endogenous 12- and 15-HETE-PEs increased then remained stable. Novel lipids believed to be precursors of 15-HETE-PEs, called 15-KETE-PEs, were identified in human monocytes. 15-KETE-PEs increased by 15 minutes and were metabolised by three hours and may therefore account for the temporal generation pattern of 15-H(p)ETE-PEs . [^{14}C] radiolabelled products of 15-LOX were generated and characterised in human monocytes. Detection of the [^{14}C] radiolabel in activated monocytes suggested that products of 15-LOX may form Michael adducts with proteins by three hours post activation. This may account for the 15-KETE-PE decrease in time-course samples.

A method was developed to synthesise 15-H(p)ETE-PE standards, to use in a new assay for their direct quantification following MS analysis. The 15-HETE-PE standard was also used to investigate its pro- *versus* anti-inflammatory effect on LPS-stimulated human monocytes. 15-HETE-PE down-regulated IL-1β, TNF-α, IL-6 and G-CSF generation, suggesting that products of 15-LOX are anti-inflammatory in human monocytes. 12-H(p)ETE-PEs were externalised in activated WT macrophages, which may be important for cell-cell interactions and re-structuring the cell membrane.

EM analysis indicated that 12/15-LOX$^{-/-}$ macrophages have a lysosomal storage disease, symptoms included the presence of lysosomal storage bodies, vacuoles and damaged mitochondria. This may be caused by detected raised levels of PE, PS, PG and PI phospholipids and cholesterol esters in 12/15-LOX$^{-/-}$ macrophages.

The data described herein provides a platform to further investigate the function of esterified products generated by 15- and 12/15-LOX.

ABBREVIATIONS

-/-	homozygous knockout (genotype)
A23187	Calcium ionophore
AA	Arachidonic Acid
AA-PEs	Arachidonyl-phosphatidylethanolamine phospholipids
BDMA	Dimethylbenzylamine
BEL	Bromoenol lactone
BSA	Bovine serum albumin
C	Carbon
Ca^{2+}	Calcium
CID	Collision-induced-decomposition
COX	Cyclooxygenase
cpm	counts per minute
CREB	cAMP response element binding
CRB	CREB-bonding protein
DDSA	Dodecenyl succinic anhydride
$_dH_2O$	Distilled water
DICE	Differentiation control elements
DMPA	Dimyristic phosphatydic acid
DMPC	Dimyristic phosphatidylcholine
DMPE	Dimyristic phosphatidylethanolamine
DMPG	Dimyristic phosphatidylglycerol
DMPS	Dimyristic phosphatidylserine
DNA	Deoxyribonucleic acid
DODE	9,12-dioxo-dodecenoic acid
DPPC	1,2-Dipalmitoyl-sn-glycero-3-phosphocholine
EET	Epoxyeicosatetraenoic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EMS	Electrospray mass spectrometry
F (amino acid)	Phenylalanine
FBS	Foetal bovine serum
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
G-CSF	Granulocyte cell stimulating factor
GM-CSF	Granulocyte macrophage cell stimulating factor
GPX	Glutathione peroxidase
HEDE	Hydoxyeicosadienoic acid
HETE	Hydroxyeicosatetraenoic acid
HETE-d8	deuterated hydroxyeicosatetraenoic acid
HETrE	Hydroxyeicosatrienoic acid
hnRNPs	Heterogeneous nuclear ribonucleoproteins
HODE	Hydroxyoctadecadienoic acid
HpODE	Hydroperoxyoctadecadienoic acid
HpETE	Hydroperoxyeicosatetraenoic acid
H(p)ETE	A combination of hydroperoxyeicosatetraenoic acid, hydroxyeicosatetraenoic acid and other eicosanoids or lipids that form hydroxyeicosatetraenoic acid following reduction.

HPLC	High performance liquid chromatography
IFNγ	Interferon-γ
IL	Interleukin
H	Hydrogen
H (amino acid)	Histidine
HNE	4- hydroxynonenal
Hn RNPs	Heterogeneous nuclear ribonucleoproteins
HUVECs	Human vein endothelial cells
HxA	Hepoxilin
Hz	Hertz
I (amino acid)	Isoleucine
IFNγ	interferon-γ
i.p.	intra peritoneal
JAK	Janus kinase
KETE	Ketoeicosatetraenoic acid
L (amino acid)	Leucine
LA	Linoleic acid
LAMP	Lysosome associated membrane protein
LBP	LPS binding protein
LC	Light chain
LOX	Lipoxygenase
LPS	bacterial lipopolysacchraride
LSD	Lysosomal storage disorder
LTA_4	Leukotriene A_4
LTB_4	Leukotriene B_4
LTC_4	Leukotriene C_4
LTD_4	Leukotriene D_4
LTE_4	Leukotriene E_4
LXA_4	Lipoxin A_4
LXB_4	Lipoxin B_4
Lys	Lysine
M (amino acid)	Methionine
MAFP	Methyl arachidonyl fluorophosphate
MAPK	Mitogen-activated protein kinase
mCD4	membrane bound CD4
MCP	Monocyte chemotactic protein
M-CSF	Macrophage cell stimulating factor
MDA	Malondialdehyde
MRM	Multi reaction monitoring
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
m/z	Mass to charge ratio
NFκB	Nuclear factor κappa-B
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NP-HPLC	Normal phase- HPLC
O_2	Oxygen
•OH	Hydroxyl radical
OOEPC	Oleyloxyethyl phosphorylcholine

Ox-PAPE	oxidised 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphatidylethanolamine
Ox-PLPC	Oxidised 1-palmitoyl-2-linoleoyl-glyceryl-phosphatidylcholine
Ox-PLs	Oxidised phospholipids
P	Phosphorus
P450	Cytochrome P450
PA	Phosphatidic acid
PACOCF3	Palmityl trifluoromethyl ketone
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PKCδ	Protein kinase Cδ
PLA	Phospholipase A
PMNL	polymorphonuclear leukocytes
RNA	Ribonucleic acid
PPAR	Peroxisome proliferator-activated receptor
PS	Phosphatidylserine
R (amino acid)	Arginine
RAG-2	Recombinase activator gene-2
RANTES	Regulated on activation normal T expressed and secreted
RO•	Alkoxyl radical
RP-HPLC	Reverse phase HPLC
SAPE	Stearoyl- arachidonyl-phosphatidylethanolamine
STATs	signal transducers and activators of transcription
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
S.epi	*Staphylococcus Epidermidis*
SES	*Staphylococcus Epidermidis* supernatant
siRNAs	Short interfering ribonucleic acids
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TYK	Tyrosine kinase
UV	Ultraviolet
W (amino acid)	Tryptophan
WT	Wild type
Y (amino acid)	Tyrosine

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CHAPTER 1

INTRODUCTION

1.1 Phospholipids

Phospholipids are considered to be the building blocks of cellular membranes, and are present in all organisms, including archae-bacteria, plants and humans (Hanahan DJ, 1997, Vance & Vance, 2002). They are involved in diverse functions, including compartmentalisation of cytoplasm, intercellular adhesion, cytoskeletal support and are precursors of lipids mediators (Small DM, 1986, Henson & Murphy, 1989). Among the most abundant phospholipids in eukaryotic cells are glycerophospholipids, molecules that consist of a glycerol backbone (three carbon sugar), with three functional groups. The first is a phosphate ester group (polar head group) by which glycerophospholipids are classified (Figure 1.1). The second and third components are either two fatty acids or a fatty acid and an alkyl hydrocarbon (Berg *et al*, 2002, Pulfer & Murphy, 2003). The fatty acid present at the sn1 position can be linked to a phospholipid via an acyl, plasmalogen or ether bond, while sn2 fatty acids are attached via an acyl bond. A diverse range of fatty acid species can be present at the sn1 or sn2 positions, all of which vary in carbon chain length and degree of un-saturation. Such fatty acids include arachidonic acid (AA) and linoleic acid (LA), which are important precursors of many lipid mediators. These fatty acids can be cleaved from phospholipids by phospholipases, in particular, phospholipase A_1 (PLA_1) and A_2 (PLA_2) specifically hydrolyse at the sn1 and sn2 positions respectively (Figure 1.2).

In mammals, the sn2 fatty acid is usually polyunsaturated and is therefore prone to oxidation by enzymes (Spiteller G, 2006). This results in the formation of biologically active molecules (Marathe *et al*, 1999, Subbanaounder *et al*, 2000, Murphy RC, 2001). Oxidation of free fatty

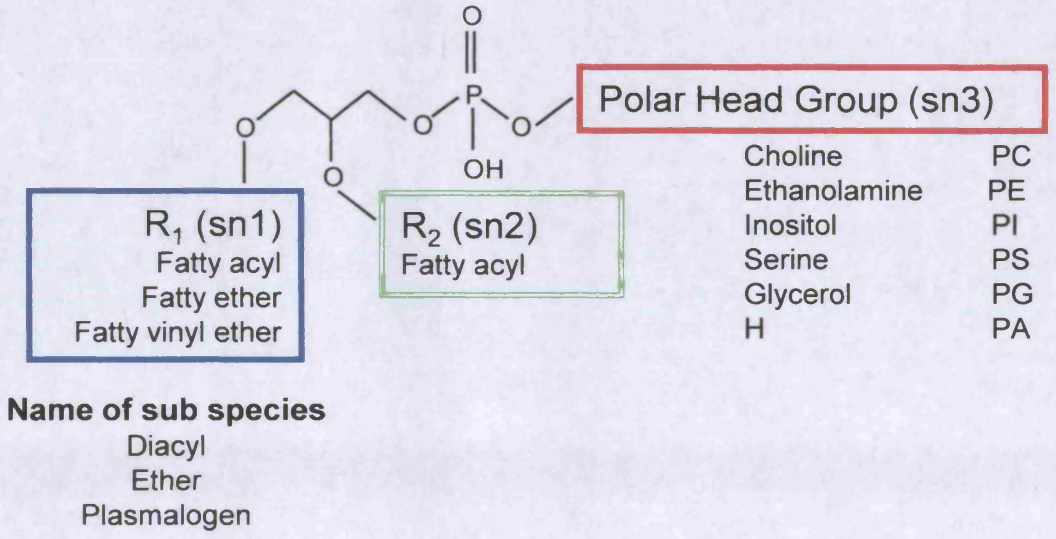


Figure 1.1. Structure of glycerophospholipids. Glycerophospholipids have fatty acyl, fatty ether or fatty vinyl ether substituents at sn1, a fatty-acyl substituent at sn2, and a polar head group at the sn3 position of glycerol (reproduced from Pulfer & Murphy, 2003, with permission (12/11/2010)).

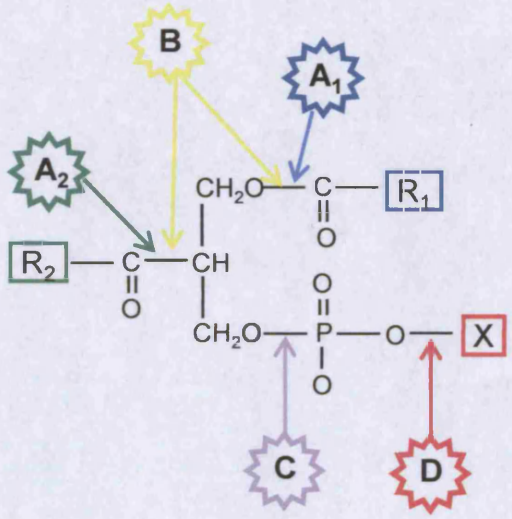


Figure 1.2. Location of glycerophospholipid hydrolysis by phospholipases. Glycerophospholipids are targeted at specific locations for hydrolysis by phospholipase enzymes, as indicated. The denoted letters apply to the phospholipase classification (adapted from Gunstone *et al,* 2007, with permission (16/11/2010)).

acids is carried out by cyclooxygenase (COX), cytochrome P450 (CyP450s) and lipoxygenase (LOX) enzymes (Figure 1.3). However, a specific LOX isoform, the 12/15-LOX, is reported to oxidise both free and esterified fatty acids (Maskrey *et al*, 2007). The main focus of this work will be oxidised lipid products of 12/15-LOX, in particular, products derived from phosphatidylethanolamine (PE) phospholipids that contain AA.

Cellular membranes are composed of bilayers with each leaflet containing a different composition of phospholipids. This asymmetric arrangement is integral to membrane function (Bevers *et al*, 1998). The inner leaflet of a bi-layer membrane is highly enriched in phosphatidylethanolamine (PE) and phosphatidylserine (PS). Their externalisation to the outer membrane leaflet can alter membrane fluidity and change how a cell is perceived by neighbouring cells. Translocation of PS and PE to the outer plasma membrane leaflet of macrophages is an important event in apoptosis. Their externalisation acts as a signal for macrophages to carry out phagocytosis and promote programmed cell death in targeted cells (Fadok *et al*, 2001). The types of phospholipid classes present in membranes is important since a change in composition has been noted in particular disease conditions such as Alzheimer's (Han *et al*, 2001). The oxidation of phospholipids by LOX alters the properties of a membrane, and is reported to play a role in events such as apoptosis and cell migration (Maccarrone *et al*, 1999 & 2000, Miller *et al*, 2001). In this thesis, phospholipid products that may be responsible for the immunological effects exerted by LOX are characterised and their biological effects are investigated.

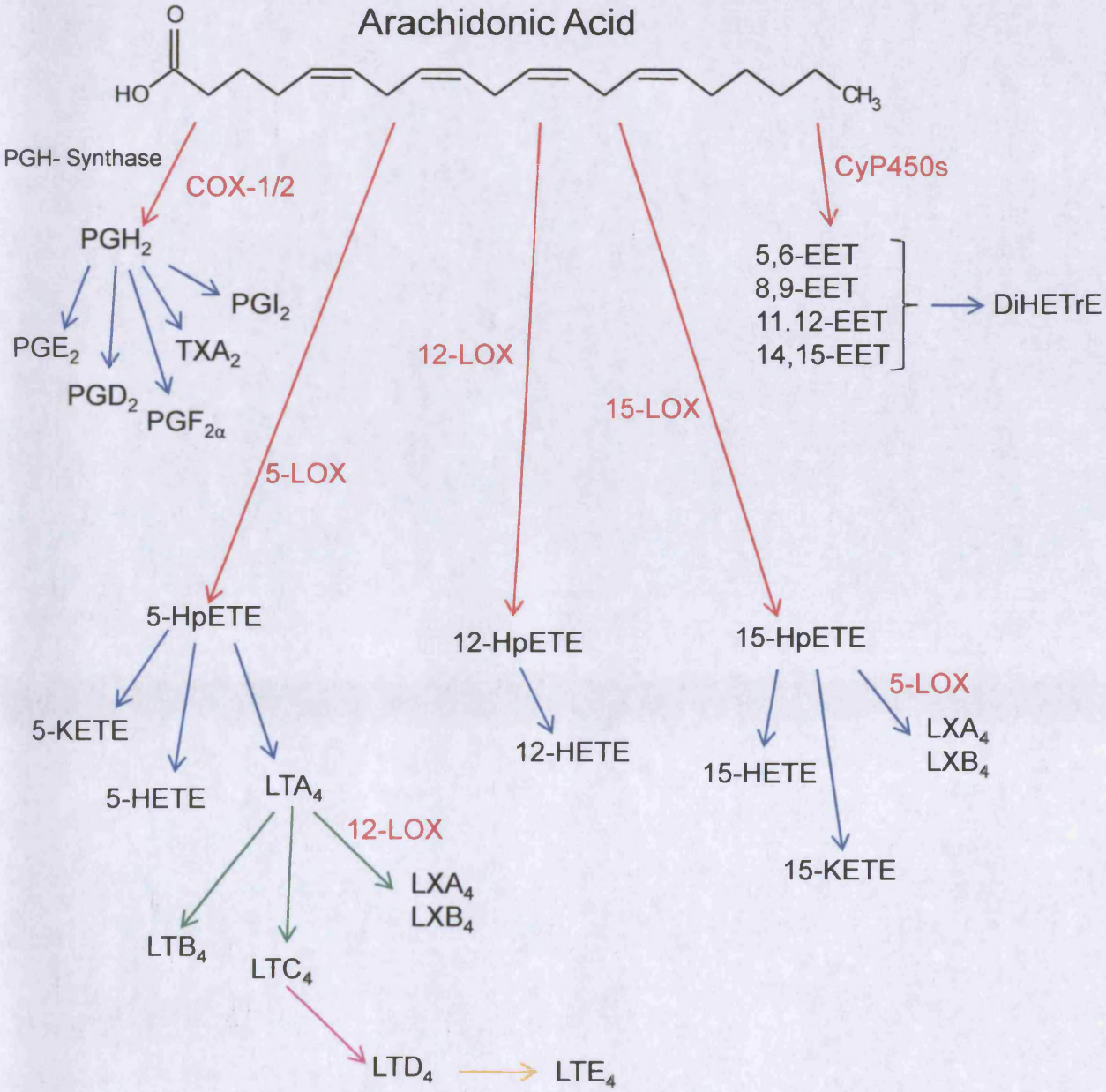


Figure 1.3. Metabolism of arachidonic acid. Arachidonic acid (AA) can be metabolised to a variety of products by LOX, COX and P450 enzymes to the indicated products (reproduced from Murphy *et al*, 2005, with permission (12/11/2010)).
Abbreviations: COX, Cyclooxygenase; LOX, lipoxygenase; Cyp450, cytochrome P450; HpETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; KETE, ketoeicosatetraenoic acid; PG, prostaglandin; TX, thromboxane; LT, leukotriene; LX, lipoxin; EET, epoxyeicosatetraenoic acid; HETrE, hydroxyeicosatrienoic acid.

1.2 Lipoxygenase enzymes

LOXs are a class of non-heme iron enzymes that stereo-specifically incorporate molecular oxygen onto the carbon chain of esterified or free polyunsaturated fatty acids (Brash AR, 1999, Kuhn *et al,* 2002, Feussner & Wasternack, 2002, Furstenberger *et al,* 2006). Oxidation by LOX is bimolecular, requiring the presence of both oxygen and a fatty acid or lipid substrate (Kuhn & O'Donnell, 2006). This reaction consists of three key steps (Figure 1.4): firstly, hydrogen is abstracted from a particular carbon (determined by the isoform of LOX), followed by delocalisation of the radical. Next, oxygen is inserted, which forms a peroxyl radical then the fatty acid is reduced, forming a peroxide (Kuhn & O'Donnell, 2006). This process is described in greater detail in Section 1.6.

1.2.1 History and classification of LOX

LOX enzymes have been identified in a variety of organisms, including higher animals, plants, lower marine life forms and a small number of bacteria (Hawkins & Brash, 1987, Brash *et al,* 1996, Grechkin A, 1998, Kuhn & Thiele, 1999, Porta & Rocha-Sosa, 2001, Vance *et al,* 2001). Only three bacterial microbes have been found to contain genes encoding LOX, namely, *Pseudomonas aeruginosa, Anabaena* (cyanobacterium) and *Nitromonas europaea* (chemolithoautotroph). In addition, *Nostoc punctiforme* (cyanbacterium) and *Sorangium cellulosum* (soil bacterium) contain LOX-like sequences (Porta & Rocha-Sosa, 2001, Zheng *et al,* 2008). As so few bacteria contain genes for LOX, their presence may be the result of horizontal gene transfer from eukaryotic microorganisms (Porta & Rocha-Sosa, 2001). However, basic eukaryotes such as yeast (e.g. *Sacharomyces cerevisiae*) do not contain genes for LOX, suggesting that these enzymes were introduced at later stages of organism evolution, followed by horizontal transfer to bacteria (Kuhn & O'Donnell, 2006).

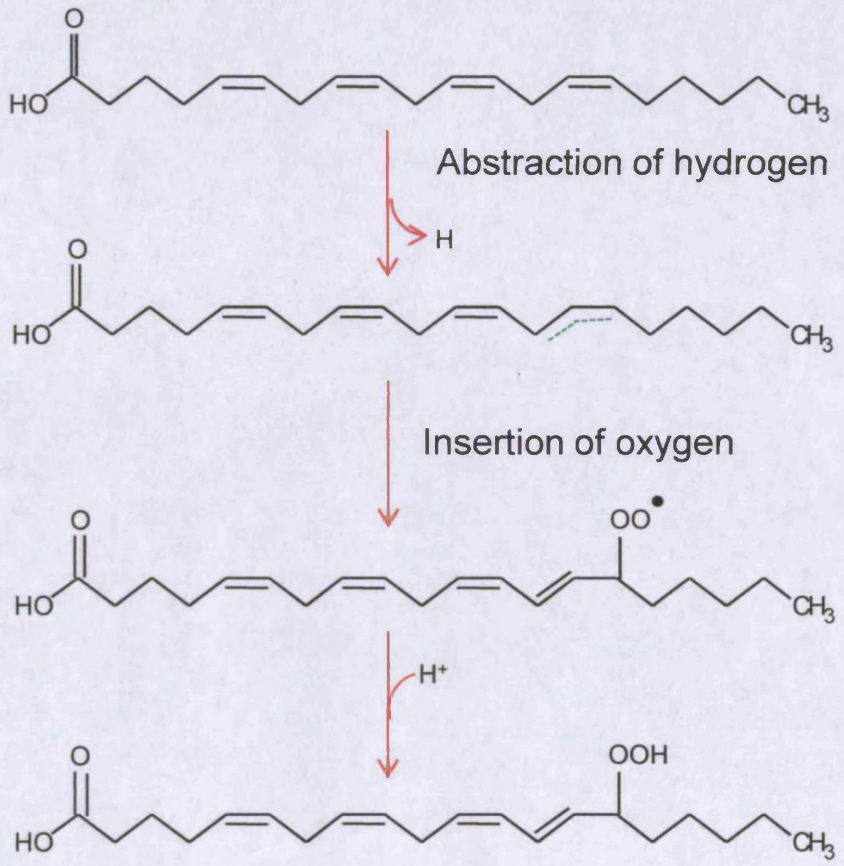


Figure 1.4. Scheme of LOX reaction. LOXs are lipid peroxidising enzymes that stero- and regio-specifically incorporate molecular oxygen into the hydrocarbon chain of polyunsaturated fatty acids. The reaction consists of three key steps as indicated (adapted from Kuhn & O'Donnell, 2006, with permission (06/12/2010)).

Much of the knowledge regarding the structure and enzymatic mechanism of LOX is derived from studies investigating soybean LOX-1, some of which date back to the 1940s (Theorell et *al,* 1947, Kuhn *et al,* 2002). At first, LOX was thought not to exist in animal cells. However, in 1974, experiments showed that 12-HETE was formed following the incubation of AA with human platelets, marking the discovery of mammalian LOX (Hamberg & Samuelsson, 1974). This was followed by detection of a rabbit reticulocyte LOX which was purified for characterization and biological studies (Schewe *et al,* 1975, Rapoport *et al,* 1979).

Currently, LOXs are categorized according to the position that oxygen is incorporated in one of their primary substrates, AA, forming hydroperoxyeicosatetraenoic acid (HpETE) (Figure 1.5) (Kuhn & Thiele 1999, Cathcart & Folcik, 2000, Walther *et al,* 2001, Kuhn & O'Donnell, 2006, Mahipal *et al,* 2007). The LOX isoforms present in mammals include: 5-LOX, 8-LOX, 12-LOX and 15-LOX (Kuhn & O'Donnell, 2006). This method of classification has become more complicated as the same peroxide products are generated by several isoforms of mammalian LOX, which are encoded by different genes and are expressed in different cell types (Figure 1.6) (Schewe *et al,* 1986, Kuhn & O'Donnell, 2006). For example, in humans there are two isoforms of 15-LOX, a leukocyte/reticulocyte 15-LOX (15-LOX1) and an epidermal type (15-LOX2) (Schewe *et al,* 1975, Brash *et al,* 1997). In this case, the method of classification used above is misleading, as while both enzymes produce the same products, their phylogenetic relatedness is low (Bryant *et al,* 1980). Indeed, the sequence homology of 15-LOX1 bears a greater similarity to the murine leukocyte 12S-LOX (12/15-LOX) (85% homology) than to 15-LOX2 (35% homology) (Brash AR, 1997, Kuhn & O'Donnell, 2006). There are also four isoforms of murine 12-LOX enzymes. As with the 15-LOXs, leukocyte 12S-LOX bears little phylogenetic relatedness to the platelet type 12S-LOX (58-65%

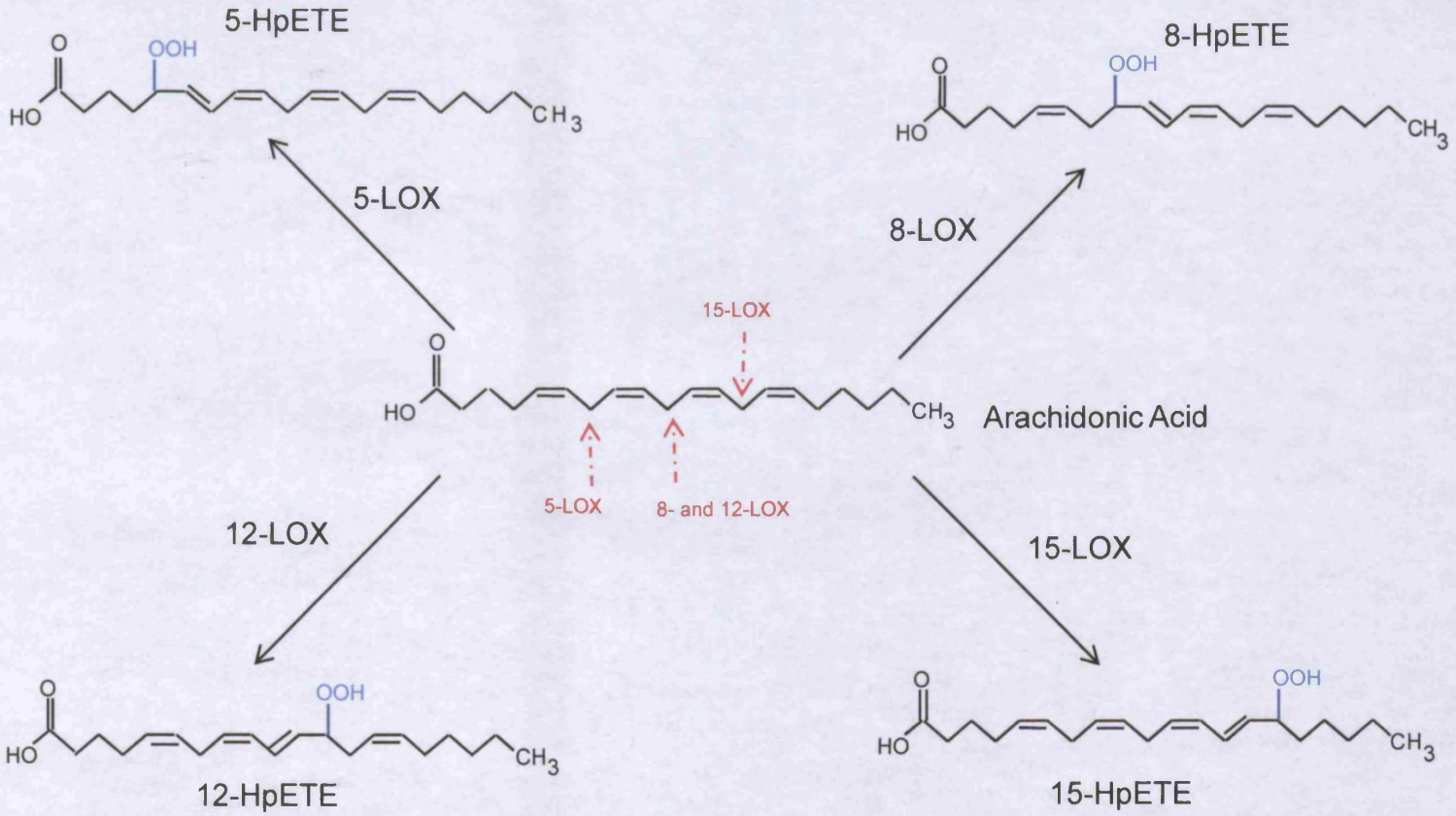


Figure. 1.5. AA can be metabolised by LOX to form specific hydroperoxy eicosanoids known as HpETEs. Individual LOX isomers are denoted according the location of oxygen insertion on AA (blue). The red arrows indicate the location of hydrogen abstraction (reproduced from McGinley & van der Donk, 2003, with permission (16/11/2010)).

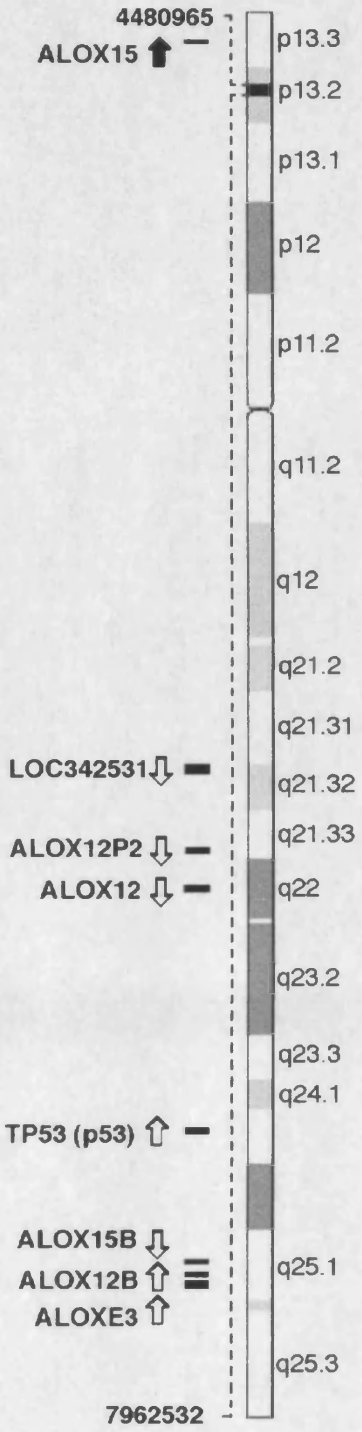


Figure. 1.6. Localisation of human LOX chromosomal genes. All human LOX genes (except for 5-LOX) lie on the short arm of chromosome 17. Multiple genes for LOX may be a consequence of gene duplication. The genes most closely related in terms of sequence homology lie closest to one another (reproduced from Kuhn & O'Donnell, 2006, with permission (06/12/2010)).

homology) or to the epidermal 12S- and 12R-LOXs (Chen *et al*, 1994, Brash *et al*, 1997). Therefore, a second method of classification has been proposed where mammalian LOXs are sorted according to their phylogenetic relatedness, rather than position of AA peroxidation (Kuhn & Thiele, 1999). According to this system of classification, there are four LOX sub-families: leukocyte 12/15-LOXs, 5-LOXs, platelet-type 12-LOX and the epidermal type LOXs (Kuhn & Thiele, 1999, Kuhn & O'Donnell, 2006). In addition to the murine 12-LOX and human 15-LOX found in leukocytes, functionally equivalent leukocyte 12/15-LOXs have been identified in rats (12-LOX), pigs (12-LOX) and rabbits (15-LOX), and all share a high degree of phylogenetic relatedness (Figure 1.7) (Yoshimoto *et al*, 1991, Watanabe *et al*, 1993, Chen *et al*, 1994, Kuhn & Thiele, 1999). In humans, there is no gene for leukocyte 12-LOX, however, genes for leukocyte 15-LOX and 12-LOX have been identified in rabbits. As the sequence of both enzymes bear a high degree of sequence homology, it is likely that they have evolved as a result of gene duplication (Berger *et al*, 1998, Kuhn *et al*, 2002).

1.3 Synthesis of 12/15-LOXs

12/15-LOX is constitutively expressed by resident peritoneal macrophages in mice, while human 15-LOX1 is expressed by immature red blood cells, eosinophils, airway epithelial cells and mast cells derived from the umbilical cord blood (Sigal *et al*, 1988, Nadel *et al*, 1991, Heydeck *et al*, 1998, Conrad DJ, 1999, Gulliksson *et al*, 2007). In addition, low expression of 15-LOX1 has been reported in alveolar macrophages, polymorphonuclear leukocytes, atherosclerotic lesions, vascular cells, the uterus and in the male reproductive system (Valone *et al*, 1980, Shannon *et al*, 1991, Natarajan *et al*, 1993 & 2002, Cheon *et al*, 2002, Sutovsky *et al*, 2004, Kuhn & O'Donnell, 2006). Alternatively, particular cell types

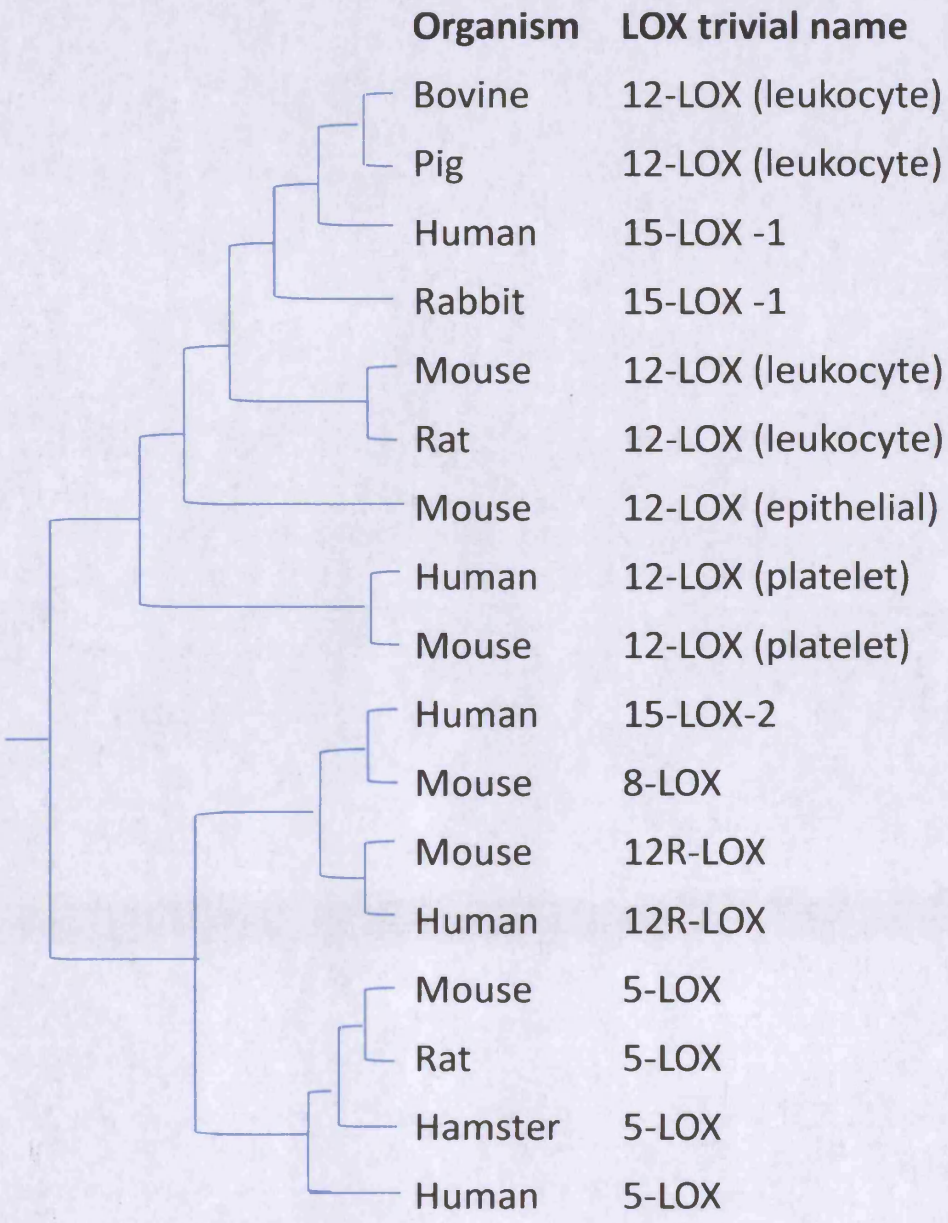


Figure 1.7 Phylogenetic tree of mammalian LOXs. LOXs were sorted according to their phylogenetic relatedness tree (reproduced from Brash AR, 1999, with permission (12/11/2010)).

can be induced to express 12/15-LOX. For example, leukocytes such as peripheral blood monocytes (human or mouse) do not express 12/15-LOX, however, expression is induced following treatment with interleukin-4 (IL-4) or IL-13 (Conrad *et al*, 1992, Levy *et al*, 1993, Heydeck *et al*, 1998, Chaitidis *et al*, 2005). Similarly, 15-LOX1 is up-regulated by this method in a human lung carcinoma cell line, A549 (Conrad *et al*, 1992, Brinckmann *et al*, 1996). In contrast, several prominent human (Mono-Mac-6, THP 1, U937, HL-60) and murine (J774A.1, P388.DI) monocytic cell lines do not express 12/15-LOX following treatment with IL-4 (Brinckmann *et al*, 1996).

1.4 Regulation of 12/15-LOX expression

Several aspects of the signalling cascade leading to the expression of 12/15-LOX have been identified. This process is initiated by IL-4 and IL-13, via the IL-4/IL-13 receptors (Brinckmann *et al*, 1996). The receptor complex for IL-4 has two sub-units, IL-4Rα and IL-4Rγ, while IL-13 binds to a receptor complex containing IL-13Rα1 and IL-4Rα (Figure 1.8) (Aman *et al*, 1996, Letzelter *et al*, 1998, Ogata *et al*, 1998, Nelms *et al*, 1999, Roy *et al*, 2002, Kuhn & O'Donnell, 2006). The binding of IL-13 and IL-4 to their respective receptor complexes results in auto-phosphorylation of members of the janus kinase (JAK) family, JAK2 and TYK2, which are bound to IL-4Rα and IL-13Rα1 respectively (Musso *et al*, 1995, Murata *et al*, 1996, Brinckmann *et al*, 1996, Roy & Cathcart, 1998, Nelms *et al*, 1999, Roy *et al*, 2002). Once phosphorylated, the JAK proteins in turn phosphorylate the receptor chains to which they are attached (Ihle *et al*, 1994, Kuhn *et al*, 2002). This leads to recruitment of signal transducers and activators of transcription (STATs), specifically STAT6 for IL-4 and STAT proteins 1α, 3, 5A, 5B or 6 for IL-13 (Heydeck *et al*, 1998, Cathcart & Folcik, 2000, Kuhn *et al*, 2002, Roy *et al*, 2002, Bhattacharjee *et al*, 2006). JAKs then phosphorylate a

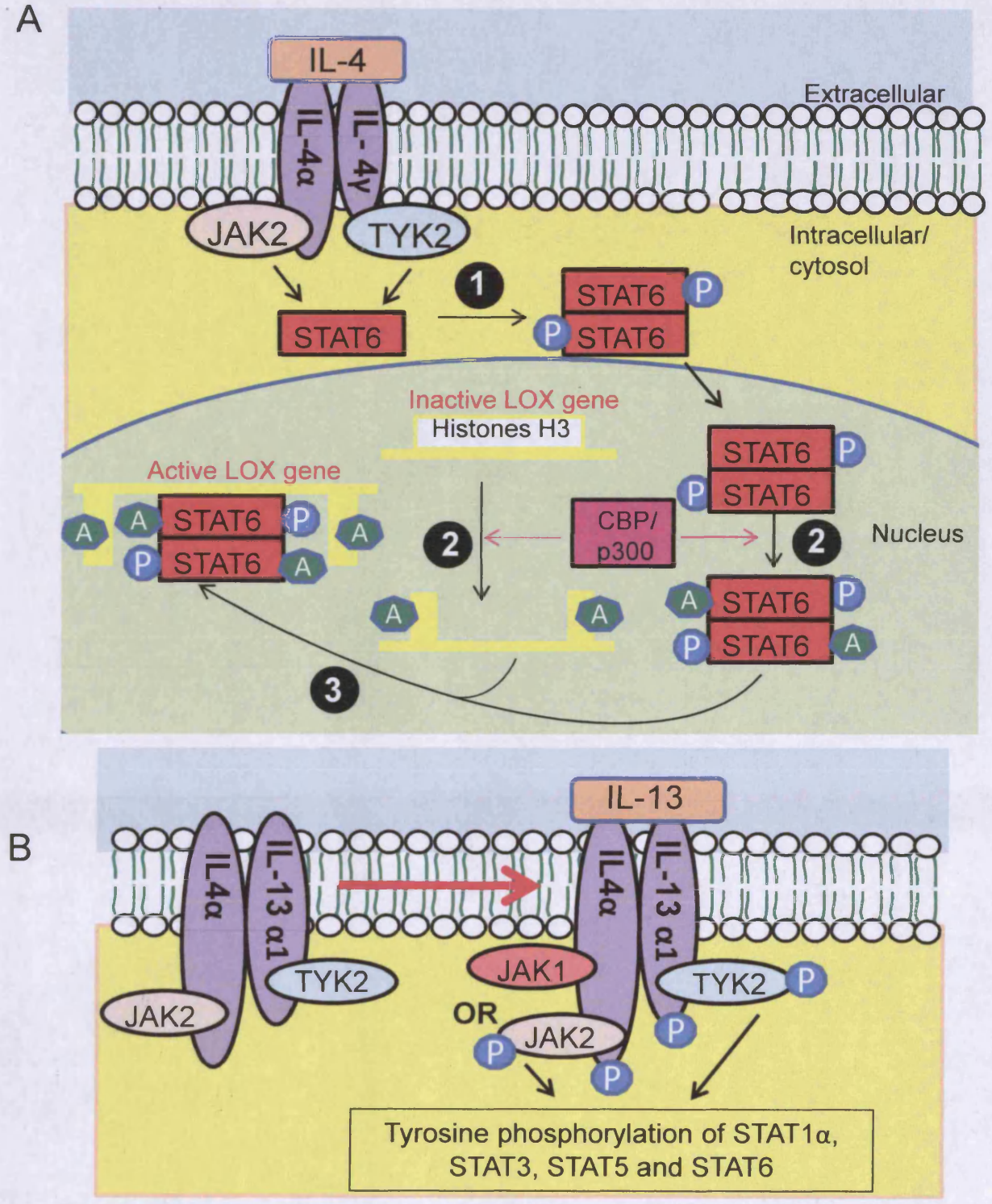


Figure 1.8. Mechanism of 12/15-LOX induced expression by IL-4 and IL-13. In response to the binding of IL-4 (A) and IL-13 (B) to their receptor complexes, JAK kinases (JAK2 and TYK2) autophosphorylate, followed by phosphorylation of the receptor chains and further signalling by STATs. The signalling cascade is made up of three steps: (1) Phosphorylation of STATs, leading to their activation; (2) Acetylation of activated STATs and nuclear histones; (3) Binding of STAT dimers to DNA promoter regions, activating transcription of 12/15-LOX (adapted from Roy *et al*, 2002, and Kuhn & O'Donnell, 2006, with permission (06/12/2010)).

tyrosine residue in the C-terminal of STATs, allowing STATs to form hetero/homo-dimers (Ihle *et al*, 1994, Roy *et al*, 1998). STATs then translocate to the nucleus and are acetylated by activated histone acetyl transferase CREB-binding protein (CBP/p300) (Conrad *et al*, 1992, Ihle *et al*, 1994, Roy *et al*, 1998, Kamitani *et al*, 2001). Simultaneously, nuclear histones are also acetylated, which de-masks the DNA promoter sequence for 12/15-LOX, allowing STATs to bind and activate gene transcription (Conrad *et al*, 1992, Heydeck *et al*, 1998, Conrad & Lu, 2000, Kamitani *et al*, 2001, Shankaranarayanan *et al*, 2001, Xu *et al*, 2003). Other regulatory elements are involved in the activation of 15-LOX, for example, following cell induction by IL-13, P38 MAPK is up-regulated, which mediates the phosphorylation of STAT1 and STAT3 (Xu *et al*, 2003). Protein kinase Cδ (PKCδ) and PI3-kinase are also involved in this process and are required for the activation of 15-LOX (Xu *et al*, 2003 & 2004, Coffey *et al*, 2004, Battacharjee *et al*, 2006).

Studies involving STAT6$^{-/-}$ and recombinase activator gene-2$^{-/-}$ (RAG-2$^{-/-}$) mice indicate that IL-4 and IL-13 may not be the only cytokines capable of inducing 12/15-LOX production *in-vitro* (Sendobry *et al*, 1991, Heydeck *et al*, 1998). Therefore, many cytokines have been investigated in terms of their ability to induce 12/15-LOX expression. It is not up-regulated by IL-6, IL-10, interferon-γ (IFNγ) or granulocyte macrophage-cell stimulating factor (GM-CSF) (Conrad *et al*, 1992, Chaitidis *et al*, 2005). Studies investigating the tissue expression of murine 12/15-LOX have shown that it is primarily generated by peritoneal macrophages, with little or no expression in macrophages from other locations (Huo *et al*, 2004). This suggests that 12/15-LOX is selectively up-regulated following the entry of peripheral monocytes into the peritoneum (Kuhn & O'Donnell, 2006). A potential cytokine involved in this process, and is therefore implicated in the induction of 12/15-LOX is fractalkine

(CX3CR1), which can trigger migration and differentiation of monocytes to resident peritoneal macrophages (Geissmann *et al*, 2003).

The activity of human 15-LOX1 is under strict regulation, albeit post-transcriptional, and/or post-translational (Kuhn *et al*, 1999b). Post-translational regulation of 15-LOX has been reported in young rabbit reticulocytes where the messenger ribonucleic acid (mRNA) of 15-LOX is expressed but no functional enzyme is present (Thiele *et al*, 1982). Similarly, mRNA of 15-LOX1 is expressed in human vein endothelial cells (HUVECs) following treatment with IL-4, however, no protein is detected (Lee *et al*, 2001b). Such negative regulation has been shown *in-vitro* by differentiation control elements (DICE), which bind to repetitive sequences in the 3' translational region of 15-LOX mRNA, thus preventing translation (Ostareck-Lederer *et al*, 1994). In addition, regulatory proteins such as heterogeneous nuclear ribonucleoproteins (hnRNPS) K and E_1, and short interfering RNAs (siRNAs) can prevent translation of 15-LOX (Ostareck *et al*, 1997, Li *et al*, 2005). 15-LOX is also regulated post-translationally by nitric oxide (NO), which is believed to interact with the catalytic iron center of LOX (Wiesner *et al*, 1996, Holzhütter *et al*, 1997). Furthermore, the association of 15-LOX to the membrane is dependent on calcium (Brinckmann *et al*, 1998).

1.5 12/15-LOX structure

The crystal structure of rabbit reticulocyte-type 15-LOX was completed to a resolution of 2.4 Å in 1997, seven years after its crystallization (Figure 1.9) (Sloane *et al*, 1990, Gillmor *et al*, 1997). The amino acid sequences of mammalian and plant LOXs are somewhat different, however, the 3D structures of the two enzyme types bear a close resemblance (Gillmor *et al*, 1997, Kuhn *et al*, 2005). Rabbit 15-LOX is a 75 kDa fatty acid made up of a single polypeptide chain folded into two domains (Kuhn *et al*, 1993, Carrol *et al*, 1993, Gilmor *et*

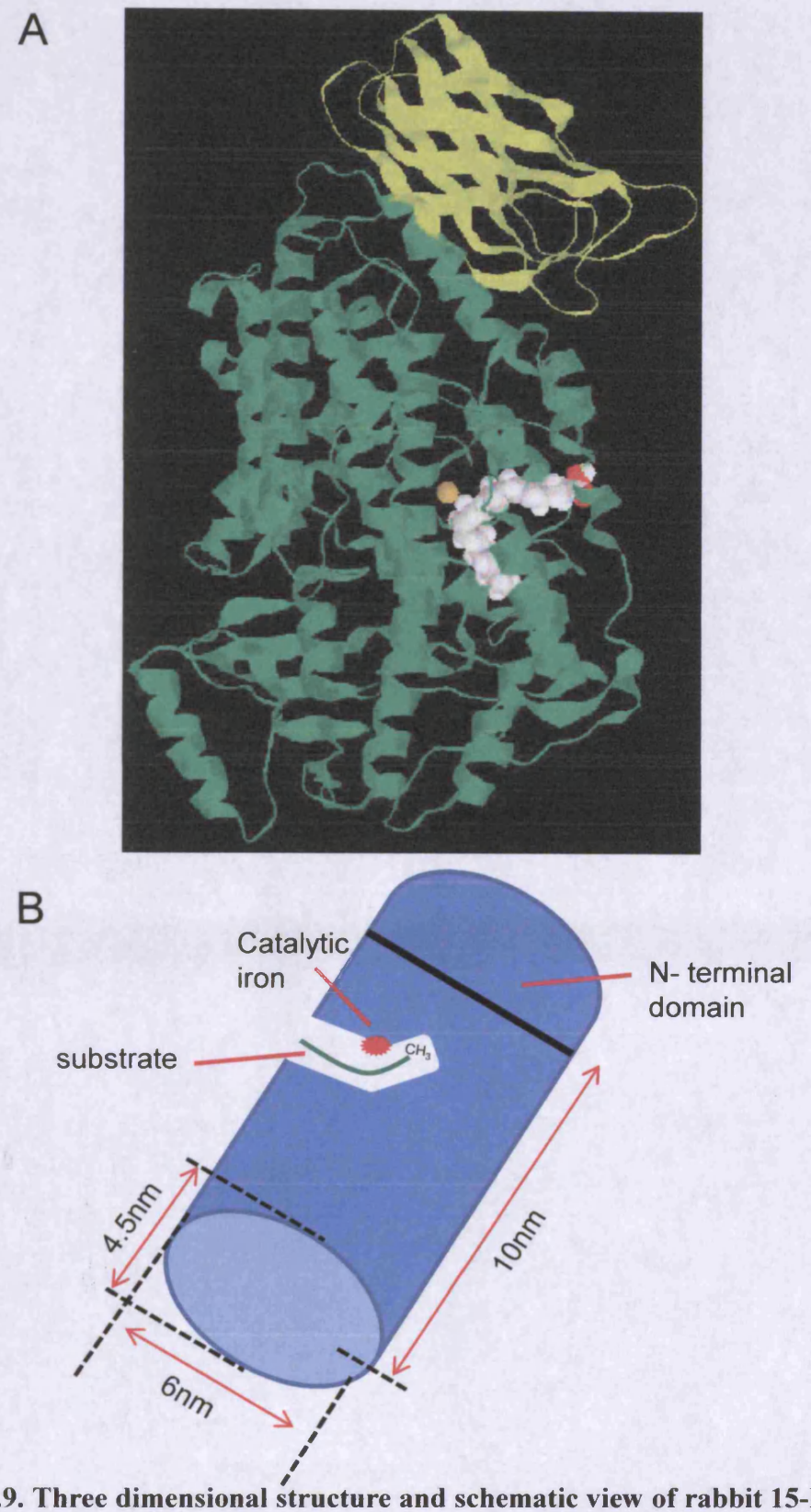


Figure 1.9. Three dimensional structure and schematic view of rabbit 15-LOX. (A) The 3D structure of rabbit 15-LOX shows the N-terminal β-barrel domain (yellow); Non-heme iron (dark yellow); catalytic domain (green), amino acids 210, 211, 601, 602 and 177–187 have not been defined (white). (B) A schematic view of rabbit 15-LOX, its shape resembles an elliptic cylinder (reproduced from Gillmor *et al*, 1997 and Kuhn *et al*, 2005, with permission, 04/12/2010)).

al, 1997). The N-terminus (110 amino acids) consists of two four-stranded anti-parallel β-sheets that interfaces with two long helices of the C-terminus via a peptide (amino acids 111-124) (Gillmor *et al*, 1997, Kuhn *et al*, 2005). Within the C-terminus (550 amino acids), is a catalytic domain containing 18 helices, separated by a small β-sheet sub-domain (Gillmor *et al*, 1997, Kuhn *et al*, 2002, Kuhn O'Donnell, 2006). The overall shape is an elliptical cylinder, with a boot shaped substrate binding cleft that is accessible from the protein surface (Figure 1.9B) (Gillmor *et al*, 1997, Kuhn *et al*, 2005). The catalytic domain contains the non-heme iron and at its core are two long helices where particular residues take on a π-helix conformation (residues 360-366 and 537-543) (Gilmor *et al*, 1997). Within the π-helixes are four of the six ligands that associate with iron, namely, H361, H366, H541 and H454. The fifth is I663 which resides in the C-terminal and the sixth (required for Fe^{3+} state) is a water molecule (Minor *et al*, 1996, Gilmor *et al*, 1997). The ferric centre and its associated ligands are pivotal for enzyme activity. Specifically, the changeable ionisation state of iron, from Fe^{2+} to Fe^{3+} is central to the ability of LOX to carry out hydrogen abstraction (McGinley & van der Donk, 2003, Kuhn *et al*, 2005).

1.6 Phospholipid and fatty acid oxidation by 12/15-LOX

12/15-LOX, induced by IL-13 and/or IL-4, remains predominantly dormant in cells as product generation requires direct activation of the enzyme (Kuhn & O'Donnell, 2006). When cells are treated with A23187, 12/15-LOX translocates from the cytosol to intracellular membranes, to metabolise phospholipids or free fatty acids liberated by PLA_2 (Figure 1.10) (Brinckmann *et al*, 1998, Christmas *et al*, 1999, Miller *et al*, 2001, Coffey *et al*, 2004, Kuhn *et al*, 2005, Maskrey *et al*, 2007). Following activation, LOX becomes associated with the plasma membrane, via calcium-dependent mechanisms (Maccarrone *et al*, 2001). During this process, both electrostatic and hydrophobic interactions take place between the hydrophobic

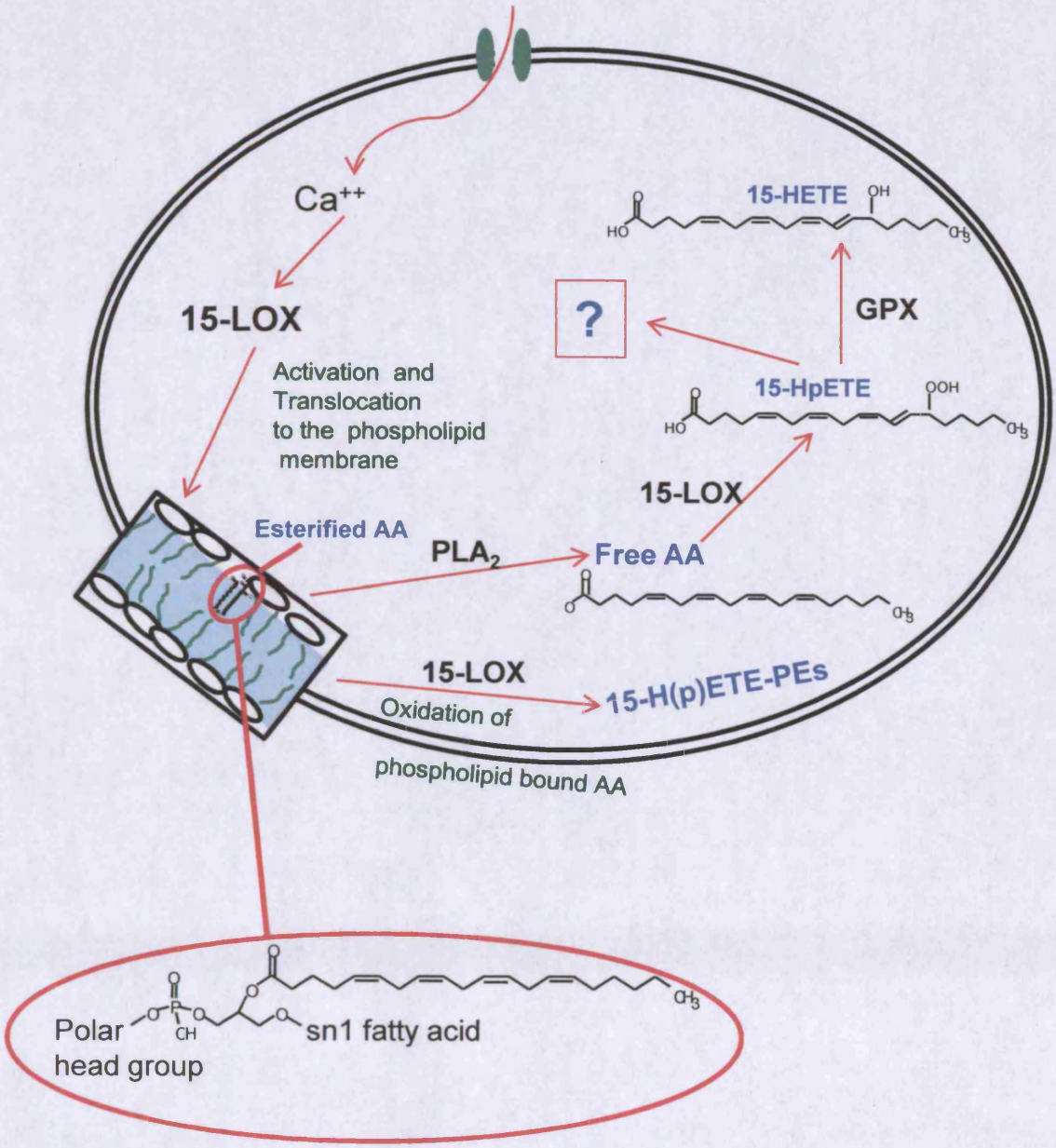


Figure 1.10 15-LOX can metabolise esterified and free AA. Increases in [Ca^{2+}] result in translocation of 15-LOX to cellular membranes, where it metabolises free and esterified AA . The same mechanism also applies to 12/15-LOX where AA is oxidised at C-12.

tail of a fatty acid and particular amino acids exposed in the binding pocket of LOX (Y15, F70 and L71) (Walther *et al*, 2002 & 2004, Kuhn & O'Donnell, 2006). Site directed mutagenesis studies have shown that specific amino acids are sequence determinants of membrane binding, namely, Y15, F70 and L71 (located in the β-barrel domain of the N-terminal) and W181 and L195 (in the catalytic domain) (Walther *et al*, 2002 & 2004, Kuhn *et al*, 2005). Structural studies suggest that there are two calcium binding sites on the enzyme, one in the β-barrel domain of the N-terminus, and the second in the catalytic domain (Kuhn *et al*, 2005). Calcium is also thought to increase substrate binding by promoting the formation of salt-bridges between acidic amino acids on the surface of 12/15-LOX and the negatively charged phospholipid head group (Rapoport *et al*, 1982, Walther *et al*, 2002 & 2004, Kuhn *et al*, 2005). A fatty acid substrate enters the active site with its methyl group, once inside the binding cleft, substrate alignment is aided by hydrophobic interactions between the methyl group and amino acids F353, I418 and I593 (Figure 1.11) (Kuhn *et al*, 1990, 1999 & 2002, Sloane *et al*, 1991, Chen & Funk, 1993, Gan *et al*, 1996, Borngraber *et al*, 1996 & 1999, Funk & Loll, 1997, Gillmor *et al*, 1997). This is also facilitated by ionic interactions between R403 and the substrate carboxyl group (Kuhn *et al*, 2002 & 2005, Gan *et al*, 1996, Gilmor *et al*, 1997). Studies involving site directed mutagenesis have indicated that particular residues are important in the activity and mechanism of LOX. For example, substitution of R403 for an uncharged amino acid decreases catalytic activity (Gan *et al*, 1996). Furthermore, in rabbit 15-LOX, the side chains of F353, I418, M419 and I593 dictate the depth of the substrate binding pocket and thus determine where the fatty acid is oxidised (Figure 1.11) (Sloane *et al*, 1990 & 1991, Borngräber *et al*, 1996 & 1999, Kuhn *et al*, 2000 & 2005, Feussner & Wasternack, 2002, Kuhn and O'Donnell, 2006).

1.6.1 Hydrogen abstraction

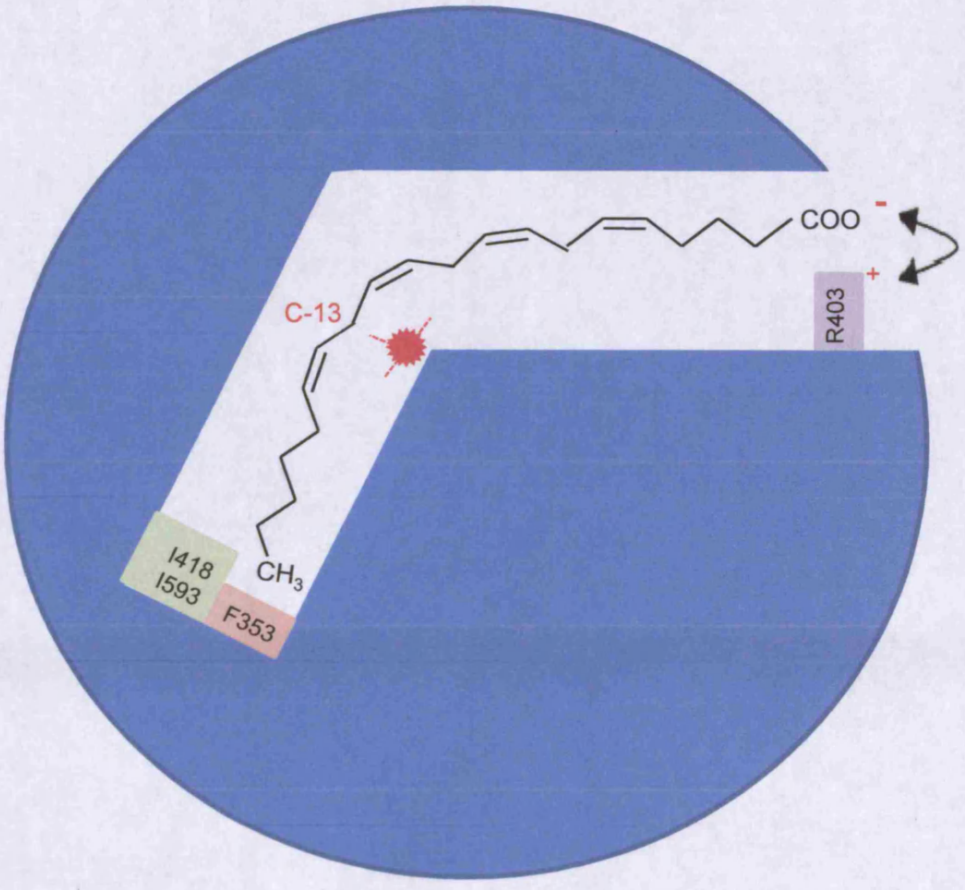


Figure. 1.11 Alignment of arachidonic acid at the 15-LOX active site. The methyl end enters first and interacts with I418, I593 and F353 while the carboxyl terminal interacts with R403 (reproduced from Kuhn *et al*, 2002, with permission (04/12/2010)).

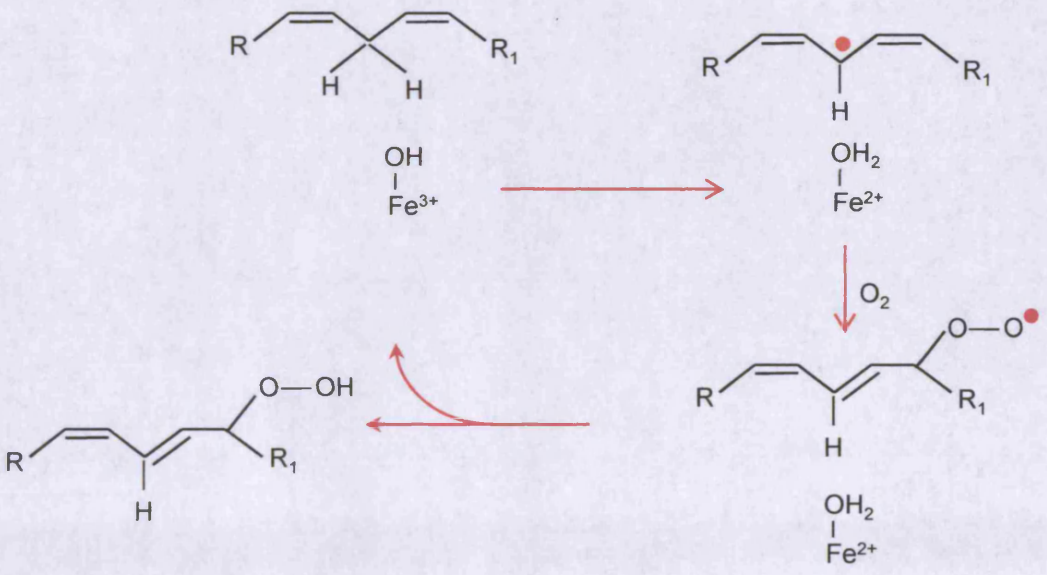


Figure 1.12. LOX reactions centre on the ability of the iron atom to transfer between a ferrous and ferric and state. Following enzyme activation, the non-heme iron becomes oxidised to a ferric state (Fe^{3+}). Hydrogen abstraction from AA reduces the iron, to the ferrous form (Fe^{2+}). Loss of hydrogen associated with Fe^{2+} results in a return of iron to a ferric state, in preparation for another round of enzyme activity (reproduced from McGinly & van der Donk, 2003, and Kuhn & O'Donnell, 2006, with permission (12/11/2010 and 04/12/2010).

Raised intracellular [Ca^{2+}] and low levels of basally generated HpETEs are required for LOX activation, which involves the oxidation of the ferrous iron (Fe^{2+}) within LOX, to the ferric (Fe^{3+}) form (Figure 1.12) (Scarrow *et al,* 1994, Coffey *et al,* 2004, Schneider *et al,* 2007, Conrad *et al,* 1992). This is the catalytically active species that can abstract hydrogen from unsaturated lipids. Initially, hydrogen is abstracted from a methylene located at C-13 or C-10, to form 15-HpETE and 12-HpETE respectively (Kuhn *et al,* 1990 & 2005, Walther *et al,* 2001). Removal of hydrogen produces a free radical, oxygen is then added regio-selectively, forming a peroxyl radical on the opposite side of the fatty acid substrate, which is finally reduced to a hydroperoxide (Figure 1.12) (Maas & Brash, 1983, Rickert & Klinman, 1999, Kuhn *et al,* 2005, Schneider *et al,* 2007). The abstraction of hydrogen from a bisallylic methylene is the rate limiting step of oxidation by 12/15-LOX (Egmond *et al,* 1973). If the catalytic iron is located between two bisallylic methylene groups, hydrogen abstraction is possible from two locations, such dual specificity makes 12/15-LOX unique from other LOX isoforms (Figure 1.13) (Bryant *et al,* 1982, Kuhn *et al,* 1990 & 2005, Walther *et al,* 2001).

1.6.2 Oxygen incorporation

The specificity of oxygen incorporation is, in part, dependent on the location of the bisallylic methylene from the methyl end of the fatty acid (Hamberg & Samuelsson, 1967, Kuhn *et al,* 1990a). However, the mechanism by which molecular oxygen is incorporated is yet to be fully characterised. Kinetic studies have concluded that oxygen is not bound to the iron of 12/15-LOX prior to the initial abstraction of hydrogen (Knapp & Klinman, 2003, Schneider *et al,* 2007). Furthermore, free diffusion of oxygen into the active site is unlikely, because of the high degree of stereo-selectivity and the depth and hydrophobic nature of the substrate pocket (Figure 1.14) (Kuhn *et al,* 2005). There are several hypotheses speculating on the nature of how oxygenation is carried out by LOX. The first is via *steric shielding*, where the

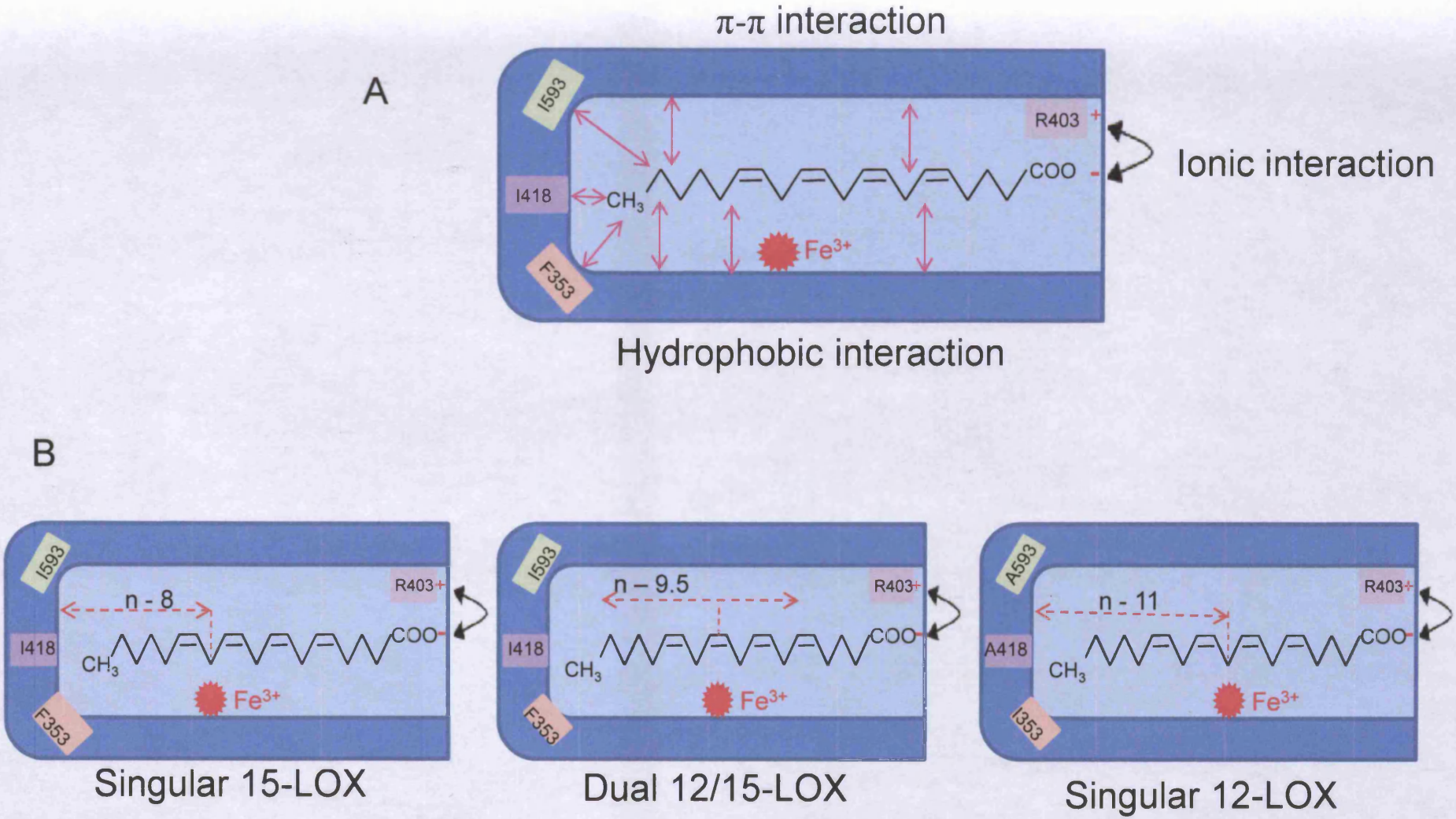


Figure 1.13 Interactions between 12/15-LOX and AA. (A) The binding forces involved contributing to substrate alignment: (i) hydrophobic interactions between amino acid side chains of 15-LOX and the substrate (double ended arrows), (ii) ionic interactions between R403 of LOX and the substrate carboxyl-terminal, and (iii) π-electron interactions between the aromatic residues of 15-LOX and double bonds within the substrate. (B) The alignment of substrates at the active site of 12-, 15- and 12/15-Lipoxygenase (Reproduced from Kuhn *et al*, 2005, with permission (04/12/2010)).

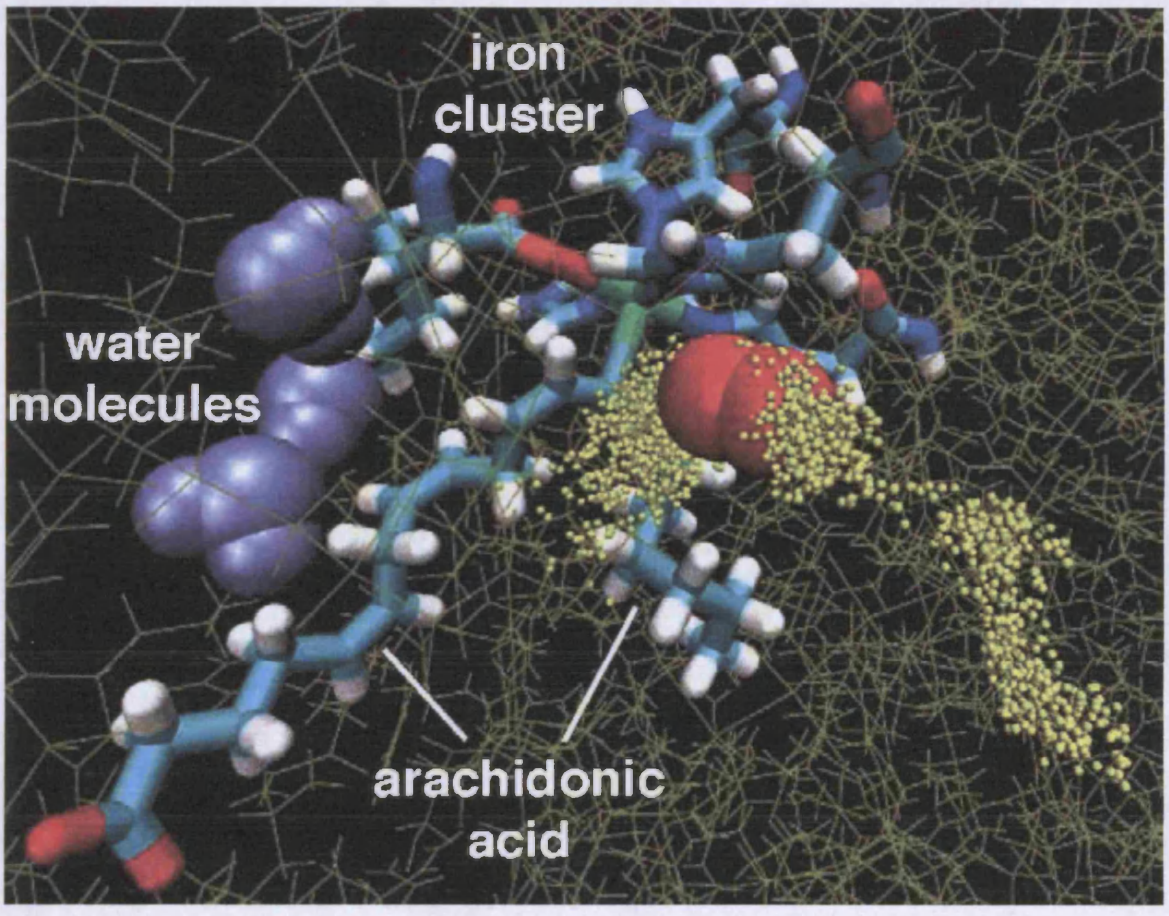


Figure 1.14 Movement of oxygen within rabbit 15-LOX. The suspected path of oxygen diffusion was studied by the use of MD simulation experiments. The yellow dots represent the path that oxygen is thought to take if entering the enzyme by diffusion, from the enzyme surface to the active site. The non-heme iron is green and the oxygen is represented by the red double spheres (Reproduced from Kuhn *et al*, 2005, with permission (04/12/2010)).

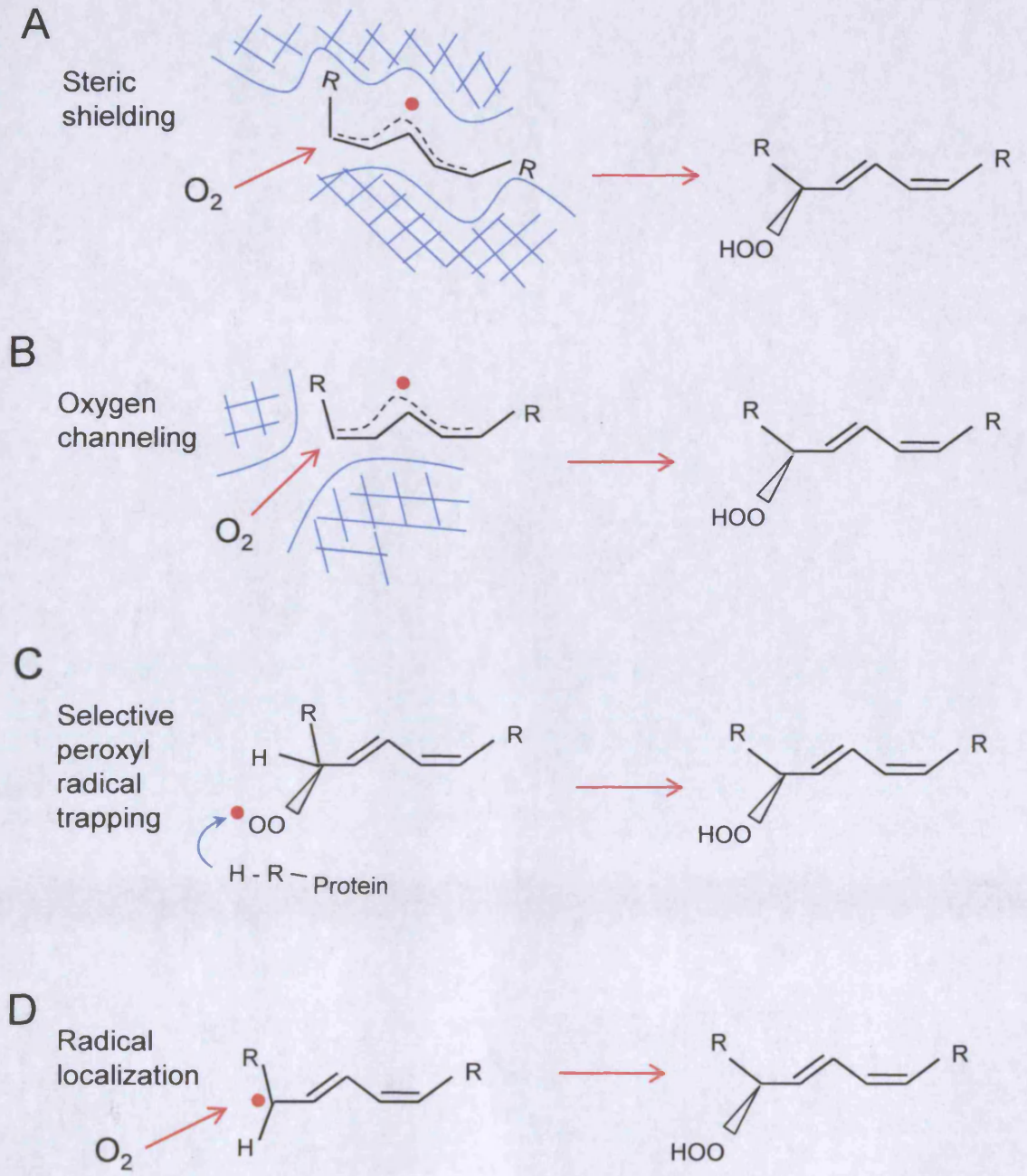


Figure 1.15. Illustration of four hypotheses for oxygenation control in LOX catalysis. (A) *Steric shielding*: All parts of the substrate are blocked, apart from the reactive center of the pentadiene radical. (B) *Oxygen channeling*: oxygen is directed from the outside to the pentadiene radical. (C) *Selective peroxyl radical trapping*: oxygen can react at all sites of the pentadiene; A hydrogen donor is strategically placed and traps the desired product by reducing, forming a hydroperoxide. (D) *Radical localization*: oxygen is induced by the enzyme to rotate around a carbon bond. It becomes trapped, leaving one carbon to react with oxygen (Reproduced from Schneider *et al*, 2007, with permission (12/11/2010)).

structure of LOX restricts the accessibility of the fatty acid to particular areas within the binding pocket (Figure 1.15 A). As a targeted methylene is rotated towards the iron during hydrogen abstraction, the face of the fatty acid may be hidden and confined. Therefore, oxygen stored in a pocket within the active site is incorporated onto the opposite face to hydrogen abstraction, forming a peroxyl radical (Gilmor *et al*, 1997, Schneider *et al*, 2007). The existence of an *oxygen channel* leading molecular oxygen directly from the cytoplasm to the site of oxygenation has also been suggested, which would ensure positional specificity during incorporation (Figure 1.15 B) (Schneider *et al*, 2007). However, research investigating the structure of LOX has not shown that such a channel exists (Boyington *et al*, 1993, Minor *et al*, 1996, Gillmor *et al*, 1997, Knapp *et al*, 2001, Kuhn *et al*, 2005). Rather than stereo-selectiveness being dependent on oxygen insertion, in an alternative hypothesis the *peroxyl radical is trapped* following interactions with a hydrogen donor that has been strategically placed by LOX. As a result, only the 'trapped' product can be reduced to the hydroperoxide (Figure 1.15 C) (Schneider *et al*, 2007). Lastly, in a process named *radical localization*, the radical is rotated by the enzyme and forced around a particular carbon bond where it is trapped, leaving only one carbon for oxygen to react with (Figure 1.15 D) (Schneider *et al*, 2007). For the latter hypothesis, the peroxyl group would travel on and off 'incorrect' carbons before arriving at the correct location. This is therefore unlikely because of the time involved for it to take place. However, this process may be important in keeping the peroxyl group trapped at a selected location for the formation of the hydroperoxide (Schneider *et al*, 2007).

1.6.3 Product dissociation and suicidal inactivation of 12/15-LOX

Following the metabolism of fatty acids, LOXs undergo suicidal inactivation (Hartel *et al*, 1982, Kim *et al*, 1989). This was believed to occur following oxidation of a particular methionine residue by the hydroperoxy products. However, site directed mutagenesis of this

methionine does not prevent inactivation (Rapoport *et al*, 1984, Gan *et al*, 1995). In rabbit 15-LOX, this process may be accompanied by covalent linkage of reaction intermediates (Rapoport *et al*, 1984, Schneider *et al*, 2007, Kishimoto *et al*, 1996, Weisner *et al*, 2003, Zhu *et al*, 2003).

1.7 Effects of 12/15-LOX products

LOXs are multifunctional enzymes, having hydroperoxide, leukotriene, lipoxin and hepoxilin synthase activities. Human and mouse 12/15-LOXs have a broad range of substrates as they can metabolise AA, LA, phospholipids, cholesterol esters, mono-, di- and tri-acylglycerols (Kuhn *et al*, 2002, Arai *et al*, 1997, Maskrey *et al*, 2007). Furthermore, rabbit and murine 12/15-LOX and can oxidise bio-membranes and lipoproteins but so far, this has only been shown *in-vitro* (Schewe *et al*, 1975, Harkewicz *et al*, 2008).

The primary activity of LOX is oxygenation, producing hydroperoxy conjugated diene lipids. AA is a major substrate for this reaction, resulting in the formation of HpETEs (Figure 1.5), alternatively, oxidation of LA produces hydroperoxyoctadecadienoic acid (HpODEs). Hydroperoxides can undergo secondary decomposition by homolytic cleavage of the peroxy group, forming alkoxyl (RO•) and hydroxyl (•OH) radicals (Nunez-Delicado *et al*, 1996). Self-rearrangement, or reactions with nearby bio-molecules result in the formation of more stable secondary peroxidation products, such as alkanes, short chain aldehydes and keto-dienes (Salzmann *et al*, 1984, Glasgow *et al*, 1986, Yokoyama *et al*, 1986). Alternatively, a rapid reduction of peroxides by glutathione peroxidases (GPXs) forms the corresponding hydroxyl group, for example, HETEs and HODEs, for AA and LA, respectively (Ursini *et al*, 1995). Hydroperoxides can also be further metabolised, for example 5- and 12/15-LOX can convert 5-HpETE and 15-HpETE respectively, to 5,6- and 14,15-epoxy leukotrienes (Figure

1.3) (Bryant *et al*, 1985, Denis *et al*, 1991). These compounds can then be used as substrates for the synthesis of more biologically relevant leukotrienes (Denis *et al*, 1991). Many metabolites of LOX are biological mediators and are implicated in inducing gene expression by activating transcription factors e.g. the mitogen-activated protein kinase (MAPK) family, nuclear factor-κB (NFκB), c-jun and the peroxisome proliferator-activated receptor (PPAR) family (Brash AR, 1999, Middleton *et al*, 2005, Huang *et al*, 1999). The effects of particular products generated by LOX are described below.

1.7.1 H(p)ETEs and H(p)ODEs

H(p)ETEs and H(p)ODEs have been implicated in signalling events such as neurotransmission, cell adhesion, differentiation and survival (Piomelli *et al*, 1987, Tang *et al*, 1996, Yu *et al*, 1995, Tang & Honn, 1994). They have regulatory properties in several cell types, affecting cell adhesion, proliferation, chemotaxis and DNA synthesis (Patricia *et al*, 2001, Rao *et al*, 1994, Reddy *et al*, 2002, Hedrick *et al*, 1999, Natarajan *et al*, 1994, Wen *et al*, 1996). H(p)ETEs and H(p)ODEs regulate these events via PKC, PPARγ, PPARδ, MAPKs, CREB, Ras translocation and G-coupled receptors (Figure 1.16) (Beckman JS, 1990, Liu *et al*, 1994, Lie *et al*, 1995, Wilson *et al*, 1996, Huang *et al*, 1999, Hsi *et al*, 2001, Shureiqi *et al*, 2003). 13-HODE and 15-HETE also have chemotactic properties, resulting in recruitment of leukocytes, thus propagating inflammatory processes (Higgs *et al*, 1981, Schwenk *et al*, 1992). However, the anti- versus pro-inflammatory nature of these products has not been fully determined, 15-HpETE for example, displays pro-inflammatory properties in rabbit skin but has also been reported to down regulate the synthesis of pro-inflammatory cytokines, such as TNF-α (Higgs *et al*, 1981, Ferrante *et al*, 1997, Ferrante & Ferrante, 2005).

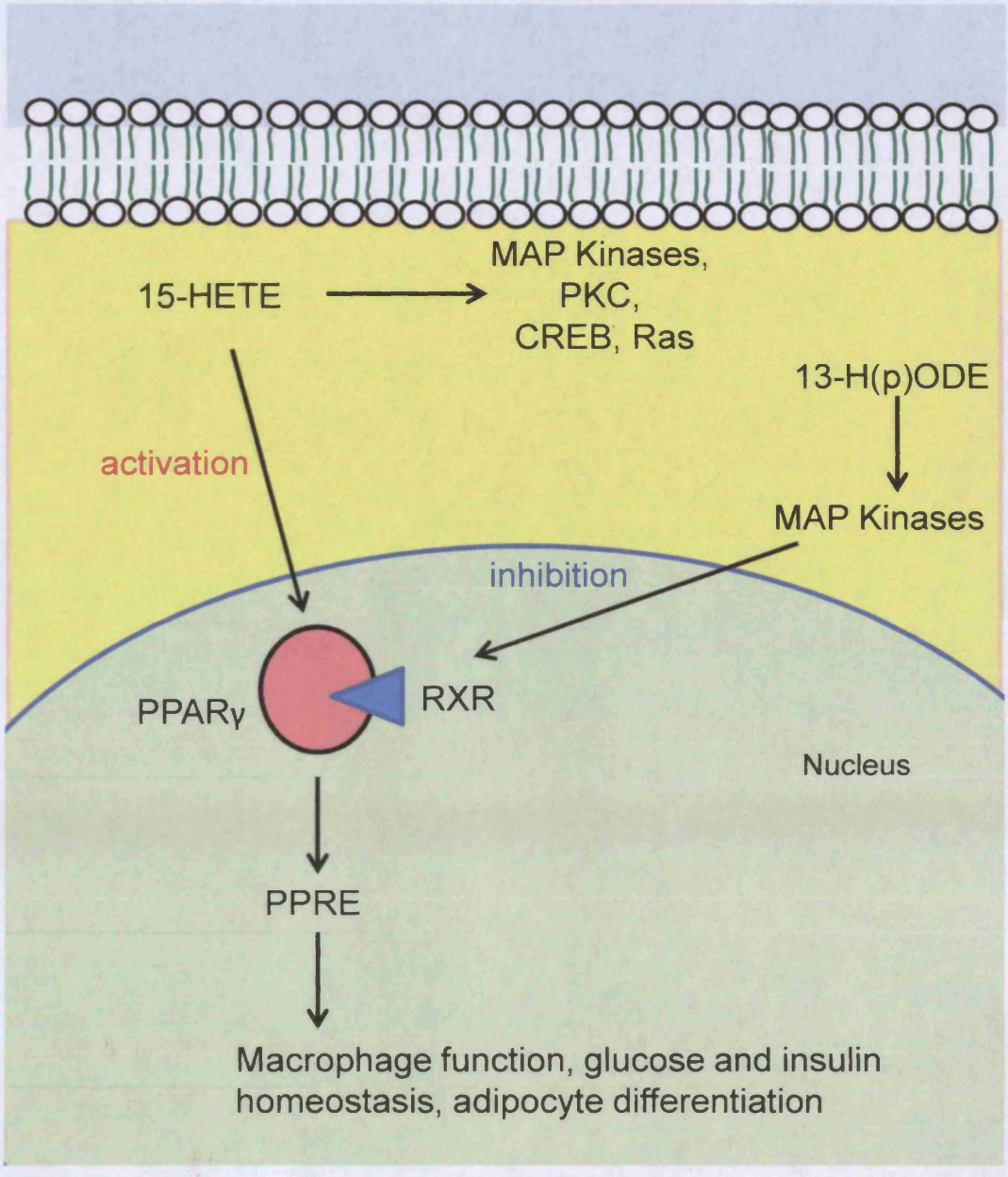


Figure 1.16 Signal transduction pathways mediated by H(p)ETEs and H(p)ODEs. 15-HETE can activate PPARγ, however, 13-H(p)ODEs can inhibit PPARγ via MAP kinase phosphorylation. 15-HETE can also activate MAP kinases, Ras, CREB and PKC intracellular signaling pathways (adapted from Kuhn & O'Donnell, 2006, with permission, (06/12/2010)).

The effect of esterified 15-HETEs on cytokine generation will be investigated in work for this thesis.

1.7.2 Leukotrienes

Leukotrienes are products of 5-LOX that differ from HpETEs and HpODEs as they are conjugated trienes, rather than dienes (Figure 1.3) (Murphy *et al*, 2005). Leukotriene A_4 (LTA_4) is synthesised following the actions of 5-LOX and 5-lipoxygenase activating protein (FLAP) on AA, LTA_4 can then be converted to LTB_4 and LTC_4. LTC_4 is further metabolised to leukotrienes D_4 (LTD_4) and E_4 (LTE_4) (Lam BK, 2003, Murphy *et al*, 2005, Peters-Golden *et al*, 2007). Leukotrienes are synthesised by many leukocytes, including monocytes/macrophages, neutrophils, mast cells, eosinophils, dendritic cells and basophils (Peters-Golden *et al*, 2007). These cells are also regulated by leukotrienes, which stimulate their production, growth and recruitment (Peters-Golden *et al*, 2007). Furthermore, they increase cell adhesion and transmigration into damaged tissue and promote leukocyte activation and survival, all actions that are consistent with a pro-inflammatory response (Ford-Hutchinson *et al*, 1980, Peters-Golden *et al*, 2007). Also, leukotrienes often have a pro-inflammatory effect in particular diseases, such as asthma, atherosclerosis, cancer and rheumatoid arthritis (Coffey & Peters-Golden, 2003, Capra V, 2004, Peters-Golden *et al*, 2007, Back M, 2009).

1.7.3 Lipoxins

Lipoxins are conjugated tetraenes and are generated by metabolism of HpETEs and certain leukotrienes, via the combined actions of 15-LOX1 and 5-LOX, or 5-LOX and 12-LOX from platelets (Serhan *et al*, 1984a, 1984b & 2005, Kuhn *et al*, 1987, Papayianni *et al*, 1995). There are two ways of forming LXA_4, the first involves the metabolism of 15-HpETE by 5-

LOX followed by a non-enzymatic addition of water (Serhan *et al*, 1990 & 1999, Pace-Asciak *et al*, 1999, McMahon *et al*, 2001, Murphy *et al*, 2005). Secondly, LTA_4 can be converted to LXA_4 and/or LXB_4 by the action of 12-LOX and addition of water at C-6 or C-14 (Serhan *et al*, 1990, Pace-Asciak *et al*, 1999, McMahon *et al*, 2001). The synthesis of lipoxins is proposed to require the interaction of several LOX isoforms, therefore, the presence of different cell types is necessary. *In-vitro,* human monocyte-derived dendritic cells incubated with IL-4 and TGF-β1, express both 15-LOX1 and 5-LOX, but they do not produce lipoxins *in-vivo* (Spanbroek *et al*, 2001). Lipoxins are generated in various tissues during *in-vivo* cell-cell interactions and have both pro- and anti-inflammatory properties (Serhan *et al*, 2005). In particular, lipoxins have anti-inflammatory effects towards neutrophils, such as inhibiting their migration, adhesion, chemotaxis and generation of superoxide (Grandordy *et al*, 1990, Colgan *et al*, 1993, Serhan *et al*, 1994, Stenke *et al*, 1994, Papaiyanni *et al*, 1996, Fiore & Serhan, 1996, Filep *et al*, 1999). In addition, lipoxins down-regulate the response of monocytes, dendritic cells, eosinophils and natural killer cells (Ramstedt *et al*, 1987, Stenke *et al*, 1994, Maddox *et al*, 1998, Bandeira-Melo *et al*, 2000). In animal models of various inflammatory conditions, such as cystic fibrosis and glomerulonephritis, lipoxins interfere with host defence by hindering leukocyte rolling and tissue infiltration and prevent tissue damage that is mediated by neutrophils (Munger *et al,* 1999, Leonard *et al*, 2002, Serhan *et al*, 2003, Karp *et al*, 2004).

1.7.4 Hepoxilins

12-LOX in human platelets and rat and/or porcine 12/15-LOX can catalyse the isomerisation of 12(S)-HpETE, to form hepoxilins A_3 and B_3 (HxA_3 and HxB_3) (Nigam *et al*, 2004). These compounds contain an epoxide group, making them chemically and biologically unstable,

thus investigating their *in-vivo* function is difficult (Pace-Asciak CR, 2009). Similar to the other products of 12/15-LOX, hepoxilins are involved in signalling. For example, they increase intracellular calcium concentration, promote secretion of glucose-induced insulin from pancreatic islet cells, open potassium channels in platelets and are involved in neuronal signalling (Pace-Asciak & Martin, 1984, Piomelli *et al*, 1987, Belardetti *et al*, 1989, Dho *et al*, 1990).

1.7.5 Oxidised products of esterified AA

Following the discovery of a reticulocyte LOX in 1974, experiments were carried out to explore its function. Investigations revealed that this LOX can oxidise PE and PC phospholipids isolated from rat liver mitochondria, as well as lysing both outer and inner mitochondrial membranes *in-vitro* (Schewe *et al,* 1975). Human and murine 12/15-LOX is reported to oxidise AA esterified to PC and PE phospholipids (Kuhn *et al,* 1990b, Maskrey *et al*, 2007). In particular, 15-HETE and 12-HETE esterified to PE-phospholipids (15-HETE-PEs and 12-HETE-PEs) have been detected in human monocytes and murine peritoneal lavages respectively (Maskrey *et al*, 2007). Furthermore, endogenous 12-HETE and 5-HETE esterified to both PE and PC have been detected in human platelets and PMN leukocytes respectively (Thomas *et al*, 2010, Clark *et al*, in preparation). To support, studies have shown that exogenously added 5-HETE is esterified primarily to triacylglycerols, followed by PC and PE phospholipid classes (Arai *et al,* 1997). However, the role of LOX in generating ox-PLs in cells is not well studied and the biological function of HETE-PEs and -PCs has not been investigated. Thus, in this thesis, the generation and potential function of these novel phospholipids are investigated.

1. 8 Biological roles of 12/15-LOX

The biological role of 12/15-LOX is not fully determined since the known products do not fully account for the biological activities of the enzyme. Our group recently found that 12-HETE is not responsible for the biological effects of 12/15-LOX in murine macrophages, particularly in the stimulation of cytokines (Dioszeghy *et al*, 2008). Therefore, other products of 12/15-LOX, such as the 12-HETE-PEs, may be involved.

1.8.1 LOX and atherosclerosis

Many groups have investigated the role of LOX isoforms and their products in atherosclerosis. The function of 12/15-LOX in this disease model is not fully understood and conflicting evidence has been reported regarding its anti- or pro-atherosclerotic role (Kuhn & Chan, 1997, Feinmark & Cornicelli, 1997, Cornicelli & Trivedi, 1999, Cathcart & Folcick, 2000, Witter & Hersberger, 2007). In humans, 12/15-LOX has been detected in atherosclerotic lesions, macrophage foamy lesions and within smooth muscle cells (Hiltunen *et al*, 1995, Hugoe *et al*, 1995, Yla-Herttuala *et al*, 1999). Studies investigating the role 12/15-LOX in rodent models suggest that the enzyme is pro-atherosclerotic. In support, LDL oxidised by LOX is atherogenic as its continual up-take by macrophages and smooth muscle cells (via scavenger receptors such as CD36) cause the formation of lipid foam cells (Bird *et al*, 1999, Boullier *et al*, 2000, Stocker & Keaney, 2004, Wittwer & Hersberger, 2007). Several observations have lead to the belief that 12/15-LOX is pro-atherogenic:

(i) *in-vitro* studies show that 12/15-LOX oxidises LDL, a reaction suggested to be of central importance in atherosclerosis (Kuhn *et al*, 1994b).

(ii) During the oxidation of LDL, secondary reactions take place that cause the oxidation of proteins such as apolipoprotein B, which alters their function (Neuzil *et al*, 1998).

(iii) Disrupting the gene for murine 12/15-LOX leads to reduced oxidation of LDL and decreased formation of atherosclerotic lesions in apoE$^{-/-}$ or LDL receptor$^{-/-}$ mice (Sun & Funk, 1996, Cyrus *et al*, 1999, George *et al*, 2001).

(iv) Consistent with a pro-atherosclerotic effect, transgenic mice that over-express 15-LOX and are deficient of the LDL receptor, became more susceptible to atherosclerosis (Harats *et al*, 2000).

(v) Finally, 15-LOX inhibitors prevent the formation of atherosclerotic lesions in cholesterol fed rabbits (Sendobry *et al*, 1996, Bocan *et al*, 1998).

There is also conflicting evidence to that stated above, implicating 12/15-LOX in an anti-atherogenic role. For example, trans-genic rabbits that over-express 15-LOX are protected from atherosclerosis (Shen *et al*, 1996). Similarly, in a macrophage cell line J774A.1 over-expressing 12-LOX, less cholesterol esters were deposited in intra-cellular stores in comparison to mock transfected controls (Belkner *et al*, 2005). Products of 15-LOX also have anti-atherogenic effects such as down-regulating the binding of platelets to the endothelium (Buchanan *et al*, 1985). Furthermore, 15-HETE inhibits the de-granulation of PMN and the synthesis of superoxide and also prevents PMN migration across the endothelium *in-vitro* (Smith *et al*, 1993, Takata *et al*, 1994a).

Of interest, many of the studies showing that 12/15-LOX is pro-atherogenic were carried out in mice, while studies investigating rabbit 15-LOX suggest that the enzyme is anti-atherogenic. Such opposing evidence may due to the different product isomers synthesised by

LOXs in the two animal models. As previously stated, murine 12/15-LOX predominantly oxidises C-12 of AA while rabbit 15-LOX oxidises at C-15. As a result, the different products isomers may have diverse, or even opposing biological properties.

1.8.2 LOX and cell maturation, differentiation and restructuring of the cell membrane

15-LOX was first discovered in rabbit reticulocytes, where expression coincides with a key step in their maturation, the degradation of mitochondria (Grullich *et al*, 2001). However, in experiments where 15-LOX was inhibited, the process of maturation was slowed but not averted. Therefore, 15-LOX may be involved with other metabolic processes that take place during reticulocyte maturation. For example, its expression is also associated with triggering ATP-dependent proteolysis (Rapoport *et al*, 1982, Schewe *et al*, 1986). Products of 15-LOX, in particular 15-HETE, regulate the proliferation of particular cells, such as Friend erythroleukemia cells (Postoak *et al*, 1990, Beckman *et al*, 1990).

12/15-LOX may also be involved in cell-cell interactions prior to phagocytosis. Evidence to support this includes enzyme translocation from the cytosol to the plasma membrane of murine peritoneal macrophages where they come into contact with apoptosing thymocytes (Miller *et al*, 2001). Furthermore, inhibiting 12/15-LOX reduces both actin polymerisation and the ability of macrophages to phagocytose apoptotic cells (Miller *et al*, 2001). 15-LOX is also suggested to play a role in apoptosis as it can oxidise bio-membranes (Schewe *et al*, 1975). In addition, the apoptosis of neuroblastoma and erythroleukemia cells is induced following LOX activation (Maccarrone *et al*, 1999 & 2000). These actions strongly support a role for 12/15-LOX in the regulation of events that require membrane re-structuring, including of cell differentiation, maturation, phagocytosis and apoptosis.

1.8.3 LOX and cancer

15-, 12/15-, 5- and platelet 12-LOX have all been implicated in cancer, but their specific roles have not been fully determined. Eicosanoid products of LOX are thought to exert their effects via PPARs (Hunter *et al*, 1985, Holtzman MJ, 1992, Shureiqi *et al*, 2003, Michalik *et al*, 2004). For particular cancers, studies show either up- or down-regulatory effects by different isomers of LOX. The role of LOX in this disease is complex due to the multiple numbers of LOX genes and therefore, the resulting number of different LOX isoforms (Firstenberger *et al*, 2003). A general consensus is that human and murine 12/15-LOX and the epithelial LOXs (15-LOX2 and 8-LOX), are anti-carcinogenic, present in healthy tissue and benign tumours, but are absent in carcinomas such as lung, breast, prostate, skin, colon and bladder tissue (Shureiqi *et al*, 1999, Shappell *et al*, 1999, Gonzalez *et al*, 2004, Subbarayan *et al*, 2005). In contrast, 5- and platelet 12-LOX are up-regulated in several cancers including skin, lung, esophageal, pancreatic and prostate (Krieg *et al*, 1995, Gao *et al*, 1999, Gupta *et al*, 2001, Jiang *et al*, 2003, Ohd *et al*, 2003, Hennig *et al*, 2005).

1.8.4. LOX and asthma

A growing number of studies indicate that 15-LOX plays a role in asthma. 15-LOX is detected in human airways and is raised in severe asthma, as is the generation of 15-HETE (Hunter *et al*, 1985, Sigal *et al*, 1991, Holtzman *et al*, 1992, Profita *et al*, 2000, Chu *et al*, 2002, Zhao *et al*, 2009). Deficiency of 12/15-LOX increases the secretion of IgA and is reported to protect mice from allergic airway inflammation (Hajek *et al*, 2008). 15-LOX is also up-regulated in human bronchial endothelial cells and in nasal epithelia, with higher

expression in people who smoke, suggesting a modulatory role in airway function (Jayawickreme *et al*, 1999).

1.8.5 LOX & inflammation

Products of LOX are frequently linked to inflammatory regulation. 15-HETE and 13-HODE are raised in particular inflammatory conditions, for example, 15-HETE is raised in human proctocolitis and inflammatory bowel disease (Donoiwitz M, 1985, Zijlstra *et al*, 1991). LOX also has a complicated role in hypertension and vascular damage as different products have opposing effects. In particular, 12-HETE and LTA_4 are pro-inflammatory. Their effects include the constriction of renal blood vessels and glomerular mesangial cells, decreasing renal blood flow and increasing the permeability of glomerular and peritubular capillaries (Navar *et al*, 1996, Imig J, 2000, Yiu *et al*, 2003, Natarajan *et al*, 2004 Imig J, 2006). Conversely, lipoxins and 15-HETE have anti-inflammatory effects in this disease (Badr *et al*, 1984, Fischer *et al*, 1992, Badr & Lakkis, 1994, Munger *et al*, 1999, Leonard *et al*, 2002, Kieran *et al*, 2004). There is additional evidence supporting that 12/15-LOX derived eicosanoids have anti-inflammatory effects. For example, metabolic products of AA increase in the cornea following injury, particularly lipoxins, which promote wound healing (Bazan *et al*, 1985, Gronert *et al*, 2005a & 2005b, Kenchgowda *et al*, 2010).

In mice, 12/15-LOX is largely expressed by resident peritoneal macrophages, which play a key role in peritoneal inflammation. In comparison to macrophages from WT mice, macrophages devoid of 12/15-LOX show increased generation of IL-1, IL-3, granulocyte macrophage cell stimulating factor (GM-CSF), and IL-17, but less RANTES (Dioszeghy *et al*, 2008, Miller *et al*, 2001, Huang *et al*, 1999, Zhao *et al*, 2002). Furthermore, during infection by *Staphylococcus epidermidis* (*S.epi*) (a bacterium largely responsible for

peritonitis), 12/15-LOX$^{-/-}$ macrophages generate more GM-CSF but less RANTES, IL-12p40 and IL-12p70 than WT cells (Dioszeghy *et al*, 2008). Importantly, infecting mice with *Staphylococcus epidermidis* also alters the synthesis of products by 12/15-LOX (Morgan *et al*, 2009). This evidence implies that 12/15-LOX is involved in regulating inflammation. In support, the function of 12/15-LOX$^{-/-}$ macrophages is altered, for example, they show a decreased ability to carry out phagocytosis of apoptotic cells. Thus, 12/15-LOX is involved in regulating inflammation, but the exact mechanism of its action is unclear.

Other anti-inflammatory events promoted by LOX, in particular by 15-HETE, include inhibiting the formation and release of superoxide from PMN leukocytes (Smith *et al*, 1993). Also, 15-HETE reduces the synthesis of LTB_4, a pro-inflammatory eicosanoid involved in experimental arthritis (Herlin *et al*, 1990, Takata *et al*, 1994b). 12/15-LOX also down-regulates the expression of NFκB, a pro-inflammatory transcription factor (Nakajima *et al*, 2001, Yuan *et al*, 2005, Appel *et al*, 2005). Thus, the reported evidence suggests that 12/15-LOX can have both pro- and/or anti-inflammatory effects during inflammation.

1.9 The synthesis of oxidised phospholipids by 12/15-LOX

A lack of methods sensitive enough to detect and quantify oxidised phospholipids has delayed work in this area until recent years. However, due to the increased availability of new generation mass spectrometers, this technique is proving to be invaluable for research investigating oxidised phospholipids. Yet, much is still unknown regarding the biological and immunological impact of specific molecular species of oxidised phospholipids.

Until recently, free H(p)ETEs were believed to be the sole products synthesised by LOX following the metabolism of AA. However, with the aid of mass spectrometry (MS), four novel lipids synthesised by 12/15-LOX were identified and characterised in IL-4-treated human monocytes (Figure 1.17) (Maskrey *et al,* 2007). These phospholipids consist of 15-HETE attached to phosphatidylethanolamine (PE), and were identified as 18:0a/15-HETE-PE, 18:0p/15-HETE-PE, 18:1p/15-HETE-PE and 16:0p/15-HETE-PE (Figure 1.18). They are generated by direct oxidation of arachidonate-containing PEs (AA-PEs) (Maskrey *et al,* 2007). Levels of 15-HETE-PEs increased following cellular activation, indicating that they are generated enzymatically. Furthermore, 12-HETE-PEs were identified in human platelets and murine peritoneal macrophages (Maskrey *et al,* 2007).

Studies using 12/15-LOX$^{-/-}$ mice indicated that the enzyme is involved in regulating inflammation (Maskrey *et al,* 2007, Dioszeghy *et al,* 2008). Specifically, 12/15-LOX modulates immune cell function, however, it does so in the absence of free H(p)ETEs, suggesting that alternative products, such as HETE-PEs, may be involved (Huang *et al,* 1999, Miller *et al,* 2001, Shappell *et al,* 2001, Zhao *et al,* 2002, Shankaranarayanan & Nigam, 2003). Recent studies have indicated that 12/15-LOX may act in a pro-resolving manner during inflammation (Gonert *et al,* 2005, Middleton *et al,* 2006, Zhao *et al,* 2009). Therefore, it is important to determine whether 15-HETE-PEs have anti-inflammatory effects. However, studies investigating the metabolism and biological importance of 15-HETE-PEs in inflammatory conditions have not been carried out.

1.10 Electrospray mass spectrometry

The study by Maskrey *et al* (2007) forms the basis of this thesis as it describes the identification of 15-HETE-PEs in human monocytes and 12-HETE-PEs in murine peritoneal

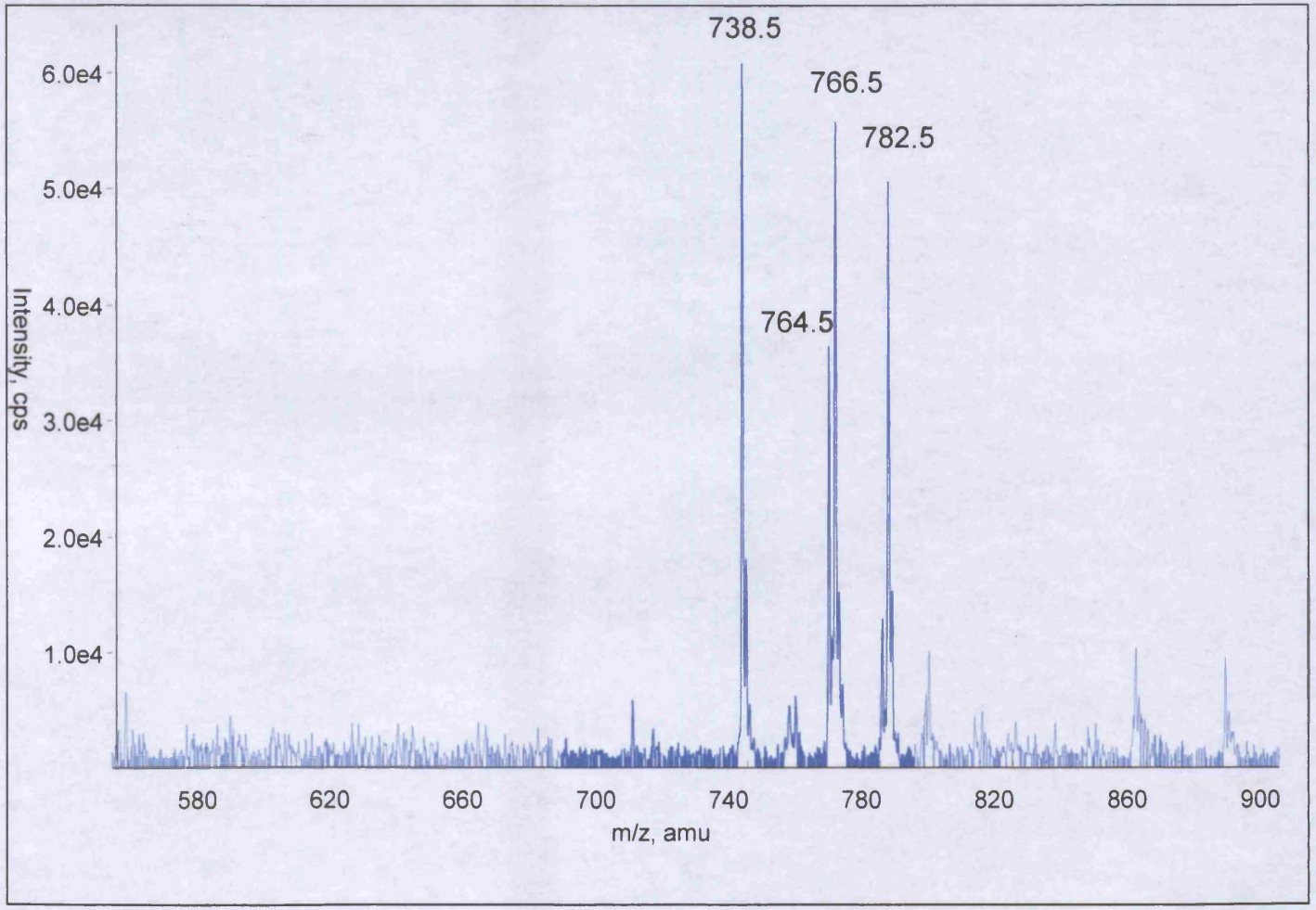
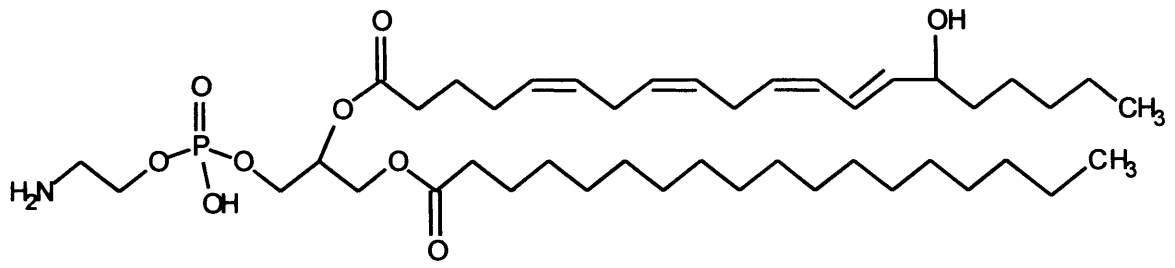
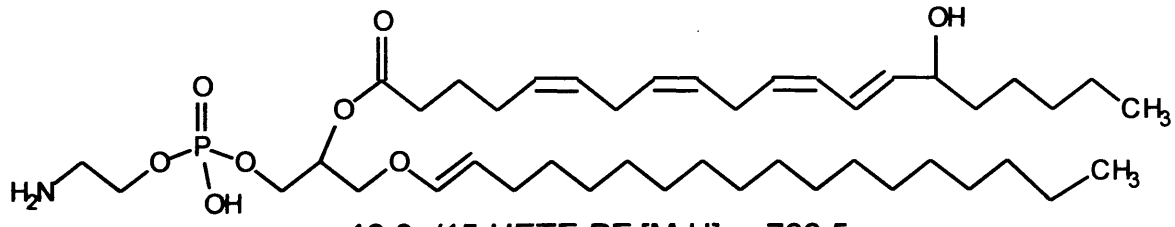


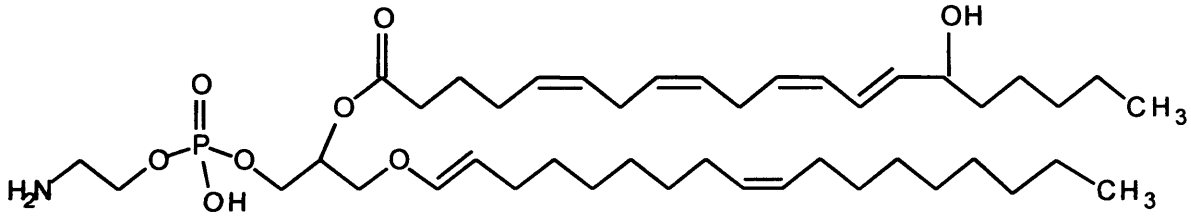
Figure 1.17 Precursor scanning identified four ions, that contain HETE, which are elevated following A23187 activation of human monocytes. Precursor scanning for HETE (*m/z* 319) identified four major parent ions: m/z 738.5, 764.5, 766.5 and 782.5 $[M-H]^-$ (Reproduced from Maskrey *et a l*, 2007, with permission (18/11/2010)).



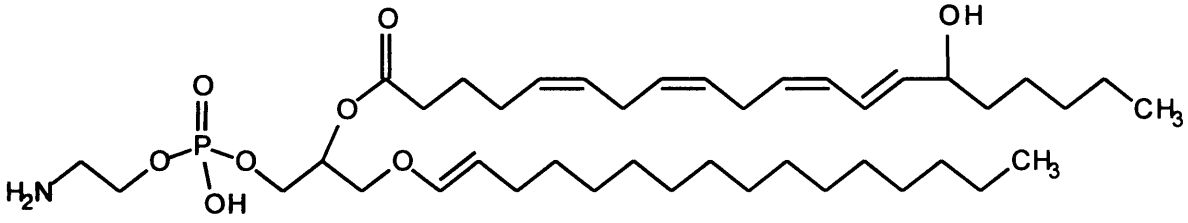
18:0a/15-HETE-PE [M-H]$^-$ = 782.5.



18:0p/15-HETE-PE [M-H]$^-$ = 766.5.



18:1p/15-HETE-PE [M-H]$^-$ = 764.5.



16:0p/15-HETE-PE [M-H]$^-$ = 738.5.

Figure 1.18 Proposed structures for four HETE-containing products of LOX in activated human monocytes. Based on comparison with synthesis standards, four 15-HETE-PEs were identified. Similar compounds were identified in platelets where 15-HETE is replaced by 12-HETE (Maskrey *et al*, 2007).

macrophages. The primary method of analysis utilized by Maskrey *et al* (2007) was electrospray mass spectrometry (EMS), which was also used for the studies described herein. The mass spectrometer used throughout these investigations was an Applied Biosystems 4000 Q-Trap, a schematic representation of which is shown in Figure 1.19. Liquid samples are introduced either by direct injection or following HPLC separation (Figure 1.20). Within the mass spectrometer, the sample is fed through a hypodermic needle at a high voltage, which causes its evaporation into an 'electrospray' (Mann *et al*, 2001, Fenn *et al*, 1989). Molecules within the electrospray are then ionised in atmosphere (Mann *et al*, 2001). The ability of an analyte to form a negative $[M-H]^-$ or positive $[M+H]^+$ ion is critical for its detection, and for this to occur it must be sufficiently polar (Mann *et al*, 2001). After ionisation, charged molecules travel through three quadrapoles (Q1, Q2 and Q3) to a detector. These consist of four rods, to which an oscillating electric field is applied (Mann *et al*, 2001). Q1 and Q3 act as mass filters, and only let stable masses through to the detector. If required, charged molecules can be contained in Q2 and undergo collision-induced-decomposition (CID) using inert gas (Maskrey & O'Donnell, 2008). The fragments then pass through Q3 to the detector. A mass spectrum can be acquired by scanning the amplitude of the electric field and recording the ions at the detector (Mann *et al*, 2001). Due to the structural arrangement of the mass spectrometer, it can be used in different ways to analyse molecules, some of which are described below.

For the investigations described herein, EMS was used in various modes. For example, purified infusions of lipid were injected directly into the mass spectrometer in order to investigate its contents; this is known as a Q1 scan (Figure 1.21A) (for example, Figure 3.3). During this analysis, only molecules that are both ionised and on a stable trajectory are detected. Mass spectrometry (MS) can also be used to investigate the fragmentation pattern of

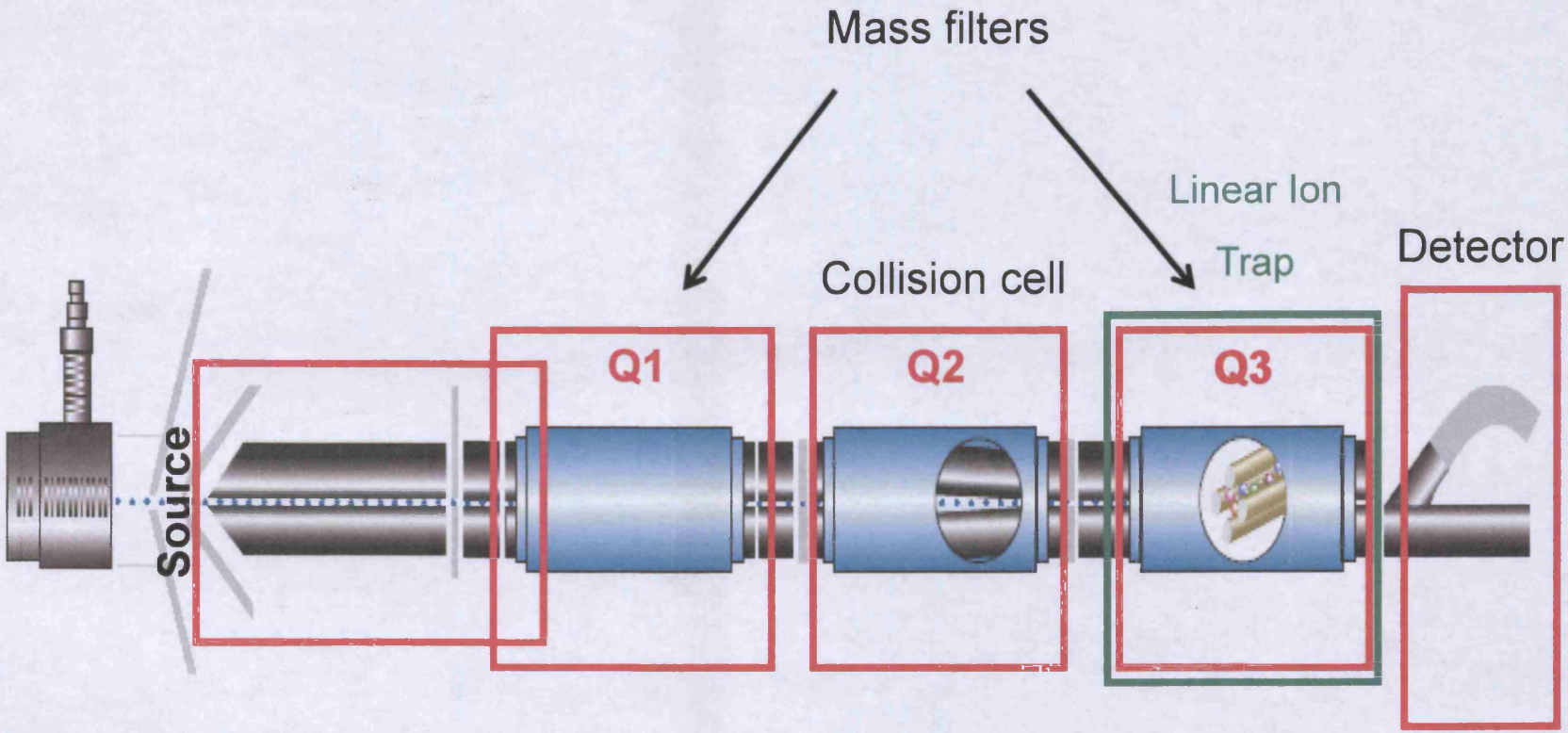


Figure 1.19 Schematic diagram of a mass spectrometer. An Applied Biosystems Q-trap 4000 mass spectrometer was used as a diagnostic tool for the work described herein. The scheme represents the general structure of this instrument (Reproduced with permission from Applied Biosystems, (16/11/2010).

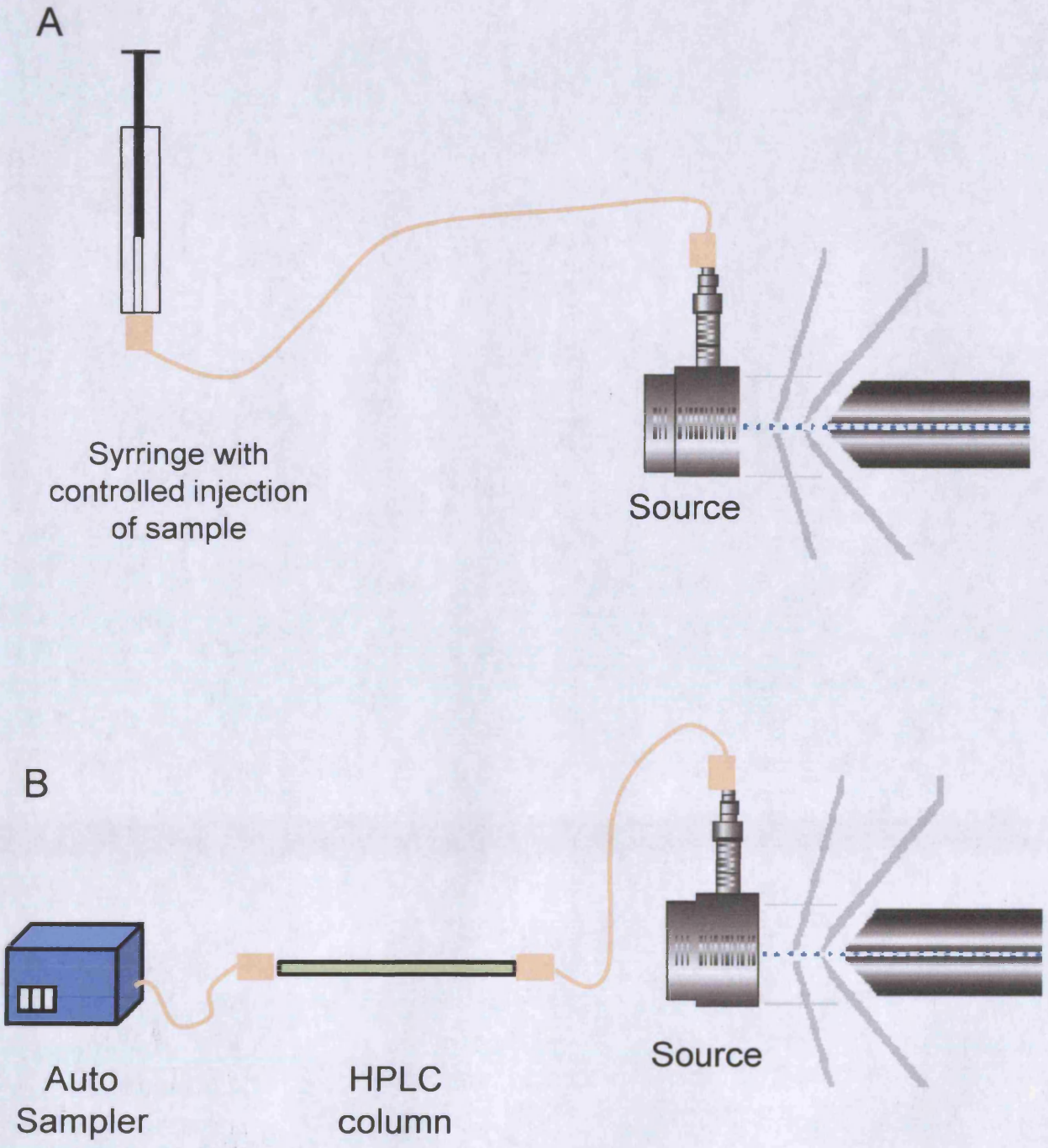


Figure 1.20 Basic illustration of direct injection (A) and HPLC separation (B) prior to MS analysis

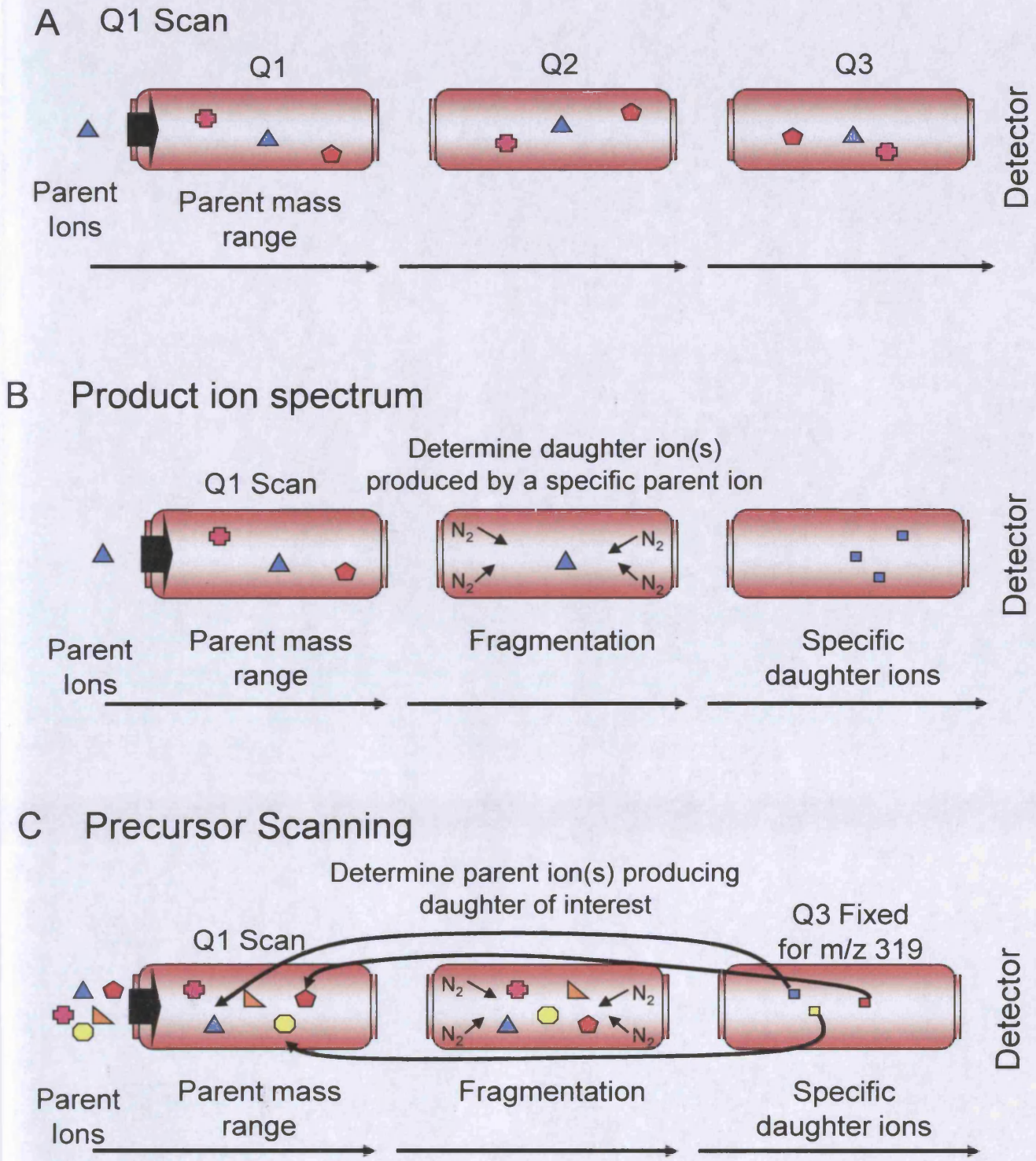


Figure 1.21 Illustration of a Q1 scan (A), product ion spectrum (B) and precursor scanning (C) by MS.

a specific analyte detected during a Q1 scan. A compound of interest can selectively undergo CID in the collision cell, the products are then released into Q3 and travel through to the detector (Figure 1.21 B) (Maskrey & O'Donnell, 2008). This is known as a product ion spectrum, an example of which can be seen in Figure 3.3 (inset). Alternatively, MS has been used in 'fishing' experiments, searching for the *m/z* of a particular structure(s) that can be fragmented into smaller molecules of known *m/z*. This mode is known as precursor scanning (Figure 1.21 B). An example is described by Maskrey *et al* (2007), where precursor scanning was used to investigate compounds that contain HETE (*m/z* 319). Lipids extracted from human monocytes were separation by liquid chromatography, followed by precursor scanning for *m/z* 319. As seen in Figure 1.17, four compounds contained this fragment, which were identified as 15-HETE-PEs. In addition to the methods described above, samples can be analysed for numerous compounds simultaneously that have specific parent and daughter *m/z,* which are consequently detected in Q1 and Q3 respectively, known as multiple reaction monitoring (MRM) transitions (Maskrey & O'Donnell, 2008, Yin *et al,* 2010). This method of analysis is known as MRM scanning (MS//MS). The use of HPLC preceding MS/MS (LC/MS/MS) has meant that individual compounds can be separated followed by their sensitive identification. Combining the known parent to daughter *m/z* transition with time of elution can be very specific to individual compounds. Thus, LC/MS/MS is a very efficient technique for phospholipid analysis.

MS has been used extensively in proteomics and its use in lipidomics, is also proving to be invaluable. MS is efficient since, multiple compounds can be analysed simultaneously and due to its sensitivity and specificity, small changes such as oxidation (a *m/z* increase of 16), can be monitored easily (Pitt & Spickett, 2008). For this reason, MS is likely to be used by many groups investigating protein or lipid oxidation. MS has been used for many purposes,

for example, to understand the mechanisms and products derived from lipid oxidation (Pulfer & Murphy, 2003, Yin *et al* 2010, Yin *et al*, 2009, Yin & Porter, 2007). Furthermore, novel compounds, as well as amino acid modifications, have been identified by MS in both research and clinical samples (Pitt & Spickett, 2008, Maskrey *et al*, 2007, Greenberg *et al*, 2006. Podrez *et al*, 2002a &b, von Schliffen *et al*, 2009, Williams *et al*, 2007, Fermandis & Wenk, 2009, Walther & Mann, 2010). Thus MS is a suitable technique for the investigations described herein.

The identification of H(p)ETE-PEs by Maskrey *et al* (2007) meant an assay was required for their direct quantification. As a soybean LOX was available that oxidises AA at C-15, existing methods were adapted in orderused to synthesise and purify a H(p)ETE-PE standard, for use in a new quantification assay. Maskrey *et al* (2007) characterised the synthesis of 15-H(p)ETE-PEs by 15-LOX but they did not characterise how 12-H(p)ETE-PEs are made by murine 12/15-LOX. Therefore, using MS and traditional chromatographic techniques, I aimed to characterise the synthesis of 12-H(p)ETE-PEs by 12/15-LOX in murine macrophages. The immunological effects of 12/15-LOX may be exerted by 12- and 15-H(p)ETE-PEs, therefore, studies were required to determine how the H(p)ETE-PEs are metabolised. Thus, the generation and metabolism of 15- and 12-H(p)ETE-PEs was investigated in human monocytes and murine macrophages respectively.

Studies in this thesis investigated whether 15- and 12-H(p)ETE-PEs may be involved in inflammatory processes. In particular, the pro- versus anti-inflammatory effect of 15-H(p)ETE-PEs was assessed by adding a synthesised 18:0a/15-H(p)ETE-PE standard to human monocytes. Also, membrane externalisation of PE and PS is believed to regulate events that require re-structuring of the membrane, such as phagocytosis. Thus, studies herein

describe whether 12-H(p)ETE-PEs were externalised in murine macrophages. Also, analysis of WT and 12/15-LOX$^{-/-}$ peritoneal macrophages by electron microscopy showed whether their ultra-structure is affected by the absence of 12/15-LOX, which may aid in determining the function of this enzyme. Briefly, the work in this thesis has attempted to characterise the synthesis and metabolism of 12- and 15-H(p)ETE-PEs generated by 12/15-LOX in murine macrophages and human monocytes, and has discussed how they may be involved in inflammatory responses.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials.

2.1.1 Chemicals.

15(*S*)-hydroxy-[S-(E,Z,Z,Z)]-5,8,11,13-eicosatetraenoic acid (15-HETE), 15(*S*)-hydroxy-11Z,13E-eicosadienoic acid (15-HEDE) and 15(*S*)-hydroxy-[S-(E,Z,Z,Z)]-5,8,11,13-eicosatetraenoic acid-d8 (15-HETE-d_8) were from Alexis Chemicals Ltd. (Nottingham, UK). 1-stearoyl-2-arachidonyl-sn-glycero-3-phosphoethanolamine (SAPE), 1-stearoyl-2-arachidonyl-sn-glycero-3-phosphocholine (SAPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE), 2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine (DMPS), 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DMPG) and 1,2-dimyristoyl-*sn*-glgbycero-3-phosphate (DMPA) were from Avanti Polar Lipids inc. (Alabaster, Alabama, USA). Human recombinant interleukin 4 (hrIL-4) was from Promega (Southampton, Hampshire, UK). Lymphoprep was from Axis Shield (Oslo, Norway). Lipopolysaccharide was from Calbiochem, (Beeston, Nottingham,UK). Bovine serum albumin was from USB (Staufen, Germany). Glutaldehyde, sodium cacodylate, osmium tetroxide, urianyl acetate, propyl oxide, araldyte CY212, dodecenyl succinic anhydride (DDSA) and dimethylbenzylamine (BDMA) were all from Agar Scientific (Stansted, Essex, UK). Kodak D-19 developer and Ilford Hypam fixer were from Piccadilly Photographic (Cardiff, Wales, UK). All other reagents were from Sigma-Aldrich company Ltd (Poole, Dorset, UK) unless otherwise stated. All Solvents used were from Fisher (Loughborough, Leicestershire, UK).

2.1.2 General buffers and solutions used.

Krebs Ringer buffer

50 mM HEPES, 120 mM NaCl, 4.71 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 26.8 mM $NaHCO_3$, 10 mM Glucose, pH 7.4

Phosphate buffered Saline (PBS)

137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4

Citrate-PBS

137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.4% (w/v) trisodium citrate, pH 7.4

Dextran-Citrate-PBS

137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.8% (w/v) trisodium citrate, 2% (w/v) Dextran, pH 7.4

Monocyte media

RPMI media, 10% (v/v) foetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine.

Extraction Solvent

1 M acetic acid:2-propanol:hexane (2:20:30 (v/v/v))

Wash buffer

0.05% Tween-20 in PBSF

Tris buffered saline

20 mM Trizma Base, 150 mM NaCl (pH 7.4)

Reagent diluent

1 % BSA in PBS buffer

TNFα blocking buffer

1 % BSA, 5 % Sucrose in PBS with 0.05 % NaN_3

TNFα reagent diluents

0.1 % BSA, 0.05 % Tween-20 in Tris buffered saline

Carbonate buffer

0.1 M sodium carbonate buffer (8.4 g $NaHCO_3$ and 3.56 g Na_2CO_3 in 1 L H_2O), pH 9.5

Cacodylate buffer

0.2 M sodium cacodylate trihydrate in H_2O, pH 7.

Embedding Resin

Epoxy resin (Araldyte CY212); Dodecenyl succinic anhydride (DDSA); Dimethylbenzylamine (BDMA). Ratio of 1:1:0.03 (w/w/w)

Reynold's lead citrate

Lead Nitrate 1.33 g, Sodium Citrate 1.76 g, H_2O 30 ml

Acetic acid stop bath

Acetic acid; H_2O. Ratio of 1:60

2.2 Methods

2.2.1 Mass spectrometry analysis.

2.2.1.1 HETE quantitation by LC/MS/MS.

Fatty acids were separated on a C18 Spherisorb ODS2, 5 μm, 150 x 4.6 mm column (Waters Ltd, Elstree, Hertfordshire, UK). The gradient used was 50 - 90% B over 20 minutes at 1 ml/min (A= water:acetonitrile:acetic acid at 75:25:0.1; B= methanol:acetonitrile:acetic acid at 60:40:0.1). Products were quantified by LC/ESI/MS/MS on a 4000 Q-Trap (Applied Biosystems, Foster City, California, USA) using specific parent to daughter transitions of $[M-H]^-$ *m/z* 319.2 (HETE) → 219 (15-HETE), 179 (12-HETE) and 115 (5-HETE), 327.2 → 226 (15-HETE-d_8), 327.2 → 184 (12-HETE-d_8) and 323.2 → 223 (15-HEDE) in a negative ion mode (Figure 2.1). The collision energies and de-clustering potentials for each analyte are below in Table 2.1. Products were quantified using 15-HETE-d_8, 12-HETE-d_8 or 15-HEDE as internal standards run in parallel under the same conditions. Standard curves with purified HETEs *versus* 15-HETE-d_8

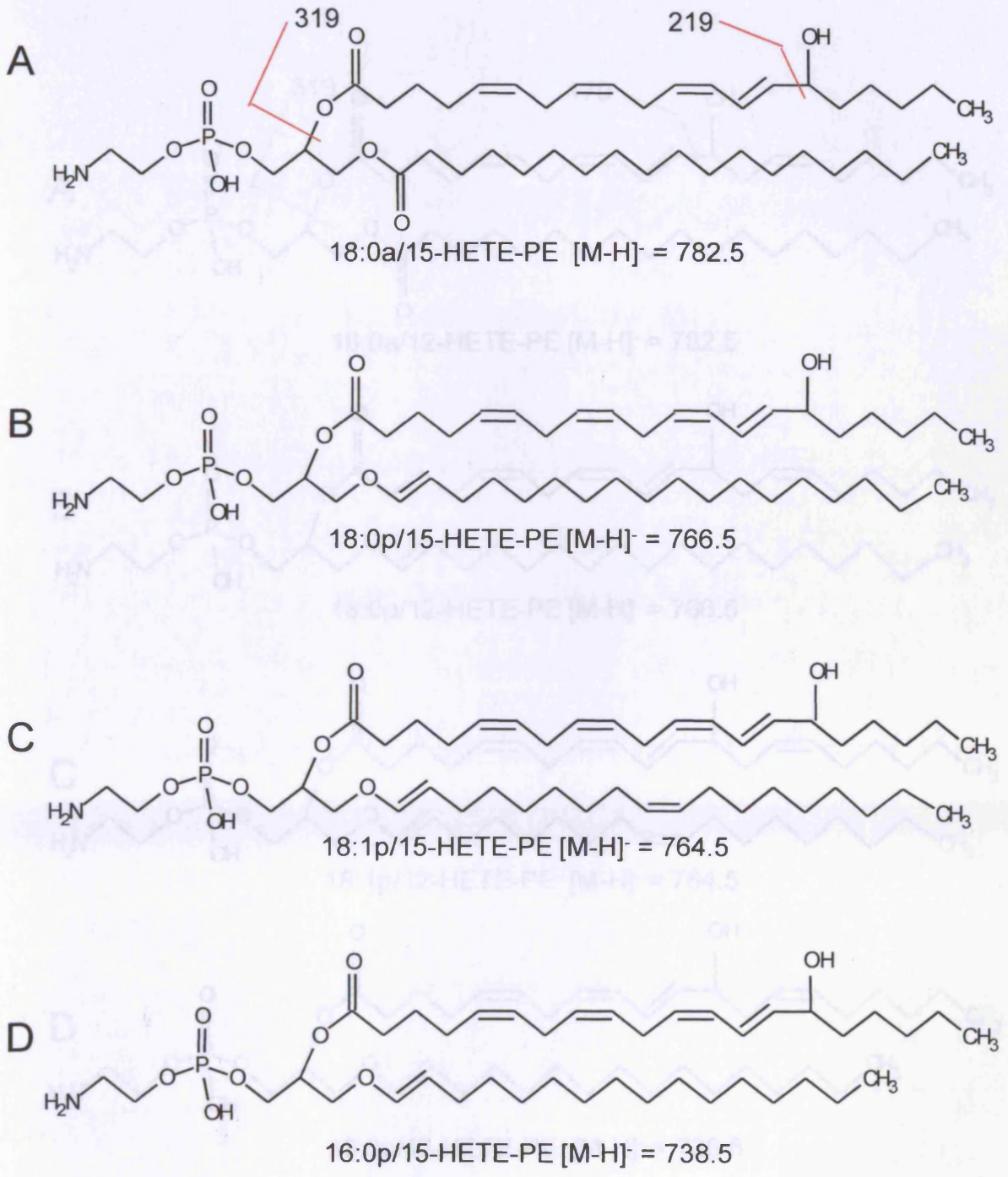
and 12-HETE-d_8 were constructed for eicosanoid quantification, allowing for detection of approximately 1 to 10 pg of each eicosanoid.

Table 2.1 Parent and daughter *m/z* and MS conditions for analytes

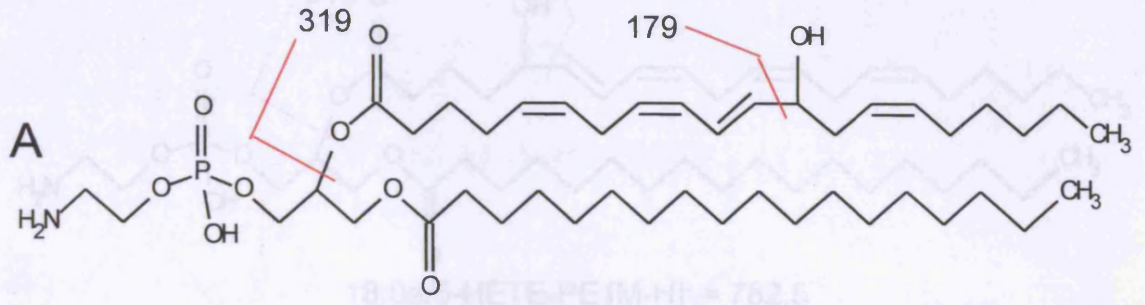
Analyte	Q1 Mass (amu)	Q3 Mass (amu)	Declustering Potential (V)	Collision Energy (V)
15-HETE-d8	327	226	-65	-16
12-HETE-d8	327	184	-85	-20
15-HEDE	323	223	-105	-28
15-HETE	319	219	-45	-20
12-HETE	319	179	-85	-20
5-HETE	319	115	-80	-22
15-KETE	317	113	-40	-24
AA	303	259	-75	-20

2.2.1.2 Phospholipid quantification by LC/MS/MS.

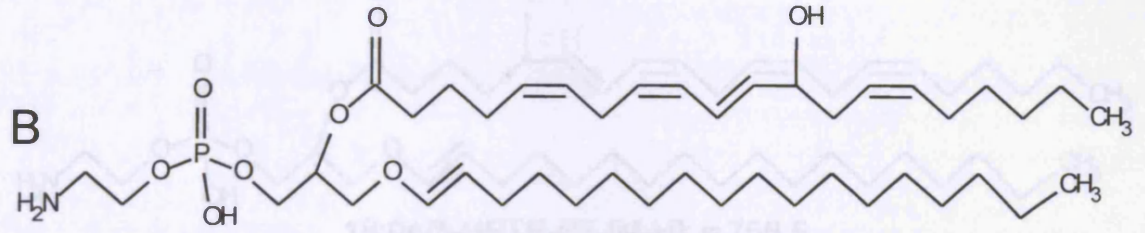
Phospholipids were separated using a Luna 3 μm C18 150 x 2 mm column (Phenomenex Ltd., Macclesfield, Cheshire, UK) based on the hydrophobicity of the sn1 fatty acid. The gradient used was 50-100 % B over 10 minutes then 100 % B for 30 minutes at 200 μl/min (A= methanol:acetonitrile:water at 6:2:2 with 1 mM ammonium acetate; B= methanol with 1 mM ammonium acetate). Products were quantified by LC/ESI/MS/MS on a Q-Trap (as above) using the specific parent to daughter transitions of *m/z* 782.5, 766.5, 764.5 and 738.5 → 219 (15-HETE-PEs) (Scheme 2.1) or 179 (12-HETE-PEs) (Scheme 2.2) or 115 (5-HETE-PEs) (Scheme 2.3) and 634 → 227 (DMPE) (Scheme 2.4) (all $[M-H]^-$). The collision energy for the HETE-PEs was -140 V and the declustering potential, -45 V. Standard curves with either purified 15-HETE-PE (generated and purified in 2.2.3) or an isomer mixture of HETE-PEs *versus* DMPE were constructed for lipid quantification, allowing for detection of approximately 1 to 10 pg of each phospholipid. These will be fully described in Chapter 3.



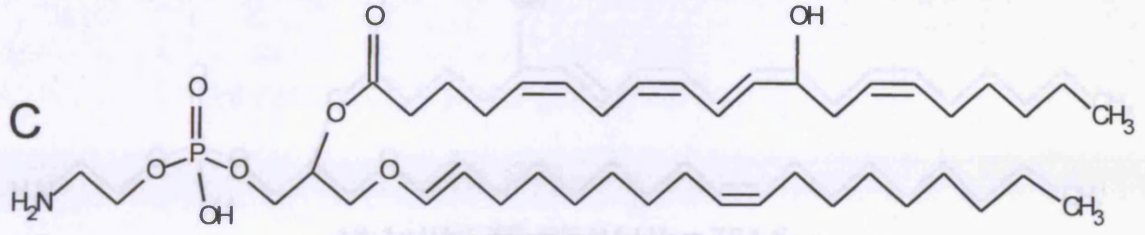
Scheme 2.1 Structures and *m/z* of 15-HETE-PEs



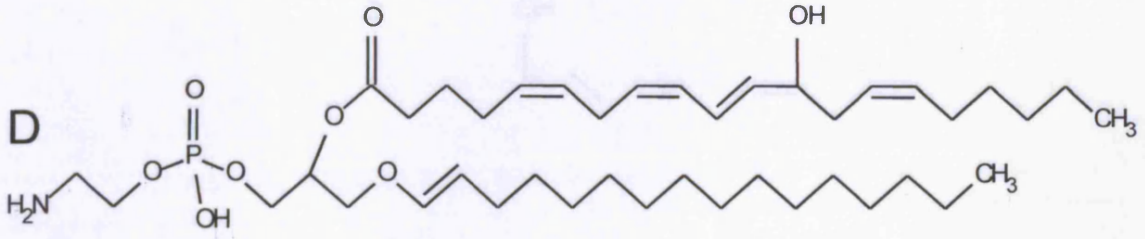
18:0a/12-HETE-PE $[M-H]^-$ = 782.5



18:0p/12-HETE-PE $[M-H]^-$ = 766.5

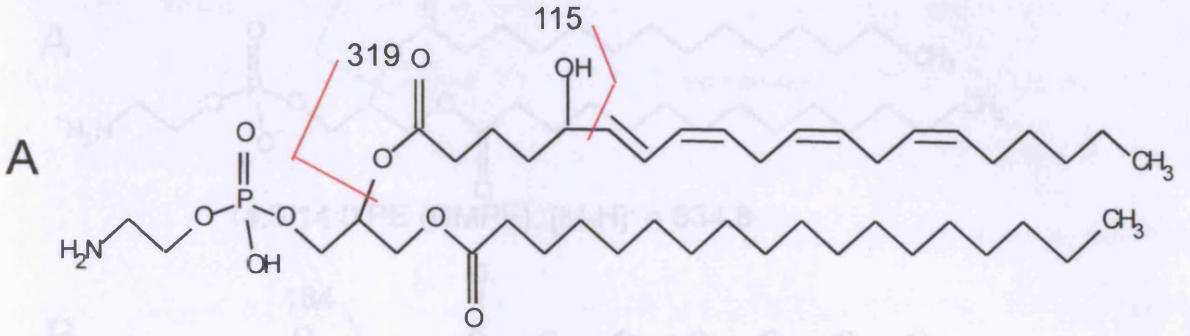


18:1p/12-HETE-PE $[M-H]^-$ = 764.5

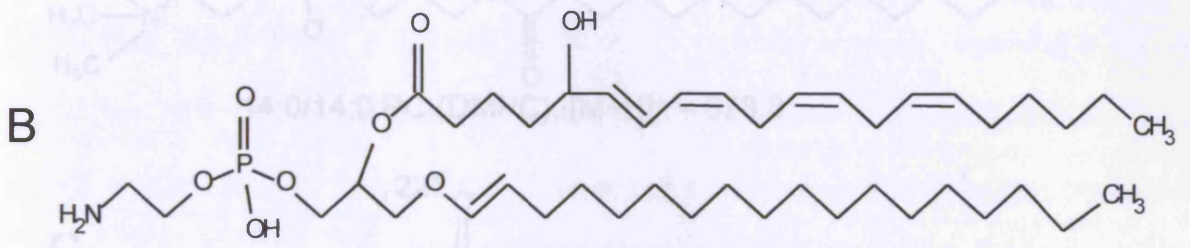


16:0p/12-HETE-PE $[M-H]^-$ = 738.5

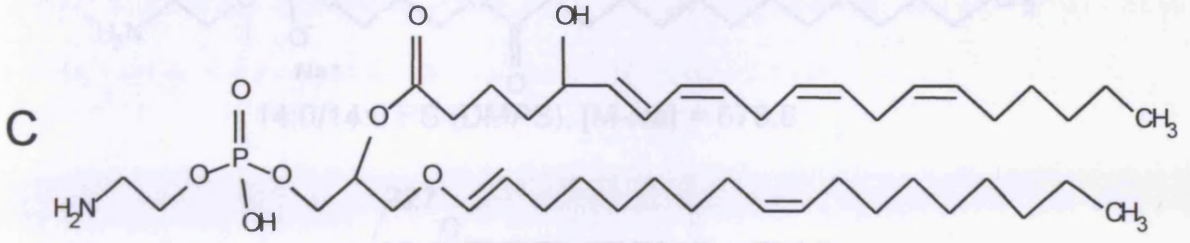
Scheme 2.2 Structures and *m/z* of 12-HETE-PEs



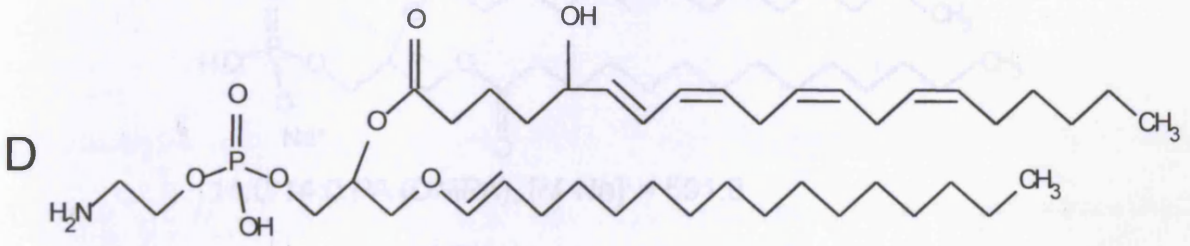
18:0a/5-HETE-PE $[M-H]^-$ = 782.5



18:0p/5-HETE-PE $[M-H]^-$ = 766.5

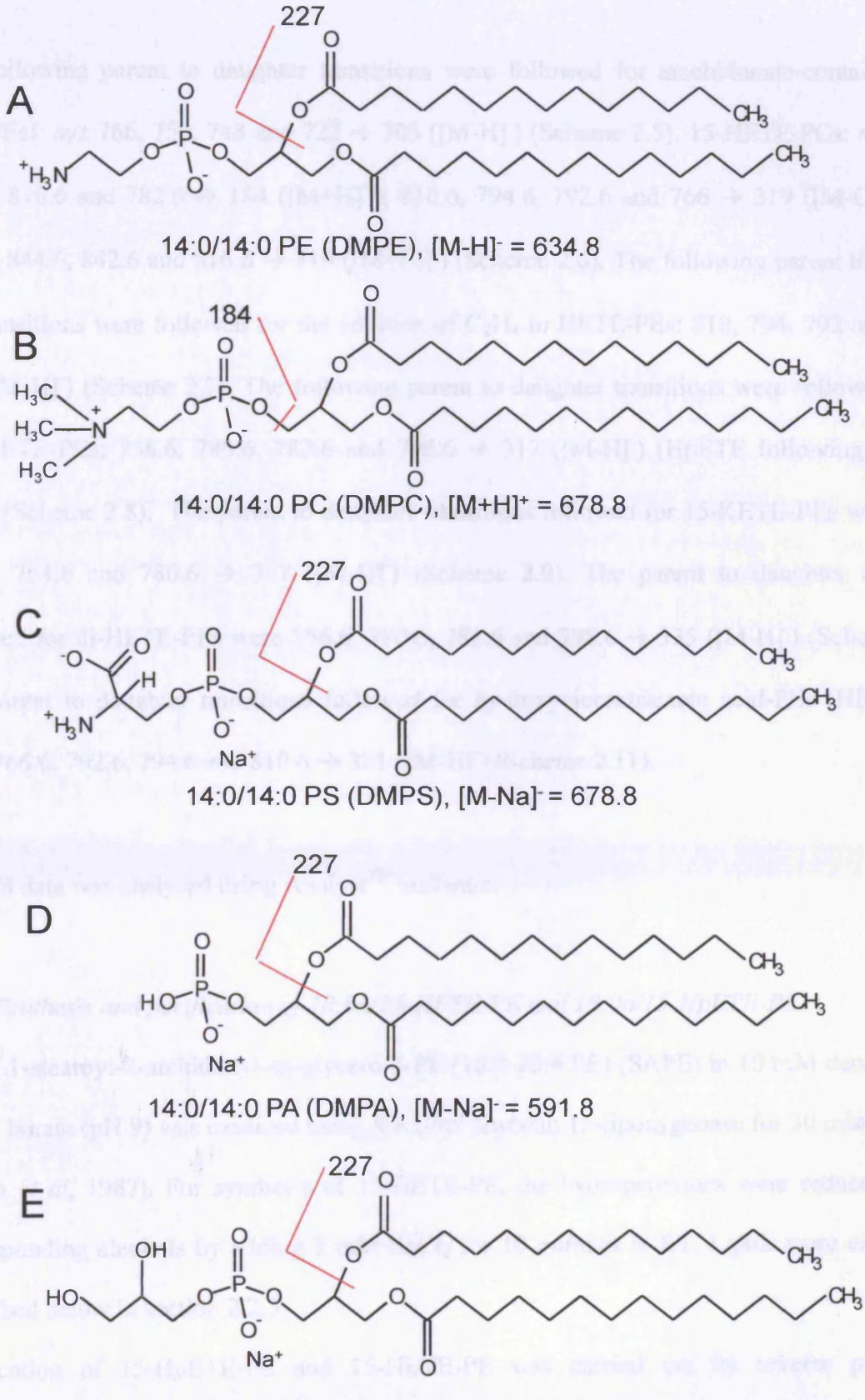


18:1p/5HETE-PE $[M-H]^-$ = 764.5



16:0p/5-HETE-PE $[M-H]^-$ = 738.5

Scheme 2.3 Structures and *m/z* of 5-HETE-PEs



Scheme 2.4 Structures and *m/z* of phospholipid standards

The following parent to daughter transitions were followed for arachidonate-containing PEs (AA-PEs): *m/z* 766, 750, 748 and 722 → 303 ($[M-H]^-$) (Scheme 2.5). 15-HETE-PCs: *m/z* 826.6, 808.6, 810.6 and 782.6 → 184 ($[M+H]^+$); 810.6, 794.6, 792.6 and 766 → 319 ($[M-CH_3]^-$) and 860.6, 844.6, 842.6 and 816.6 → 319 ($[M+Cl]^-$) (Scheme 2.6). The following parent to daughter ion transitions were followed for the addition of C_2H_4 to HETE-PEs: 810, 794, 792 and 766 → 347 ($[M-H]^-$) (Scheme 2.7). The following parent to daughter transitions were followed for the 15-HpETE-PEs: 756.6, 780.6, 782.6 and 798.6 → 317 ($[M-H]^-$) (HpETE following a loss of H_2O), (Scheme 2.8). The parent to daughter transitions followed for 15-KETE-PEs were 736.6, 762.6, 764.6 and 780.6 → 317 ($[M-H]^-$) (Scheme 2.9). The parent to daughter transitions followed for di-HETE-PEs were 756.6, 780.6, 782.6 and 798.6 → 335 ($[M-H]^-$) (Scheme 2.10). The parent to daughter transitions followed for hydroxyeicosatrienoic acid-PEs (HETrE-PEs) were 766.6, 792.6, 794.6 and 810.6 → 321 ($[M-H]^-$) (Scheme 2.11).

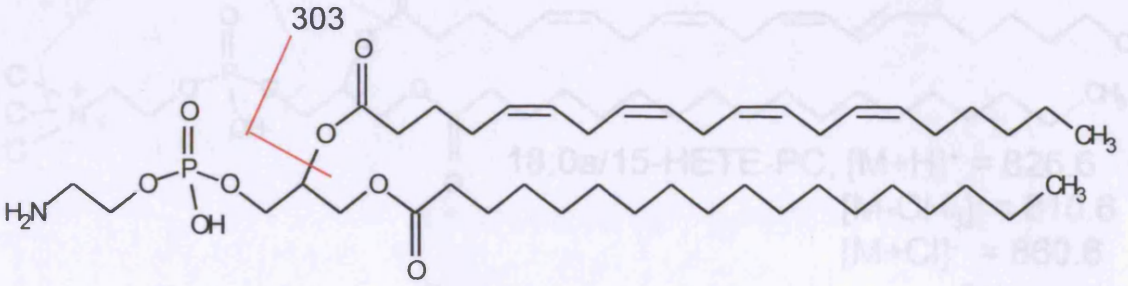
All MS data was analysed using Analyst™ software.

2.2.2 Synthesis and purification of 18:0a/15-HETE-PE and 18:0a/15-HpETE-PE.

1 mM 1-stearoyl-2-archidonyl-sn-glycero-3-PE (18:0-20:4 PE) (SAPE) in 10 mM deoxycholate/ 0.2 M borate (pH 9) was oxidized using 8 KU/ml soybean 15-lipoxygenase for 30 minutes at RT (Brash *et al*, 1987). For synthesis of 15-HETE-PE, the hydroperoxides were reduced to their corresponding alcohols by adding 1 mM $SnCl_2$ for 10 minutes at RT. Lipids were extracted as described below in section 2.2.3.

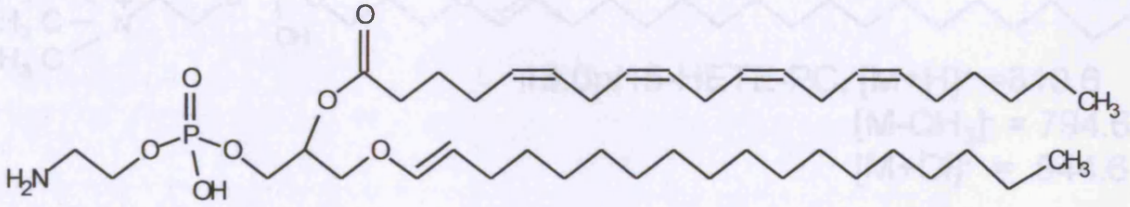
Purification of 15-HpETE-PE and 15-HETE-PE was carried out by reverse phase high performance liquid chromatography (RP-HPLC) using a 5 μm spherisorb ODS2 C18, 4.6 x 150

A



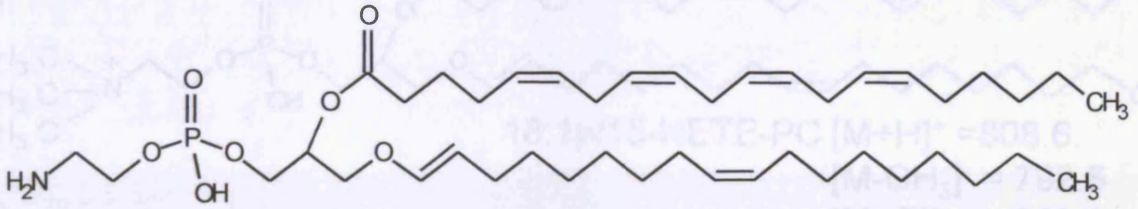
18:0a/AA-PE, $[M-H]^-$ =766.6

B



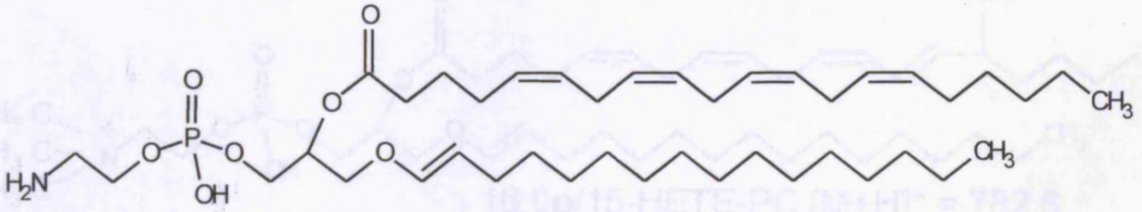
18:0p/AA-PE, $[M-H]^-$ = 750.6

C



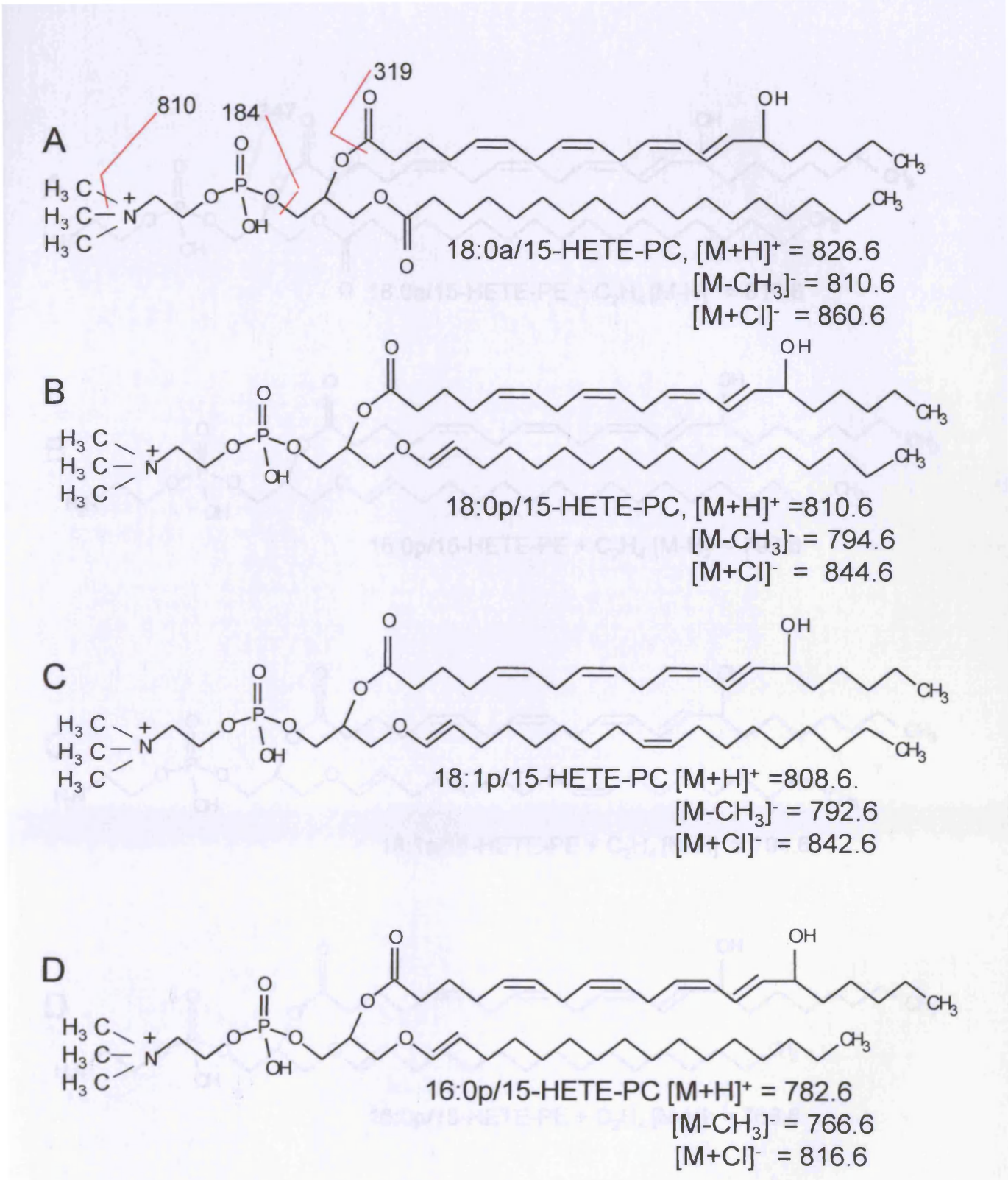
18:1p/AA-PE, $[M-H]^-$ = 748.6

D

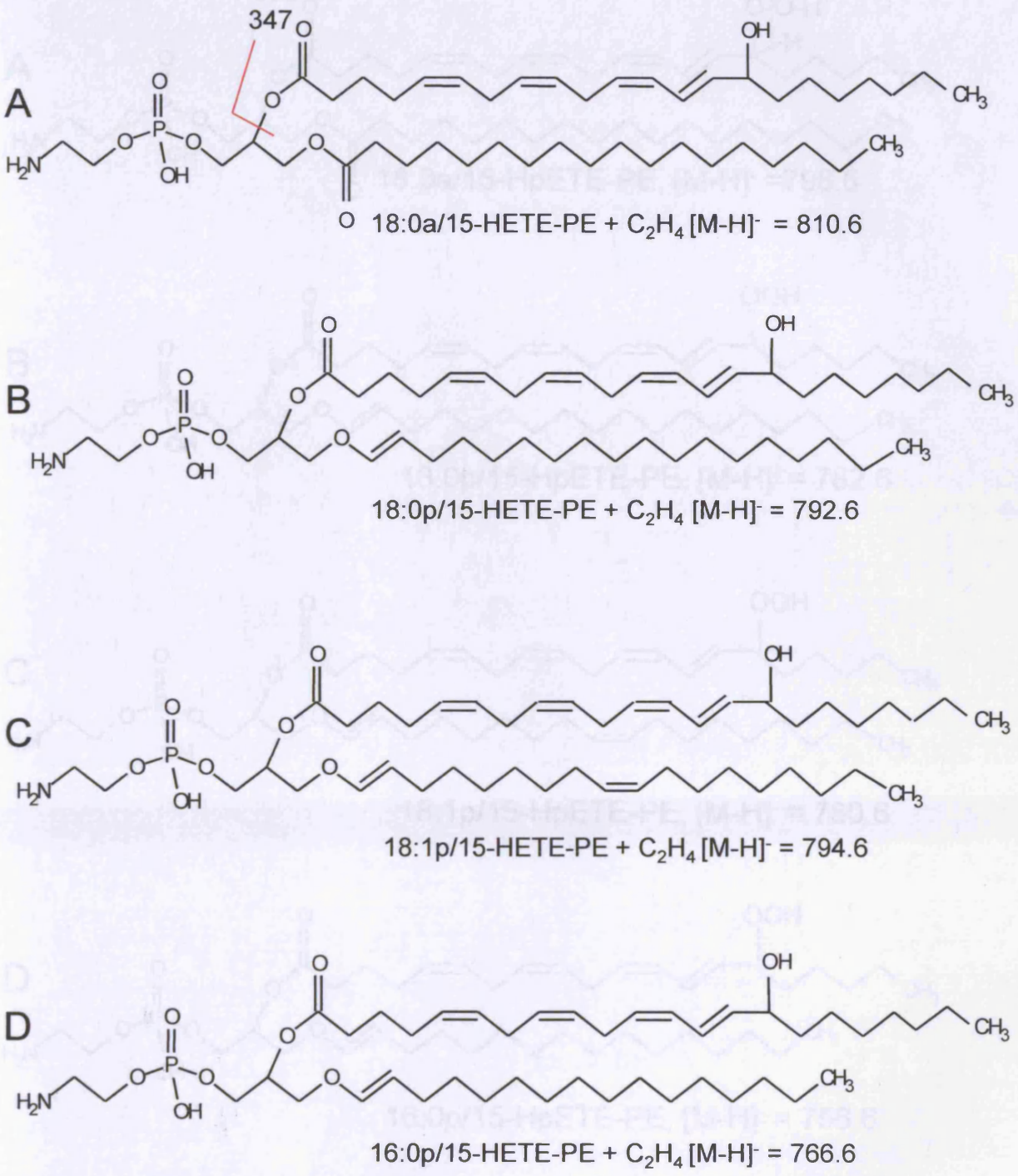


16:0p/AA-PE, $[M-H]^-$ = 722.6

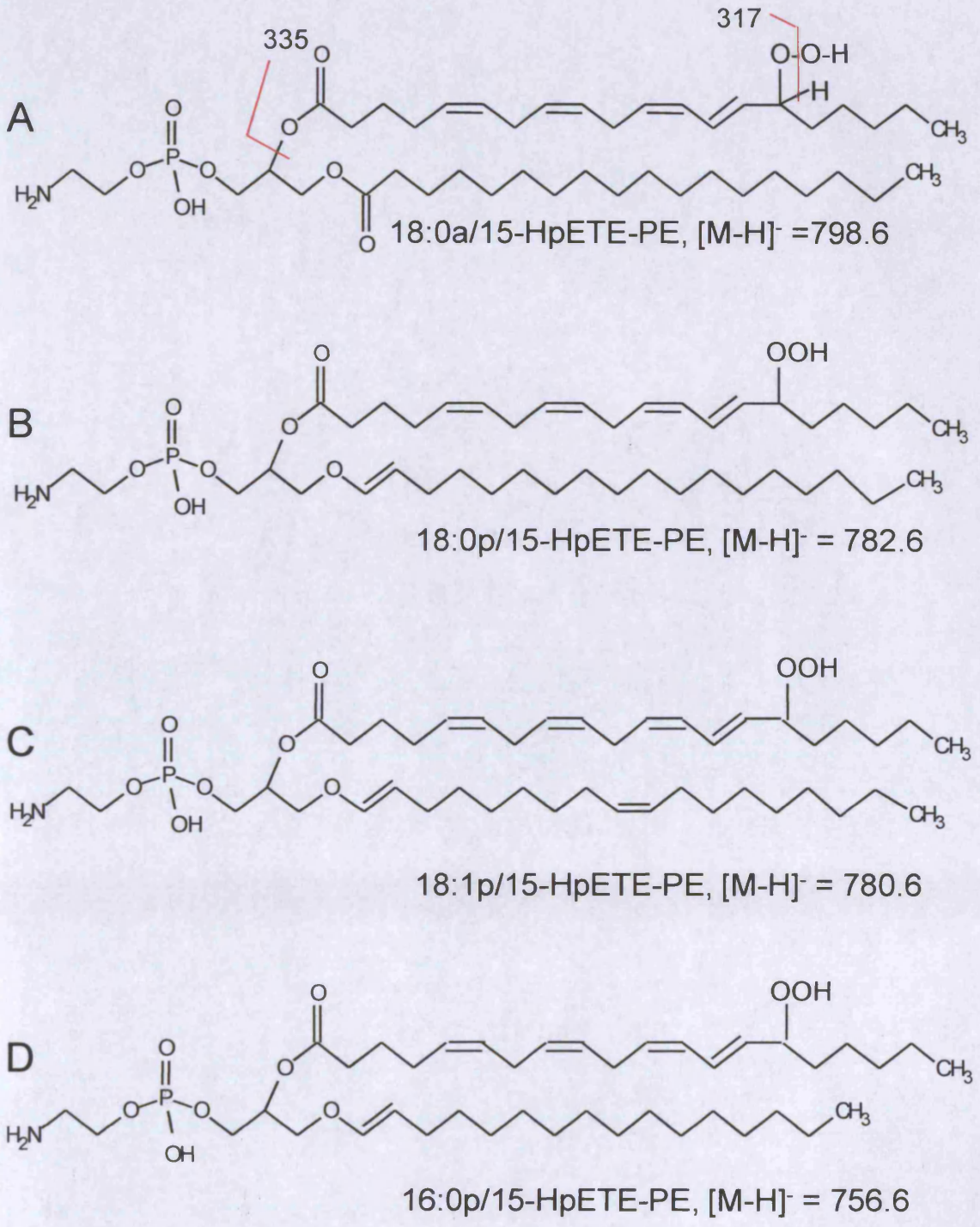
Scheme 2.5 Structures and *m/z* of AA-PEs



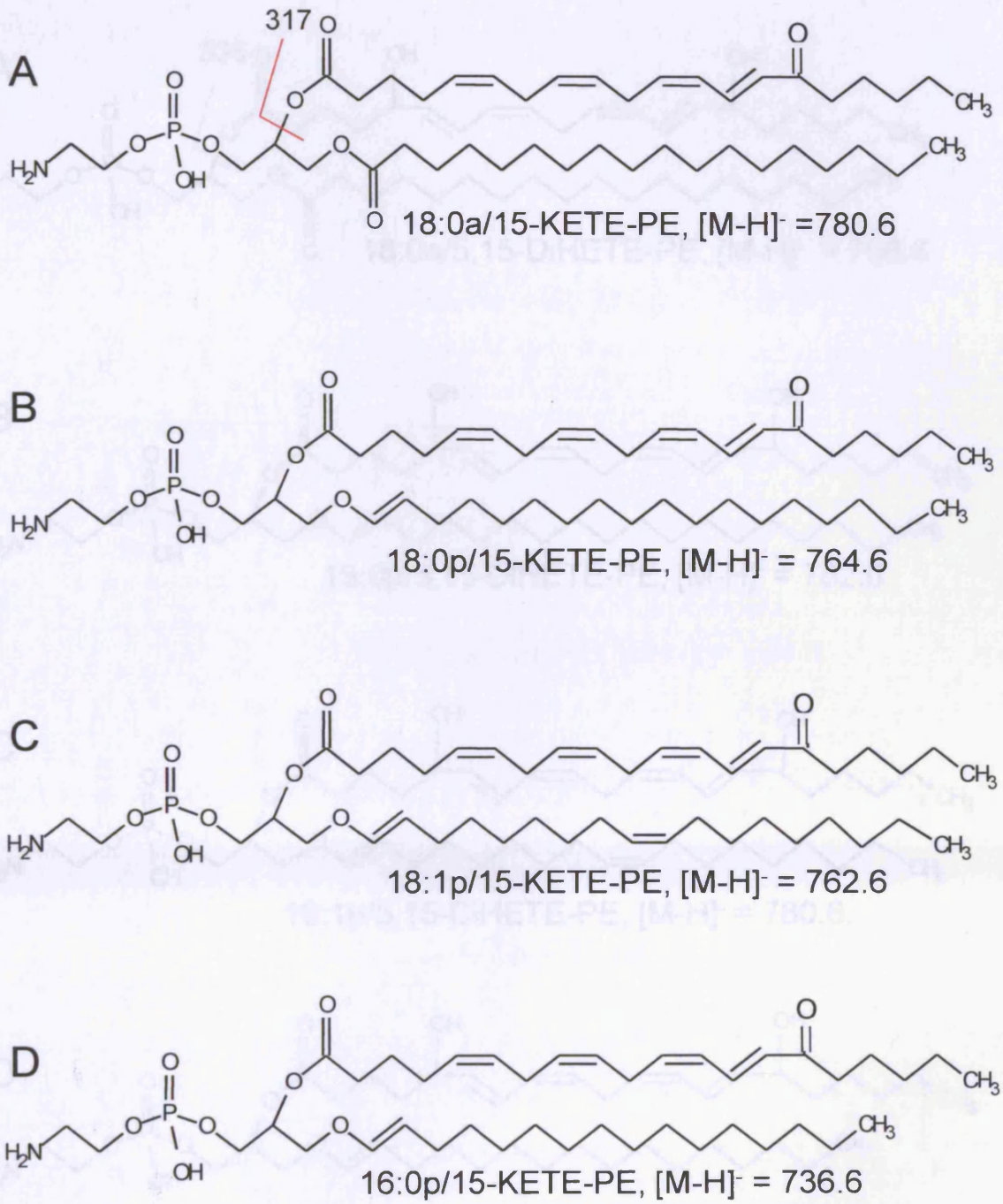
Scheme 2.6 Structures and *m/z* of 15-HETE-PCs



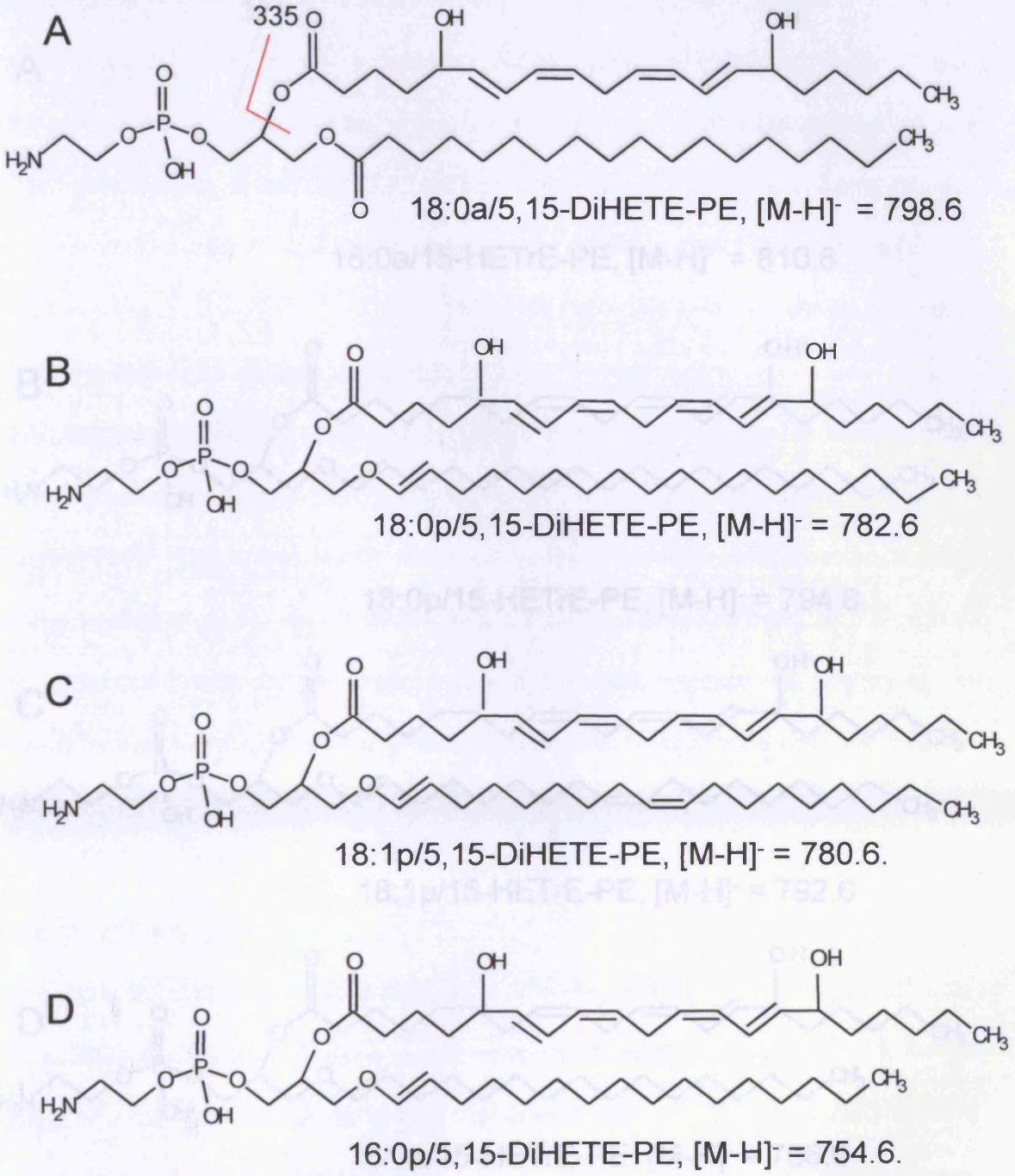
Scheme 2.7 Structures and *m/z* of 15-HETE-PE following C_2H_4 elongation



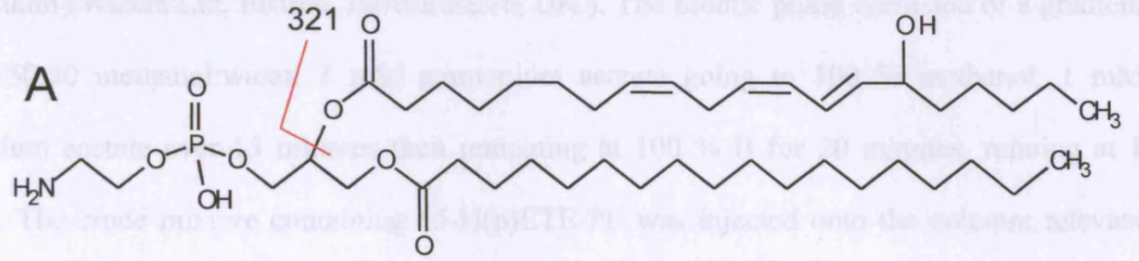
Scheme 2.8 Structures and *m/z* of 15-HpETE-PEs



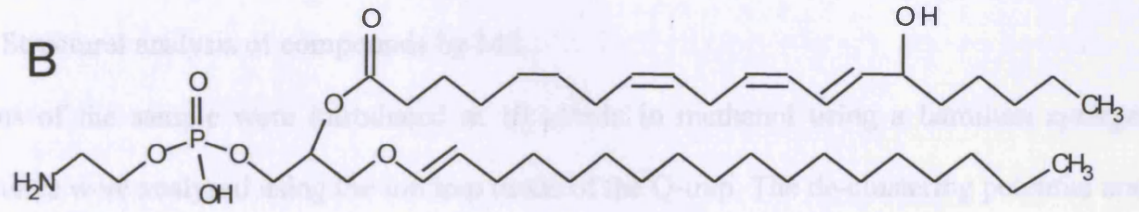
Scheme 2.9 Structures and *m/z* of 15-KETE-PEs



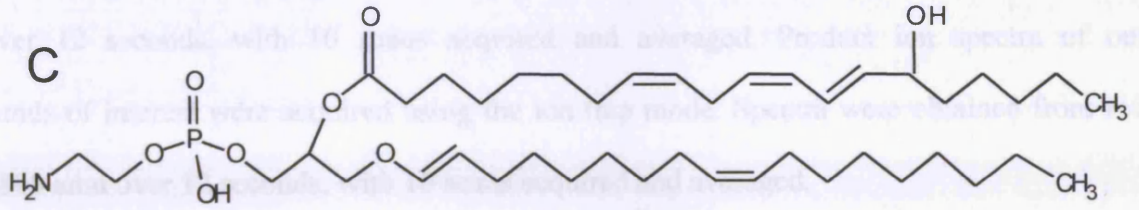
Scheme 2.10 Example structures and *m/z* of di-HETE-PEs, those shown are of 5, 15- of di-HETE-PEs



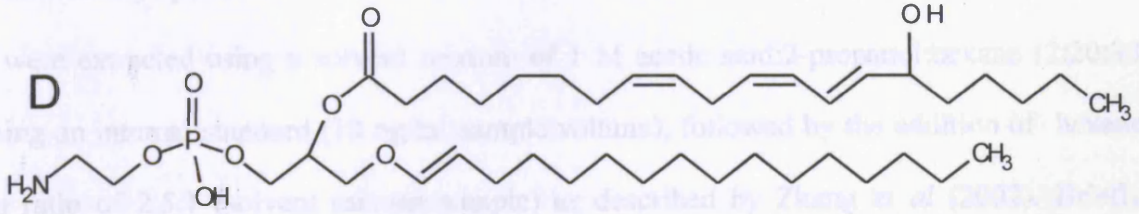
18:0a/15-HETrE-PE, $[M-H]^-$ = 810.6



18:0p/15-HETrE-PE, $[M-H]^-$ = 794.6



18:1p/15-HETrE-PE, $[M-H]^-$ = 792.6



16:0a/15-HETrE-PE, $[M-H]^-$ = 766.6

Scheme 2.11 Structures and *m/z* of 15-HETrE-PEs

mm column (Waters Ltd, Elstree, Hertfordshire, UK.). The mobile phase consisted of a gradient of (A) 50:50 methanol:water, 1 mM ammonium acetate going to 100 % methanol, 1 mM ammonium acetate over 15 minutes then remaining at 100 % B for 20 minutes, running at 1 ml/min. The crude mixture containing 15-H(p)ETE-PE was injected onto the column; relevant peaks were collected, combined, dried down under N_2 gas, then re-suspended in methanol.

2.2.2.1 Structural analysis of compounds by MS.

Dilutions of the sample were introduced at 10 µl/min in methanol using a hamilton syringe. Compounds were analysed using the ion trap mode of the Q-trap. The de-clustering potential and collision energy were -140 V and -45 V, respectively. Q1 scans were carried out in negative mode out to determine overall purity of compounds, spectra were obtained from *m/z* 500 to 1000 amu over 12 seconds, with 10 scans acquired and averaged. Product ion spectra of our compounds of interest were acquired using the ion trap mode. Spectra were obtained from *m/z* 100 to 800 amu over 12 seconds, with 10 scans acquired and averaged.

2.2.3 Extraction of lipids.

Lipids were extracted using a solvent mixture of 1 M acetic acid:2-propanol:hexane (2:20:30) containing an internal standard (10 ng/ml sample volume), followed by the addition of hexane, all at a ratio of 2.5:1 (solvent mixture:sample) as described by Zhang *et al* (2002). Briefly, following the addition of samples to extraction mixture, they were mixed vigorously by vortex then centrifuged for 5 minutes and the upper organic layer was removed into clean glass vials. A second extraction step was carried out by the addition of hexane at a ratio of 2.5:1 (solvent

mixture:sample). The organic layers were pooled and dried under N_2 flow or under vacuum, re-suspended in methanol and stored at -70 °C.

2.2.4 Chromatographic analysis of 18:0a/15-HETE-PE.

2.2.4.1 Normal phase HPLC-UV of phospholipid classes.

Phospholipid classes were isolated from samples by normal phase HPLC (NP-HPLC). Lipid extracts re-suspended in normal phase solvents (50:50 of solvents A:B (A, hexane:2-propanol, 3:2; B, solvent A:H_2O, 94.5:5.5)) were separated on a Spherisorb S5W 4.6 x 150-mm column (Waters Ltd, Elstree, Hertfordshire, UK) using a gradient of 50-100 % B over 25 minutes (A, hexane:2-propanol, 3:2; B, solvent A:H_2O, 94.5:5.5) at a flow rate of 1.5 ml/min (Dugan LL, 1986). Absorbance was monitored at 205 nm, and products were identified by retention time by comparison with to a mixture of standard phospholipids. The PE phospholipids eluted at around 5 minutes and were collected. Samples were dried down under N_2, then re-suspended in methanol.

2.2.4.2 HETE isomer determination.

The PE fraction collected from NP-HPLC was subjected to base-hydrolysis. The samples were dried under N_2 and re-suspended in 1.5 ml propan-2-ol. An equal volume of 0.2 M NaOH was added to the samples followed by incubation for 30 minutes at 60 °C under N_2. The samples were acidified to pH 3.0 and then the fatty acids extracted twice using 3 ml hexane. The upper organic layers were combined, dried under N_2 and finally resuspended in methanol. All the HETE isomers were analysed by RP- LC/MS/MS as described above in 2.2.2.1.

2.2.4.3 Chiral analysis of HETE isomers for enantiomer determination.

15-HETE and 12-HETE was purified from cell extracts using the method described in 2.2.2.1, dried under N_2 then re-suspended in the chiral phase mobile phase (hexane:2-propanol:acetic acid, 100:5:0.1), and injected onto a Chiralcel OD 0.46 x 25-cm column (Chiral Technologies Ltd, Exton, PA) with isocratic separation at 1 ml/min with absorbance monitored at 235 nm. HETE chirality was identified by comparison with 12- and 15- R and S HETE standards (Cayman Chemical, Michigan, USA).

2.2.5 NMR.

NMR analysis of 15-HETE-PE was carried out in collaboration with Dr Michaela Serpil and Professor Chris McGuigan, School of Pharmacy, Cardiff University. ^{1}H-NMR, ^{31}P-NMR and ^{13}C Pendent -NMR were recorded at 500, 200 and 125 MHz respectively on a Bruker Avance 500 spectrometer using CD_3OD as the solvent. Chemical shifts are reported in parts per million (ppm, δ units) and *J* values are given in Hertz (Hz).

2.2.6 Isolation and activation of human monocytes.

2.2.6.1 Isolation of monocytes from human buffy coats.

All blood donations were with approval from the Cardiff University Research Ethics Committee. Isolation of human monocytes was carried out under sterile conditions. 50 ml buffy-coat blood obtained from the Welsh Blood Service, was diluted 1:1 with Dextran-Citrate-PBS. The red cells were left to sediment for 1-2 hours then the upper plasma layer removed and under-layered with Lymphoprep at a ratio of 2:1 (supernatant:Lymphoprep) and centrifuged at 800 g for 20 minutes at 4 °C. The interface (containing monocytes) was collected and diluted 1:1 with chilled

PBS/citrate (0.4 % w/v citrate, pH 7.4), then centrifuged at 400 g for 10 minutes at 4 °C. The pellet was washed with citrate-PBS, centrifuged at 400 g for 5 minutes at 4 °C, discarding the supernatant to remove contaminating platelets. This was repeated five times. Following the final spin, the pellet was re-suspended in 2 ml monocyte medium (RPMI 1640 (Invitrogen, Paisley, UK), containing 10 % FCS, 100 U/ml penicillin, 100 μg/ml streptavidin, 2 mM glutamine). Cells were counted by trypan blue exclusion on a haemocytometer. The cell stock was diluted to 10^8 cells/ml with monocyte medium, 10^8 cells were seeded per T75 flasks (Nunc Brand, Fisher Scientific UK, Loughborough, Leicestershire, UK). The monocytes were allowed to adhere by incubating the flasks at 37 °C for 2 hours, 5 % CO_2. The cells were washed with RPMI 1640 then fresh monocyte media and 700 pM human recombinant interleukin 4 (hrIL-4) (Promega, Southampton, Hampshire, UK) was added prior to a second 72 hour incubation. Monocytes were harvested by removing and combining the monocyte containing medium from flasks and centrifuging at 500 g for 5 minutes, 4 °C, then re-suspended in Krebs buffer. Cells were counted by trypan blue exclusion then diluted to 4 x 10^6 cells/ml for use in experiments.

2.2.6.2 Activation of human monocytes to generate LOX-derived esterified and free eicosanoids. Monocytes were activated with 10 μM A23187 at 37 °C and incubated for various time periods depending on the experiment in question. All samples underwent lipid extraction, some following reduction by adding 1 mM $SnCl_2$ for 10 minutes at room temperature (RT). In some experiments, cells and supernatant were separated by centrifugation at 290 g for 5 minutes (4 °C). For some experiments, cells were spiked with 170 ng 15-HETE-d_8 or 15-HETE-PE or 15-HpETE-PE prior to activation. The effect of PLA_2 inhibitors (50 nM bromoenol lactone (BEL), 10 μM palmityl trifluromethyl ketone ($PACOCF_3$), 2 μM oleyloxyethyl phosphorylcholine

(OOEPC)) and PAF-AH inhibitor (150 μM methylarachidonyl-fluorophosphonate (MAFP)) on 15-HETE-PE synthesis was investigated by the addition of PLA_2 inhibitors 10 minutes prior to activation. For some experiments, reduction was carried out using 10 mM $NaCNBH_3$ for 1 hour at 37 °C (Hazen *et al*, 1997) followed by lipid extraction. For all samples, 10 ng per 4 x 10^6 cells of DMPE and 15-HETE-d_8 and/or 15-HEDE were added as internal standards, following reduction.

2.2.7 Isolation and activation of murine macrophages.

C7567/BL6 wild type and 12/15-$LOX^{-/-}$ mice were culled by Schedule 1 methods using CO_2 asphyxiation. Peritoneal lavages were carried out using 2 ml PBS. Lavages from mice were pooled (unless otherwise specified), pelleted by centrifugation at 500 g for 5 minutes at 4 °C, re-suspended in monocyte medium. Cells were counted by trypan blue exclusion and either used directly or and seeded in flasks at 100 x 10^6 cells/ml to isolate the macrophages by adhesion. For this, cells were incubated for 2 hours at 37 °C, 5 % CO_2. The monocyte medium was removed and the adhered macrophages washed once with RPMI media, fresh monocyte medium was added to the flasks and the macrophages released by gentle scraping. Macrophages were pelleted as described above, washed and pelleted in PBS, re-suspended in Krebs buffer, counted, and diluted to 4 x 10^6 cells/ml for experiments. Cells were activated with 10 μM A23187 at 37 °C and incubated for time periods depending on the experiment in question. All samples underwent lipid extraction, some following reduction by adding 1 mM $SnCl_2$ for 10 minutes at RT. In some experiments, cells were re-suspended in Krebs buffer made using $H_2{}^{18}O$, rather than regular Krebs prior to activation. In several experiments, control and activated cells were incubated with 1 mg/ml Sulfo-NHS-Biotin (Pierce Ltd, Rockford, USA) for 10 minutes, at RT; unbound Sulfo-

NHS-Biotin was removed by centrifuging samples at 550 g for 5 minutes, followed by re-suspending the cell pellets in Krebs buffer, reduction and lipid extraction. In other experiments, cells were pelleted using a micro-centrifuge by spinning at 290 g for 5 minutes (4 °C), the supernatant was then subjected to ultracentrifugation at 100,000 g for 1 hour (4 °C) in a Beckman L8-60M (SW55TI rotor). All pellets were re-suspended in PBS, reduced, and lipids extracted. For all samples, 10 ng per 4 x 10^6 cells of DMPE and 12-HETE-d_8 were added as internal standards, following reduction.

2.2.8 Phospholipid profiling of WT and 12/15-LOX$^{-/-}$ murine macrophages.

Macrophages from WT and 12/15-LOX$^{-/-}$ mice were isolated as described above. Harvested macrophages were re-suspended in 0.5 ml Krebs buffer and the lipids were extracted. PE, PC, PI, PA and PG phospholipids were profiled by LC/ESI/MS/MS on a 4000 Q-Trap as described in 2.2.2.2, using the specific parent to daughter transitions shown in Tables 2.2. to 2.7. Relative levels of lipids were determined by comparison to internal standards with the following parent to daughter transitions *m/z* 634 → 227 (DMPE) $[M-H]^-$, 678 → 184 (DMPC) $[M+H]^+$, 591 → 227 (DMPA) $[M-Na]^-$ and 665 → 227 (DMPG) $[M-Na]^-$ (Scheme 2.4). PS-phospholipid profiling was carried out by flow injection using the phospholipid solvent system (as described in 2.2.2.2) running at 50:50 A:B, 1 ml/min for 6 minutes. Products were profiled using an internal standard, with parent to daughter transition of *m/z* 678 → 227 (DMPS) $[M-Na]^-$.

Analysed Transition [M-H]$^-$	PE Phospholipid Structure	Fatty acid analysed by MS
662/255	30:0 (16:0/14:0-PE)	16:0
688/255	32:1a (16:0/16:1-PE)	16:0
690/255	32:0a (16:0/16:0-PE)	16:0
698/279	34:2p (18:2/14:0-PE)	18:2
700/281	34:1p (18:1/16:0-PE)	18:1
702/281	34:1e (18:1/16:0-PE)	18:1
702/283	34:0p (18:0/16:0-PE)	18:0
714/281	34:2a (18:1/16:1-PE)	18:1
714/255	34:2a (16:0/18:2-PE)	16:0
716/281	34:1a (18:1/16:0-PE)	18:1
718/255	34:0a (16:0/18:0-PE)	16:0
722/303	36:4p (16:0/20:4-PE)	20:4
726/281	36:2p (18:1/16:0-PE)	18:1
728/281	36:1p (18:1/18:0-PE) or 36:2e(18:1/18:1-PE)	18:1
730/283	36:0p (18:0/18:0-PE)	18:0
730/281	36:1e (18:1/18:0-PE)	18:1
738/303	36:4a (16:0/20:4-PE)	20:4
740/255	36:3a (16:0/20:3-PE)	16:0
742/281	36:2a (18:1/16:0-PE)	18:1
744/281	36:1a (18:1/18:0-PE)	18:1
746/327	38:6e (16:0/22:6-PE)	22:6
746/283	36:0a (18:0/18:0-PE)	18:0
748/303	37:6 (17:2/20:4-PE)	20:4
748/327	38:6e (16:0/22:6-PE)	22:6
748/329	38:5p (16:0/22:5-PE)	22:5
750/303	38:4p (18:0/20:4-PE)	20:4
750/331	38:5e (16:1/22:4-PE)	22:4
752/307	38:3p (18:1/20:2-PE)	20:2
752/303	38:4e (20:4/18:0-PE)	20:4

Table 2.2 PE Phospholipid profiling transitions

Analysed Transition [M-H]$^-$	PE Phospholipid Structure	Fatty acid analysed by MS
754/309	38:2p (18:1/20:1-PE)	20:1
754/307	38:3e (18:1/20:2-PE)	20:2
756/309	38:1p, (18:0/20:1-PE or 38:2e (18:1/20:1-PE)	20:1
758/311	38:0p (18:0/20:0-PE)	20:0
762/327	38:6a (16:0/22:6-PE)	22:6
762/283	38:6a (18:0/20:6-PE)	18:0
764/281	38:5a (18:1/20:4-PE)	18:1
764/255	38:5a (16:0/22:5-PE)	16:0
766/303	38:4a (18:0/20:4-PE)	20:4
768/305	38:3a (18:0/20:3-PE)	20:3
768/281	38:3a (18:1/20:2-PE)	18:1
770/281	38:2a (18:1/20:1-PE)	18:1
772/327	39:8 (17:2/22:6-PE)	22:6
772/283	38:1a (18:0/20:1-PE)	18:0
774/283	38:0a (18:0/20:0-PE)	18:0
774/327	40:6p (18:0/22:6-PE)	22:6
776/329	40:5p (18:0/22:5-PE)	22:5
776/327	40:6e (18:0/22:6-PE)	22:6
778/303	40:4p (20:0/20:4-PE)	20:4
778/331	40:4p (18:0/22:4-PE) or 40:5e (18:1/22:4-PE)	22:4
788/327	40:7 (18:1/22:6-PE)	22:6
790/327	40:6a (18:0/22:6-PE)	22:6
792/303	40:5a (20:1/20:4-PE)	20:4
794/331	40:4a (18:0/22:4-PE)	22:4
810/327	42:8 (20:2/22:6-PE)	22:6
812/327	42:9 (20:3/22:6-PE)	22:6
814/327	42:8 (20:2/22:6-PE)	22:6
818/327	42:6a (20:0/22:6-PE)	22:6

Table 2.2 continued (PE Phospholipid profiling transitions)

Analysed Transition $[M+H]^+$	PC Phospholipid Structure	Fatty acid also analysed by MS in negative mode
678/184	28:0	-
692/184	32:0a	-
701/184	-	-
703/184	-	-
704/184	30:1a (14:0/16:1-PC)	14:0
706/184	30:0a (14:0/16:0-PC)	14:0 & 16:0
717/184	32:0p	-
718/184	32:0p (14:0/18:0-PC) or 32:1e (14:0/18:1-PC)	14:0
720/184	32:0e (16:0/16:0-PC)	16:0
727/184	-	-
729/184	-	-
730/184	32:2a (16:1/16:1-PC)	16:1
731/184	-	-
732/184	32a:1 (16:0/16:1-PC)	16:0 & 16:1
734/184	32a:0 (16:0/16:0-PC)	16:0
735/184	-	-
736/184	-	-
744/184	34:2e (16:0/18:2-PC)	18:2
746/184	34:1e (16:0/18:1-PC)	18:1
748/184	34:0e (16:0/18:0-PC)	18:0
758/184	34:2e (16:0/18:2-PC)	16:0 & 18:2
760/184	34:1a (16:0/18:1-PC)	16:0 & 18:1
762/184	34:0a (16:0/18:0-PC)	16:0 & 18:0
763/184	-	-
764/184	-	-
772/184	36:1p or 36:2e	-
774/184	36:1e (18:0/18:1-PC)	18:1
782/184	36:4a (16:0/20:4-PC)	16:0 & 20:4
784/184	36:3a (16:0/20:3-PC)	16:0 & 20:3
785/184	-	-
786/184	36:2a (18:1/18:1-PC)	18:1
788/184	36:1a (18:0/18:1-PC)	18:0 & 18:1
790/184	36:0a (18:0/18:0-PC) or 38:6p (18:0/20:6-PC)	18:0
792/184	38:5p or 38:6e	-

Table 2.3 PC Phospholipid profiling transitions

Analysed Transition $[M+H]^+$	PC Phospholipid Structure	Fatty acid analysed MS in negative mode
806/184	38:6a (16:0/22:6-PC)	16:0 & 22:6
807/184	-	-
808/184	38:5a (16:0/22:5-PC)	16:0 & 22:5
810/184	38:4a (18:0/20:4-PC)	18:0 & 20:4
811/184	-	-
812/184	38:3a (18:1/20:2-PC)	18:1 & 20:2
814/184	38:2a (18:1/20:1-PC)	18:1 & 20:1
816/184	38:1a (18:1/20:0-PC)	18:1 & 20:0
818/184	40:6p (18:0/22:6-PC)	18:0 & 22:6
820/184	40:5p (18:0/22:5-PC)	22:5
832/184	40:0e (20:0/20:0-PC)	20:0
834/184	40:6a (18:0/22:6-PC)	18:0 & 22:6
835/184	-	-
836/184	40:5a (20:1/20:4-PC)	20:1 & 20:4
839/184	-	-
862/184	42:6a (20:0/22:6-PC)	20:0 & 22:6

Table 2.3 Continued (PC Phospholipid profiling transitions)

Analysed Transition $[M-H]^-$	PI Phospholipid structure	Fatty acid analysed by MS
857/303	36:4 (16:0/20:4-PI)	20:4
859/305	36:3 (16:0/20:3-PI)	20:3
863/283	36:1 (18:0/18:1-PI)	18:0
881/327	38:6 (16:0/22:6-PI)	22:6
883/329	38:5 (16:0/22:5-PI)	22:5
885/283	38:4 (18:0 /20:4-PI)	18:0
887/281	38:3 (18:1/20:2-PI)	18:1
909/283	40:6 (18:0/22:6-PI)	18:0

Table 2.4 PI Phospholipid profiling transitions

Analysed Transition $[M-H]^-$	PA Phospholipid Structure	Fatty acid analysed by MS
591/227	28:0 (14:0/14:0-PA)	14:0
597/283	29:4 (18:0/11:4-PA)	18:0
619/227	32:0 (14:0/18:0-PA)	14:0
645/253	32:1 (16:0/16:1-PA)	16:1
647/255	32:0 (16:0/16:0-PA)	16:0
667/303	34:4 (14:0/20:4-PA)	20:4
671/279	34:2 (16:0/18:2-PA)	18:2
673/281	34:1 (16:0/18:1-PA)	18:1
675/283	34:0 (16:0/18:0-PA)	18:0
695/303	36:4 (16:0/20:4-PA)	20:4
699/281	36:2 (18:1/18:1-PA)	18:1
701/281	36:1 (18:0/18:1-PA)	18:1
703/283	36:0 (18:0/18:0-PA)	18:0
719/327	38:6 (16:0/22:6-PA)	22:6
723/303	38:4 (18:0/20:4-PA)	20:4
727/309	38:2 (18:1/20:1-PA)	20:1
729/303	39:8 (19:4/20:4-PA)	20:4
747/327	40:6 (18:0/22:6-PA)	22:6
749/303	40:5 (20:1/20:4-PA)	20:4
751/303	40:4 (20:0/20:4-PA)	20:4

Table 2.5 PA Phospholipid profiling transitions

Analysed Transition $[M-H]^-$	PS Phospholipid Structure	Fatty acid analysed by MS
760/281	34:1 (16:0/18:1-PS)	18:1
782/303	36:4 (16:0/20:4-PS)	20:4
784/305	36:3 (16:0/20:3-PS)	20:3
786/281	36:2 (18:1/18:1-PS)	18:1
788/281	36:1 (18:0/18:1-PS)	18:1
788/283	36:1 (18:0/18:1-PS)	18:0
790/283	36:0 (18:0/18:0-PS)	18:0
806/327	38:6 (16:0/22:6-PS)	22:6
808/329	38:5 (16:0/22:5-PS)	22:5
810/283	38:4 (18:0/20:4-PS)	18:0
810/303	38:4 (18:0/20:4-PS)	20:4
812/307	38:3 (18:1/20:2-PS)	20:2
814/281	38:2 (18:1/20:1-PS)	18:1
816/309	38:1 (18:0/20:1-PS)	20:1
818/311	38:0 (18:0/20:0-PS)	20:0
834/327	40:6 (18:0/22:6-PS)	22:6
836/303	40:5 (20:1/20:4-PS)	20:4
838/303	40:4 (20:0/20:4-PS)	20:4
840/305	40:3 (20:0/20:3-PS)	20:3
842/307	40:2 (20:0/20:2-PS)	20:2
844/309	40:1 (20:0/20:1-PS)	20:1
846/311	40:0 (20:0/20:0-PS)	20:0
862/327	42:6 (20:0/22:6-PS)	22:6
864/329	42:5 (20:0/22:5-PS)	22:5
866/331	42:4 (20:0/22:4-PS)	22:4

Table 2.6 PS Phospholipid profiling transitions

Analysed Transition $(M-H)^-$	PG Phospholipid Structure	Fatty acid followed by MS
667/303	29:6 (9:2/20:4-PG)	20:4
671/279	27:4 (9:2/18:2-PG)	18:2
675/283	29:2 (18:0/11:2-PG)	18:0
703/283	31:2 (18:0/13:2-PG)	18:0
719/283	32:1 (18:0/14:1-PG)	18:0
721/255	32:0 (16:0/16:0-PG)	16:0
749/255	34:0 (16:0/18:0-PG)	16:0
773/281	36:2 (18:1/18:1-PG)	18:1
793/327	38:6 (16:0/22:6-PG)	22:6
797/303	38:4 (18:0/20:4-PG)	20:4

Table 2.7 PG Phospholipid profiling transitions

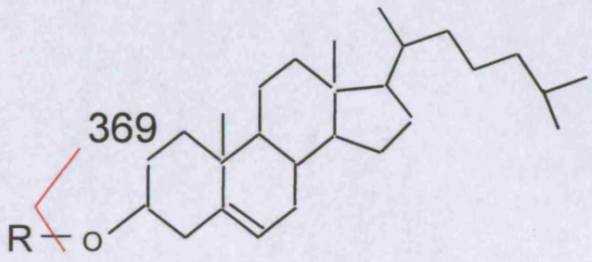
2.2.9 Cholesterol ester profiling in WT and 12/15-LOX$^{-/-}$ murine macrophages.

Precursor mass spectra were obtained operating in positive mode. Samples were introduced at 10 µl/min in methanol using a hamilton syringe. The de-clustering potential and collision energy were -140 V and -45 V respectively. Spectra were obtained from *m/z* 100 - 1000 atomic mass units over 12 seconds with 10 MCA scans acquired. Cholesterol esters were then detected by LC/MS/MS, having adapted a method described by Ferreira *et al* (2009). Cholesterol esters were separated on a C18 ODS2, 5 µm, 150 x 4.6 mm column (Waters Ltd, Elstree, Hertfordshire, UK) using an isocratic method with mobile phase propan-2-ol:acetonitrile:ammonium acetate (60:40:4) at 1 ml/min. Products were profiled by LC/ESI/MS/MS using the specific parent to daughter transitions of *m/z* 668, 666, 682, 690, 706, 642, 640, 670 708, 714 and 730 → 369.1 (cholesterol) ($[M+NH_4]^+$) (Scheme 2.12). The collision energy for cholesterol esters was -33 V and the declustering potential, -91 V.

2.2.10 Investigation of cytokine production in response to 15-HETE-PE.

2.2.10.1 Generation of DPPC and 15-HETE-PE liposomes.

10 mM DPPC, and 1µM 15-HETE-PE was prepared in chloroform. The sample was dried down under N_2 flow then incubated under vacuum at room temperature for 1 hour. Lipids were re-hydrated to their original concentration by the addition of RPMI media. To create liposomes the sample was freeze-thawed 10 times using liquid N_2 and warm water. Liposomes with diameters of above 100 nm were excluded from the lipid emulsion using a Lipofast^{TM} (Avestin, Toronto, Canada) extruder.



R=

16:0-	$[M+NH_4]^+ = 642.8$
16:1-	$[M+NH_4]^+ = 640.8$
18:0-	$[M+NH_4]^+ = 670.8$
18:1-	$[M+NH_4]^+ = 668.8$
18:2-	$[M+NH_4]^+ = 666.8$
18:2OH-	$[M+NH_4]^+ = 682.8$
20:4-	$[M+NH_4]^+ = 690.8$
20:4OH-	$[M+NH_4]^+ = 706.8$
22:6-	$[M+NH_4]^+ = 714.8$
22:6OH-	$[M+NH_4]^+ = 730.8$

Scheme 2.12 Structures and *m/z* of cholesterol esters

2.2.10.2 Cellular uptake of 15-HETE-PE.

Human monocytes were isolated from buffy coats as described above, 5 x 10^7 leukocytes in monocyte medium were seeded in 24-well plates and incubated for 2 hour at 37 °C, 5 % humidity. The adhered monocytes were washed once with RPMI medium, then fresh RPMI medium (0.5 ml) was added to each well. Cells were incubated with 1 μM 15-HETE-PE, introduced either in methanol, in liposomes or in BSA solution (2 mg/ml BSA dissolved in RPMI), for various time points. At each time point, the supernatant from each well was removed into extraction buffer. To recover the adhered cells fresh RPMI medium was added to the wells, which were gently scraped then the medium was removed into extraction buffer. Following extraction, samples were re-suspended in methanol.

2.2.10.3 Cytokine production by human monocytes in response to LPS and 15-HETE-PE.

Following human leukocyte isolation 5 x 10^7 cells were adhered to 24-well plates. Monocytes were cultured with 10 ng/ml LPS, with and without 20 or 40 μg (50 or 100 μM) SAPE or 15-HETE-PE for 24 hours. Supernatant was removed and centrifuged at 290 g. Cytokine levels were measured using enzyme linked immunosorbent assay (ELISA) kits from R&D (Abingdon, UK) and BD Biosciences (Oxford, UK).

Briefly, for R&D kits, DYNEX Immulon 4HBX 96 well plates (Fisher Scientific, Loughborough, Leicestershire, UK) were coated overnight with a capture antibody in PBS buffer overnight at either RT or 4°C. Plates were washed then blocked for one hour at RT with either 1 or 5% BSA in PBS (or for TNFα, TNFα blocking buffer was used). Following washing, a

standard curve of the appropriate cytokine and each sample, suitably diluted in reagent diluent (TNFα reagent diluent was used for TNFα) were added to plates and incubated at RT for 2 hours. Plates were washed, followed by the addition of the detection antibody, in reagent diluent for 2 hours at RT. Following washing, wells were incubated with avidin-horseradish peroxidase conjugate in reagent diluent for 20 minutes a RT, plates were washed again and SureBlue TMB microwell peroxidase substrate (KPL Inc., Gaithersburg, Maryland, USA) was added and allowed to develop. Substrate development was stopped by acid addition, then plates were analysed using a multiskan spectrum plate reader (Thermolabsystems, Thermo Fisher Scientific Inc., Waltham, MA. USA).

Briefly, for BD Bioscience kits, Immulon plates were coated overnight with a capture antibody in carbonate buffer overnight at 4 °C. Plates were washed then blocked for one hour at RT with 5% BSA in PBS. Following washing, a standard curve of the appropriate cytokine and each sample, suitably diluted in reagent diluent were added to plates and incubated at RT for 2 hours. Plates were washed, followed by the addition of the detection antibody and avidin-horseradish peroxidase conjugate, in reagent diluent for 1 hour at RT. Plates were washed again and SureBlue TMB substrate was added and allowed to develop. Substrate development was stopped by acid addition, then plates were analysed using a multiskan spectrum plate reader (Thermolabsystems, Thermo Fisher Scientific Inc., Waltham, MA. USA).

2.2.11 Investigating association of HETE-PEs with proteins.

2.2.11.1 Generation of radiolabelled HETE-PEs.

Following the isolation of human monocytes as described above, the adhered monocytes were incubated with hrIL-4 at 37 °C for 48 hours. Following incubation, cells were scraped, counted by trypan blue exclusion then separated into two flasks. 700 pM hrIL-4 was added to one flask and to the other, 700 pM hrIL-4 and either 100 μM ^{14}C-ethanolamine hydrochloride (13.8 mCi/mmol) (Moravek Biochemicals Inc., Brea, CA, USA), 1 μM ^{14}C-SAPE (56 mCi/mmol) (ARC UK LTD, Cardiff, UK), or 0.5 μM ^{14}C-arachidonic acid (57 mCi/mmol) (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Following 24 hours cells were scraped, then washed twice with PBS. The cells were counted by trypan blue exclusion and each was diluted to 4 x 10^6 cells/ml in Krebs buffer. Using A23187, the cells were activated for 15 minutes or 3 hours. Following each time-point, lipids from samples were either immediately extracted or were reduced prior to extraction, using 1 mM $SnCl_2$ for 10 minutes (RT) or 10 mM $NaCNBH_3$ for 1 hour at (37 °C). Lipids were then extracted using hexane, both the organic and aqueous phases were kept for analysis, the organic layers were dried under N_2 flow then re-suspended in methanol. To determine their ^{14}C radioactive content, samples were analysed for 10 minutes on a Tri-Carb 2100 TL liquid scintillation analyser (Packard, Global Medical Instrumentation Inc, Ramsey, Minnesota, USA).

The organic fraction underwent LC/MS/MS analysis as described above in section 2.2.2.2, 30 second fractions were collected over times corresponding to the elution of the HETE-PEs and their parent compounds, which were then subject to scintillation counting. To investigate which of the phospholipid classes contained the ^{14}C radiolabel, the organic fraction was also analysed by NP- HPLC, 1 minute fractions were collected and subject to scintillation counting. Alternatively, proteins from the aqueous samples were precipitated by incubating with 10 %

TCA for 30 minutes, on ice, followed by a centrifugation at 17,500 g for 15 minutes at 4 °C. Supernatant was removed and pellet was re-suspended in Krebs buffer. Both pellet and supernatant was analysed by scintillation counting.

2.2.12 Transmission electron microscopy of WT and 12/15-LOX$^{-/-}$ cells.

2.2.12.1 Sample Preparation.

Wild type and 12/15-LOX$^{-/-}$ mice were sacrificed and peritoneal lavages were carried out with PBS. Lavage cells, pelleted in eppendorfs, were submerged in cacodylate buffer containing 2.5 % glutaldehyde and stored at 4 °C overnight. Samples were washed twice for 15 minutes with 0.1 M cacodylate buffer then re-suspended in 1% osmium tetroxide in 0.2 M cacodylate buffer and incubated at room temperature for 1 hour. Samples were washed four times for 15 minutes with H_2O followed by staining with 0.5 % urianyl acetate in dH_2O for 1 hour at RT. All samples were dehydrated by re-suspending in a series of solutions containing increased percentage of ethanol, each for 15 minutes: 50 %, 70 %, 80 %, and 90 % followed by 3 times with 100 % ethanol. Samples were transferred to glass vials and re-suspended in propyl oxide. Resin infiltration was carried out by re-suspending samples in 1:1 pre-mixed embedding resin and propyl oxide overnight, at room temperature, leaving the vials open to allow evaporation of the propyl oxide. Cell samples were immersed further with fresh embedding resin and transferred into plastic moulds. Cell/tissue pellets were allowed to settle to the bottom of the moulds, following 2 hours at RT, samples were transferred to an oven set at 60 °C where they were left for approximately 48 hours.

2.2.12.2 Cutting and sectioning of resin pellets.

90 nm sections were cut from 3 different pellet locations using a Reichert-Jung Ultracut E microtome. Sections were then mounted onto naked grids. Grids were stained using 2 % urianyl acetate for 10 minutes, washed twice with distilled water followed by staining with Reynold's lead citrate for 5 minutes and an additional two washes with dH_2O. Samples were dried on filter paper then analysed by transmission electron microscopy, on a Philips EM208.

2.2.12.3 Development of EM pictures.

Kodak EM 2289 film (Agar Scientific, Stansted, Essex, UK) was developed for 3.5 minutes, at 20 °C in Kodak D-19 developer, diluted 1:2 with H_2O. Films were then fixed by incubating for 30 seconds in an acetic acid stop bath, followed by 4 minutes in Ilford Hypam fixer, diluted 1:3 with H_2O. Films were washed under running water for 20 minutes then dried.

2.2.13 Statistical Analysis.

Data is presented as the mean +/- SEM. of three independent experiments. Statistical analysis of differences was carried out by Students t-test or by one-way ANOVA with a Tukey post hoc test to compare all pairs of data sets. A p-value of less than 0.05 was considered to be significant.

CHAPTER 3

SYNTHESIS, PURIFICATION AND CHARACTERISATION OF 18:0a/15-HETE-PE AND 18:0a/15-HpETE-PE

3.1 Introduction

The availability of oxidised phospholipid standards is key to investigating their biological function and equally important is the ability to quantify these lipids. Many studies have been carried out using mixtures of oxidised phospholipids (Ox-PLs), generated either by air oxidation or by exposure to reactive nitrogen species (Watson *et al*, 1997, Podrez *et al*, 1999, Podrez *et al*, 2002, Von Schlieffen *et al*, 2009, Cherepanova *et al*, 2009, Walton *et al*, 2003, Leitinger *et al*, 1999). The use of crude phospholipid mixtures is a major weakness in the experiments described in these studies, as it is difficult to determine which phospholipid oxidation product is the cause of an exerted effect. Thus, when investigating the effect of a particular phospholipid on cell function, it is important that it is free of contaminants, such as additional bio-active lipids.

In the studies listed above, no individual molecular species of Ox-PL were quantified. However, the total amount of Ox-PLs was evaluated by calculating the amount of total lipid peroxide products, or by estimation, based on how much substrate was used. Alternatively, Ox-PLs were quantified using microphosphorus assays (Gao *et al*, 2006, Chen *et al*, 2008). The importance of knowing the quantity and concentration of specific lipids used in an experiment cannot be overlooked. Using estimated or inaccurate concentrations may lead to experiments being carried out under non-physiological conditions and consequently result in incorrect or misleading data. Thus far, a lack of oxidised phospholipid standards has meant

that their direct quantification by MS has not been possible. Up to now, Ox-PLs were indirectly quantified in our studies by calculating the amount of eicosanoids, following their release from phospholipids by base hydrolysis (Maskrey *et al*, 2007). The structures of Ox-PLs are more complex and unstable than the un-oxidised lipid substrates, as a result, Ox-PLs are more challenging to study.

To enable the quantification of 15-HpETE-PEs and 15-HETE-PEs, purified forms of these lipids are required as standards. In 1987, a study was published describing the use of soybean LOX to oxidise fatty acids esterified to PC (Brash *et al*, 1987). As soybean LOX is commercially available, the method described by Brash *et al* was adapted to generate our own phospholipids of interest, specifically, PEs containing 15-HETE at the sn2 position (Van Os *et al*, 1981).

3.1.1 Method development for the synthesis of 15-HpETE-PE and 15-HETE-PE.

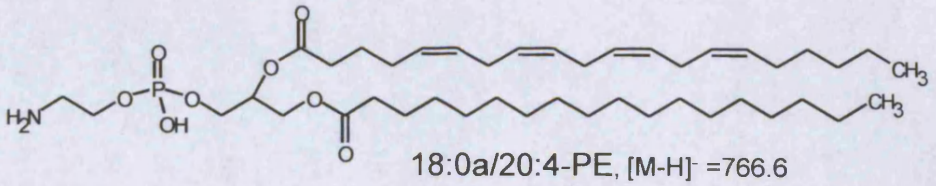
The structures of 15-HETE-PEs previously detected in human monocytes are shown in Scheme 2.1 (Maskrey *et al*, 2007). 15-HpETE-PEs differ by the presence of a peroxyl group at C-15, rather than a hydroxide (Scheme 2.8). The four 15-HETE-PEs differ from each other by the identity of their sn1 fatty acid or by the type of bond by which they are connected to the phospholipid. One is attached via a diacyl bond (Scheme 2.1 A), while the remaining three are plasmalogens (Scheme 2.1 B-D). To generate the diacyl 15-H(p)ETE-PE, 15-LOX can be used to oxidise 1-stearoyl-2-arachidonyl-sn3-phosphatidylethanolamine (SAPE) (Scheme 2.5 A), available from Avanti as a pure lipid. However, the substrates for the three plasmalogen lipids are only available as a mixture of 'brain plasmalogen PEs'. Therefore, while all four lipids can be synthesised, the plasmalogens would require additional

purification. Attempting to isolate particular lipids from a complex mixture would be a far greater challenge than synthesising and isolating one product from a single substrate. Scheme 3.1 describes the proposed method for the synthesis of 18:0a/15-HpETE-PE and 18:0a/15-HETE-PE. Before a synthesised compound is used, its identity must be thoroughly established by structural characterisation. It must also be analysed to ensure that it is free of contaminants, which could potentially interfere with the biological activity of the lipid.

The discovery of four 15-H(p)ETE-PEs in human monocytes made it a requirement to have a pure standard to enable direct quantification of these esterified lipids by MS and also, for use in experiments investigating their biological function (Maskrey *et al,* 2007). As described in Chapter 1, cellular LOX enzymes generate HpETEs that are rapidly reduced to the corresponding hydroxyl compounds (HETEs) by glutathione peroxidases (Ursini & Bindoli, 1987). As a result, both 15-HpETE-PE and 15-HETE-PE standards were required.

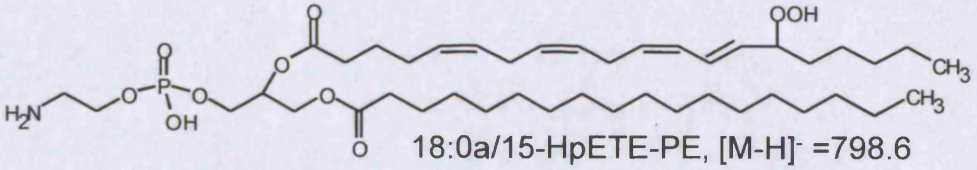
3.1.2 Aims

- To synthesise 18:0a/15-H(p)ETE-PE of a pure quality for use as a standard for quantification and biological studies.
- To confirm the structure of the product using a combination of techniques, including NMR, MS and traditional chromatographic techniques.



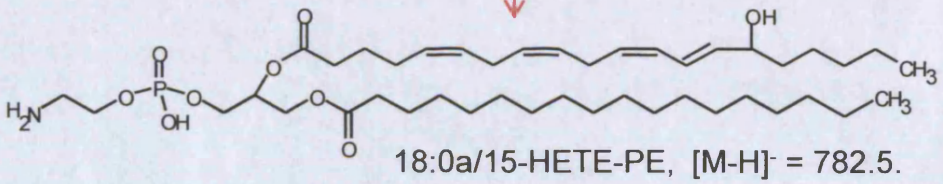
18:0a/20:4-PE, $[M-H]^-$ =766.6

Oxidation of SAPE by 15-Lipoxygnease (Soybean Type V)



18:0a/15-HpETE-PE, $[M-H]^-$ =798.6

Reduction by $SnCl_2$*



18:0a/15-HETE-PE, $[M-H]^-$ = 782.5.

Removal of lipids by hexane extraction

Purification of 15-HpETE-PE / 15-HETE-PE from crude lipid mixture by RP-HPLC

Evaporation of hexane by drying under N_2

Re-suspension into a known volume of methanol

Analysis by MS

Calculation of amount by UV

* For the synthesis of 15-HETE-PE only

Scheme 3.1. Method for the synthesis and purification of 15-HpETE-PE and 15-HETE-PE

3.2 Results

3.2.1 Structural characterisation of 18:0a/15-HpETE-PE and 18:0a/15-HETE-PE.

3.2.1.1 UV analysis

Following the oxidation of SAPE by LOX, the reaction products were extracted and analysed by UV. Products had a λ-max at 235 nm, indicating the presence of a conjugated diene chromophore (Figure 3.1). 15-HpETE-PE and 15-HETE-PE contain a conjugated diene while SAPE does not, thus the results show that SAPE was successfully oxidised. Next, 15-HETE-PE and 15-HpETE-PE were purified by reverse phase HPLC, monitoring elution at a wavelength of 235 nm. As indicated, both oxidised products eluted at approximately 21 minutes, clearly separating from the substrate lipid, SAPE (Figure 3.2).

3.2.1.2 Mass spectrometry analysis

Following purification, the products were analysed by MS, a single ion with *m/z* of 782.6 or 798.6 $[M-H]^-$ were seen for the hydroxy and hydroperoxy phospholipids respectively (Figures 3.3 and 3.4). The figure insets show the product ion spectra of the phospholipids, demonstrating daughter ions diagnostic of 15-HETE at *m/z* 219 and 175, the daughter ions of 15-HpETE were at 317 and 219. The fragment with *m/z* of 283 corresponds to stearic acid, present at the sn1 position.

3.2.1.3 ^{1}H, ^{31}P and ^{13}C NMR analysis of 15-HETE-PE

To confirm the structure of 15-HETE-PE using an additional method, the purified lipid was analysed by multinuclear NMR. The result of these analyses was consistent with the 18:0a/15-HETE-PE structure. ^{1}H-NMR analysis identified the protons adjacent to the conjugated diene, specifically, the double doublet at δ 6.56 (1H, J = 15.02 and 11.02 Hz) (labelled as H_A), the triplet at δ 6.02 (1H, J = 10.55 Hz) (H_B) and the double doublet at δ 5.68

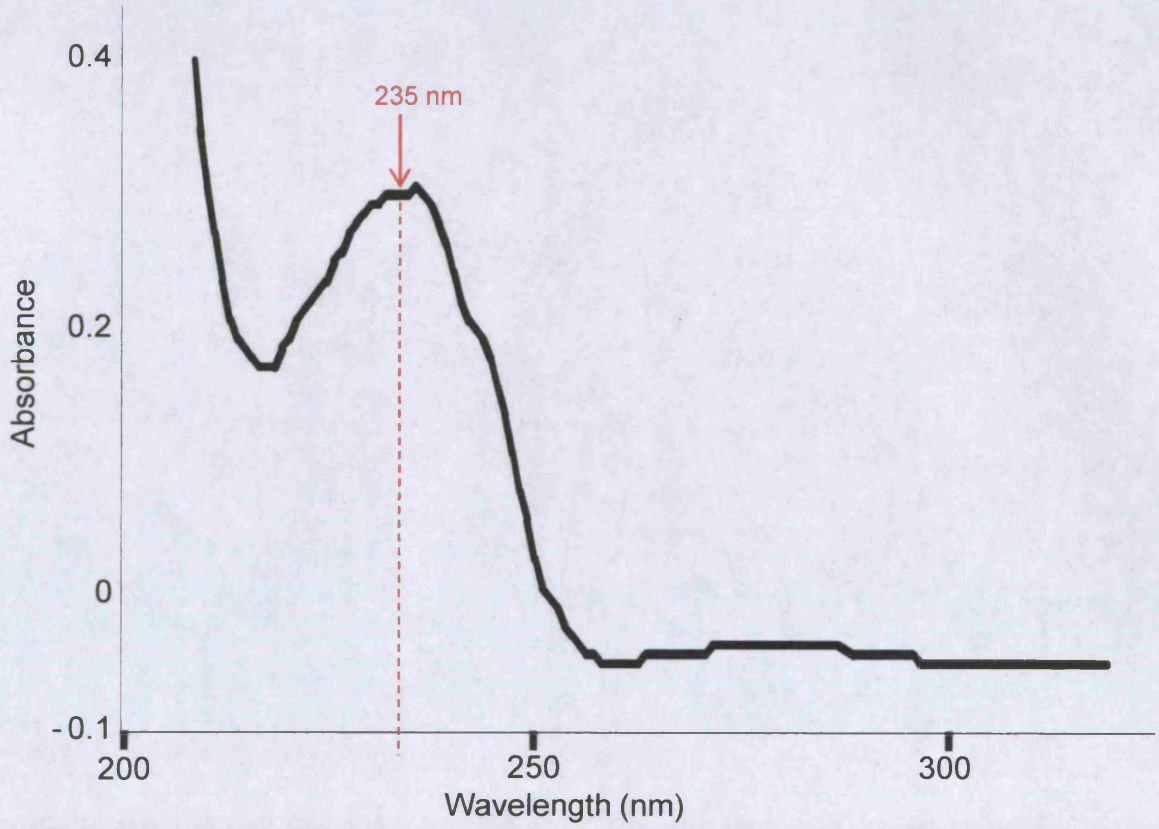


Figure 3.1 Products of SAPE oxidation by LOX have an absorbance maxima at 235 nm, confirming the presence of a conjugated diene. SAPE was oxidised using soybean LOX followed by $SnCl_2$ reduction. The lipid products were extracted then analysed using a UV spectrometer prior to purification.

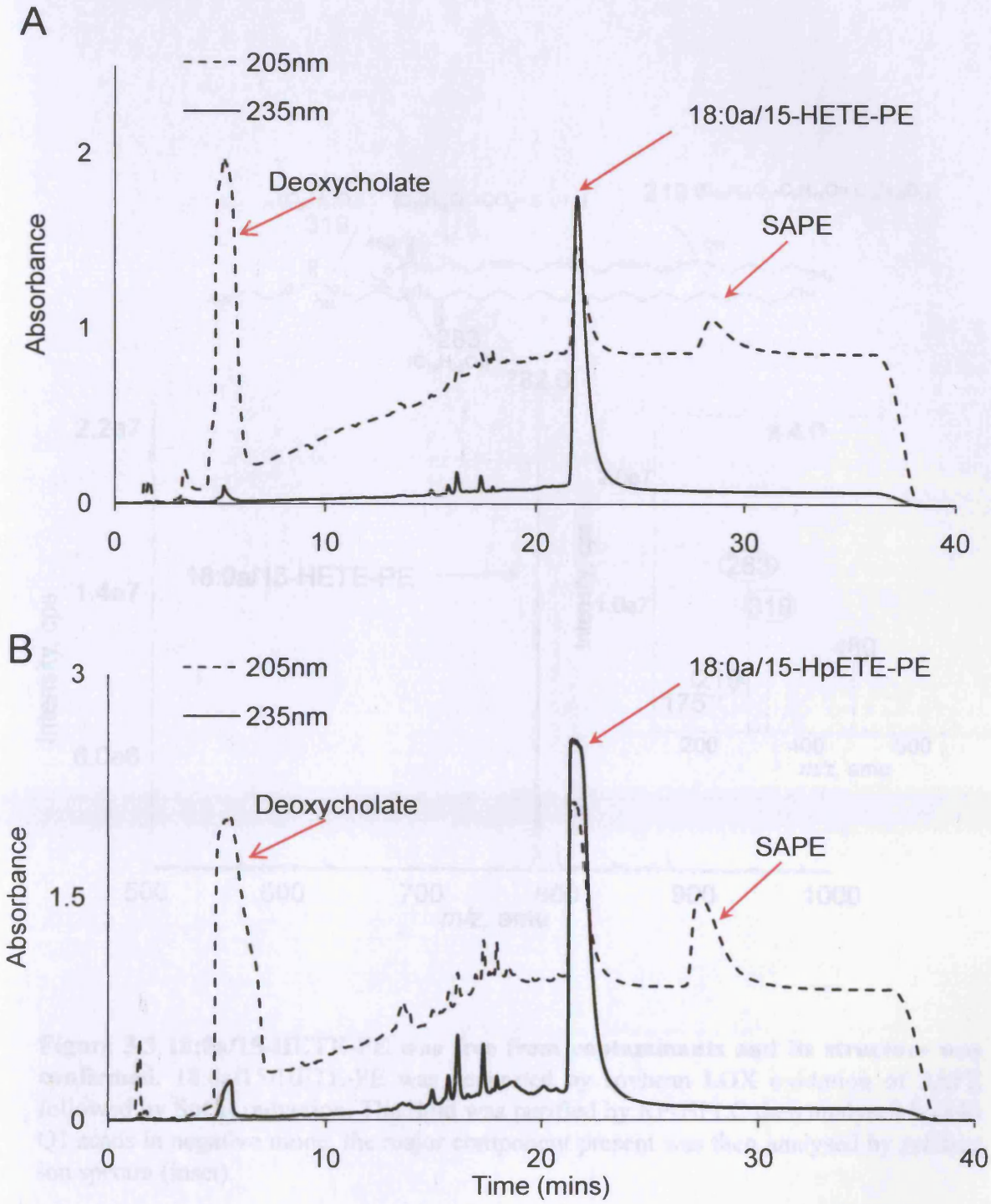


Figure 3.2. 18:0a/15-HETE-PE and 18:0a/15-HpETE-PE separates from SAPE during RP-HPLC. 15-HpETE-PE was synthesised by oxidising SAPE using soybean LOX, followed by $SnCl_2$ reduction to generate 15-HETE-PE. Both lipids were extracted then underwent purification and analysis by UV-RP-HPLC. A) UV-RP-HPLC purification of 15-HETE-PE. B) UV-RP-HPLC purification of 15-HpETE-PE.

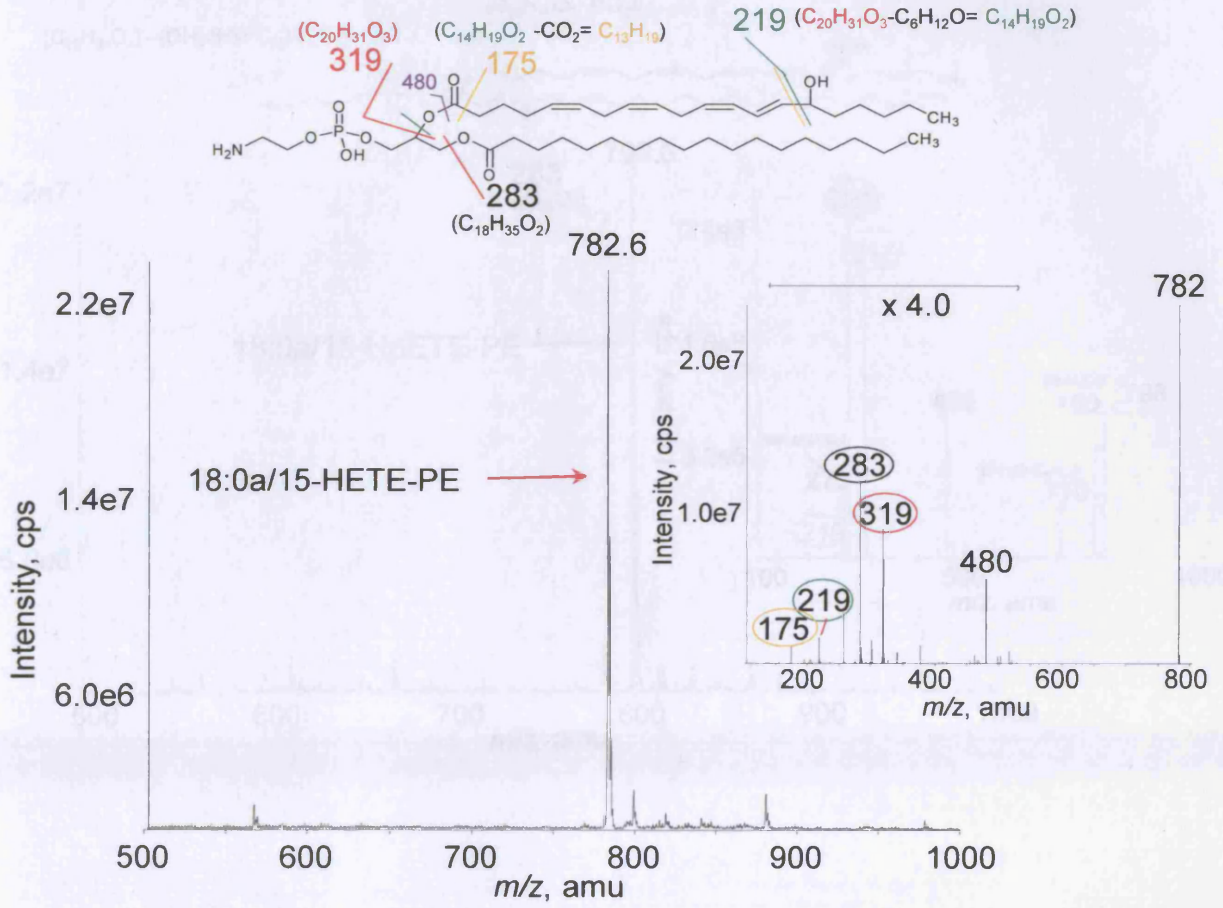


Figure 3.3 18:0a/15-HETE-PE was free from contaminants and its structure was confirmed. 18:0a/15-HETE-PE was generated by soybean LOX oxidation of SAPE followed by $SnCl_2$ reduction. The lipid was purified by RP-HPLC then analysed by MS Q1 scans in negative mode, the major component present was then analysed by product ion spectra (inset).

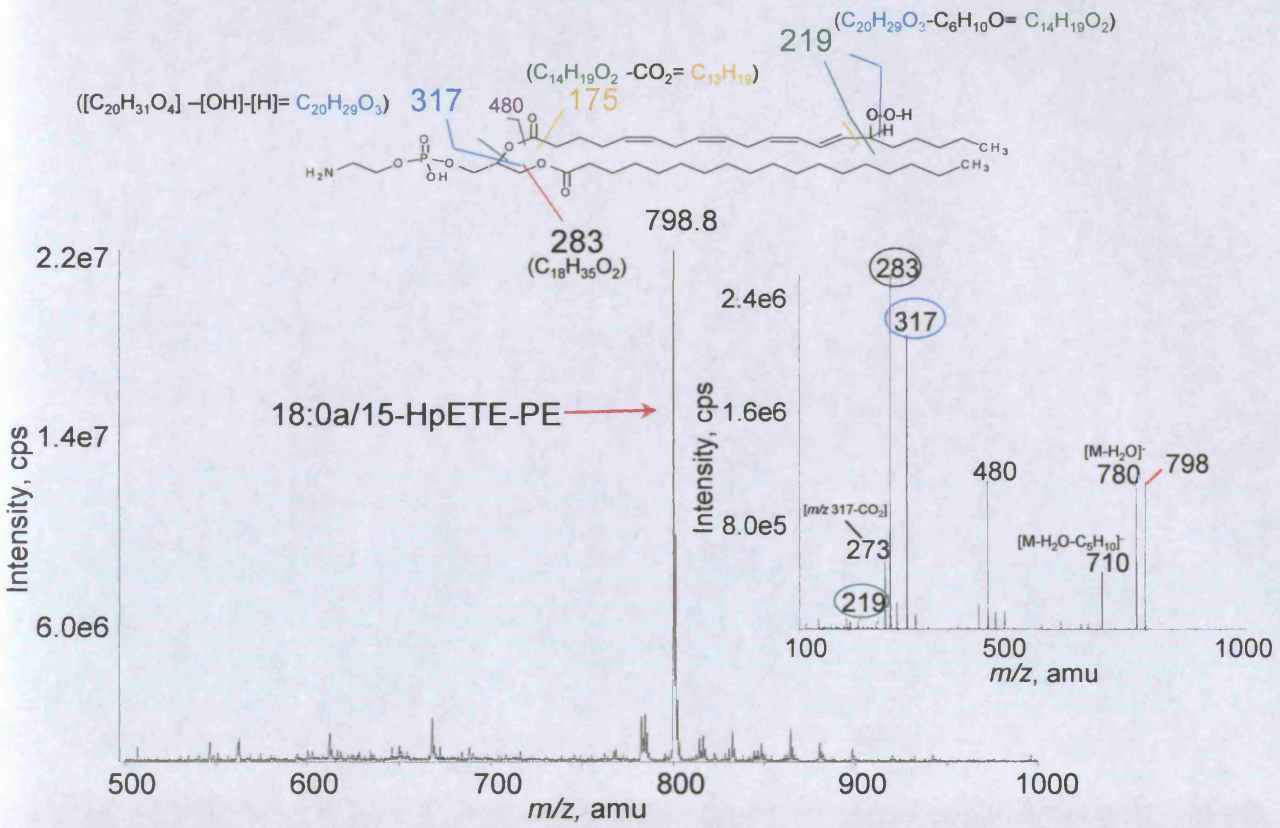


Figure 3.4 18:0a/15-HpETE-PE was free from contaminants and its structure was confirmed. 18:0a/15-HpETE-PE was generated by soybean LOX oxidation of SAPE. The lipid was purified by RP-HPLC then analysed by MS Q1 scans in negative mode, the major component present was then analysed by product ion spectra (inset).

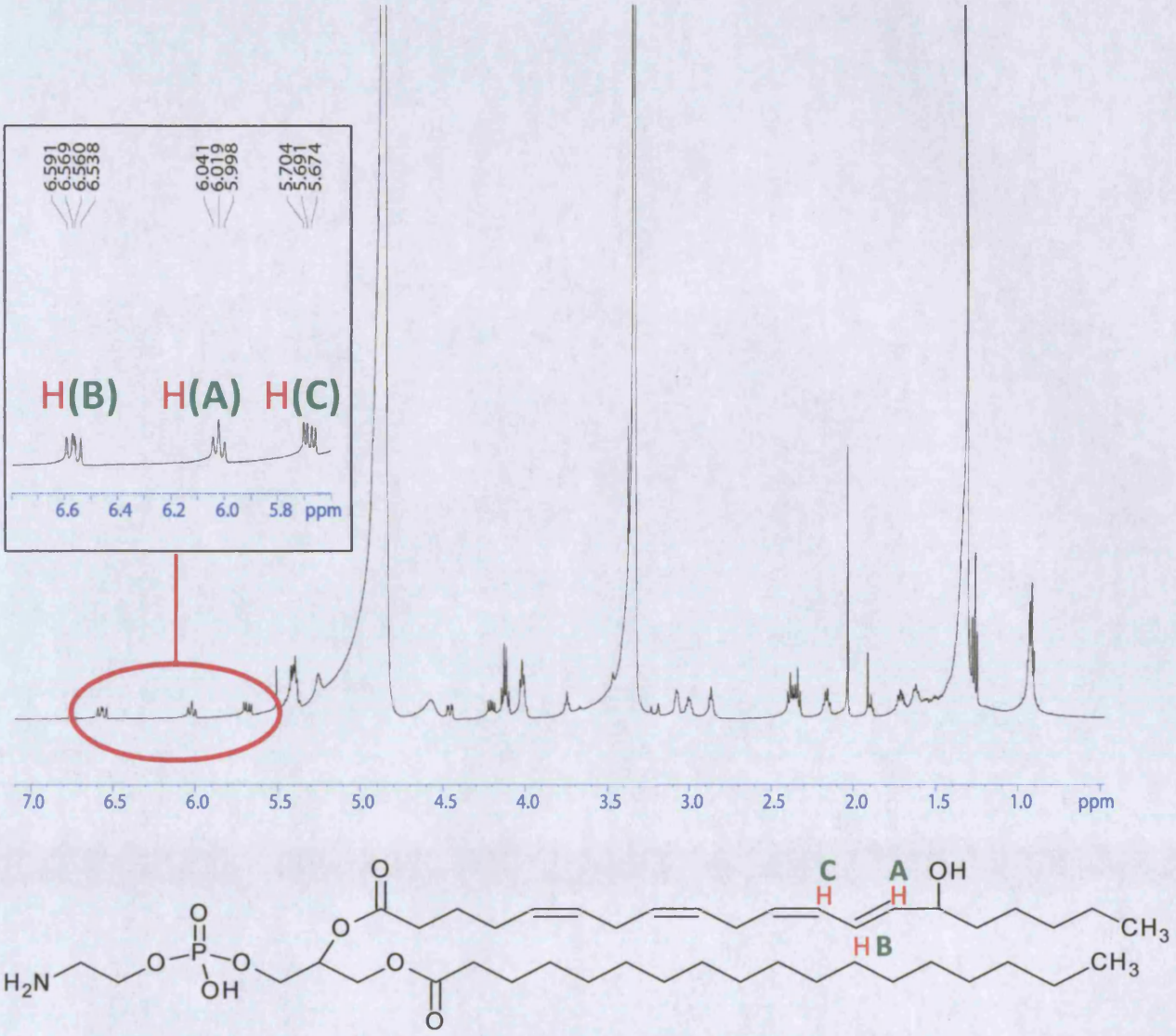
(1H, J = 14.8 Hz and 6.75 Hz) (H_C) (Figure 3.5). For ^{13}C-NMR, signals at δ 138.07, 130.53, 130.05, 129.94, 129.55, 129.47, 128.80, and 126.21 confirm the presence of the alkene bonds and the conjugated diene (Figure 3.6). However, the signals corresponding to the carbonyl groups were not detected, probably due to the very low concentrations of the sample. The ^{31}P-NMR spectrum showed that 15-HETE-PE was free of phosphorus containing contaminants such as detergents (Figure 3.7). Importantly, NMR analysis confirmed the presence of the conjugated diene which is formed during oxidation. As synthesis of 15-HpETE-PE only differs to that of 15-HETE-PE by excluding the reduction step, 15-HpETE-PE was not analysed by NMR.

3.2.1.4 Analysis of 15-HETE-PE by chromatographic techniques

To confirm the positional isomer of HETE present in the synthesised lipid, traditional chromatographic techniques were used. Purified 15-HETE-PE underwent saponification to release the fatty acids and the extracted products were analysed by LC/MS/MS for all HETE transitions. The HPLC traces confirm that the only HETE isomer present is 15-HETE (Figure 3.8). 15-HETE was then collected during RP-HPLC separation and its chirality was determined by separation on a Chiralcel OD column (as described in section 2.2.4.3). The sample was compared to 15R- and 15S-HETE standards and found to contain mainly the S-isomer, as expected from the enantiomeric specificity of soybean 15-LOX (Figure 3.9).

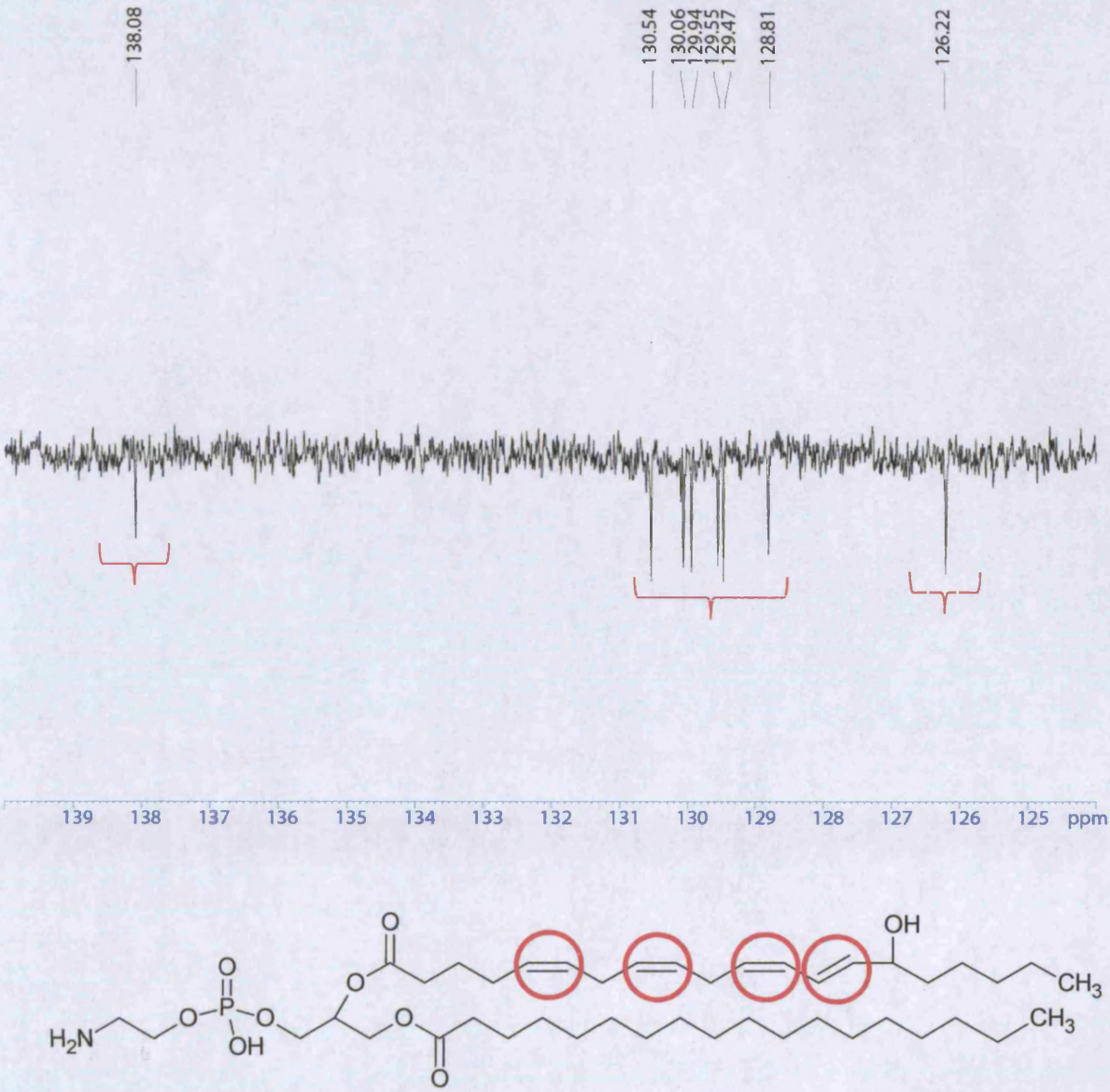
3.2.2 Product yield of 15-HETE-PE and 15-HpETE-PE.

To synthesise 18:0a/15-HETE-PE and 18:0a/15-HpETE-PE, SAPE was oxidised using soybean LOX (Type V), with or without reduction by $SnCl_2$ respectively. From 20 mg of SAPE substrate, there was a product yield of approximately 6 mg of purified 15-HpETE-PE and 15-HETE-PE, calculated using UV. This is equivalent to a 30% yield of product.



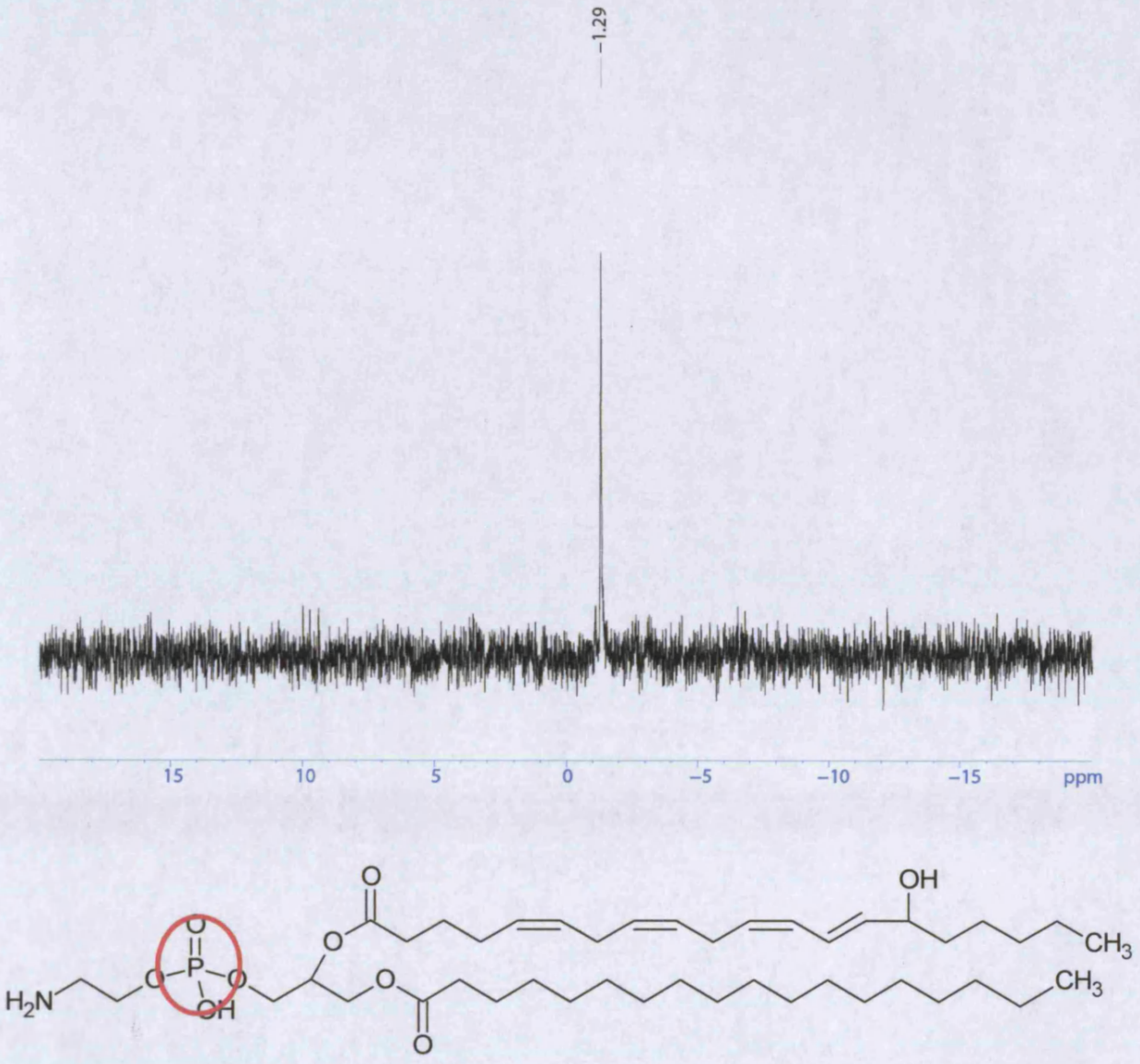
^{1}H-NMR (500 MHz CD_3OD) δ 6.56 (1H, dd, *J* =15.02 and 11.02 Hz), 6.02 (1H, t, *J* = 10.55 Hz), 5.68 (1H, dd, *J* = 14.8 Hz, 6.75 Hz), 5.40 (5H, m), 5.25 (1H, m), 4.48 (1H, dd, *J* = 12.35 and 3.25 Hz), 4.20 (1H, dd, *J* = 12.35 and 6.65 Hz), 4.12 (1H, m), 4.02 (4H, m), 3.07 (2H, m), 3.00 (2H, m), 2.86 (2H, m), 2.37 (4H, m), 2.15 (2H, m), 1.70 (2H, m), 1.71 (2H, m), 1.27 (32H, m), 0.92 (6H, m).

Figure 3.5 ^{1}H-NMR of 15-HETE-PE. 18:0a/15-HETE-PE was synthesised by soybean LOX oxidation of SAPE followed by $SnCl_2$ reduction. The lipid was purified by RP-HPLC, dried under gaseous N_2 then re-suspended in deuterated chloroform. 15-HETE-PE was analysed by ^{1}H-NMR (500 MHz) on a Bruker Avance 500 MHz spectrometer at 25 °C. The inset shows the signals that correspond to three hydrogen atoms near the conjugated diene (H(A), H(B) and H(C)).



^{13}C-NMR (125MHz CD_3OD). δ 138.07, 130.53, 130.05, 129.94, 129.55, 129.47, 128.80, 126.21, 73.34, 71.91 (J_{CP}=7.75Hz), 64.83 (J_{CP}=5.75Hz), 63.63, 38.5, 34.92, 34.49, 33.08, 33.01, 30.78, 30.63, 30.47, 30.44, 30.21, 27.54, 27.01, 26.59, 26.30, 26.01, 25.93, 23.72.

Figure 3.6 ^{13}C-Pendant NMR of 15-HETE-PE. 18:0a/15-HETE-PE was synthesised by soybean LOX oxidation of SAPE followed by $SnCl_2$ reduction. The lipid was purified by RP-HPLC, dried under gaseous N_2 the re-suspended in deuterated methanol. 15-HETE-PE was analysed by ^{13}C-Pendant NMR spectroscopy on a Bruker Avance 500 MHz at 25 °C.



^{31}P-NMR (202 MHz CD_3OD) δ -1.29.

Figure 3.7 ^{31}P NMR of 15-HETE-PE. 18:0a/15-HETE-PE was synthesised by soybean LOX oxidation of SAPE followed by $SnCl_2$ reduction. The lipid was purified by RP-HPLC, dried under gaseous N_2 then re-suspended in deuterated methanol. 15-HETE-PE was analysed by ^{31}P-NMR (202 MHz) on a Bruker Avance 500 MHz spectrometer at 25 °C.

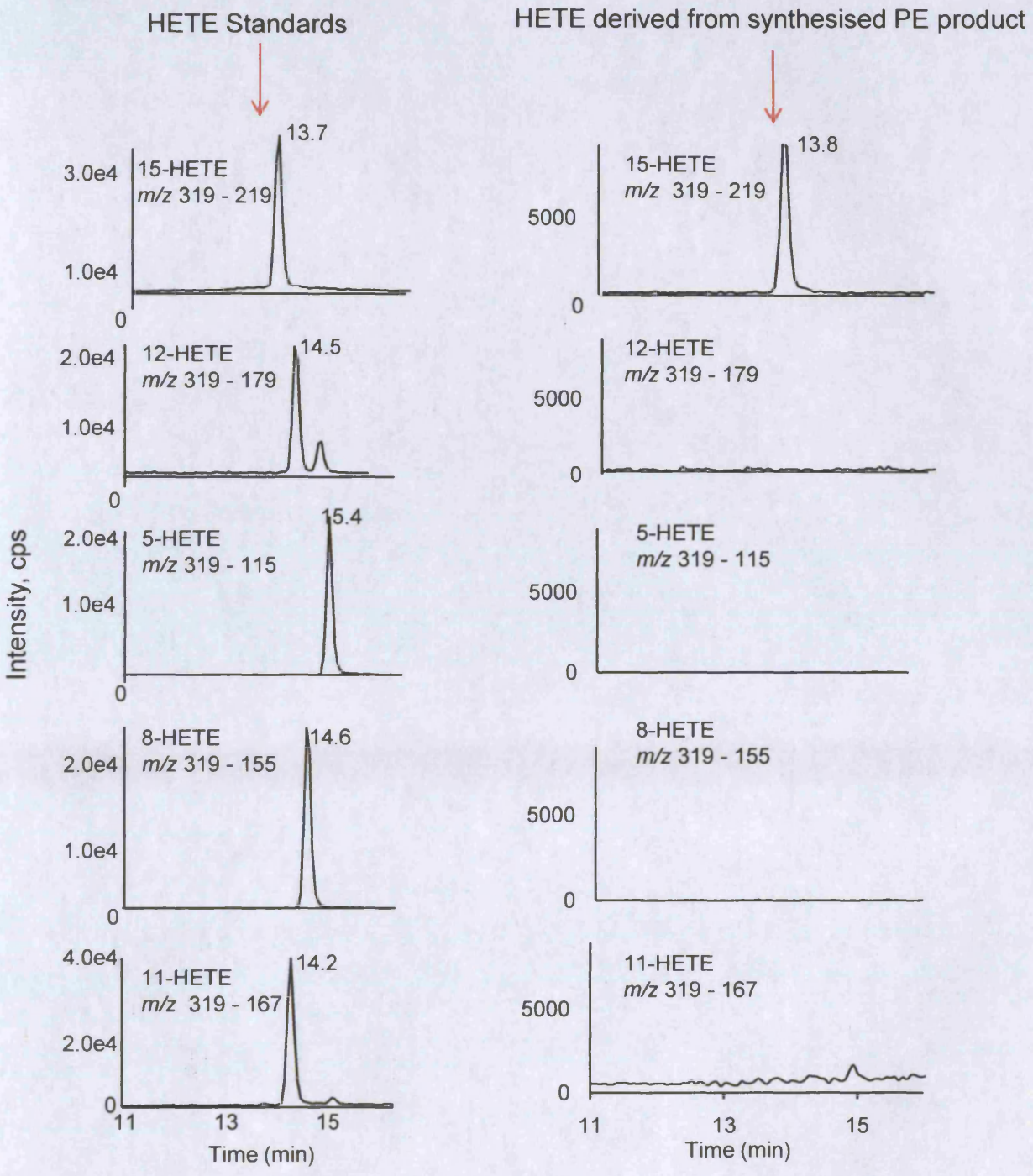


Figure 3.8 Synthesised lipid contains only 15-HETE. 18:0a/15-HETE-PE was synthesised by soybean LOX oxidation of SAPE followed by $SnCl_2$ reduction. The lipid was purified by RP-HPLC then underwent base hydrolysis to release the HETE. The fatty acids were extracted followed by LC/MS/MS analysis.

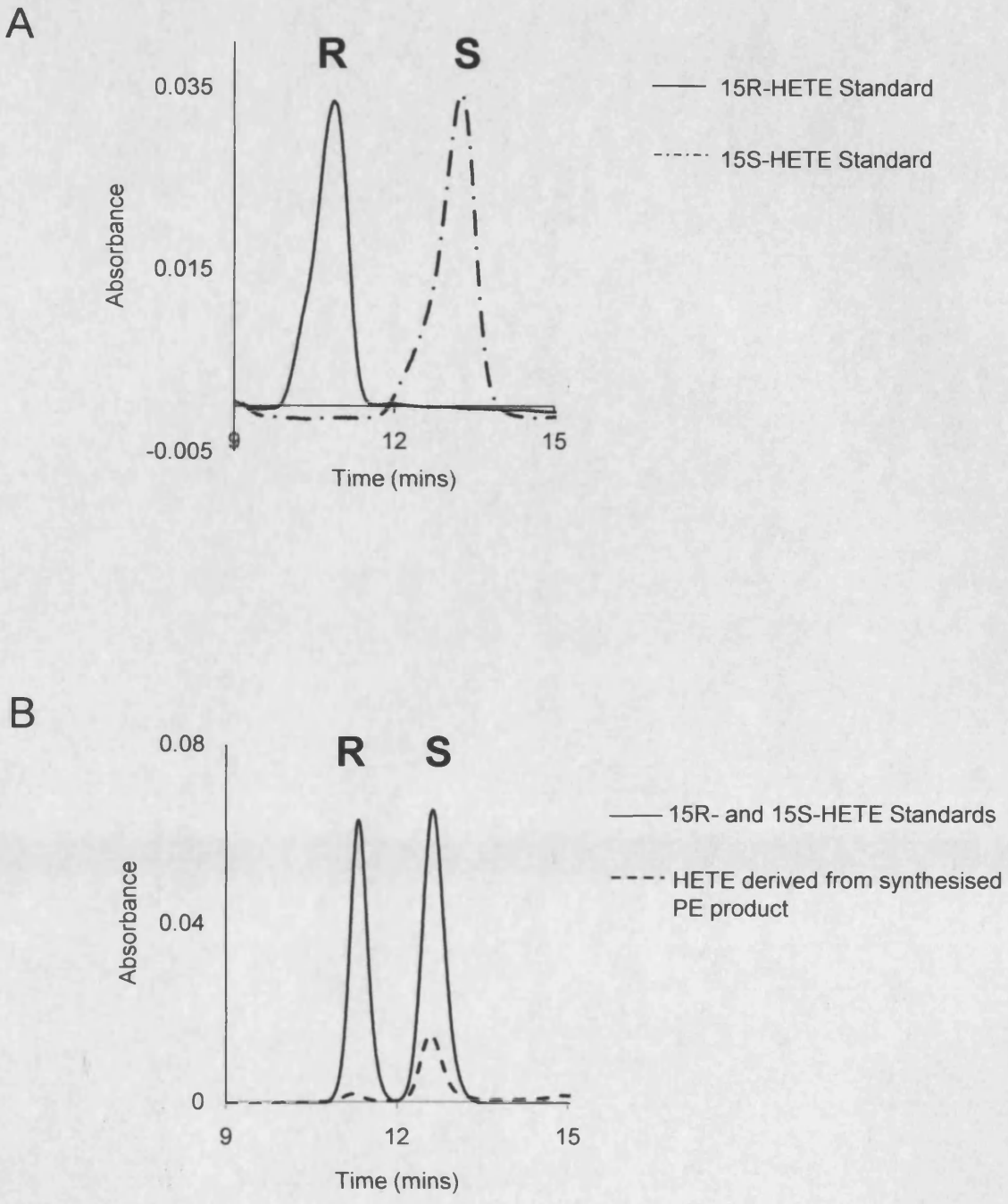


Figure 3.9 Synthesised 15-HETE-PE contains largely 15S-HETE. 15R and 15S-HETE were analysed by chiral phase chromatography individually (A). 18:0a/15-HETE-PE was synthesised by soybean LOX oxidation of SAPE followed by $SnCl_2$ reduction. The lipid was purified by RP-HPLC then underwent base hydrolysis to release the HETE. 15-HETE was collected during RP-HPLC then analysed by chiral phase HPLC and compared to combined 15R- and 15S-HETE standards (B).

3.2.3 Synthesised 18:0a/15-H(p)ETE-PE standards have the same elution time as 18:0a/15-H(p)ETE-PEs from human monocytes.

Prior to their use as standards, LC/MS/MS analysis of the synthesised 18:0a/15-H(p)ETE-PEs were compared to that of 18:0a/15-H(p)ETE-PEs derived from human monocytes, to confirm that they were the same phospholipids. Human monocytes were activated using A23187 for 15 minutes followed by lipid extraction either directly, or after $SnCl_2$ reduction. LC/MS/MS analysis showed that both human monocyte derived, and the synthesised 18:0a/15-HETE-PE and 18:0a/15-HpETE-PE standards have the same retention time. This confirms that they are the same lipids (Figures 3.10 and 3.11).

3.2.4 Development of an assay for phospholipid quantification by MS.

A new method for quantifying phospholipids following MS analysis is required. The assay described herein calculates the amount of 15-H(p)ETE-PE in samples by comparing the analyte peak area to the peak area of an internal standard. To account for differences in lipid recovery during the extraction procedure, an internal standard (Di-myristoyl phosphatidylethanolamine (DMPE)) was added to biological samples prior to extraction. DMPE was used as an internal standard as it is the closest phospholipid in structure to 15-HETE-PEs that is not present in the samples analysed for this thesis. Prior to quantifying 15-H(p)ETE-PE in samples, a standard curve was constructed where varying amounts (0 - 10,000 pg) of 15-HETE-PE, or 15-HpETE-PE, was added to a fixed amount of DMPE (100 pg). Thus, different ratios of 15-H(p)ETE-PE *vs* DMPE were present in each sample. Following LC/MS/MS analysis, the ratio of the integrated area of 15-H(p)ETE-PE:DMPE was plotted against the ratio of pg 15-H(p)ETE-PE:DMPE injected (Figures 3.12 and 3.13). A standard curve generated for 15-HETE-PE using a daughter ion of 219 can only be used to quantify 15-HETE-PE products (rather than other HETE isomers), as 219 is a fragment

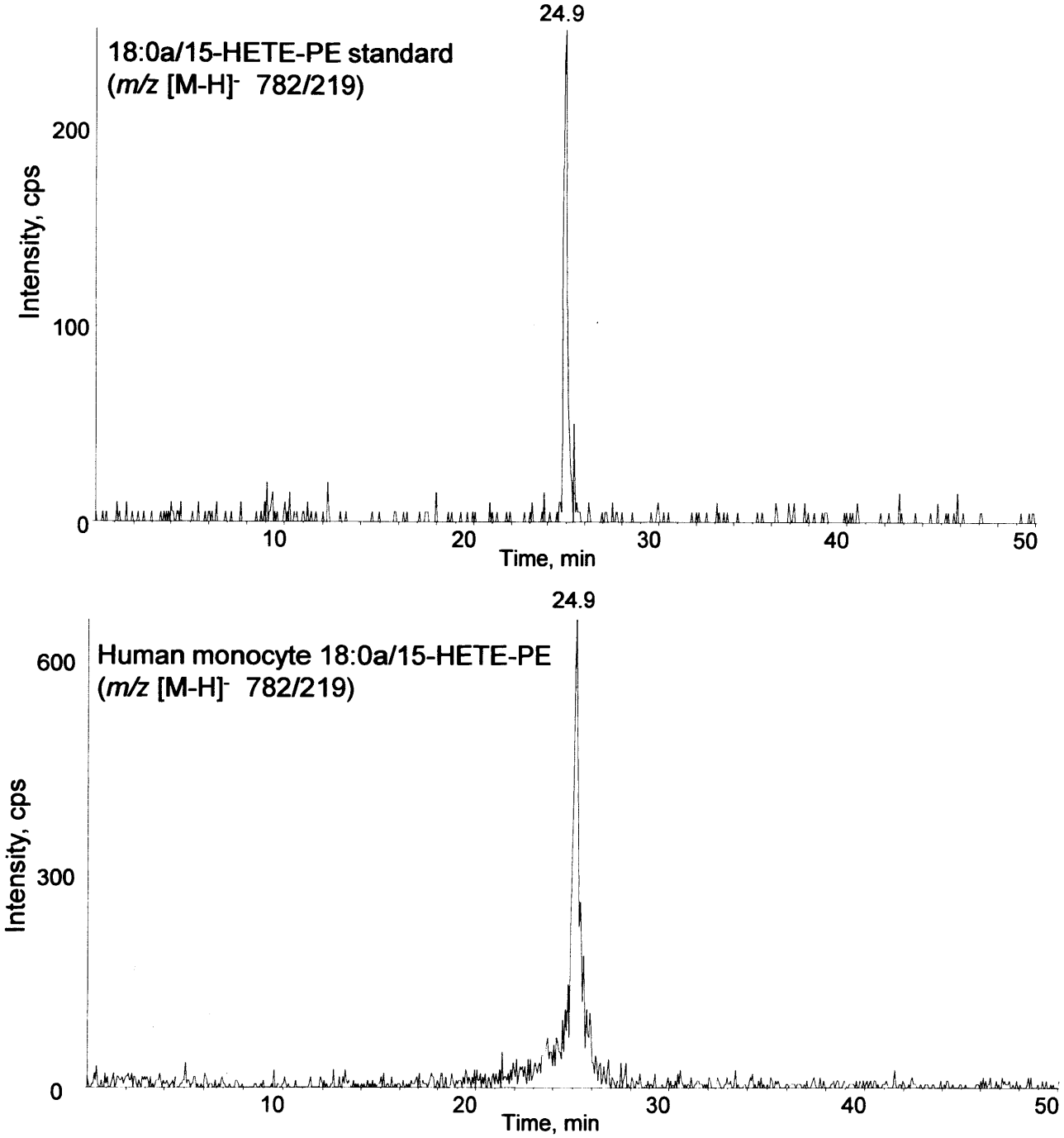


Figure 3.10. Human monocyte 18:0a/15-HETE-PE has the same retention time as a 18:0a/15-HETE-PE standard. Human monocytes were activated for 15 minutes using A23187, followed by reduction with $SnCl_2$ and lipid extraction. Lipids from monocyte samples and a 18:0a/15-HETE-PE standard were analysed by LC/MS/MS.

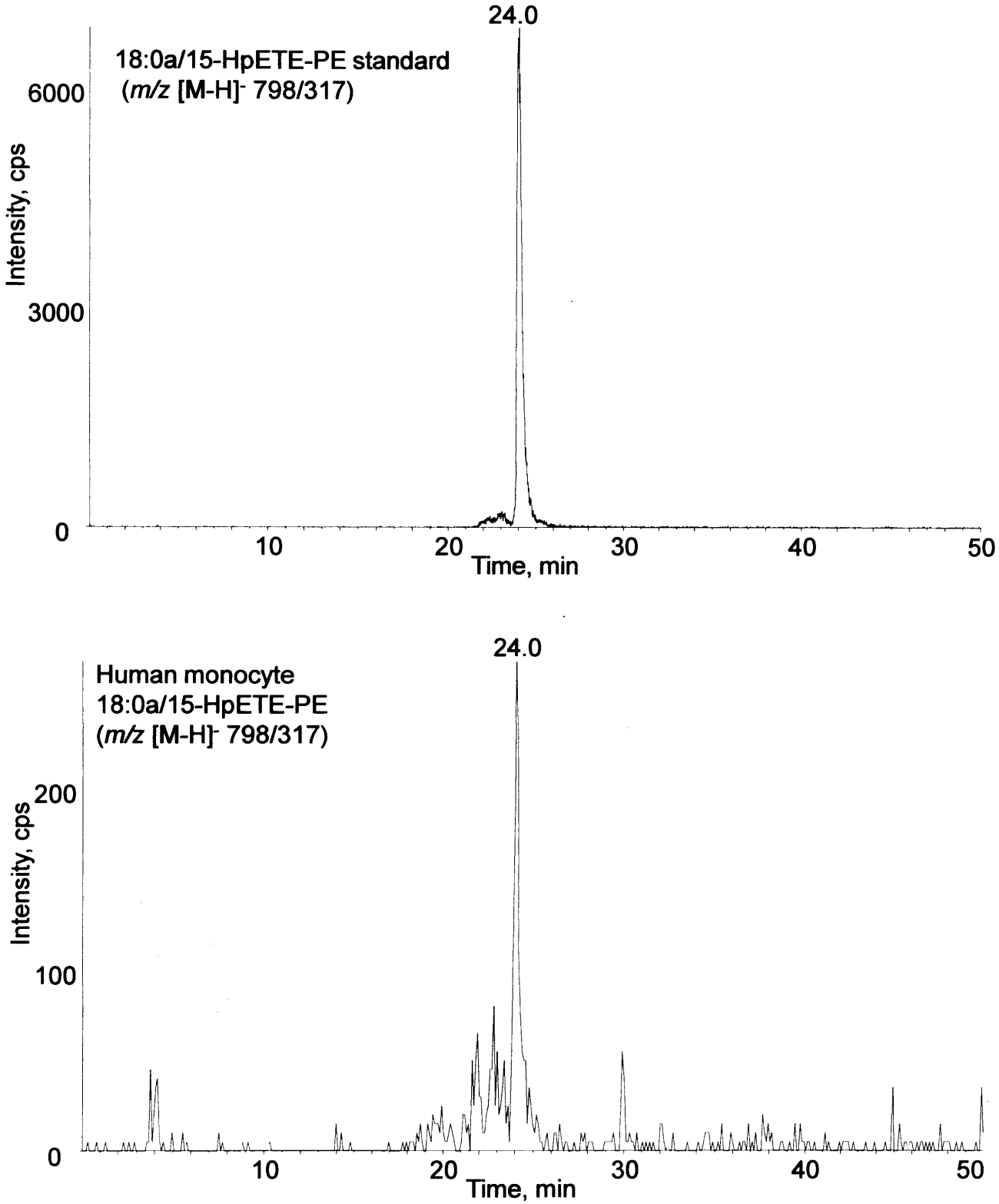


Figure 3.11. 18:0a/15-HpETE-PE from human monocytes has the same retention time as the 18:0a/15-HpETE-PE standard. Human monocytes were activated with 10 μM A23817 for 15 minutes followed by and lipid extraction. Monocyte samples and 18:0a/15-HpETE-PE standard were analysed by LC/MS/MS.

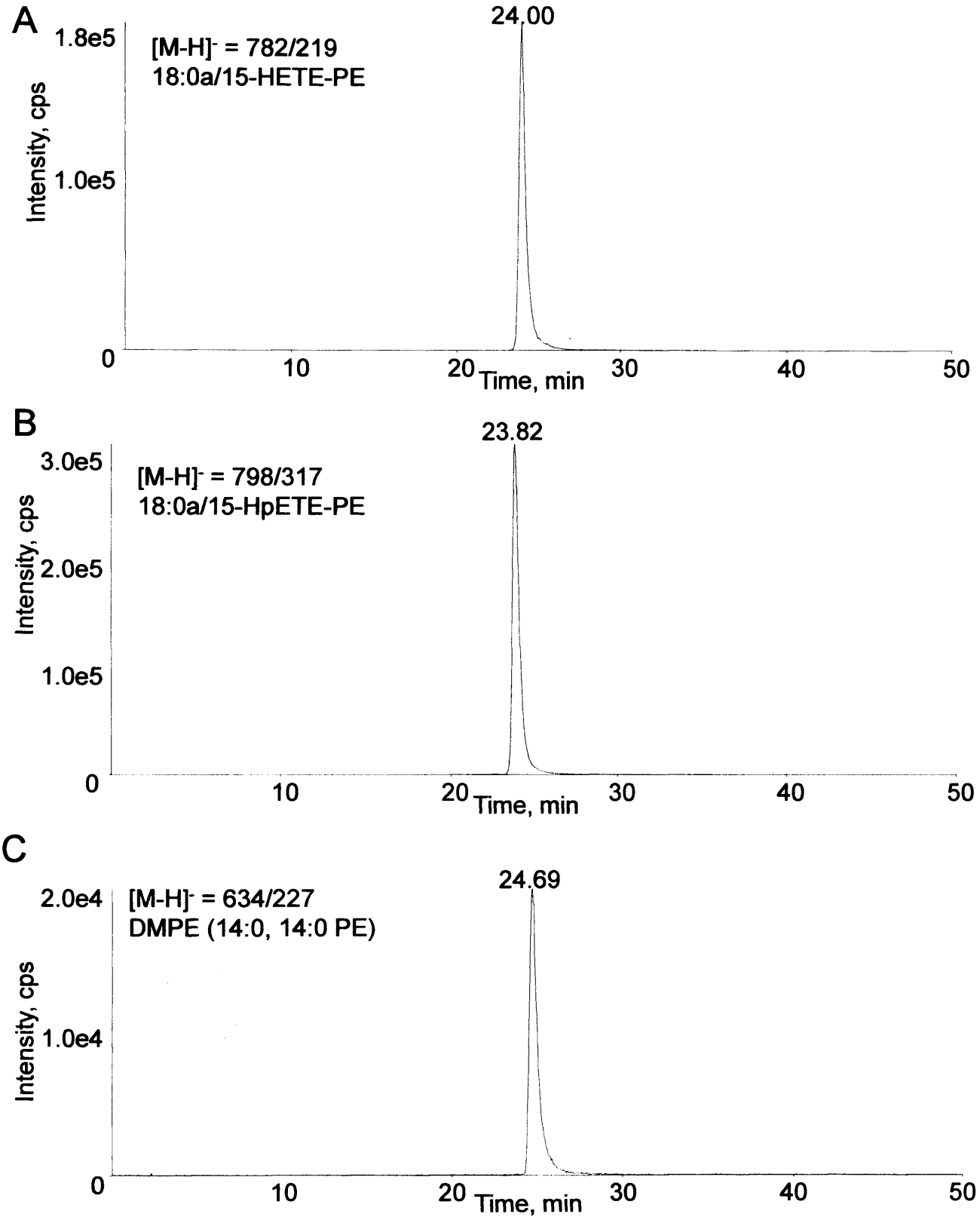


Figure 3.12. Representative chromatograms of synthesised 15-HETE-PE, 15-HpETE-PE and DMPE standards. A synthesised and purified 15-HETE-PE (A), 15-HpETE-PE (B) and a DMPE standard (C.) were analysed by LC/MS/MS. To generate a standard curve the ratio of peak areas of detected 15-HETE-PE or 15-HpETE-PE and DMPE ((A/C) or (B/C)) is plotted against the known ratio of pg injected.

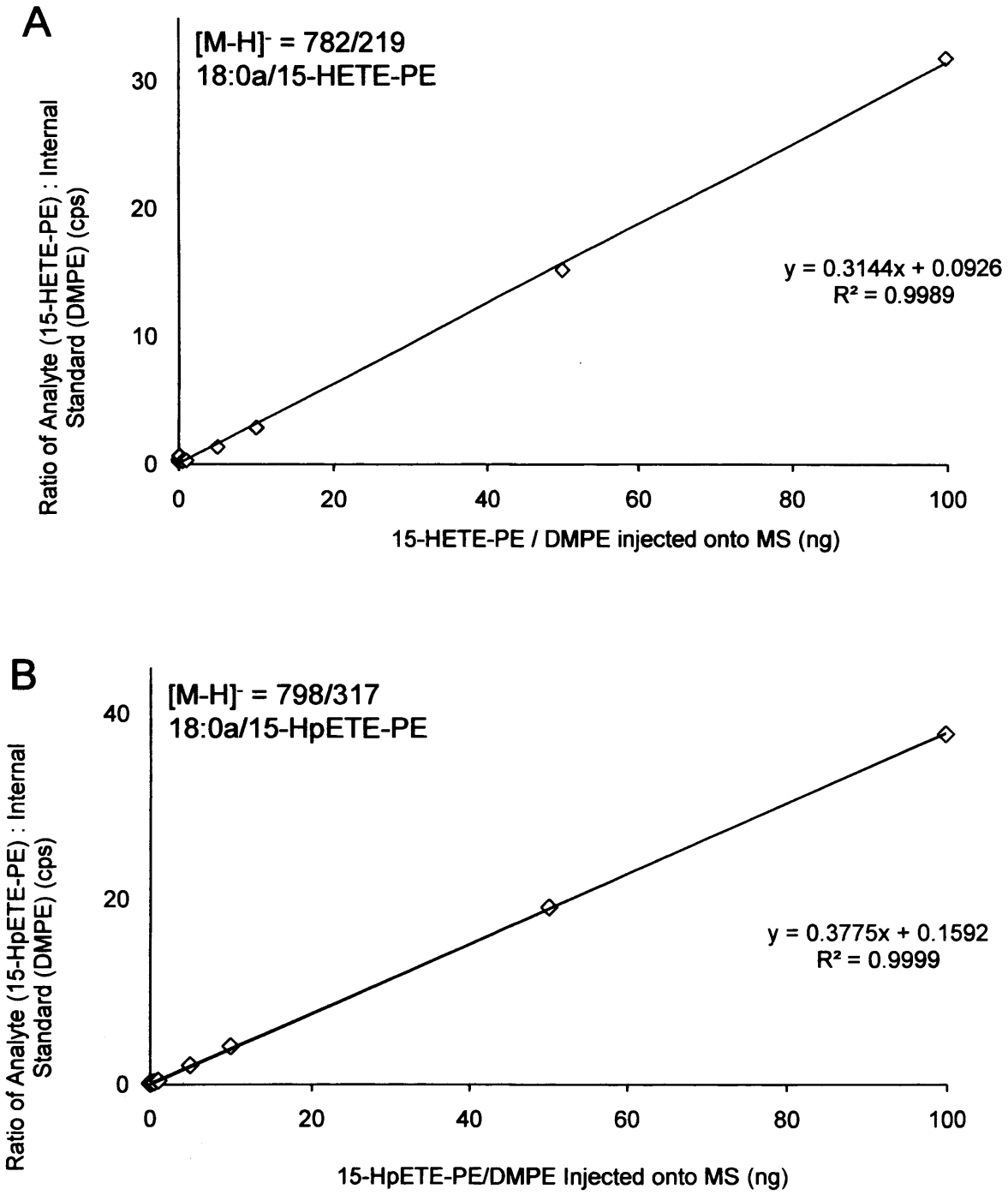


Figure 3.13. Representative standard curve for 15-HETE-PE and 15-HpETE-PE. A synthesised and purified 15-HETE-PE and a DMPE standard were analysed by LC/MS/MS. To generate a standard curve the ratio of peak areas of detected 15-HETE-PE and DMPE is plotted against the known ratio of actual quantity injected. The gradient from the standard curve can then be used to quantify 15-HETE-PE in test samples.

specific to 15-HETE. However, a standard curve formed using a daughter ion of 319 could be applied in the quantification of all PE-HETE isomers.

The standard curves were linear over a 200-fold range (50 to 10,000 pg) and the equations for the lines of regression were $y = 0.3144x$ for 15-HETE-PE and $y = 0.3775x$ for 15-HpETE-PE. In addition, the line slopes did not vary significantly from unity ($r^2 = 0.9989$ to 0.9999 respectively) (Figure 3.13). The lower limit of detection, determined by a minimum signal to noise ratio of approximately 4:1, was approximately 25 pg. The gradient of the generated 15-H(p)ETE-PE standard curve can be used to quantify 15-H(p)ETE-PEs in test samples using the following formula:

$$\text{Analyte (ng)} = \left(\frac{\text{Analyte Peak Area}}{\text{Internal Standard Peak Area}}\right) \text{X} \left(\frac{\text{Internal Standard (ng)}}{\text{Gradient from standard curve}}\right)$$

3.3 Discussion

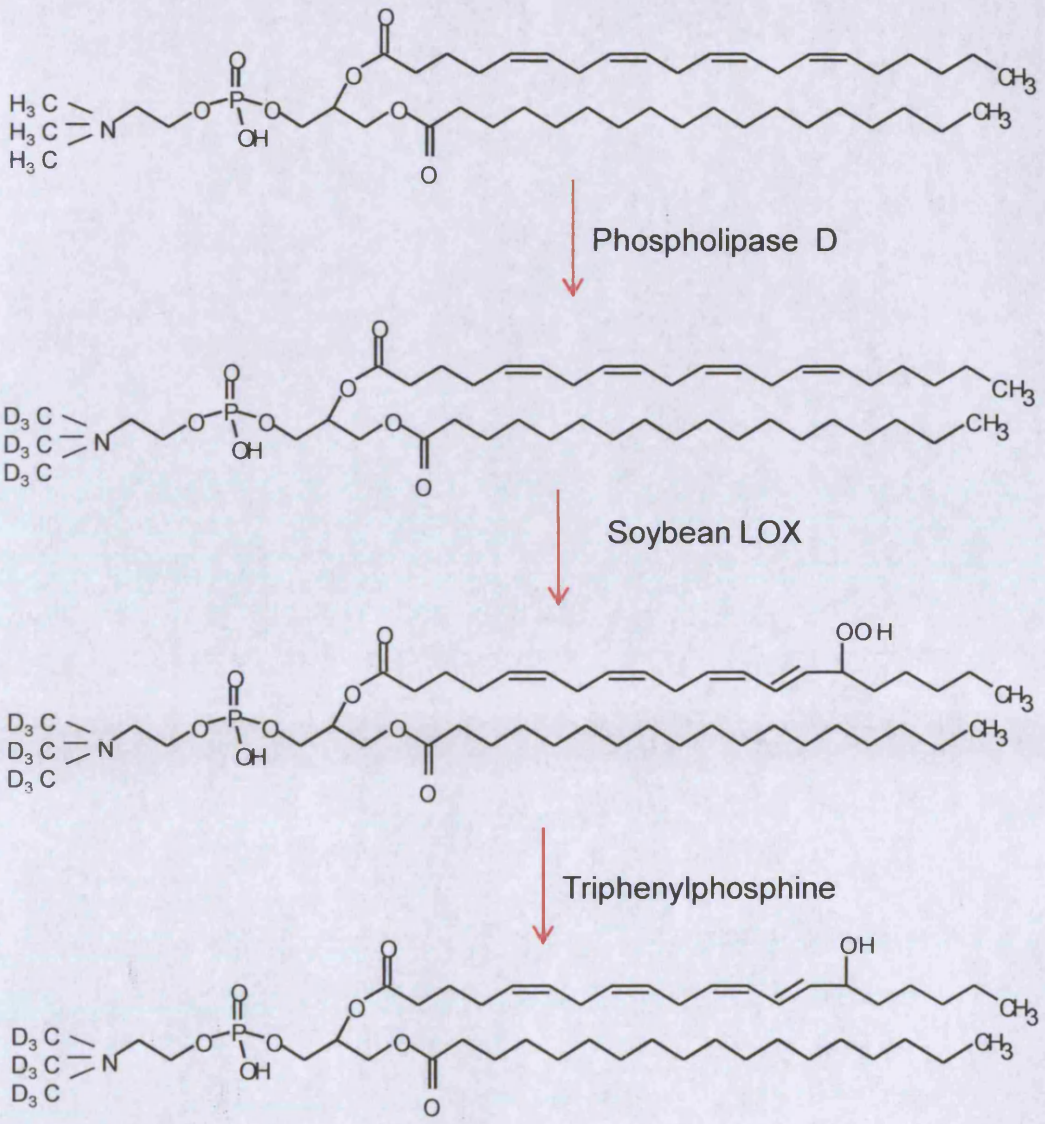
The work described in this chapter illustrates the successful adaptation of a published method to synthesise our own lipids of interest (Brash *et al*, 1987). Furthermore, the structures of the generated products were confirmed using techniques comparable to those used in the same published article, which included analysis by UV, RP-HPLC, MS, NMR and chiral HPLC.

This chapter has also described a new assay for the quantification of 15-H(p)ETE-PEs, which is more accurate than methods used up to now. Previous studies have not calculated amounts of individual molecular species of phospholipid products, or they were quantified indirectly by calculating the amount of sn2 fatty acids following base hydrolysis (Watson *et al*, 1997, Podrez *et al*, 1999, Podrez *et al*, 2002, Von Schlieffen *et al*, 2009, Cherepanova *et al*, 2009, Walton *et al*, 2003, Leitinger *et al*, 1999, Maskrey *et al*, 2007). Also, the quantification methods used in these referenced articles did not take into account the amount of product lost during lipid extraction. This is addressed by the new quantification method described in this chapter, by adding the internal standard (DMPE) to samples during lipid extraction. Furthermore, using the hexane:isopopanol-based extraction solvent the recovery of lipids is approximately 76-83%, which is greater than that achieved when using the Bligh and Dyer extraction method (60-62%) (Morgan *et al*, 2010). In publications by Podrez *et al* (2002a & 2002b), a similar assay was used. Particular oxidised PC lipids (such as 9-hydroxy-12-oxo-10-dodecenoic acid (HODA-PC) and 9-keto-12-oxo-10-dodecenoic acid (KODA-PC)) were quantified with the aid of synthesised standards, some of which are available from Avanti. However, lipid extraction was carried out using the Bligh and Dyer method, followed by the addition of the internal standard. Conversely, adding a known amount of standard to samples before lipid extraction (as described in this chapter) ensures that the efficiency of extraction is

taken into consideration during quantification. Therefore, the amount of lipids present can be calculated with greater accuracy.

Currently, DMPE is used as an internal standard as structurally, it is the most similar phospholipid to HETE-PEs available that is not present in human monocytes and murine macrophages, which are the samples used throughout this thesis. The extraction efficiency of DMPE is also at 76-83% using the hexane:isopropanol based extraction, similar to that of the HETE-PEs (Morgan *et al*, 2010). However, as the structures of DMPE and the HETE-PEs are different their extraction efficiency will not be exactly the same. A more suitable standard would be a deuterated PE-HETE, which would have an identical extraction efficiency and could also be analysed by LC/MS/MS. Previous researchers have synthesised deuterated hydroperoxide and hydroxide -PC lipids, (Milne *et al*, 2005) (Scheme 3.2). This method could be adapted to incorporate deuterium onto the amine of a PE head group. Initial experiments attempting to synthesise deuterated 15-HETE-PEs have been carried out by members of the O'Donnell research group, but were not successful. Nonetheless, efforts to synthesise these standards are ongoing through collaboration with members of the research group involved in the development of this technique (Milne *et al*, 2005).

In addition to 15-H(p)ETE-PEs, other phospholipid-HETE isomers require quantification, including 12-HETE-PEs detected in murine macrophages and 5-HETE-PEs in neutrophils. The 15-HETE-PE standard curve can be generated following MS analysis, using a daughter ion of *m/z* 219 or 319. 219 is a fragment that is specific to 15-HETE, therefore, a standard curve generated using this daughter ion can be used specifically to quantify 15-HETE-PEs. However, a standard curve generated using a daughter ion of 319 (the *m/z* of HETE $[M-H]^-$) could be used to quantify all the HETE isomers. During RP-HPLC analysis of samples, 15-, 12- and 5-HETE-PEs can be distinguished as they elute at different times, in this order



Scheme 3.2 Synthesis of d_9-1-steroyl, 2-archidonyl-sn-glycero-3–PC hydroperoxide (-OOH) and alcohol (-OH) internal standards. The PC head groups were cleaved by phospholipase D in the presence of d_9-Choline. The phospholipids were oxidised using soybean LOX then reduced using triphenylphosphine (reproduced from Milne *et al,* 2005, with permission (06/12/2010)).

(Morgan *et al*, in preparation). 12-HETE-PE or 5-HETE-PE can then be selected during MS analysis for quantification. However, in some instances, HPLC peaks overlap and on these occasions the selected peak area of analytes may not be a true reflection of the actual amount present. Analysing each HETE isomer to its specific daughter ion fragment would allow greater accuracy during quantification, for example, using daughter ions with *m/z* of 179 for 12-HETE-PEs and 115 for 5-HETE-PEs. Quantification by this method would require the synthesis of 12- and 5-HETE-PE standards. However, there are no purified LOX enzymes that can oxidise efficiently at C-12 and C-5 of AA. A rabbit 15-LOX has been successfully isolated and also, a recombinant platelet 12-LOX, but no mammalian 5-LOX. These enzymes would not be suitable for synthesising large quantities of lipid standards as mammalian isoforms of LOX undergo rapid suicidal inactivation. As a result, very little amounts of products are synthesised, which are insufficient to use in biological studies (O'Donnell, unpublished). A 5-LOX has been isolated from potatoes but results from preliminary experiments show that this enzyme also oxygenates at C-15 of AA and only generates a small yield of 5-HETE-PEs (Clark *et al*, unpublished).

Since this method of synthesising 15-H(p)ETE-PE was developed, an alternative method of synthesising phospholipid standards has established by the O'Donnell research group, by adapting different published methods (Yin *et al*, 2008, Milne *et al*, 2005). This involves the air oxidation of substrates such as SAPE in the presence of a vitamin E analogue, which prevents the formation of secondary oxidation products. All HpETE-PE isomers are formed during this process, specifically a mixture of 5-, 8-, 9-, 11-, 12- and 15-HpETE-PEs (O'Donnell *et al*, unpublished). They are reduced using $SnCl_2$ to form HETE-PEs if required. The relative proportion of each isomer is determined by separation using a Discovery C-18 column, and the overall UV absorbance is used to calculate the amount of each isomer (Milne

et al, 2005). Using the mixed HETE isomer standard with the quantification assay described in this chapter, standard curves of each HETE isomer are generated. Thus, each HETE-PE can be analysed during MS, using its specific daughter ion fragment. Therefore, all esterified HETE products can be quantified. This method has also been used to generate HETE-PC products, using SAPC as the substrate. In addition, oxidised products of linoleic acid (LA) and docosahexaenoic acid (DHA) (both free and esterified) have been synthesised using the air oxidation method. Thus, the majority of analytes investigated by the O'Donnell research group can now be directly quantified (Morgan *et al*, 2010).

As a direct result of the experiments described in this chapter, 15-H(p)ETE-PEs can now be quantified directly and with greater accuracy. Results will therefore be more reliable in comparison to experiments where products were quantified indirectly, or by estimation. Greater accuracy during quantification will mean that physiological concentrations of 15-H(p)ETE-PEs can be determined and therefore applied during biological experiments. Using one oxidised phospholipid in experiments is more specific than using a mixture of oxidised phospholipid molecular species. Thus, published results will give a truer account of the concentrations and actions of these phospholipids.

The synthesised 15-H(p)ETE-PE standards and the quantification assay described in this chapter was used in the subsequent chapters of this thesis. Specifically, 15-H(p)ETE-PEs were quantified in human monocytes (Chapter 4) and human peritoneal lavages (Chapter 7). Furthermore, 12-H(p)ETE-PEs were quantified in murine macrophages using the air oxidised standard described above (Chapter 6). Finally, the synthesised 15-HETE-PE standard was added to human monocytes to investigate potential anti- or pro- inflammatory actions (Chapter 7).

CHAPTER 4

CHARACTERISATION OF 15-H(p)ETE-PE METABOLISM FOLLOWING ACTIVATION OF 15-LOX

4.1 Introduction

Products of 12/15-LOX may be involved in regulating inflammation, but the identities of the specific lipid products remain unknown (Kuhn & O'Donnell, 2006). Oxidation of AA by LOX generates HpETEs that contain a hydroperoxide group and these are believed to be rapidly reduced to hydroxides by GPx enzymes (McGinley & van der Donk, 2003, Hamberg & Samuelsson, 1974, Ursini & Bindoli, 1987). Peritoneal macrophages from mice lacking 12/15-LOX (12/15-LOX$^{-/-}$) show abnormalities in regulating cytokine production, both basally and following infection with *S.epi* (Dioszeghy *et al*, 2008). The addition of 12-HETE and 12-HpETE to 12/15-LOX$^{-/-}$ mice infected with *S.epi*, did not restore wild type (WT) phenotype (Dioszeghy *et al*, 2008). This suggests that alternative products formed by LOX may be responsible for its biological effects. While esterified products of 15-LOX known as 15-H(p)ETE-PEs have been identified in human monocytes, their metabolism has not been characterised (Maskrey *et al*, 2007). Given that 12/15-LOX may have important implications in several inflammatory disorders, it is important to investigate what happens to 15-H(p)ETE-PEs following their generation. This may lead to the identification of products that are responsible for the effects exerted by 12/15-LOX.

A study by Maskrey *et al* (2007) concentrated on characterising the initial generation of 15-H(p)ETE-PEs in human monocytes and established several points concerning these lipids.

(1) Following activation, approximately 30% of 15-H(p)ETE is esterified.

(2) Esterified 15-H(p)ETE is predominantly associated with PE phospholipids.

(3) Four 15-H(p)ETE-PEs are generated following monocyte activation (Scheme 2.1).

(4) H(p)ETE derived from human monocyte 15-H(p)ETE-PE consists mainly of the 'S' enantiomer.

(5) 15-H(p)ETE-PEs are formed by direct oxidation of AA-containing PE lipids (AA-PEs), and not by esterification of 15-H(p)ETE.

Following the discovery and structural characterisation of human monocyte 15-H(p)ETE-PEs by Maskrey *et al* (2007), the studies described in this chapter address the issue of how 15-H(p)ETE-PEs are metabolised following their generation. In addition, the quantification assay developed in Chapter 3 will be used for the first time, to directly quantify these novel phospholipids.

4.1.1 Aims

Establishing what happens to 15-H(p)ETE-PEs following their synthesis may have indicated how they are involved in complex immune processes. Therefore, the studies described in this chapter aimed to:

- Describe how 15-H(p)ETE-PEs are metabolised in human monocytes.

4.2 Results

4.2.1 15-H(p)ETE-PEs increase following monocyte activation and are metabolised by 3 hours following their synthesis.

The temporal generation of 15-H(p)ETE-PE generation by human monocytes was determined. Monocytes were activated for 10 to 180 minutes, and lipids reduced using $SnCl_2$ before extraction. Importantly, this reduction step converts all 15-HpETE to 15-HETE, thus detected 15-HETE-PEs is the total of esterified LOX products. Lipids were extracted then analysed by LC/MS/MS. 15-H(p)ETE-PEs increased significantly by 10 minutes following activation and then declined by 180 minutes (Figure 4.1). The results suggest that 15-H(p)ETE-PEs are metabolised following their synthesis.

4.2.2 *15-H(p)ETE-PE metabolism does not involve hydrolysis of 15-H(p)ETE, conversion of the PE head group to PC or elongation of 15-H(p)ETE.*

There are several possibilities for the metabolism of 15-H(p)ETE-PEs, including cleavage or elongation of the Sn2 fatty acid, or conversion of the PE head group to PC or PS. To investigate whether the decrease in 15-H(p)ETE-PEs may have been due to cleavage of the sn2 fatty acid by PLA_2, time-course samples were analysed by LC/MS/MS for free 15-H(p)ETE. The results showed that free 15-H(p)ETE increased by 10 minutes, but then remained the same throughout the time-course (Figure 4.2). Constant levels of free 15-H(p)ETE suggested either that free 15-H(p)ETE was not metabolised following its synthesis, or that free 15-H(p)ETE was simultaneously cleaved and re-esterified. To investigate this latter possibility, monocyte samples were spiked with free 15-HETE-d_8 then activated for time-points of 10 - 180 minutes. The lipids were then extracted and analysed by LC/MS/MS. 15-HETE-d_8 levels did not change throughout

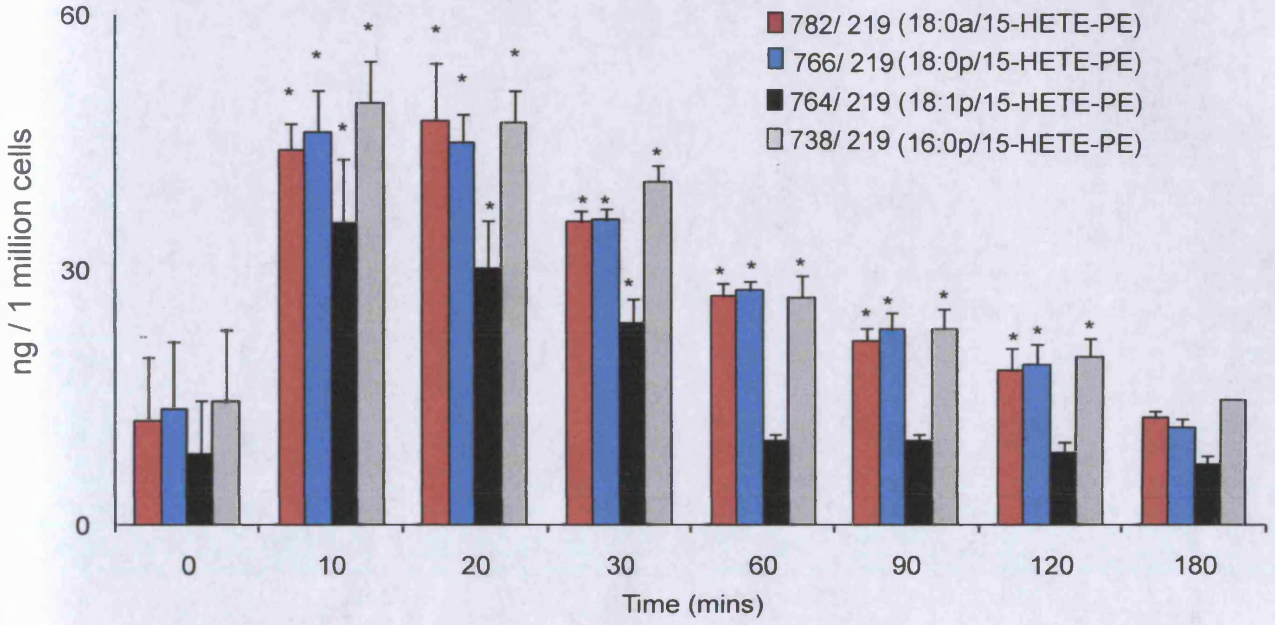


Figure 4.1. 15-H(p)ETE-PEs are metabolised to unknown products by 3 hours. Human monocytes were activated with 10 μM A23187 for various time points followed by reduction with $SnCl_2$. Lipids were extracted and analysed by LC/MS/MS (n = 3, mean ± SEM). *Students t-test, $p < 0.05$, with one-tailed Mann-Whitney test, the difference of each analyte is significant in comparison to its relative control.

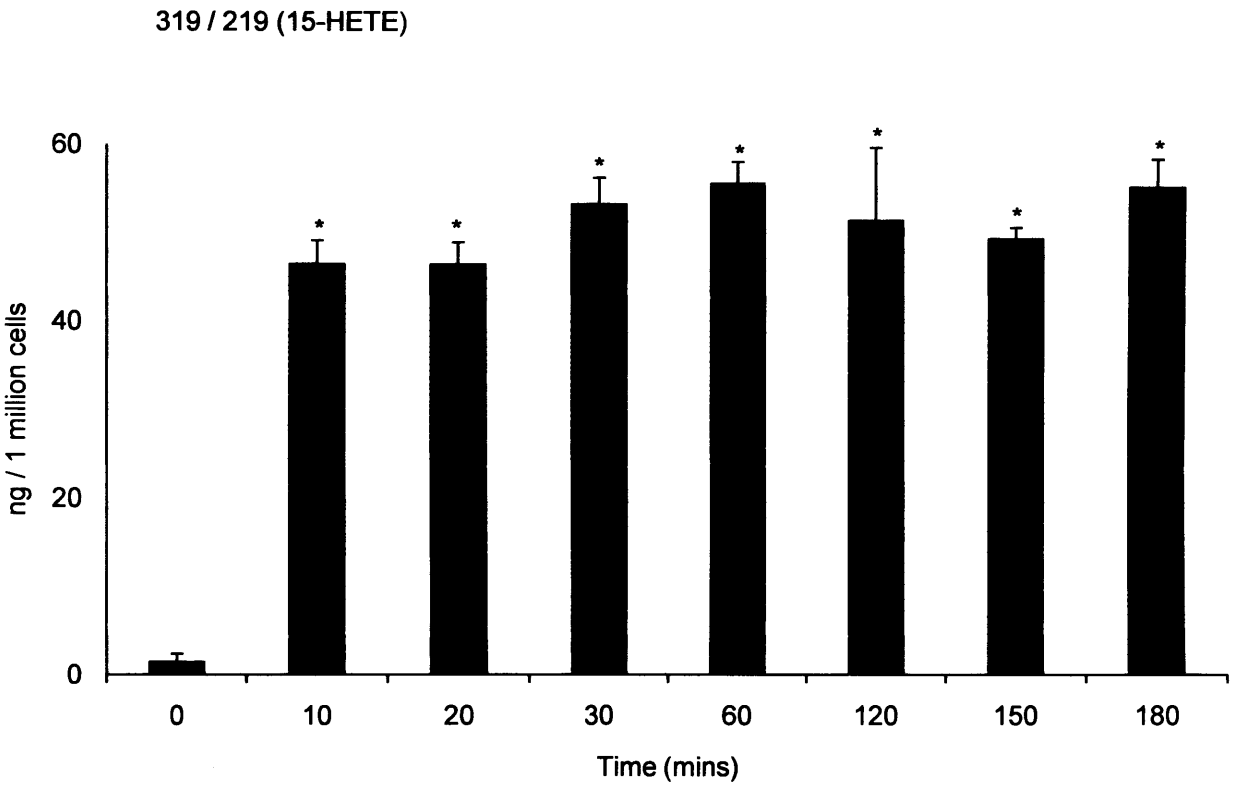


Figure 4.2 15-H(p)ETE remains stable following synthesis. Human monocytes were activated with 10 μM A23187 for various time points then reduced with $SnCl_2$. Lipids were extracted and analysed by LC/MS/MS (n = 3, mean ± SEM). *Students t-test, $p < 0.05$, the difference is significant in comparison to the control sample.

the time-course, which indicates that 15-H(p)ETE is unlikely to undergo simultaneous cleavage and re-esterification (Figure 4.3). To further confirm that 15-H(p)ETE is not being hydrolysed from 15-H(p)ETE-PEs, human monocytes were incubated with PLA_2 inhibitors and a PAF-AH inhibitor, prior to activation. Levels of 15-H(p)ETE-PEs increased following activation, then returned to baseline by three hours (Figure 4.4), similar to the activated monocyte time-course shown in Figure 4.1. If hydrolysis of 15-H(p)ETE had been a method of 15-H(p)ETE-PE metabolism, inhibiting PLA_2 enzymes have resulted in levels of 15-H(p)ETE-PEs remaining the same throughout the time-course following their synthesis, rather than declining to basal levels. Therefore, the metabolism of 15-H(p)ETE-PEs does not involve hydrolysis of 15-H(p)ETE by PLA_2.

The decrease in 15-H(p)ETE-PEs seen in Figure 4.1 may have been due to conversion of the phospholipid head group from PE to PC or PS, by PE methyltransferase or PS synthase, respectively (Berg *et al*, 2002) (Schemes 4.1 and 4.2). In order to study conversion to PC, a 18:0a/15-HETE-PC standard was synthesised. SAPC was oxidised using soybean LOX, followed by $SnCl_2$ reduction and lipid extraction. The 18:0a/15-HETE-PC product was then analysed by MS to determine its *m/z* and elution time. LC/MS/MS analysis and product ion spectra were acquired in both positive and negative modes, which confirmed the identity and elution time of 18:0a/15-H(p)ETE-PC (Figure 4.5). 16:0p, 18:0p and 18:1p/15-H(p)ETE-PCs were also synthesised by oxidising a mixture of brain plasmalogen PC phospholipids using soybean LOX, but the product ion spectra were inadequate for identification and their elution times could not be confirmed. Consequently, monocyte samples were analysed for 18:0a/15-HETE-PC only.

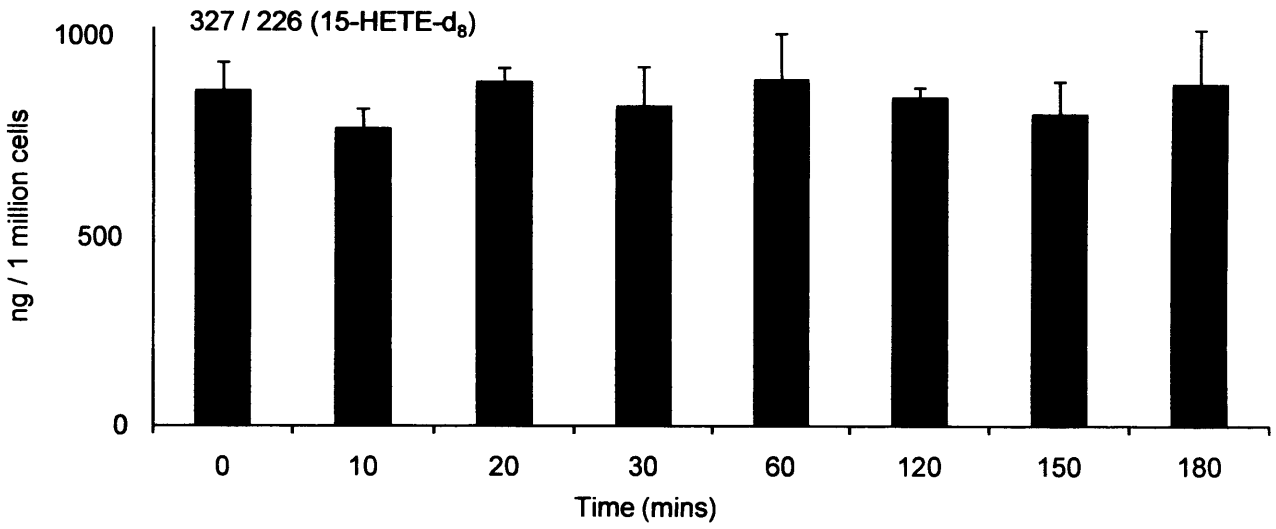


Figure 4.3 15-HETE-d_8 is not esterified into phospholipids of human monocytes. 15-HETE-d_8 was added to human monocytes prior to activation with 10 μM A23187. Samples were activated for various time-points followed by lipid extraction then analysis by LC/MS/MS (n = 3, mean ± SEM).

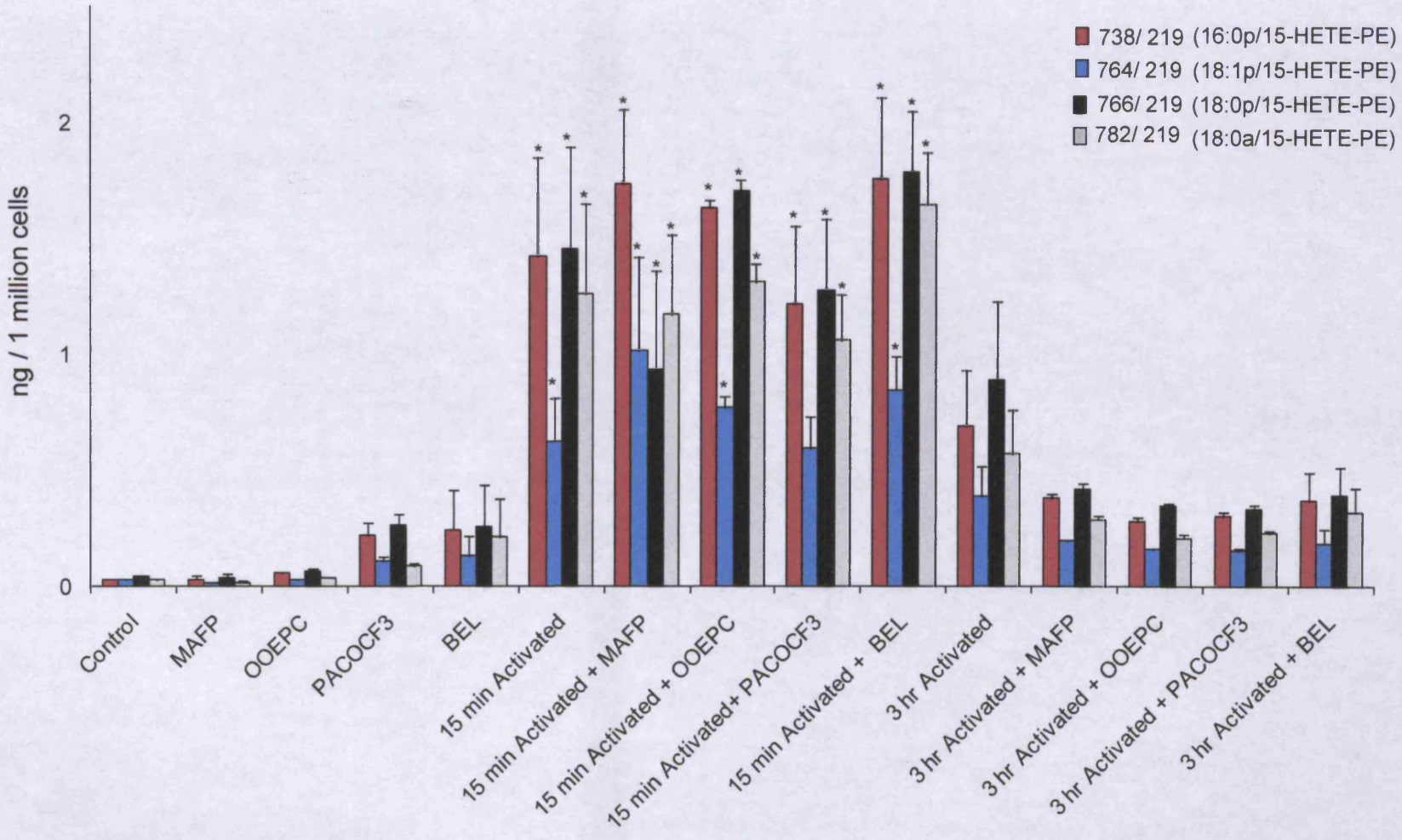
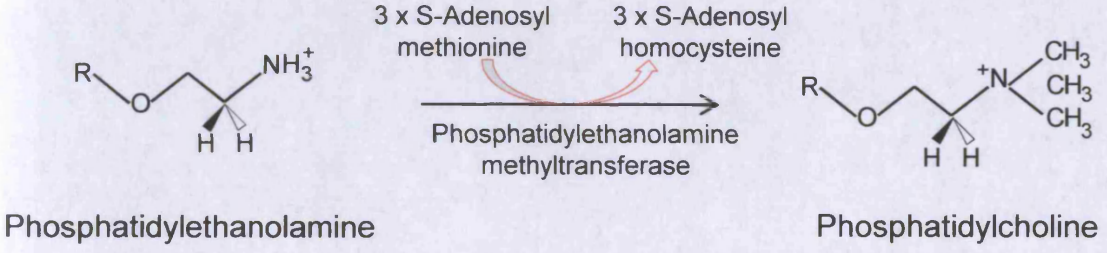


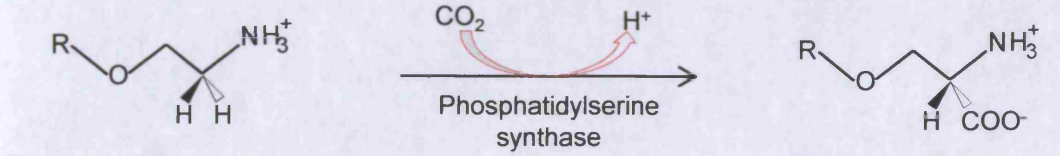
Figure 4.4 15-H(p)ETE-PEs are not metabolised by PLA_2 or PAF-AH enzymes. Human monocytes were activated with 10 μM A23187 for 15 minutes and 3 hours following incubation with the PLA_2 inhibitors, OOEPC, BEL and $PACOCF_3$ or the PAF-AH inhibitor, MAFP. Samples were reduced with $SnCl_2$ followed by lipid extraction and LC/MS/MS analysis (n = 3, mean ± SEM). * Students t-test, $p < 0.05$ with one-tailed Mann-Whitney test, the difference of each analyte is significant in comparison to its relative control .



Phosphatidylethanolamine

Phosphatidylcholine

Scheme 4.1 Conversion of the phospholipid head group from phosphatidylethanolamine to phosphatidylcholine.



Phosphatidylethanolamine

Phosphatidylserine

Scheme 4.2 Conversion of the phospholipid head group from phosphatidylethanolamine to phosphatidylserine

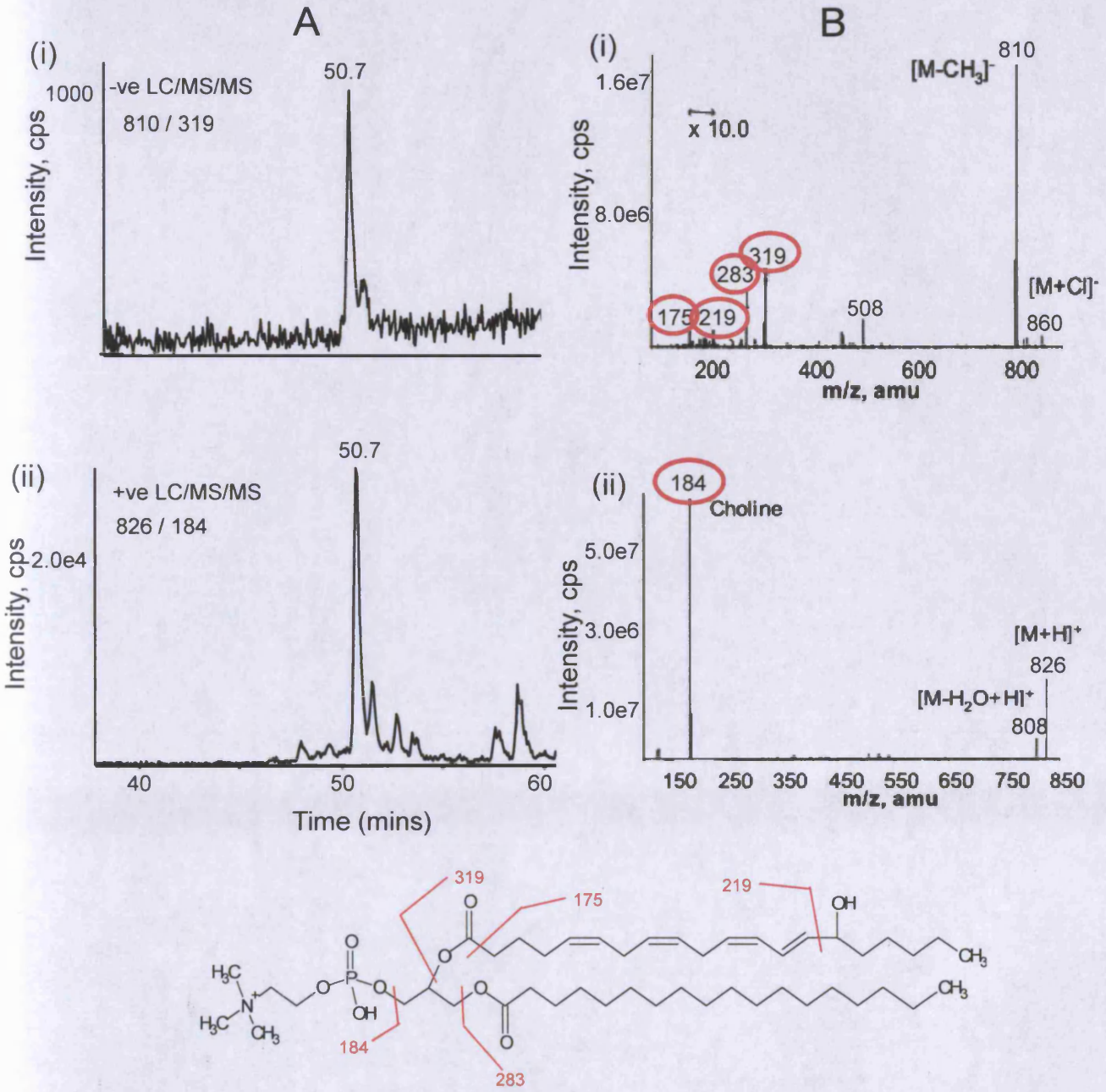


Figure 4.5 18:0a/15-H(p)ETE-PC standard was successfully synthesised. SAPC was oxidised using soybean LOX, then reduced with $SnCl_2$ to form 18:0a/15-HETE-PC. The lipid product was extracted and its elution time and identity was confirmed by LC/MS/MS analysis (A) and product ion spectra (B) in both negative (i) and positive modes (ii).

To investigate the generation of 18:0a/15-HETE-PC, human monocytes were activated using A23187 for up to 180 minutes followed by reduction, lipid extraction then analysis by LC/MS/MS. 18:0a/15-H(p)ETE-PC increased by 10 minutes then returned to basal levels by three hours, similar to 15-H(p)ETE-PEs, however, the signals were very weak indicating low abundance (Figure 4.6). This suggests that 18:0a/15-H(p)ETE-PC may be a minor product of 15-LOX and not formed following the conversion of the 18:0a/15-HETE-PE head group to PC. Since PE can also be converted to PS, samples were also analysed by LC/MS/MS for transitions equivalent to 15-HETE-PSs but the correct peaks could not be identified due to low abundance (data not shown), therefore conversion of the PE head group to PS cannot be excluded but is presumed unlikely. Overall, the results suggest that conversion of the 15-H(p)ETE-PE head group to PC or PS is not a likely route of their metabolism, at least to PC.

Elongation of the sn2 fatty acid (H(p)ETE) by the addition of C_2H_4 was also investigated as a possible route for 15-H(p)ETE-PE metabolism (Rosenthal & Hill, 1986). Theoretical transitions for 15-H(p)ETE-PE + C_2H_4 were calculated and then monocyte lipids were analysed by LC/MS/MS (Scheme 2.7). If 15-H(p)ETE was undergoing elongation, levels of 15-H(p)ETE-PEs + C_2H_4 would be expected to rise after 10 minutes, corresponding with the decline seen for 15-H(p)ETE-PEs in Figure 4.1. However, over 180 minutes levels of 15-H(p)ETE-PE + C_2H_4 did not change significantly. A small increase was seen during the same timescale as activation but signals were weak, indicating low abundance. The results suggest that elongation of 15-H(p)ETE is not a route of 15-H(p)ETE-PE metabolism (Figure 4.7).

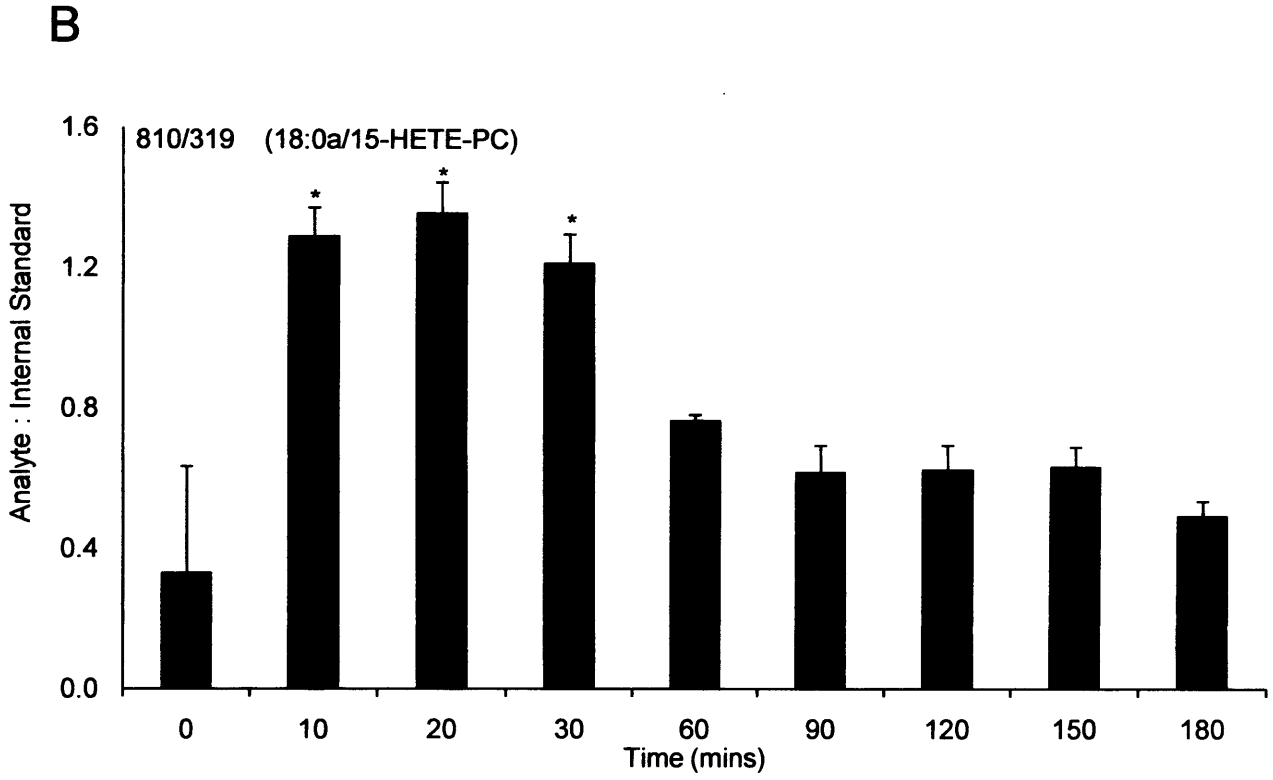


Figure 4.6. Metabolism of 15-H(p)ETE-PE is unlikely to involve conversion of the PE head group to PC. Human monocytes were activated with 10 μM A23187 for various time points followed by reduction with $SnCl_2$. Lipids were extracted and analysed by LC/MS/MS for 18:0/15-HETE-PC (n = 3, mean ± SEM). *Students t-test, $p < 0.05$ with one-tailed Mann-Whitney test, the difference is significant in comparison to the control sample..

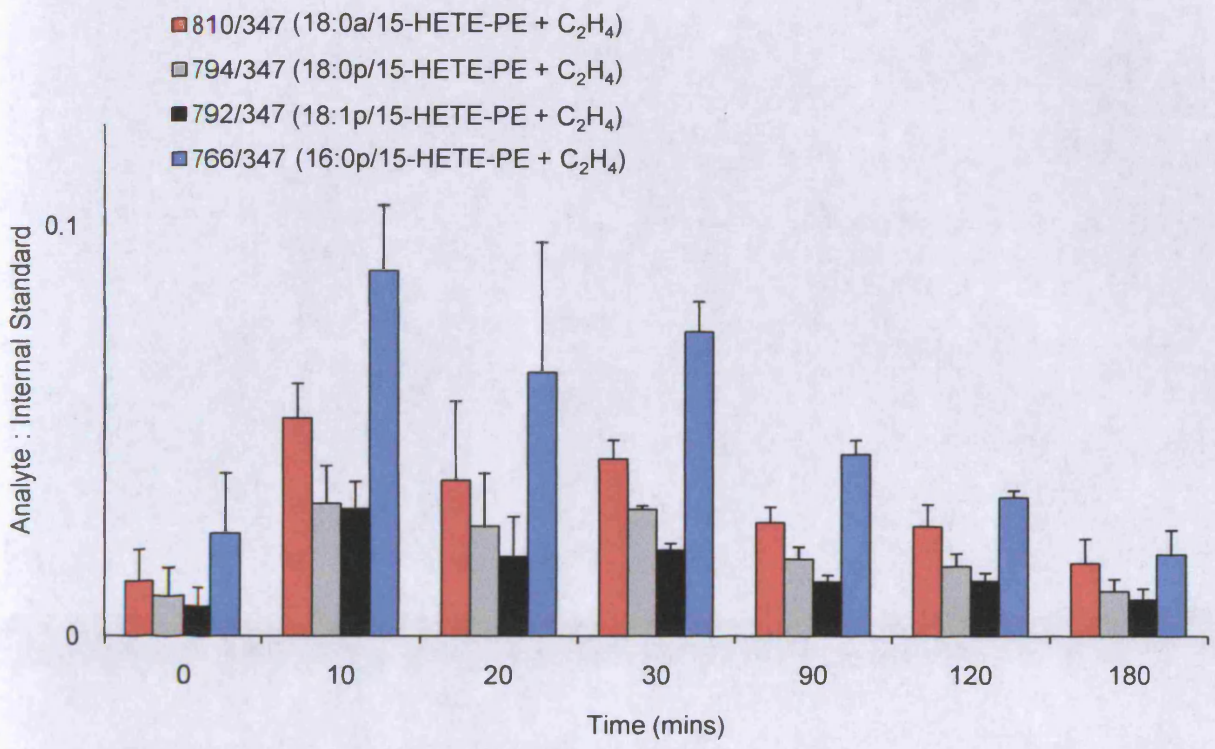


Figure 4.7. Metabolism of 15-H(p)ETE-PE is unlikely to involve chain elongation of H(p)ETE. Human monocytes were activated with 10 μM A23187 for up to 180 minutes. Samples were reduced with $SnCl_2$ followed by lipid extraction. Theoretical transitions with calculated *m/z* corresponding to an elongation of C_2H_4 to H(p)ETE portion were calculated and analysed by LC/MS/MS (n = 3, mean ± SEM).

4.2.3 Products of 15-LOX are not all endogenously reduced to 15-HETE-PEs.

Previous studies have shown that HpETE products of LOX are reduced to HETEs by GPx enzymes (Ursini *et al*, 1987). Therefore, to aid detection of 15-LOX products in human monocytes, lipid extracts were routinely reduced using $SnCl_2$, which stabilised all HpETE-PEs to more stable HETE-PEs. However, as the methods of 15-H(p)ETE-PE metabolism described above were excluded, it is possible that 15-HpETE-PEs were not endogenously reduced to 15-HETE-PE in human monocytes. It was previously shown that there is a loss of cytosolic glutathione in rat hepatocytes following activation with A23187 (Olafsdottir *et al*, 1988). Therefore, following A23187 activation of human monocytes, the amount of GPx present may be insufficient to reduce all of the products synthesised by LOX. If lipids were extracted from human monocytes with no prior reduction, then the detected 15-HETE-PEs would be due to endogenous reduction by GPx. Following $SnCl_2$ treatment, detected 15-HETE-PEs are made up of both endogenous 15-HETE-PEs and additional lipid products of 15-LOX that can be chemically reduced to 15-HETE-PEs. Therefore, levels of 15-HETE-PEs would be higher in $SnCl_2$ treated samples.

The amount of 15-HETE-PE generated endogenously in monocyte samples was investigated. Human monocytes were activated for up to 180 minutes, at each time-point half of each sample was immediately removed into lipid extraction buffer (non-reduced). The remaining half was reduced by $SnCl_2$ prior to lipid extraction, then samples were analysed by LC/MS/MS. The results show that products of 15-LOX are not all endogenously reduced to form 15-HETE-PEs. Throughout the time-course, 27 to 38% of 15-LOX products were endogenously reduced to 15-HETE-PEs and levels remained constant following their initial synthesis (Figure 4.8). In samples reduced using $SnCl_2$, 15-H(p)ETE-PEs increased by 15 minutes then declined by 180 minutes.

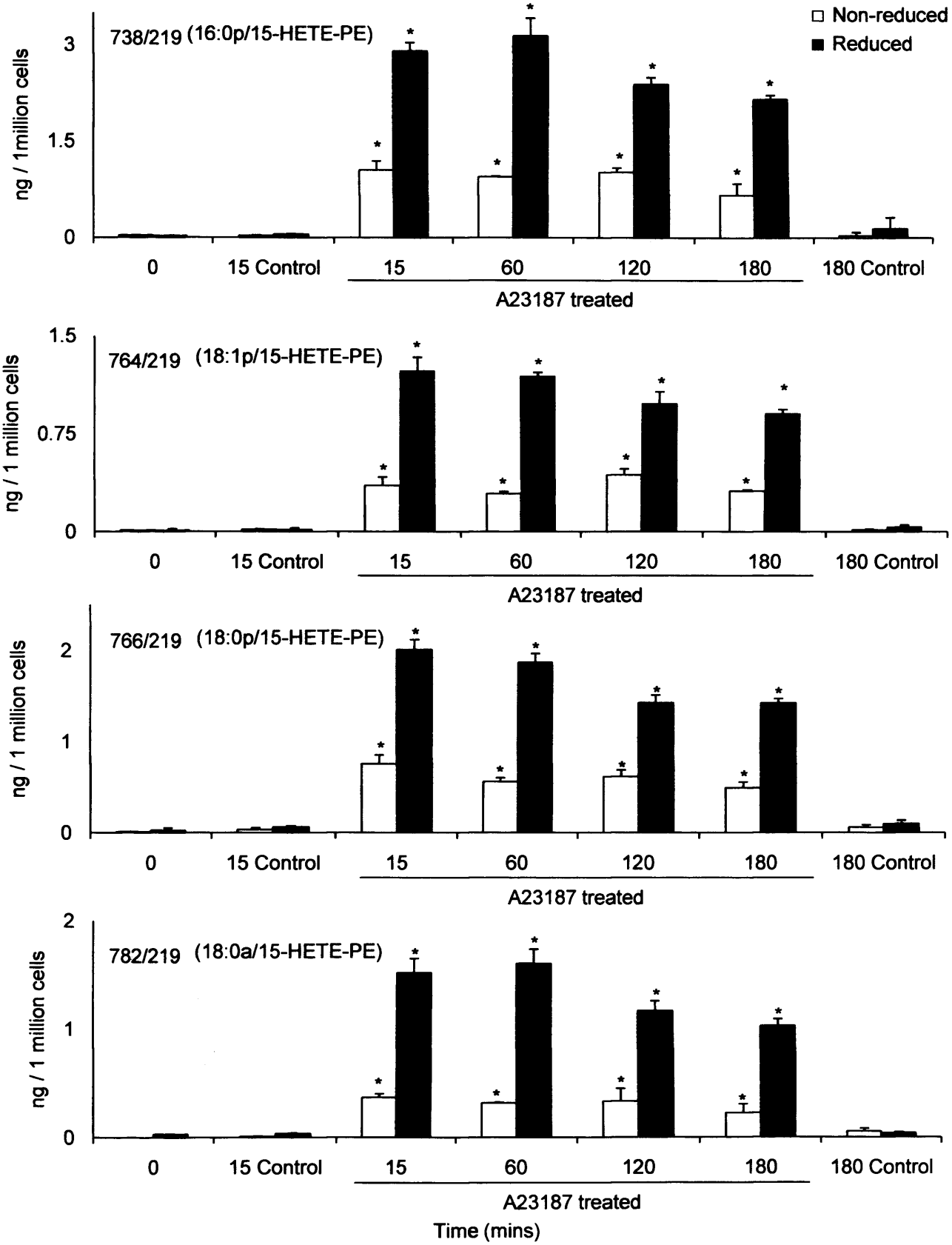


Figure 4.8. Products of 15-LOX are not all endogenously reduced to 15-HETE-PE. Monocytes were activated with 10 μM A23187 for various time points. Samples underwent lipid extraction either directly or following reduction with $SnCl_2$. Lipids were analysed by LC/MS/MS (n = 3, mean ± SEM). □ Non-reduced, ■ Reduced. * Students t-test, $p < 0.05$ with one-tailed Mann-Whitney test, the difference of each analyte is significant in comparison to its relative control.

As endogenous 15-HETE-PEs remained stable, the 15-H(p)ETE-PE decrease seen in reduced samples is likely to be due to the metabolism of a 15-HETE-PE precursor.

4.2.4 15-HpETE-PEs are unlikely to be the precursors of 15-HETE-PEs that are metabolised in time-course samples.

To identify lipids in human monocytes that can be reduced to form 15-HETE-PEs, the time-course samples generated for Section 4.2.3 were analysed by LC/MS/MS for transitions corresponding to possible 15-HETE-PE precursors. Since 15-LOX is reported to synthesise 15-HpETE, which is also known to form 15-HETE following reduction, 15-HpETE-PEs were possible precursors of 15-HETE-PEs ($[M-H]^-$ *m/z* 756, 780, 782 and 798 to a daughter ion of 317) (Scheme 2.8) (Ursini *et al*, 1987). 15-HpETE-PEs were detected in non-reduced monocyte samples, but their levels were very low and in some cases, undetectable (as for 16:0p/15-HpETE-PE) (Figures 4.9B to 4.12B). If 15-HpETE-PEs were precursor lipids of 15-HETE-PEs synthesised following $SnCl_2$ reduction, then the difference in 15-HpETE-PEs between reduced and non-reduced samples would be expected to correlate with the variation seen for 15-HETE-PEs. However, results from the quantification of 15-HpETE-PEs showed that their levels were too low to account for the differences in 15-HETE-PEs between reduced and non-reduced samples.

4.2.5 15-KETE-PEs are formed following 15-LOX activation and are metabolised to unknown products.

Free 15-ketoeicosatetraenoic acid (15-KETE) (also known as 15-oxoETE) has been detected in human monocytes and 12-KETE is reported to form 12-HETE following reduction (Falgueyret *et*

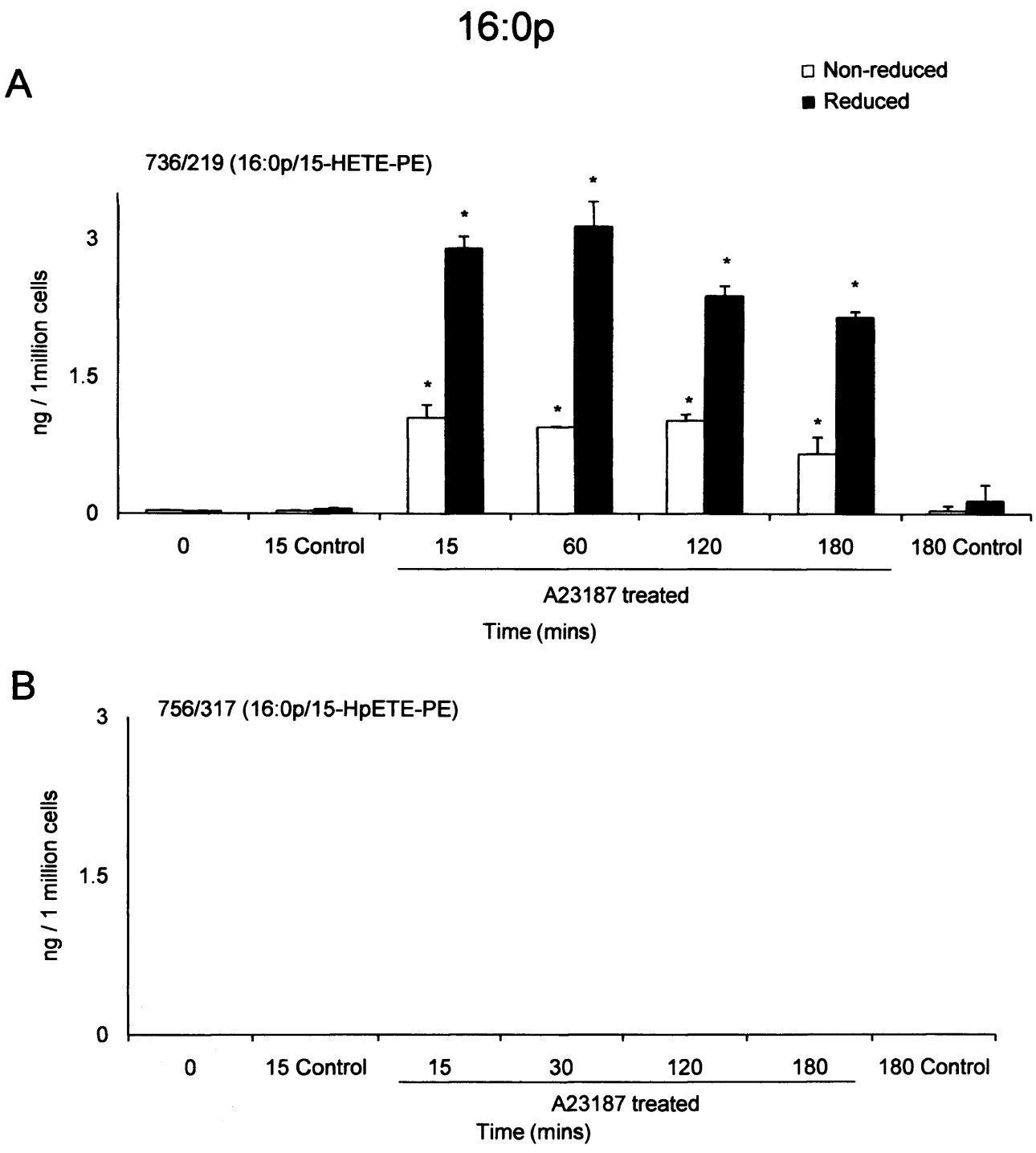


Figure 4.9 16:0p/15-HpETE-PE is unlikely to be the 16:0p 15-HETE-PE precursor that is metabolised by 3 hours. Monocytes were activated with 10 μM A23187 for various time points. Samples underwent lipid extraction either directly or following reduction with $SnCl_2$. Lipids were analysed by LC/MS/MS. 15-HETE-PE (A), 15-HpETE-PE (B) products of 16:0p phospholipids (n = 3, mean ± SEM). □ Non-reduced, ■ Reduced. * Students t-test, $p < 0.05$ with one-tailed Mann-Whitney test, the difference of each analyte is significant in comparison to its relative control.

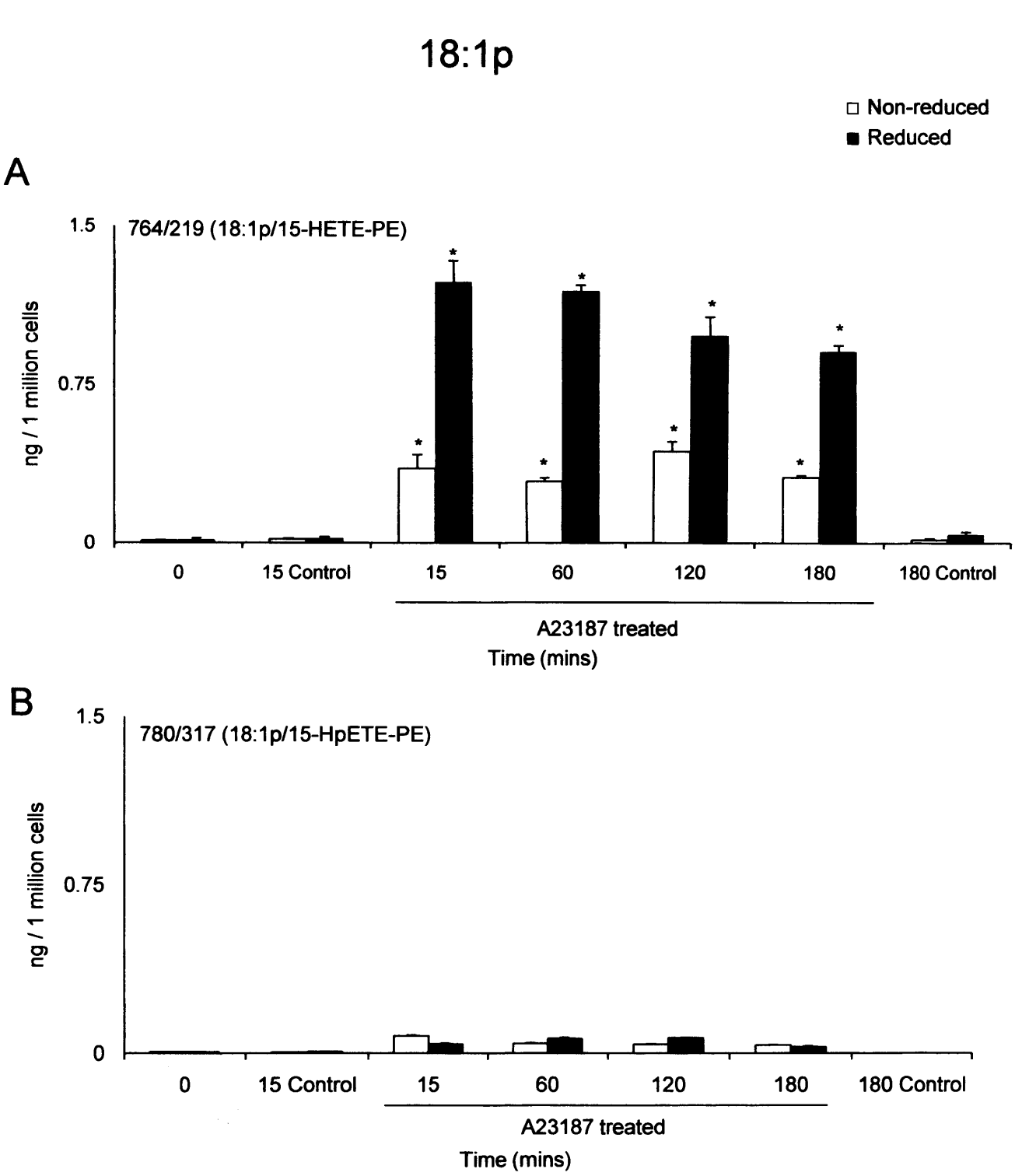


Figure 4.10 18:1p/15-HpETE-PE is unlikely to be the 18:1p 15-HETE-PE precursor that is metabolised by 3 hours. Monocytes were activated with 10 μM A23187 for various time points. Samples underwent lipid extraction either directly or following reduction with $SnCl_2$. Lipids were analysed by LC/MS/MS. 15-HETE-PE (A), 15-HpETE-PE (B) products of 18:1p phospholipids (n = 3, mean ± SEM). □ Non-reduced, ■ Reduced. * Students t-test, $p < 0.05$ with one-tailed Mann-Whitney test, the difference of each analyte is significant in comparison to its relative control.

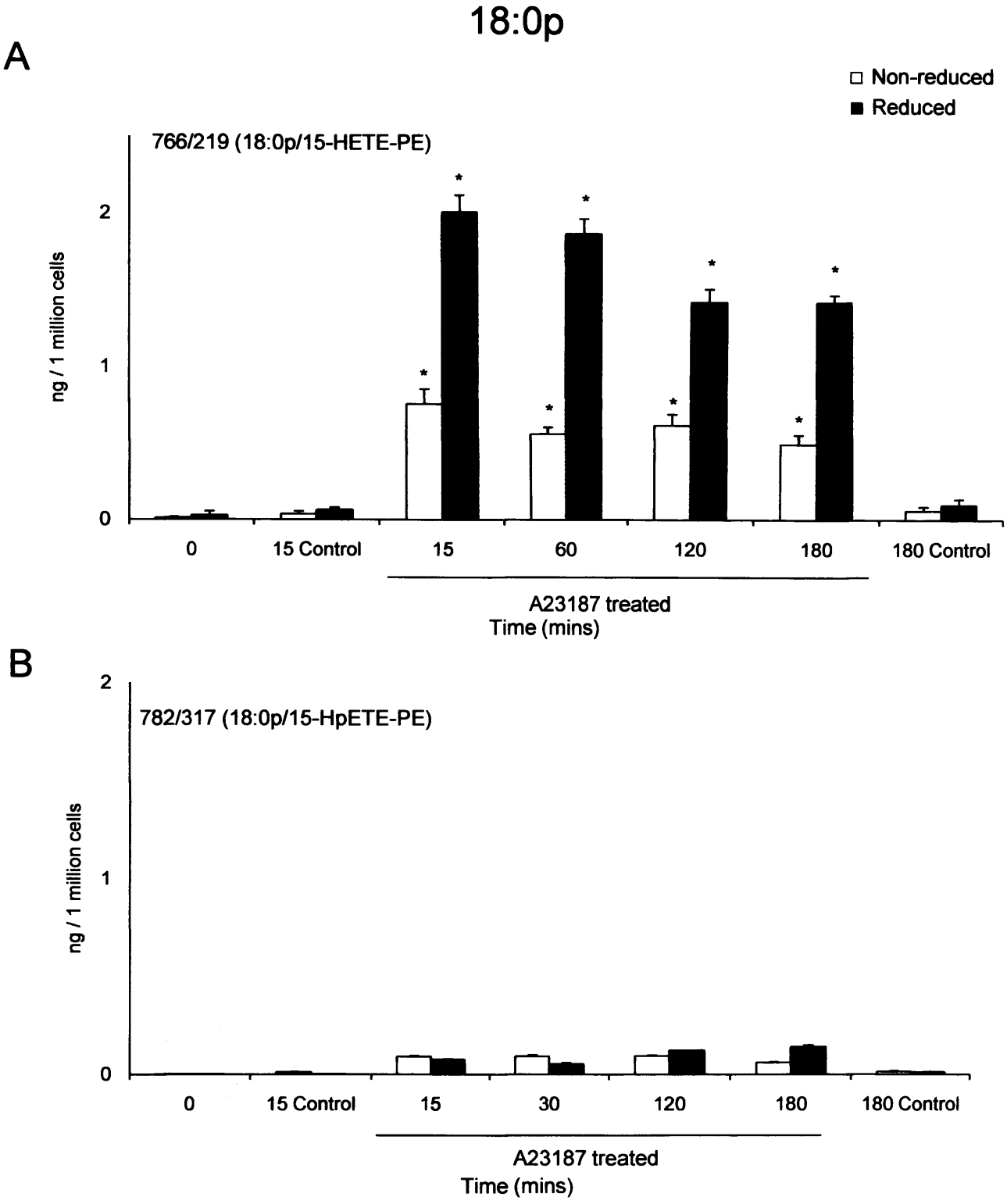


Figure 4.11 18:0p/15-HpETE-PE is unlikely to be the 18:0p 15-HETE-PE precursor that is metabolised by 3 hours. Monocytes were activated with 10 μM A23187 for various time points. Samples underwent lipid extraction either directly or following reduction with $SnCl_2$. Lipids were analysed by LC/MS/MS. 15-HETE-PE (A), 15-HpETE-PE (B) products of 18:0p phospholipids (n = 3, mean ± SEM). □ Non-reduced, ■ Reduced. * Students t-test, $p < 0.05$ with one-tailed Mann-Whitney test, the difference of each analyte is significant in comparison to its relative control.

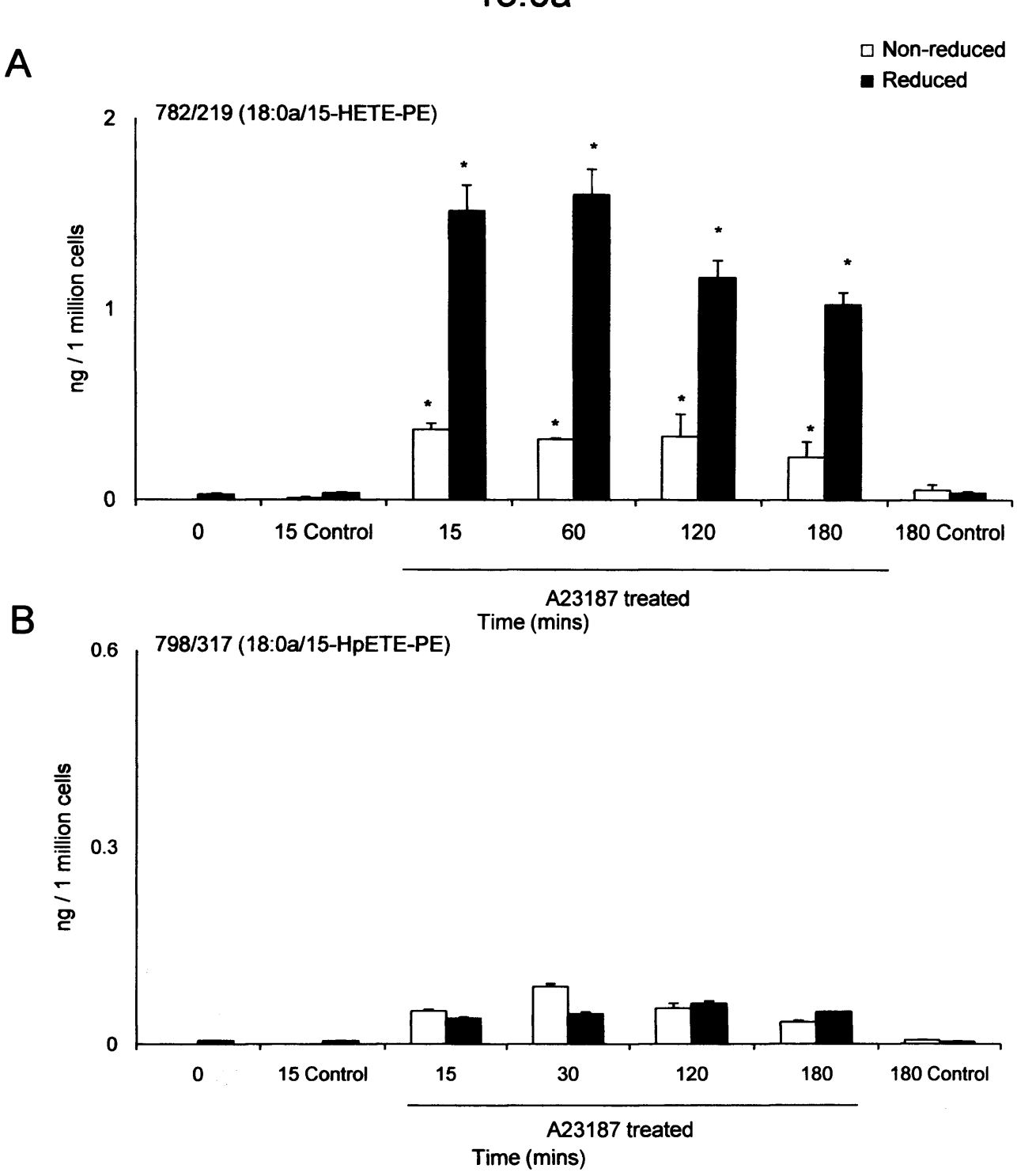


Figure 4.12 18:0a/15-HpETE-PE is unlikely to be the 18:0a 15-HETE-PE precursor that is metabolised by 3 hours. Monocytes were activated with 10 μM A23187 for various time points. Samples underwent lipid extraction either directly or following reduction with $SnCl_2$. Lipids were analysed by LC/MS/MS. 15-HETE-PE (A), 15-HpETE-PE (B) products of 18:0a phospholipids (n = 3, mean ± SEM). □ Non-reduced, ■ Reduced. * Students t-test, $p < 0.05$ with one-tailed Mann-Whitney test, the difference of each analyte is significant in comparison to its relative control.

al, 1990, Wei *et al*, 2007). Therefore, other potential lipid precursors of 15-HETE-PE include the carbonyl containing 15-KETE-PEs ($[M-H]^-$ *m/z* 736, 762, 764 and 780 to a daughter ion of 317) (Scheme 2.9). Therefore, the presence and identity of 15-KETE-PEs were investigated in human monocytes prior to their analysis in time-course samples. Monocytes were activated for 15 minutes with A23187 then the lipids were extracted and analysed by LC/MS/MS. Product ion spectra were acquired at the apices of detected peaks (Figures 4.13 to 4.16 A). Product ion spectra of the particular peaks highlighted in Figures 4.13-16 contained fragments that are also seen in the product ion spectra of a 15-KETE standard, shown in a published study (*m/z* 113, 203, 219, 255, 273 and 299, labelled in green) (Murphy *et al*, 2005). In particular, fragments typical of 15-KETE-PEs were seen in these product ion spectra, confirming their identity.

Next, monocyte time-course samples were analysed for 15-KETE-PEs. The results show that 15-KETE-PEs increased in non-reduced samples following activation, then declined by three hours (Figure 4.17). Consistent with the criteria of the 15-HETE-PE precursor, 15-KETE-PEs diminished following reduction, which corresponded to the increase in levels of 15-HETE-PEs. The results therefore suggest that 15-KETE-PEs form 15-HETE-PEs following reduction, and that they may be the lipids undergoing metabolism during time-course experiments.

4.2.6 18:0a/15-HpETE-PE forms 18:0a/15-KETE-PE following its addition to monocytes.

15-KETE-PEs are novel lipids and how they are synthesised by cells is not known. They may be formed by three pathways, (i) directly following the action of 15-LOX on AA-PE, (ii) following metabolism of the 15-HpETE-PE peroxyl group to a carbonyl, (iii) following enzymatic conversion of 15-HETE (Scheme 4.3) (McMurry, J, 2000, Bergholte *et al,* 1987, Wei *et al,*

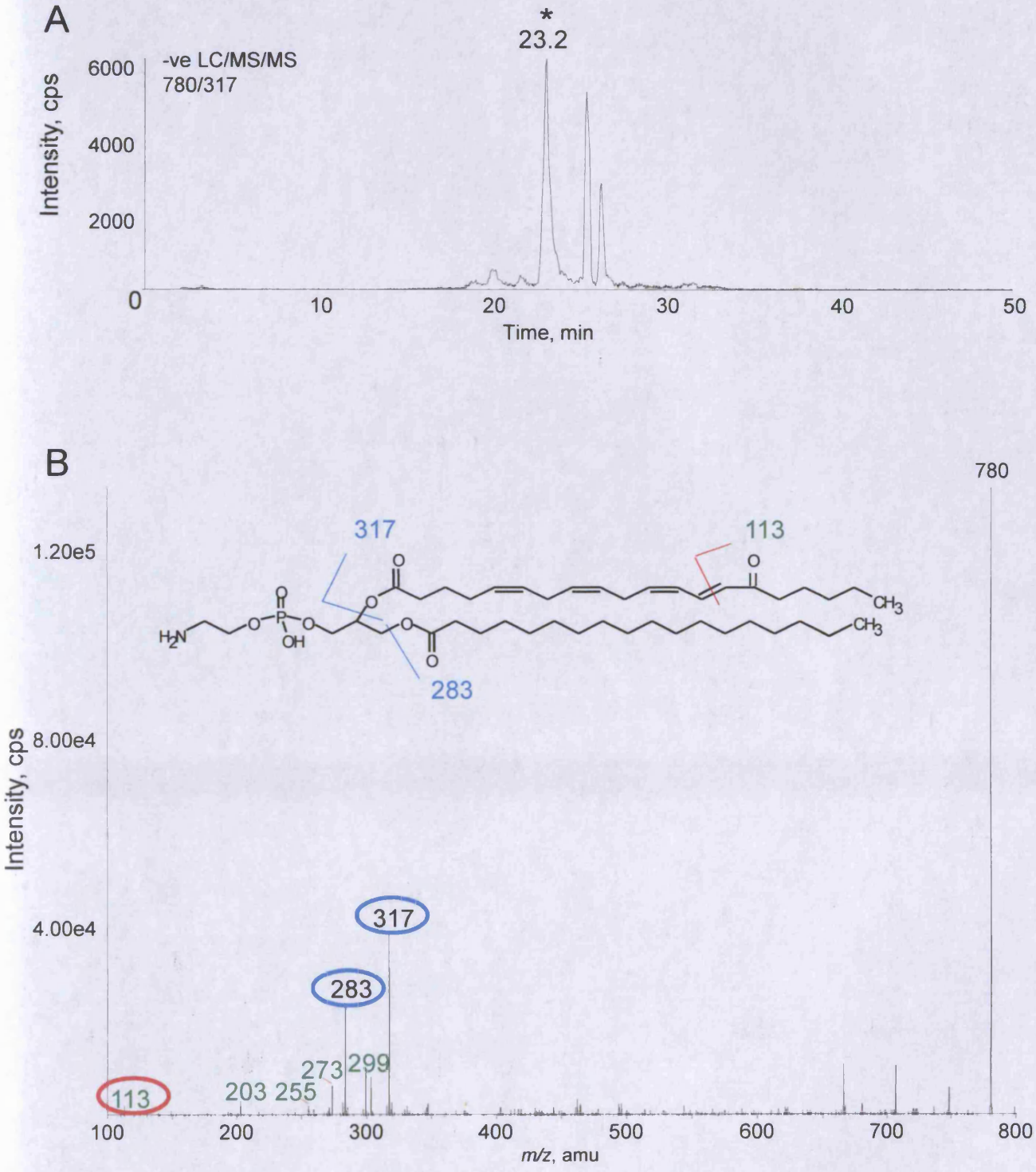


Figure 4.13 LC/MS/MS trace and product ion spectrum of 18:0a/15-KETE-PE. Non-reduced time-course samples were analysed by LC/MS/MS (A), at the peak apex (*) product ion spectra were taken (B). Fragments labeled in green are typical of 15-KETE , as published by Murphy *et al* (2005).

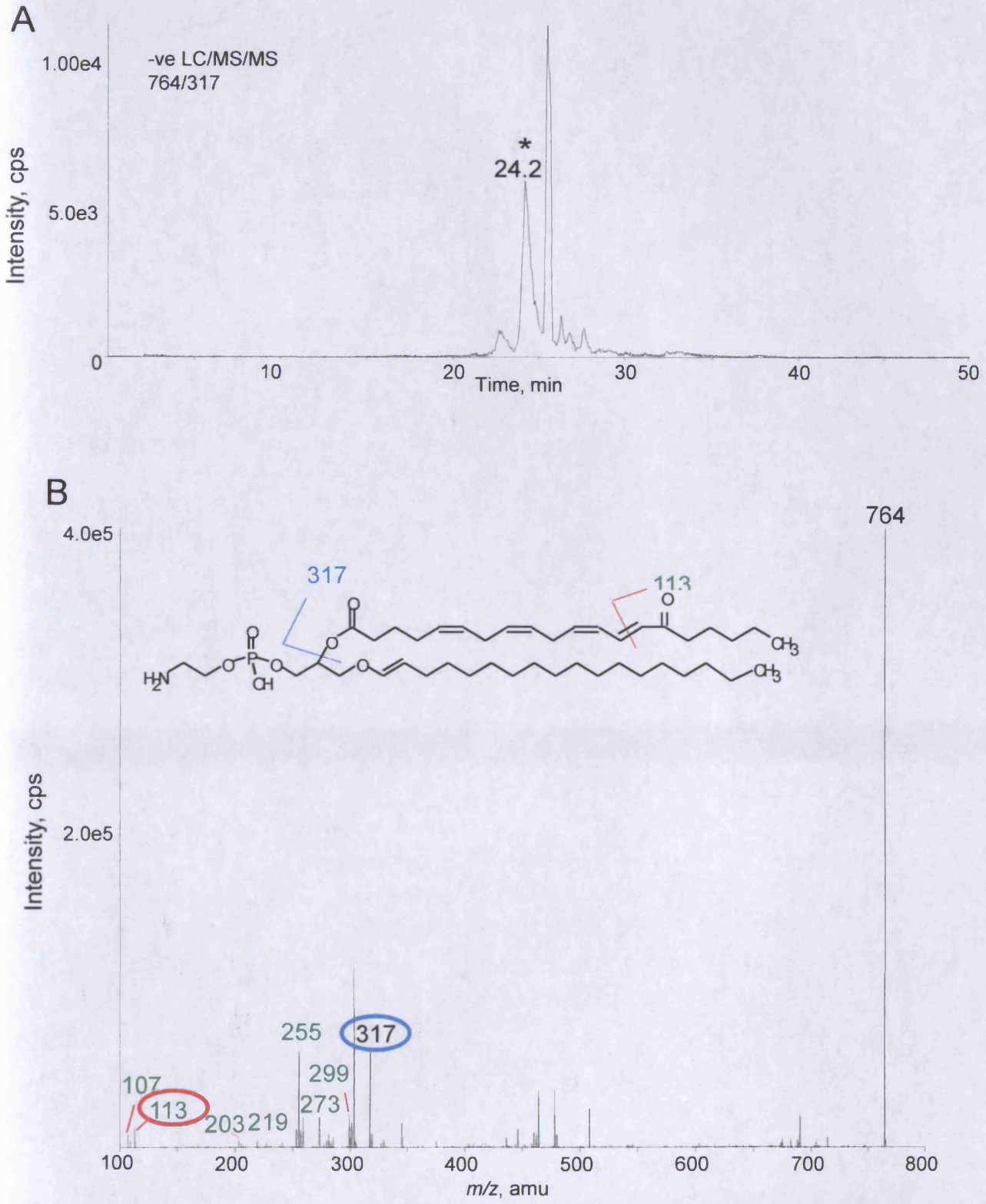


Figure 4.14 LC/MS/MS trace and product ion spectrum of 18:0p/15-KETE-PE. Non-reduced time-course samples were analysed by LC/MS/MS (A), at the peak apex (*) product ion spectra were taken (B). Fragments labeled in green are typical of 15-KETE, as published by Murphy *et al* (2005).

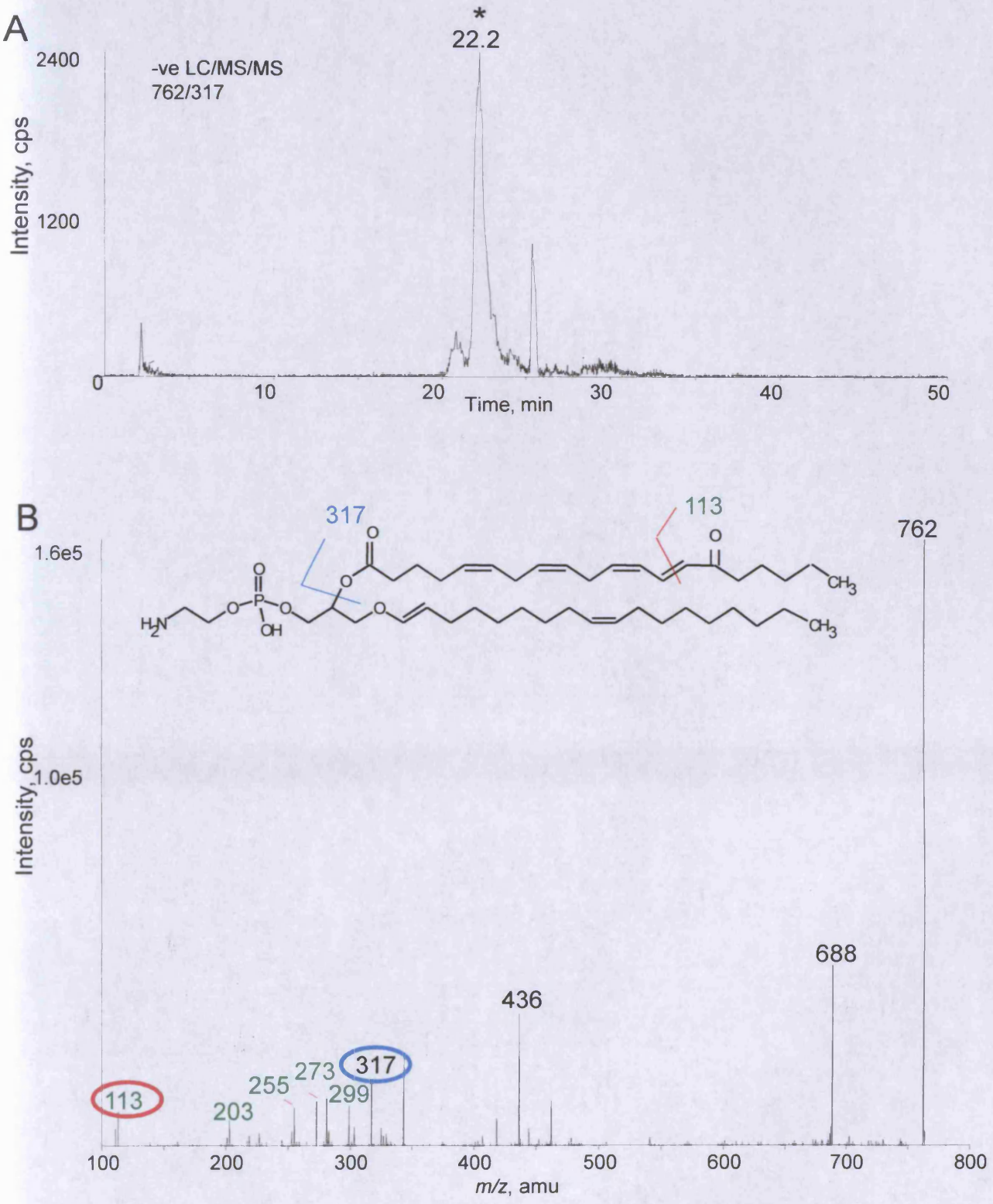


Figure 4.15 LC/MS/MS trace and product ion spectrum of 18:1p/15-KETE-PE. Non-reduced time-course samples were analysed by LC/MS/MS (A), at the peak apex (*) product ion spectra were taken (B). Fragments labeled in green are typical of 15-KETE, as published by Murphy *et al* (2005).

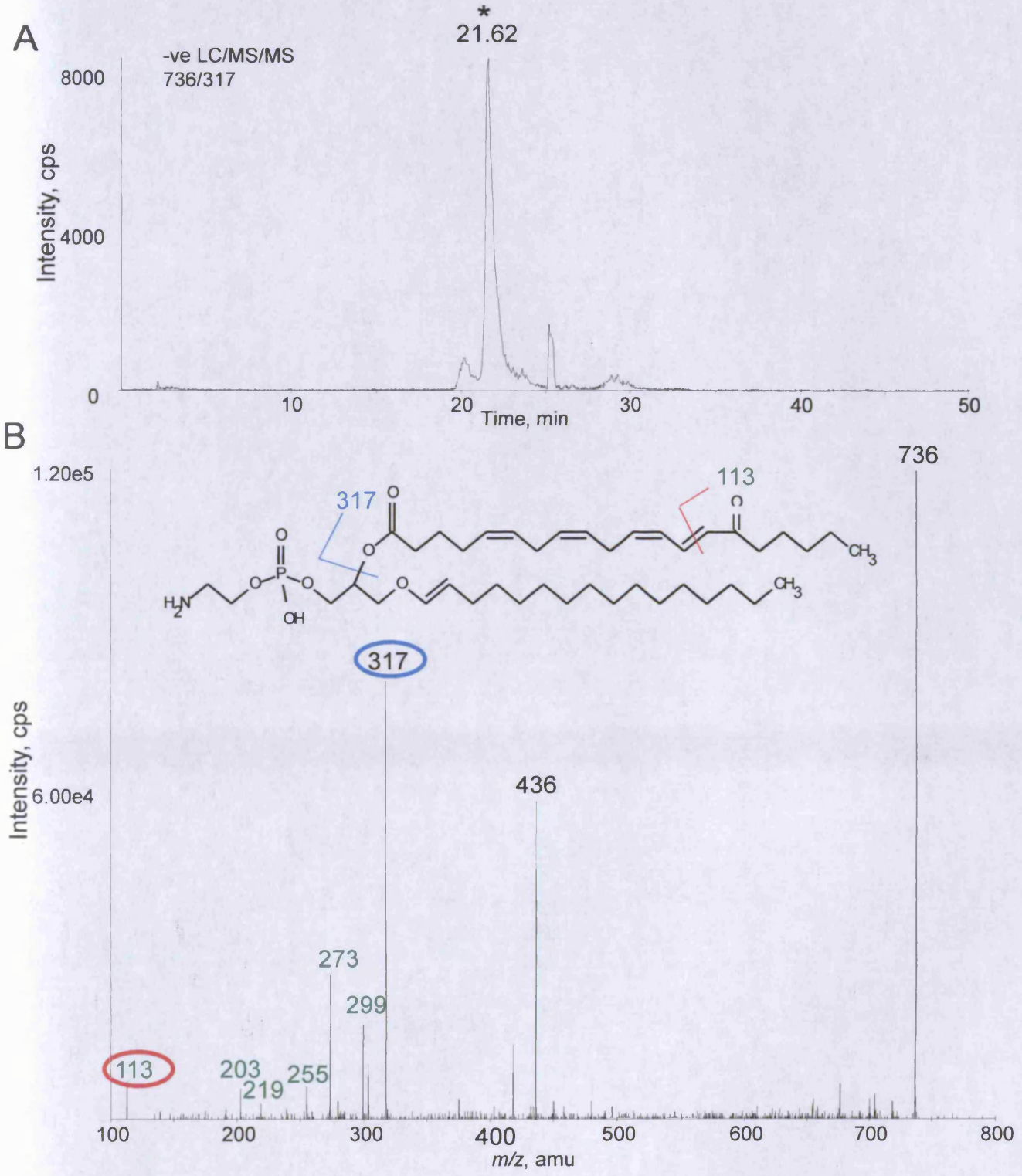


Figure 4.16 LC/MS/MS trace and product ion spectrum of 16:0p/15-KETE-PE. Non-reduced time-course samples were analysed by LC/MS/MS (A), at the peak apex (*) product ion spectra were taken (B). Fragments labeled in green are typical of 15-KETE, as published by Murphy *et al* (2005).

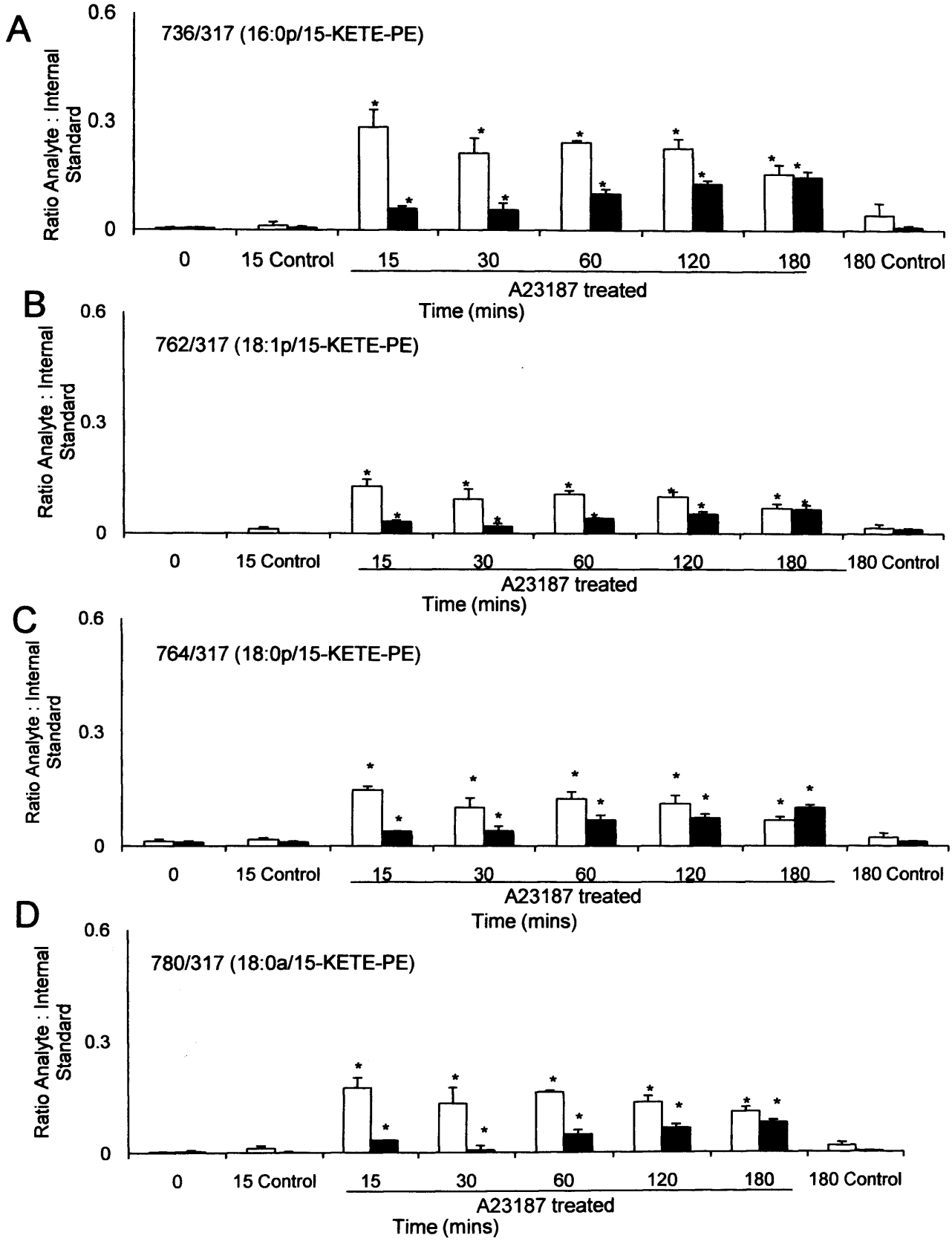
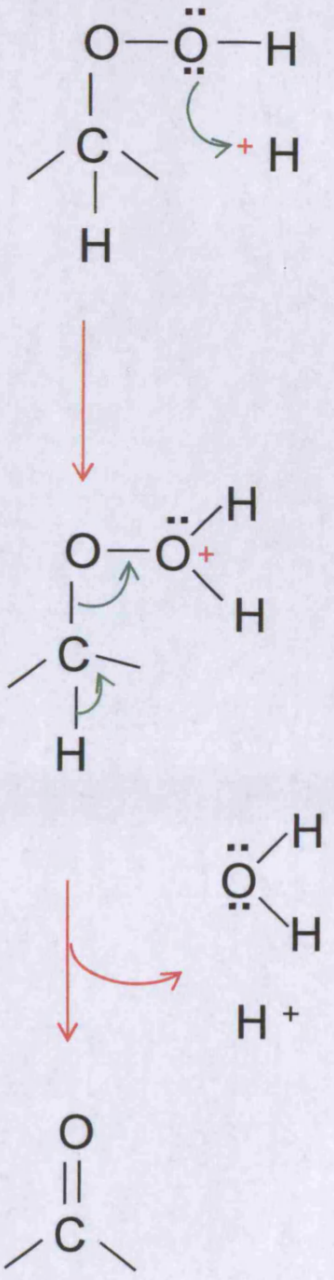


Figure 4.17 15-KETE-PEs are generated following activation of 15-LOX. Monocytes were activated with 10 μM A23187 for various time points. Samples underwent lipid extraction either directly or following reduction with $SnCl_2$. Lipids were analysed by LC/MS/MS for 15-KETE-PE (n = 3, mean ± SEM). □ Non-reduced, ■ Reduced. * Students t-test, $p < 0.05$ with one-tailed Mann-Whitney test, the difference of each analyte is significant in comparison to its relative control.



Scheme 4.3 Formation of carbonyl following the decomposition of a peroxyl group (adapted from McMurry J, 2000, with permsssion (04/12/2010).

2009). Previous studies have shown that free 12-HpETE and 5-HpETE can be converted enzymatically to 12- and 5-KETE respectively, thus supporting the second method of conversion (Piomelli *et al*, 1988, Zarini & Murphy, 2003). As a 15-HpETE-PE standard was available, studies investigated whether monocytes can convert exogenously added 15-HpETE-PE to 15-KETE-PE. 18:0a/15-HpETE-PE was added to human monocytes, followed by lipid extraction and analysis by LC/MS/MS. In cell-free buffer samples containing 18:0a/15-HpETE-PE, 18:0a/15-KETE-PE was also detected, as well as a small amount of 18:0a/15-HETE-PE (Figure 4.18). This is likely to be due to the instability of 15-HpETE-PE standard, which may spontaneously degrade to these lipids in storage. However, 18:0a/15-HpETE-PE added to human monocytes was fully metabolised, and corresponded with a significant increase in 18:0a/15-KETE-PE, and a small increase in 18:0a/15-HETE-PE (Figure 4.18). The results suggest that monocytes can convert exogenously added 15-HpETE-PE to 15-KETE-PE.

4.2.7 15-KETE-PEs are metabolised to unknown products by three hours following their generation.

Results in non-reduced time-course samples shown in Figures 4.9-4.12 C suggested that 15-KETE-PEs are metabolised following their synthesis. The 15-KETE-PEs could be metabolised to two potential products, hydroxyeicosatrienoic acid-PEs (HETrE-PEs) (Scheme 2.10) ($[M-H]^-$ *m/z* 738, 766, 768 and 784 to 321) and/or di-HETE-PEs (Scheme 2.11) ($[M-H]^-$ *m/z* 738, 780, 782 and 798 to 335), therefore levels of these products were investigated in monocyte time-course samples described in Section 4.2.3. If 15-KETE-PEs were converted to these products, they would be expected to increase during the later time-points, corresponding to the decrease in detected 15-KETE-PEs. A large fold increase was seen following activation for di-HETE-PEs in

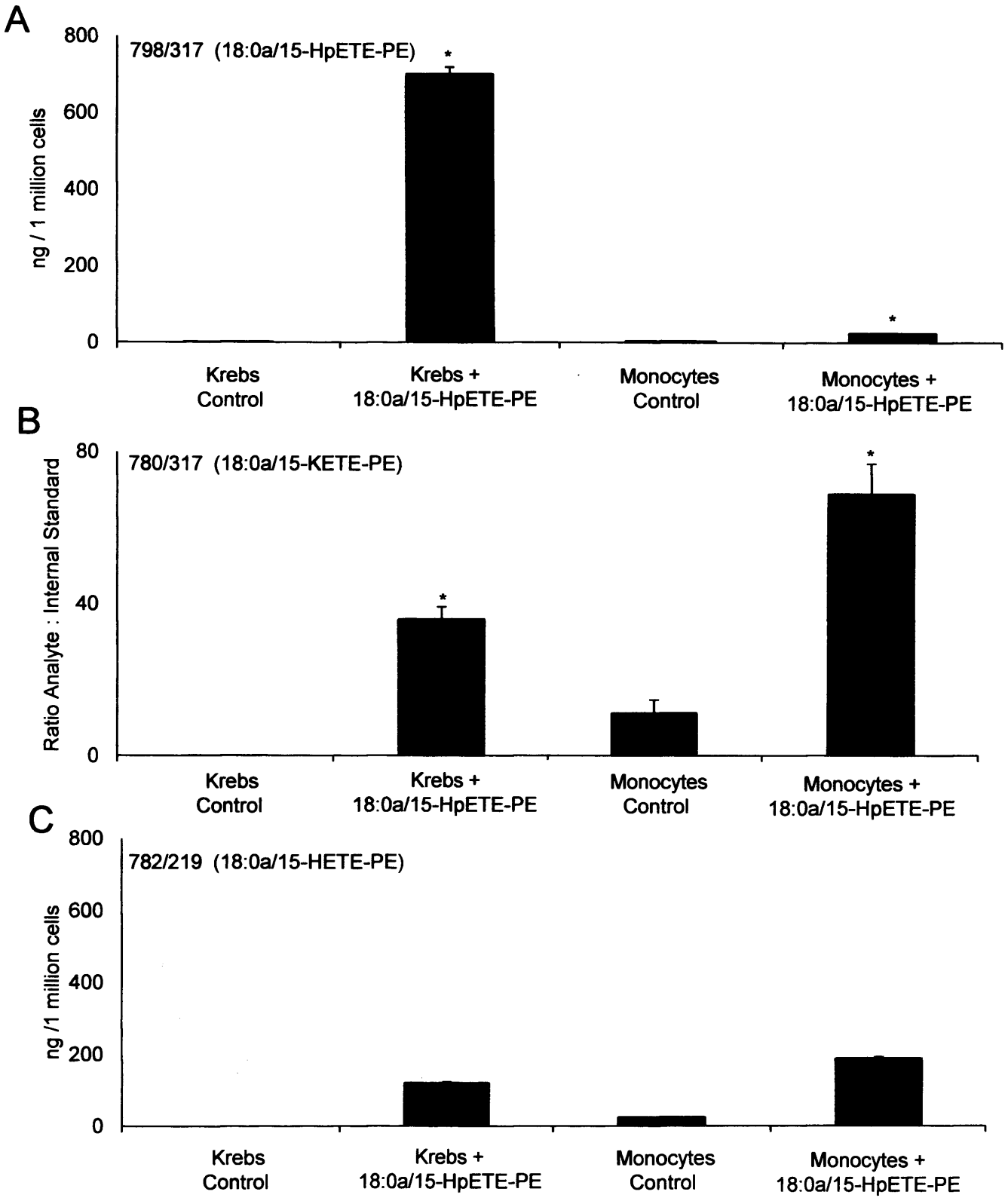


Figure 4.18 18:0a/15-HpETE-PE can be converted to 18:0a/15-KETE-PE by monocytes. 18:0a/15-HpETE-PE was added to human monocytes followed by immediate lipid extraction. Samples were analysed by LC/MS/MS for 18:0a/15-HpETE-PEs (A), 18:0a/15-KETE-PEs (B) and 18:0a/15-HETE-PEs (C) (n = 3, mean ± SEM). To note, no samples were reduced by $SnCl_2$.*Students t-test, $p < 0.05$, the difference of each analyte is significant in comparison to its relative control

non-reduced and reduced samples and for HETrE-PEs in reduced samples only. However, levels of di-HETE-PEs and HETrE-PEs remained constant throughout the time-course (Figure 4.19). The daughter ion used during LC/MS/MS analysis for di-HETE-PEs, *m/z* 335, is also the *m/z* of several other analytes, including several potential isomers of di-HETEs and leukotriene B_4. The large increase following activation may be due to the analysis of multiple lipids with the same parent to daughter ion transition and retention time. Though, the results suggest that 15-KETE-PEs are not metabolised to HETrE-PEs or di-HETE-PEs as levels of these compounds do not increase throughout the time-course.

4.2.8 15-H(p)ETE-PEs are principally retained by monocytes while free 15-H(p)ETE is secreted following cellular activation.

Products of 12/15-LOX are suspected to be involved in regulating inflammation, possibly by acting as signalling molecules. Therefore, experiments were carried out to assess whether 15-H(p)ETE-PEs were secreted or retained by monocytes following their generation. Monocytes were activated for 15 minutes, then free and cell-associated 15-H(p)ETE-PEs were separated by centrifugation. Samples were reduced followed by lipid extraction and analysis by LC/MS/MS. 15-H(p)ETE-PEs were mostly retained by monocytes following activation, conversely, free 15-H(p)ETE was primarily secreted (Figure 4.20). The results suggest that if 15-H(p)ETE-PEs are involved in signalling, then they may have autocrine or intracrine actions. However, as free 15-H(p)ETE were mostly secreted, it could have paracrine signalling properties.

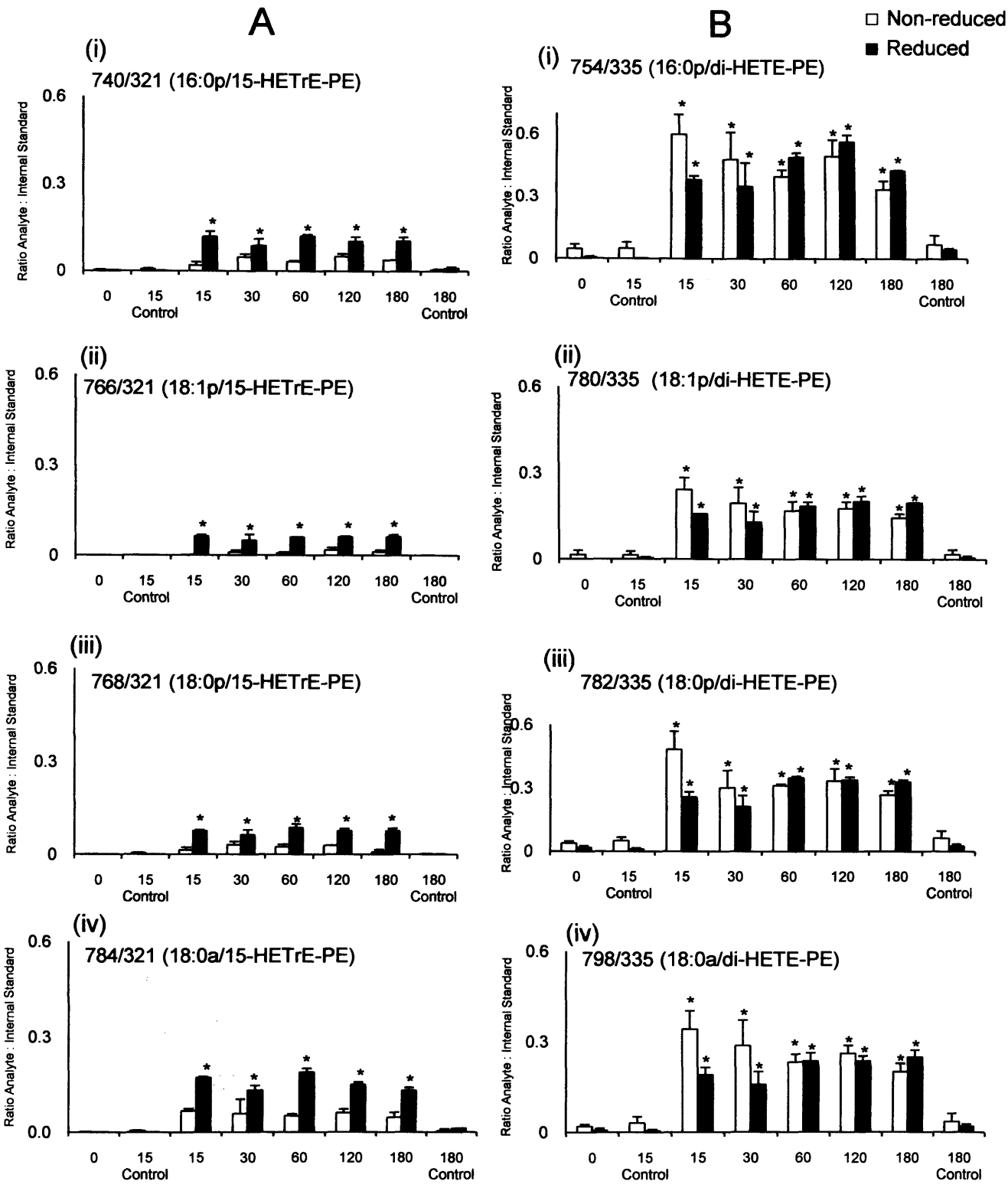


Figure 4.19 15-KETE-PEs are not metabolised to 15-HETrE-PEs or di-HETE-PEs. Monocytes were activated with 10 μM A23187 for various time points. Samples underwent lipid extraction either directly or following reduction with $SnCl_2$. 15-HETrE-PE (A) and di-HETE-PE (B) products for 16:0p (i), 18:1p (ii), 18:0p (iii) and 18:0a (iv) phospholipids were analysed by LC/MS/MS (n = 3, mean ± SEM). * Students t-test, $p < 0.05$ with one-tailed Mann-Whitney test, the difference of each analyte is significant in comparison to its relative control.

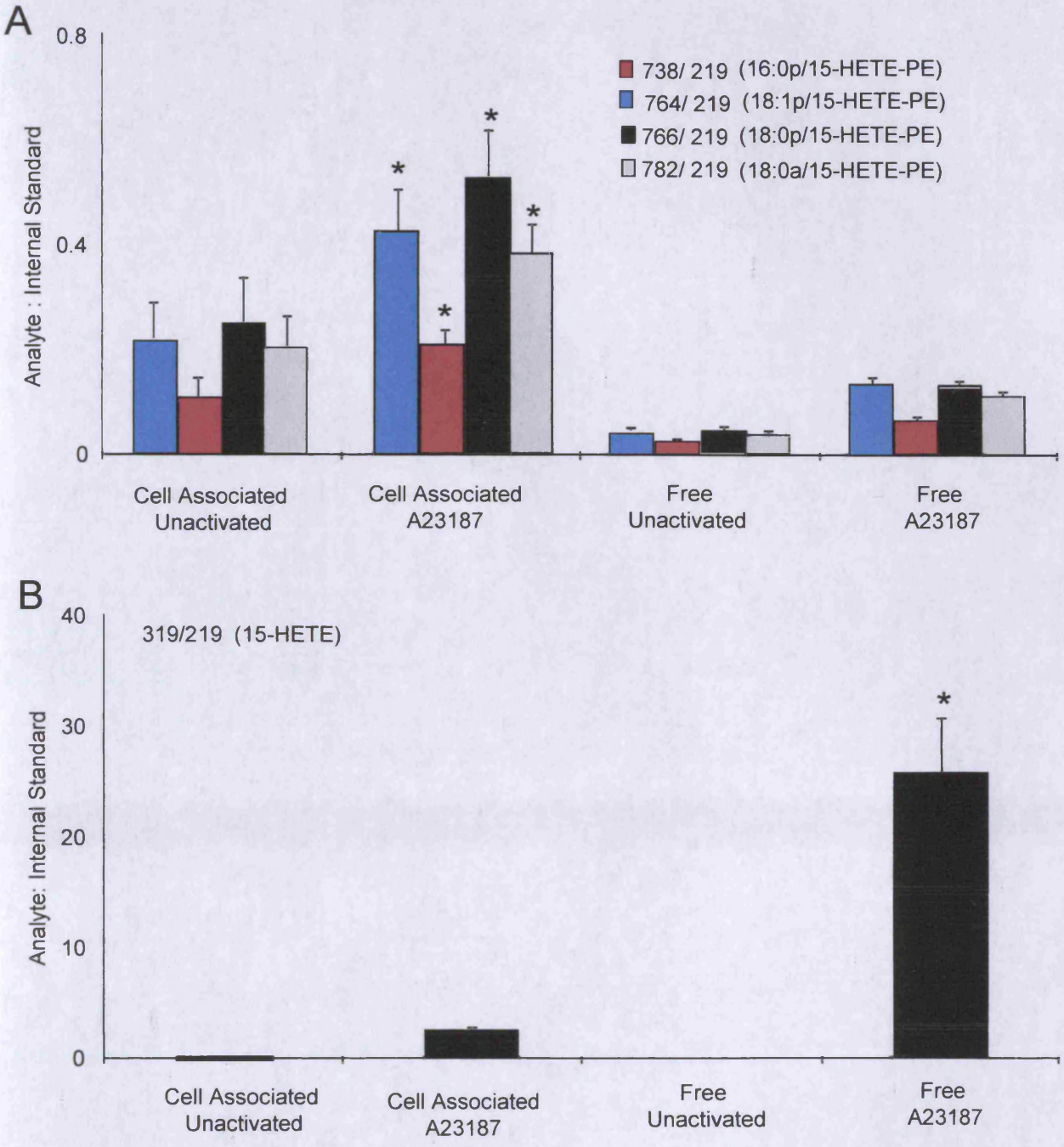


Figure 4.20. 15-H(p)ETE-PEs are retained by monocytes following their generation while free 15-H(p)ETE is secreted. Human peripheral blood monocytes were activated with 10 μM A23187 for 15 minutes. Lipids from the supernatant and cellular extracts were reduced then extracted and analysed by LC/MS/MS for 15-H(p)ETE-PEs (A) and 15-H(p)ETE (B) (n = 3, mean ± SEM). * Students t-test, $p < 0.05$, the difference of each analyte is significant in comparison to its relative control.

4.3 Discussion

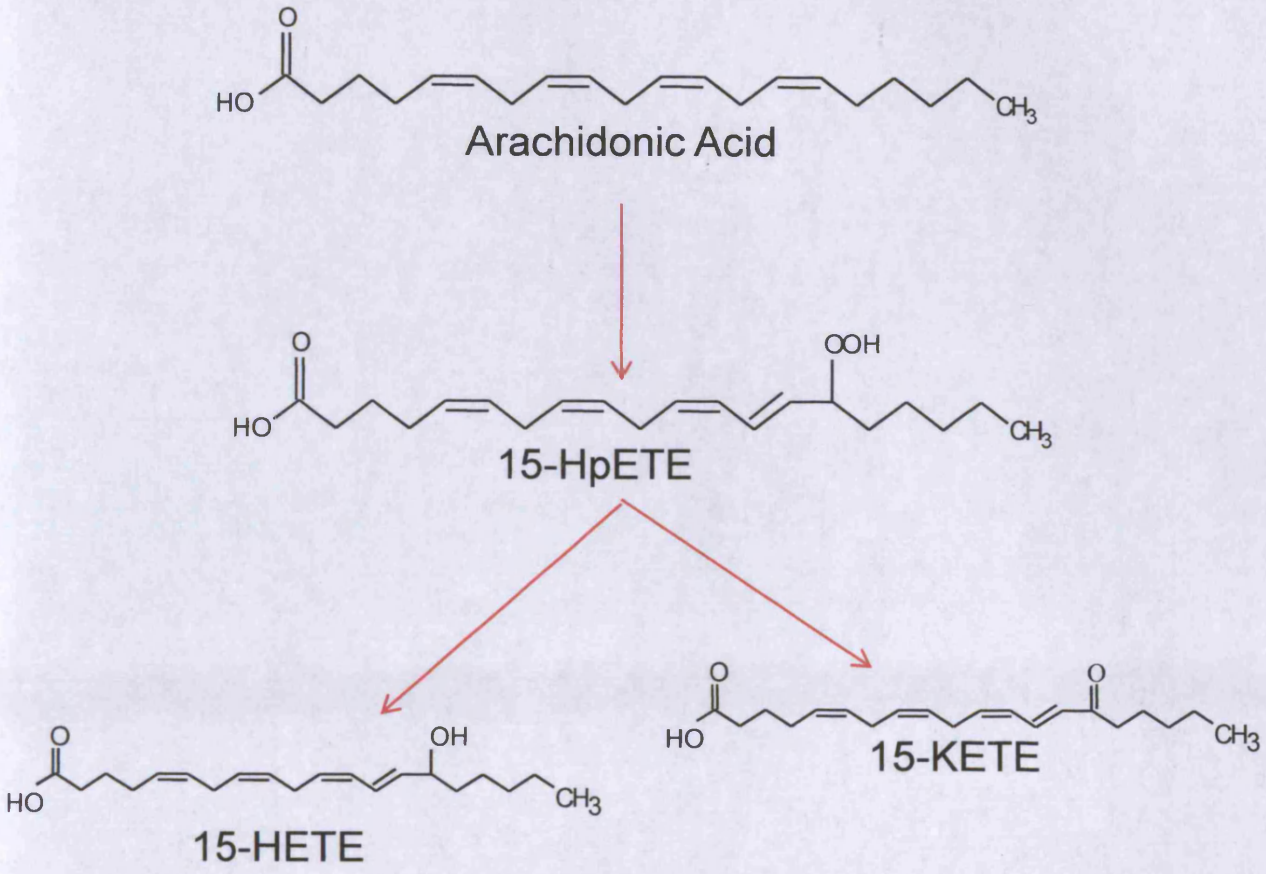
The primary aim of experiments described in this chapter was to characterise the synthesis of 15-H(p)ETE-PEs in human monocytes and establish how they are subsequently metabolised. Following activation, 15-H(p)ETE-PEs in reduced samples reached maximum levels by 10 minutes which then decreased by 180 minutes (Figure 4.1). The decrease in these lipids was suspected to be due to their metabolism. Several routes of metabolism were investigated and then excluded, including hydrolysis of 15-H(p)ETE-PE by PLA_2 to release 15-H(p)ETE, conversion of the PE-head group to PC, and elongation of the sn2 fatty acid (H(p)ETE) by the addition of C_2H_4 (Figures 4.2-4.7). The results from these experiments suggested that 18:0a/15-HETE-PC may also be a product of 15-LOX (Figure 4.6). This is supported by results published in Maskrey *et al* (2007), where a small amount of 15-HETE was shown to be esterified to PC in activated human monocytes. 18:0a/15-HETE-PC was not quantified in monocyte time-course samples as DMPE was used as an internal standard. The ionisation efficiencies of PC and PE are very different, PC compounds ionise more efficiently in positive mode but 15-HETE can only ionise in negative mode (Pulfer & Murphy, 2003). Therefore, 15-HETE-PCs were analysed in negative mode with reduced detection. The use of DMPC as an internal standard would account for this issue of ionisation and using the synthesised 18:0a/15-HETE-PC standard, this lipid could be quantified in human monocytes. However, conversion of the PE head group to PC was ruled out as a route for 15-H(p)ETE-PE metabolism as it did not follow the expected temporal pattern. Conversion of PE to PS was not excluded as the correct LC/MS/MS peaks could not be identified; this also suggests that there was no significant formation of PS-HETEs by 15-LOX (data not shown).

The above mechanisms of metabolism were excluded, therefore, subsequent experiments investigated whether oxidised lipid products of 15-LOX were being effectively endogenously reduced to 15-HETE-PE by GPxs. Analysis of non-reduced time-course samples showed that endogenous 15-HETE-PEs accounted for approximately 30% of the total 15-HETE-PEs detected in $SnCl_2$ reduced samples (Figure 4.8). The remaining 70% of 15-HETE-PEs were likely to be due to unidentified lipids that form 15-HETE-PEs following chemical reduction. Therefore, investigations were carried out to identify lipids synthesised by 15-LOX that can be reduced to form 15-HETE-PEs. 15-HpETE-PEs were possible lipid precursors of 15-HETE-PEs but very little was detected in monocyte time-course samples (Figures 4.9-4.12) (Schewe *et al*, 1986). Data in Figure 4.17 showed that 18:0a/15-HpETE-PE is very unstable and rapidly metabolised when added to human monocytes, which is consistent with such little detection of 15-HpETE-PE in human monocytes. Next, levels of 15-KETE-PEs were investigated in human monocytes. Results from time-course experiments showed that detected temporal pattern of generated 15-KETE-PEs were consistent with what would be expected from suspected lipid precursors of 15-HETE-PEs. Importantly, in non-reduced time-course samples 15-KETE-PEs increased by 15 minutes following activation then declined, similar to levels of 15-HETE-PEs in $SnCl_2$ reduced time-course samples. 15-KETE-PEs also diminished following reduction, corresponding to a simultaneous increase in 15-HETE-PEs. This suggests that 15-KETE-PEs are the likely precursors of 15-HETE-PEs but they are metabolised throughout the time-course to unknown products. Possible products of 15-KETE-PE metabolism include secondary oxidation products, this could be investigated in future experiments.

As there are no 15-KETE-PE standards, the identity of 15-KETE-PEs in monocyte samples was validated by comparing their product ion spectra to spectra of a 15-KETE standard as shown in a previous study (Murphy *et al*, 2005). The identity of 15-KETE-PEs could be confirmed by an alternative method. 15-KETE-PEs in non-reduced activated human monocytes could be isolated by RP-HPLC, followed by base hydrolysis or treatment with PLA_2 enzymes to release the fatty acids. Free 15-KETE could then be identified by either MS analysis or HPLC, by comparing with a 15-KETE standard, available from Cayman Chemical. Hydrolysis using PLA_2 enzymes may be more appropriate for this experiment as it specifically releases the sn2 fatty acid. This method is also considered to be a less 'harsh' than base hydrolysis, which may itself decompose the KETE-lipids (Chaitidis *et al*, 1998).

In this chapter it was proposed that 15-KETE-PEs are novel lipids formed following 15-LOX activation in human monocytes. However, the mechanism of their formation is unknown. They may be synthesised by two ways:

(i) Via metabolism of 15-HpETE-PEs synthesised by 15-LOX (as shown Scheme 4.3). 18:0a/15-HpETE-PE added exogenously to monocytes was rapidly metabolised and coincided with an increase in 15-KETE-PE (Figure 4.17). Furthermore, 15-HpETE can be converted to 15-KETE in the presence of human prostacyclin and thromboxane synthases, therefore, 15-KETE-PEs could be formed directly from 15-HpETE-PEs (Yeh *et al*, 2007). In support of this route of metabolism, the direct conversion of HpETEs to KETEs, has also been described in murine macrophages and the nervous system of *Aplysia* (12-HpETE to 12-KETE) (Piomelli *et al*, 1988, Zarini & Murphy, 2003). It is also possible that HpETE could be converted to KETE non-enzymatically as shown in Scheme 4.4 (McMurry J, 2000).



Scheme 4.4 The formation of 15-HpETE by 15-LOX-1
(adapted from Claesson *et al,* 2008 and Yeh *et al,* 2007, with permission (06/12/2010 and 13/12/2010)).

(ii) Alternative routes have been reported for the synthesis of KETEs. Some studies report that KETEs can be generated enzymatically from HETE. For example, 15-KETE is formed after 15-hydroxyprostaglandin dehydrogenase (PGDH)-mediated oxidation of 15-HETE in R15L-cells and the same reaction is also carried out by PGDH isolated from rabbit lungs (Bergholte *et al,* 1987, Wei *et al,* 2009). 5-HETE can also be converted to 5-KETE by 5-hydroxyeicosanoid dehydrogenase (5-HEDH) in neutrophils (Powell and Rokach, 2005). Importantly, a PGDH has been detected in human monocytes that is reported not only to convert lipoxin hydroxyl groups to carbonyls, but also, converts 15-HETE to 15-KETE (Wei *et al,* 2009, Romano M, 2006, Maddox *et al,* 1998). Therefore, it is possible that 15-KETE-PEs are generated following the metabolism of 15-HETE-PE.

(iii) 15-KETE-PEs may be synthesised directly by 15-LOX.

Under anaerobic conditions, soybean LOX is reported to synthesise 15-KETE instead of 15-HpETE (de Laclos & Borgeat, 1988). This supports the hypothesis that mammalian 15-LOX may also be able to synthesise 15-KETE. To investigate whether 15-LOX can directly generate 15-KETE-PEs, an isolated rabbit 15-LOX could be used in an anaerobic reaction with pure SAPE and/or brain plasmalogen-PEs. Analysis of lipids generated would indicate whether 15-HpETE-PEs or 15-KETE-PEs can be direct products of 15-LOX.

Products of 12/15-LOX are believed to be involved in inflammation, possibly by acting as signalling molecules, therefore, it is important to identify these products and to determine how their effects are exerted (Kuhn & O'Donnell, 2006). 15-H(p)ETE-PEs (which herein includes the 15-KETE-PEs) were retained by monocytes following LOX activation. This suggests that

esterified products of 15-LOX may be involved in autocrine signalling. Free 15-KETE is also believed to have signalling properties. Previous studies report that 15-KETE is chemotactic and inhibits the proliferation of human vascular vein endothelial cells (HUVECs) and breast cancer cells (Sozzani *et al*, 1996, O'Flaherty *et al*, 2005, Wei *et al*, 2009). Furthermore, two 15-KETE analogs, 5-KETE and 15d-PGJ_2 (15-deoxy-$\Delta_{12,14}$-prostaglandin J_2), regulate cell apoptosis and proliferation by acting as agonists of PPARγ. 5-KETE and 15d-PGJ_2 also affect interactions between macrophages and endothelial cells, which may have implications in particular inflammatory disorders including atherosclerosis and asthma (Li *et al*, 2004). 15-KETE-PEs may have similar properties which could be investigated in future experiments.

To conclude, the results in this chapter have indicated that approximately 30% of 15-LOX products are endogenously reduced to 15-HETE-PEs following 15-LOX activation in human monocytes. Furthermore, 15-KETE-PEs are generated following 15-LOX activation and are metabolised to unknown products. Many studies have described the attachment of lipid products to proteins via associations between carbonyl and amine groups, known as Schiff bases and Michael adducts. In particular, one study has shown that free 15-KETE and 5-KETE can form Michael adducts with glutathione (Lee *et al*, 2007). A covalent association with proteins could have accounted for the decline in 15-KETE-PEs throughout the time-course and was thus investigated in Chapter 5.

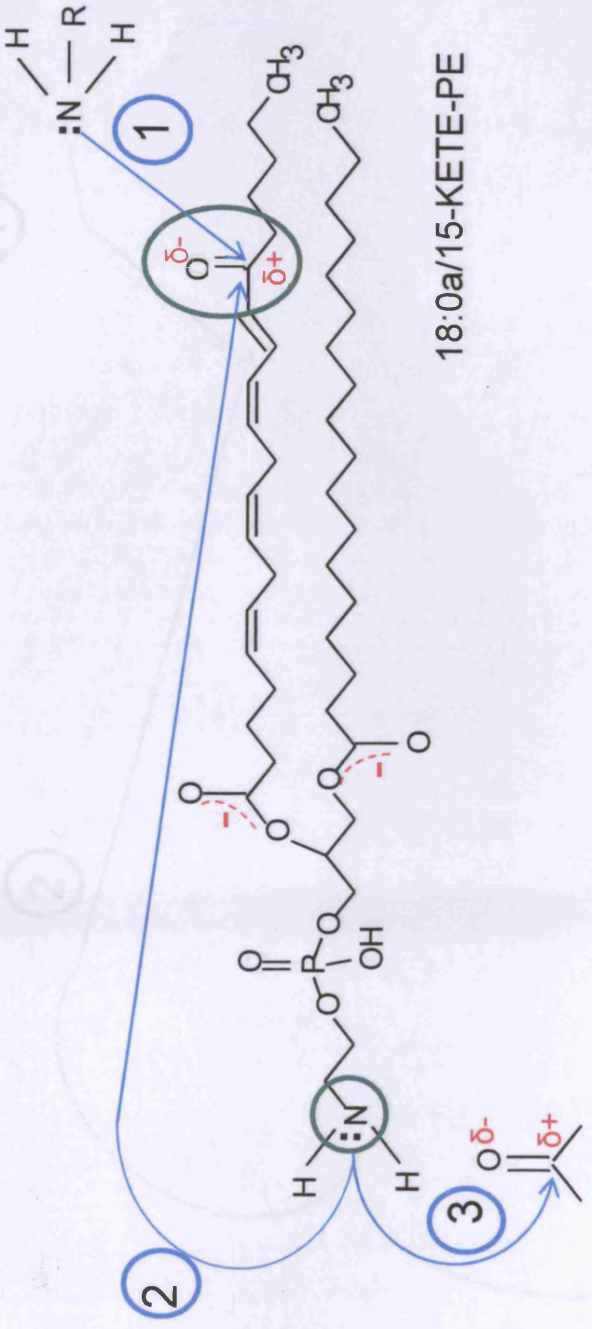
CHAPTER 5

INCORPORATION OF [^{14}C] INTO PRODUCTS OF 15-LOX

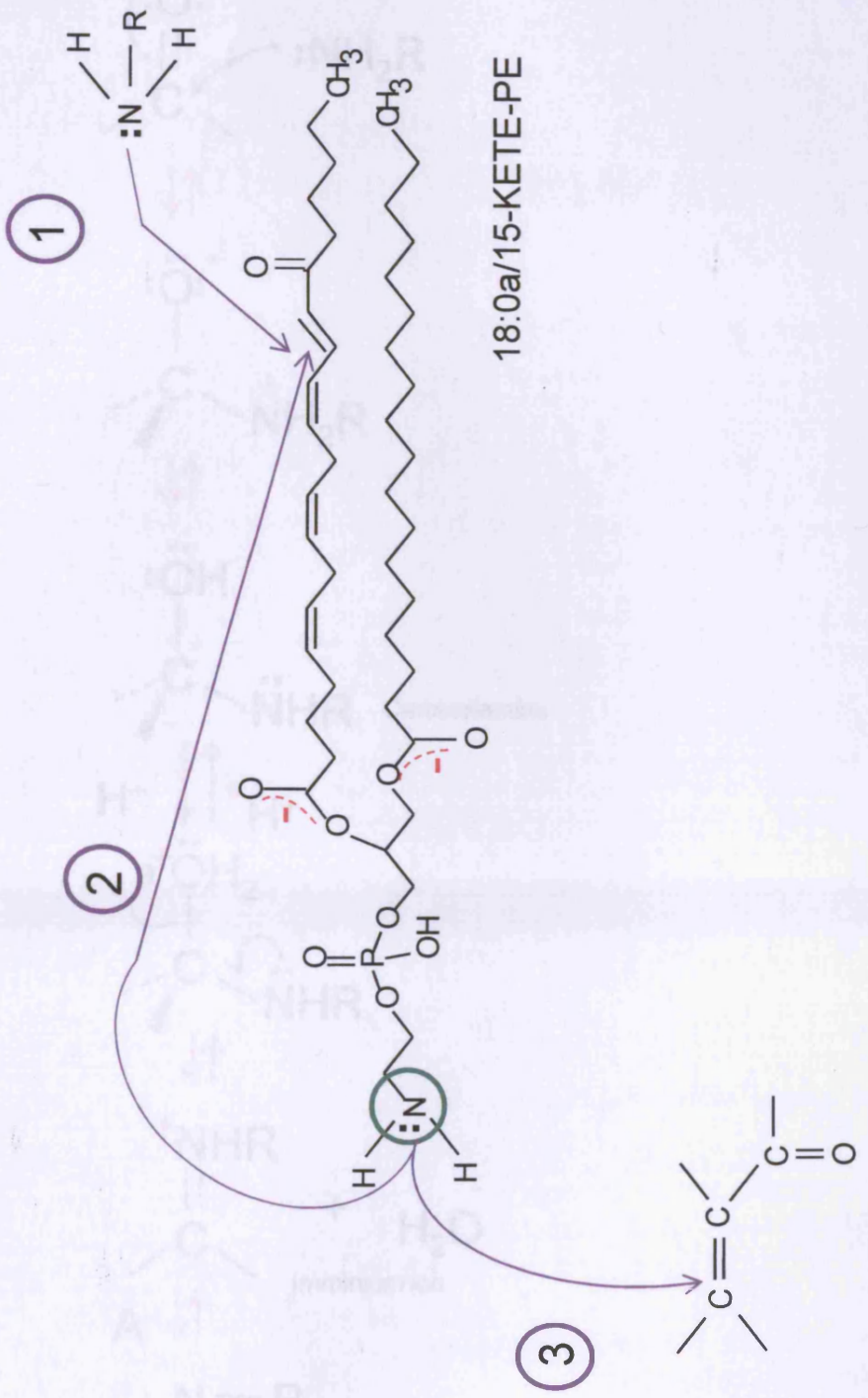
5.1 Introduction

In the previous chapter, 15-KETE-PEs were shown to be synthesised in human monocytes following the activation of 15-LOX. 15-KETE-PEs were metabolised within 3 hours of their synthesis. This may have been due to the formation of covalent adducts with proteins, known as Schiff bases or Michael adducts. Schiff bases are formed when a carbonyl group undergoes nucleophilic attack by a primary amine (Scheme 5.1) (McMurry *et al*, 2000). In particular, 15-KETE-PEs have a carbonyl group at C-15 of the sn2 fatty acid that could form a Schiff base as it has a defined δ+ and δ– charge separation. A second carbonyl group is located near the glycerol backbone, but this is unlikely to form a Schiff base as the negative charge is dissociated over the ester bond (Scheme 5.1) (McMurry *et al*, 2000). Alternatively, Michael adducts are formed when a primary amine attacks particular alkene bonds that are located near a carbonyl group, which are called α,β-unsaturated ketones (McMurry *et al*, 2000) (Scheme 5.2). The carbonyl group of sn2 fatty acid in 15-KETE-PEs is an α,β-unsaturated ketone, and the alkene bond is located between C-13 and C-14 (Scheme 2.9). The mechanisms for Schiff base and Michael adduct formation are shown in Schemes 5.3 and 5.4 respectively.

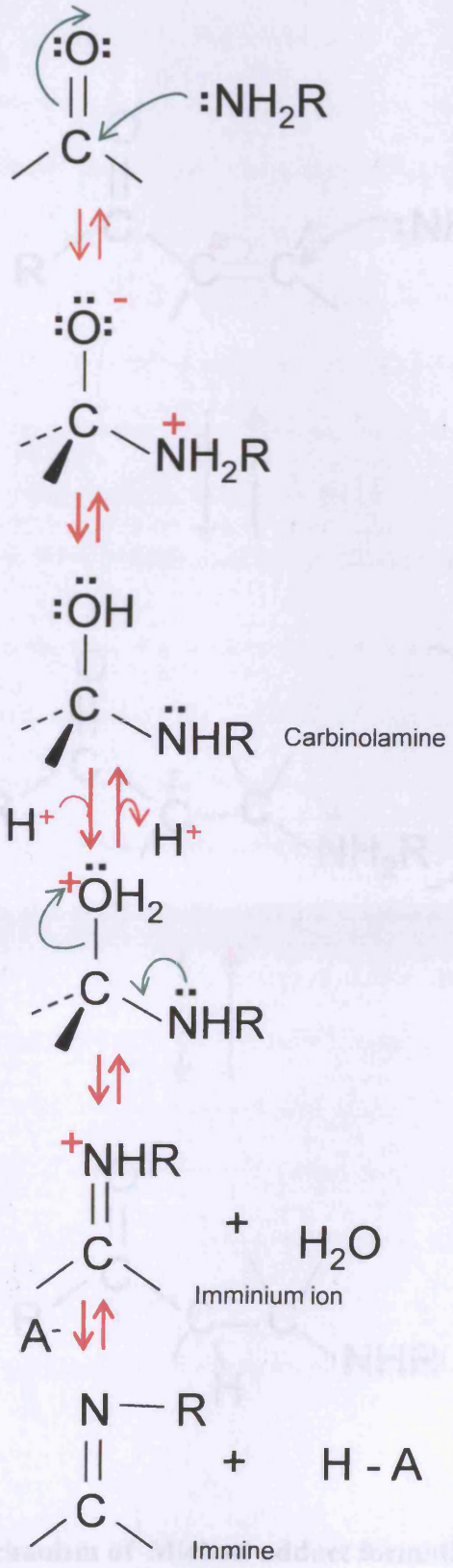
Electrophilic lipids can form Schiff bases and Michael adducts with peptides and proteins. Examples include, 4–hydroxynonenal (HNE), malondialdehyde (MDA), 9,12-dioxo-dodecenoic acid (DODEs), isoketals, levuglandins and prostaglandins, which all bind covalently to proteins (Requena *et al*, 1997, Grimsrud *et al*, 2007, Vila *et al*, 2008, Aldini *et*



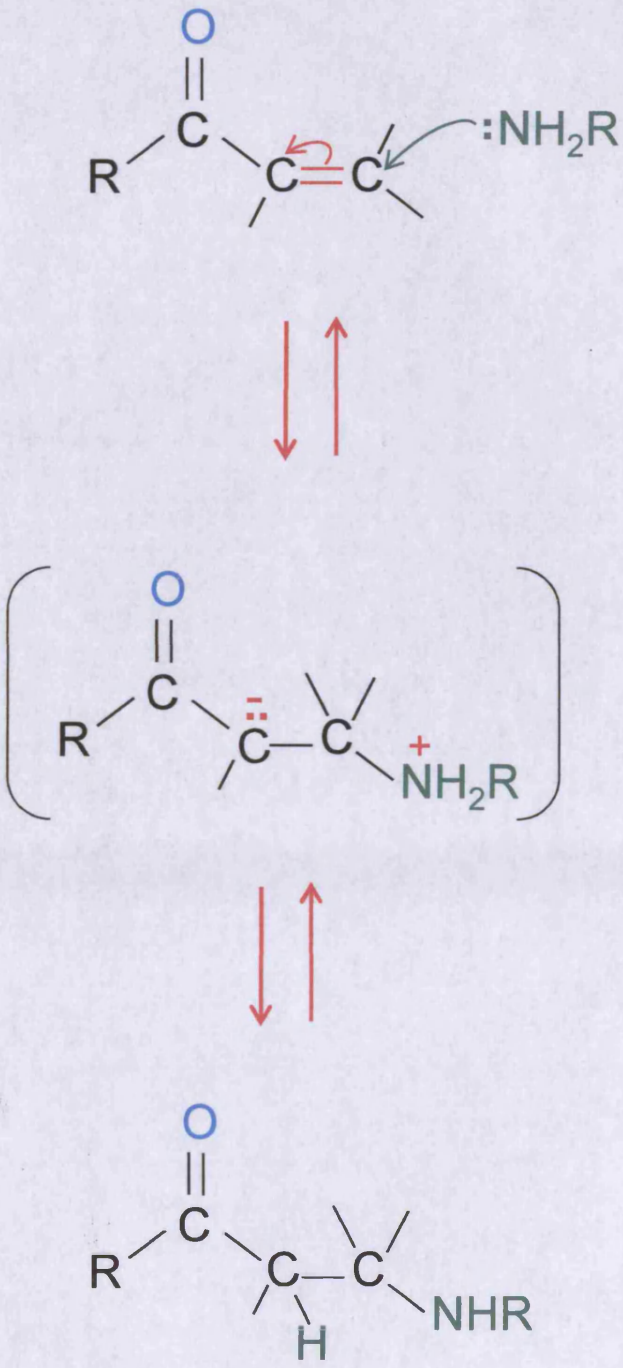
Scheme 5.1 Positions in 15-KETE-PE susceptible to nucleophilic attack that form Schiff bases. A carbonyl group is susceptible to nucleophilic attack by primary amines present in lysine, or other PE/PS phospholipids (1). A carbonyl can also be attacked by an amine present within the same phospholipid (2). The amine of a PE/PS phospholipid head group can attack carbonyl groups of nearby compounds (3).



Scheme 5.2 Positions in 15-KETE-PE susceptible to nucleophilic attack that can form Michael adducts. An alkene bond near a carbonyl group is susceptible to nucleophilic attack by a primary amines present in lysine, or other PE/PS phospholipids (1). An alkene bond can also be attacked by an amine present within the same phospholipid head group (2). The amine of a PS/PE phospholipid head group can attack an alkene bonds in nearby compounds (3).



Scheme 5.3 Mechanism of imine (Schiff base) formation by reaction of a primary amine group with an aldehyde/ ketone (adapted from McMurry *et al*, 2000, with permission (04/12/2010)).



Scheme 5.4 Mechanism of Michael adduct formation by reaction of a primary amine group with an α, β-unsaturated ketone (adapted from McMurry *et al*, 2000 with permission (04/12/2010)).

al, 2006, Williams *et al*, 2007, Bernoud-Hubac *et al*, 2004, Boutaud *et al*, 2001). Phospholipids can also form Schiff bases with proteins, as reported for oxidised products of 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphatidylethanolamine (Ox-PAPE) and 1-palmitoyl-2-linoleoyl-glyceryl-phosphatidylcholine (Ox-PLPC) (Szapacs *et al*, 2008, Gugiu *et al*, 2008). Furthermore, PE and PS phospholipids can carry out nucleophilic attack due to the amine present in their head group. Thus, 15-KETE-PEs could form Schiff bases or Michael adducts within the same phospholipid, or could form phospholipid-phospholipid, or phospholipid-protein complexes (Schemes 5.1 and 5.2) (Jain & Sohet *et al*, 1984, Hazen *et al*, 1999, Wachel *et al*, 2006, Mattila *et al*, 2008, Bach *et al*, 2009a, Bach *et al*, 2009b).

The vast number of proteins and lipids in cells makes it a major challenge to identify which particular proteins form adducts with 15-KETE-PEs in human monocytes. Furthermore, as protein-lipid and lipid-lipid interactions have not been widely studied, the technologies designed to analyse these products are limited. Previous studies on other electrophilic lipids have investigated protein adduct formation using radioactive isotopes such as ^{14}C and ^{3}H (Kang *et al*, 1999, Horkko *et al*, 1997, Bull *et al*, 1996, Vandel Huevel *et al*, 1991, Rudolph *et al*, 2009). In these approaches, radiolabelled lipids are added to culture medium, then incorporated into cell membranes. Radiolabelled 15-KETE-PE or 15-HpETE-PE do not exist, however, [^{14}C]-SAPE, [^{14}C]-AA and [^{14}C]-ethanolamine are available. In an attempt to establish whether products of 15-LOX form adducts with cellular proteins, these these substrates were utilised to induce cells to generate radiolabelled phospholipid oxidation products that could then be studied for protein adduct formation.

5.1.1 Aims.

- To establish a method where 15-LOX in human monocytes could be induced to synthesise ^{14}C radiolabelled products.
- To explore whether radiolabelled oxidised products of 15-LOX could form adducts with proteins.

5.2 Results

5.2.1 Monocytes treated with [^{14}C]-ethanolamine hydrochloride generated more [^{14}C]-15-H(p)ETE-PEs, in comparison to monocytes incubated with [^{14}C]-AA or [^{14}C]-SAPE.

[^{14}C]-ethanolamine hydrochloride, [^{14}C]-AA or [^{14}C]-SAPE may be used by cells to generate [^{14}C]-radiolabelled products of 15-LOX. However, due to variations in their size and hydrophobicity, the efficiency of their incorporation into membrane phsopholipids may vary. Human monocytes were incubated for 24 hours with 200 μM [^{14}C]-ethanolamine hydrochloride (13.8 mCi/mmol), 200 μM [^{14}C]-ethanolamine hydrochloride (112 mCi/mmol), 10 μM [^{14}C]-AA (57 mCi/mmol) or 1 μM [^{14}C]-SAPE (56 mCi/mmol), to investigate which resulted in the largest generation of [^{14}C]-15-H(p)ETE-PEs. After harvesting, monocytes were activated for 15 minutes using A23187, followed by reduction, then lipid extraction. The elution time of 15-H(p)ETE-PEs was confirmed by analysing samples by LC/MS/MS. Next, to isolate the 15-H(p)ETE-PEs, samples underwent RP-HPLC where fractions were collected at intervals of one minute. They were then analysed by scintillation counting to calculate the amount of [^{14}C]-15-H(p)ETE-PEs present. As the monocyte samples were reduced, the [^{14}C]-15-H(p)ETE-PEs detected are a combination of [^{14}C]-15-HpETE-PEs, [^{14}C]-15-KETE-PEs as well as endogenous [^{14}C]-15-HETE-PEs. Monocytes incubated with [^{14}C]-ethanolamine hydrochloride (112 mCi/mmol) generated the highest amount of [^{14}C]-15-H(p)ETE-PEs (73.2 pmol), followed by monocytes incubated with [^{14}C]-ethanolamine hydrochloride (13.8 mCi/mmol) (4.5 pmol), [^{14}C]-AA (2.8 pmol) and lastly, [^{14}C]-SAPE (1 pmol) (Figure 5.1). Therefore, [^{14}C]-ethanolamine hydrochloride (112 mCi/mmol) was used in all subsequent experiments. Scheme 5.5 shows how [^{14}C]-ethanolamine is incorporated into PE.

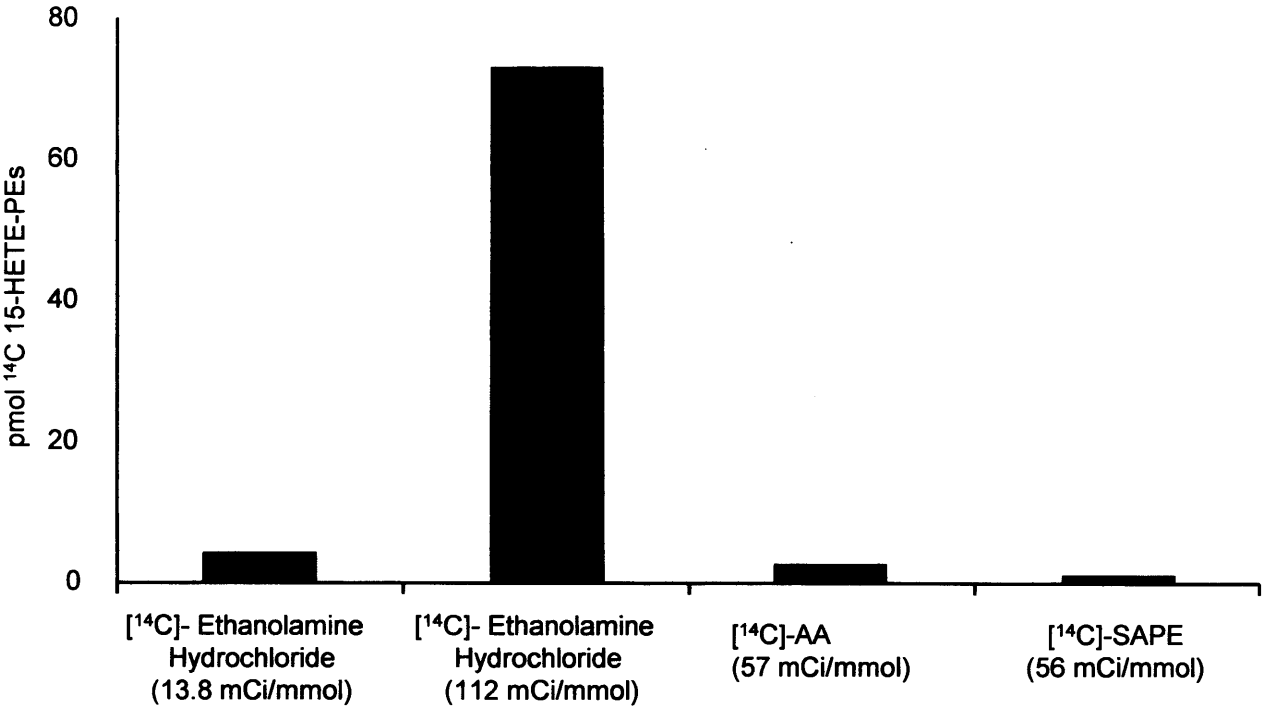
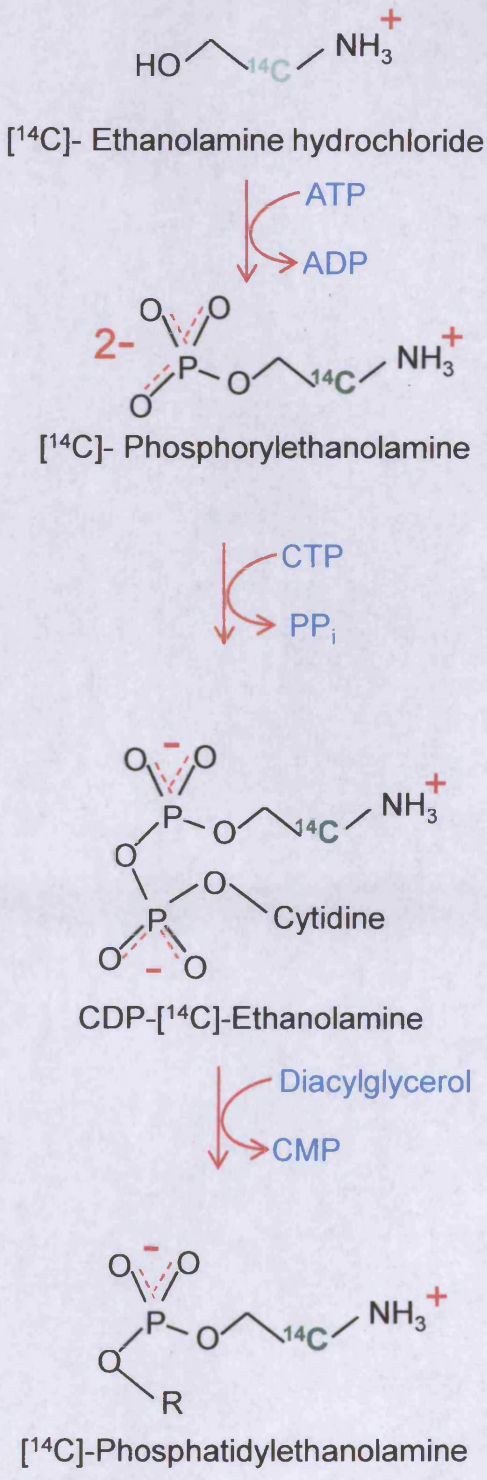


Figure 5.1 Comparison of different strategies used to generate [^{14}C]-15-HETE-PEs. IL-4 induced human monocytes were incubated for 24 hours with either 200 μM [^{14}C]-ethanolamine hydrochloride (13.8 mCi/mmol), 200 μM [^{14}C]-ethanolamine hydrochloride (112 mCi/mmol), 10 μM [^{14}C]-AA or 1 μM [^{14}C]-SAPE. The monocytes were harvested, activated with A23187 then reduced. The lipids were extracted and analysed by LC/MS/MS. Samples underwent RP-HPLC where fractions were collected every 1 minute for times corresponding to the elution of 15-HETE-PEs. The fractions were then analysed by scintillation counting and the amount of [^{14}C-]15-HETE-PEs per 1 million cells was calculated (n=1).



Scheme 5.5 Incorporation of [^{14}C]-ethanolamine hydrochloride into phosphatidylethanolamine.

5.2.2 [^{14}C]-treated monocytes generate [^{14}C]-AA-PEs and [^{14}C]-15-H(p)ETE-PEs.

To characterise fully the generation of [^{14}C]-AA-PEs and [^{14}C]-15-H(p)ETE-PEs, IL-4 treated human monocytes were incubated for 24 hours with [^{14}C]-ethanolamine hydrochloride (112 mCi/mmol). Following their incubation with the radiolabel, human monocytes were harvested and activated with A23187 for 15 minutes, followed by reduction then lipid extraction. Sample analysis by LC/MS/MS confirmed that 15-LOX activation was not affected by incubation with [^{14}C]-ethanolamine hydrochloride (Figure 5.2). LC/MS/MS transitions for [^{14}C]-AA-PEs and [^{14}C]-15-HETE-PEs were also analysed, however, this approach was not useful, as they only have a mass increase of two in comparison to the ^{12}C isomers they elute the same time as naturally occurring isotope peaks of the non-radiolabelled products (data not shown). Due to low incorporation of [^{14}C], the correct peaks could not be identified by LC/MS/MS. However, MS analysis confirmed the elution times of the non-radiolabelled AA-PEs and 15-H(p)ETE-PEs in these samples (Figures 5.3A and 5.4A). An alternative method of analysing radioactivity is by scintillation counting. To calculate the amount of [^{14}C]-AA-PEs and [^{14}C]-15-H(p)ETE-PEs in control and activated monocytes, phospholipids were separated by RP-HPLC and fractions of both samples were collected at intervals of thirty seconds. Each fraction was then analysed by scintillation counting. Radioactivity traces of both unactivated and activated samples showed peaks corresponding to the elution times of AA-PEs and 15-HETE-PEs (Figures 5.3B and 5.4B). Following activation, levels of radioactive lipids eluting at the retention times of 15-HETE-PEs were seen to increase. This indicates that [^{14}C]-ethanolamine hydrochloride was incorporated into membrane phospholipids, which was then oxidised by LOX to form H(p)ETE-PEs. As a known quantity of [^{14}C]-ethanolamine hydrochloride was added to monocytes, the amount of [^{14}C] incorporated into 15-H(p)ETE-PEs can be calculated as follows. The total amount of

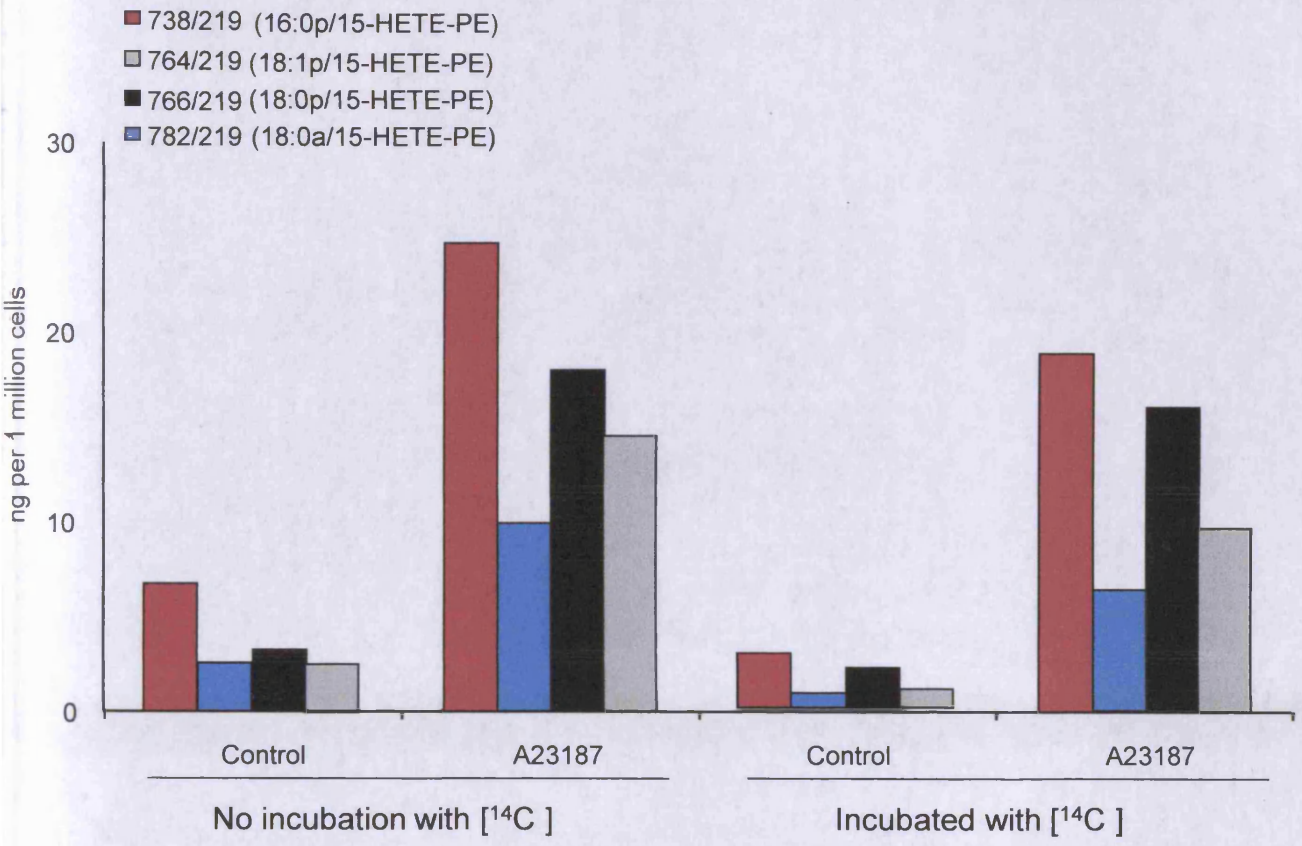


Figure 5.2. There is no statistical difference in 15-HETE-PE generated by activated human monocytes following incubation with and without [^{14}C]-ethanolamine hydrochloride. IL-4 stimulated human monocytes were incubated for 24 hours with and without [^{14}C]-ethanolamine hydrochloride. Cells were harvested, activated with 10 μM A23187 for 15 minutes followed by reduction and lipid extraction (n=1). Samples were analysed by LC/MS/MS. No statistical difference was seen during comparison of 15-HETE-PE generation between both A23187 treated samples using Students T-Test with one-tailed Mann-Whitney test.

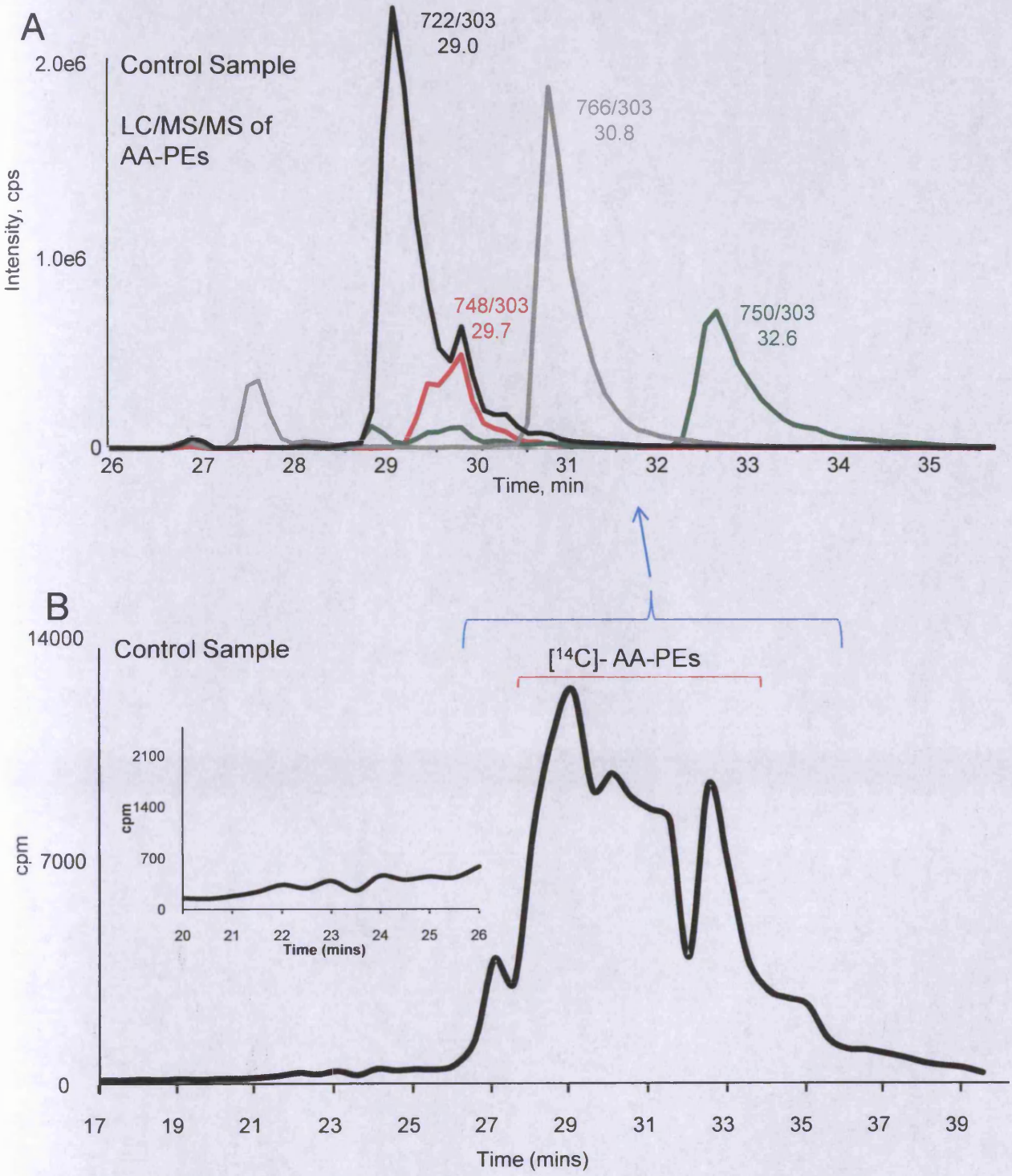


Figure 5.3 [^{14}C]-AA-PEs are detected following incubation of monocytes with [^{14}C]-ethanolamine hydrochloride. IL-4 induced monocytes were incubated for 24 hours with [^{14}C]-ethanolamine hydrochloride. Monocytes were harvested, followed by reduction then lipid extraction. The control sample was analysed by LC/MS/MS and the elution times of AA-PEs were recorded (A). During RP-HPLC, fractions were collected at intervals of 30 seconds, which were then analysed by scintillation counting for the detection of [^{14}C]-AA-PEs (B).

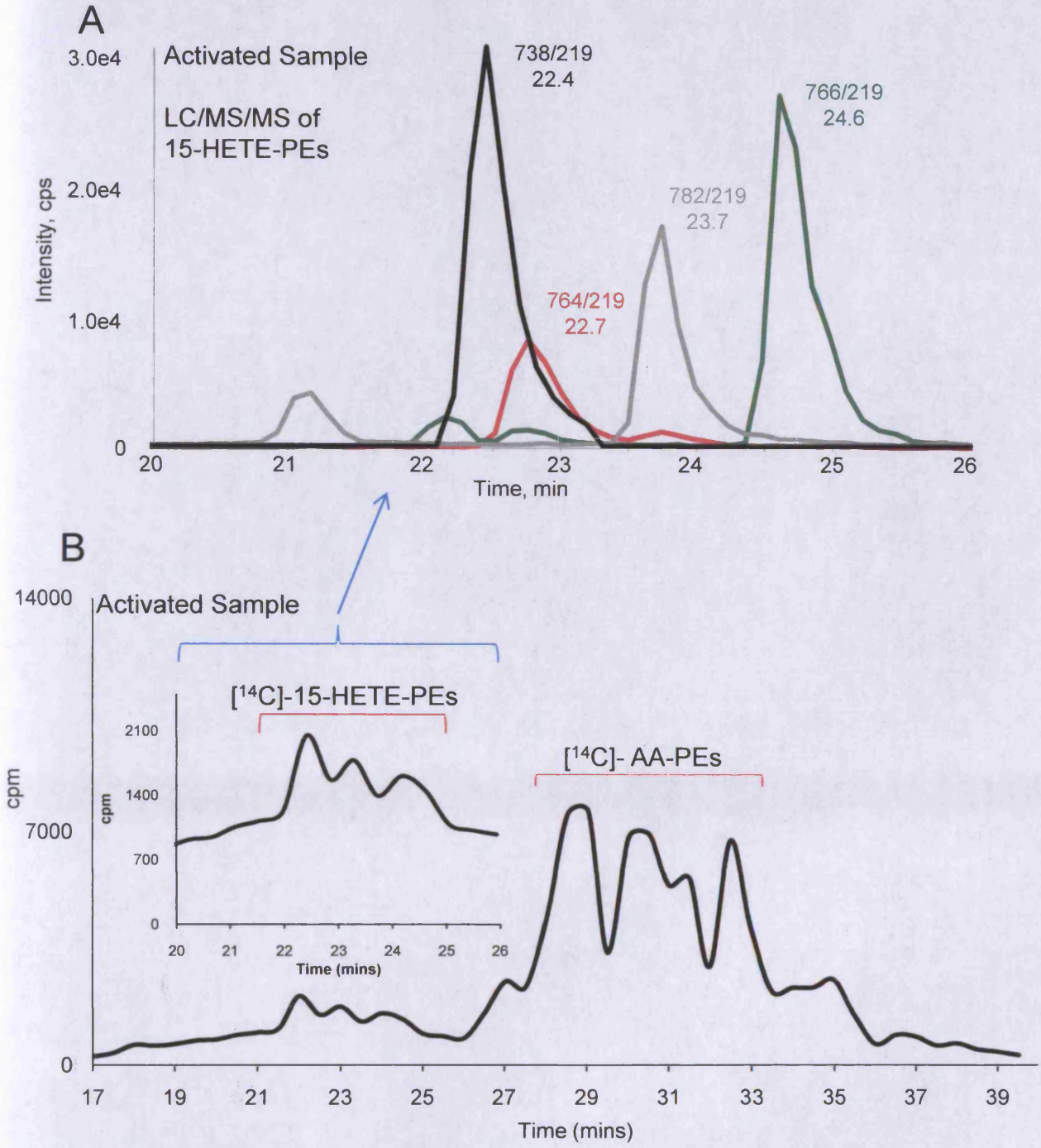


Figure 5.4. [^{14}C]-15-HETE-PEs are detected in activated monocytes following incubation with [^{14}C]-ethanolamine hydrochloride. IL-4 induced monocytes were incubated for 24 hours with [^{14}C]-ethanolamine hydrochloride. Monocytes were harvested, activated for 15 minutes with A23187, followed by reduction then lipid extraction. The activated sample was analysed by LC/MS/MS and the elution times of 15-H(p)ETE-PEs were recorded (A). During RP-HPLC, fractions were collected at intervals of 30 seconds, which were then analysed by scintillation counting for the detection of [^{14}C]-15-H(p)ETE-PEs (B). As samples were reduced detected 15-HETE-PEs and [^{14}C]-15-HETE-PEs also includes reduced 15-KETE-PEs and 15-HpETE-PEs.

15-HETE-PEs was first quantified following analysis by LC/MS/MS (Figure 5.2). Next, the total of detected [^{14}C]-15-H(p)ETE-PEs was calculated from the radioactivity traces of activated samples (Figure 5.4B). In this was, it was seen that 2.7% of 15-H(p)ETE-PEs were radiolabelled in activated monocytes, equivalent to 0.00036% of the total amount of radioactivity added.

5.2.3 [^{14}C]-ethanolamine hydrochloride is specifically incorporated into PE phospholipids.

[^{14}C]-ethanolamine hydrochloride is incorporated into the PE pool, however, as shown in Schemes 4.1 and 4.2 (Chapter 4), PE can be converted to PC or PS. To assess which phospholipid classes contained the [^{14}C] radiolabel, the control sample generated in section 5.2.2 underwent normal phase–HPLC (NP-HPLC). Scintillation counting of fractions from LC elution showed that [^{14}C] was exclusively incorporated into PE phospholipids (Figure 5.5). The total amount of PE in monocyte samples was calculated by UV using an egg PE standard (not shown) and the amount of radiolabelled PE was calculated following scintillation count analysis (Figure 5.5). 1.5% of the PE phospholipid pool was radiolabelled, this incorporation is equivalent to 0.02% of the total amount of radioactivity added.

5.2.4 Preliminary investigations support the association of proteins with [^{14}C]-radiolabelled lipids synthesised by 15-LOX.

In human monocytes, 15-LOX has been shown to oxidise AA-PEs to synthesise 15-HpETE-PEs and 15-KETE-PEs (Chapter 4). Similarly, in monocytes incubated with [^{14}C]-ethanolamine hydrochloride, [^{14}C]-AA-PEs may be oxidised by 15-LOX to synthesise [^{14}C]-15-HpETE-PEs and [^{14}C]-15-KETE-PEs. Experiments were carried out to investigate

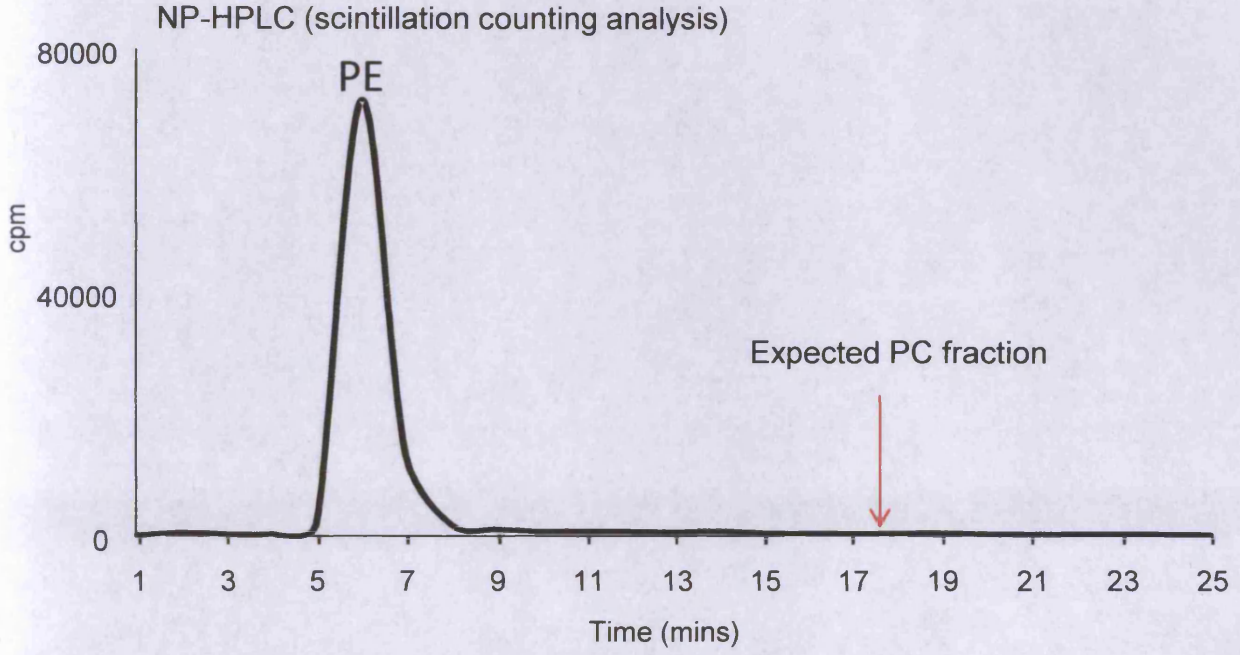


Figure 5.5 [^{14}C]-ethanolamine hydrochloride is exclusively incorporated into the PE phospholipids. IL-4 induced monocytes were incubated for 24 hours with [^{14}C]-ethanolamine hydrochloride. Following cell harvest, monocytes were reduced followed by lipid extraction. Samples were analysed by UV during NP-HPLC (A), fractions were collected at intervals of one minute, which were then analysed by scintillation counting (B).

whether radiolabelled oxidised products of 15-LOX could bind to proteins, and whether this could account for some of the decrease in 15-KETE-PEs as seen in Figure 4.17. IL-4 treated human monocytes were incubated for 24 hours with [^{14}C]-ethanolamine hydrochloride, harvested then activated using A23187 for 15 minutes or 3 hours. At each time point, samples were split into three. One third was removed immediately into extraction solvent (non-reduced), the remaining sample was split into two and either reduced for 10 minutes with $SnCl_2$ or 1 hour with $NaCNBH_3$, followed by lipid extraction. Two different reductants were used as the strength of a reducing agent is believed to affect the stabilisation of adducts. For example, $NaCNBH_3$ is less nucleophilic than reducing agents such as $NaBH_4$ therefore, its rate of reduction rate is slower, allowing the stabilisation of both Schiff bases and Michael adducts (Baxter & Reitz, 2003). Strong reducing agents such as $NaBH_4$ and $SnCl_2$ reduce ketones to hydroxyl groups, thus stabilising Michael adducts only (Szapacs, *et al* 2008). Therefore, using $SnCl_2$ and $NaCNBH_4$ as reductants, the formation of both Schiff bases and Michael adducts can be investigated. Following lipid extraction, the aqueous phase of monocyte samples were used to investigate whether proteins had formed adducts with [^{14}C]-labelled products of 15-LOX.

5.2.4.1 [^{14}C]-radiolabelled lipid products of 15-LOX are likely to covalently bind with proteins following cell activation.

To investigate whether radiolabelled lipid products of 15-LOX bind to proteins, the aqueous phase of samples generated in 5.2.4 underwent TCA precipitation. They were then centrifuged for 15 minutes to separate protein-associated (pellet) and non-protein-associated (supernatant) compounds. The pellets and supernatants of samples were analysed by scintillation counting. The amount of radiolabelled lipids associated with proteins increased

from control to 3 hours in all samples (Figure 5.6). The largest rise was seen between 0 minutes and 3 hours for $NaCNBH_3$ treated samples, where the percentage of [^{14}C] associated with proteins increased from 6.5 to 11%. The percentage of radiolabel associated with the protein fraction increased in both $SnCl_2$ and $NaCNBH_4$ treated samples, this is primarily due to the formation of Michael adducts between oxidised lipids and proteins/peptides. Therefore, covalent association of 15-KETE-PEs to proteins may account for the decrease in 15-KETE-PEs seen in time-course experiments (Chapter 4).

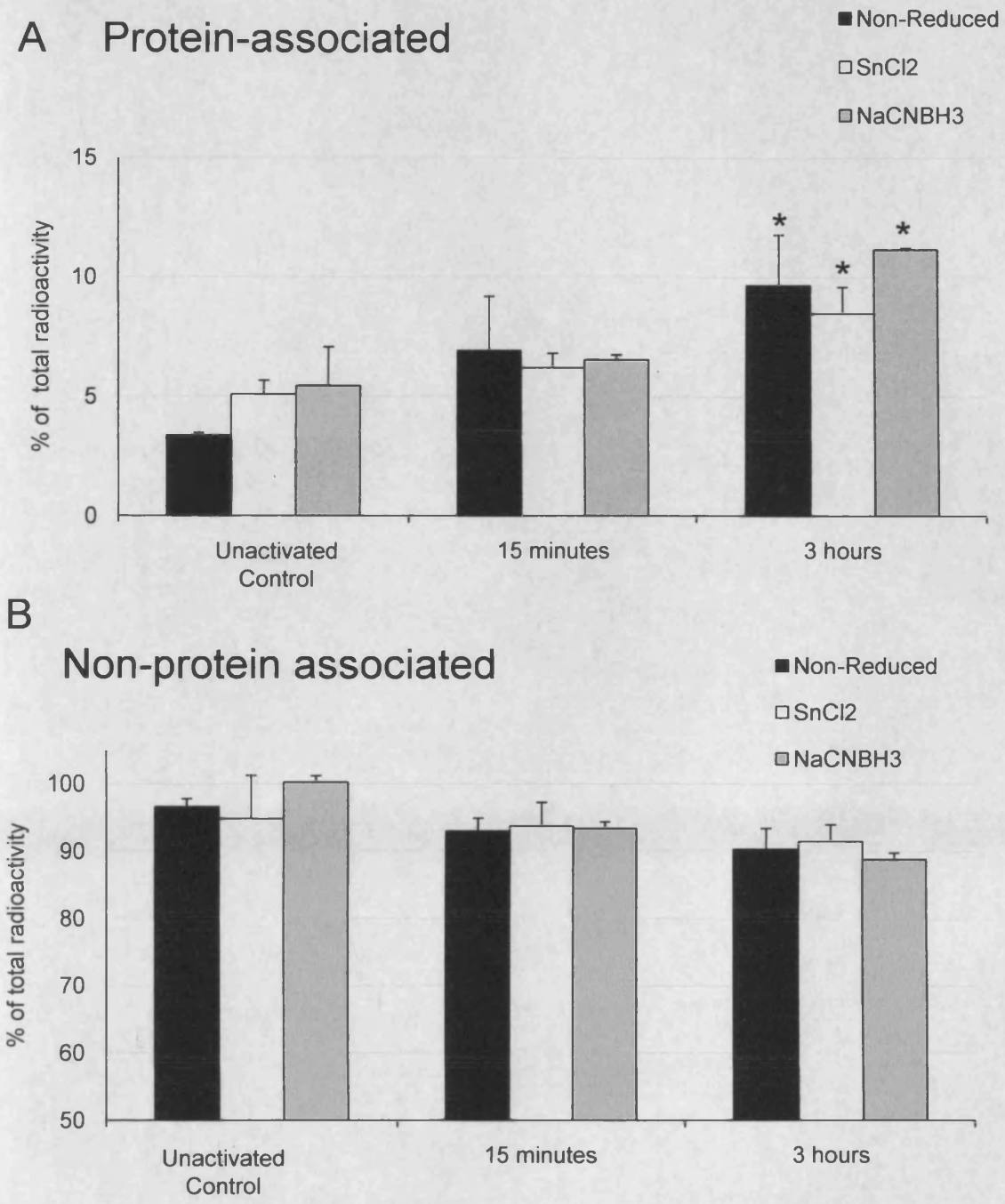


Figure 5.6 Radiolabelled lipid products of 15-LOX bind with proteins following cell activation. IL-4 induced human monocytes were incubated with [^{14}C]-ethanolamine hydrochloride, following harvest, cells were activated for 15 minutes or 3 hours with A23187. Samples underwent lipid extraction immediately or were firstly reduced using $SnCl_2$ or $NaCNBH_3$. The aqueous fraction was subjected to TCA precipitation followed by centrifugation, to separate protein associated and non-protein associated compounds. Samples were then analysed by scintillation counting and their percentage of radioactivity was calculated in the protein associated (A) and non-protein associated (B) fractions (n = 3, mean ± SEM). *Students t-test, $p < 0.05$, the difference of each analyte is significant in comparison to its relative unactivated control.

5.3 Discussion

To investigate whether products of 15-LOX could bind covalently with proteins, [^{14}C]-ethanolamine hydrochloride was used to generate radiolabelled AA-PEs, which were subsequently oxidised by 15-LOX. To ensure incorporation of the [^{14}C] radiolabel, the presence of [^{14}C]-AA-PEs and [^{14}C]-15-HETE-PEs (which also contained reduced [^{14}C]-15-KETE-PEs and [^{14}C]-15-HpETE-PEs) in human monocytes was firstly characterised. The results indicate that only a small proportion of [^{14}C] is incorporated into membrane PE phospholipids (2.7%). Furthermore, only 0.00036% of the total [^{14}C] radiolabel added to monocytes was present in the 15-H(p)ETE-PE pool. Thus, if [^{14}C]-15-KETE-PEs bind covalently to proteins, the amount of radiolabelled products would be even less, therefore identifying bound proteins using this method may be challenging. Using [^{14}C]-ethanolamine hydrochloride with a higher specific activity may aid in identifying covalently bound products but, the [^{14}C]-ethanolamine hydrochloride used in this chapter (112 mCi/mmol) has the highest specific activity available for this compound. Alternatively, incubating monocytes with a higher concentration of [^{14}C]-ethanolamine hydrochloride could increase the amount of [^{14}C] incorporated. However, a concentration of 200 μM [^{14}C]-ethanolamine hydrochloride was used and increasing it further could result in activating monocytes prior to the addition of A23187. The use of two alternative [^{14}C] containing compounds, [^{14}C]-AA and [^{14}C]-SAPE was investigated in studies for this chapter. However, they were not incorporated into membrane phospholipids as efficiently as [^{14}C]-ethanolamine hydrochloride. Other radiolabelled compounds include [^{3}H]-ethanolamine hydrochloride and [^{3}H]-AA, but [^{3}H] is a weaker radioactive isotope than [^{14}C] and is therefore not a suitable alternative to [^{14}C]-ethanolamine hydrochloride.

Schiff bases are reactive and require stabilisation prior to analysis (McMurry T, 2000, Tallman *et al*, 2006, Szapacs *et al*, 2008, Held *et al*, 1979). In particular, a mild reducing agent such as $NaCNBH_3$ is required to stabilise Schiff bases. Conversely, $SnCl_2$ is a strong reductant that will only stabilise Michael adducts (Isawa & Mukaiyama, 1987). In time-course experiments described in Chapter 4, 15-KETE-PEs declined by 3 hours following activation of 15-LOX. Levels of radiolabel in the protein-associated fraction increased by 3 hours following activation in both $SnCl_2$ and $NaCNBH_3$ treated samples. The difference in protein-associated radiolabel between $SnCl_2$ and $NaCNBH_3$ treated samples represents the formation of Schiff bases. Therefore, the results suggest that lipid products of 15-LOX form primarily Michael adducts with proteins, which could account for the decline seen in 15-KETE-PEs. Previous studies support the formation of covalent bonds between lipids and proteins, which may also be important immunologically. For example, aminophospholipid antibodies specifically recognise and bind to oxidised cardiolipins and oxidised 1-palmitoyl-2-linoleyl-phosphatidylcholine (ox-PLPC) that have formed covalent bonds with proteins such as β_2 glycoprotein 1 (Horkko *et al*, 1996, Horkko *et al*, 1997). In addition, recent reports have shown that oxidised PS, PE and PG phospholipids form Schiff bases with antimicrobial peptides (Mattila *et al*, 2008, Yang *et al* 2008, Eberard *et al*, 2009, Wan *et al*, 2007). This evidence supports the association of oxidised phospholipids with proteins and peptides. In addition, lipid electrophiles can act as signalling mediators by forming Michael adducts with proteins. Examples include 15-deoxy-12,14-prostaglandin J_2 (14-deoxy-PGJ_2) to NFκB, and 4-HNE to the inhibitor of κBα (IκB), heat shock factor (HSF), PPAR and MAPK (Rudoph & Freeman, 2009, Cernuda-Morollon *et al*, 2001, Ji *et al*, 2001, Coleman *et al*, 2007, Jacobs & Marnett, 2007, Zhang *et al*, 2006).

Future work could include identifying peptides and/or proteins that bind to the oxidised phospholipids generated by 15-LOX. Proteins in samples could be separated by SDS-PAGE, the gels could then be cut and analysed by scintillation counting. This may show whether radiolabelled proteins are present and using a protein marker would give an indication of their size. Alternatively, proteins bound to oxidised phospholipids could be extracted using biotinylated phospholipid and fatty acid probes. Specifically, phospholipids tagged with biotin could be oxidised, allowed to bind to proteins then the biotin group could be used to extract the attached complexes from samples. The products could be digested using trypsin followed by identification using LC/MS/MS (Figure 5.7) (Tallman *et al*, 2006, Szapacs *et al*, 2008). This technique has previously been used to identify 21 proteins that bind to ox-PLPC (Szapacs *et al*, 2008). In addition, a biotinylated probe has been used to investigate the binding of proteins to Ox-PAPE in aortic endothelial cells. In that study, 20 proteins were shown to bind Ox-PAPE-N-biotin, but as of yet, they have not been identified (Gugiu *et al*, 2008). A biotinylated free acid HETE probe has been used for a similar purpose. Proteins attached to biotinylated 15-HETE were sequenced and the fragments identified corresponded to actin and the α-subunit of liver mitochondrial ATP synthase (Kang *et al*, 1999).

Several publications have investigated the formation of covalent bonds between proteins and lipid electrophiles or short chain aldehydes. Of particular interest are reports showing that 15-HpETE binds covalently to BSA *in-vitro* (Refsgaard *et al*, 2000, Liu & Wang, 2005). Furthermore, a precursor of 12-HETE, such as 12-HpETE or 12-KETE, is also suggested to bind to proteins in platelet microsomes (Wilson *et al*, 1979). In these publications, the adducts were either Schiff bases, Michael adducts, or formed following the metal ion catalysis of a peroxide, forming a peroxyl radical that can react directly with lysine residues (Refsgaard *et al*, 2000, Kato *et al*, 1992, Burcham *et al*, 1996, Esterbauer *et al*, 1991). All

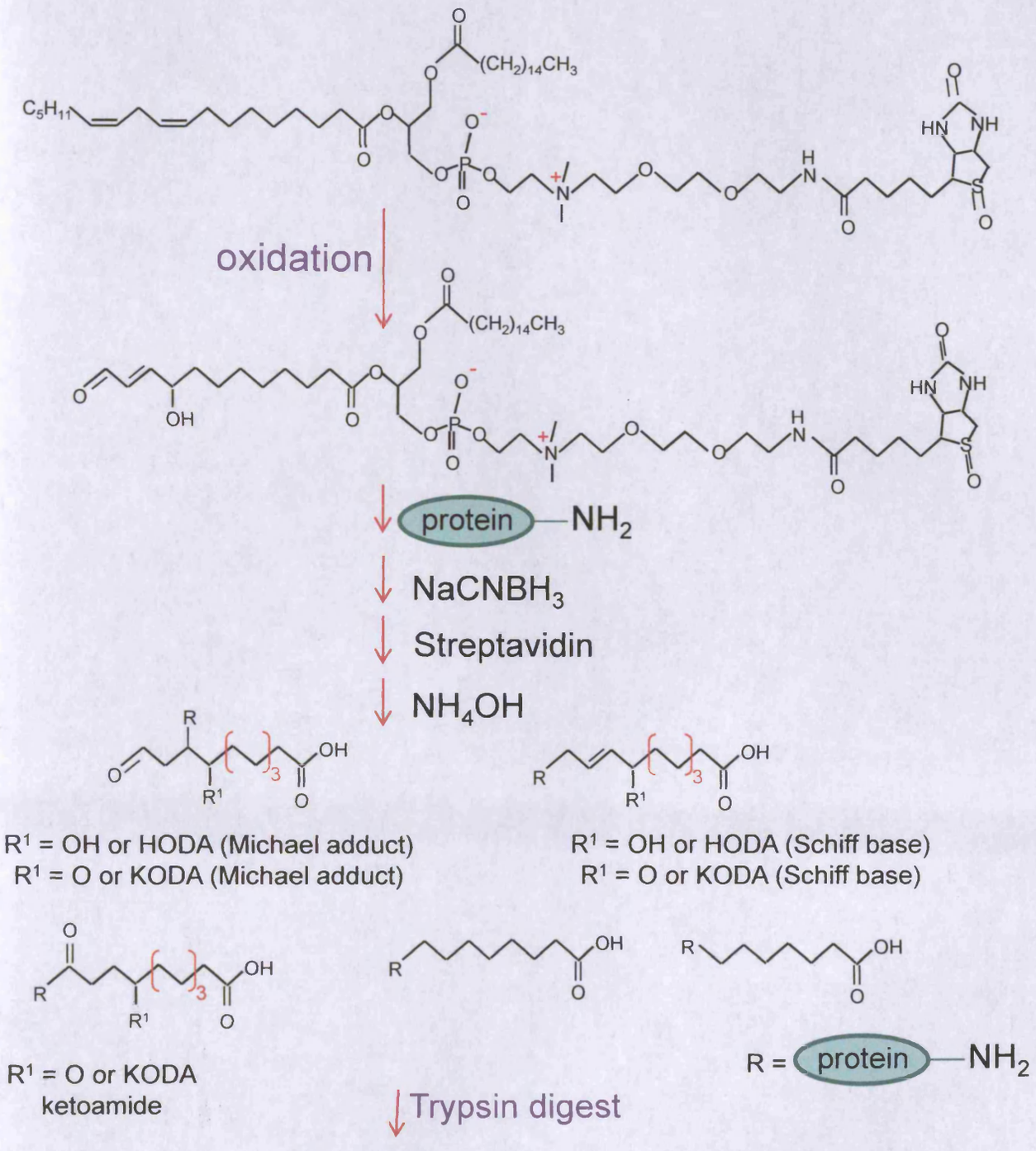


Figure 5.7. The isolation of proteins using biotinylated-PLPC. Oxidized biotin-PLPC can be added to samples of individual proteins or cellular extracts. The phospholipid forms a Schiff bases or Michael adducts, which are stabilised by reduction with $NaCNBH_3$. A streptavidin column is used to extract complexes bound by biotin. Hydrolysis using ammonia cleaves away the protein from the rest of the biotinylated lipid. The protein can then be digested using trypsin, followed by LC/MS/MS analysis for identification (adapted from Szapacs *et al,* 2008, with permission (13/12/2010).

three types of adducts could potentially be formed between proteins and oxidised products of 15-LOX in human monocytes.

Various proteins have been identified as targets of oxidised lipids. Among the most abundant proteins that bound to biotinylated ox-PLPC were albumin, alpha-1-antitrypsin, apolipoprotein A1, A2, A4 and E and complement C3 (Szapacs *et al*, 2008). Table 5.1 lists some metabolites derived from AA that covalently bind to proteins. This list includes examples of adducts that may be similar to complexes formed by products of 15-LOX. Importantly, both free and esterified 13-HpODEs, can covalently bond with amine groups of polypeptides, as well as to PE or PS, thus supporting that HpETE-PEs may be able to form similar adducts (Fruebis *et al*, 1992). PE and PS phospholipid head groups can also form Schiff bases with sugars and oxidised cholesterol products (Levi *et al*, 2008, Nakagawa *et al*, 2005, Hazen *et al*, 1999, Wachel *et al*, 2006, Bach *et al*, 2009a & 2009b). Multiple esterified or free oxidised products of 15-LOX may be able to bind to one protein. For example, a particular report investigated the binding of a 13-HpODE decomposition product, 9,12-dioxo-10(E)-dodecenoid acid (DODE) with cytochrome C. This study showed that up to four DODE molecules could bind to one molecule of cytochrome C at one time (Williams *et al*, 2007). This may be taken into consideration when investigating the binding of 15-LOX products to proteins. These studies show that free eicosanoids can certainly bind covalently with proteins, but as of yet, this has not been investigated for esterified eicosanoids generated by LOXs.

The biological role(s) of these adducts remains largely unknown. In one study, an IgM monoclonal antibody known as EO6 was shown to bind to Schiff base adducts formed between oxidised phospholipids and lysine residues of apoB (Friedman *et al*, 2002). This is proposed to prevent the uptake of ox-LDL by macrophages, and may therefore be important

AA metabolite	Bound Protein	References
Levuglandin E_2 (LGE_2)	Prostaglandin H synthase (PGHS)-1 -2, histones and ubiquitin	Lecomte *et al* ,1990, Boutoud *et al*, 2001, Iyer *et al*, 1989
Prostaglandin H_2 (PGH_2)	albumin and unidentified	Maclouf *et al*, 1980, Fizpatrick & Gorman 1977, Eling *et al*, 1977, Anderson *et al*, 1979, Crutchley *et al*, 1979
Thromboxane A_2 (TXA_2)	albumin and unidentified	Maclouf *et al*, 1980, Fizpatrick & Gorman, 1977
Prostaglandin A_1 (PGA_1)	glutathione by glutathione – S-transferases	Ham *et al*, 1975 Cagen *et al*, 1975 Chaudhari *et al*, 1978 Spearman *et al*, 1985
15-keto-PGF2α	glutathione by glutathione – S-transferases	Ham *et al*, 1975, Cagen *et al*, 1975, Chaudhari *et al* 1978, Spearman *et al*, 1985
4-hydroxynonenal (HNE)	albumin, heat shock proteins, fatty acid binding proteins, IκB, PPAR, MAPK and many others	Requena *et al*, 1997 Aldini *et al*, 2006, Vila *et al*, 2008, Grimsrud *et al*, 2007 Ji *et al*, 2001, Coleman *et al*, 2007, Jacobs & Marnett, 2007, Zhang *et al*, 2006
malondialdehyde (MDA)	spectrin and Apoliprotein B, PE and PS headgroups	Requena *et al*, 1997, Jain & Shohet, 1984, Jain & Hochstein, 1980, Fogelman *et al*, 1980
9,12-dioxo-10(E)-dodecenoid acid (DODE)	Cytochrome C	Williams *et al*, 2007
13-HpODEs (free and esterified)	PE head groups	Fruebis *et al*, 1992
Isoletals	PE head groups	Bernoud-Hubac *et al*, 2004

Table 5.1. Metabolites of AA implicated in the formation of Schiff bases or Michael adducts

in atherosclerosis (Friedman *et al*, 2002). Therefore, there is evidence to support that covalent adducts formed by oxidised phospholipids, may have immunological importance.

In summary, hydroperoxides such as free HpODEs or HpETEs form Michael adducts with amines, polypeptides, PE and PS head groups, therefore it is likely that esterified HpETEs or KETEs synthesised by LOX may also form similar adducts. Furthermore, the formation of covalent bonds between oxidised products of LOX and proteins could account for the disappearance of 15-KETE-PEs seen in Chapter 4. To further investigate the association of radiolabelled products of 15-LOX with proteins initial studies could include using SDS-PAGE to separate protein adducts. This may indicate whether this line of investigation is worth pursuing. If so, then synthesis of a biotinylated AA-PE standard (for subsequent oxidation by LOX) may also be important to identify proteins/peptides that bind to esterified LOX products.

CHAPTER 6

CHARACTERISATION OF ESTERIFIED PRODUCTS GENERATED BY MURINE MACROPHAGE 12/15-LOX

6.1 Introduction

The studies described in the previous chapter investigated products of 15-LOX in human monocytes. Analogous lipids generated by 12/15-LOX have also been detected in murine macrophages (Maskrey *et al*, 2007). Murine 12/15-LOX preferably oxygenates AA at C-12 and its highest expression is in resident peritoneal macrophages (Sun & Funk, 1996). Studies on C57/BL6 mice lacking 12/15-LOX (12/15-LOX$^{-/-}$) have shown that their peritoneal macrophages do not generate either free or esterified 12-HETEs (Sun & Funk, 1996, Maskrey *et al*, 2007). In these mice, total blood cells counts are normal and there are no differences in organ morphology (Sun & Funk, 1996). However, their macrophages show some immunological alterations, including decreased expression of CD-36 in response to IL-4, reduced expression of IL-12 following LPS stimulation and impaired phagocytosis (Huang *et al*, 1990, Zhao *et al*, 2002, Miller *et al*, 2001).

12-HETE-PEs were identified in peritoneal macrophages, and their levels increased following activation with A23187 (Scheme 2.2) (Maskrey *et al*, 2007). These novel esterified products of 12/15-LOX may have immunological significance and their absence may be responsible for at least some of the immune defects in macrophages from 12/15-LOX$^{-/-}$ mice. Therefore, it was important to characterise their generation and metabolism as part of characterising their function.

The phospholipid composition of membranes is important and their trafficking between the inner and outer membrane leaflet can influence events such as apoptosis and phagocytosis. PS and PE are externalised in membranes during apoptosis in order to target cells for phagocytosis (Shiratsuchi *et al*, 1998). Also, there are studies suggesting that oxidation of PS is required prior to their externalisation and for subsequent apoptosis of particular cell types (Tyurina *et al,* 2004a, Jain S, 1985). 12-H(p)ETE-PEs may play a similar function, since phagocytosis by 12/15-LOX$^{-/-}$ macrophages is impaired. Therefore, the externalisation of 12-H(p)ETE-PEs was investigated in this chapter.

6.1.1 Aims

In light of the recent discovery of 12-HETE-PEs in murine macrophages, the studies described in this chapter have:

- Characterised the synthesis, and investigated the metabolism, of 12-H(p)ETE-PEs in murine peritoneal macrophages.
- Investigated their cellular location both basally and following macrophage activation.
- Considered the biological role of 12-H(p)ETE-PEs in murine macrophages. In particular, studies investigated whether 12-H(p)ETE-PEs were externalised following their synthesis.

6.2 Results

6.2.1 12-H(p)ETE-PEs generated basally are cell-associated in peritoneal lavages.

The cellular location of 12-H(p)HETE-PEs was investigated in peritoneal lsavages of WT and 12/15-LOX$^{-/-}$ mice. Cells were firstly pelleted by centrifugation to separate free and cell-associated 12-H(p)ETE-PEs. Next, the supernatant was ultra-centrifuged to separate free and vesicle-associated 12-H(p)ETE-PEs. Samples were reduced using $SnCl_2$, followed by extraction and analysis by LC/MS/MS. The results show that basal 12-H(p)ETE-PEs were entirely cell-associated, and that these lipids are not generated by 12/15-LOX$^{-/-}$ mice (Figure 6.1).

6.2.2 12-H(p)ETE-PEs increase in activated peritoneal lavages.

The levels of 12-H(p)ETE-PEs in control and A23187 activated peritoneal lavage samples were determined. Samples were activated and then the lipids reduced, extracted and analysed by LC/MS/MS. Control murine lavages contained approximately 5.5 ± 0.2 ng 12-H(p)ETE-PEs compared with 18.5 ± 1.03 ng free 12-H(p)ETE per mouse (mean ± SEM, n = 3). Following activation, 12-H(p)ETE-PEs increased to 98.8 ± 17 ng and free 12-H(p)ETE were 123 ± 11 ng per mouse (mean ± SEM, n = 3). The results show that 12-H(p)ETE-PEs and 12-H(p)ETE are present in control lavage samples and increase following activation (Figure 6.2).

6.2.3 12-H(p)ETE-PEs are primarily retained by macrophages following cellular activation.

In order to determine whether 12/15-LOX products are secreted following their generation, activated macrophages were separated from the total lavage by centrifugation. The lipids were reduced, extracted and analysed by LC/MS/MS. Following activation, 12-H(p)ETE-PEs were mostly cell-associated but were also present in the supernatant (Figure 6.3 A). In contrast, free

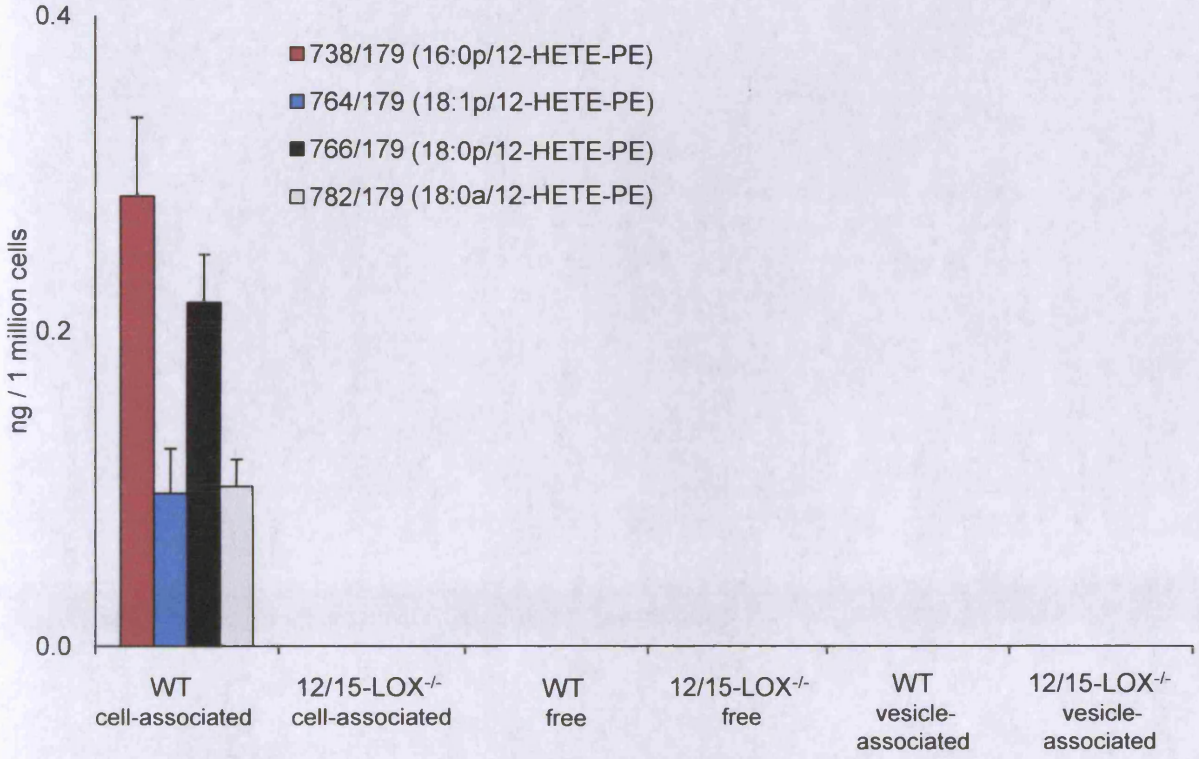


Figure 6.1 Basal 12-H(p)ETE-PEs are cell-associated. Cells in murine peritoneal lavages were pelleted by centrifugation and the supernatant was then ultra-centrifuged. All samples were treated with $SnCl_2$ to reduce the lipids, which were then extracted and analysed by LC/MS/MS (n = 3, mean ± SEM).

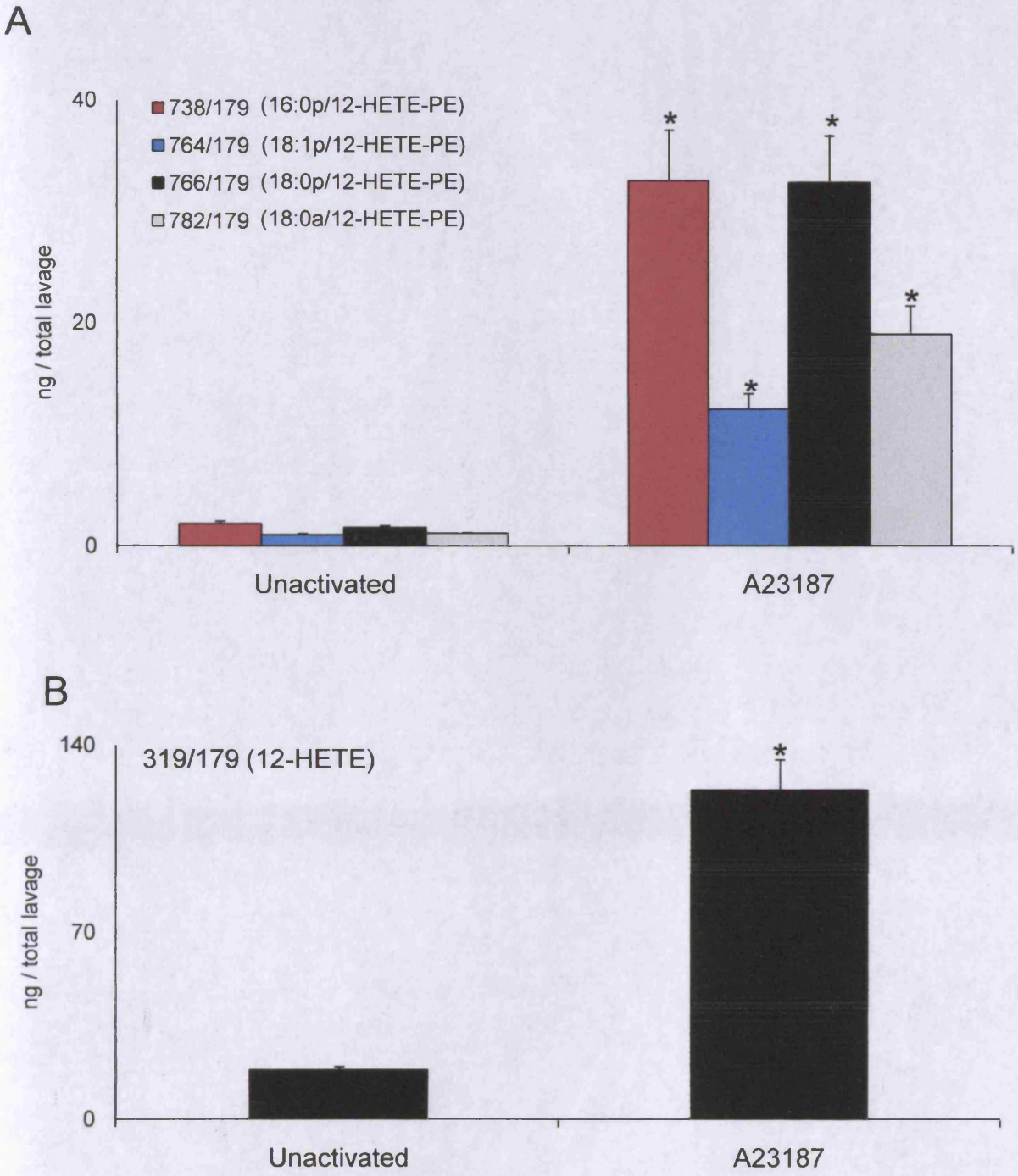


Figure 6.2 12-H(p)ETE-PEs increase following activation in peritoneal lavages. Murine peritoneal lavages were activated with 10 μM A23187 for 15 minutes, samples were treated with $SnCl_2$ reduce the lipids, then underwent lipid extraction. 12-H(p)ETE-PEs (A) and 12-H(p)ETE (B) were analysed by LC/MS/MS (n = 3, mean ± SEM). *Students t-test, $p < 0.05$, the difference of each analyte is significant in comparison to its relative control.

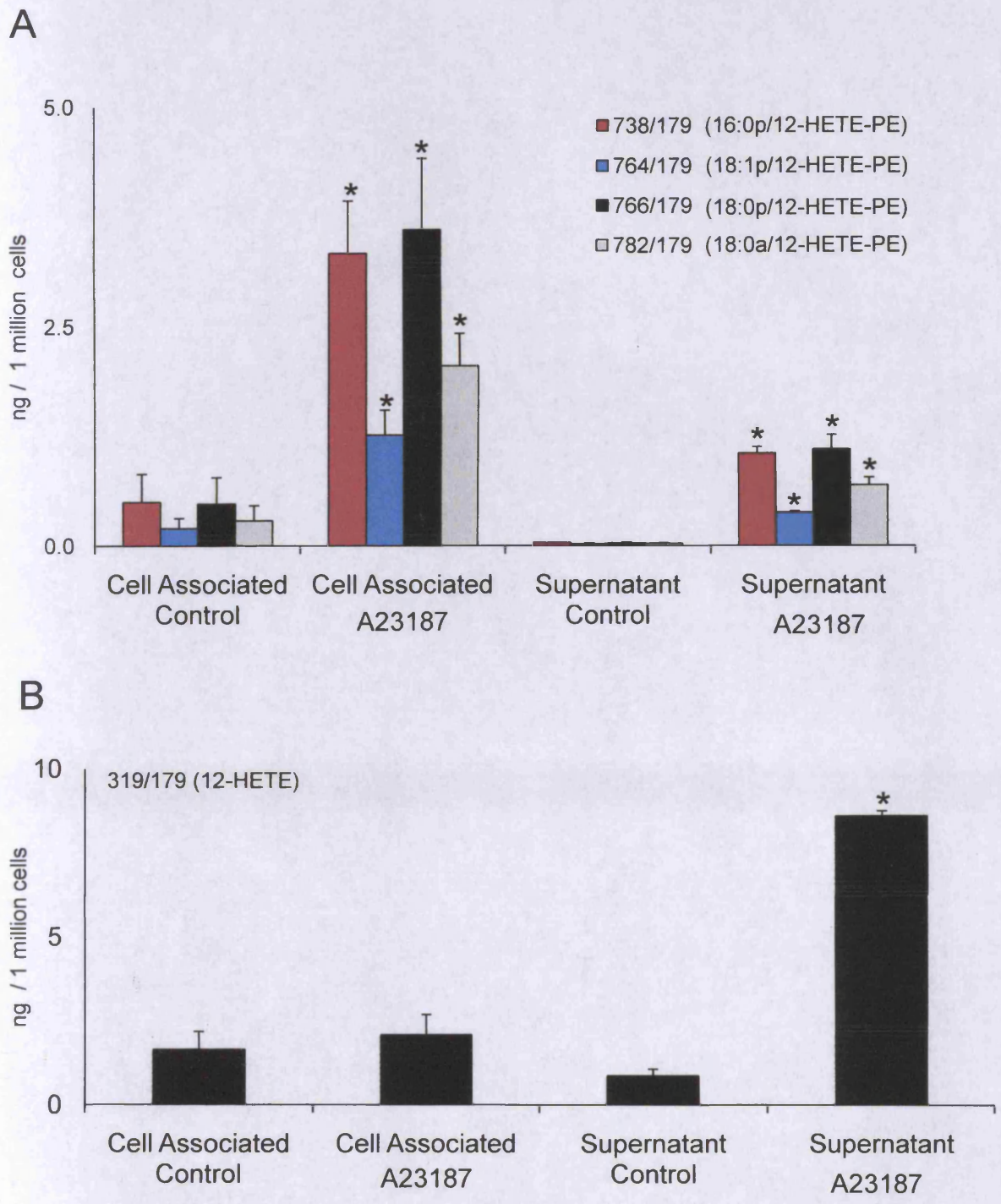


Figure 6.3 12-H(p)ETE-PEs are primarily cell-associated following activation. Peritoneal macrophages were isolated from murine lavages then activated with 10 μM A23187 for 15 minutes, lipids were reduced using $SnCl_2$, extracted and analysed by LC/MS/MS for 12-H(p)ETE-PEs (A) and 12-H(p)ETE (B) (n = 3, mean ± SEM). *Students t-test, $p < 0.05$, the difference of each analyte is significant in comparison to its relative control.

12-H(p)ETE was mainly secreted (Figure 6.3 B). Products of 12/15-LOX may be involved in cellular signalling, regulating events such as inflammation, therefore the results suggest that 12-H(p)ETE-PEs may have both para- and autocrine effects while 12-H(p)ETE may only have paracrine effects.

6.2.4 12S-H(p)ETE is present in the PE phospholipid fraction.

12-HETE is the primary free and esterified HETE isomer formed by murine 12/15-LOX but the chirality of esterified 12-HETE-PEs has not been determined (Maskrey *et al*, 2007). Therefore, experiments were carried out to confirm which HETE enantiomer is synthesised by murine 12/15-LOX. Lavage samples activated using A23187 were reduced with $SnCl_2$ followed by lipid extraction. The PE fraction was then isolated by NP-HPLC, and then the fatty acids released using base hydrolysis, extracted and analysed by LC/MS/MS. 12-H(p)ETE was the most prevalent H(p)ETE isomer present, however, 15-H(p)ETE was also detected, as confirmed using different isomer HETE standards (Figure 6.4 A-J). 12-H(p)ETE was isolated by RP-HPLC then analysed by chiral HPLC. Comparison with 12S- and 12R-HETE standards showed that 12S-H(p)ETE was the only enantiomer present (Figure 6.5). Therefore, the results indicate that murine 12/15-LOX forms some 15-H(p)ETE-PEs but largely synthesises 12S-H(p)ETE-PEs.

6.2.5 12-H(p)ETE-PEs are formed by direct oxidation of membrane phospholipids.

12-HpETE-PEs could be synthesised within the cells by two pathways. The first is by direct oxidation of membrane phospholipids (Scheme 6.1). Alternatively, LOX could oxidise free AA forming free 12-HpETE, which would then be re-esterified (Scheme 6.2). During this pathway, PLA_2 hydrolysis involves the incorporation of oxygen from water into AA. Buffers can be

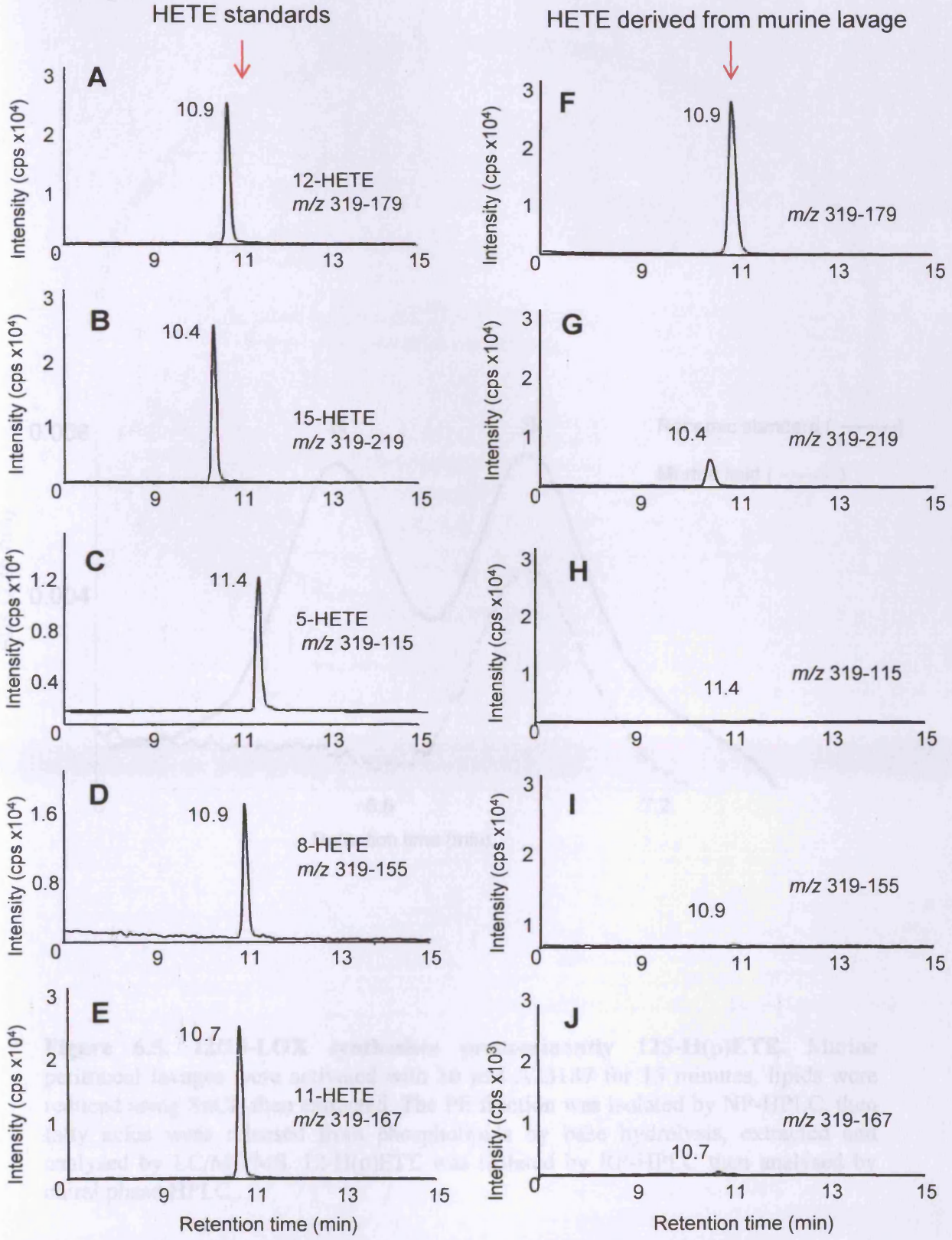


Figure 6.4. 12-H(p)ETE is the main product in the PE fraction according to the peak apex. Murine peritoneal lavages were activated with 10 μM A23187 for 15 minutes, lipids were reduced using $SnCl_2$ and extracted. The PE fraction was isolated by NP-HPLC, then fatty acids were released from phospholipids by base hydrolysis, extracted and analysed by LC/MS/MS. Murine generated HETEs were compared to HETE standards (A-J) (n=10).

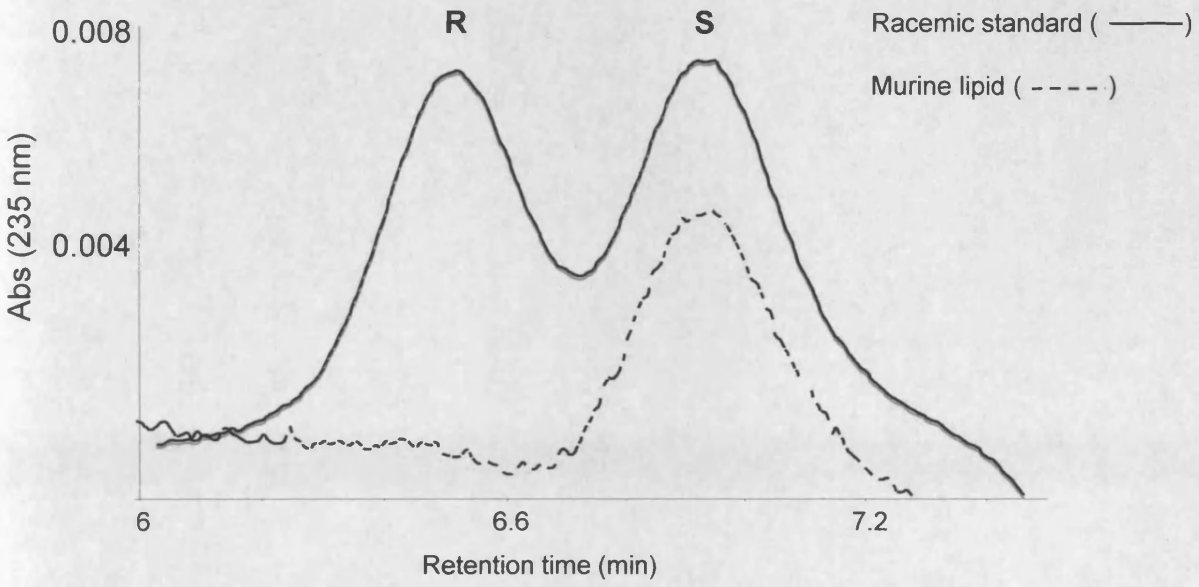
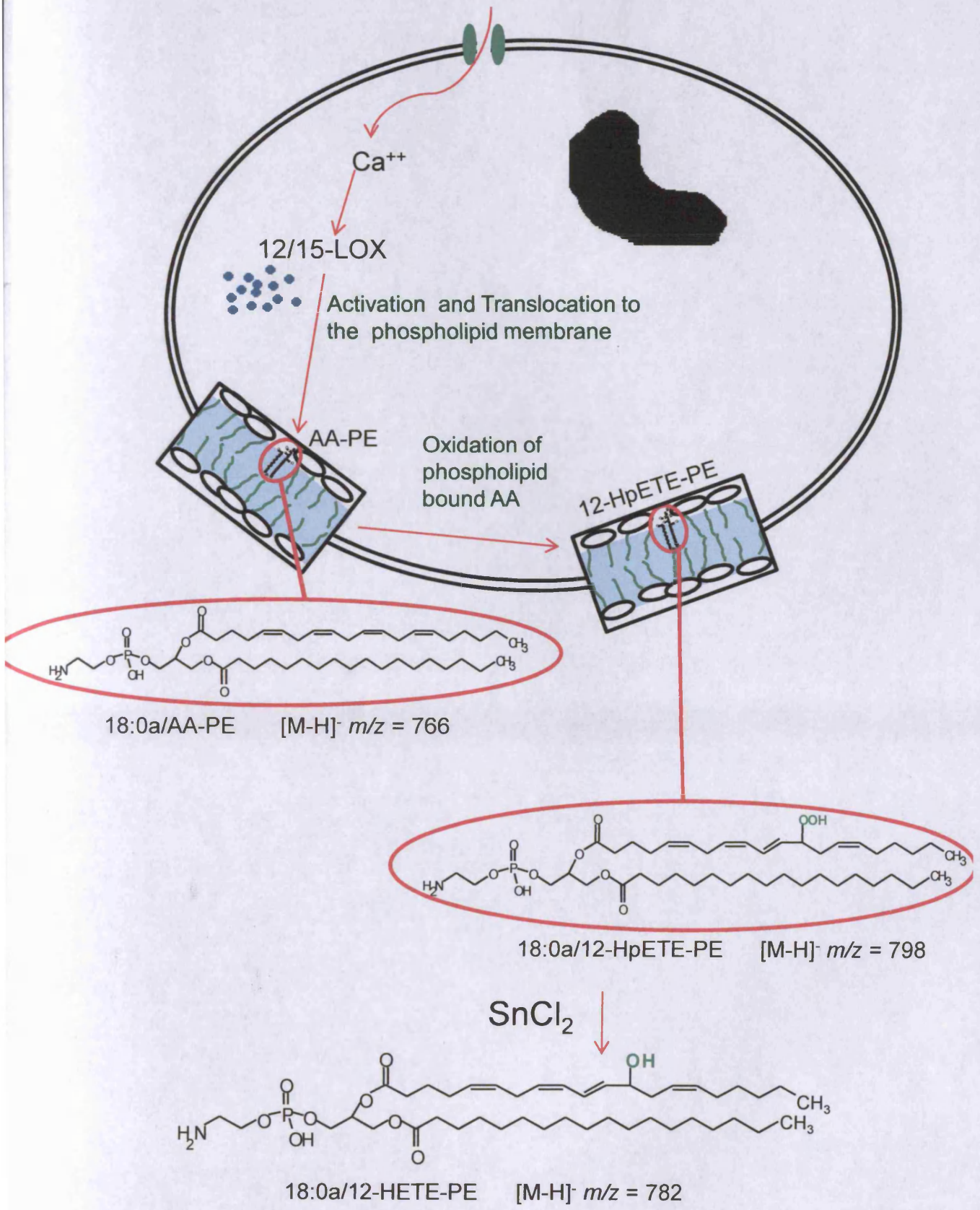
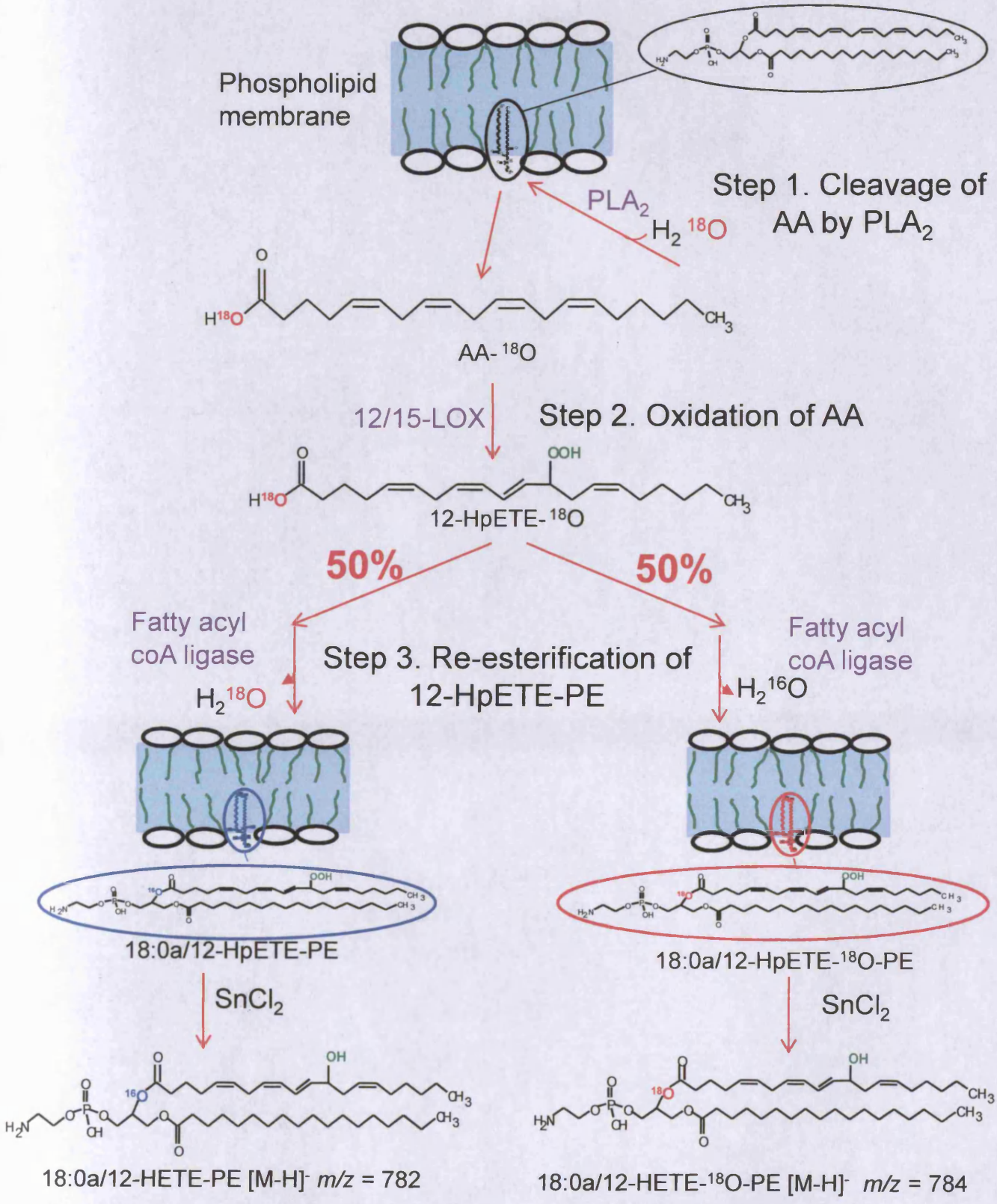


Figure 6.5. 12/15-LOX synthesises predominantly 12S-H(p)ETE. Murine peritoneal lavages were activated with 10 μM A23187 for 15 minutes, lipids were reduced using $SnCl_2$ then extracted. The PE fraction was isolated by NP-HPLC, then fatty acids were released from phospholipids by base hydrolysis, extracted and analysed by LC/MS/MS. 12-H(p)ETE was isolated by RP-HPLC then analysed by chiral phase-HPLC .



Scheme 6.1 Synthesis of 12-HpETE-PE by direct oxidation.



Scheme 6.2 Synthesis of 12-HpETE-PE and 12-HpETE-^{18}O -PE by re-esterification of 12-HpETE or 12-HpETE-^{18}O respectively.

generated using 'heavy' water ($H_2{}^{18}O$), and thus, activation of cells in this buffer would result in incorporation of heavy oxygen (^{18}O) into AA, generating esterified and free HETEs with a mass increase of two. These products can subsequently be analysed by LC/MS/MS. During re-esterification, there is a 50% chance that the ^{18}O label will be lost, and therefore, 50% of 12-H(p)ETE-PE will be labelled (Scheme 6.2). To determine if 12-HpETE-PEs are formed by direct oxidation or by re-esterification of 12-HpETE, macrophages were activated in krebs buffer made up with ^{18}O water. The lipids were reduced then extracted. LC/MS/MS analysis showed that the isotope label was successfully incorporated into free 12-H(p)ETE (data not shown), however, ^{18}O-labelled 12-H(p)ETE-PEs were not detected, indicating that 12-H(p)ETE-PEs are generated by direct oxidation of phospholipids (Figure 6.6).

6.2.6 12-H(p)ETE-PEs are metabolised within 3 hours to unknown products.

Next, the metabolism of 12-H(p)ETE-PEs from murine 12/15-LOX were investigated. Peritoneal macrophages were activated for a maximum of 180 minutes, at each time-point, half of each sample was removed immediately into lipid extraction buffer while the remaining half was reduced prior to lipid extraction. Lipids were then analysed by LC/MS/MS. 30% of 12/15-LOX products were endogenously reduced to 12-HETE-PEs throughout the time-course (non-reduced samples) (Figure 6.7). Endogenous 12-HETE-PEs declined throughout the time-course, almost returning to baseline by 180 minutes (Figure 6.7). Following $SnCl_2$ treatment, detected 12-H(p)ETE-PEs are made up of both endogenous 12-HETE-PEs and additional lipid products of 12/15-LOX that can be chemically reduced to 12-HETE-PEs. Therefore, levels of 12-H(p)ETE-PEs would be higher in $SnCl_2$ treated samples. 12-H(p)ETE-PEs were also metabolised by 180 minutes, however, the decrease in endogenous 12-HETE-PEs was not enough to account for the

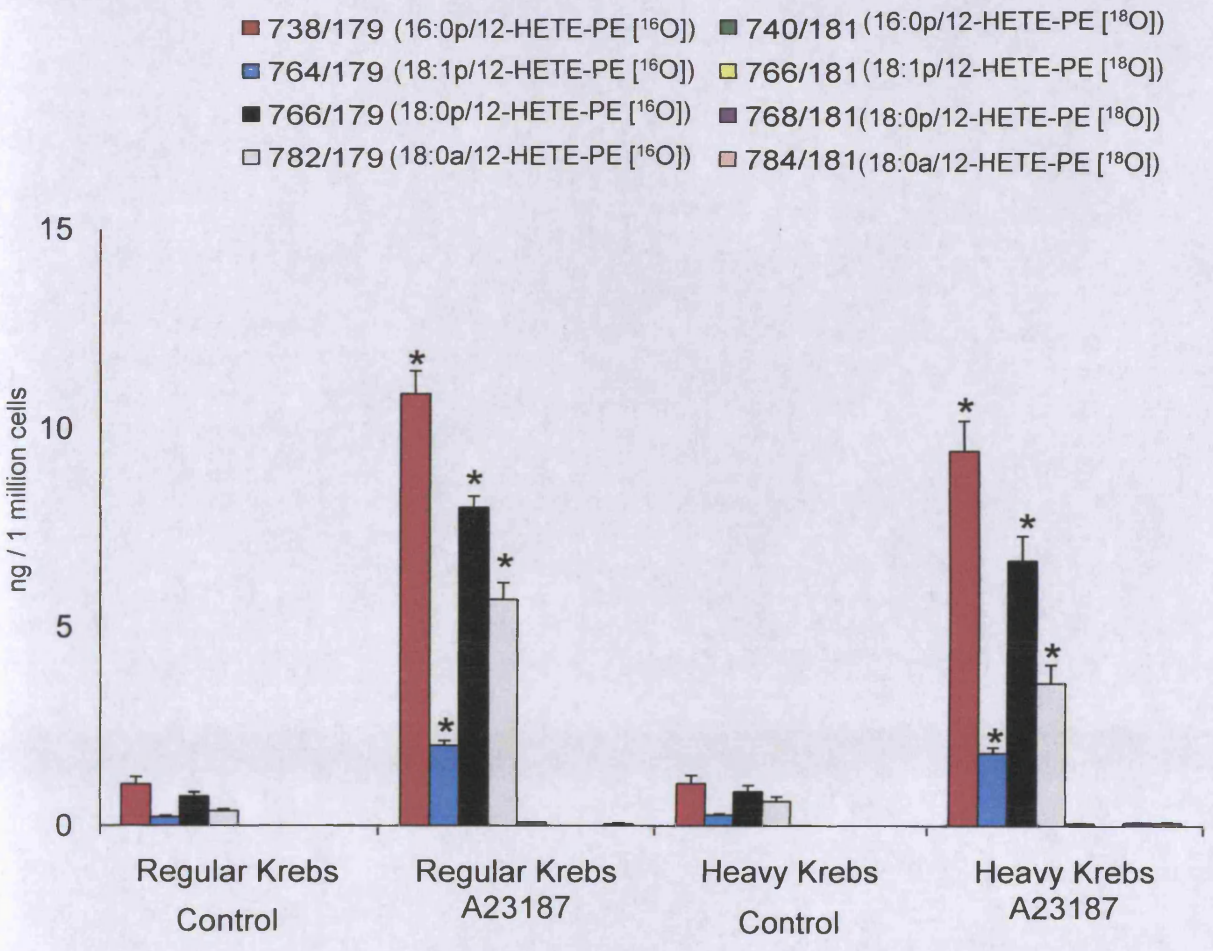


Figure 6.6 12-H(p)ETE-PEs are formed by direct oxidation of AA-PEs. Macrophages from murine lavages were activated with 10 μM A23187 for 15 minutes in krebs buffer generated with either with regular or ^{18}O labelled water. Lipids were reduced using $SnCl_2$ followed by extraction and LC/MS/MS analysis. Samples were analysed for label incorporation into 12-H(p)ETEs (A), 12-H(p)ETE-PEs (B) (n = 3, mean ± SEM). *Students t-test, $p < 0.05$, the difference of each analyte is significant in comparison to its relative control.

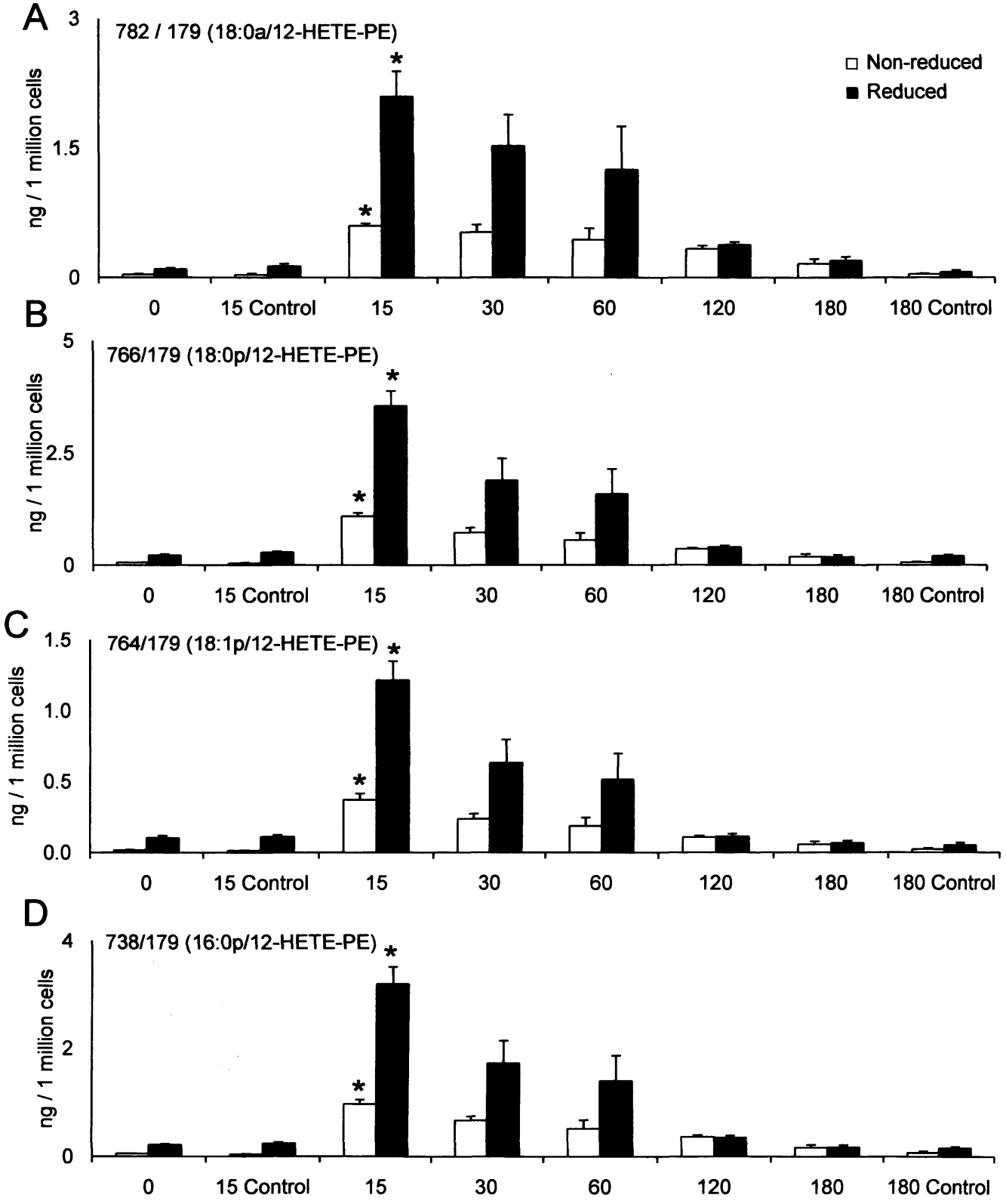


Figure 6.7 12-H(p)ETE-PEs are metabolised by three hours to unknown products. Macrophages isolated from mouse lavages were activated with 10 μM A23187 for various time-points. Lipids from all samples were extracted, half following $SnCl_2$ reduction. Analysis of 18:0a (A), 18:0p (B), 18:1p (C) and 16:0p (D) 12-H(p)ETE-PEs was carried out by LC/MS/MS (n = 3, mean ± SEM). □ Non Reduced ■ Reduced. *Students t-test, $p < 0.05$, with Mann-Whitney test, the difference of each analyte is significant in comparison to its relative control.

decrease in 12-H(p)ETE-PEs in reduced samples. The results suggest that both 12-HETE-PEs and a non-reduced lipid precursor, such as 12-HpETE-PEs or 12-KETE-PEs are metabolised in activated macrophages. In contrast, endogenous free 12-HETE increased following activation then remained stable throughout the time-course (Figure 6.8).

6.2.7 12-H(p)ETE-PEs are externalised in the plasma membrane of WT macrophages following activation.

Previous studies have shown that externalisation of PS and PE phospholipids is required to induce apoptosis in particular cell types prior to their phagocytosis (Shiratsuchi *et al*, 1998). Furthermore, oxidation of phospholipids may be required before they are externalised (Tyurina *et al*, 2004a, Jain SK, 1985). In order to determine whether 12-H(p)ETE-PEs are externalised to the outer leaflet of the membrane bi-layer following activation, peritoneal macrophages were incubated with sulfo-NHS-biotin, which binds to primary amine groups of PE and PS phospholipids on the exterior of cells. Samples were reduced and the lipids extracted. Phospholipids bound to biotin can be followed by MS as their *m/z* increases by 226 amu. 12-H(p)ETE-PEs were externalised following cellular activation of WT macrophages (Figure 6.9 A). However, 12-H(p)ETE-PEs were not detected at all in macrophages from 12/15-LOX$^{-/-}$ mice. Macrophage samples were also analysed for the AA-PEs. In 12/15-LOX$^{-/-}$ macrophages, AA-PEs were externalised, which did not occur in WT cells (Figure 6.9 B). The results suggest that following activation of WT macrophages, 12-H(p)ETE-PEs are selectively externalised, and in the absence of 12/15-LOX, the un-oxidised lipids (AA-PEs) are externalised in their place.

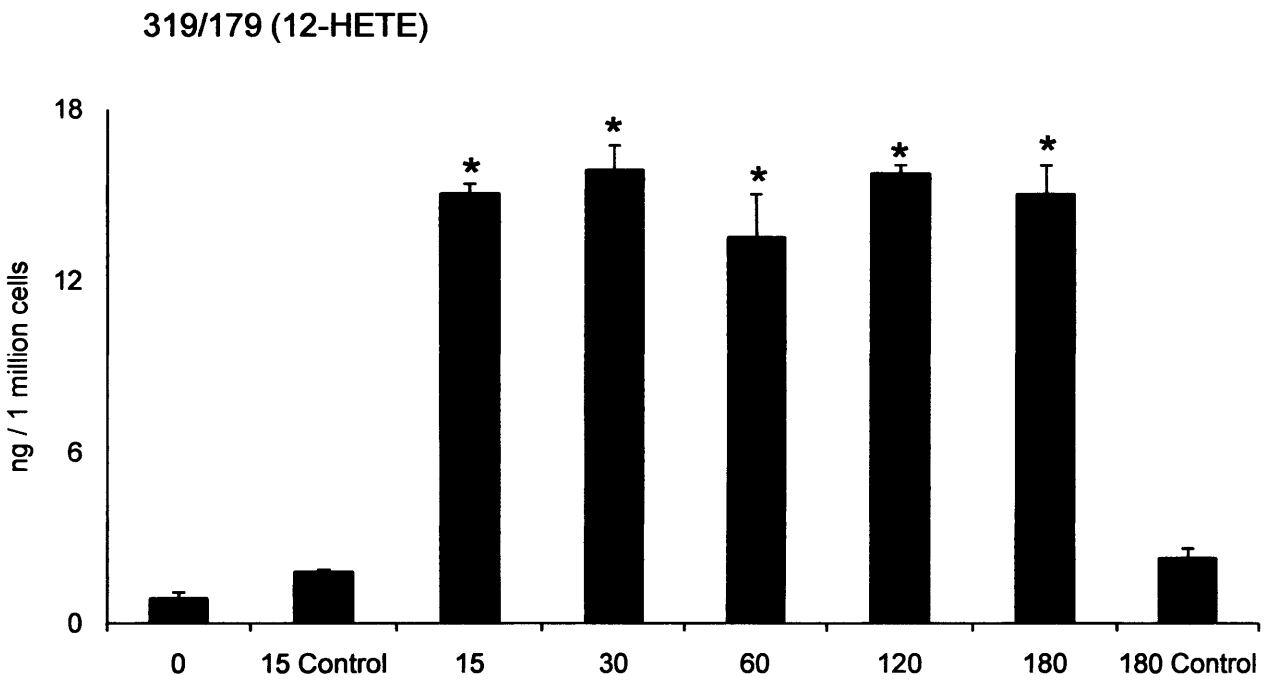


Figure 6.8 Endogenous 12-HETE remains stable following activation. Macrophages from mouse lavages were activated with 10 μM A23187 for various time-points. Lipids were extracted and endogenous 12-HETE was analysed by LC/MS/MS (n = 3, mean ± SEM). *Students t-test, $p < 0.05$, the difference is significant in comparison to the control.

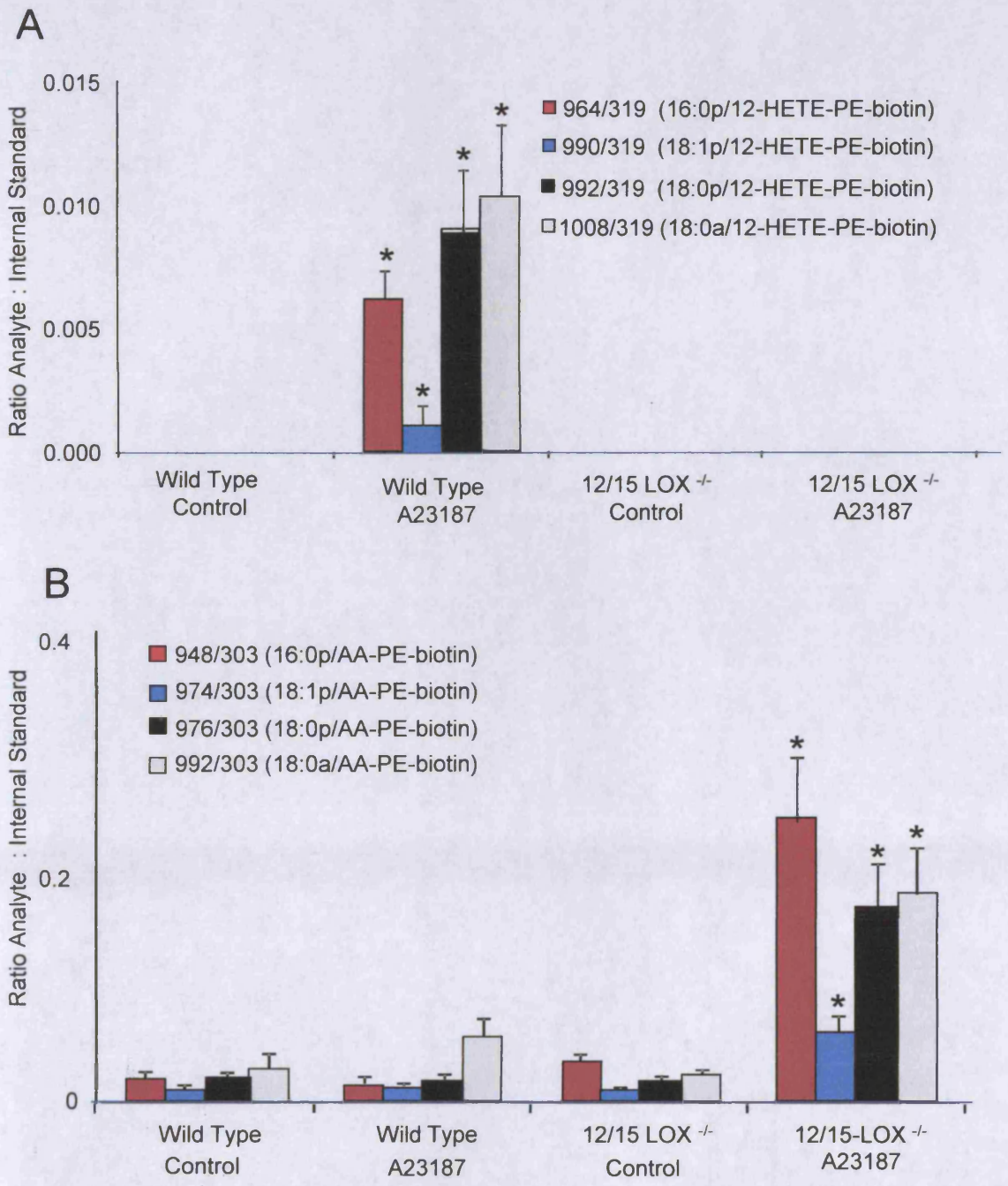


Figure 6.9 12-H(p)ETE-PEs are externalised following activation in macrophage plasma membrane. Murine macrophages were activated with A23187 and externally exposed PE were biotinylated. Samples were then reduced using $SnCl_2$, followed by lipid extraction and LC/MS/MS analysis. Levels of biotinylated 12-H(p)ETE-PEs (A) and AA-PEs (B) were determined before and after activation in WT or 12/15-LOX$^{-/-}$ peritoneal macrophages (n = 3, mean ± SEM). *Students t-test, $p < 0.05$, the difference of each analyte is significant in comparison to its relative control.

6.3 Discussion

12-H(p)ETE-PEs generated by 12/15-LOX were recently identified by Maskrey *et al* (2007) in murine macrophages following A23187 activation. 12/15-LOX is suspected to play a role in inflammatory disorders such as atherosclerosis, however, the products responsible have not been identified. Following the discovery of 12-H(p)ETE-PEs, it is important to characterise their generation and metabolism, and establish how they may be involved in inflammatory disorders.

The results in this chapter have shown that macrophage 12-H(p)ETE-PEs are derived entirely from direct oxidation (rather than esterification of free 12-H(p)ETE), similar to 15-H(p)ETE-PEs in human monocytes (Figure 6.6) (Maskrey *et al,* 2007). Interestingly, this is different to platelet 12-LOX esterified phospholipids, which are generated by esterification of 12-HETE (Thomas *et al*, 2010). This suggests that platelet 12-LOX and murine 12/15-LOX synthesise the same products but by a different mechanism. However, human 15-LOX and murine 12/15-LOX generate their products by the same mechanism. In addition, previous studies have shown that oxidised cholesterol ester products of rabbit reticulocyte 15-LOX are also the result of direct oxidation, this supports that 12/15-LOX enzymes synthesise their products using similar mechanisms (Belkner *et al*, 1998).

Following this, the metabolism of 12-HpETE-PEs in murine macrophages was investigated. The trend seen in reduced macrophage time-course samples was similar to results seen in Chapter 4 for human monocytes, where the lipid precursors of 15-HETE-PEs were identified as 15-KETE-PEs and were the primary lipids metabolised in human monocytes (Figures 4.9- 4.12). The lipid precursor of 12-HETE-PE in mice may be 12-HpETE-PE or 12-KETE-PE but as of yet, this has

not been investigated. If the products are identified as either of these lipids, product ion spectra and LC/MS/MS retention times require validation using standards. 12-HETE-PE and 12-HpETE-PE standards can be generated using air oxidation techniques (as described in Chapter 3), however, 12-KETE-PEs cannot be generated in this way and are not available commercially (Yin *et al*, 2008).

In time-course experiments, endogenous 12-HETE-PEs were metabolised by 180 minutes, this is different to results obtained from human monocytes where only the lipid precursors were metabolised (Figures 4.8 and 6.7). The metabolism of 12-HETE-PEs and their precursors may be via PLA_2 cleavage of the sn2 fatty acid. However, levels of endogenous 12-HETE were stable throughout the time-course. This is similar to results in human monocytes where 15-HETE remained stable in time-course samples and hydrolysis by PLA_2 was ruled out as a method of 15-HETE-PE and 15-KETE-PE metabolism. However, to exclude the metabolism of 12-H(p)ETE-PEs by PLA_2 enzymes, experiments are required using inhibitors of PLA_2. 12/15-LOX translocates to the plasma membrane following activation, suggesting that 12-HpETE-PEs are likely to be synthesised from plasma membrane derived phospholipids (Miller *et al*, 2001). Studies in Chapter 5 determined that 15-H(p)ETE-PEs or 15-KETE-PEs may bind to proteins. It may therefore be relevant to investigate whether 12-H(p)ETE-PEs (or 12-KETE-PEs) can form covalent associations with membrane proteins. Covalent bonding could account for the decrease in the unidentified products of 12/15-LOX. The results suggest that 12/15-LOX products from mice may be metabolised differently to 15-LOX products generated by human monocytes. This may be related to the pro- versus anti-inflammatory effect of 12/15-LOX in mice and rabbits respectively. For example, 12/15-LOX is believed to be anti-atherogenic in rabbits but pro-

atherogenic in mice, suggesting that murine leukocyte 12-LOX and rabbit 15-LOX may not be functionally equivalent (Kuhn *et al*, 1994b, Neuzil *et al*, 1998, Sun & Funk, 1996, Shen *et al*, 1996, Smith *et al*, 1993, Takata *et al*, 1994a). Future experiments could therefore compare the effect of 12-H(p)ETE-PEs and 15-H(p)ETE-PEs in both murine and rabbit models of atherosclerosis.

12-H(p)ETE-PEs are externalised by activated WT macrophages (Figure 6.9). This may indicate an involvement in apoptosis or interactions with other cells. The externalisation of 12-H(p)ETE-PEs may also alter membrane fluidity, which could affect phagocytosis and migration. Movement of PE and PS phospholipids to the outer leaflet of plasma membranes is suggested to be a marker of cell death and is required for phagocytosis of apoptotic cells (Mirnikjoo *et al*, 2009, Fadok *et al*, 2001, Diaz *et al*, 1999, Shiratsuchi *et al*, 1998). Furthermore, oxidation of PS phospholipids has been described prior to their externalisation, a process which was also essential for the phagocytosis of particular cells (Tyurina *et al,* 2004a, Jain SK, 1985). Therefore, externalised 12-H(p)ETE-PEs could be required for macrophages to carry out phagocytosis. In support of these studies, treatment of leukemia HL-60 cells with an anti-oxidant, to prevent the peroxidation of PS, also blocked PS externalisation and phagocytosis (Tyurina *et al*, 2004b). Furthermore, hepatoma HepG2 cells are reported to undergo apoptosis when exposed to exogenous PE. Thus externalised 12-H(p)ETE-PEs in macrophage membranes may have a similar effect on cells that are targeted for phagocytosis (Yao *et al*, 2009).

12/15-LOX has been linked to cellular actions that require re-structuring of the cell membrane. Once such example is during phagocytosis, where macrophages from 12/15-LOX$^{-/-}$ mice fail to

properly phagocytose apoptotic thymocytes (Miller *et al*, 2001). The results in this chapter show that 12-H(p)ETE-PEs are externalised following cellular activation. This could alter membrane fluidity and explain how 12/15-LOX is involved in events such as phagocytosis. In addition, phosphorylation of F-actin during phagocytosis requires the activity of 12/15-LOX (Rice *et al*, 1998, Tang *et al,* 1993, Liu *et al,* 1995, Honn & Tang *et al,* 1992, Honn *et al,* 1994, Miller *et al*, 2001 & 2003, Tang & Honn, 1994, Kang *et al,* 1999). Actin is a major component of the cytoskeleton and its re-structuring is pivotal to actions requiring movement of cell membranes (Berg *et al*, 2002). Therefore, future experiments could include investigating if 12-H(p)ETE-PEs are involved in the phosphorylation of F-actin. Furthermore, as HETE is reported to form a complex with proteins thought to be subunits of actin, experiments could also show whether 12-H(p)ETE-PEs can bind to F-actin (Kang et al, 1999). 12/15-LOX is reported to translocate to the plasma membrane of macrophages and dendritic cells following activation (Andersson *et al,* 2006, Miller *et al*, 2001, Zhu *et al*, 2003). This supports the action of 12/15-LOX on membrane phospholipids, where product generation may have a direct affect on membrane fluidity and structure. While studies show that 12/15-LOX, and therefore, 12-H(p)ETE-PEs, may be involved in re-structuring the cellular membrane, the importance of this is yet to be assessed. However, as 12/15-LOX is only significantly expressed in peritoneal macrophages its role is likely to be specific to this subset of cells (Sun & Funk, 1996).

If 12/15-LOX, and therefore 12-H(p)ETE-PEs, are involved in actin polymerisation then they may also have an effect on cell migration. Over-expression of 12/15-LOX in endothelial cells transfected with the gene results in the up-regulation of VCAM-1, which increases the adhesiveness of cells'. A similar result was observed when endothelial cells were induced with

IL-4, a cytokine used to induce 12/15-LOX in human monocytes (Lee *et al*, 2001, Vitta *et al,* 1999). As discussed above, PE externalisation alters the properties of a cell membrane, which may also affect the ability of cells to adhere to one another. Therefore, externalised 12-H(p)ETE-PEs may be involved in interactions between macrophages and endothelial cells. The up-regulation of VCAM by 12-H(p)ETE-PEs has not been investigated as of yet, but may be worth pursuing to determine the role of 12-H(p)ETE-PEs in migration and cell-cell interactions. In particular, migration assays comparing the attachment of 12/15-LOX$^{-/-}$ and WT macrophages to surface ligands expressed by epithelial cells, would investigate whether 12-H(p)ETE-PEs are involved in macrophage-epithelial interactions. In support that such cell-cell interactions may take place, oxidised PS phospholipids in macrophage membranes have been shown to interact with the CD-36 receptor of apoptotic cells undergoing phagocytosis (Greenberg *et al*, 2006). In particular, externalisation of a decomposed hydroperoxide group from oxidised phospholipids may assist the physical contact of cells with pattern recognition receptors, known as the lipid whisker model (Figure 6.10) (Greenburg *et al,* 2008).

In addition to 12-H(p)ETE-PE externalisation in murine macrophages, platelet 12-H(p)ETE-PEs are externalised following activation (Thomas *et al*, 2010). Interestingly, in a patient suffering from the bleeding disorder Scott Syndrome (where membrane lipid scrambling is impaired), platelet 12-HETE-PEs are not externalised following activation (Thomas *et al*, 2010). This suggests that scramblases are involved in 12-H(p)ETE-PE externalisation. Lipid oxidation is also reported to affect aminophospholipid translocases and PS scrambling, causing the externalisation of PS phospholipids (Tyurina *et al,* 2004a). However, little is known about the structure and mechanism of floppases and scramblases, and most of the current research investigating the

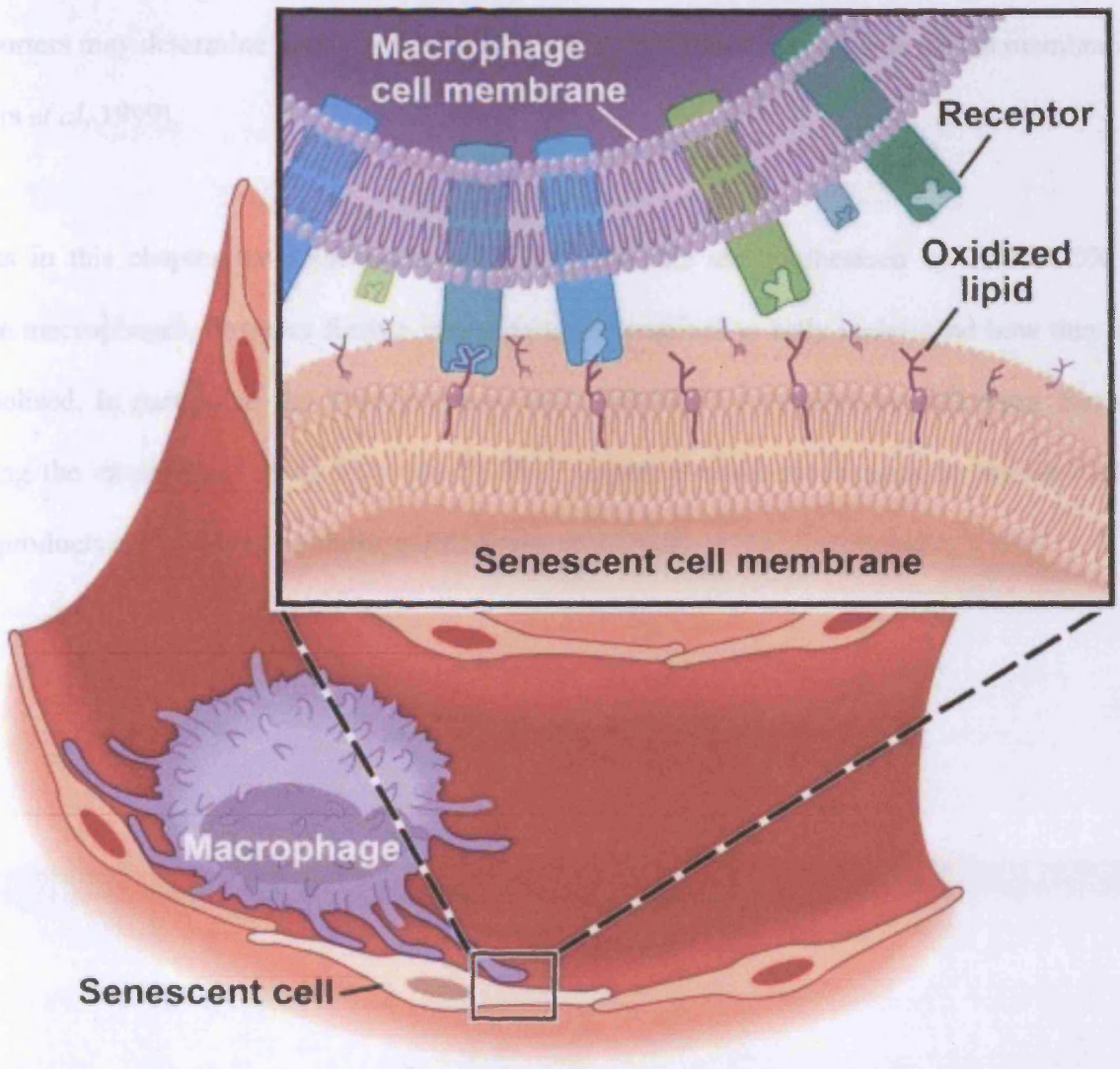


Figure 6.10. Schematic representation of the lipid whisker model. Cell membranes of apoptotic cells have oxidised phospholipids with sn-2-oxidised fatty acid acyl chains protruding into the extracellular space. This conformation allows them to interact with scavenger receptors and other pattern recognition receptors on the surface of macrophages (reproduced from Greenburg *et al,* 2008, with permission (12/11/2010)).

externalisation of lipids has concentrated on PS and not PE. The use of inhibitors for these transporters may determine how 12-H(p)ETE-PEs are moved to the outer-side of cell membranes (Bevers *et al*, 1999).

Results in this chapter have shown how 12-H(p)ETE-PEs are synthesised by 12/15-LOX in murine macrophages, however further investigation is required to fully understand how they are metabolised. In particular, the lipid precursor of 12-HETE-PEs requires identification. Results showing the externalisation of 12-H(p)ETE-PEs suggest a novel mechanism by which 12/15-LOX products are involved in altering membrane properties.

CHAPTER 7

CELLULAR UPTAKE OF 15-HETE-PE, ITS EFFECT ON CYTOKINE GENERATION AND ITS SYNTHESIS DURING HUMAN BACTERIAL INFECTION

7.1 Introduction

The generation of murine 12-HETE-PEs in acute inflammatory processes was recently characterised in three *in-vivo* models, (i) peritonitis in response to *S.epi* supernatant (SES), (ii) live *S.epi* infection, or (iii) following OVA sensitization of the lung (Figures 7.1 and 7.2) (Morgan *et al*, 2009). Results showed that levels of 12-HETE-PEs were reduced in murine peritoneal lavages following SES and live *S.epi* infection, but raised in ova lung allergy. These results indicate that 12/15-LOX is regulated during both Th1 and Th2 driven immune responses.

The presence of 15-HETE-PEs has not been investigated in human inflammatory disorders. Patients undergoing peritoneal dialysis as a treatment for renal failure are prone to bacterial infections, such as by *S.epi*. Lavage fluid taken from patients with suspected bacterial infections could be analysed by MS for 15-HETE-PEs, to determine whether levels of these phospholipids are altered during bacterial infection in humans.

Following their generation, 12- and 15-HETE-PEs remain cell-associated, which suggests that they may play a role in intracellular signalling (Chapters 4 and 6). This may include stimulating the synthesis of cytokines. As described in Chapter 3, a 15-HETE-PE standard

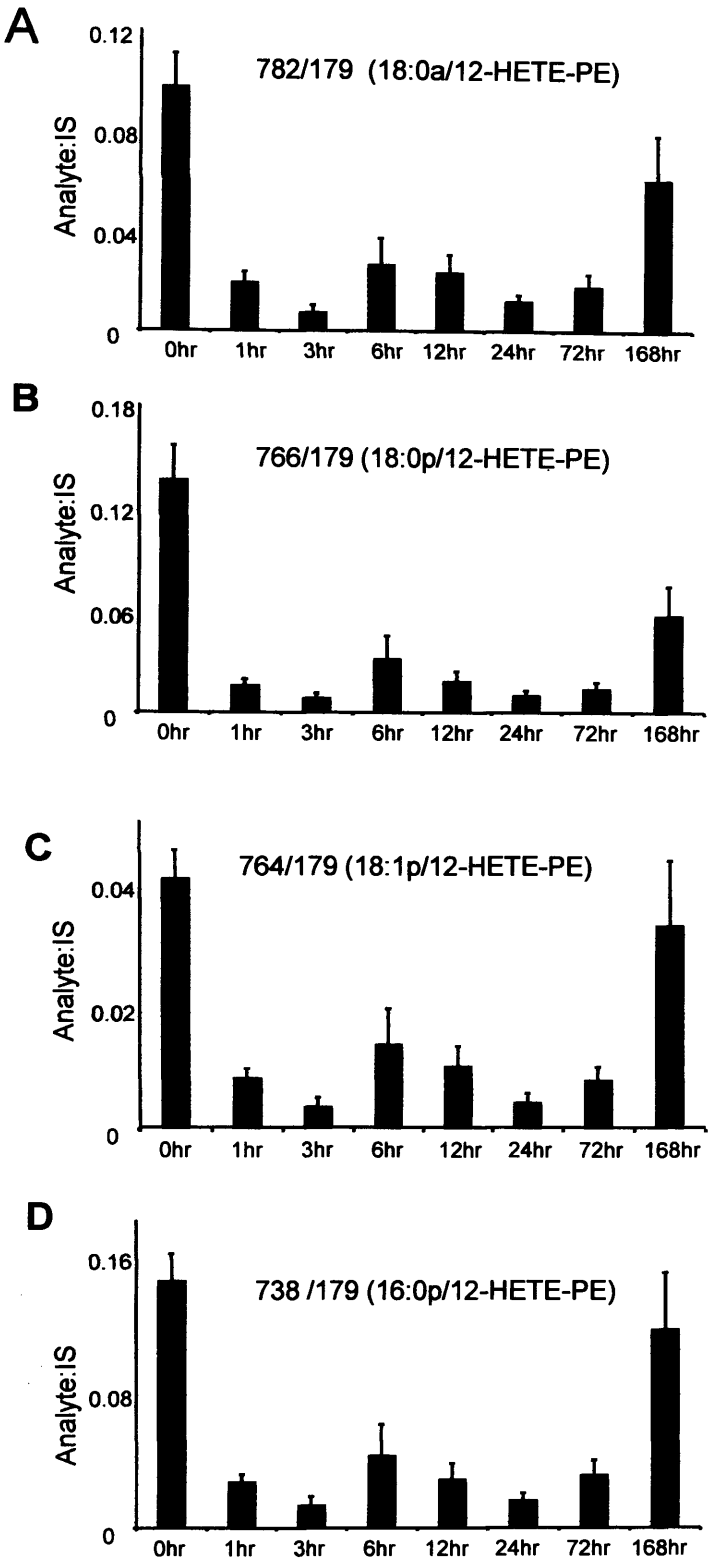


Figure 7.1 12-HETE-PEs decrease in SES peritonitis during inflammation, then recover by day 7. 12-HETE-PEs from peritoneal lavages of SES-infected mice were profiled post infection at various times by LC/MS/MS (n = 5) (Reproduced from Morgan *et al*, 2009, with permission (12/11/2010).

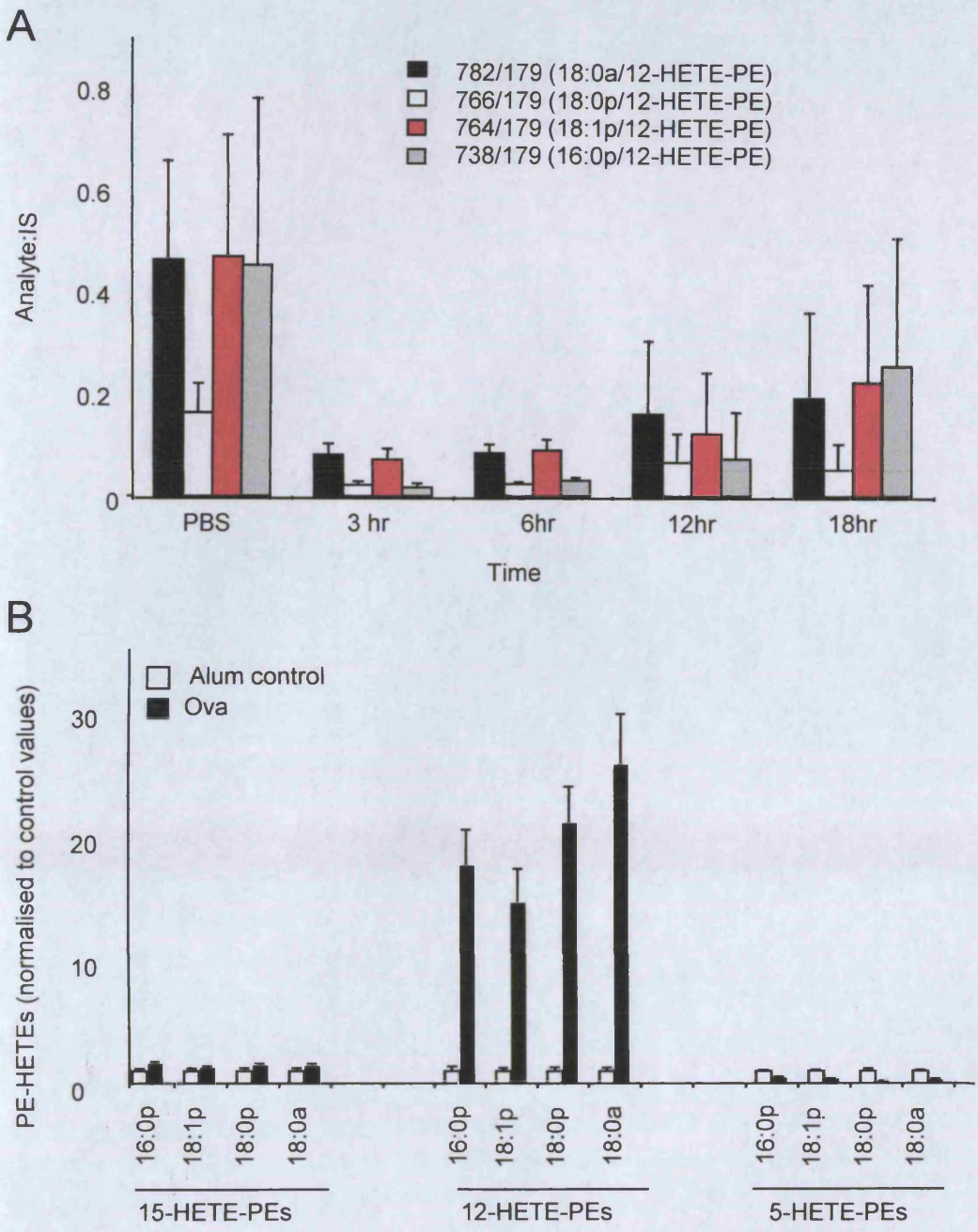


Figure 7.2 12-HETE-PEs decrease in live bacterial peritonitis, but are elevated in Ova lung allergy. (A) 12-HETE-PEs from peritoneal lavages of S.epi-infected mice were profiled post infection at various times by LC/MS/MS (n = 3 - 5). (B) 12-HETE-PEs were profiled in lung homogenate from Ova-sensitized mice after 6 days of Ova challenge (intranasally), using LC/MS/MS (n = 3, mean = ± SEM) (Reproduced from Morgan *et al*, 2009, with permission (12/11/2010).

was synthesised, which can be used in biological experiments. Before investigating its effect on cytokine generation, studies were conducted to determine the best method for its delivery and incorporation into cultured cells. Three methods were tested.

(i) The lipids were added to cells in methanol solution.

(ii) Liposomes were used. These are well known as vehicles of different substances, they have been utilised in gene therapy, drug delivery and as immune adjuvants (Figure 7.3) (Griesenbach *et al*, 1998, Liu D, 1991, Abra RM, 1984, Gregoriadis *et al*, 1996, Bennett JE, 1996). However, their application as lipid transporters has not been explored.

(iii) Albumin-lipid complexes were tested. Carrier proteins such as serum albumin can also transport substances across cell membranes. Albumin binds reversibly and with high affinity to a diverse selection of ligands, including fatty acids, drugs, metals, amino acids and heme containing compounds (Varshney *et al*, 2010).

The above methods were adapted for our own investigations, to introduce 15-HETE-PE to cells. Following the successful incorporation of 15-HETE-PE, experiments then investigated its biological function. Oxidised phospholipids are reported to affect cytokines generated by LPS stimulated cells (von Schlieffen *et al*, 2009, Erridge *et al*, 2007, Erridge *et al*, 2008, Walton *et al*, 2003). Therefore, the effect of 15-HETE-PE on cytokine generation was investigated in monocytes activated by LPS.

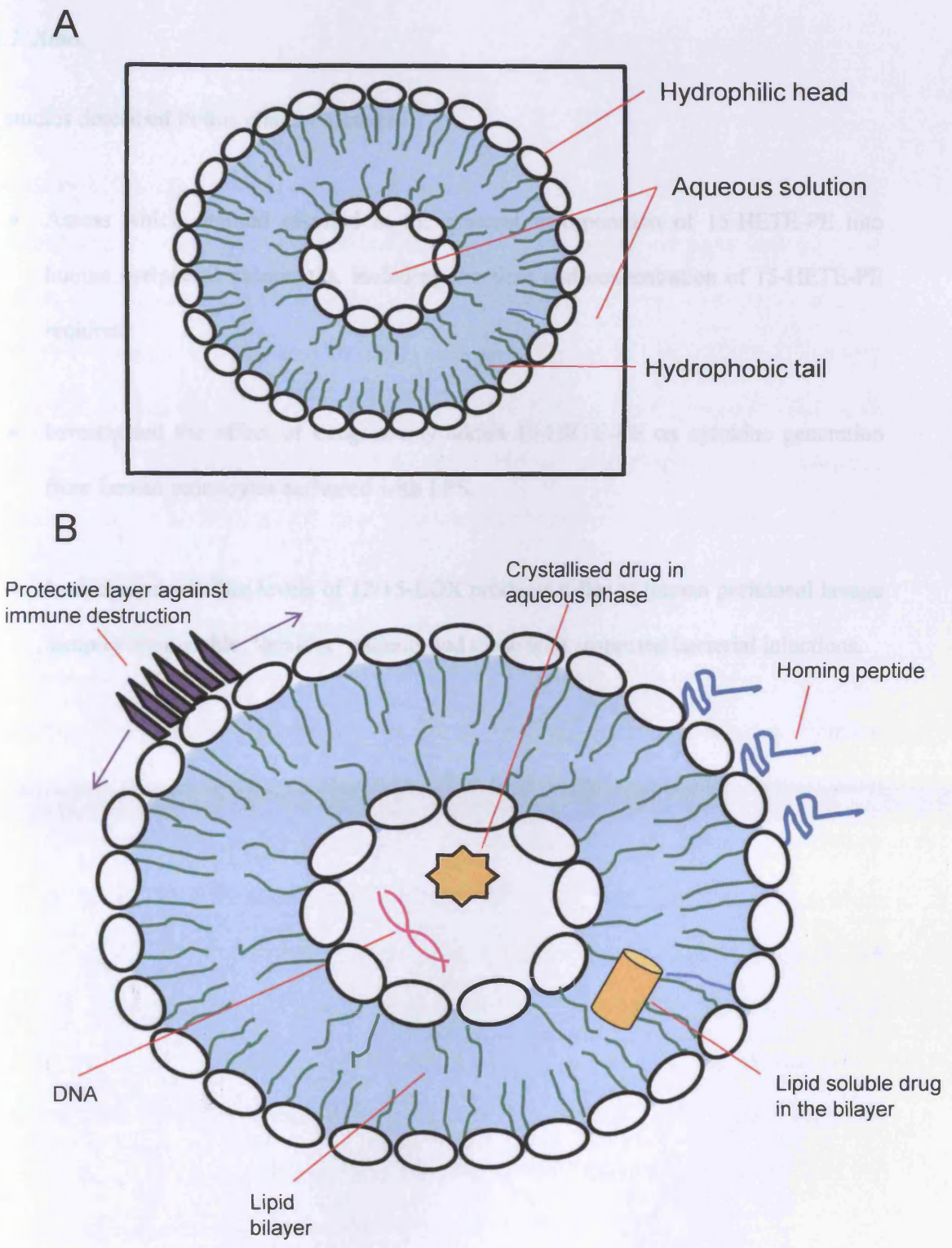


Figure 7.3 Liposomes are vesicles of bilayer phospholipids used to transport various substances. (A) Liposomes are formed from a lipid bilayer which encapsulate aqueous solutions. (B) Liposomes can be adapted for use as carriers of many substances such as drugs and DNA.

7.1.1 Aims

In studies described in this chapter aimed to:

- Assess which method resulted in the greatest incorporation of 15-HETE-PE into human peripheral monocytes, including the time and concentration of 15-HETE-PE required.

- Investigated the effect of exogenously added 15-HETE-PE on cytokine generation from human monocytes activated with LPS.

- Investigated whether levels of 12/15-LOX products differ in human peritoneal lavage samples from stable, 'healthy' patients and those with suspected bacterial infections.

7.2 Results

7.2.1 Synthesis of liposomes containing 15-HETE-PE.

Liposomes of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 15-HETE-PE were generated using a freeze-thaw method and vesicles larger than 100 nm were removed by an extruder. The liposomes were analysed by MS to ensure incorporation of both DPPC and 15-HETE-PE. A negative ion Q1 scan revealed ions corresponding to DPPC $[M\text{-}CH_3]^-$ (*m/z* 718) and a DPPC adduct $[M+Cl]^-$ (*m/z* 768) (Figure 7.4 A). Positive ion Q1 scans showed peaks corresponding to DPPC $[M+Na]^+$ (*m/z* 756) and $[M+H]^+$ (*m/z* 734) (Figure 7.4 B). 15-HETE-PE was not seen in the negative Q1 scan, which is likely due to high concentrations of DPPC causing ion suppression. However, LC/MS/MS analysis of the sample confirmed the presence of this lipid in the liposome preparations (Figure 7.5).

To confirm the identity of peaks seen in Q1 scans, product ion spectra were obtained. Negative product ion spectra of DPPC ions 718 and 768 showed similar fragmentation patterns, both containing a fragment of *m/z* 255, which corresponds to palmitic acid (Figure 7.6 A and B). It should be noted that the chlorine adduct, *m/z* 768, is not seen in its product ion spectrum due to its complete fragmentation. A positive product ion spectrum of 734 confirmed the presence of the PC head group (*m/z* 184) (Figure 7.6 C). A negative product ion spectrum of *m/z* 782 was also carried out to confirm the presence and identity of 15-HETE-PE. The spectrum contained all the typical fragments of 15-HETE-PE (*m/z* 283 (stearic acid), 319, 219 and 175 (15-HETE)) (Figure 7.7). These results confirm that the generated liposomes exclusively contained DPPC and 15-HETE-PE.

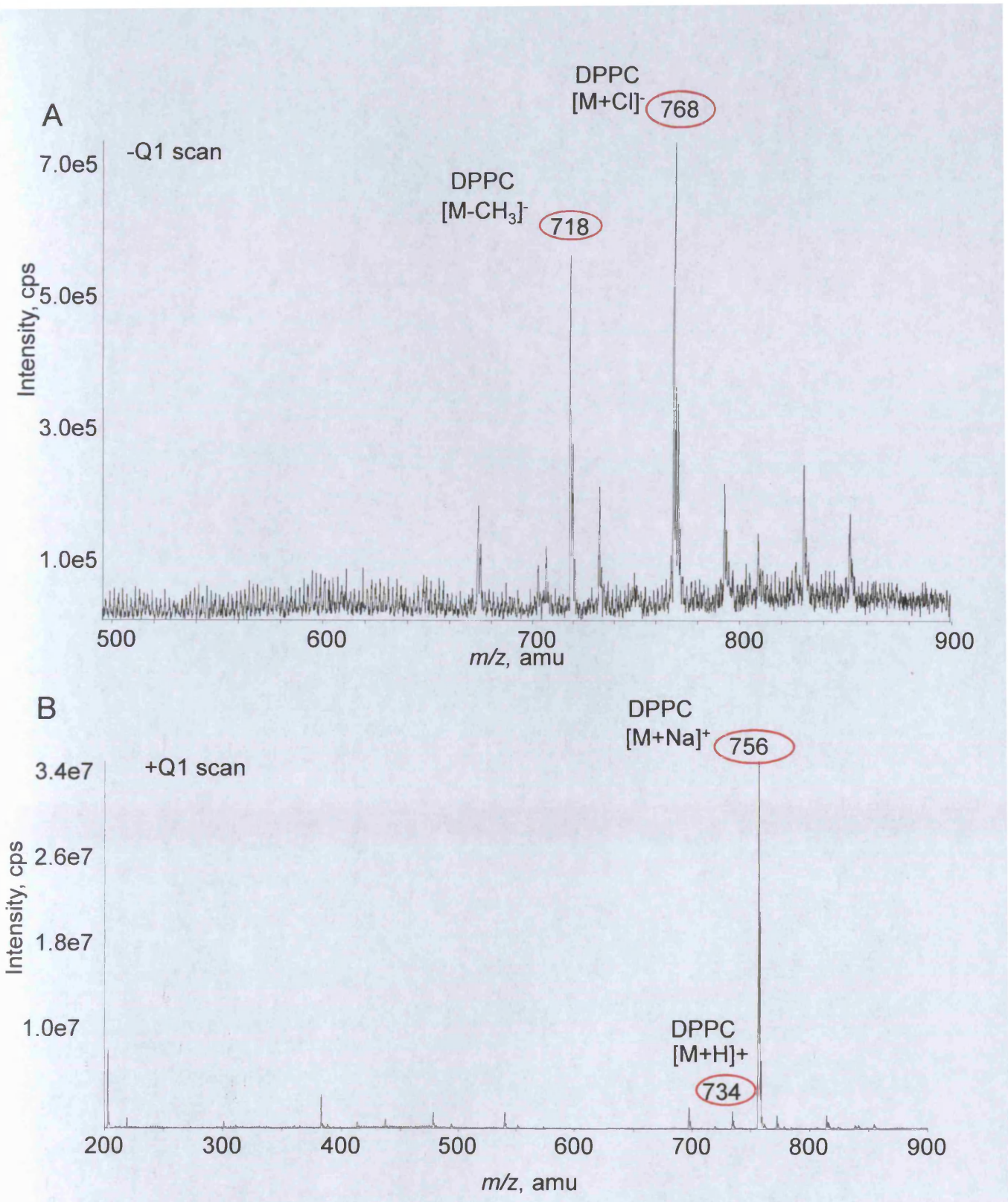


Figure 7.4 Confirmation that synthesised liposomes contain DPPC. Liposomes were made using a freeze thaw method then analysed by MS. Q1 scans of a 1 in 1000 diluted liposome sample in negative mode (A) and positive mode (B) confirm presence of DPPC.

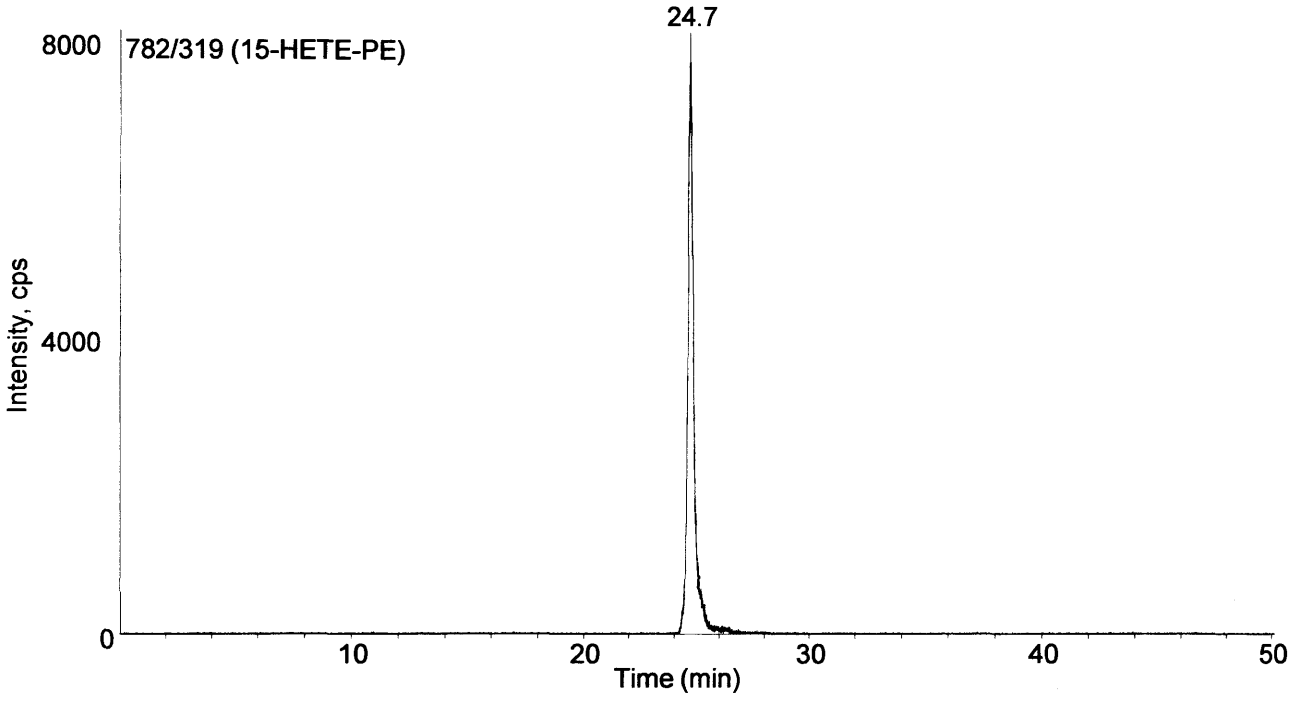


Figure 7.5 Confirmation that synthesised liposomes contain 15-HETE-PE. Liposomes were made using a freeze thaw method. A sample was analysed by LC/MS/MS to confirm the presence of 15-HETE-PE.

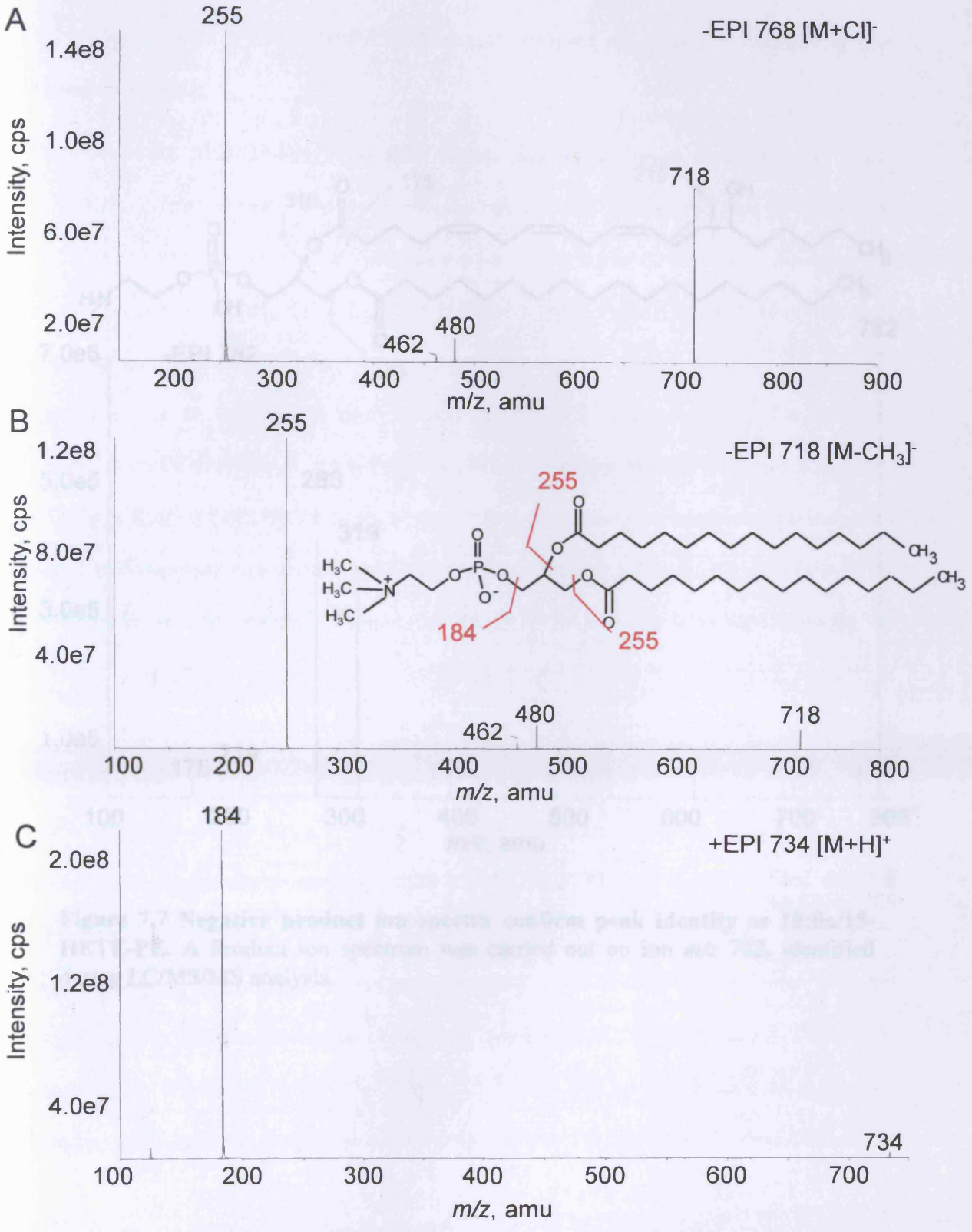


Figure 7.6 Negative and positive product ion spectra confirm peak identity as DPPC. Product ion spectra were carried out on ions identified during Q1 scan analysis of liposome sample. (A) 768 $[M+Cl]^-$, (B) 718 $[M-CH_3]^-$ and (C) 734 $[M+H]^+$.

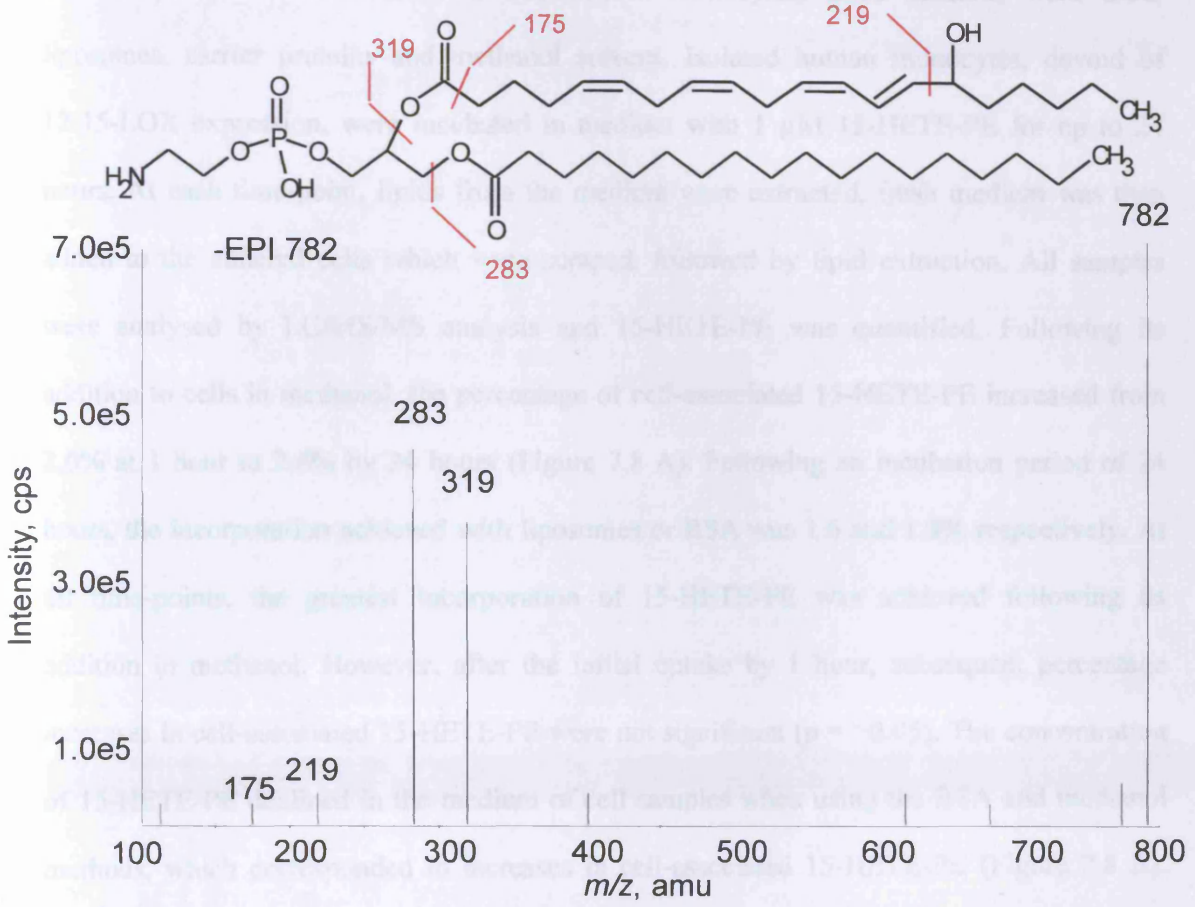


Figure 7.7 Negative product ion spectra confirm peak identity as 18:0a/15-HETE-PE. A Product ion spectrum was carried out on ion *m/z* 782, identified during LC/MS/MS analysis.

7.2.2 The addition of 15-HETE-PE in methanol achieved the largest incorporation into human monocytes.

To incorporate 18:0a/15-HETE-PE into human monocytes, three methods were used: liposomes, carrier proteins and methanol solvent. Isolated human monocytes, devoid of 12/15-LOX expression, were incubated in medium with 1 μM 15-HETE-PE for up to 24 hours. At each time-point, lipids from the medium were extracted, fresh medium was then added to the adhered cells which were scraped, followed by lipid extraction. All samples were analysed by LC/MS/MS analysis and 15-HETE-PE was quantified. Following its addition to cells in methanol, the percentage of cell-associated 15-HETE-PE increased from 2.0% at 1 hour to 2.6% by 24 hours (Figure 7.8 A). Following an incubation period of 24 hours, the incorporation achieved with liposomes or BSA was 1.6 and 1.8% respectively. At all time-points, the greatest incorporation of 15-HETE-PE was achieved following its addition in methanol. However, after the initial uptake by 1 hour, subsequent percentage increases in cell-associated 15-HETE-PE were not significant ($p = >0.05$). The concentration of 15-HETE-PE declined in the medium of cell samples when using the BSA and methanol methods, which corresponded to increases in cell-associated 15-HETE-PE (Figure 7.8 B). When comparing the combined concentration of 15-HETE-PE in cells and medium, 60-85% of the initial 15-HETE-PE concentration is unaccounted for. This suggests that 15-HETE-PE may decompose when stored at temperatures of 37 °C or it readily precipitates onto the plastic sides of tissue culture wells. A higher concentration of 15-HETE-PE was detected in the medium of liposome samples in comparison to BSA and methanol methods, suggesting that liposome complexes may reduce its decomposition and precipitation. The results indicate

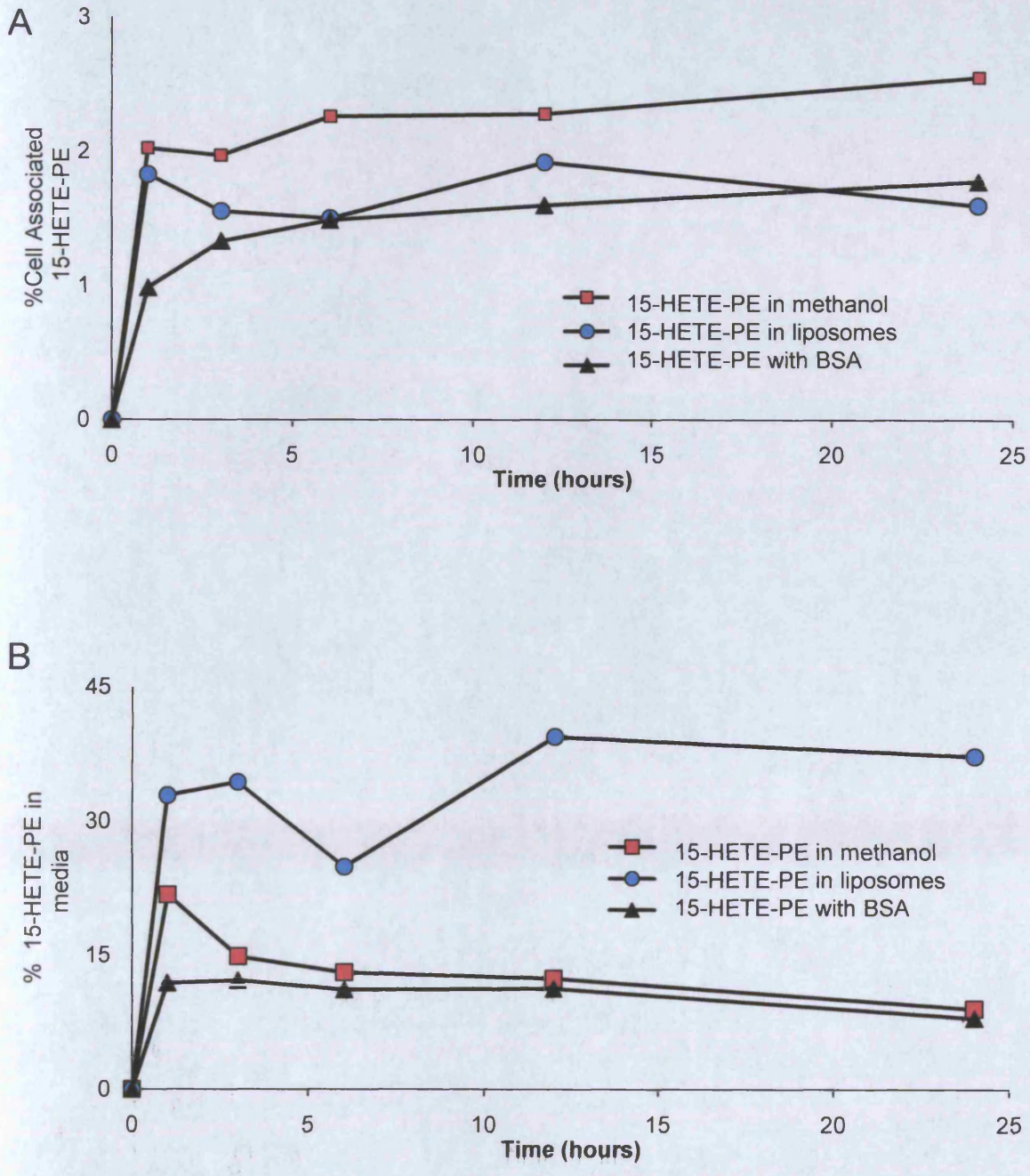


Figure 7.8 The addition of 15-HETE-PE in methanol resulted in the largest incorporation. 1 μM 15-HETE-PE was added to monocytes either in methanol (■), DPPC liposomes (●) or in BSA solution (▲) for various time-points. Lipids were extracted from both media and cells and analysed by LC/MS/MS. The percentage of 15-HETE-PE was calculated: A) % cell associated 15-HETE-PE (B) % 15-HETE-PE present in the media.

that the addition of 15-HETE-PE in methanol gives the greatest incorporation into human monocytes, although overall incorporation was very low.

7.2.3 Incorporation of 15-HETE-PE into human monocytes is dose-dependent.

To determine the optimum concentration of 15-HETE-PE required for incorporation, monocytes were incubated for 1 hour, with 0.4 to 40 μg (1 to 100 μM) 15-HETE-PE. Lipids from cells and the surrounding medium were extracted then analysed by LC/MS/MS. The uptake of 15-HETE-PE was dose-dependent up to incubation with 20 μg (50 μM) 15-HETE-PE, but above this, incorporation began to plateau (Figure 7.9). The concentration of cell-associated 15-HETE-PE increased from 0.005 to 1.45 μg following incubation with 0.4 to 40 μg respectively (Figure 7.9 A). The actual incorporation following incubation with 40 μg (100 μM) was greater than at 20 μg (50 μM) but the overall percentage of incorporation declined from 6.2 to 3.7% (Figure 7.9 B). This suggests that the addition of 15-HETE-PE at concentrations of above 50 μM were approaching the maximum concentration for lipid uptake using this method. Therefore, concentrations of 50 and 100 μM 15-HETE-PE were used to investigate their effect on cytokine generation in LPS treated human monocytes.

7.2.4 Cytokine generation is dose-dependently reduced by 15-HETE-PE.

Next, the effect of 15-HETE-PE on cytokine generation was investigated. Human monocytes were incubated for 24 hours in tissue culture wells containing media with 50 or 100 μM 15-HETE-PE or the un-oxidised lipid, SAPE, in the presence or absence of LPS. Incubation for 24 hours was required as cytokine levels do not typically increase until several hours

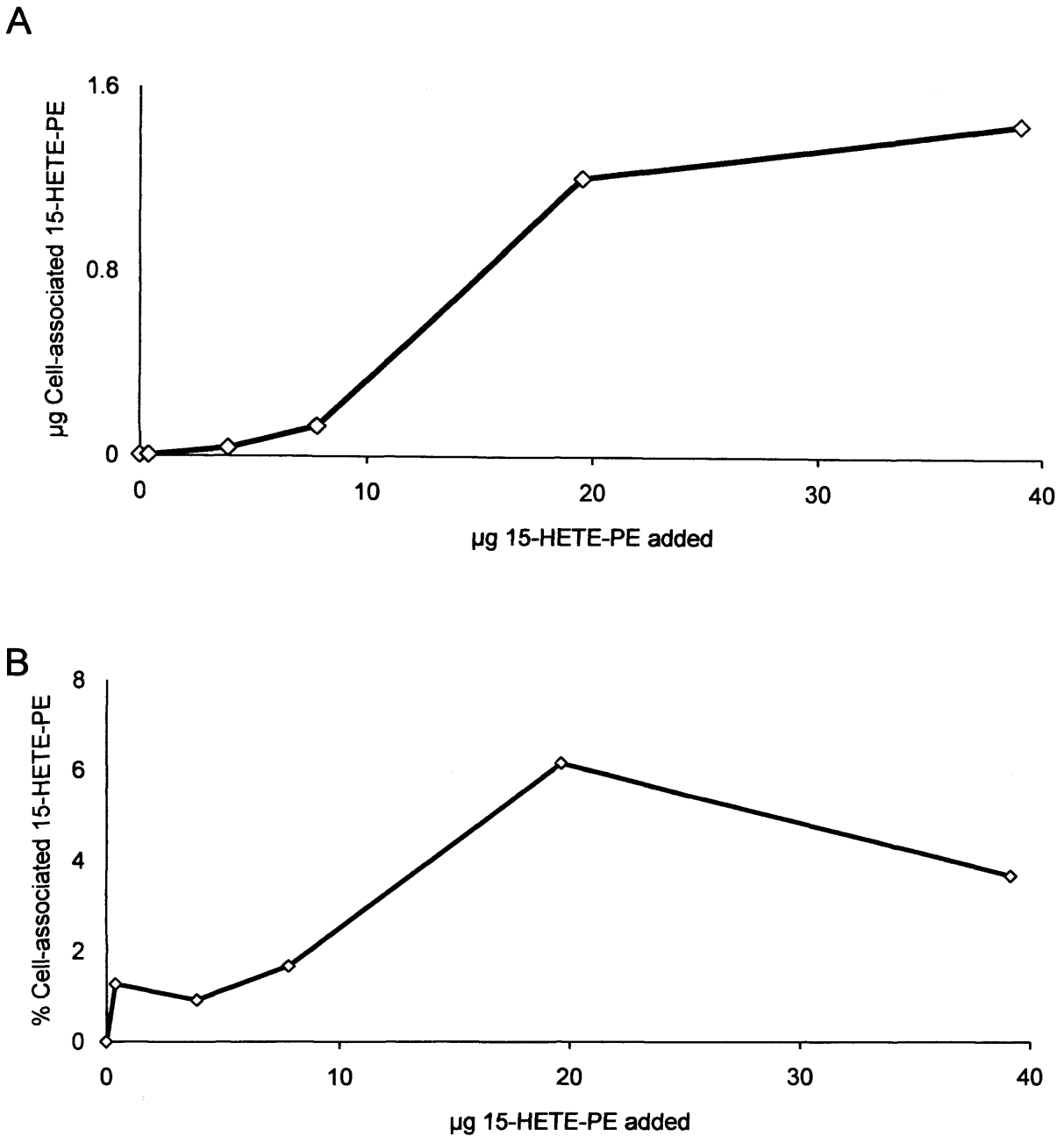


Figure 7.9 Incorporation of 15-HETE-PE is dose-dependent. Human monocytes were incubated for one hour with increasing concentrations of 15-HETE-PE. Cell-associated lipids were extracted and analysed by LC/MS/MS. The amount of cell-associated 15-HETE-PE was calculated (A) and also the overall percentage of incorporation (B).

following cell stimulation (Janeway *et al*, 2005). Following 24 hours, the medium from each well was transferred to eppendorf tubes and centrifuged to remove cellular debris. Medium was then analysed for generation of cytokines by ELISA. In control samples, no cytokine generation was seen following the addition of 15-HETE-PE or SAPE, however, LPS stimulated a strong generation of G-CSF, IL-1β, IL-6, TNF-α and IL-10 (Figures 7.10-7.14). The addition of 15-HETE-PE to un-stimulated monocytes resulted in decreased generation of G-CSF, IL-1β, IL-6, IL-8 and MCP-1 (Figures 7.10-7.12 & 7.15-7.16), however, this was not dose-dependent. In addition, it may not be specific to 15-HETE-PE as IL-1β, IL-6 and MCP-1 generation was also deceased following treatment with 50 μg SAPE. The addition of 15-HETE-PE to LPS-stimulated monocytes resulted in dose-dependent inhibition of G-CSF, IL-1β, IL-6 and TNF-α, while SAPE was without effect (Figures 7.10-7.13). IL-10 was also dose-dependently reduced following the addition of 15-HETE-PE. However, a similar result was seen in response to SAPE, suggesting that inhibition of IL-10 generation was not specific to 15-HETE-PE (Figure 7.14). LPS failed to stimulate the synthesis of IL-8 and RANTES, and levels did not significantly change following the addition of 15-HETE-PE or SAPE (Figures 7.15 & 7.17). MCP-1 generation was reduced by LPS, a response that was further reduced by both 15-HETE-PE and SAPE (Figure 7.17). The results indicate that 15-HETE-PE down-regulates LPS stimulated generation of G-CSF, IL-1β, IL-6 and TNF-α, and may also down-regulate generation of G-CSF, IL-1β, IL-6, IL-8 and MCP-1 in un-stimulated cells.

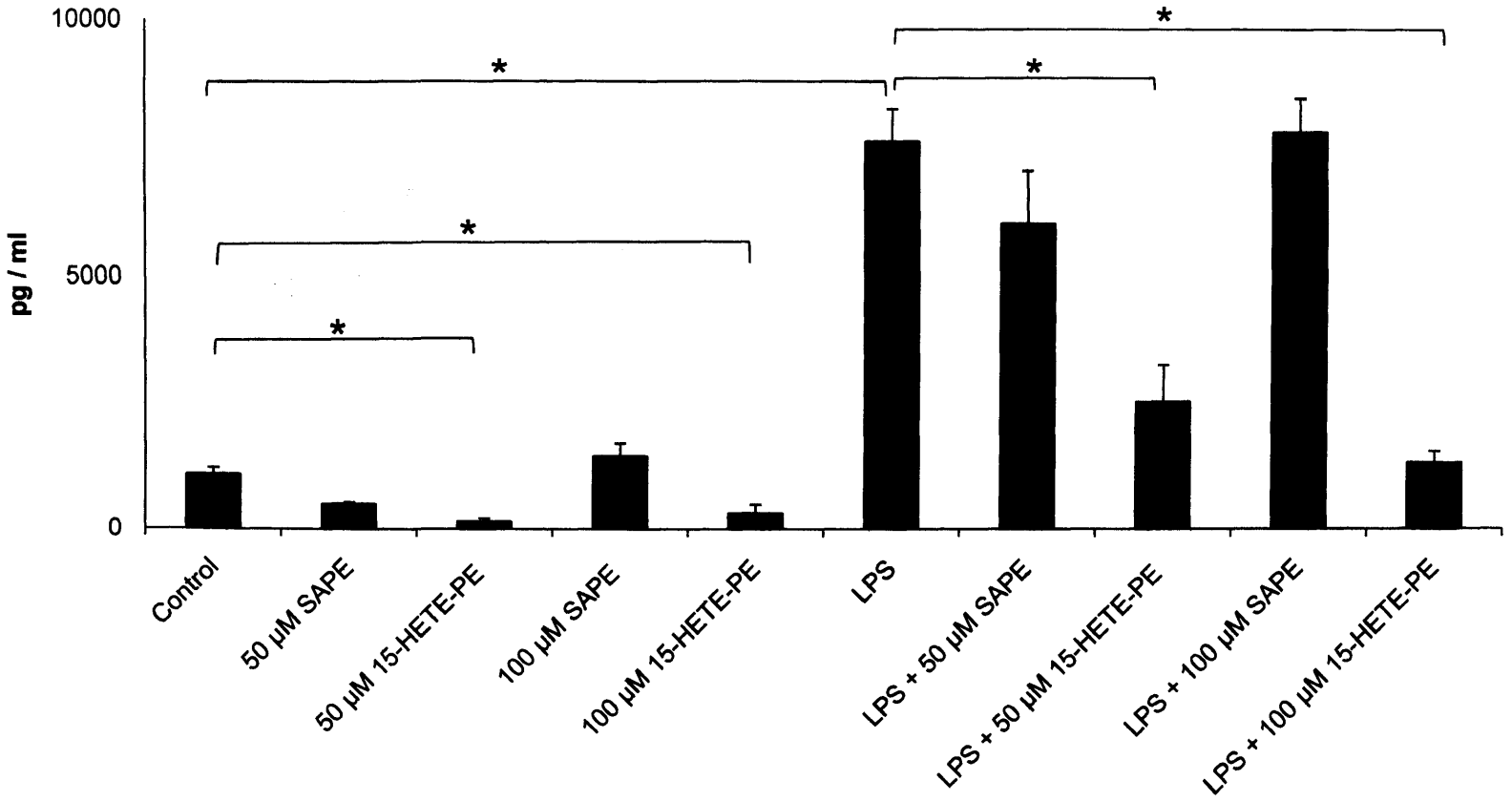


Figure 7.10 LPS stimulated G-CSF is dose-dependently reduced by 15-HETE-PE. Control and LPS stimulated monocytes were incubated with 50 and 100 µM 15-HETE-PE or SAPE for 24 hours. The media supernatant was analysed by ELISA for cytokine generation (n = 3, mean ± SEM). *Students t-test, $p < 0.05$.

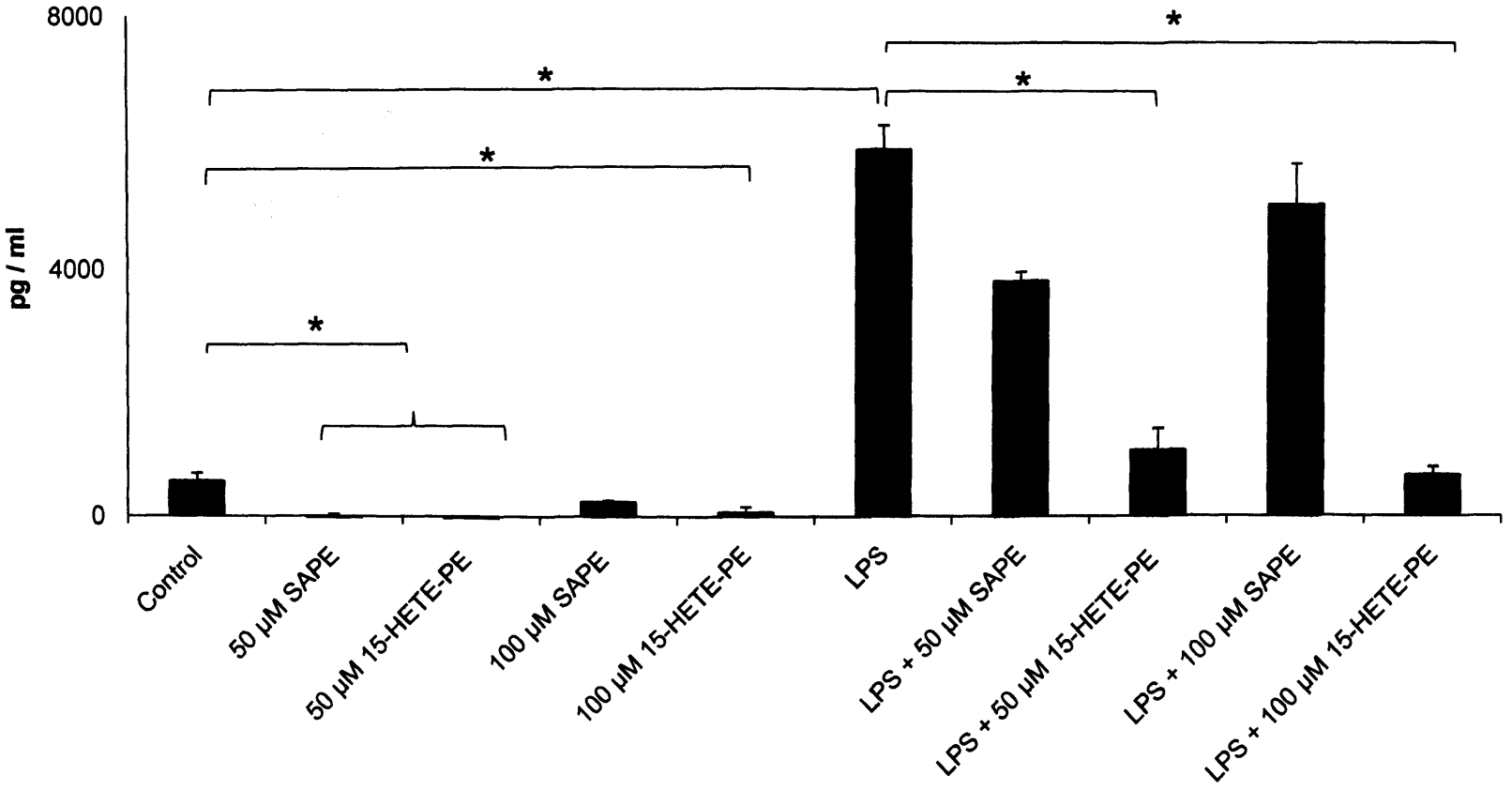


Figure 7.11 LPS stimulated IL-1β is dose-dependently reduced by 15-HETE-PE. Control and LPS stimulated monocytes were incubated with 50 and 100 µM 15-HETE-PE or SAPE for 24 hours. The media supernatant was analysed by ELISA for cytokine generation (n = 3, mean ± SEM). *Students t-test, $p < 0.05$.

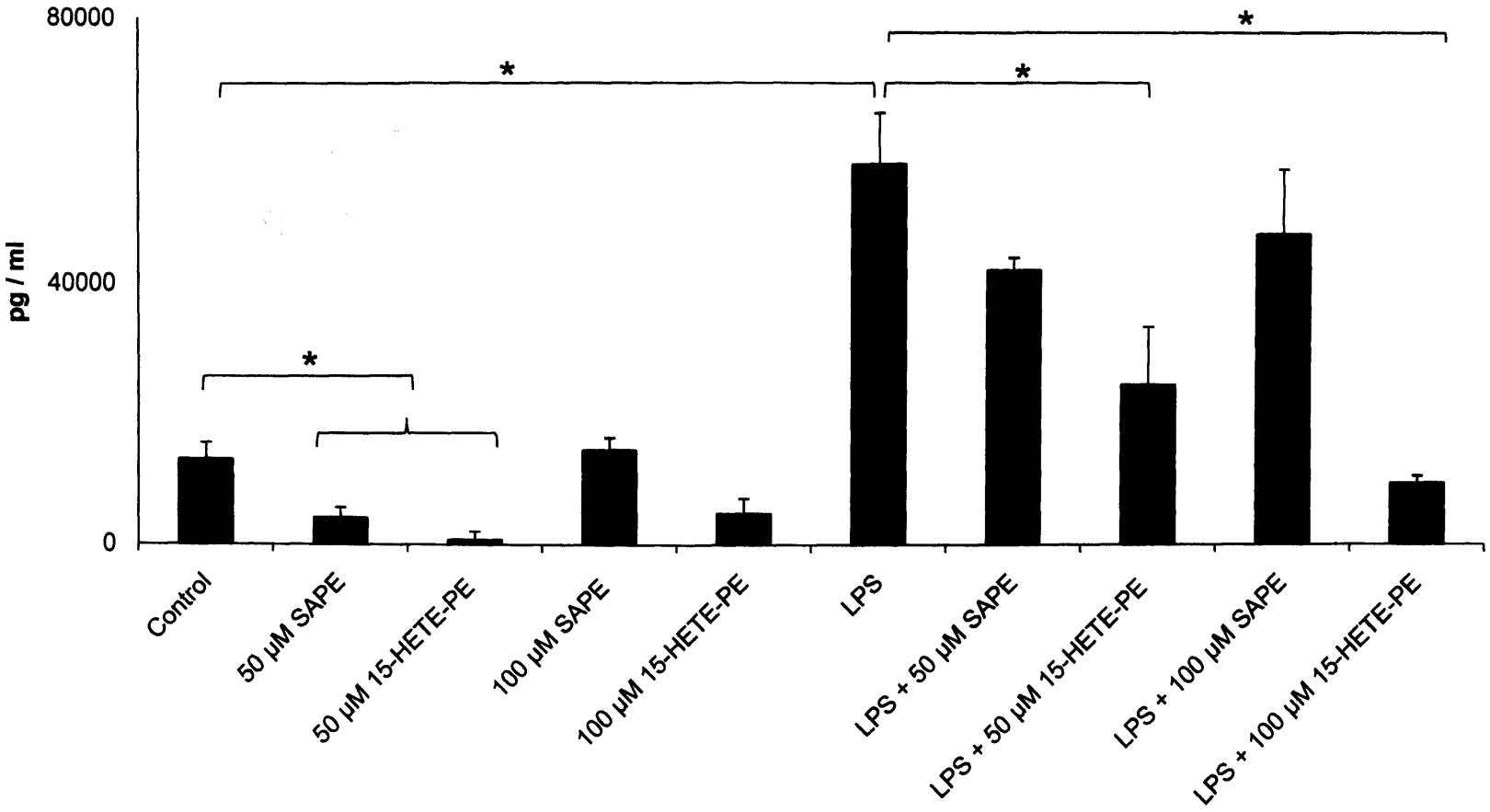


Figure 7.12 LPS stimulated IL-6 is dose-dependently reduced by 15-HETE-PE. Control and LPS stimulated monocytes were incubated with 50 and 100 µM 15-HETE-PE or SAPE for 24 hours. The media supernatant was analysed by ELISA for cytokine generation (n = 3, mean ± SEM). *Students t-test, $p < 0.05$.

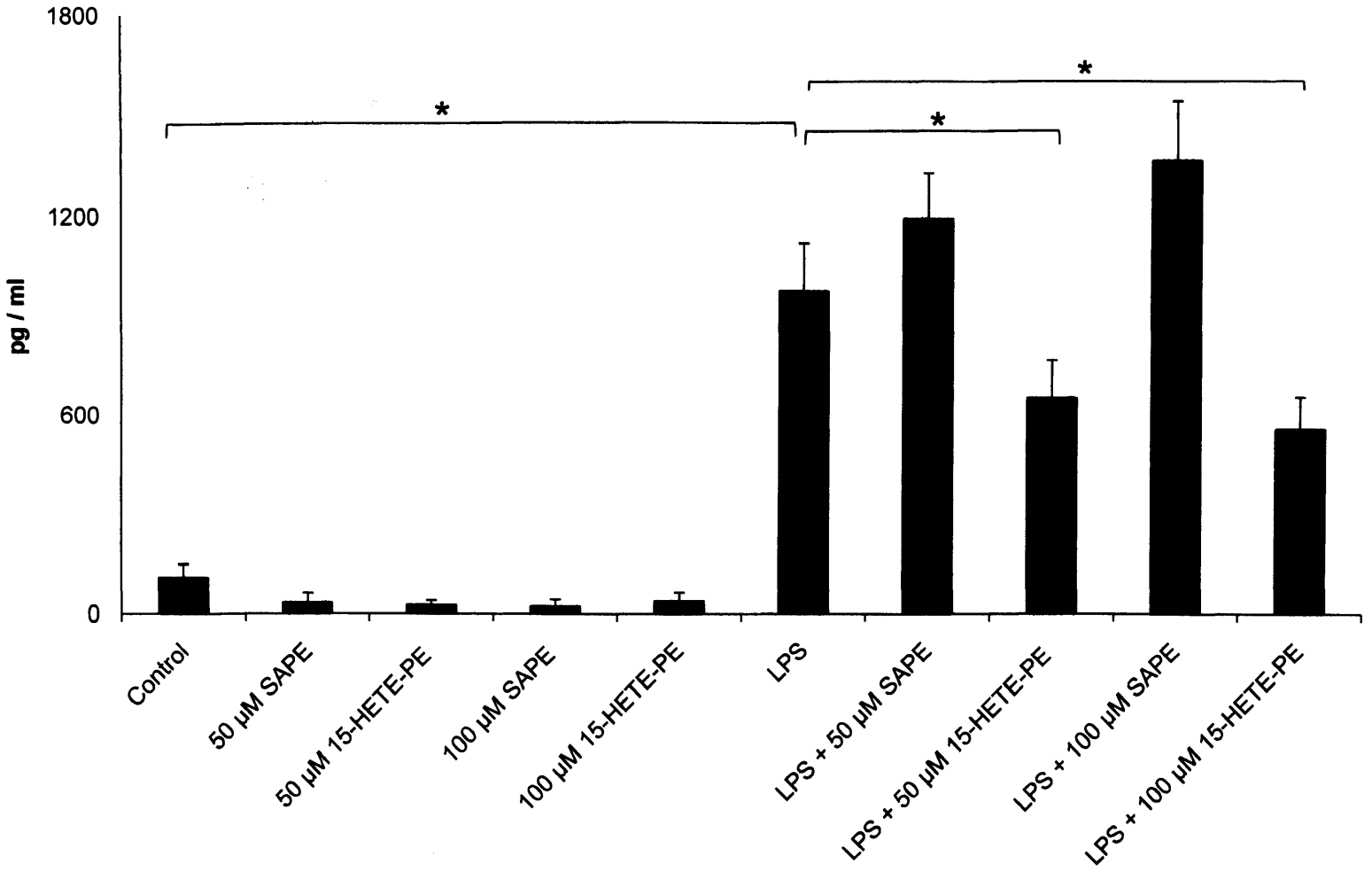


Figure 7.13 LPS stimulated TNF-α is dose-dependently reduced by 15-HETE-PE. Control and LPS stimulated monocytes were incubated with 50 and 100 µM 15-HETE-PE or SAPE for 24 hours. The media supernatant was analysed by ELISA for cytokine generation (n = 3, mean ± SEM). *Students t-test, $p < 0.05$.

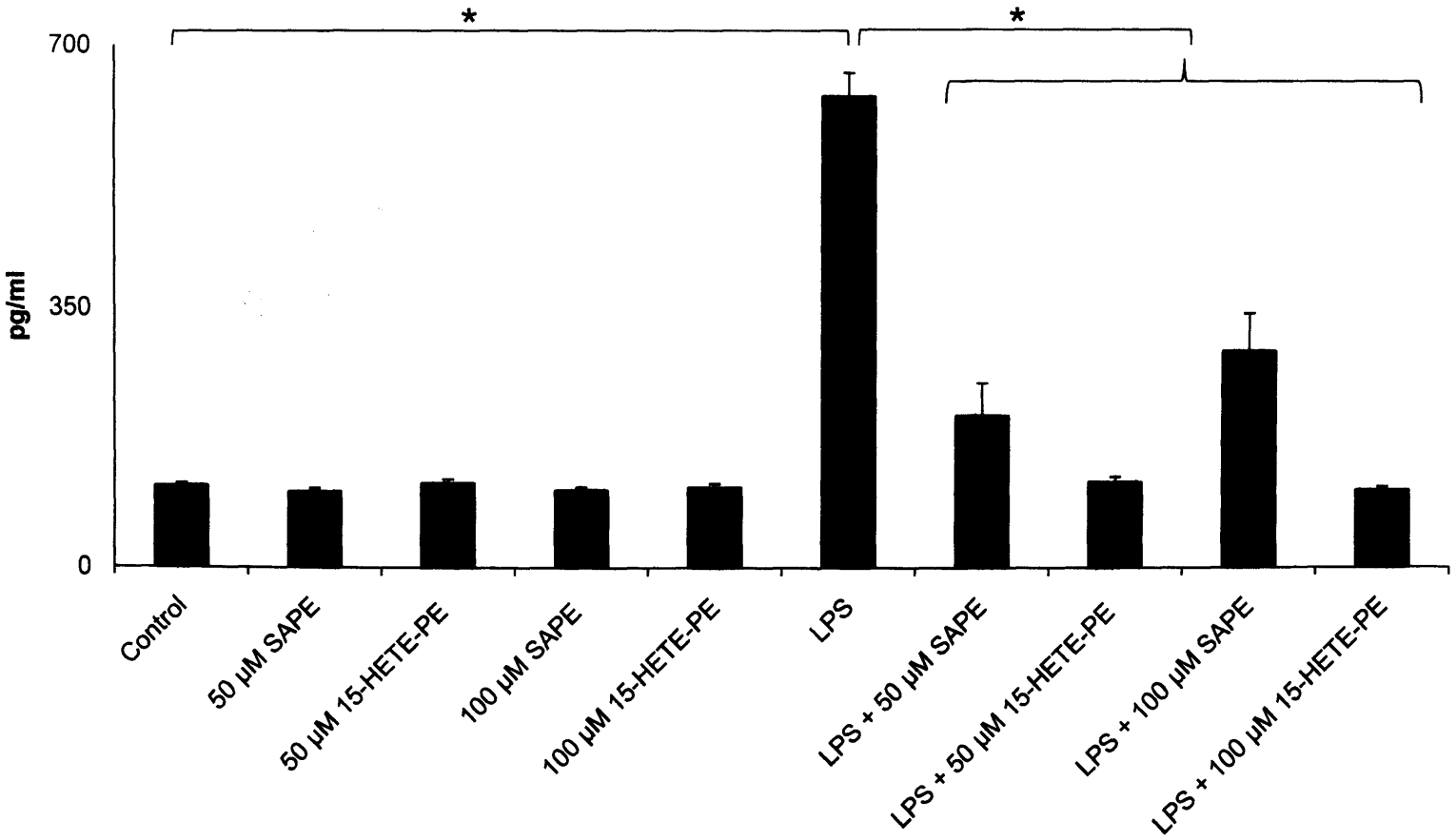


Figure 7.14 LPS stimulated IL-10 is not specifically reduced by 15-HETE-PE. Control and LPS stimulated monocytes were incubated with 50 and 100 µM 15-HETE-PE or SAPE for 24 hours. The media supernatant was analysed by ELISA for cytokine generation (n = 3, mean ± SEM). *Students t-test, $p < 0.05$.

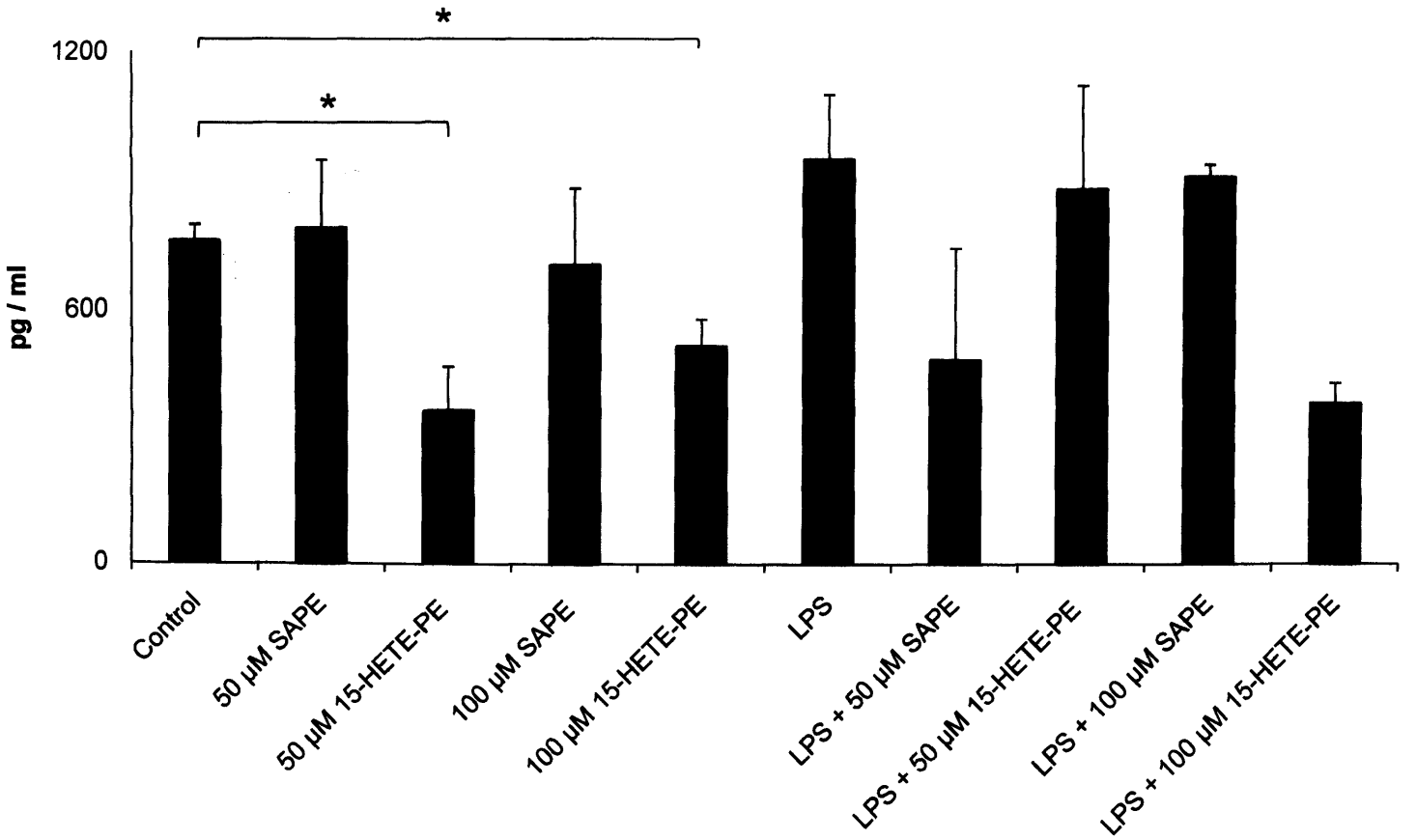


Figure 7.15 IL-8 generation decreases following the addition 15-HETE-PE to unstimulated monocytes. Control and LPS stimulated monocytes were incubated with 50 and 100 µM 15-HETE-PE or SAPE for 24 hours. The media supernatant was analysed by ELISA for cytokine generation (mean ± SEM, n=3).

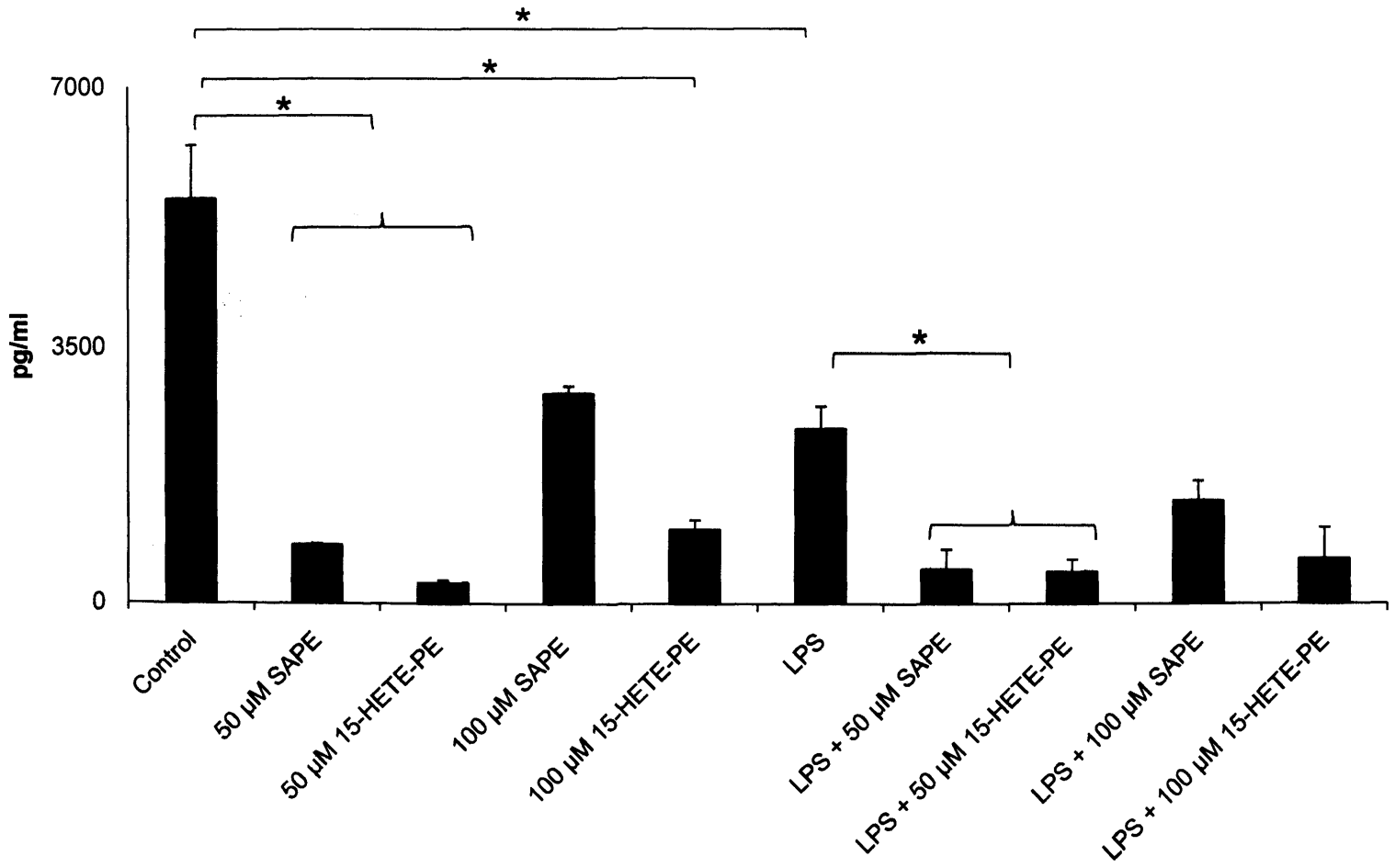


Figure 7.16 MCP-1 generation decreases following the addition 15-HETE-PE to unstimulated monocytes. Control and LPS stimulated monocytes were incubated with 50 and 100 µM 15-HETE-PE or SAPE for 24 hours. The media supernatant was analysed by ELISA for cytokine generation (n = 3, mean ± SEM). *Students t-test, $p < 0.05$.

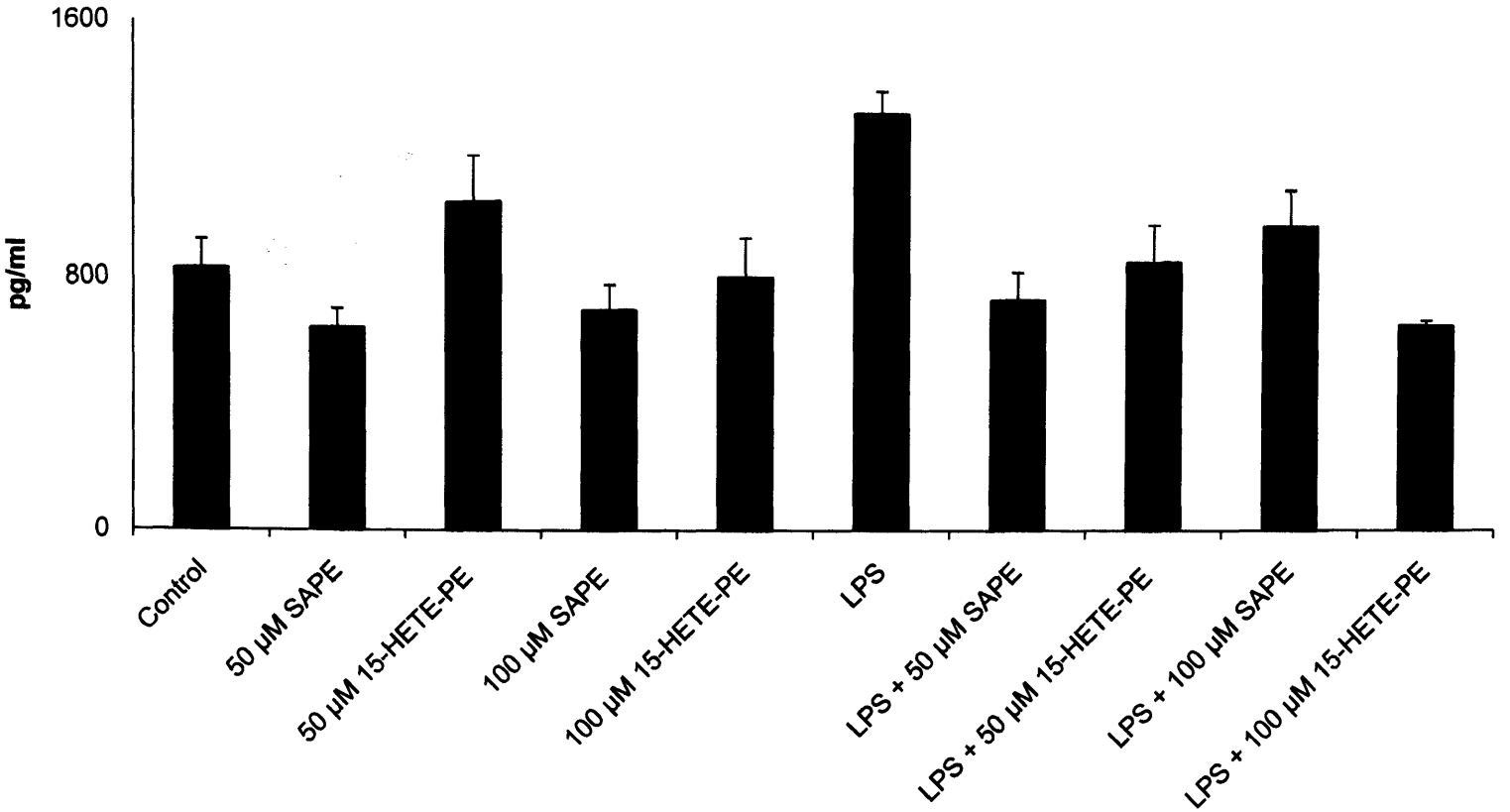


Figure 7.17 RANTES is not affected by the addition of LPS or 15-HETE-PE. Control and LPS stimulated monocytes were incubated with 50 and 100 µM 15-HETE-PE or SAPE for 24 hours. The media supernatant was analysed by ELISA for cytokine generation (mean ± SEM, n=3).

7.2.5 Levels of 15-HETE-PEs in control and bacterial-infected human peritoneal lavages are variable.

The generation of HETE-PEs during bacterial infections in humans has not been investigated. Lavage samples from patients undergoing peritoneal dialysis (stable controls) and individuals with suspected bacterial infections underwent lipid extraction and LC/MS/MS analysis. As part of routine diagnosis, bacterial cultures of patient lavage samples assessed whether they had bacterial infections and also identified the type of bacterium. Small levels of 15-HETE-PEs were detected in lavage fluid of stable control patients that were comparatively increased in the presence of particular gram-positive bacterial infections (P1-P3) (Figure 7.18). The results are contrary to that reported in studies of a mouse model *S.epi* infection, which is also a gram-positive bacterium, where 15-HETE-PE decreased following infection (Figures 7.1 and 7.2 A). Infection with *Haemolytic Streptococci α* (P2) showed the greatest increase of 15-HETE-PE, followed by infection with *Coryneform* (P3) and a small increase was seen in response to *Haemolytic Streptococci* β (P1). 15-HETE-PEs were not raised following infection by the gram-negative bacteria, *Proteus Vulgaris* and *Klebsiella* (P4 and P5). Increases in 15-HETE-PE were also seen in N3 and N4, namely samples from cases where bacterial infection was initially suspected but tested negative during diagnosis by bacterial cultures. This suggests that these patients may have been infected with a bacterium outside the scope of routine bacterial cultures or had undergone an alternative immune challenge. To date, samples testing positive for bacterial infection have all been caused by different bacterial types, therefore only one sample has been collected for each identified bacterium. Furthermore, patients identified as 'stable controls' have underlying health conditions and are therefore not 'true' naïve controls as used in murine disease models. Thus, the results are

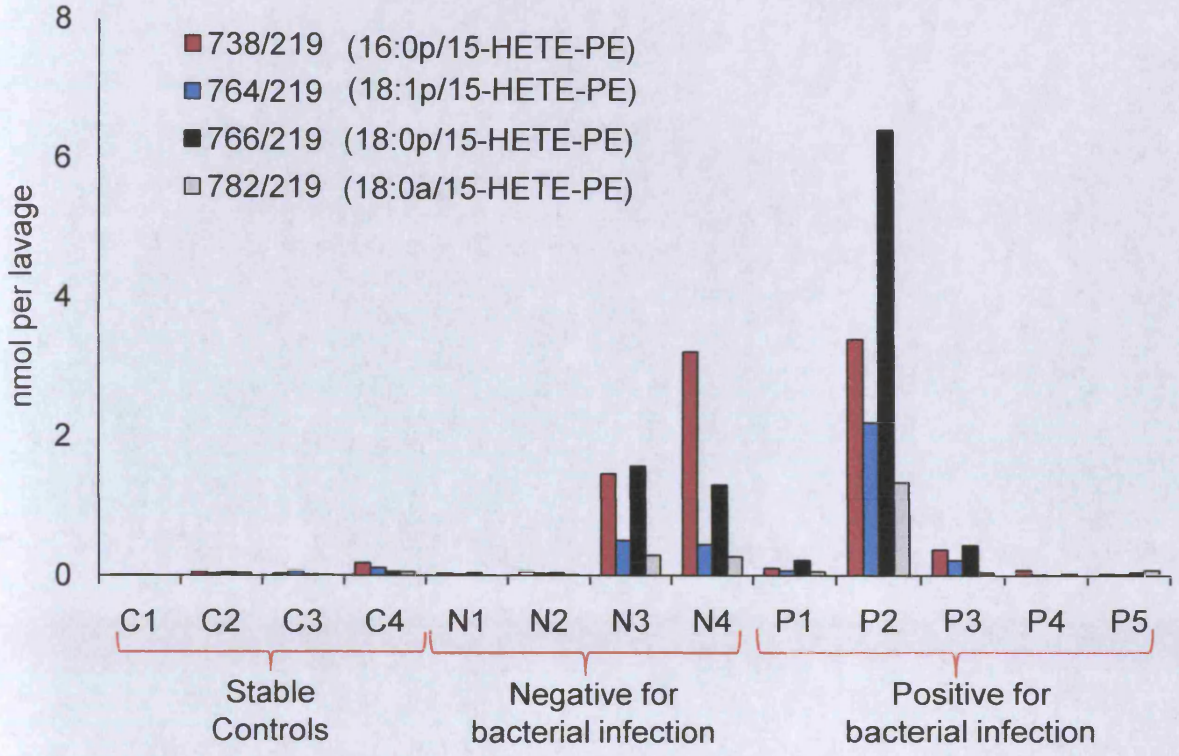


Figure 7.18 15-HETE-PE may be generated in response to infection by particular gram-positive bacteria. Lipids were extracted from lavage samples of patients undergoing peritoneal dialysis- stable controls, or patients with suspected bacterial infection. Samples were analysed by LC/MS/MS for 15-HETE-PE. Formal identification of infected bacteria: P1: *Haemolytic Streptococci* β (gram +ve), P2: *Haemolytic Streptococci* α (gram +ve); P3: *Coryneform* (gram +ve), P4: *Proteus Vulgaris* (gram -ve); P5: *Klebsiella (*gram -ve).

preliminary, merely indicating whether 15-HETE-PEs may be raised or decreased in response to particular bacteria. When multiple samples showing the same trend have been collected, the significance of these results can be verified.

7.3 Discussion

As 15-HETE-PEs may be involved in regulating immune responses, the investigations described in this chapter were aimed at incorporating 15-HETE-PE into human monocytes in order to investigate their effect on cytokine generation (Morgan *et al*, 2009). The addition of 15-HETE-PE in methanol was the most effective method for incorporation into monocytes, and concentrations of 50 and 100 μM 15-HETE-PE resulted in the highest percentage uptake. 1.2 and 1.45 μg of 15-HETE-PE (per one million monocytes) was cell associated following incubation with 50 and 100 μM 15-HETE-PE respectively (Figure 7.9A). In comparison, 2.5 ng of 15-HETE-PE is generated endogenously per one million monocytes (Figure 4.8). Incubating cells with lipid concentrations of 100 μM may not seem physiological, however, only a small percentage of 15-HETE-PE becomes cell-associated (6.2% for 50 μM). While levels of incorporation are low, depending on the location of lipid uptake, the local membrane concentration of 15-HETE-PE may be high. In Chapter 4, 15-H(p)ETE-PEs synthesised by 15-LOX were shown remain within monocytes, thus following local generation, they may also be present in high levels. In Chapter 5, the incorporation of three radioactive compounds into monocytes was compared - ethanolamine, AA and SAPE. The results showed that the smallest compound, ethanolamine was the one most successfully incorporated. Therefore, so little incorporation of 15-HETE-PE may be due to its structure and amphipathic nature. Numerous research groups investigating the biological function of phospholipids are using concentrations in the high μM range for their experiments, some of which use a mixture of oxidised phospholipids (Asai *et al*, 2009, von Schlieffen *et al*, 2009, Treede *et al*, 2009, Ferrante & Ferrante, 2005). In these studies, the addition of high concentrations was required to cause an effect. Furthermore, preliminary experiments for this chapter involved incubating

human monocytes with nM concentrations of 15-HETE-PE and SAPE, but neither lipid affected the generation of LPS stimulated cytokines (data not shown). The results indicated that the lipids were not taken-up sufficiently by cells to exert an effect. Therefore, as so little is actually incorporated, incubating cells with high concentrations of lipid is required to ensure biological effect. However, as the percentage of incorporation was less following incubation with 100 μM than that achieved with 50 μM, there may be an upper limit for lipid incorporation. In addition, 100 μM was the highest concentration investigated as increasing the concentration further would risk cell lysis. The greatest amount of lipid incorporation took place within the first hour and did not increase significantly at following time-points. The most suitable method for introducing lipids to human monocytes, was successfully identified and experiments also established the amount of cell-associated 15-HETE-PE at each of the investigated time-points and concentrations.

The next aim was to assess the effect of 15-HETE-PE on cytokine generation. 15-HETE-PE treatment of un-stimulated monocytes resulted in down-regulation of G-CSF, IL-1β, IL-6, IL-8 and MCP-1. Furthermore, release of G-CSF, IL-1β, IL-6 and TNF-α was dose-dependently reduced by 15-HETE-PE. These cytokines are considered to be pro-inflammatory and are involved in wound repair. In particular, IL-6, TNF-α and IL-1β are up-regulated during the acute-phase response and are key elements in early healing events (Singer & Clark, 1999, Janeway *et al*, 2005). While wound repair requires the initial presence of these cytokines, prolonged presence of IL-1β, TNF-α and IL-6 can have detrimental effects on tissue (Mast & Schultz, 1996, Howells GL, 1995). For example, their elevation is reported in chronic

conditions such as atherosclerosis and rheumatoid arthritis (Touitou & Kone-Paut, 2008, Ishihara & Hirano, 2002, Kleeman *et al*, 2008, Kirii *et al*, 2003, Kamari *et al*, 2007, Bode *et al*, 1999, Ahmed *et al*, 2000, Niemand *et al*, 2003, Tarnuzzer & Schultz, 1996, Wallace & Stacey, 1998). G-CSF is foremost a growth factor, produced by endothelial cells and macrophages. It is suspected to play a key role in regulating the number and activity of circulating neutrophils during an inflammatory response (Liesechke *et al*, 1994, Bronchud *et al*, 1988, Metcalf D, 2008, Morstyn *et al* 1988). G-CSF has also been linked to the expression of TNF-α and IL-1β, in a process known as the colony-stimulating factor (CSF)- network (Hamilton JA, 2008, Campbell *et al*, 1991, Leizer *et al*, 1990). Up-regulation of these cytokines by LPS in our samples is consistent with their pro-inflammatory functions. The down-regulatory effect of 15-HETE-PE on this small selection of cytokines supports an anti-inflammatory role for 15-LOX in human monocytes, consistent with its anti-atherosclerotic effect in rabbits (Serhan *et al*, 2003). Products of 12/15-LOX oxidised at the C-12 position of AA are frequently shown to be pro-inflammatory in mice, while products oxidised at C-15 are often anti-inflammatory (Sasson & Eckel, 2006, Shen *et al*, 1996, Smith *et al*, 1993, Takata *et al*, 1994a). Additional evidence to support this includes the down-regulation of LPS stimulated TNF-α following the addition of 15-HpETE to mono mac 6 cells (Ferrante *et al*, 1997, Ferrante & Ferrante, 2005). Furthermore, the treatment of cultured monocytes and a J774A.1 cell line with 12-HETE, resulted in the increased expression of IL-6 and TNF-α mRNA, a pro-inflammatory effect (Wen *et al*, 2007). Importantly, reduced generation of G-CSF, IL-1β, Il-6 and TNF-α will reduce the number of neutrophils and pro-inflammatory signals present at an inflammatory site. This may be significant in chronic conditions such as

atherosclerosis and rheumatoid arthritis, where an original beneficial inflammatory response becomes harmful over time and attenuates any damage undertaken by cells.

Significantly, the effect of cytokine generation following LPS stimulation is specific to 15-HETE-PE and not its un-oxidised precursor SAPE, indicating that the action of 12/15-LOX is required. Several articles have recently been published regarding LPS-stimulated cytokines and the effect of oxidised phospholipids on their generation, in particular oxidised PC. The incubation of human umbilical vein endothelial cells with air-oxidised PC, PE, PG, PS and PA phospholipids, have been shown to reduce expression of E-selectin (von Schlieffen *et al*, 2009). Multiple oxidised-PC lipids (1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (OxPAPC), 1-palmitoyl-2-azelaoyl-*sn*-glycero-3-phosphocholine (PAzPC), 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine (PGPC), 1-Palmitoyl-2-(5,6-epoxy isoprostane E2)-*sn*-glycero-3-phosphocholine (PEIPC), 1-Palmitoyl-2-(5- oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC)), and other oxidised phospholipids (oxidised 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoglycerol (OxPAPG), oxidised 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphate (OxPAPA), oxidised 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoserine (OxPAPS), and oxidised 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine (OxPAPE)) reduce LPS-stimulated TNF-α, IL-1α and IL-β (von Schlieffen *et al*, 2009, Erridge *et al*, 2008). This is consistent with results reported in this chapter. The inhibition of LPS signalling by oxidised phospholipids has recently been reviewed by C. Erridge (2009), who describes at what stage oxidised phospholipids are likely to exert their effect (Figure 7.19). Oxidised phospholipids may block LPS signalling by (i) inhibiting the binding of LPS to LPS binding protein (LBP), (ii) blocking the association of LBP to membrane bound CD-14 (mCD-14) or, (iii) following the association of LBP to

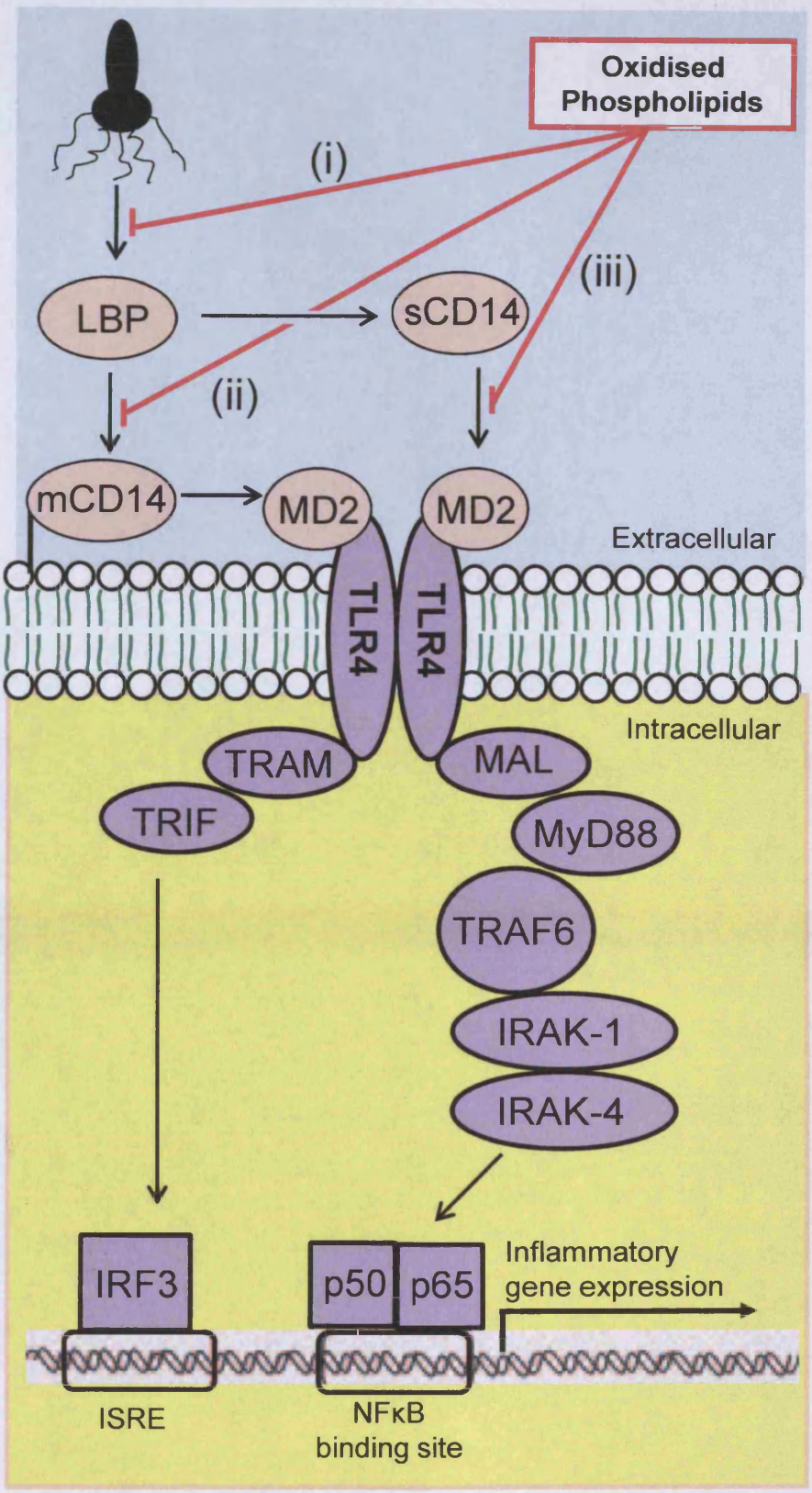


Figure 7.19 Locations where LPS signaling may be inhibited by oxidised phospholipids. Oxidised phospholipids inhibit the binding of LPS to LBP (i), mCD14 (ii), and MD2 (iii), preventing signaling via TLR4. Intracellular signalling is unaffected (reproduced from Erridge, C. 2009, (12/11/2010) with permission).

soluble CD-14 (sCD-14), its association to the MD2 subunit of TLR4 can be blocked (Erridge *et al*, 2007, Erridge *et al*, 2008, Walton *et al*, 2003). Although mechanisms for HETE-PE inhibition of cytokine generation have not yet been investigated, they may include some of these. It is also important to note, as generation of particular cytokines was seen in the absence of LPS stimulation there is likely to be an additional, if not an alternative pathway, by which 15-HETE-PE may exert its effect.

The results have shown that IL-10 generation is also dose-dependently inhibited by 15-HETE-PE, but the response was non-specific as the un-oxidised lipid was also diminished. The lack of effect on IL-8 and RANTES generation and the reduction in MCP-1 following LPS-activation is inconsistent with reported literature. Not only has LPS been shown to stimulate the generation of these cytokines in human monocytes, LPS stimulated IL-8 generation is reduced following the addition of oxidised-PAPC (Sica *et al,* 1999, Erridge *et al*, 2007). These inconsistencies may have been due to sample degradation following multiple episodes of freeze-thawing.

In order to gain a broader view of events that are affected by 15-HETE-PE, a larger range of cytokines require analysis. A method suitable for this purpose is a FACS based technique known as luminex that allows the simultaneous analysis of approximately 30 cytokines. 12/15-LOX regulates additional cytokines to those reported in this results chapter, for example, 12/15-LOX up-regulates a subunit of IL-12, IL-12p40 (Middleton *et al,* 2005). But it is unknown whether 15-HETE-PEs are involved in this process. 12/15-LOX is critical for the production of IL-12 in LPS stimulated murine macrophages, and stimulation of IL-12p40

by LPS is defective in 12/15-LOX$^{-/-}$ mice (Zhao *et al*, 2002, Middleton *et al*, 2005). Furthermore, peritoneal macrophages from 12/15-LOX$^{-/-}$ mice show altered expression of several cytokines. For example, they generate less CCL5/RANTES but more IL-1, GM-CSF, IL-3 and IL-17 in comparison to WT cells (Dioszeghy *et al*, 2008). In addition, 12/15-LOX$^{-/-}$ macrophages stimulated with SES generate more GM-CSF, but less IL-12p40, IL-12p70 and RANTES (Dioszeghy *et al*, 2008). These studies and results in this thesis support a role for 12/15-LOX in the regulation of multiple cytokines. Future work could include comparing the effect of 15- and 12-HETE-PE on these cytokines in human monocytes and murine macrophages. However, currently, 18:0a/12-HETE-PE is only available within an isomer mixture of HETE-PEs, as discussed in Chapters 3 and 6. Carrying out studies investigating the addition of 15- and 12-HETE-PE to the same cell model may show whether they exert different effects. This may define more clearly the pro- or anti- inflammatory role of 12/15-LOX in humans and mice.

The data from human peritoneal lavage samples suggest that 15-HETE-PEs may be generated in response to immune challenges from particular gram-positive bacteria. However, each sample that tested positive for bacteria showed very different concentrations of 15-HETE-PEs. The number of patient samples must be significantly increased to validate this data; such small numbers of samples make it difficult to draw any other conclusions. There are several drawbacks in the analysis of this data, such as the way it was analysed and how the results have been expressed. In particular, levels are expressed in nmol/per total lavage, but normalising according to the number of macrophages would be considered more correct (Figure 7.18). Where possible, FACS analysis was carried out on samples to determine the

number of macrophages, but they varied vastly between samples. Also, only two control samples could be analysed by FACS and both showed very different results. Due to this, the data expressed this way was skewed with no correlation whatsoever. The data has therefore been presented in a way in which one conclusion at least may be drawn, that 15-HETE-PEs were raised in response to some gram-positive bacteria, in comparison to the control samples.

These samples were from patients with underlying health conditions rather than healthy individuals, and for the patients with identified bacterial infections, it is not known at what stage of the bacterial infection they were at when the lavage was carried out. In addition, it cannot be certain that the 'stable control patients' and patients that tested negative for bacterial infections had any other underlying infections. For example, 15-HETE-PEs were raised in two of the samples that tested negative for screened bacteria, but due to what? Investigating the generation of 12-HETE-PE in response to various gram-positive and negative bacteria using mouse models may show whether different bacteria cause a different profile generation of 12-HETE-PE.

In summary, a method was described for inducing low level incorporation of 15-HETE-PE into human monocytes. The effect of 15-HETE-PE on cytokine generation was then investigated. Generated IL-1β, IL-6, TNF-α and G-CSF were down-regulated following the addition of 15-HETE-PE, suggesting that 12/15-LOX products may have anti-inflammatory properties in human monocytes.

CHAPTER 8

ULTRA-STRUCTURAL ANALYSIS AND PHOSPHOLIPID PROFILING OF PERITONEAL MACROPHAGES FROM WT AND 12/15-LOX$^{-/-}$ MICE

8.1 Introduction

Phospholipids are major constituents of membrane structures and changes in their composition may affect cellular/organelle formation as well as their function. 12/15-LOX may play a role in membrane function as macrophages from 12/15-LOX$^{-/-}$ mice are unable to carry out phagocytosis as efficiently as WT macrophages (Miller *et al*, 2001). This may be due to alterations in actin phosphorylation and polymerisation, which ultimately alters the re-organisation of the cytoskeleton (Kang *et al*, 1999, Rice *et al*, 1998, Tang & Honn, 1997).

In Chapter 6, 12/15-LOX was shown to be involved in altering the phospholipid membrane composition by generating oxidised PEs which are externalised. Changing the lipid profile of membranes may alter their physical properties and could therefore affect cellular processes that require re-organisation of the membrane, such as migration and phagocytosis, events that have previously been linked to 12/15-LOX (Miller *et al*, 2001 & 2003, Kang *et al*, 1999, Rice *et al*, 1998, Tang & Honn, 1997). In one study, cells devoid of 12/15-LOX had increased cellular deposition of cholesterol esters, which were alternatively degraded in cells where 12/15-LOX was present (Belkner *et al,* 2005). 15-LOX1 has been linked to the oxidation of cholesteryl arachidonate, which could be a method of metabolising cholesterol esters (Belkner *et al,* 1991). Importantly, an excess of lipids in cells may have implications on cellular membrane composition and structure. Differences in phospholipid levels between WT and 12/15-LOX$^{-/-}$ macrophages have not been investigated, but this could be done using MS. Furthermore, altered phospholipid composition may cause ultra-structural differences,

which could be observed by electron microscopy (EM). EM allows the visualisation of entire cell structures as well as the organelles, all of which are defined by phospholipid membranes.

8.1.1 *Aims*

MS was used to assess any differences in phospholipid profile between WT and 12/15-LOX$^{-/-}$ macrophages, and whether they could account for any physiological differences seen during EM analysis.

- To determine if there were ultra-structural differences between peritoneal macrophages from WT and 12/15-LOX$^{-/-}$ mice.
- To characterise the phospholipid and cholesterol ester profiles of peritoneal macrophages from WT and 12/15-LOX$^{-/-}$ mice.

8.2 Results

8.2.1 Peritoneal macrophages from 12/15-LOX$^{-/-}$ mice show ultra-structural abnormalities consistent with a lysosomal storage disease.

To compare the ultra-structure of WT and 12/15-LOX$^{-/-}$ peritoneal macrophages, lavages from naïve mice were pooled, pelleted and prepared for analysis by transmission EM (as described in section 2.2.12). The general structure of macrophage cell populations from three separate pooled preparations is shown in Figures 8.1 to 8.3. Figure 8.1 shows whole macrophages from WT mice. Highlighted by black arrows are healthy-looking mitochondria, defined by their small, compact size and defined cristae. In contrast, mitochondria from 12/15-LOX$^{-/-}$ macrophages appear swollen and granular (Figure 8.2). 12/15-LOX$^{-/-}$ macrophages also contain a large number of vacuoles (yellow arrows) and suspected lysosomal storage bodies that appear as dark inclusions (red arrows); some of these inclusions have double membranes which are an indication of autophagosomes (blue arrows). Vacuoles and suspected lysosomal storage bodies can also be seen in WT macrophages but in fewer numbers. The same structures are seen in enlarged pictures of WT and 12/15-LOX$^{-/-}$ macrophages (Figure 8.3). Structural analysis of 12/15-LOX$^{-/-}$ macrophages suggest that their physiology is consistent with cells showing symptoms of lysosomal storage disease (LSD). The lack of visual 'swirls' which are usually associated with such disorders, indicate that this may be a secondary condition, rather than a primary LSD.

8.2.2 Statistical analysis suggests that glycerophospholipid profiles of WT and 12/15-LOX$^{-/-}$ macrophages may account for their structural differences.

In order to determine whether the structural differences seen between WT and 12/15-LOX$^{-/-}$ macrophages could be due to altered phospholipid composition, macrophages were isolated

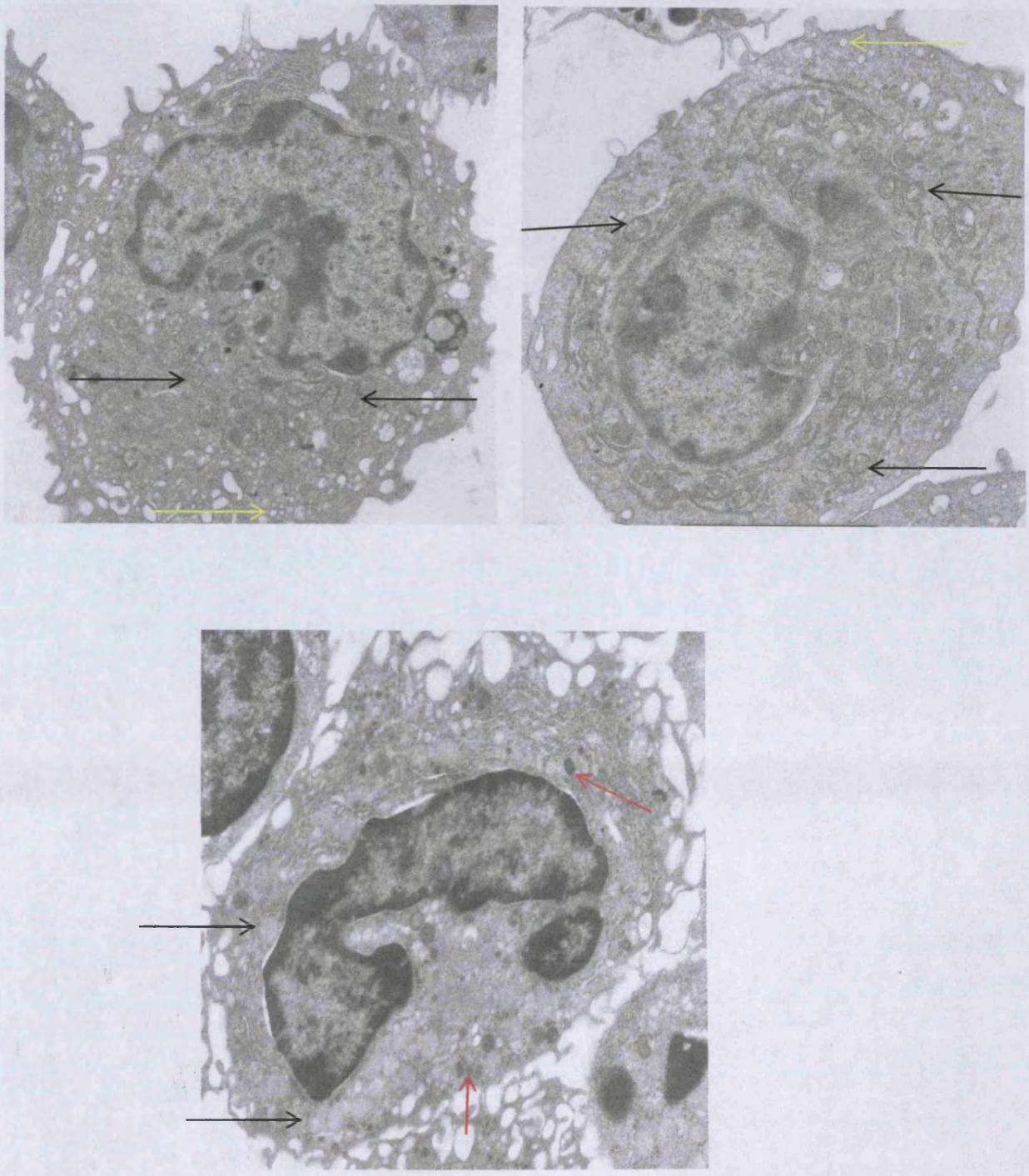


Figure 8.1 EM analysis of WT peritoneal macrophages. Peritoneal lavage samples from WT mice were fixed and prepared for EM analysis. Sections were analysed by TEM at x10,000 magnification. Arrows indicate areas of particular interest, including healthy mitochondria (black), and few lysosomal storage bodies (red) and vacuoles (yellow).

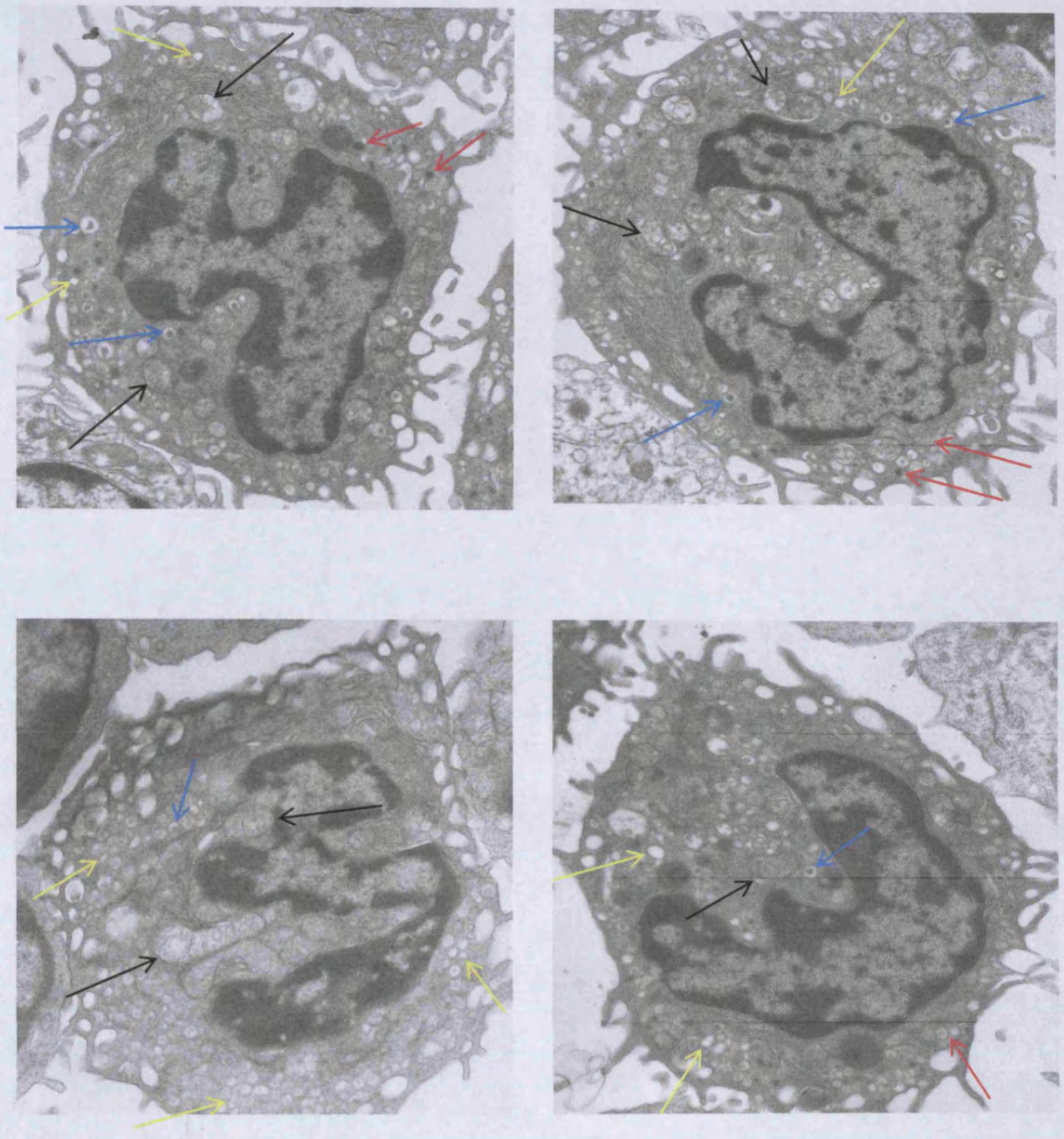


Figure 8.2 Analysis of 12/15-LOX$^{-/-}$ peritoneal macrophages suggests the presence of LSD. Peritoneal lavage samples from 12/15-LOX$^{-/-}$ mice were fixed and prepared for EM analysis. Sections were analysed by TEM at x10,000 magnification. Arrows indicate areas of particular interest, including abnormal mitochondria (black), and numerous autophagosomes (blue), lysosomal storage bodies (red) and vacuoles (yellow).

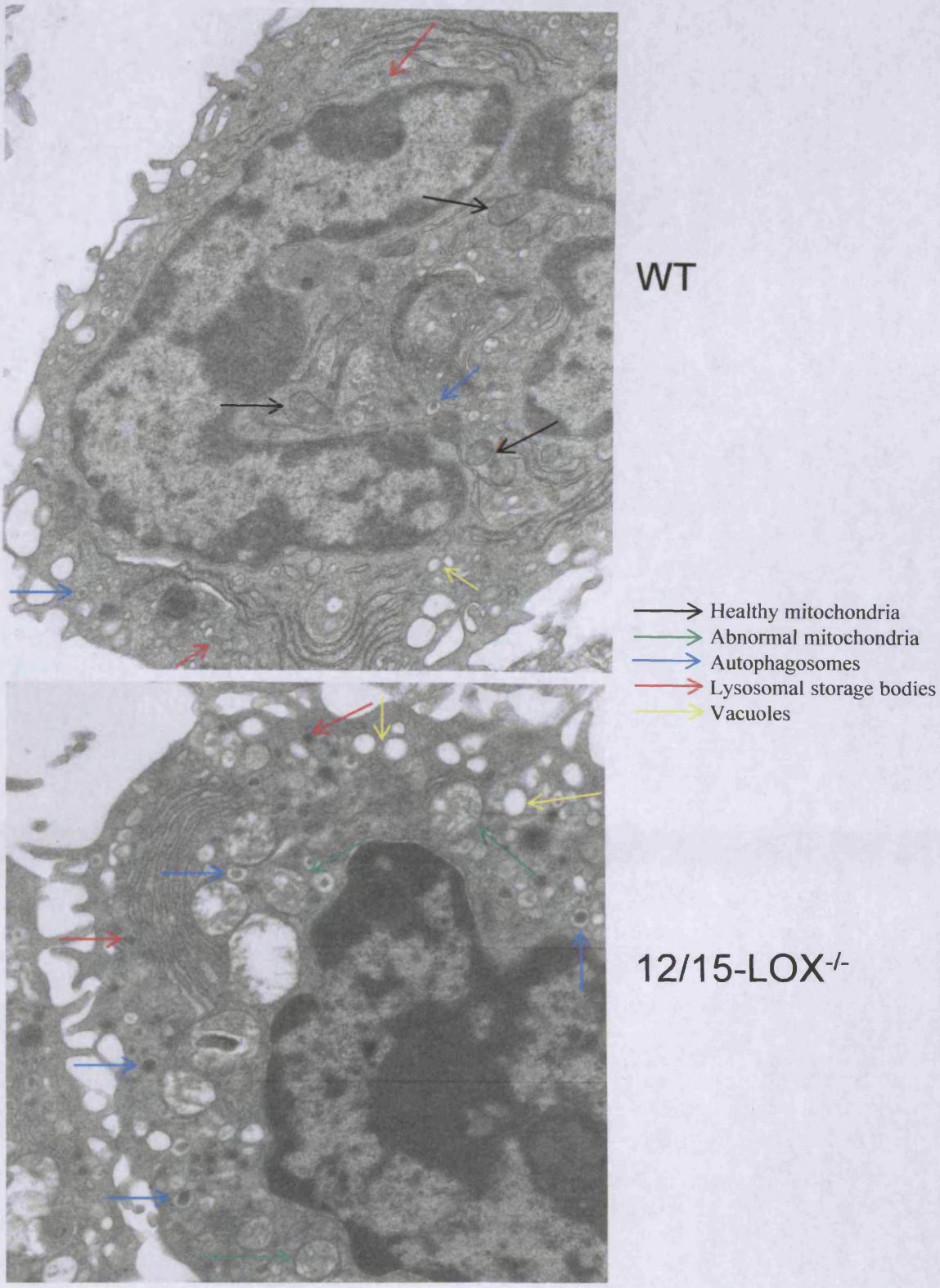


Figure 8.3 Close inspection of macrophages suggest the presence of LSD in 12/15-LOX$^{-/-}$ mice. Peritoneal lavage samples from WT and 12/15-LOX$^{-/-}$ mice were fixed and prepared for EM analysis. Sections were analysed by TEM at x20,000 magnification. Arrows indicate areas of particular interest, including healthy mitochondria (in WT) (black), abnormal mitochondria (in 12/15-LOX$^{-/-}$) (green), and numerous autophagosomes (blue), lysosomal storage bodies (red) and vacuoles (yellow).

from peritoneal lavage samples by adhesion followed by lipid extraction. Samples were analysed by LC/MS/MS for many phospholipids commonly found in membranes, including PE, PC, PI, PG, PA and PS. Very few of the analysed phospholipids showed an individual statistical difference between WT and 12/15-LOX$^{-/-}$ (Figures 8.4 to 8.9). For the PE, PS, PG and PI phospholipids that were statistically different following T-test analysis (P= <0.05), the highest levels were seen in 12/15-LOX$^{-/-}$ macrophages. Conversely, individual PC phospholipids that showed a statistical difference were all greater in WT macrophages. However, one-way ANOVA analysis with a Tukey post hoc test to compare all pairs of phospholipid data sets from WT and 12/15-LOX$^{-/-}$ phospholipids showed that combined levels of detected PE, PS and PG phospholipids were significantly higher in 12/15-LOX$^{-/-}$ macrophages in comparison to macrophages from WT mice (P=<0.05). Conversely, analysis by one-way ANOVA with a Tukey post hoc test showed that combined levels of PC phospholipids were significantly higher in WT macrophages (P=<0.05). The results suggest that different glycerophospholipid profiles may contribute to the ultra-structural differences between WT and 12/15-LOX$^{-/-}$ macrophages.

8.2.3 Combined levels of cholesterol esters are higher in 12/15-LOX$^{-/-}$ peritoneal macrophages.

In addition to differences in glycerophospholipid profiles of WT and 12/15-LOX$^{-/-}$ macrophages, alternative lipids, such as cholesterol esters, may also be involved in the structural abnormalities. Importantly, 15-LOX is reported to oxidise cholesteryl arachidonate, and in the absence of 12/15-LOX, there is increased cellular deposition of cholesterol esters (Belkner *et al*, 2005). Therefore profiles of cholesterol esters were investigated in WT and 12/15-LOX$^{-/-}$ macrophages. Samples of macrophages isolated by adhesion from WT and

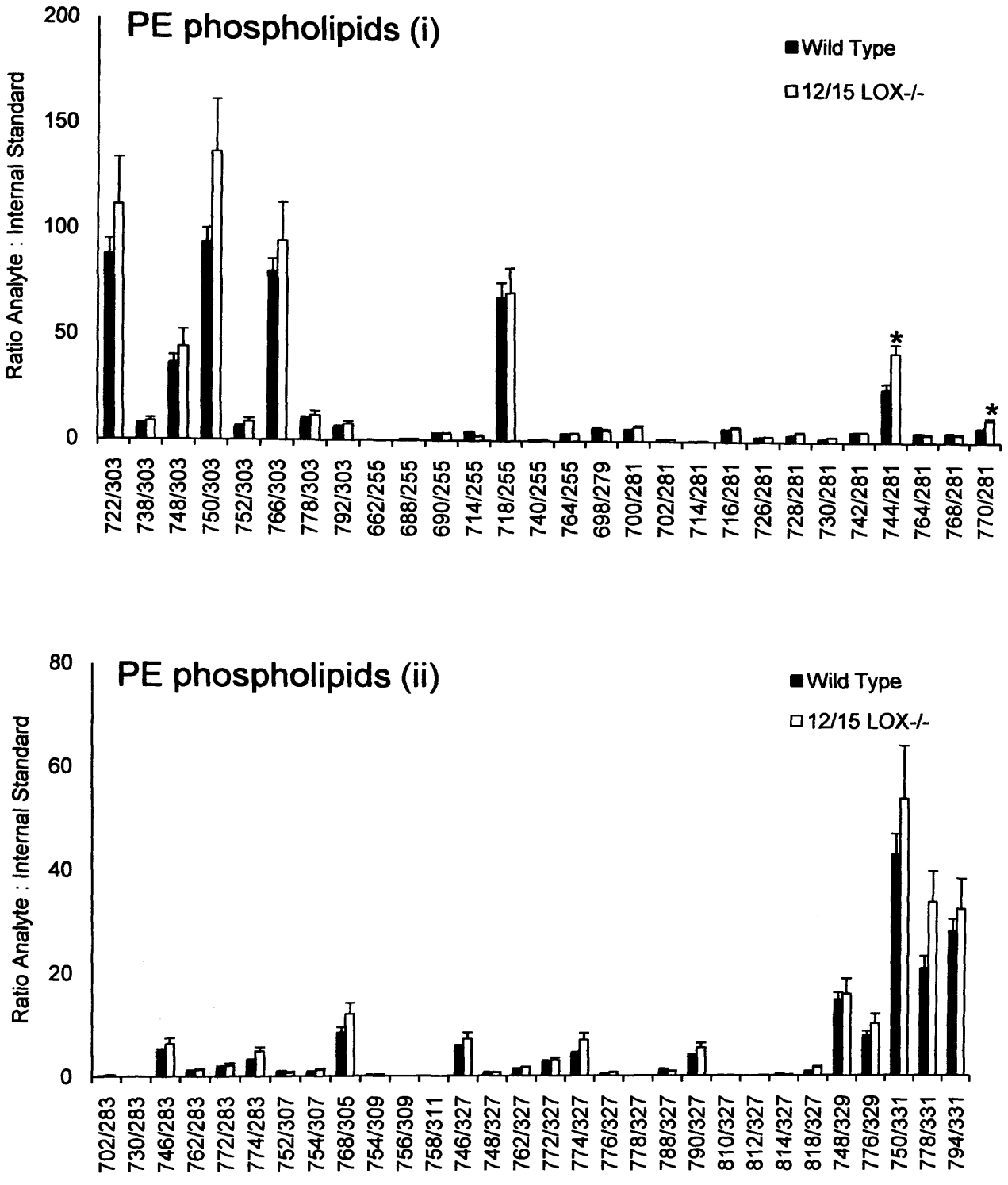


Figure 8.4 Differences between the PE phospholipid profiles of WT and 12/15-LOX$^{-/-}$ macrophages are significant following ANOVA analysis. Peritoneal macrophages from WT and 12/15-LOX$^{-/-}$ mice were isolated by adhesion prior to lipid extraction. PE phospholipids were analysed by LC/MS/MS (n=8, mean ± S.E.). *Students t-test, $p<0.05$. The overall difference in PE phospholipids between WT and 12/15-LOX data sets is significant following analysis by one-way ANOVA with a Tukey post hoc test, $p<0.05$.

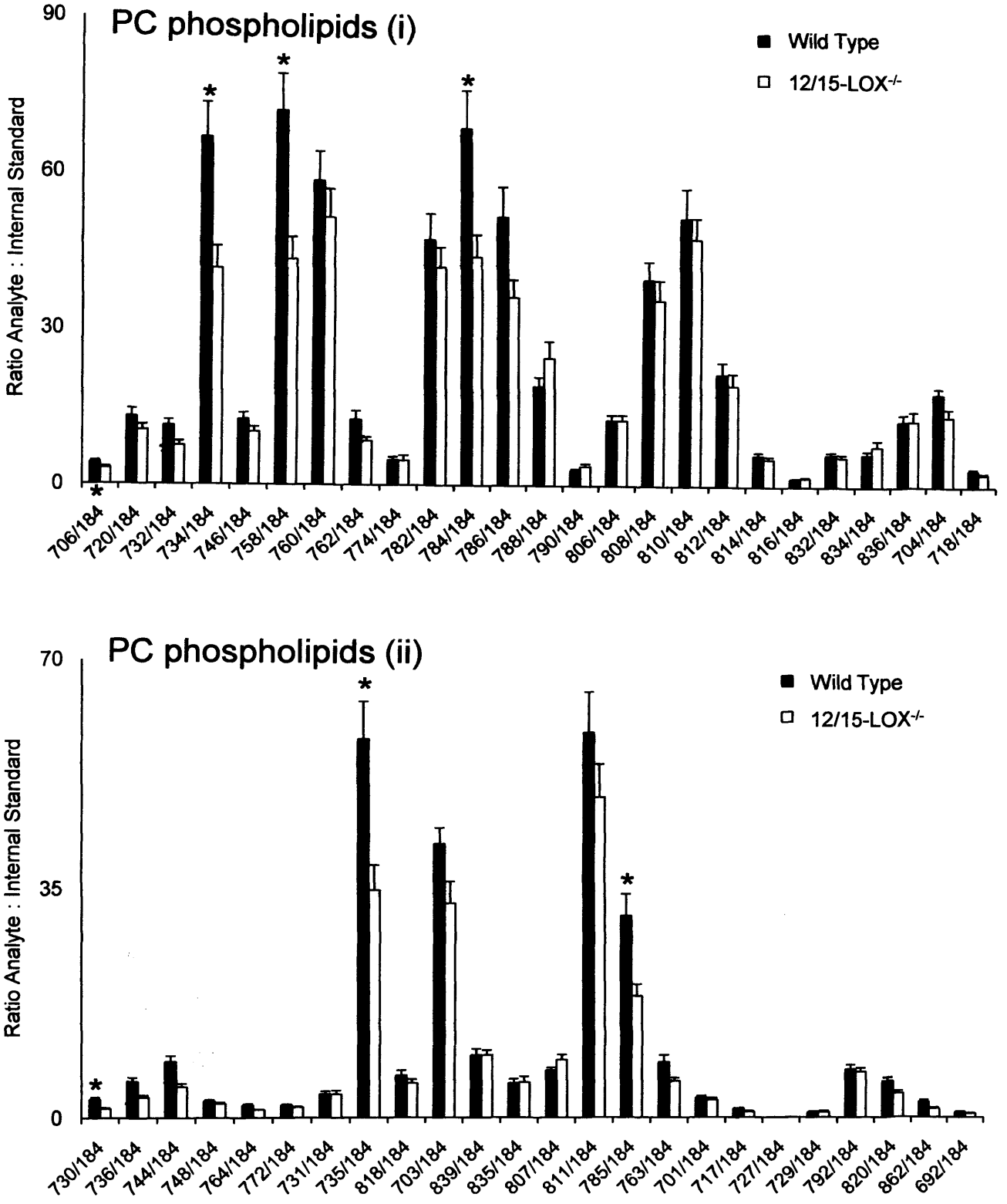


Figure 8.5 Differences between the PC phospholipid profiles of WT and 12/15-LOX$^{-/-}$ macrophages are significant following ANOVA analysis. Peritoneal macrophages from WT and 12/15-LOX$^{-/-}$ mice were isolated by adhesion prior to lipid extraction. PC phospholipids were analysed by LC/MS/MS (n=8, mean ± S.E.). *Students t-test, $p<0.05$. The overall difference in PC phospholipids between WT and 12/15-LOX data sets is significant following analysis by one-way ANOVA with a Tukey post hoc test, $p<0.05$.

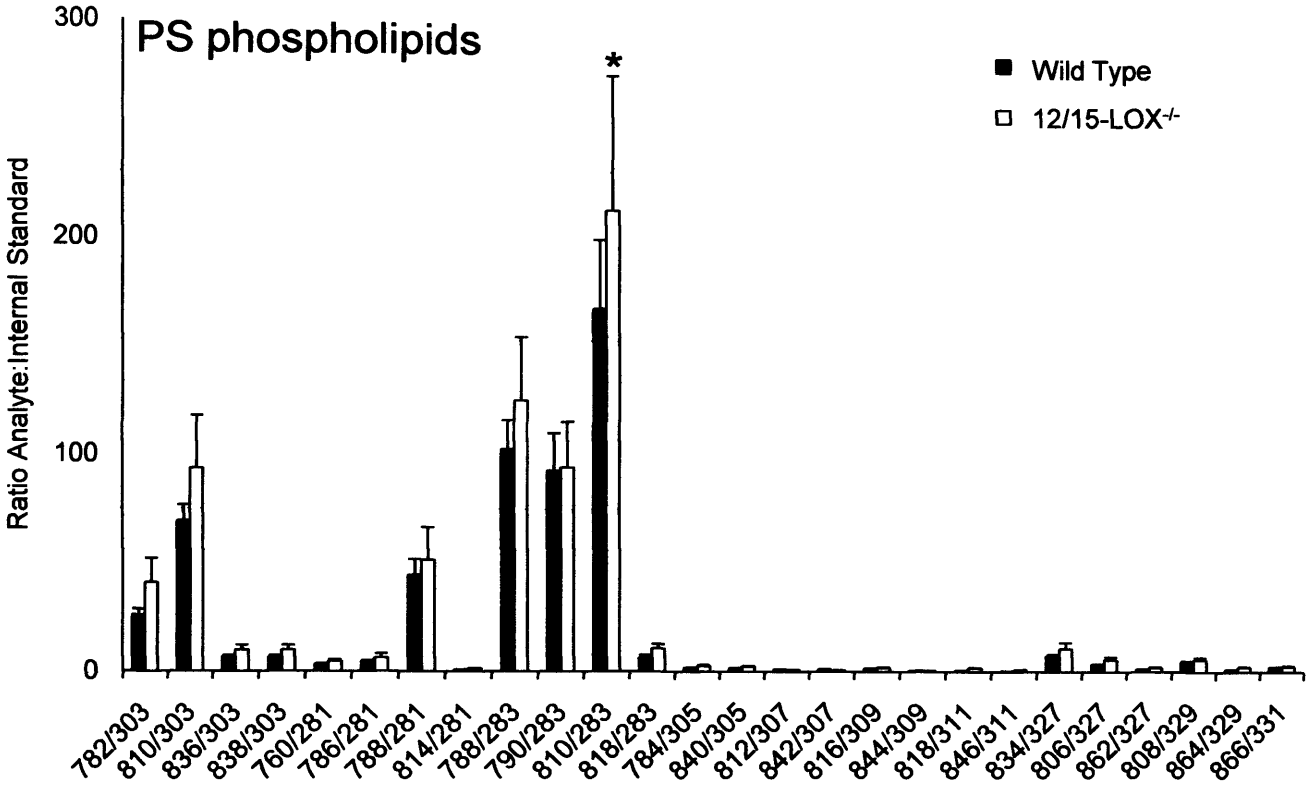


Figure 8.6 Differences between the PS phospholipid profiles of WT and 12/15-LOX$^{-/-}$ macrophages are significant following ANOVA analysis. Peritoneal macrophages from WT and 12/15-LOX$^{-/-}$ mice were isolated by adhesion prior to lipid extraction. PS phospholipids were analysed by direct injection MS/MS (n=8, mean ± S.E.). *Students t-test, $p<0.05$. The overall difference in PS phospholipids between WT and 12/15-LOX data sets is significant following analysis by one-way ANOVA with a Tukey post hoc test, $p<0.05$.

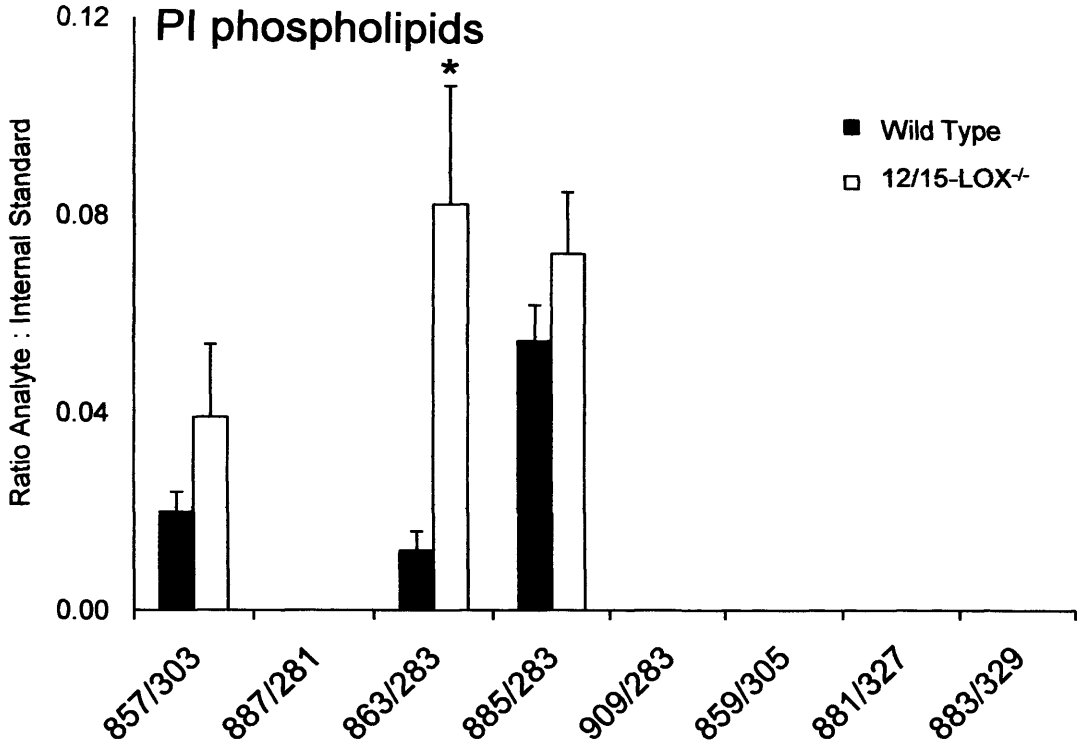


Figure 8.7 There are few significant differences between the PI phospholipid profiles of WT and 12/15-LOX$^{-/-}$ macrophages. Peritoneal macrophages from WT and 12/15-LOX$^{-/-}$ mice were isolated by adhesion prior to lipid extraction. PI phospholipids were analysed by LC/MS/MS (n=8, mean ± S.E.). *Students t-test, $p<0.05$.

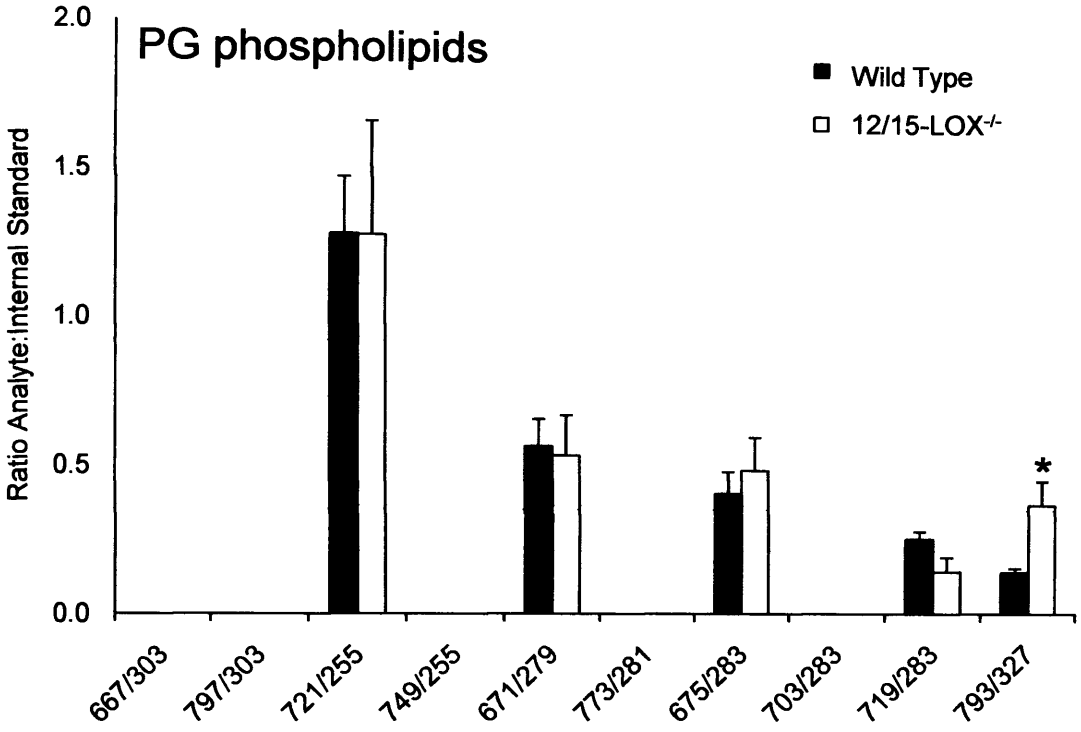


Figure 8.8 Differences between the PG phospholipid profiles of WT and 12/15-LOX$^{-/-}$ macrophages are significant following ANOVA analysis. Peritoneal macrophages from WT and 12/15-LOX$^{-/-}$ mice were isolated by adhesion prior to lipid extraction. PG phospholipids were analysed by LC/MS/MS (n=8, mean ± S.E.). *Students T-Test, $p<0.05$. The overall difference in PG phospholipids between WT and 12/15-LOX data sets is significant following analysis by one-way ANOVA with a Tukey post hoc test, $p<0.05$.

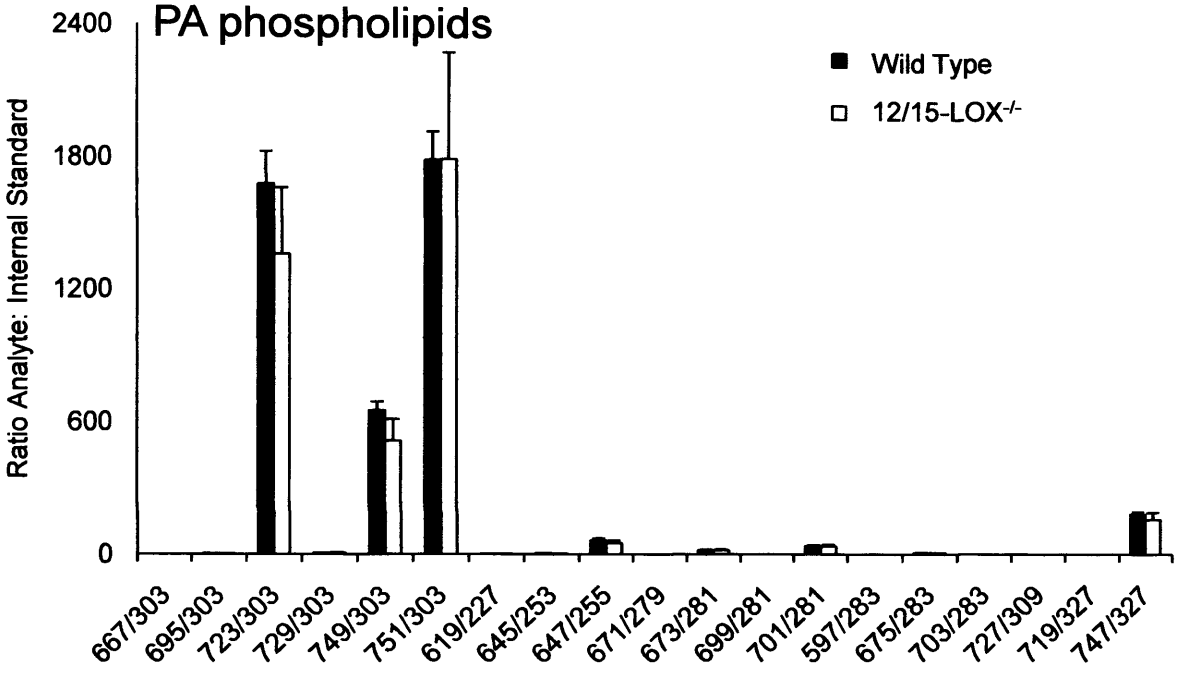


Figure 8.9 There are few significant differences between the PA phospholipid profiles of WT and 12/15-LOX$^{-/-}$ macrophages. Peritoneal macrophages from WT and 12/15-LOX$^{-/-}$ mice were isolated by adhesion prior to lipid extraction. PA phospholipids were analysed by LC/MS/MS (n=8, mean ± S.E.).

12/15-LOX$^{-/-}$ mice were analysed by LC/MS/MS for cholesterol esters. In 12/15-LOX$^{-/-}$ macrophages there were small increases in cholesterol esters, when the levels of phospholipids were combined the increases were significant in comparison to levels detected in WT, following analysis by a one-way ANOVA with a Tukey post hoc test ($p=<0.05$). Therefore, these differences may also contribute to the ultra-structural abnormalities seen in 12/15-LOX$^{-/-}$ macrophages (Figure 8.10).

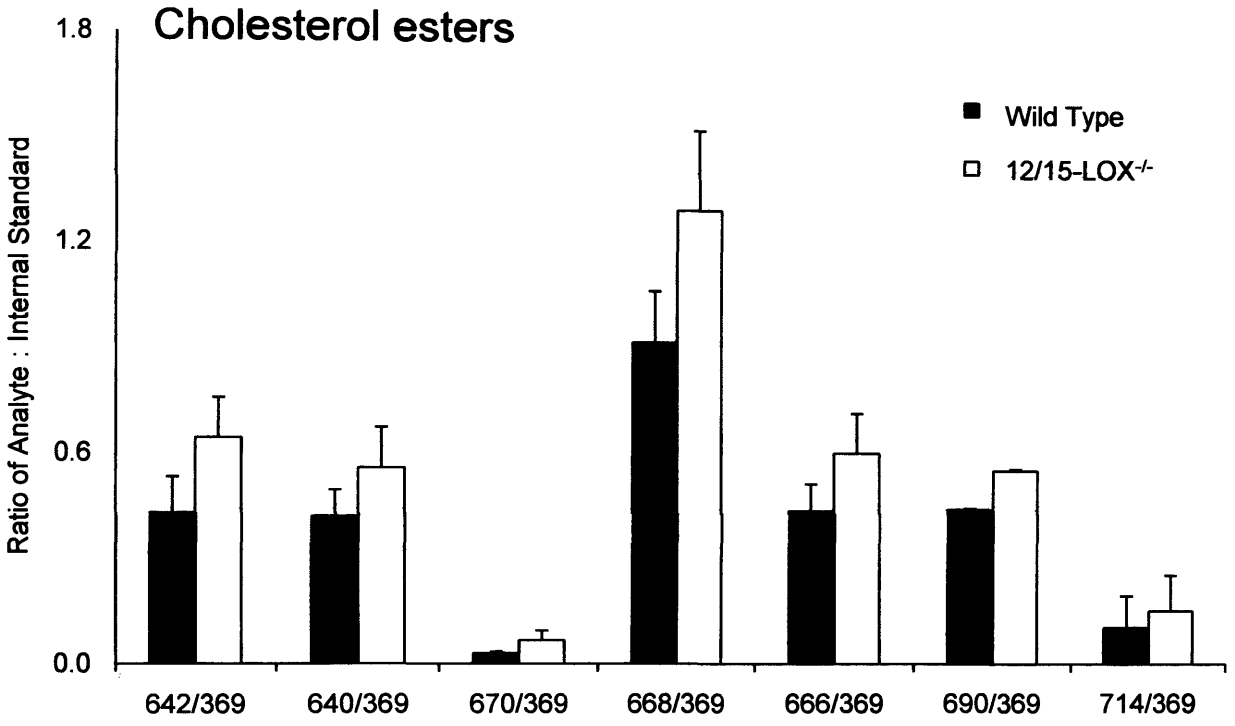


Figure 8.10 Differences between the cholesterol ester profiles of WT and 12/15-LOX$^{-/-}$ macrophages are significant following ANOVA analysis. Peritoneal macrophages from WT and 12/15-LOX$^{-/-}$ mice were isolated by adhesion prior to lipid extraction. Cholesterol esters were analysed by LC/MS/MS (n=8, mean ± S.E.). The overall difference in cholesterol esters between WT and 12/15-LOX data sets is significant following analysis by one-way ANOVA with a Tukey post hoc test, $p<0.05$.

8.3 Discussion

To investigate structural differences between WT and 12/15-LOX$^{-/-}$ macrophages, murine peritoneal samples were analysed by EM. Multiple differences were identified between them, including abnormal mitochondria, multiple lysosomal storage bodies and suspected autophagosomes, which are all consistent with LSDs. Lysosomes, are small vesicular organelles, their primary function being to merge with late endosomes, to absorb and re-disperse their content (Mullock *et al*, 1998, Bright *et al*, 2007, Kiselyov & Muallem, 2007). The process of endosomal degradation is carried out by numerous lipid and protein hydrolases. Mutations in these enzymes result in a build-up of undigested proteins, lipids and sugars that can be seen as dark inclusions during EM analysis, known as lysosomal storage bodies (Kiselyov & Muellem, 2007, Ponder & Haskins, 2007). Numerous dark inclusions were seen in 12/15-LOX$^{-/-}$ macrophages that are likely to be lysosomal storage bodies.

Lysosomes play an important role in autophagy, which is defined as "a cells' digestion of its own organelles and cytoplasmic content" (Meijer & Codogno, 2004, Cuervo *et al*, 2005). This process involves the formation of autophagic vesicles, which encapsulate organelles destined for destruction (Figure 8.11) (Kiselyov & Muellem, 2007). Autophagy is required for the rapid clearance of oxidised proteins and organelles, and is thought to mediate a specialised form of cell death that is distinct from necrosis and apoptosis (Sherz-Shovsal & Elazar, 2007, Gozuacik & Kimchi, 2004). Both lysosomes and autophagy are important regulators of mitochondrial health by mediating mitochondrial turnover. The mitochondria seen in 12/15-LOX$^{-/-}$ macrophages appear swollen and granular, consistent with signs that they are 'old' and damaged, long overdue to undergo autophagy. The phenotype of cells showing signs of LSD resembles that of aged cells, abnormally structured mitochondria and the presence of lysosomal storage bodies are typical of this condition (Kiselyov & Muellem,

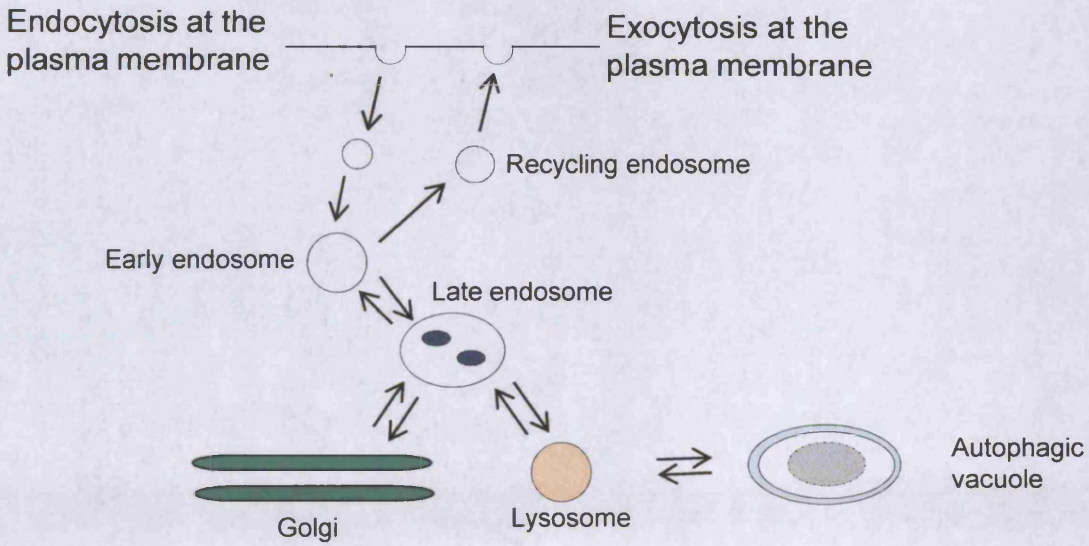


Figure 8.11. The endocytic pathway, lysosomes and autophagy. Endocytosed material enters endocytic vesicles (early endosomes). The material is sorted and if designated for destruction, it is transported to late endosomes which fuse with lysosomes for digestion and absorption. Autophagy is also driven by lysosomes which fuse with autophagic vacuoles (reproduced from Kiselyov & Muallem, 2008, with permission (04/12/2010)).

2007). There are several common dysfunctions leading to LSDs: 1) Mis-sorting by enzymes resulting in secretion of material, rather than their targeted digestion by lysosomes. 2) Endocytosed material is not broken down due to defective lysosomal enzymes or supporting proteins. 3) Hydrolysed products are not effectively secreted from lysosomes. 4) pH regulation within lysosomes is defective (Kiselyov & Muallem, 2008).

When autophagy is prevented, mitochondria become damaged, due to the oxidative nature of reactions that take place within this organelle. For example, during cellular signalling mitochondria are important in buffering Ca^{2+} concentrations. During these buffering events, much of the damage caused to mitochondria is due to reactive oxygen species that are generated by components of the oxidative chain (Bruce *et al*, 2004, Peterson OH, 2004, Brookes *et al*, 2004, Giorgio *et al*, 2005, Kiselyov & Muellem, 2007). Usually, damaged lipid and protein components trigger mitochondrial-dependent cell death, where cells undergo fragmentation followed by engulfment by autophagic vacuoles (Brookes *et al*, 2004, Giorgio *et al*, 2005, Kiselyov & Muellem, 2007). Blocking autophagy leads to an accumulation of aged, partially dysfunctional and fragmented mitochondria, making host cells more susceptible to mitochondrial-mediated damage (Figure 8.12) (Ravikumar *et al*, 2004, Boya *et al*, 2005, Settembre *et al*, 2008, Jennings *et al*, 2006, Zhao *et al*, 2003). There is an increased number of shortened mitochondria with enlarged christae that lose their ability to function as Ca^{2+} buffers during signalling, thus making cells in cases of LSD more sensitive to the pro-apoptotic effects of Ca^{2+} (Jennings *et al*, 2006, Kiselyov *et al*, 2007). A progressive increase in autophagic vacuoles is in accordance with disproportionate organelle damage and degradation, recognised as 'autophagic stress' (Kiselyov *et al*, 2007).

In 12/15-$LOX^{-/-}$ macrophages, autophagosomes were distinguished as inclusions with double membranes. Confirming the identity of autophagosomes needs to be carried using antibodies

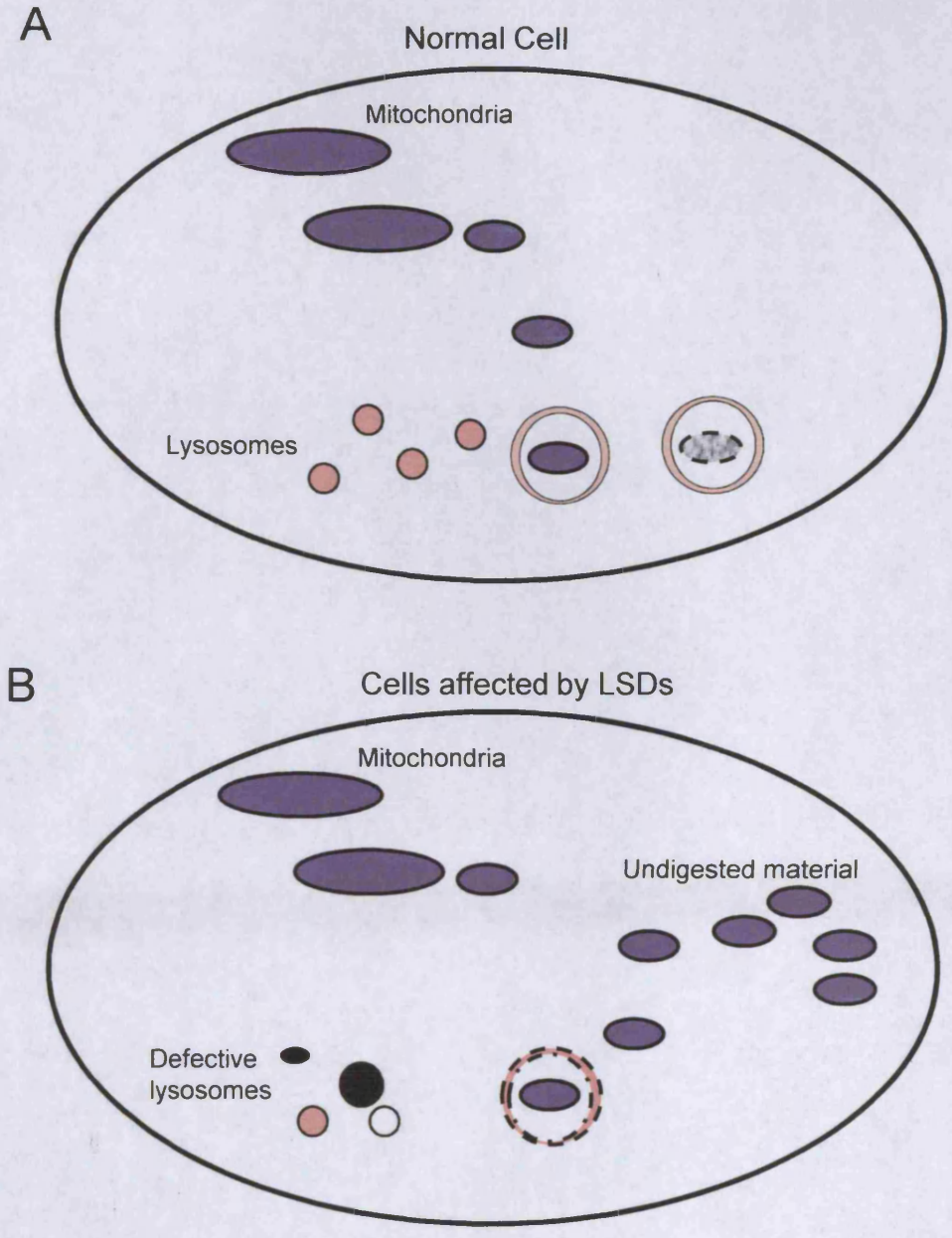


Figure 8.12 Fragmentation of mitochondria in LSDs. A: In normal cells, fragmented mitochondrial debris are removed from the cytoplasm by autophagy and are destined for degradation by lysosomes. B: In LSD-affected cells, autophagy is suppressed through unknown mechanisms; fragmented mitochondria accumulate while the mitochondria that remain have altered functional capacities (reproduced from Kiselyov K, 2007, with permission (04/12/2010)).

for monodancylcadaverine, autophageic light chain 3 (LC-3), and lysosome associated membrane protein-1 and -2 (LAMP-1 and LAMP-2), followed by analysis by confocal microscopy (Kiselyov & Muellem, 2007, Klionsky DJ, 2005, Miedel *et al*, 2008). Primary LSDs are commonly associated with the observation of 'swirls' in cells, but these were absent in 12/15-LOX$^{-/-}$ macrophages (Alroy & Ucci, 2006). The lack of 'swirls' suggests that the dark inclusions, identified as storage bodies, are not the primary storage compartment for this undigested material. To confirm whether these dark inclusions were at one point part of the endocytic pathway, cells can be loaded with colloidal gold, which can then be processed for analysis by EM (Miedel *et al*, 2008).

There are many known LSDs, all resulting in the accumulation of undigested material, whether they are lipids, proteins or carbohydrates, in vacuole and/or non-vacuole inclusions. The identification of storage bodies in the cytoplasm of peripheral blood mononuclear cells (PBMCs) and skin biopsies is an invaluable tool for the diagnosis of LSDs (Wisniewski KE, 1986). In particular, glycerosphingolipids and cholesterol have been shown to accumulate in a diverse collection of LSDs (Futerman & Van Meer, 2004). In some disorders, a build up of undigested lipids leads to an increase in lipid rafts, disturbing the dynamics of lysosomal membranes, in particular, their ability to bind with autophagosomes (Sobo *et al*, 2007). This is of interest as 12/15-LOX is an enzyme that oxidises lipid membranes, and therefore its absence may lead to the accumulation of undigested products. A lipid LSD is indicated by the presence of swirls, however, they were not observed in macrophages from 12/15-LOX$^{-/-}$ mice, this and the presence of vacuoles and inclusions indicate that it may be a protein storage disorder.

To speculate the cause of this LSD I can extrapolate from the knowledge gained during the experiments described in these chapters, as well as information from published articles. In

Chapter 6, it was shown that in the absence of 12/15-LOX there are no 12-HETE-PE products. If these lipids are involved in events downstream of their synthesis, then their absence will affect other cellular processes. In addition, in Chapter 5 it was demonstrated that 15-HETE-PEs may form Schiff bases or Michael adducts with proteins. It is possible that this process is required for the removal of particular protein/peptides. This could account for the presence of storage bodies in 12/15-LOX$^{-/-}$ macrophages. However, as the combined levels of cholesterol esters, PE, PS and PG phospholipids were raised in 12/15-LOX$^{-/-}$ macrophages, the storage bodies may be due to a lipid excess. Identification of the contents within the storage bodies may indicate the type of LSD present.

A growing number of reports show that externalisation of oxidised phospholipids, such as 12-HETE-PEs in murine macrophages (as described in Chapter 6), is required as a signal for the phagocytosis of apoptotic cells (Shiratsuchi *et al*, 1998, Yao *et al*, 2009). 15-LOX can also oxidise organellar preparations of mitochondria, rough and smooth ER, golgi and peroxisomes *in-vitro* (Van Leyen *et al*, 1998). Thus, oxidation of organelle membranes may also be required to signal their destruction or recycling via pathways such as autophagy. 12/15-LOX is generally considered to be a soluble cytosolic protein, however, when it integrates into membrane lipids it takes on properties of an integral membrane protein (Vijayvergiya *et al*, 2004). During this, 12/15-LOX produces pore-like structures in the membranes that can be seen by EM (Vijayvergiya *et al*, 2004). Following the integration of 12/15-LOX, membranes become permeable and the lumenal contents are released. Significantly, incubation of 15-LOX with AA containing lipid liposomes also leads to vesicle permeablisation (van Leyen *et al*, 1998, Maccarrone *et al*, 2001b, Walther *et al*, 2004, Vijayvergiya *et al*, 2004). However, pore like structures were not seen in WT or 12/15-LOX$^{-/-}$ cells, but 12/15-LOX may still be involved in autophagy as a previous study has shown that

15-LOX inhibitors retard the breakdown of reticulocyte mitochondria (Grullich *et al,* 2001). Also, over-expression of 15-LOX leads to the collapse of mitochondrial pH gradient (Vijayvergiya *et al,* 2004). Importantly, 15-LOX has been linked to the degradation of mitochondria in maturing reticulocytes and in differentiating lens fibres (Schewe *et al,* 1975, Schewe *et al,* 1977, Bassnett & Beebe, 1992, Basnett & Mataic, 1997, Dahm *et al,* 1997). In addition, 15-LOX may also be involved in the degradation of peroxisomes, which is also suggested to occur by autophagy (Yokota *et al,* 2001). There may be a relationship between LOX and mitochondrial function in particular cells, but as 12/15-LOX$^{-/-}$ mice appear normal and the enzyme is only expressed in a small subset of leukocytes, it is unlikely to play a major role in mitochondrial regulation. In support of this, it is important to note that both abnormal and healthy mitochondria were present in 12/15-LOX$^{-/-}$ macrophages. If 12/15-LOX is involved in mitochondrial autophagy, possibly in the fragmentation process prior to its incorporation into endosomes, it may account for the aged and deformed state of some mitochondria in 12/15-LOX$^{-/-}$ macrophages.

Cells from many distinct LSDs have very similar degenerative phenotypes, suggesting that regardless of the underlying genetic cause of the disorder, they all share common downstream mechanisms (Kiselyov *et al,* 2007). A frequent observation in these disorders is the poor condition of particular tissues, including the spleen, liver and thyroid (Beltroy *et al,* 2005). In a study carried out by colleagues of the O'Donnell group (Cardiff, UK), WT and 12/15-LOX$^{-/-}$ mice were sacrificed at 30 weeks, and particular organs, including the liver and spleen were extracted and weighed. No significant difference was seen on weight comparisons of these organs and it is yet to be seen whether they differ in overall phospholipid content (not shown). Muscle and adipose tissue samples were also taken for EM and histological analysis.

The results from this study may support the aforementioned conclusion that the disorder in 12/15-LOX$^{-/-}$ macrophages is indicative of a LSD.

Lipid profiling of 12/15-LOX$^{-/-}$ macrophages concluded that the combined levels of PE, PS and PG phospholipids and cholesterol esters were higher, while combined levels of PC were lower, in comparison to levels in WT macrophages. Therefore, an excess of cholesterol esters and/or PE, PS and PG phospholipids may account for the undigested material in lysosomal storage bodies. Alternatively, if 12/15-LOX products are confirmed as forming Schiff base and/or Michael adducts with proteins or peptides in WT cells, then it may be investigated as to whether the same proteins/peptides are the stored material in 12/15-LOX$^{-/-}$ macrophages. Previous studies have reported 12/15-LOX$^{-/-}$ macrophages show defective migration and phagocytosis of apoptotic thymocytes (Miller *et al*, 2001 & 2003). As stated above, combined levels of PEs and PSs, which are on the inside of the plasma membrane, were increased in 12/15-LOX$^{-/-}$ macrophages, while levels of PCs, which are on the outside, were decreased (Figures 8.4-8.6). The phospholipid composition of membranes is integral to their function, therefore, these changes may contribute to the altered capability of 12/15-LOX$^{-/-}$ macrophages to migrate and carry out phagocytosis.

To conclude, this chapter has described the possible identification of LSDs in 12/15-LOX$^{-/-}$ mice using EM analysis methods. Further investigations will clarify the mechanisms by which this condition comes about, taking us a step nearer to understanding the primary role of 12/15-LOX in macrophages.

CHAPTER 9

GENERAL DISCUSSION

The studies described herein have investigated the metabolism and biological function of particular LOX products, 12-H(p)ETE-PEs and 15-H(p)ETE-PEs, generated by murine macrophages and human monocytes respectively. Furthermore, to accurately quantify 15-H(p)ETE-PEs, a new quantification assay was developed, for which, a pure 15-H(p)ETE-PE standard was synthesised (Chapter 3). Previous studies have suggested that 12/15-LOX is involved in inflammation. Therefore, experiments were carried out to investigate how 12- and 15-H(p)ETE-PEs may be involved in inflammatory processes. In particular, studies investigated the effect of a synthesised 15-H(p)ETE-PE standard on cytokines generated by LPS stimulated human monocytes (Chapter 7).

Direct quantification of phospholipids is important for accurate data. Up to now, many studies have been carried out using mixtures of ox-PLs, which were quantified either by 'calculated estimation' based on the amount of initial substrate used, or by detecting the total amount of peroxide products (Watson *et al*, 1997, Podrez *et al*, 1999, Podrez *et al*, 2002, Von Schlieffen *et al*, 2009, Cherepanova *et al*, 2009, Walton *et al*, 2003, Leitinger *et al*, 1999). Alternatively, ox-PLs have been quantified indirectly, such as quantification of fatty acids following base hydrolysis (Maskrey *et al*, 2007, Morgan *et al*, 2009). These methods of phospholipid quantification are not as accurate as directly quantifying individual molecular phospholipid species. This issue was addressed in Chapter 3, where a new method was developed to directly quantify 15-H(p)ETE-PEs following analysis by LC/MS/MS (Section 3.2.4). For this assay, a pure 15-H(p)ETE-PE standard was required therefore, it was synthesised by adapting a method described by Brash *et al* (1987) (Scheme 3.1.).

The new method of quantification accounts for discrepancies in ionisation efficiency between the internal standard (DMPE) and the analyte in question, but it cannot account for any differences in extraction efficiency. Currently, DMPE is the closest lipid in structure to the HETE-PEs that does not exist in human monocytes or murine macrophages. Therefore, it is the most suitable internal standard for HETE-PE quantification. Nevertheless, to account for variations during extraction, deuterated 12- and 15-HETE-PEs are required as internal standards. The structure of deuterated HETE-PEs would be more similar to native HETE-PEs and their extraction efficiency would be identical, thus providing greater accuracy during quantification. However, due to the complexity of the structures, synthesising deuterated HETE-PEs may be challenging.

Similar quantification assays have now been used to quantify alternative HETE-PE isomers and phospholipids containing LA and DHA following the synthesis of appropriate standards (O'Donnell *et al*, unpublished). Future directives could include synthesising 15-KETE-PE standards to enable the quantification of these lipids in human monocytes and to investigate their function and metabolism. The adaptation of a method described by Brash *et al* (1987) to synthesise 15-H(p)ETE-PEs, and the subsequent use of these lipids to directly quantify 15-H(p)ETE-PEs in biological samples has been an central part of this thesis. Importantly, this assay could be used by other research groups to quantify many alternative lipids analysed by MS.

Studies report that 15-LOX has anti-inflammatory effects in rabbits, while 12/15-LOX is pro-inflammatory in mice (Kuhn & Chan, 1997, Feinmark & Cornicelli, 1997, Cornicelli & Trivedi, 1999, Cathcart & Folcick, 2000, Witter & Hersberger, 2007). Therefore, experiments were carried out to investigate the effect of 18:0a/15-HETE-PE on human monocytes (Chapter 7). 18:0a/15-HETE-PE had an anti-inflammatory effect on monocytes by down-

regulating LPS stimulated TNF-α, IL-1β, IL-6 and G-CSF generation (Figures 7.11-7.14). As monocytes were shown to synthesise 15-KETE-PEs it will also be relevant to investigate whether these lipids also down-regulate cytokine generation (Chapter 4). However, a 15-KETE-PE standard is not yet available. The addition of 12-HETE-PEs to LPS-stimulated murine macrophages may confirm whether 12/15-LOX has a pro- or anti-inflammatory effect on cytokines in mice. Furthermore, treating human monocytes with the murine isomer, 18:0a/12-HETE-PE, may show whether the position of oxidation alters its biological action. However, these experiments would require a pure 12-HETE-PE standard, which is also not available. Meanwhile, a standard of mixed 18:0a/HETE-PE isomers may be used in preliminary work investigating the effect of HETE-PEs on induced inflammation in animal models. For example, atherosclerotic mouse/rabbit models, or mice infected with live *S.Epi.* or SES, could be treated with either the HETE-PE isomer mix or the 18:0a/15-HETE-PE, to investigate whether their effects are different. The results described herein contribute to evidence previously published suggesting that 15-LOX in humans and 12/15-LOX in mice may have opposing effects. The future experiments described above will be important in determining whether this is the case, and thus, may aid in the development of therapies for inflammatory conditions, such as atherosclerosis.

The synthesis of 15-H(p)ETE-PEs in human monocytes was previously described by Maskrey *et al* (2007), therefore, the temporal generation of 12-H(p)ETE-PEs was investigated in murine macrophages (Chapter 6). The results described herein show that products of human monocyte 15-LOX and murine macrophage 12/15-LOX are synthesised similarly. However, generated 15-HETE-PEs and 12-HETE-PEs may be metabolised differently. In human monocytes, novel lipids were identified as 15-KETE-PEs, which form 15-HETE-PEs following chemical reduction (Figures 4.13-4.17). 12/15-LOX may generate

the equivalent murine phospholipids, 12-KETE-PEs, future studies could investigate this. The mechanism of 15-KETE-PE synthesis is unknown, they may be formed directly by LOX, following metabolism of the peroxide (15-HpETE-PE), or enzymatic conversion of 15-HETE-PE (Scheme 4.3). Under anaerobic conditions, soybean LOX is reported to synthesise 15-KETE in place of 15-HpETE, therefore, 15-LOX could also directly synthesise KETE-PEs (de Laclos & Borgeat, 1988). This could be investigated using an isolated rabbit 15-LOX. Similar experiments could be carried out to examine whether 12/15-LOX directly synthesises 12-KETE-PEs or 12-HpETE-PEs, however, murine 12/15-LOX has not been isolated as of yet. Alternative LOX isomers may synthesise KETE-PE lipids, which may have important effects in their respective sites of generation, and thus, may also be investigated in the future.

Following their synthesis, 15-KETE-PEs are metabolised to unknown products. Several pathways of metabolism have been rejected, including conversion of the PE head group to PC and elongation of the sn2 fatty acid (HpETE/KETE). To investigate potential products formed following 15-KETE-PE synthesis, time-course samples were analysed by precursor scans for the sn1 fatty acids (16:0p, 18:1p, 18:0p and 18:0a) and also, for the PE-head group. However, no compounds containing fragments with these *m/z* were identified (data not shown). Therefore, work was carried out to investigate whether products of 15-LOX could bind covalently with proteins (Chapter 5). The results suggest that phospholipids oxidised by 15-LOX may form Michael adducts with proteins. As these covalently bound products increase by 3 hours, they could account for the 15-KETE-PE decrease seen in time-course samples (Figure 5.6). Thus, the results herein contribute to previous evidence reporting the formation of protein-lipid bonds. Future work could include identifying protein targets of phospholipids oxidised by 15- and 12/15-LOX. To begin, experiments could investigate

proteins that have been previously identified in association with ox-PLs (Horkko *et al*, 1996, Horkko *et al*, 1997, Mattila *et al*, 2008, Yang *et al* 2008, Eberard *et al*, 2009, Wan *et al*, 2007, Gugiu *et al*, 2008, Szapacs *et al*, 2008).

12/15-LOX may be involved in processes that require alteration of the cell membrane, such as migration or phagocytosis. Results in Chapter 6 showed that 12-HETE-PEs were preferentially externalised instead of their un-oxidised counterparts (AA-PEs) in activated murine macrophages (Figure 6.9). Phospholipid externalisation may alter membrane fluidity therefore allowing processes such as phagocytosis and/or migration to occur. Thus, the results described herein explain how the absence of these ox-PLs may lead to the abnormal phagocytotic ability of 12/15-LOX$^{-/-}$ macrophages (Miller *et al*, 2001). Interestingly, in particular cell types, phospholipid oxidation is required prior to their externalisation (Mirnikjoo *et al*, 2009, Fadok *et al*, 2001, Diaz *et al*, 1999, Shiratsuchi *et al*, 1998, Tyurina *et al*, 2004a, Jain SK, 1985). Externalised ox-PLs may have an alternative immunological function. For example, externalised ox-PCs and ox-cholesterols have been identified as ligands for receptors such as CD-36, which may have important consequences in atherosclerosis (Greenberg *et al*, 2006, Podrez *et al*, 2003). Externalised HETE-PEs could also act as ligands for similar receptors. Future work could include investigating whether 15-H(p)ETE-PEs and 15-KETE-PEs are externalised in human monocytes, this may clarify whether the actions of 15-H(p)ETE-PEs are similar to 12-H(p)ETE-PEs synthesised by murine macrophages. 12-H(p)ETE-PEs are externalised in human platelets, thus it may be of interest to investigate whether products of alternative LOX isoforms are externalised in their respective cell types (Thomas *et al*, 2010). To support a role for 12/15-LOX in membrane function, future work could include migration assays, which may show whether cell movement is directly affected by 12/15-LOX, and therefore, 12- and/or 15-H(p)ETE-PEs.

Phospholipid oxidation by 12/15-LOX may be a method of changing the properties of cellular membranes, thus, inhibiting oxidation may alter membrane function. 15-LOX is reported to oxidise various cell membranes, which include the mitochondria, rough and smooth ER, golgi, peroxisomes and plasma membranes (Van Leyen *et al*, 1998, Schewe *et al*, 1975, Schewe *et al*, 1977, Yokota *et al*, 2001). Therefore, in the absence of 12/15-LOX, the functional properties of multiple organelle membranes, in addition to the plasma membrane, may be altered. Ultra-structural analysis of peritoneal macrophages from 12/15-LOX$^{-/-}$ mice showed that their macrophages have abnormalities consistent with LSDs, which may be due to variations in total cellular lipid content (Chapter 8). However, the nature of the ultra-structural abnormalities suggests the presence of a secondary protein LSD (Figure 8.2 & 8.3). Particular aspects of interest regarding the altered ultra-structure of 12/15-LOX$^{-/-}$ macrophages was firstly, the presence of lysosomal storage bodies and secondly, the abnormal mitochondria. Further work is required to determine whether the absence of 12/15-LOX is responsible for these malformations. In addition, the undigested material within lysosomal storage bodies requires identification. If it is identified as protein, then it is possible that products of 12/15-LOX act by targeting these proteins for destruction in WT macrophages via the formation of Michael adducts. Furthermore, the lysosomal storage bodies may have been part of the autophagy system. If this is confirmed, 12/15-LOX may then be linked to mitochondrial maintenance and turnover. Therefore, 12/15-LOX may play a role in altering the properties of membranes within macrophages, which may be via oxidation and externalisation of phospholipids. The structural abnormalities of 12/15-LOX$^{-/-}$ macrophages reported in Chapter 8 have not been seen previously. EM could be used to investigate whether the absence of LOX causes structural abnormalities in cells containing different isoforms of LOX. In particular, it may be of interest to develop a rabbit 15-LOX$^{-/-}$ as

the enzyme may be more functionally equivalent to human 15-LOX, in comparison to murine 12/15-LOX.

The generation of HETE-PEs in humans following bacterial infection has not previously been investigated. Therefore, as part of a long-term study, lavage samples from patients with suspected bacterial infections were analysed for the presence of 15-H(p)ETE-PEs. The results suggested that 15-H(p)ETE-PEs may be raised following infection by gram-positive bacteria. However, the number of patient samples needs to be increased in order to confirm these preliminary results. In addition, lavage samples from patients that are commencing peritoneal dialysis as a treatment for renal failure are required, they may provide a better indication of 15-H(p)ETE-PE levels in patients that are free from infection. Data gathered during this long-term study may contribute to understanding the role of 15-H(p)ETE-PE in human inflammatory conditions. Previous work carried out by our group has shown that 12-HETE-PE synthesis is altered following bacterial infection and in OVA lung allergy (Morgan *et al*, 2009). Until more data from human studies can be gathered, short term studies involving animal models are invaluable. In particular, as rabbit and human leukocyte LOX both oxidise AA at C-15, future work could include investigating the effect of 15-H(p)ETE-PEs in rabbits with inflammation induced by bacteria.

In the experiments described herein, human monocytes and murine macrophage samples were activated using calcium ionophore (A23187). While this agonist is frequently used it is not physiological. Experiments using a physiological agonist may more closely simulate events that take place *in-vivo*. Therefore, investigations were carried out attempting to activate human monocytes and murine macrophages via their surface IgG and IgE receptors (von Bubnoff *et al*, 2002). However, HETE-PEs were not synthesised consistently (data not

shown). Multiple experiments showed conflicting results, therefore, there remains no physiological agonist for 12/15-LOX in human monocytes and murine macrophages. However, finding a physiological agonist will be important for future experiments investigating products and the effects of 12/15-LOX.

To conclude, the data described herein has provided a platform to further investigate the function of esterified products generated by 15- and 12/15-LOX. Future studies may determine if products of 12/15-LOX are involved in inflammatory disorders such as atherosclerosis, and whether their effects are pro- or anti-inflammatory. In particular, if products of 12/15-LOX have anti-inflammatory effects then future studies may investigate the potential therapeutic benefit of 15-H(p)ETE-PEs, and their use as treatments for inflammatory conditions.

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