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**The effect of eicosapentaenoic acid on brain and platelet
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ionisation tandem mass spectrometry-based analysis.**

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The effect of eicosapentaenoic acid on brain and platelet produced bioactive lipid mediators

Adnan Ahmed MIR

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Eicosapentaenoic acid, docosapentaenoic acid, eicosanoids, endocannabinoids, liquid chromatography tandem mass spectrometry, brain, platelets

Abstract

Eicosapentaenoic acid (EPA) is a polyunsaturated fatty acid (PUFA) with neuroprotective and cardioprotective properties. It is thought that some of the actions of EPA may be attributed to its elongated metabolite, the PUFA docosapentaenoic acid (DPA). Docosahexaenoic acid (DHA) and arachidonic acid (AA) are bioactive PUFA ubiquitously expressed in neural tissues. EPA and AA can be converted by cyclooxygenase (COX) to prostanoids and by lipoxygenase (LOX) to hydroxy fatty acids. PUFA can also be converted to ethanolamides in the brain. These mediators are involved in physiological and pathological processes in many bodily systems.

The purpose of this study was to examine the production of eicosanoids, hydroxy fatty acids and fatty acid ethanolamides in young and aged rat brain following EPA or DPA enriched diets. The effects of specific PUFA on human platelet eicosanoid production were also investigated as these mediators play a role in adhesion and aggregation. Liquid chromatography coupled to tandem mass spectrometry (LC/ESI-MS/MS) assays were developed and used to measure lipid mediators in rat brain and human platelets.

Ageing in rat brain was accompanied with several changes in the prostanoid and hydroxy fatty acid profiles. Supplementing the diet with EPA or DPA at a daily dose of 200 mg/kg for 8 weeks prevented these changes and decreased levels of PGE₂. DPA changed the profile of hydroxy fatty acids synthesised in the brain tissue of young animals. This study has shown that levels of eicosapentaenoylethanolamide (EPA-EA) increase in the brain as a result of ageing and that this is accompanied by an increase in levels of anandamide. Feeding aged animals EPA or DPA further increased the levels of EPA-EA but prevented any change in the level of anandamide.

Niacin is used to treat hypercholesterolaemia although it is associated with an unpleasant PGD₂ mediated skin flush. This exploratory study has shown that human platelets treated with niacin did not show any changes in their prostanoid and hydroxy fatty acid profiles. Platelets treated with EPA showed increased production of TXB₃ and 12-HEPE. Niacin augmented the effects of EPA on human platelet mediator synthesis.

Overall, this study has demonstrated that EPA can change brain and platelet lipid mediator synthesis and has provided evidence that could explain some of the neuroprotective and cardioprotective actions of this PUFA.

Peer-reviewed published papers

1. M Masoodi, **AA Mir**, NA Petasis, CN Serhan, A Nicolaou. Simultaneous lipidomic analysis of three families of bioactive lipid mediators leukotrienes, resolvins, protectins and related hydroxy fatty acids by liquid chromatography/electrospray tandem mass spectrometry (2008) *Rapid Commun Mass Spectrom* **22**, 75-83.

Book chapters

1. A Nicolaou, M Masoodi, **AA Mir**. Lipidomic analysis of prostanoids by liquid chromatography electrospray tandem mass spectrometry (2008) in *methods in molecular biology*, Humana Press Inc, Totowa, NJ – in press.

Conference contributions

1. **AA Mir**, M Masoodi, NA Petasis, CN Serhan, A Nicolaou. ESI/LC-MS/MS lipidomic analysis of hydroxy fatty acid derivatives of polyunsaturated fatty acids. 41st BMSS, September 2007, Edinburgh (oral presentation).

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List of Abbreviations

2-AG	2-arachidonyl glycerol
AA	arachidonic acid
Abh4	α/β -hydrolase 4
ACC	acetyl-CoA-carboxylase
A-EA	arachidonylethanolamide (anandamide)
ALA	α -linolenic acid
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionisation
BBB	blood brain barrier
cAMP	cyclic adenosine monophosphate
CB	cannabinoid receptor
CI	collision induced
CLA	conjugated linoleic acid
CNS	central nervous system
COX	cyclooxygenase
CYP450	cytochrome P450
DGLA	dihomo- γ -linolenic acid
DHA	docosahexaenoic acid
DHA-EA	docosahexaenylethanolamide
DiHETE	dihydroxy-eicosatetraenoic acid
DP	prostaglandin D receptor
DPA	docosapentaenoic acid
EI	electron ionisation
EP	prostaglandin E receptor

EPA	eicosapentaenoic acid
EPA-EA	eicosapentaenoylethanolamide
ESI	electrospray ionisation
FAAH	fatty acid amide hydrolase
FAB	fast atom bombardment
FAS	fatty acid synthase
FLAP	5-lipoxygenase activating protein
FP	prostaglandin F receptor
GABA	γ -aminobutyric acid
GLA	γ -linoleic acid
HDHA	hydroxy docosahexaenoic acid
HEPE	hydroxy eicosapentaenoic acid
HETE	hydroxy eicosatetraenoic acid
HODE	hydroxy octadecadienoic acid
HpDHA	hydroperoxy docosahexaenoic acid
HpEPE	hydroperoxy eicosapentaenoic acid
HpETE	hydroperoxy eicosatetraenoic acid
IL	interleukin
IP	prostacycline receptor
JELIS	Japanese EPA lipid intervention study
JNK	c-jun-N-terminal kinase
KS	Kolmogorov-Smirnov
LA	linoleic acid
LC	liquid chromatography
LC/ESI-MS/MS	liquid chromatography tandem mass spectrometry

LOD	limit of detection
LOQ	limit of quantitation
LOX	lipoxygenase
LPS	bacterial lipopolysaccharide
LT	leukotriene
LTP	long term potentiation
LX	lipoxygenase
MALDI	matrix assisted laser desorption ionisation
MPC	membrane phospholipid composition hypothesis
MS/MS	tandem mass spectrometry
<i>m/z</i>	mass to charge ratio
NAE	<i>N</i> -acyl ethanolamide
NAPE	<i>N</i> -acylphosphatidylethanolamine
NAPE-PLD	<i>N</i> -acylphosphatidylethanolamine phospholipase D
NFκB	nuclear factor κB
PA	palmitic acid
PD	prostaglandin D receptor
PLP	proteolipid protein
PG	prostaglandin
PGDS	prostaglandin D synthase
PGES	prostaglandin E synthase
PLA₂	phospholipase A ₂
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species

Rv	resolvin
SA	stearic acid
SSRI	selective serotonin reuptake inhibitor
THC	tertahydrocannabinol
TLC	thin layer chromatography
TNF-α	tumour necrosis factor- α
TOF	time of flight
TP	thromboxane receptor
TRPV	vanilloid receptor
TXAS	thromboxane A synthase
TX	thromboxane

Chapter 1 : Introduction

1.1 Polyunsaturated fatty acids

The general structure of a fatty acid incorporates a long saturated or unsaturated, linear or branched, hydrocarbon chain with, a carboxyl group at one end and a methyl group at the other.

Unsaturated fatty acids can be monounsaturated or polyunsaturated (PUFA). Double bonds in the hydrocarbon chain allow for stereoisomerism, thus, unsaturated fatty acids can assume *cis* or *trans* configuration. Figure 1.1 illustrates the structure of eicosapentaenoic acid (EPA), a PUFA with all *cis* configuration and octadecadienoic acid, a PUFA with both *cis* and *trans* configuration.

Figure 1.1. Chemical structure of all-*cis* 5,8,11,14,17- eicosapentaenoic acid (A) and *cis* 9, *trans* 11-octadecadienoic acid (B).

Table 1.1 gives an overview of the different forms of PUFA nomenclature, highlighting common PUFA by systematic name, trivial or common name and by shorthand notation.

Table 1.1. Nomenclature of some common polyunsaturated fatty acids (PUFA). Each fatty acid is denoted by its systematic name, trivial name and shorthand notation

Systematic name	Trivial name	Shorthand notation
<i>cis</i> 9, <i>trans</i> 11- Octadecadienoic acid	Conjugated linoleic acid (CLA)	18:2 n -7
All- <i>cis</i> 5,8,11,14- Eicosatetraenoic acid	Arachidonic (AA)	20:4 n -6
All- <i>cis</i> 5,8,11,14,17- Eicosapentaenoic acid	Eicosapentaenoic (EPA)	20:5 n -3
All- <i>cis</i> 4,7,10,13,16,19- Docosahexaenoic acid	Docosahexaenoic (DHA)	22:6 n -3

1.1.1 Fatty acid biosynthesis

The first step towards *de novo* fatty acid biosynthesis involves activation of acetyl-CoA to its CoA thiolester, malonyl-CoA. This activation step is dependent upon the enzyme acetyl-CoA carboxylase (ACC). The malonyl-CoA generated by ACC forms the source of nearly all of the carbons of the fatty acyl chain. In most cases, only the first two carbon atoms of the chain arise from a different source, namely acetyl-CoA, which is termed the primer molecule. The chain then grows through a cyclical reaction pathway catalysed by a group of enzymes termed fatty acid synthase (FAS). Each complete cycle increases the chain length by 2C units (Gurr *et al.*, 2002, Bloch and Vance, 1977).

Although acetyl-CoA is a common primer molecule the other major primer found in mammalian tissue is butyryl-CoA (Singh *et al.*, 1984). The use of propionyl-CoA or branched primers permits the formation of odd chain lengths or branch chain fatty acids respectively (Gurr *et al.*, 2002).

The elongation of acyl chains is dependent upon FAS and this enzyme functions best on those chains longer than 4C units. From this point elongation will rapidly proceed to an acyl chain 16C units in length. Chains of 16C or above are not easily elongated possibly as a result of steric hindrance. The typical end product of animal FAS enzymes is thus palmitic acid (PA; 16:0). Further elongation of PA is then determined by a separate class of enzymes termed the elongases (Gurr *et al.*, 2002).

1.1.1.1 Polyunsaturated fatty acid biosynthesis

PUFA synthesised by animals are found to contain double bonds at the $\Delta 9$, $\Delta 6$, $\Delta 5$ and $\Delta 4$ positions. Thus, animals have been shown to express desaturase enzymes corresponding to these positions. *De novo* synthesis primarily yields PA which may then be elongated in the endoplasmic reticulum to stearic acid (SA; 18:0). The action of $\Delta 9$ desaturase on PA yields the *n*-7 fatty acid palmitoleic acid (16:1*n*-7). Subsequent desaturation and elongation of SA gives rise to the *n*-9 family of PUFA.

Whilst animals are able to form some PUFA *de novo* they cannot synthesise linoleic acid (LA; 18:2*n*-6) or α -linolenic acid (ALA; 18:3*n*-3). Animals lack the enzymes Δ^{12} desaturase and Δ^{15} desaturase and so cannot introduce double bonds at these positions. Thus, LA and ALA are termed essential fatty acids as they can only be derived in animals from the dietary intake of plants (Gurr *et al.*, 2002).

Essential fatty acids can be elongated or further desaturated by animal cells to form distinct families of PUFA. LA can be desaturated to form γ -linolenic acid (GLA; 18:3 n -6) which is then elongated to form dihomo- γ -linolenic acid (DGLA; 20:3 n -6). Sequential desaturation and elongation of LA yields the n -6 family of PUFA which includes arachidonic acid (AA; 20:4 n -6). In contrast, the consumption of ALA results in the synthesis of the n -3 family of PUFA. Thus ALA can be desaturated and elongated to yield EPA, docosapentaenoic acid (DPA; 22:5 n -3) and DHA.

PUFA can be shortened through β oxidation to reverse the elongation pathway (Luthria *et al.*, 1996, Mohammed *et al.*, 1997). Thus, the Sprecher pathway outlines an alternative route to the synthesis of DHA. According to this pathway, DPA (22:5 n -3) is first elongated to yield 24:5 n -3. This fatty acid is then desaturated at the Δ 6 position and chain shortened by β oxidation in the peroxisome resulting in the formation of DHA. Thus, β oxidation can result in the retroconversion of PUFA (Rosenthal *et al.*, 1991).

The principle of retroconversion can be applied to other PUFA resulting in the formation of chain shortened fatty acids. Thus, it has been shown that the n -3 isomer of DPA (22:5 n -3) can be shortened in the peroxisome to yield EPA and that 24:5 n -6 can be shortened to yield the n -6 isomer of DPA (22:5 n -6) (Benistant *et al.*, 1996, Brossard *et al.*, 1996). Figure 1.2 provides a summary of PUFA biosynthesis.

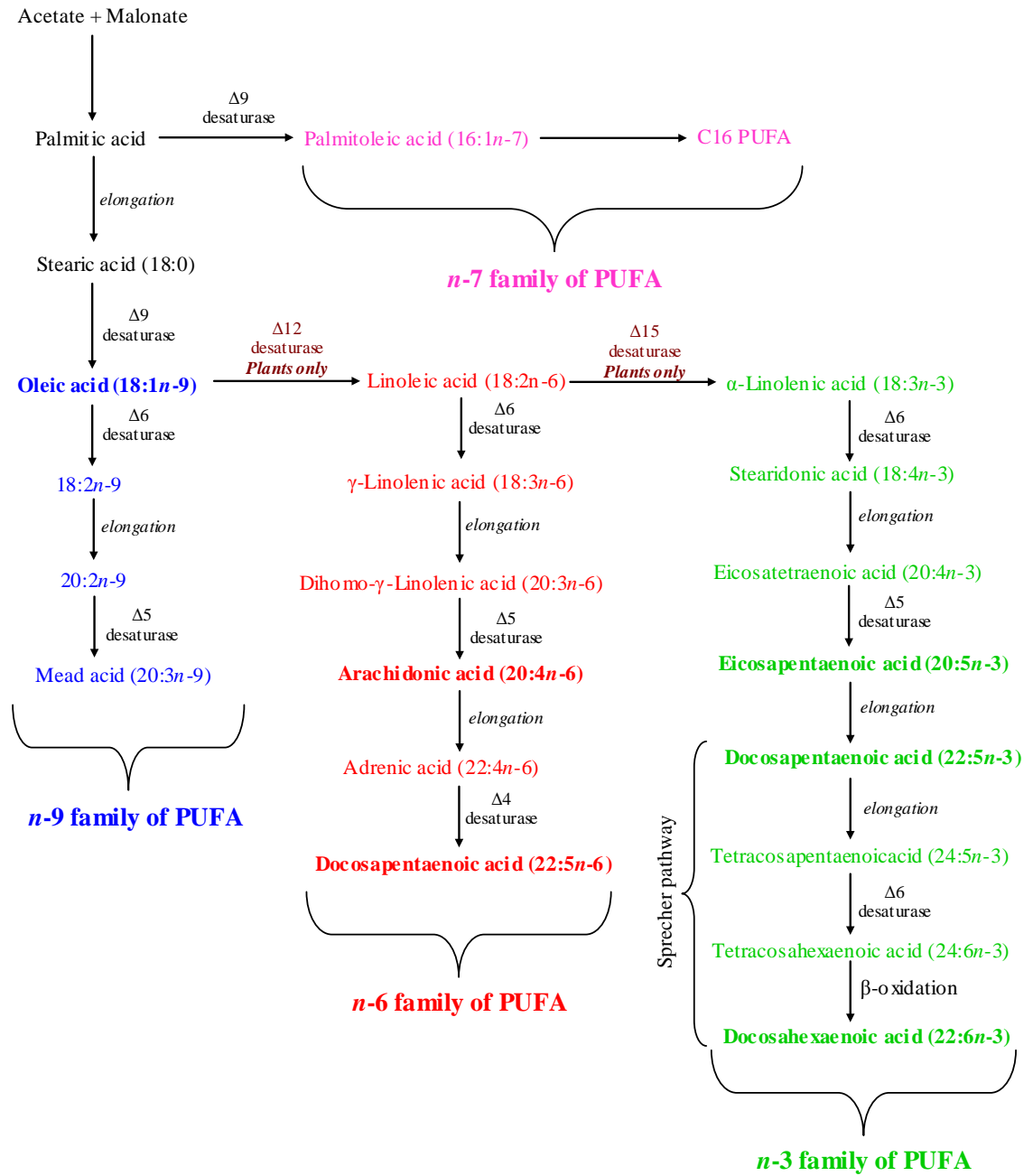


Figure 1.2. Biosynthesis of the different families of polyunsaturated fatty acids (PUFA) (adapted from (Nicolaou and Kokotos, 2004)).

1.2 Eicosanoids and other bioactive lipid mediators

The eicosanoids are biological oxidation products of C20 PUFA including AA, DGLA and EPA which give rise to a wide variety of products of remarkable physiological activity (Beare-Rogers *et al.*, 2001). Enzymatic oxidation by cellular cyclooxygenase (COX) yields cyclic endoperoxides from which prostaglandins, thromboxanes and prostacyclin can be synthesised.

Lipoxygenase (LOX) will catalyse synthesis of hydroxy fatty acids such as the hydroxyeicosatetraenoic acids, hydroxyeicosapentaenoic acids, leukotrienes and lipoxins from C20 PUFA. Cytochrome P450 (CYP450) oxygenation of PUFA will result in the formation of hydroxy fatty acids and fatty acid epoxides.

Other bioactive lipid mediators include the hydroxyoctadecadienoic acids derived from LA which is an 18C PUFA. EPA can be metabolised to form the newly discovered resolution phase interaction products or resolvins. DHA can also be converted to neuroprotectins. Metabolites of the eicosanoids and other bioactive lipid mediators include the isoprostanes and dihydro-isoprostanes.

1.2.1 Prostanoids

The prostanoids are a potent group of bioactive lipid mediators that include the prostacyclines, thromboxanes and prostaglandins. Prostanoids are intricately involved with the inflammatory process and regulate many physiological processes.

Prostanoids can only be formed from free fatty acids. Fatty acids are liberated mainly from phospholipids by the actions of a group of enzymes collectively termed phospholipases A₂ (PLA₂).

1.2.1.1 Prostanoid structure and nomenclature

Structurally prostaglandins are unsaturated carboxylic acids, consisting of a 20C skeleton that also contains a five member ring. Each prostaglandin is named using the prefix PG followed by a letter (A-K) indicating the nature and position of substituents on the cyclopentanone ring. Thromboxane A (TXA) contains an unstable bicyclic oxygenated ring structure, while thromboxane B (TXB) has a stable oxane ring (Hamberg *et al.*, 1975). Prostacyclin (PGI) has an oxygen bridge between C6 and C9 and is structurally unstable in biological systems being transformed to the stable metabolite 6 keto-PGF_{1 α} (Whittaker *et al.*, 1976).

The number of double bonds on the acyl chain of each prostaglandin is determined by the precursor PUFA. Prostaglandins derived from DGLA have only one double bond in their acyl chain and are collectively known as prostaglandins of the 1-series. Prostaglandins of the 2-series are derived from AA and have two double bonds within their acyl chain. Prostaglandins derived from EPA contain three double bond within their acyl chain and belong to the 3-series. Figure 1.3 outlines the structures of prostaglandins E and F of all three series and their precursor PUFA.

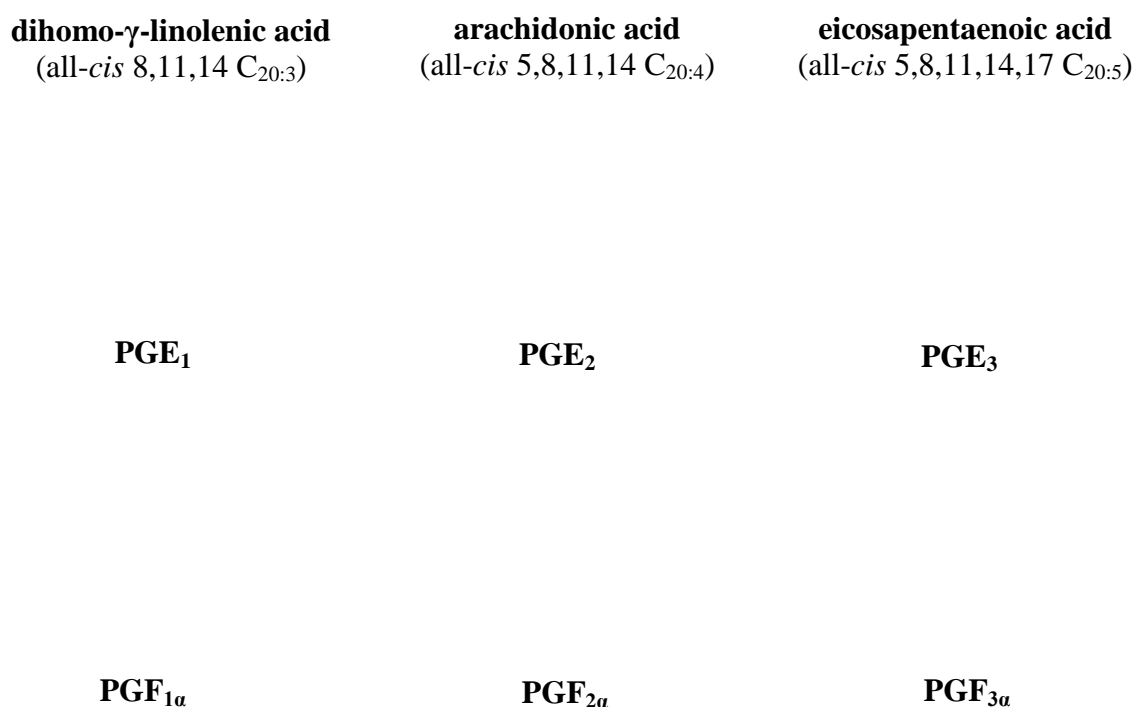


Figure 1.3. Structures of prostaglandins E and F of the 1-, 2- and 3- series and their precursor polyunsaturated fatty acids (adapted from (Nicolaou and Kokotos, 2004)).

1.2.1.2 Phospholipase A₂

In general, PLA₂ activities are broadly divided into three types, secretory Ca²⁺-dependent (sPLA₂), cytosolic Ca²⁺-dependent (cPLA₂) and cytosolic Ca²⁺-independent (iPLA₂) (Dennis, 1997, Six and Dennis, 2000). Cytosolic cPLA₂ consists of three isozymes that exhibit significant selectivity for AA containing phospholipids. Experiments with cPLA₂ knockout mice have confirmed the importance of these enzymes to eicosanoid production (Sapirstein and Bonventre, 2000, Uozumi and Shimizu, 2002).

The iPLA₂ enzymes are not thought to be selective for arachidonate containing phospholipids. These enzymes are thought to play a role in lipid membrane remodelling. There are studies that suggest that AA is liberated from different cellular pools, thus by regulating fatty acid remodelling iPLA₂ may regulate AA availability (Winstead *et al.*, 2000).

The secreted sPLA₂ are a large family of enzymes that hydrolyse fatty acids without showing any specificity for AA. These enzymes regulate many physiological functions including digestion of dietary phospholipids and regulation of the immune response. It has recently been shown that sPLA₂ enzymes play a key role during inflammation where AA is liberated (Balestrieri and Arm, 2006, Lambeau and Gelb, 2008).

Overall it is thought that there is significant interplay between the different PLA₂ variants. It is known for example that sPLA₂ enzymes significantly amplify the actions of cPLA₂. Moreover, phospholipid remodelling by iPLA₂ may make AA more readily available to the other PLA₂ enzymes (Balsinde *et al.*, 2002, Six and Dennis, 2000, Balestrieri and Arm, 2006). The role of PLA₂ enzymes in the liberation of fatty acids is fundamental to the subsequent synthesis of eicosanoids. Manipulation of these enzymes could have significant downstream effects by modulating eicosanoid formation. This makes these enzymes a desirable target for pharmacological intervention (Laye and Gill, 2003, Zalewski *et al.*, 2005).

1.2.1.3 Cyclooxygenase

The cyclooxygenases are a group of isozymes that convert liberated C20 PUFA to the cyclic endoperoxide PGH₂ which is then subsequently converted into the prostanoids.

COX can also oxygenate other PUFA including C18 PUFA such as linoleate and γ -linolenate (Kulmacz *et al.*, 2003).

Initially, the cyclooxygenase activity of the enzyme inserts molecular oxygen into the PUFA chain to yield a cyclopentane ring known as PGG₂. Subsequent peroxidase activity of the enzyme reduces PGG₂ to its 15 hydroxy analogue PGH₂. Thus the COX enzyme is composed of two active sites, a cyclooxygenase site and a heme with peroxidase activity (Gurr *et al.*, 2002, Kulmacz *et al.*, 2003).

To date only three variants of the COX enzyme have been identified and characterised. COX-1 is constitutively expressed in many mammalian cells and tissues and is thought to be involved in the general regulation of physiological events. COX-2 is present at low basal levels in inflammatory cells and is strongly induced by inflammatory stimuli (Gurr *et al.*, 2002). The abundance and activity of COX-3 is at present unclear but it is thought that this isozyme is also involved with inflammation. COX-3 may also play a role in pain perception as drugs such as acetaminophen have been shown to inhibit this isozyme without significantly affecting levels of COX-1 or COX-2 (Chandrasekharan *et al.*, 2002, Flower and Vane, 1972).

1.2.1.4 Prostanoid synthases

Cyclic endoperoxide, PGH_2 , is transformed to the individual prostanoids by a series of specific prostanoid synthases as shown in Figure 1.4. In the central nervous system, lipocalin type prostaglandin D synthase (PGDS) catalyses the isomerisation of PGH_2 to PGD_2 (Urade and Eguchi, 2002). Prostaglandin E synthase (PGES) isomerises PGH_2 to PGE_2 (Samuelsson *et al.*, 2007).

$\text{PGF}_{2\alpha}$ can be synthesised from either PGE_2 , PGD_2 or PGH_2 and has four potential stereoisomers. PGE 9-ketoreductase catalyses the conversion of PGE_2 to $\text{PGF}_{2\alpha}$ and PGD 11-ketoreductase converts PGD_2 to $\text{PGF}_{2\alpha}$. PGH 9, 11-endoperoxide reductase catalyses the conversion of PGH_2 to $\text{PGF}_{2\alpha}$ (Suzuki-Yamamoto *et al.*, 1999).

Prostacyclin synthase (PGIS) converts PGH_2 to PGI_2 which is then non-enzymatically hydrolysed to the stable metabolite 6-keto $\text{PGF}_{1\alpha}$ (Weksler *et al.*, 1977). Thromboxane A synthase (TXAS) catalyses the formation of TXA_2 which then breaks down to the stable but inactive TXB_2 (Ullrich *et al.*, 2001).

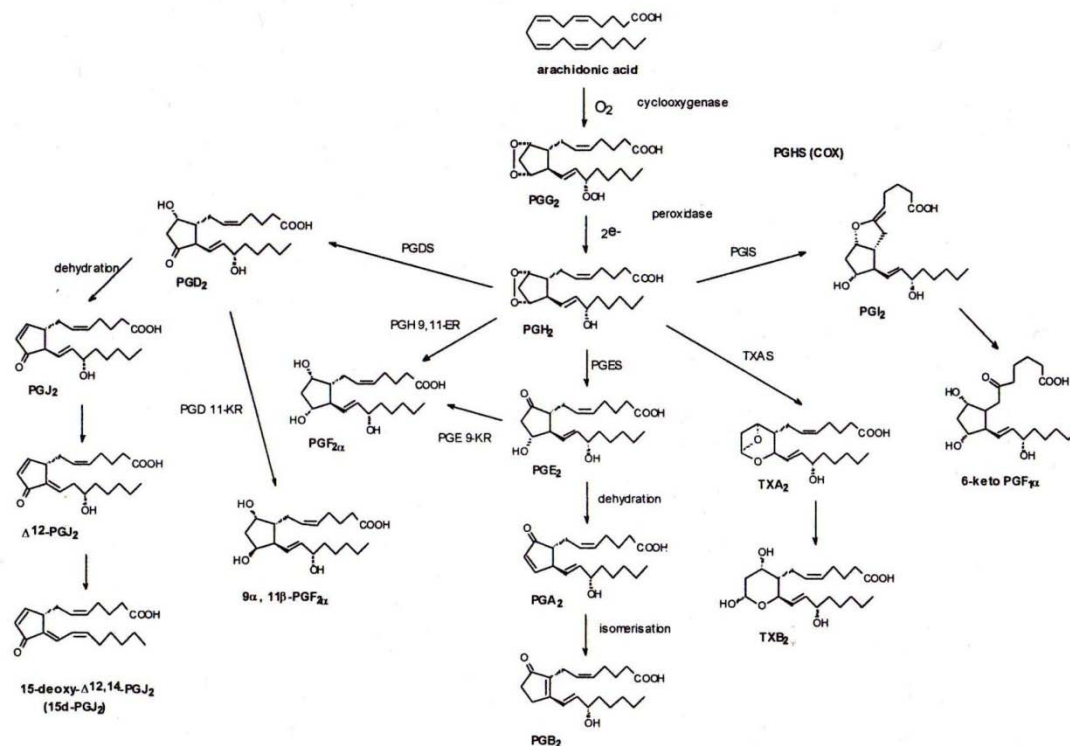


Figure 1.4. Conversion of arachidonic acid to various prostanoids via the cyclooxygenase (COX) pathway. PGHS, prostaglandin endoperoxide H synthase. PGDS, prostaglandin D synthase. PGES, prostaglandin E synthase. PGH, 9, 11-ER, prostaglandin H 9, 11-endoperoxide reductase. PGE 9-KR, prostaglandin E 9-ketoreductase. PGD 11-KR, prostaglandin D 11-ketoreductase. TXAS, thromboxane A synthase. PGIS, prostacycline synthase. (Adapted from Nicolaou and Kokotos, 2004).

1.2.1.5 Prostanoid receptors

Prostanoids are ligands of specific G-protein coupled receptors. In terms of structure, they have seven transmembrane domains typical of G protein coupled receptors (Narumiya *et al.*, 1999). The receptors are designated DP for PGD; EP₁, EP₂, EP₃ and EP₄ for PGE; FP for PGF; TP for thromboxane; and IP for prostacycline (Breyer and

Breyer, 2001, Breyer *et al.*, 2001). Whilst each prostanoid has greatest affinity for its own receptor there is considerable cross reactivity. The agonist affinity for DP for example is $\text{PGD}_2 \gg \text{PGE}_2 \gg \text{PGF}_{2\alpha}$ hence DP agonists can mediate a variety of physiological effects (Breyer *et al.*, 2001, Narumiya and Fitzgerald, 2001).

1.2.1.6 Prostanoid catabolism

Prostanoids are locally acting and considered to be formed or inactivated within the same or neighbouring cells prior to release in circulation as inactive metabolites. Essentially there are two enzymes responsible for the inactivation of prostanoids. The primary catabolic pathway involves oxidation of the 15(*S*)-hydroxyl group by 15-hydroxyprostaglandin dehydrogenase followed by reduction of the Δ^{13} double bond by Δ^{13} -15-ketoprostaglandin reductase. The resulting 15-keto and 13, 14-dihydro keto metabolites have greatly reduced biological activity. A separate catabolic pathway for thromboxane involves the oxidation of TXB_2 at C11 by 11-hydroxythromboxane B_2 dehydrogenase (Tai *et al.*, 2002).

1.2.2 Isoprostanes

Isoprostanes are a group of prostaglandin-like compounds that are synthesised by free radical catalysed peroxidation of AA and other PUFA. Unlike prostaglandins, isoprostanes do not require COX for their formation (Morrow *et al.*, 1992a). An important structural distinction between isoprostanes and prostaglandins is that the former contains side chains predominantly in the *cis* configuration whereas the latter contains side chains predominantly in the *trans* configuration (Morrow *et al.*, 1990).

Lipid peroxidation of AA will give rise to a family of PGF₂-isoprostanes. The F₂-isoprostanes are a group of 64 compounds isomeric in structure to COX derived PGF_{2α} (Montuschi *et al.*, 2004). Other products of the isoprostane pathway are also formed by rearrangement of labile PGH₂ like isoprostane intermediates. These include the E₂- and D₂- isoprostanes and the cyclopentenone A₂ and J₂ isoprostanes (Kavirasan *et al.*, 2009). Figure 1.5 describes the synthesis of different families of isoprostanes. The 15-series isoprostanes are commonly and frequently referred to as 8-iso-PGF_{2α}.

Isoprostanes have potent biological activity and are thought to mediate oxidative injury. 8-iso-PGF_{2α} and 8-iso-PGE₂ are the most abundant isoprostanes and function as vasoconstrictors. Isoprostanes have also been shown to affect renal function where they decrease blood flow and reduce the glomerular filtration rate (Imig, 2000). Isoprostane levels have been shown to increase in a number of disorders associated with oxidative stress. It has been found that levels of isoprostane in the body fluctuate on a daily basis and that these mediators are implicated in inflammation, cardiovascular disease, diabetes, pulmonary disease and neurological disorders (Hoffman *et al.*, 1997, Janssen, 2004, Kavirasan *et al.*, 2009).

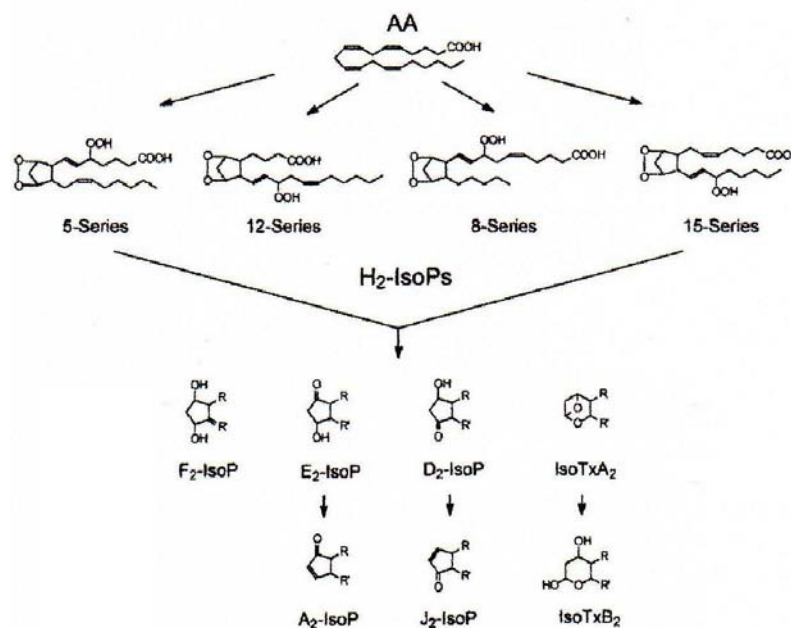


Figure 1.5. Lipid peroxidation of arachidonic acid (AA) forms four PGH₂ like intermediate regioisomers. These are reduced to four F ring regioisomers. Rearrangement can give rise to isoprostanes (IsoP) of different series (adapted from (Montuschi *et al.*, 2004)).

1.2.3 The biological activity of prostanoids

Prostanoids have been shown to be ubiquitously present within the body where they are thought to regulate many of its homeostatic functions. In the lung PGE₂ may act as a bronchodilator (Miller, 2006). TXB₂ has been implicated as having a major role to play in disease states such as asthma where this prostanoid is thought to induce constriction of the airway (Arakida *et al.*, 1999).

PGD₂ is thought to protect against excitotoxic injury (Liang *et al.*, 2005b). PGD₂ will inhibit platelet aggregation and relax vascular smooth muscle (Giles and Leff, 1988).

Prostacyclin is a vasodilator and also has anti-aggregator effects. It is the most powerful anti-aggregator known and has physiological effects directly opposite to those of TXB₂ (Youdim *et al.*, 2000, Hoffbrand *et al.*, 2005).

The prostanoids play various roles in the digestive system. PGE₂ regulates many physiological functions of the gut including mucosal protection, gastrointestinal secretion and motility, it is implicated in the pathophysiology of inflammatory bowel disease and colorectal neoplasia (Dey *et al.*, 2006).

The prostanoids are known to play a fundamental role in reproductive biology. During parturition prostanoids cause myometrial contraction and have been used to induce labor. Prostaglandin synthase inhibitors have conversely been used to delay delivery in preterm labor (Slater *et al.*, 2002). Prostanoids are also essential for the normal development of the foetus. Towards the latter stages of pregnancy prostanoids play a pivotal role in the formation of normal heart tissue. Prostanoids are also fundamental to gametogenesis and play a role in fertilisation (Richards, 2005).

1.2.4 Leukotrienes and hydroxy fatty acids

Leukotrienes and hydroxy fatty acids are produced through the actions of all LOX isoforms on C18 or C20 PUFA. Further enzyme action can yield tri hydroxy and epoxy metabolites each with distinct pharmacological properties.

1.2.4.1 The lipoxygenases

Lipoxygenases are non-heme, iron containing dioxygenase enzymes that recognise a 1-*cis*, 4-*cis* pentadiene structure of PUFA and incorporate one molecule of oxygen to produce hydroperoxy acids (Yamamoto *et al.*, 2005). The first discovered isoform of lipoxygenase was 12-LOX. To date there are four known types of human 12-LOX. These include platelet type 12(*S*)-LOX, leukocyte type 12(*S*)-LOX, epidermis type 12(*S*)-LOX and 12(*R*)-LOX (Burger *et al.*, 2000, Sun *et al.*, 1998). Whilst all types of 12-LOX oxygenate C20 PUFA such as AA they yield isomers with different stereochemical properties.

Since the discovery of 12-LOX other isozymes of LOX including 5-LOX and 15-LOX have also been found (Manev *et al.*, 2000, Turk *et al.*, 1982). 8-LOX has been identified in mouse epidermis (Furstenberger *et al.*, 2002). There are currently two known types of human 15-LOX, epithelial 15-LOX and reticulocyte 15-LOX (Deschamps *et al.*, 2007, Brash *et al.*, 1997). Whilst 12-LOX is fundamental to the platelet it has been found that 5-LOX is essential for leukotriene (LT) synthesis and is therefore necessary for a robust immune system (Samuelsson *et al.*, 1987).

Although 5-LOX is a member of the arachidonate lipoxygenase family, it differs in certain critical aspects from other members of the same family, in that it requires interaction with a specific activating protein, 5-lipoxygenase activating protein (FLAP) in order to initiate oxygenation of AA in cells (Dixon *et al.*, 1990, Martel-Pelletier *et al.*, 2004). The exact role of FLAP in 5-LOX activity is not yet fully understood (Miller *et al.*, 1990).

1.2.4.2 Lipoxygenase derived mediators

Addition of molecular oxygen to AA through LOX forms hydroperoxy eicosatetraenoic acid (HpETE) (Fruteau De Lacos *et al.*, 1987). HpETE can then be reduced to form hydroxyeicosatetraenoic acid (HETE) or undergo further LOX activity to form diHETE (Yamamoto *et al.*, 1997). Figure 1.6 outlines the products of AA LOX metabolism.

LTA₄ is an epoxy fatty acid formed via a double catalytic step involving 5-LOX. LTA₄ is highly unstable and is quickly hydrolysed by LTA₄ hydrolase (also known as LTB₄ synthase) to LTB₄ (Jakschik and Kuo, 1983). Leukotrienes are not readily formed in the absence of FLAP due to their reliance on 5-LOX (Dixon *et al.*, 1990, Miller *et al.*, 1990). Lipoxins (LX) are trihydroxy-eicosatetraenoic acids formed through the action of 15-LOX on 15-HpETE. The resulting epoxy intermediate is hydrolysed by lipoxin B₄ hydrolase or lipoxin A₄ hydrolase to form lipoxin B₄ or lipoxin A₄ respectively (Serhan, 2005).

Figure 1.6. The conversion of arachidonic acid (AA) to hydroperoxyeicosatetraenoic acids (HpETE) hydroxyeicosatetraenoic acids (HETE) and lipoxins (LX) through lipoxygenases (LOX). Leukotrienes are formed by 5-LOX and 5-lipoxygenase activating protein (FLAP). (Adapted from (Nicolaou and Kokotos, 2004).

Whilst AA can undergo LOX metabolism to yield HpETE and HETE as well as leukotrienes (LT) and LX other PUFA can also undergo LOX metabolism. EPA and DHA will both readily undergo oxidative metabolism via LOX. EPA will be oxidised to hydroperoxy eicosapentaenoic acid (HpEPE) which metabolises to hydroxyeicosapentaenoic acids (HEPE) and trihydroxy derivatives termed “resolvins”

(Rv) of the E series. DHA is converted to neuroprotectins of the D series (PD1) via a hydroxyl intermediate or resolvins (RvD1) in the presence of aspirin (Serhan *et al.*, 2004). LA will be oxidised either by COX or LOX to isomers of hydroxyoctadecadaenoic acid (HODE) (Kaduce *et al.*, 1989, Reinaud *et al.*, 1989).

1.2.4.3 Resolvins and protectins

Resolvins (resolution phase interaction products) are a novel class of lipid mediators associated with the anti-inflammatory actions of EPA (Serhan, 2004, Serhan *et al.*, 2004). These mediators are mainly found to be synthesised from EPA or DHA in the presence of aspirin. The various resolvins and protectins formed from AA are outlined in Figure 1.7.

The synthesis of EPA derived resolvins depends upon the stereochemistry of the intermediate metabolite 18-HEPE. Resolvins of the E series can only form from 18(*R*)-HEPE. Although this isomer is produced naturally it has been found that aspirin is able to acetylate COX leading to a two fold increase in synthesis of 18(*R*)-HEPE (Serhan *et al.*, 2000). Subsequent hydroxylation of 18*R*-HEPE by 5-LOX in leukocytes yields the intermediate 5*S* hydroperoxy 18*R* hydroxy EPE which is then reduced to form resolvin E2 (RvE2) or converted to resolvin E1 (RvE1) through further action of 5-LOX (Serhan and Chiang, 2008).

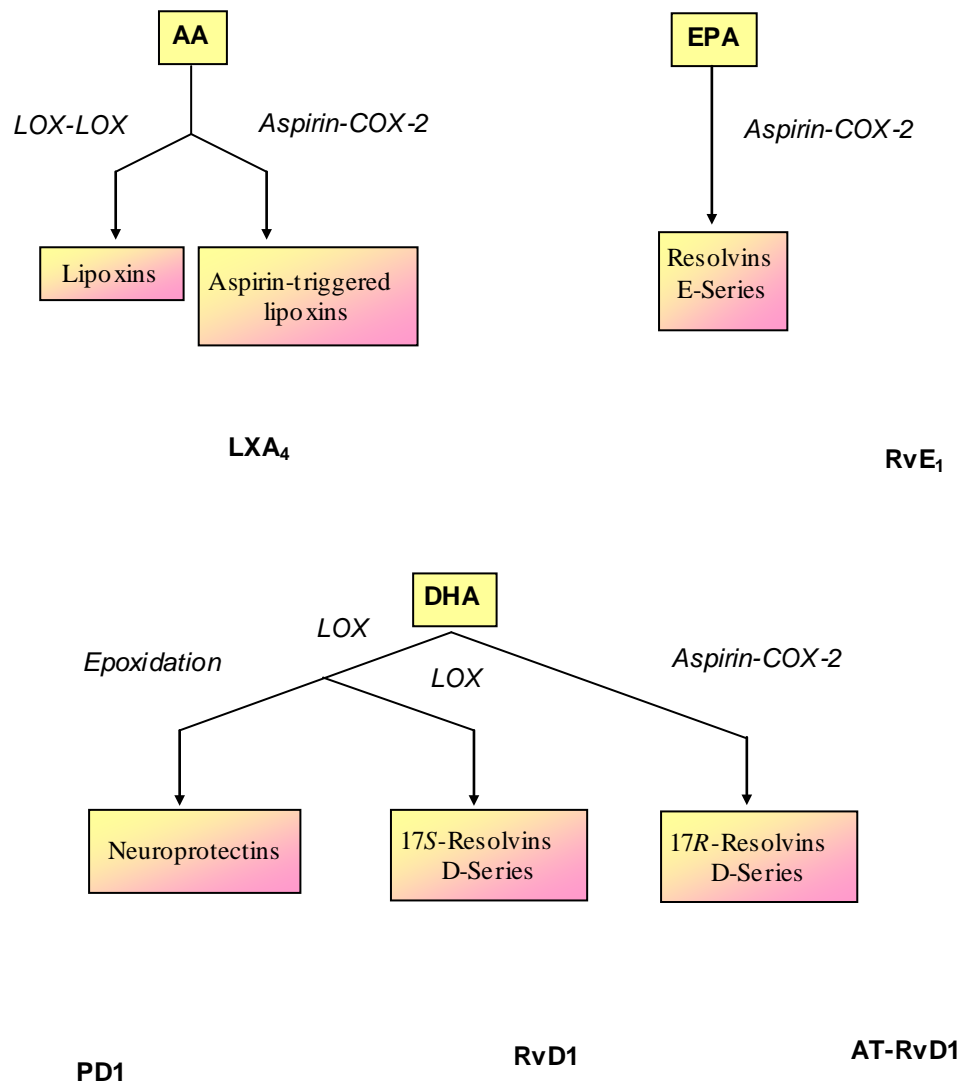


Figure 1.7. Biosynthetic routes for lipoxins, resolvins and protectins. The key enzymes (aspirin-acetylated COX-2 and Lipoxygenase (LOX)) are indicated to emphasize relationships between the pathways that use arachidonic acid (AA), eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) to generate these mediators. Structures of major representatives of each series, namely lipoxin A4 (LXA₄), resolvin E1 (RvE1), protectin (PD1), Resolvin D1 (RvD1) and aspirin triggered resolvin D1 (AT-RvD1), are illustrated (adapted from (Ariel and Serhan, 2007)).

Aspirin triggered epimers also readily form from DHA to give resolvins of the D series as shown in Figure 1.7. COX-2 converts DHA to 13-hydro(peroxy) DHA (13-HpDHA) but in the presence of aspirin this switches to 17*R*-oxygenation leading to the synthesis of 17-HpDHA and aspirin triggered RvD1. In the absence of aspirin DHA is converted through LOX initiated mechanisms to the 17*S* isomer of RvD1 (Hong *et al.*, 2003). The stereochemistry of both 17*R* and 17*S* resolvins D1 has been established and confirmed through total organic synthesis (Sun *et al.*, 2007b).

Endogenous DHA is also converted to a triene containing structure via LOX. The LOX product 17*S*-HpDHA is converted to an epoxide that is enzymatically converted to 10, 17*S*-docosatriene or neuroprotectin D1 (PD1) (Hong *et al.*, 2003). 17*S*-HpDHA can also be reduced to form 17*S* hydroxy DHA (17*S*-HDHA) and so this can be taken as a biomarker for the precursor of PD1.

1.2.5 Cytochrome P450 derived mediators

CYP450 enzymes are membrane bound heme containing oxidases that can operate as a third possible pathway of PUFA metabolism (Mcnaught and Wilkinson, 1997). The CYP450 super family of enzymes is expressed in the liver but several CYP enzymes can also be found in other tissues. This is one route through which LOX metabolites can be converted to HETE, leukotrienes or lipoxins (Elbekai and El-Kadi, 2006).

PUFA can serve as both substrates and inhibitors of the CYP450 family of enzymes. In inflamed tissue, where levels of free fatty acid are elevated, both AA and EPA are thought to potently inhibit CYP450 (Yao *et al.*, 2006). As with COX and LOX metabolism EPA will give rise to different oxygenated products as a result of CYP

metabolism when compared to AA and other PUFA (Fer *et al.*, 2008). A summary of the LOX and CYP450 derived oxidation products of PUFA is outlined in Figure 1.8. Figure 1.9 shows the structures of the different bioactive LOX and CYP450 derived metabolites of AA, EPA, DHA and LA.

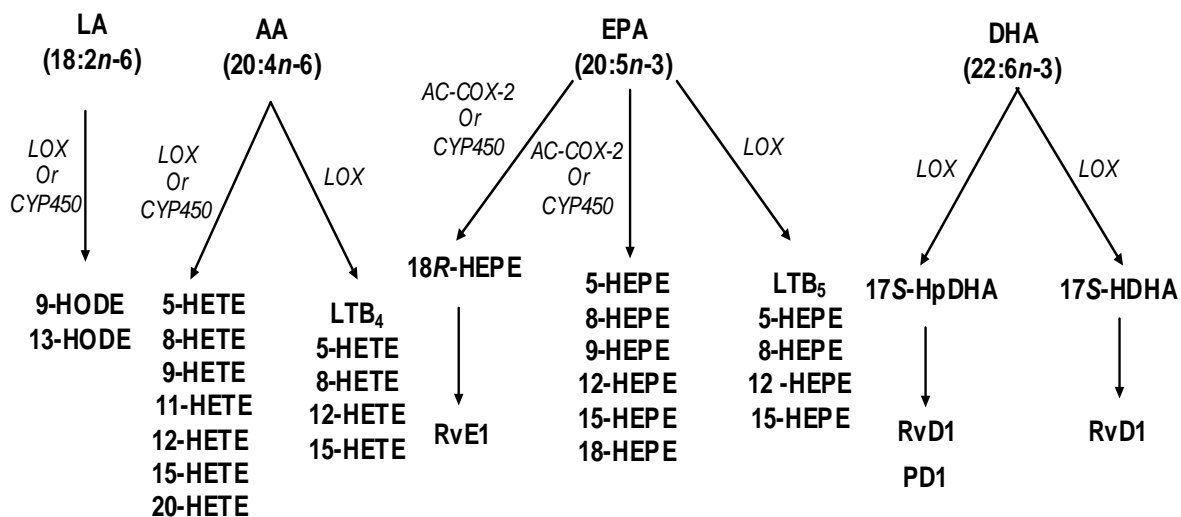


Figure 1.8. Schematic outline of hydroxy fatty acids, leukotrienes, resolvins RvE1 and RvD1, and protectin PD1 produced by linoleic acid (LA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) via lipoyxygenase (LOX), cytochrome P450 (CYP450), acetylated cyclooxygenase (AC-COX-2) or free radical catalysed pathways. (Adapted from (Masoodi *et al.*, 2008).

Figure 1.9. Structures of hydroxy fatty acids derived from (A) linoleic acid, (B) arachidonic acid, (C) eicosapentaenoic acid, (D) docosahexaenoic acid; hydroxyoctadecadienoic acid (HODE), hydroxyeicosatetraenoic acid (HETE), hydroxyeicosapentaenoic acid (HEPE), leukotriene (LT), resolvin (Rv), protectin (PD). (Adapted from (Masoodi *et al.*, 2008).

1.2.6 The biological activities of the hydroxy fatty acids

The LOX and CYP450 metabolites of PUFA exert a variety of effects upon surrounding tissues. Leukotrienes were first isolated from leukocytes where they were found to elicit chemotactic responses as well as initiate release of various inflammatory mediators (Ford-Hutchinson *et al.*, 1980a, Ford-Hutchinson *et al.*, 1980b, Samuelsson *et al.*, 1980, Smith *et al.*, 1980). Leukotrienes have also been found to constrict smooth muscle and play an active role in asthma (O'byrne, 1997).

The bioactivity of the HETE isomers is less clear than the other hydroxy metabolites. It is known that 12 HETE plays a role in the development of many cancers and that this hydroxy fatty acid also has a vaso constrictive effect and has been implicated in cardiovascular and renal disease (Honn *et al.*, 1994, Yiu *et al.*, 2003). The complex interactions of these mediators are reflected by the fact that they can display both pro-inflammatory and anti-inflammatory effects. Their actions are different in vitro compared to in vivo and they may have pharmacologically opposite effects in different animal species (Wittwer and Hersberger, 2007).

HODE are derived from the actions of COX/LOX or CYP450 on linoleic acid and are thought to have vasorelaxant properties working in balance with the constrictive effects of PGF_{2α} (Pomposiello *et al.*, 1998, Furstenberger *et al.*, 2002). 9 and 13-HODE are also commonly used as markers for oxidative stress (Yoshida *et al.*, 2006).

Resolvins modulate inflammation and cell mediated injury. Resolvins are likely to play roles in central and peripheral tissues and are involved in physiological and pathological processes (Serhan, 2004, Serhan *et al.*, 2004). It is known that DHA is metabolised to

10,17*S*-docosatrienes and 17*S*-resolvins and that EPA can form resolvins of the E series. The bioactive metabolites of DHA are called docosanoids. Docosanoids have neuroprotective effects and may be called neuroprotectins. They are involved not only in modulating the effects of eicosanoids but also in regulating both leukocyte trafficking and down-regulation of cytokines.

1.3 The Endocannabinoids

The class of cannabinoids includes all those compounds that are structurally related to tetrahydrocannabinol (THC), the main psychoactive substance found in the cannabis plant. Biologically, cannabinoids are described as those compounds that are natural ligands of the cannabinoid receptors. The class of endocannabinoids includes only those cannabinoids that are naturally produced within the body. The first endocannabinoids to be discovered were the fatty acid ethanolamide arachidonyl ethanolamide or anandamide (A-EA) and 2-arachidonyl glycerol (2-AG), isolated from brain (Devane *et al.*, 1992, Sugiura *et al.*, 1995). Other identified endocannabinoids include 2-arachidonyl glyceryl ether (noladin ether) and arachidonoyl ethanolamine (virodhamine) (Porter *et al.*, 2002).

1.3.1 The cannabinoid receptors

To date, two types of cannabinoid receptor have been identified, cloned and characterised. The type 1 receptor (CB1) was cloned in 1990 (Gerard *et al.*, 1991) and the type 2 receptor (CB2) was cloned in 1993 (Munro *et al.*, 1993). The cannabinoid receptors are coupled to a G protein complex and are composed of hydrophobic transmembrane segments connected by alternating extracellular and intracellular loops

(Bramblett *et al.*, 1995, Onaivi *et al.*, 1996). Research is currently underway to determine the presence of other subtypes of cannabinoid receptor. CB1 and CB2 receptors have been found in many mammals (Ong and Mackie, 1999, Pertwee, 1997).

The CB1 receptors are primarily found in the brain (Herkenham *et al.*, 1990) and adipose tissue (Bensaid *et al.*, 2003) but are also found in the myocardium (Bonz *et al.*, 2003), vascular endothelium (Liu *et al.*, 2000) and sympathetic nerve terminals (Gelfand and Cannon, 2006, Ishac *et al.*, 1996).

Conversely, CB2 receptors have been found mainly in peripheral tissues and particularly in immune cells (Munro *et al.*, 1993). Recent work has also demonstrated the presence of the CB2 receptor in central nervous tissue where it is thought they may control cell proliferation. Unlike CB1 ligands, the ligands of CB2 receptors in the brain are not psychoactive (Ashton and Glass, 2007, Fernandez-Ruiz *et al.*, 2007).

A-EA is a natural ligand of both the CB1 and CB2 receptors (Felder *et al.*, 1993). 2-AG is also a known ligand of both these receptors although it exhibits lower affinity for CB1 than A-EA (Sugiura and Waku, 2000). Noladin ether has much higher affinity for CB1 than CB2 receptors (Hanus *et al.*, 2001). virodhamine is a full agonist of the CB2 receptor but a partial agonist of the CB1 receptor (Porter *et al.*, 2002).

1.3.2 Other endocannabinoid receptors

Apart from the cannabinoid receptors A-EA is a known ligand of the vanilloid receptor (TRPV1), the peroxisome proliferator-activated receptor α (PPAR α) (Sun *et al.*, 2007a) and the peroxisome proliferator-activated receptor γ (PPAR γ) (Bouaboula *et al.*, 2005).

TRPV1 can be found throughout the brain and is known to be involved in the pain pathway (Toth *et al.*, 2005). It is thought that many of the neuroprotective actions of A-EA may be mediated through the TRPV1 receptor (Veldhuis *et al.*, 2003). The PPARs are ligand activated transcription factors that have been described in the brain (Moreno *et al.*, 2004). PPARs in the brain have been implicated in neural cell differentiation and death as well as in inflammation and neurodegeneration (Cimini *et al.*, 2005).

1.3.3 Biosynthesis of the fatty acid ethanolamides

Structurally A-EA is an *N*-acylethanolamide (NAE) or fatty acid amide thought to be primarily derived in the brain from an ethanolamine phospholipid such as phosphatidylethanolamine via an acyl transferase step in response to increased calcium concentration (Hansen *et al.*, 2000, Di Marzo *et al.*, 1994). *N*-acyltransferase transfers PUFA from the sn-1 position of a donor 1-*O*-acyl phospholipid, such as phosphatidylcholine, to the ethanolamine head group of phosphatidylethanolamine resulting in the synthesis of *N*-acylphosphatidylethanolamine (NAPE). NAE is then generated from the hydrolysis of NAPE through the action of *N*-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) (Di Marzo *et al.*, 1994). This route of NAE synthesis is known as the transacylation phosphodiesterase pathway and is summarised in Figure 1.10.

Another pathway for the synthesis of NAE was discovered following the knockout of NAPE-PLD in mice (Leung *et al.*, 2006). The enzyme α/β -hydrolase 4 (Abh4) has been identified as a NAPE selective hydrolase that will de-acylate NAPE to generate a glycerophosphate NAE intermediate which is then rapidly cleaved to release the corresponding NAE (Simon and Cravatt, 2006).

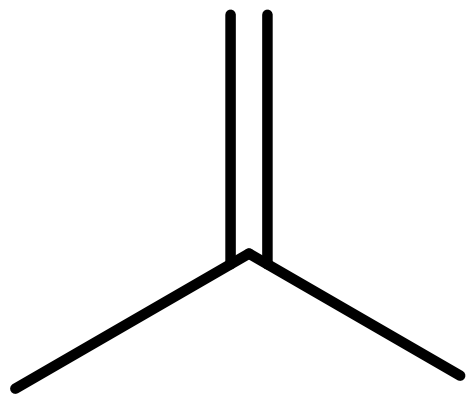


Figure 1.10. The transacylation phosphodiesterase pathway in the synthesis of anandamide. (Adapted from (Nicolaou and Kokotos, 2004).

1.3.4 Metabolism of fatty acid ethanolamides

In vivo A-EA displays only weak and transient cannabinoid properties and is quickly metabolised (Willoughby *et al.*, 1997). Enzymatic action by fatty acid amide hydrolase (FAAH) metabolises A-EA to AA and ethanolamine; this enzyme has the same effect on other NAEs metabolising them to the corresponding parent fatty acid and ethanolamine (Ueda *et al.*, 2000). It has been shown that mice lacking FAAH are severely impaired in their ability to metabolise A-EA and they exhibit a 15 fold augmented level of endogenous brain A-EA (Cravatt *et al.*, 2001). FAAH is distributed in several brain regions (Hillard *et al.*, 1995) as well as other organs such as the liver, kidney, lung, spleen and heart (Schmid *et al.*, 1983, Ueda *et al.*, 2000). FAAH is a principle regulator of A-EA and other NAE and is therefore a key player in the endocannabinoid system.

To date two variants of FAAH have been identified in humans. These are termed FAAH-1 and FAAH-2 respectively. These enzymes share 20% sequence identity but FAAH-1 is much more efficacious in the metabolism of NAEs compared to FAAH-2 (Wei *et al.*, 2006). Compared to other fatty acid ethanolamides A-EA has been shown to be the preferred substrate for this enzyme (Kurahashi *et al.*, 1997, Lang *et al.*, 1999). Oleamide hydrolysis by the enzyme was found to be at 31-78% the rate for A-EA hydrolysis (Ueda *et al.*, 2000). The hydrolytic rate for FAAH was found to be in the order A-EA>oleamide>myristamide>palmitamide (Ueda *et al.*, 2000).

Apart from undergoing hydrolysis A-EA and other NAEs can also act as substrates for COX and LOX. Structural studies have confirmed that the hydroxyl group on A-EA and other NAEs, is a key requirement for cyclooxygenation (Kozak *et al.*, 2003). It has

been reported that human recombinant COX-2 but not COX-1 can oxygenate A-EA in a manner analogous to that seen with AA to produce a novel group of compounds collectively termed prostamides (Yu *et al.*, 1997).

1.3.5 Biological actions of anandamide and other fatty acid ethanolamides

A-EA was first isolated from porcine brain tissue in 1992 and was shown to have cannabimimetic properties (Devane *et al.*, 1992). It was found to induce analgesia, hypothermia, hypomotility and hypotension (Calignano *et al.*, 1998, Crawley *et al.*, 1993, Smith *et al.*, 1994). Moreover, A-EA also possessed pro-apoptotic and anti-tumorigenic properties (Maccarrone *et al.*, 2000, Pisanti *et al.*, 2007). Like the plant derived cannabinoids A-EA was found to stimulate both CB1 and CB2 receptors and thus became the first discovered endocannabinoid in mammalian brain.

Since the discovery of A-EA other NAEs have also been found in the brain and peripheral tissues. Oleamide, the fatty acid amide derivative of oleic acid, was identified and characterised in the cerebrospinal fluid of sleep deprived cats and was proposed to be a sleep inducer (Cravatt *et al.*, 1995). The ethanolamide derivative of docosahexaenoic acid (DHA-EA) has been discovered, although not characterised to the extent of A-EA (Farrell and Merkler, 2008). To date eicosapentaenoylethanolamide (EPA-EA), the ethanolamide derivative of EPA, has not been detected in rat brain. Figure 1.11 compares the structures of A-EA, DHA-EA and EPA-EA.

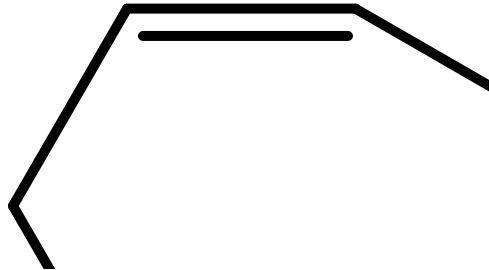


Figure 1.11. Structures of anandamide (A-EA), docosahexaenoyl ethanolamide (DHA-EA) and eicosapentaenoyl ethanolamide (EPA-EA).

1.4 Polyunsaturated fatty acids and brain function

The dry weight of an adult brain is 50% - 60% lipid with 35% of the lipid content being accounted for by PUFA (Yehuda *et al.*, 1999, Haag, 2003). DHA and AA are the most abundant PUFA in the mammalian central nervous system (CNS) and are specifically concentrated in the membrane lipids of brain gray matter (Innis, 2008). Changes in levels of brain PUFA have been linked to several pathologies including neurodegenerative disease (Lim *et al.*, 2005, Sharon *et al.*, 2003) and mood disorders (Green *et al.*, 2005). PUFAs are also essential for the normal growth and development of the human brain (Innis, 2008, Jamieson *et al.*, 1999, Martinez and Mougan, 1998).

1.4.1 The blood brain barrier

Unlike other vessels in the body, endothelial cells surrounding capillaries in the brain are not porous. The endothelial cells of brain capillaries are tightly bound preventing unregulated movement across the capillary wall. The brain cannot therefore obtain material from the plasma by a nonspecific filtering process. Instead, molecules within brain capillaries must be moved through the endothelial cells by diffusion and active transport, as well as by endocytosis and exocytosis (Aird, 1948). This regulated movement of material from plasma into brain tissue poses as a very selective blood brain barrier (BBB) where only specific molecules are permitted to enter the brain.

1.4.2 Bioactive lipid mediators in the brain

The eicosanoids and hydroxy fatty acids are abundant in neural tissue where they regulate many physiological functions and take part in cell signalling. These mediators are synthesised from PUFA within neural membranes.

1.4.2.1 Cyclooxygenase, prostanoids and isoprostanes in the brain

COX-1 and COX-2 are constitutively expressed in brain tissue. COX-1 metabolites are involved in several homeostatic processes whereas it is thought COX-2 is rapidly induced as a result of inflammatory mediators (Phillis *et al.*, 2006). More recently, the discovery of a third isoform, COX-3 has been made (Chandrasekharan *et al.*, 2002). It has been suggested that acetaminophen, a powerful analgesic and anti-pyretic, may work by blocking COX-3 (Botting and Ayoub, 2005).

PGD₂ has been shown to have a wide range of beneficial effects on cerebral vasculature. As confirmed by previous experiments it is the most abundant PG in the brain protecting against excitotoxic injury and regulating functions such as sleep, temperature and nociception (Liang *et al.*, 2005b).

The role of PGF_{2α} in the brain is unclear although it is thought to be involved with neurotransmission and signalling mechanisms (Dorman, 1991). In one study, injection of PGF_{2α} into the brain resulted in reduced gastric secretion and the potency of this effect was second only to PGE₂ (Puurunen, 1983). In another study PGF_{2α} appeared to exacerbate hypoxic injury in cultured neuronal cells, these effects were mirrored by PGE₂ and PGD₂ (Li *et al.*, 2008). Thus, PGF_{2α} is expressed in neuronal tissue and may play a role in neurodegeneration and cell signalling.

PGE₂ is produced at high levels in the injured CNS where it is generally considered a cytotoxic mediator for inflammation (Cimino *et al.*, 2008, Levi *et al.*, 1998). Several studies have found that levels of PGE₂ increase during various neurological disorders such as Alzheimer's disease or amyotrophic lateral sclerosis (Hoshino *et al.*, 2007, Liang *et al.*, 2005a, Liang *et al.*, 2008). Activation of the EP2 receptor has been shown to induce apoptosis in cell cultures of rat hippocampal neurones (Takadera *et al.*, 2004) and to exacerbate neurotoxicity in cultured rat cortical neurones (Takadera and Ohyashiki, 2006).

The isoprostanes are potent constrictors of cerebral arterioles released after brain injury (Hoffman *et al.*, 1997). It has been reported that levels of 8-*iso*-PGF_{2α} can increase 10 fold over baseline following brain injury (Hoffman *et al.*, 1996). This finding confirms

that there is substantial lipid peroxidation following brain trauma and that isoprostanes produce dramatic vasoconstriction leading to hypoperfusion (Hoffman *et al.*, 1997).

1.4.2.2 Lipoxygenase and hydroxy fatty acids in the brain

12-LOX is the most abundant lipoxygenase in brain (Bendani *et al.*, 1995) although neural tissue and cells also express 5-LOX and 15-LOX (Phillis *et al.*, 2006, Zhang *et al.*, 2006).

Neural tissues will actively form leukotrienes in the presence of FLAP. Following neural injury there is up-regulation of leukotriene synthesis and corresponding 5-LOX levels (Ohtsuki *et al.*, 1995, Tomimoto *et al.*, 2002). The brain will also synthesise thromboxanes which together with the leukotrienes have potent vascular effects (Minamisawa *et al.*, 1988). Leukotrienes also mediate chemotaxis of leukocytes and disruption of the BBB making it more permeable (Di Gennaro *et al.*, 2004). The leukotrienes will increase free radical production in the brain as well as potentiate cytokine production (Tassoni *et al.*, 2008).

The physiological roles of HETE in the brain are still largely unknown. 12-HETE is thought to modulate voltage sensitive calcium channels and prevent calcium overload following ischaemia (Murphy *et al.*, 1995). In this way it is reported that 12-HETE may have a neuroprotective effect (Hampson and Grimaldi, 2002). Conversely, 12-HETE has also been shown to have neurotoxic effects that contribute towards the development of neurodegenerative disorders such as Alzheimer's disease (Lebeau *et al.*, 2004).

It has been suggested that both 15-HETE and 11-HETE can be synthesised in brain by an unidentified mechanism involving COX. Cultured mouse neurones have been shown to stop producing these hydroxy fatty acids after incubation with indometacin, a COX inhibitor (Oomagari *et al.*, 1991). Thus levels of these compounds may be elevated in the brain owing to oxygenation by COX, LOX or CYP 450.

The effects of HODE on the brain are not well understood although it has been shown that pre-treatment of cultured cells with 13-HODE blocks cell signalling effects by 12-HETE (Liu *et al.*, 1995). Thus 13-HODE may work in balance with 12-HETE in the brain. The effects of 9-HODE are not described in the brain although both isomers of HODE have been used as markers of oxidative stress within neuronal tissue (Liu *et al.*, 1998, Niki and Yoshida, 2005).

The brain is known to synthesise resolvins and protectins as a result of injury. RVD1 and PD1 have been detected following stroke and are thought to participate in the resolution phase of inflammation (Hong *et al.*, 2007).

1.4.2.3 Fatty acid ethanolamides in the brain

The fatty acid ethanolamides are thought to have a neuromodulatory effect in the brain (Di Marzo, 1999). They have been shown to inhibit cAMP and block voltage sensitive Ca^{2+} channels thereby counteracting depolarisation (Felder *et al.*, 1993, Maccarrone *et al.*, 2002a). Thus A-EA has been shown to impair working memory through a reduction in long term potentiation (LTP) (Terranova *et al.*, 1995). CB1 receptor agonists such as A-EA have been shown to induce bradycardia and hypotension (Lake *et al.*, 1997) as well as acts as a smooth muscle relaxants (Pertwee, 1997). These actions of A-EA are

thought to be mediated through downregulation of autonomic nervous functions (Di Marzo *et al.*, 1998). Thus the fatty acid ethanolamides influence brain function and may have a neuroprotective role by inhibiting excitotoxicity (Milton, 2002, Veldhuis *et al.*, 2003).

The cerebral cortex, hippocampus and basal ganglia are richly populated with CB1 receptors and these receptors have been shown to mediate cognition, memory and motor function (Moldrich and Wenger, 2000). There is also evidence to show that stimulation of CB1 receptors in several areas of the central nervous system ameliorates perception of pain thereby producing an antinociceptive effect (Svizenska *et al.*, 2008). The neuromodulatory actions of the fatty acid ethanolamides are thought contribute towards their analgesic effects (Walker *et al.*, 1999). It has been shown that FAAH inhibitors lead to up-regulation of fatty acid ethanolamide which induces pain relief (Yao *et al.*, 2006).

It has been shown that there is a dramatic increase in A-EA and related fatty acid ethanolamides following ischaemic insult to the brain, a condition where COX-2 is induced (Muthian *et al.*, 2004). It is not clear if treatment of animals with a FAAH inhibitor stimulates prostamide synthesis and whether such an approach may offer novel therapeutic advantage in management of ischaemic insult. Fatty acid amides also interact with many neurotransmitter systems including those related to acetylcholine (Domino, 1981, Mule *et al.*, 2007), dopamine (Martin *et al.*, 2008), serotonin (Bambico *et al.*, 2007) and γ -aminobutyric acid (GABA) (Rossi *et al.*, 2008).

It is thought that the endocannabinoid system plays a key role in neuroprotection with scope for novel therapeutic developments (Panikashvili *et al.*, 2005, Veldhuis *et al.*, 2003). Research is currently underway to target cannabinoid receptors in the treatment of conditions such as Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis, HIV encephalitis, closed head injury, and granulomatous amebic encephalitis (Cabral and Griffin-Thomas, 2008).

1.4.3 Polyunsaturated fatty acids as antioxidants in the brain

Hyperoxia is known to cause neurotoxicity through an accumulation of reactive oxygen species (ROS) and is thought to be one factor contributing towards neurodegeneration (Mosley *et al.*, 2006, Chavko *et al.*, 2003). ROS are generated by multiple mechanisms and give rise to radicals that can damage neurones and brain microvasculature (Hall and Bosken, 2009, Halliwell, 2006). ROS have also been shown to reduce mitochondrial membrane potential leading to impaired energy production in the brain followed by apoptosis (Galindo *et al.*, 2003, Marchetti *et al.*, 1996, Vergun *et al.*, 2001).

Neural tissue is particularly susceptible to oxidative stress because of its high oxygen consumption (Ritchie, 1967). Oxidative stress has been found to be a major factor in the ageing process (Ames *et al.*, 1993). It has been found that there is a build up of oxidative stress with age and that this is accompanied by an age related deficit in learning probably due to neurodegeneration (Liu *et al.*, 2003). Thus, increased levels of oxidative stress as a result of ageing can lead to neurodegeneration and cognitive decline. Several studies have demonstrated that regular intake of antioxidants can reduce age related cognitive decline (Morris *et al.*, 2002, Paul *et al.*, 2007, Ueda *et al.*, 2005).

It has been shown that PUFA are easily oxidised and are therefore able to react with free radicals and act as antioxidants (Richard *et al.*, 2008). It has been found that the efficiency with which any PUFA acts as an antioxidant is not only related to the degree of desaturation but also the microenvironment of the PUFA and the length of the PUFA chain (Higdon *et al.*, 2001, Cosgrove *et al.*, 1987, Yazu *et al.*, 1996). In this regard it has been found that *n*-3 PUFA have the best antioxidant properties (Richard *et al.*, 2008).

1.4.4 The importance of polyunsaturated fatty acids to neural membranes and memory

Neurons transmit signals across synapses in the brain with varying efficiency. The efficiency of the signal across the synapse is referred to as the synaptic strength and the natural variation in synaptic strength is referred to as synaptic plasticity (Riout-Pedotti *et al.*, 2007). It is thought that memories are stored as patterns of synaptic strength (Dobrunz, 1998, Smolen, 2007). Long term potentiation (LTP) can influence synaptic strength and facilitate memory formation (Dobrunz, 1998). Measuring the ability to sustain LTP is therefore a widely accepted model for encoded learning and memory (Bliss and Collingridge, 1993, Izquierdo, 1994, Smolen, 2007).

Oxidative stress as a result of ageing is known to inhibit LTP (McGahon *et al.*, 1999c). Dietary intake of EPA has been shown to restore LTP in aged animals (McGahon *et al.*, 1999a, McGahon *et al.*, 1999b, McGahon *et al.*, 1999c). The ability of PUFA to reverse age related deficits in LTP is thought to be related to membrane fluidity (McGahon *et al.*, 1999b).

The fluidity of membranes may be crucial to their role as effective gatekeepers to the cell. Membrane fluidity refers to the physical state of the fatty acyl chains comprising the membrane bilayer structure as well as the mobility of the various components embedded within the membrane (Yehuda *et al.*, 2002). During ageing the cell membrane is depleted of PUFA and there is an associated increase in cholesterol leading to rigidity (Alvarez *et al.*, 1993, Hashimoto *et al.*, 1999, Ulmann *et al.*, 2001). Thus, ageing, accompanied by elevated oxidative burden characterised by increased rigidity of the membrane, affects the normal physiological functioning of the cell which is apparent through memory impairment and decreased LTP (Yehuda *et al.*, 2002).

The importance of PUFA to the integrity and functioning of neural membranes can be gauged through experiments with senescence accelerated mice (Kumar *et al.*, 1999). These mice show reduced activity of the desaturase enzyme as a result of ageing and subsequent depletion of PUFA in their membranes. These mice exhibit age induced impairments such as memory loss and learning disabilities. Thus PUFA are critical to membrane fluidity which in turn is critical to proper cell functioning and maintenance of memory and learning functions.

1.5 Polyunsaturated fatty acids and platelet function

1.5.1 The role of platelets in the cardiovascular system

The circulatory system has evolved several mechanisms by which it carefully controls loss of blood from a site of injury. All of these mechanisms involve platelets which are involved in homeostasis, leading to the formation of thrombi. As they lack a nucleus, platelets themselves are not histologically true cells but are fundamental to the clotting process and are intricately involved in maintaining vessel tone (Thomas S, 2006).

Like other cells of the human body, platelets are derived from haematopoietic stem cells found in the bone marrow. The stem cells differentiate into megakaryoblasts that eventually give rise to megakaryocytes. The cytoplasm of the megakaryocyte breaks up and the individual fragments formed are termed platelets. The body is continuously producing platelets and the platelet count is carefully maintained giving an indication of their importance to the circulatory system (Hoffbrand *et al.*, 2005).

Platelets are able to participate in the clotting process by adhering to the vessel wall or by aggregating at the site of blood loss (O'brien, 1961). The processes of adhesion and aggregation are mediated through various tissue factors and blood coagulation factors (Macfarlane, 1948). Lack of these factors gives rise to many clotting disorders which if left untreated can result in death through haemorrhage.

1.5.2 The role of prostanoids in platelet biology

Upon activation platelets are able to synthesise prostanoids which then exert a variety of local effects. The main prostanoid produced by platelets is TXA₂ (Hamberg *et al.*, 1975) but other prostanoids synthesised by platelets include PGD₂ (Oelz *et al.*, 1977) and PGE₂ (Lagarde *et al.*, 1979).

1.5.2.1 Thromboxane and prostacycline balance

TXA₂ is part of a major platelet positive feedback loop causing secondary amplification of platelet activation reinforcing the process of aggregation thereby augmenting plug formation (Packham *et al.*, 1987). The generated TXA₂ increases the free calcium ion concentration in a cAMP dependent manner through inhibiting adenylate cyclase

leading to aggregation and adhesion of platelets (Hoffbrand *et al.*, 2005). TXA₂ not only activates platelets but also has powerful vasoconstrictive activity stemming the flow of blood to a particular area (Altiere *et al.*, 1986). This can have major implications for organs with high oxygen requirements such as the brain where lack of blood flow can lead to ischaemia and infarction.

PGI₂ augments formation of cAMP by stimulating adenylate cyclase in a manner opposite to that of thromboxane. This causes a drop in free calcium ion concentration attenuating adhesion and aggregation (Gorman *et al.*, 1978). PGI₂, generated by vascular endothelial cells, works in balance with TXA₂ and has physiologically the opposite effects (Bunting *et al.*, 1983). PGI₂ is a vasodilator and a potent inhibitor of platelet aggregation thereby ensuring smooth and uninterrupted blood flow (Gorman *et al.*, 1977, Moncada and Vane, 1979).

1.5.2.2 Other prostanoids synthesised by platelets

PGD₂ is actively synthesised by platelets and is a known inhibitor of aggregation. This prostaglandin also induces peripheral vasodilatation by relaxing smooth muscle vasculature (Oelz *et al.*, 1977, Braun and Schror, 1992). Thus PGD₂ prompts peripheral flushing and nasal congestion which limit its use as an anti-thrombotic therapeutic agent (Gray *et al.*, 1992, Heavey *et al.*, 1984).

PGE₂ is also formed by platelets and exhibits concentration dependent effects. At high concentrations this prostaglandin inhibits platelet aggregation in a cAMP dependent manner but at lower concentration it acts to augment aggregation caused by other agents (Armstrong, 1996).

1.5.2.3 Platelet prostanoid receptors

TXA₂ exerts its effects through the TP receptor which can be found within the membrane of the platelet and also around smooth muscle. Whilst the primary ligand for these receptors is thromboxane they are also activated by PGH₂ and PGF_{2α} giving these prostanoids thrombotic properties (Tymkewycz *et al.*, 1991).

The actions of PGI₂ are mediated via specific cell surface IP receptors (Siegl *et al.*, 1979). In addition to prostacyclin it has been found that PGE₁ and PGE₂ also interact with platelet IP receptors (Miller and Gorman, 1979).

PGD₂ exerts its effect on the platelet by activating the protein coupled DP1 receptor (Giles *et al.*, 1989). This prostanoid is also a partial agonist of TP receptors. PGE₂ and PGF_{2α} can additionally act as ligands of the DP1 receptor, their activities being weaker than that of PGD₂ (Breyer *et al.*, 2001, Narumiya *et al.*, 1999).

The actions of PGE₂ on the platelet are complicated by the identification of at least four receptor subtypes (EP₁ to EP₄) (Paul *et al.*, 1998). PGE₂ is a known agonist of all these receptor subtypes but has different affinities and this may help explain its concentration dependent dual mode of action on the platelet (Armstrong, 1996).

1.5.3 Hydroxy fatty acids in platelet biology

Human platelets are able to synthesise HETE from AA, HEPE from EPA and HODE from LA (Burger *et al.*, 2000, Morita *et al.*, 1983). Oxygenation of AA and EPA is primarily at the 12C position and catalysed by 12-LOX.

1.5.3.1 The role of platelet type 12-lipoxygenase

The major pathway of AA metabolism in human platelets proceeds via platelet type 12-lipoxygenase (hp-12-LOX) to form 12-HpETE (Funk *et al.*, 1990). The other isoforms of 12-LOX have relatively low genetic similarity with hp-12-LOX and also produce isomers of differing stereochemistry (Burger *et al.*, 2000, Kuhn and O'donnell, 2006, Yamamoto *et al.*, 2005). In the mouse, leukocyte type 12-LOX will form 8(*S*)-HETE and 9(*S*)-HODE from AA and LA respectively but platelet type 12-LOX will form these isomers in the *R* configuration. Additionally, leukocyte 12-LOX will form 15-HETE but this product is only formed in small amounts from the platelet isoform (Yamamoto *et al.*, 1997, Burger *et al.*, 2000).

1.5.3.2 The biological actions of hydroxy fatty acids synthesised by platelets

A variety of cardiovascular activities have been reported for 12-HETE ranging from acting as a vasoconstrictor to directly affecting diuretic function (Natarajan and Nadler, 1991, Nozawa *et al.*, 1990). Overall 12-HETE has been demonstrated to raise blood pressure and has been detected in significantly higher concentrations in the blood of hypertensive rats (Chang and Su, 1985, Stern *et al.*, 1996). Elevated levels of 12-HETE have also been found in hypertensive humans (Gonzalez-Nunez *et al.*, 2001). In spite of the fact that 12-HETE has been shown to affect vascular tone its actions upon the platelet remain largely undetermined although it is thought that 12-HETE may play an important role in mediating the thrombotic process (Ozeki *et al.*, 1998, Katoh *et al.*, 1998).

Studies investigating the effects of LA on the cardiovascular system have demonstrated the anti thrombotic properties of both 13-HODE and 9-HODE (Truitt *et al.*, 1999). In

vitro experiments have shown that addition of 13-HODE to stimulated platelets decreases adherence to arterial vessel walls and inhibits platelet aggregation (Tloti *et al.*, 1991).

More recent work has indicated that 13-HODE and 9-HODE work at least in part by inhibiting platelet COX-1 thereby attenuation formation of the pro-aggregatory TXA₂. 13-HODE is reported to be a much more powerful inhibitor of COX than 9-HODE, both isomers showed no effect on the actions of hp-12-LOX (Truitt *et al.*, 1999).

It has been reported that 15-HETE is able to selectively inhibit hp-12-LOX without affecting COX (Vanderhoek *et al.*, 1980). This effect of 15-HETE is unique since experiments performed with other isomers failed to demonstrate inhibition (Vanderhoek *et al.*, 1980). Thus there exist feedback mechanisms within the platelet that carefully control hydroxy fatty acid synthesis.

1.5.4 Niacin as a lipid lowering agent

Nicotinic acid (niacin) is a water soluble B vitamin the structure of which is shown in Figure 1.12. Given at relatively high doses niacin is known to lower cholesterol and has been used in the management of hypercholesterolemia (Altschul *et al.*, 1955, Drexel, 2007, Robinson *et al.*, 2001). Ingestion of niacin causes a vasocutaneous flushing reaction commonly referred to as the niacin flush (Andersson *et al.*, 1977). The niacin flush is described as an undesirable experience causing intense itching and burning of the skin which lasts for up to 70 min after ingestion (Warady *et al.*, 1989). The flush is known to be mediated through PGD₂ which is thought to be released by cells in the skin causing vasodilatation (Morrow *et al.*, 1992b).

Figure 1.12. The chemical structure of nicotinic acid (niacin)

1.5.4.1 Niacin and platelet eicosanoids

Previous work has shown that niacin can stimulate release of PGI₂ from endothelial cells and reduce platelet aggregation (Walldius and Wahlberg, 1985). Niacin will also induce serotonin release from the platelet which is thought to augment the flushing response (Papaliadis *et al.*, 2008). To date there have not been any studies investigating the link between niacin and platelet derived eicosanoids.

1.5.4.2 The effect of eicosapentaenoic acid on prostaglandin D₂ production

The exact source of PGD₂ during the niacin flush remains unknown but several studies have implicated macrophages and skin Langerhans cells as the chief source of this prostaglandin (Kamanna and Kashyap, 2008, Meyers *et al.*, 2007).

EPA has been shown to inhibit the synthesis and release of PGD₂ from macrophages during inflammation in the lung (Mickleborough *et al.*, 2009). The same holds true for the release of prostanoids from saphenous vein endothelial cells (Urquhart *et al.*, 2001). Stimulating endothelial cells with calcium results in the synthesis of PGD₂ and other

prostanoids but incubation of the cells with EPA attenuates these changes (Urquhart *et al.*, 2001). Other work has shown that EPA will actively decrease PGD₂ release from cultured human mast cells by blocking the actions of COX-2 (Obata *et al.*, 1999). Thus it appears as though EPA is able to attenuate PGD₂ synthesis and release from different cells.

1.5.5 The influence of dietary PUFA on platelets

It has been shown that an altered platelet membrane fatty acid profile can result in defective aggregation (Marra *et al.*, 1998). It is therefore evident that incubating platelets with different PUFA can have profound effects downstream on the clotting process.

The platelet plays a key role in the formation of thrombi and in the progression of cardiovascular disease. It has already been established that eicosanoids directly affect the clotting process and by manipulating these metabolites through the diet it may be possible to alter the course of many cardiovascular pathologies.

Studies have shown that by supplementing the diet with GLA there is a significant increase in the levels of dihomo- γ -linolenic acid in circulating plasma and in the membranes of platelets (Laidlaw and Holub, 2003, Marra *et al.*, 1998). Studies involving dietary supplementation of EPA and DHA show that the expression of these PUFA increases both in serum lipids and in platelet phospholipids (Mori *et al.*, 2000, Nieuwenhuys and Hornstra, 1998). This increase in *n*-3 content of the phospholipids occurs at the expense of AA and adrenic acid. Significantly, as the concentration of *n*-3 in the diet increases so does the proportion of these fatty acids in the phospholipids

(Nieuwenhuys and Hornstra, 1998). Thus by changing the distribution of PUFA in membrane fatty acids the profile of eicosanoids downstream is also altered. In the case of platelets this means that aggregation or adhesion can be attenuated thereby helping prevent thrombus formation.

The aggregatory series-3 prostanoids derived from EPA are thought to be less potent than the corresponding series-2 prostanoids derived from AA (Wada *et al.*, 2007). Thus TXA₃ is thought to be less aggregatory than TXA₂ whereas PGI₃ and PGI₂ are thought to be equally anti-aggregatory (Von Schacky *et al.*, 1985a, Von Schacky *et al.*, 1985b). If consumption of EPA favours production of the series-3 prostanoids from platelets a protective effect against cardiovascular disease can be achieved.

1.6 The properties of eicosapentaenoic acid and its therapeutic applications

The influence of diet on disease pathology and prognosis was first identified within Inuit communities living in Greenland. These communities regularly consumed oily fish thereby increasing their intake of *n*-3 PUFA which were thought to contribute towards an unusually low incidence of cardiovascular disease. These communities typically had a lower incidence of psoriasis, asthma and rheumatoid arthritis, all diseases with an inflammatory component (Horrobin, 1987).

Similar findings were made amongst Japanese people living in coastal towns where fish formed a major part of the staple diet (Hirai *et al.*, 1989). Thus *n*-3 PUFAs became the focus of attention towards developing a strategy to decrease the incidence of many inflammatory diseases.

EPA belongs to the *n*-3 family of PUFA and is derived mainly through dietary intake of oily fish (Nettleton, 1991). Regular consumption of EPA has been shown to have a protective effect in many disease states including arthritis (Volker *et al.*, 2000), cardiovascular disease (Kohro and Yamazaki, 2009) and schizophrenia (Peet, 2003) as well as cancers (Rhodes *et al.*, 2003, Yang *et al.*, 2004) and other diseases with an inflammatory component (Danno *et al.*, 1993, Koto *et al.*, 2007).

Selected actions of EPA are thought to include the ability of this PUFA to modulate cytokine release, inhibit the synthesis of pro-inflammatory mediators from AA, affect signal transduction pathways and influence membrane composition and function (Larsson *et al.*, 2004). There is still however controversy about the mechanism of action of EPA. Many of the properties of this PUFA are difficult to evaluate as it is often present as a mixture of different fatty acids in dietary sources. Furthermore, this PUFA and its mediators are susceptible to oxidative attack and need to be stored with antioxidants which may interfere with clinical outcome (Lytle *et al.*, 1992). EPA can also be metabolically shortened or elongated to other PUFA. Elongation of EPA yields *n*-3 DPA and so some of the actions of EPA may be mediated through this PUFA (Spector *et al.*, 1983).

1.6.1 Bioactivity of eicosapentaenoic acid and its mediators

EPA has been shown to be an inhibitor of COX (Obata *et al.*, 1999, Ringbom *et al.*, 2001), its overall effect probably to decrease the 2-series PG derived from AA (Mascioli *et al.*, 1989). EPA has also been shown to inhibit Δ^5 desaturase thereby attenuating metabolic synthesis of AA (Barham *et al.*, 2000, Dias and Parsons, 1995, Gronn *et al.*, 1992). In one study it was found that EPA attenuated the effects of inflammation by

inhibiting PLA₂ expression which prevented release of AA and the formation of the inflammatory prostanoids (Song *et al.*, 2007).

The series-3 prostanoids of EPA are both chemically and functionally distinct from the series-2 prostanoids derived from AA. PGE₃ for example is much less efficient in promoting the release of pro-inflammatory cytokines such as IL-6 than PGE₂ (Bagga *et al.*, 2003). Moreover, PGE₂ enhances response to mitogenic and inflammatory stimuli more potently than PGE₃ (Bagga *et al.*, 2003). Furthermore, AA derived eicosanoids have more potent effects on myocardial arrhythmia compared to those derived from EPA (Li *et al.*, 1997). AA derived TXB₂ is a potent vasoconstrictor and platelet activator whereas EPA derived TXB₃ is much less potent (Schmitz and Ecker, 2008, Weber *et al.*, 1986).

It has been found that in platelets EPA derived HEPES and AA derived HETEs are similar in potency and have similar actions (Takenaga *et al.*, 1986). The EPA derived series-5 LTs however are thought to be much less potent than their AA derived series-4 counterparts (Chang *et al.*, 1989, Tatsuno *et al.*, 1990). LTB₄ for example is thought to be significantly more potent than LTB₅ in the enhancement of natural killer cell activity (Chang *et al.*, 1989). Furthermore, LTB₄ will actively augment production of IL-1 and calcium mobilisation in human blood monocytes whereas LTB₅ has no significant effect (Tatsuno *et al.*, 1990).

Other potential metabolites of EPA include its fatty acid ethanolamide EPA-EA. This compound is the structural analogue of A-EA. To date EPA-EA has not been found in rat brain tissue although it has been described in piglets (Berger *et al.*, 2001). There are

no documented studies examining the relationship of EPA-EA with intake of high purity EPA. Based on the diverse roles of A-EA it is envisaged that EPA-EA could also be a biologically important molecule.

1.6.2 The effects of eicosapentaenoic acid on cell signalling

EPA has been shown to affect cell function by directly interacting with signalling mechanisms controlling the inflammatory process. In particular EPA has been shown to modulate pro-inflammatory signalling processes (Sadeghi *et al.*, 1999) whilst simultaneously up regulating anti-inflammatory processes (Cassatella *et al.*, 1993, De Waal Malefyt *et al.*, 1991). These effects of EPA are thought to be attributed to the actions of this PUFA on the release of cytokines (Cassatella *et al.*, 1993, De Waal Malefyt *et al.*, 1991, Sadeghi *et al.*, 1999).

Changes in LTP are thought to be mediated through an increase in pro-inflammatory cytokines such as IL-1 β which in turn stimulate the mitogen-activated protein c-jun-N-terminal kinase (JNK) (Barry *et al.*, 2005, Curran *et al.*, 2003). Rising levels of ROS trigger inflammatory changes in the brain and it has been found that ROS increase with age leading to a decline in LTP (Murray and Lynch, 1998).

EPA has been shown to inhibit age related increase in ROS and has also been shown to block JNK activation downstream (Richard *et al.*, 2008, Moon *et al.*, 2007). Thus age related decrease in LTP, due to accumulation of ROS, are reversed by EPA (Lonergan *et al.*, 2002).

Intake of EPA has also been shown to decrease production of circulating pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α) (Sadeghi *et al.*, 1999) and this effect is active even when EPA is taken only during an inflammatory event. The cytokine IL-10 is known to attenuate inflammation by suppressing production of pro-inflammatory cytokines such as TNF- α , IL-1 β and IL-6 as assessed in human monocytes and murine macrophages (Cassatella *et al.*, 1993, De Waal Malefyt *et al.*, 1991). Levels of IL-10 following inflammatory changes are increased in rats fed EPA over a 5 week period (Sadeghi *et al.*, 1999). Thus EPA has been shown to inhibit inflammation by blocking pro-inflammatory cytokines and augmenting levels of IL-10.

1.6.3 The effects of eicosapentaenoic acid on protein and gene expression

It has been shown that EPA is able to modulate gene transcription thereby influencing the synthesis of potent enzymes thought to be involved with inflammation (Novak *et al.*, 2003). It has also been shown that EPA affects mitochondrial function at a gene level preventing cellular apoptosis (Lonergan *et al.*, 2004). These actions of EPA illustrate how this PUFA is able to significantly affect downstream cellular processes through interaction at gene level.

The *n*-3 PUFA are known to interact with PPARs thereby regulating gene expression and subsequent release of inflammatory cytokines (Gottlicher *et al.*, 1993, Xu *et al.*, 1999). EPA has also been shown to influence cytokine formation in stimulated cancer cells through interaction with PPAR γ (Kawashima *et al.*, 2008). The protective effects of EPA in human colon cancer cells have been directly linked to the affinity of this PUFA to PPAR γ (Allred *et al.*, 2008). In addition to modulating cytokine release it has

recently been shown that EPA regulates cholesterol biosynthesis and fatty acid metabolism through interaction with PPAR α (Sugiyama *et al.*, 2008). Thus, many of the actions of EPA can be explained through downstream gene modulation as a result of PPAR activation. The significance of the interaction between EPA and PPAR is only recently being realised.

Nuclear factor κ B (NF κ B) is a transcription factor that controls release of several cytokines, chemokines, adhesion molecules and inducible effector enzymes (Schmitz and Ecker, 2008, Gilmore, 1999, Hayden *et al.*, 2006). Modulation of NF κ B has been suggested as an effective therapeutic strategy for combating diseases such as asthma, arthritis and a host of other inflammatory conditions (Chen *et al.*, 1999). There is currently considerable interest in evaluating the true extent to which NF κ B influences physiological and patho-physiological processes (Ghosh and Karin, 2002).

EPA has been found to inhibit NF κ B activity directly by decreased degradation of the inhibitory subunit of the transcription factor in human monocytes (Novak *et al.*, 2003, Zhao *et al.*, 2004). In another study it was found that oxidised derivatives of EPA were able to inhibit NF κ B in aortic murine endothelial cells (Mishra *et al.*, 2004). Thus by influencing levels of NF κ B EPA can attenuate inflammation at gene level.

EPA has also been shown to induce the proteolipid protein (PLP) gene in glioma cells (Salvati *et al.*, 2004). PLP is a major protein component (25%) of CNS myelin (Eto *et al.*, 1971). PLP is therefore essential for the formation of myelin sheaths which in turn are essential for proper brain function (Boison and Stoffel, 1989). It has been shown that EPA induces PLP through a cAMP dependent pathway and that AA is unable to

demonstrate the same activity (Salvati *et al.*, 2004). Thus EPA influences brain development at a gene level.

BCL-2 is an anti-apoptotic protein that plays a role in maintaining the integrity of the mitochondrial membrane (Yang *et al.*, 1997). Phosphorylation of BCL-2 by JNK antagonises its anti-apoptotic effect (Bassik *et al.*, 2004), thus, JNK is able to destabilise the mitochondrial membrane (Lonergan *et al.*, 2004). It has been shown that phosphorylation of BCL-2 is increased during inflammation as a result of increased JNK expression. This leads to the loss of cytochrome C from the mitochondrial membrane triggering apoptosis (Yang *et al.*, 1997, Kluck *et al.*, 1997). One way of measuring cell death through apoptosis therefore is by measuring the expression of cytochrome C in the cytosol.

Inflammation leads to elevated levels of free cytochrome C. When EPA has been taken however, the expression of cytochrome C in the cytosol is significantly lower (Lonergan *et al.*, 2004). This implies that EPA is interfering with the process of cell death possibly by preventing phosphorylation of BCL-2 through decreased expression of JNK and subsequently attenuating release of cytochrome C.

1.6.4 Eicosapentaenoic acid and memory

It is already known that regular intake of EPA can prevent a drop in LTP due to inflammation and ageing. It has also been shown that EPA will prevent build up of ROS and therefore help maintain the fluid nature of cell membranes. Taken together, these effects of EPA should prevent memory loss.

In order to determine the memory protective effects of EPA rats were fed a diet containing 0.2% ethyl EPA or 1% ethyl EPA for 5 weeks after which they were injected directly into the brain with IL-1 β to induce inflammation (Song and Horrobin, 2004). The IL-1 β injection resulted in significantly impaired spatial learning and memory as assessed by the Morris water maze. This study showed that 1% EPA significantly attenuated memory impairment in those rats injected with IL-1 β . In addition to this EPA seemed to dose dependently reverse the IL-1 β induced increase in turnover of noradrenaline. Conversely, neither concentration of EPA affected the turnover of noradrenaline or serotonin in those animals injected with saline. Thus EPA appeared to significantly attenuate memory impairment induced by central IL-1 β administration without affecting the neurotransmitter systems of control animals.

1.6.5 Eicosapentaenoic acid in the treatment of affective disorders

There has been a lot of interest recently in the treatment of schizophrenia and depression with EPA. There is much evidence to show that the metabolism of phospholipids and PUFAs is abnormal in schizophrenic patients and therefore the pathology of this disease may be related to fatty acid intake (Du Bois *et al.*, 2005, Schwarz *et al.*, 2008, Horrobin *et al.*, 1991).

The membrane phospholipid composition (MPC) hypothesis of schizophrenia proposes that an alteration in the phospholipid species of the cell membrane will affect the fluidity of the membrane thereby modulating vital cell functions such as the activity of ion channels as well as ligand receptor biology (Horrobin, 1998). This in turn can affect neurotransmission thereby causing neuropsychiatric disorder such as schizophrenia. It

is thought that PLA₂ levels may be higher in the cells of schizophrenics than normal individuals thereby depleting DHA and AA in cell membranes, a feature thought to be common in schizophrenia (Arvindakshan *et al.*, 2003, Gattaz *et al.*, 1990).

It has already been described how EPA restores fluidity in cell membranes and has an inhibitory effect on PLA₂. EPA also directly affects the noradrenergic and serotonergic systems in the brain without affecting the dopaminergic system (Song and Horrobin, 2004). Owing to the perceived mechanisms of action of EPA several clinical trials have been conducted to investigate the possibility of treating schizophrenia and depression with this fatty acid.

Initially, trials were conducted with only small cohorts. In one such trial schizophrenic patients in India were given 2g EPA in addition to their normal medication (Shah *et al.*, 1998). The trial lasted 3 months and involved only 10 patients. Although the sample size was small this study showed that addition of EPA to the diet reduced hallucinatory behaviour with a significant 29% improvement in symptoms when compared to control. This study then paved the way for more research and larger scale studies with EPA for treatment of schizophrenia.

Following on from previous work two relatively large trials were conducted initially examining the effects of both EPA and DHA as an adjunct to ongoing treatment on schizophrenia but subsequently focussing on EPA supplementation alone (Peet *et al.*, 2001). A total of 45 patients were included in the first study of which 15 were given 2g EPA per day, 15 were given 2g DHA per day and 15 were given inactive placebo daily.

This study showed that after 3 months of supplementation EPA, but not DHA caused a significant improvement in symptoms compared to control.

Having decided to focus on EPA as a result of previous data a further trial was set up in India to look into the effects of giving EPA alone, without any other medication to schizophrenic individuals. Patients were to receive either 2g EPA per day or placebo for a total of 3 months (Peet *et al.*, 2001). During the course of the trial ethical considerations meant that many of the patients had to commence traditional anti-psychotic medication to prevent worsening of their symptoms. Interestingly, the entire placebo group had to take anti-psychotic medication by the end of the trial but 6 out of 14 patients taking EPA were not on medication at the end of the experiment and 4 of these had no need for medication throughout the trial period.

After establishing the effectiveness of EPA in the treatment of schizophrenia a further large scale trial involving 115 patients and lasting over 3 months was initiated (Peet and Horrobin, 2002). This time patients received either 1g, 2g or 4g EPA per day. EPA was given as an adjunct to existing antipsychotic treatment. In general all the subgroups taking EPA at all doses showed improvement compared to placebo. This was especially the case in those patients taking the antipsychotic clozapine. In this case the response achieved through EPA supplementation, especially 2g per day, was substantially better than with placebo

Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), is currently licensed in the UK as an effective anti depressant in major depressive illness and is used to treat millions of people worldwide. It has been shown that taking 1g EPA per day for 8

weeks has the same effect on depressive illness as 20mg Fluoxetine per day (Jazayeri *et al.*, 2008). The same work also found that a combination of Fluoxetine with EPA had superior effects to either compound given alone.

1.7 Mass spectrometry in bioanalysis

1.7.1 Principles of mass spectrometry

Mass spectrometry is used in the identification and structural characterisation of ionised species. Material is ionised and passed through a changing electromagnetic field. The magnitude and direction of the electric field will result in the deflection of the ions into a specific path or beam. The degree of deflection is directly linked to the charge on the ion and its molecular mass. Ions are separated according to their mass to charge ratio (m/z) and the output plotted as a spectrum.

1.7.1.1 Methods of ionisation

There are several variations in the method of ionisation, these include the use of laser as in matrix assisted laser desorption ionisation (MALDI) or strong electric fields as in electrospray ionisation (ESI). Other methods of ionisation include fast atom bombardment (FAB) atmospheric pressure chemical ionisation (APCI), electron ionisation (EI), and chemical ionisation (CI). ESI is the method of choice when analysing eicosanoids and other bioactive lipids (Murphy *et al.*, 2005).

1.7.1.2 Types of analysers

Once ions have been formed they need to be separated by analysers according to their masses which must be determined. These include time of flight (TOF) analysers,

magnetic analysers or quadrupole analysers. TOF analysers work on ions that are expelled from the source as distinct bundles through an intermittent process such as laser desorption. Magnetic analysers apply a perpendicular magnetic field to the direction of travel of ions thereby influencing trajectory. The quadrupole operates by oscillating ions through a rapidly changing field into a stable trajectory. Ions thus travel as a wave through the quadrupole. Altering the parameters of the quadrupole will allow specific ion selection with all other ions being ejected from the quadrupole (Hoffman and Stroobant, 2002).

1.7.1.3 Detectors

Different types of detectors exist, which can be classified in either of two categories. The photographic plate and the Faraday cage allow a direct measurement of the charges that reach the detector, whereas the electron or photomultiplier detectors and array detectors increase the intensity of the signal (Hoffman and Stroobant, 2002).

1.7.2 Principles of electrospray ionisation tandem mass spectrometry

In an ESI system, solvent containing the analytes is pumped through a narrow capillary. An electric field is generated at the outflow of the capillary by applying a potential difference between the end of the capillary and a counter electrode. As the solvent emerges out of the capillary the electric field induces a charge accumulation which will eventually break to form highly charged droplets. The end of the capillary is heated to aid ion formation (Hoffman and Stroobant, 2002).

Tandem mass spectrometry (MS/MS) refers to a setup of two analysers operating simultaneously to isolate ions of interest. Figure 1.13 summarises the pairing of ESI to tandem mass spectrometry (ESI-MS/MS) in a triple quadrupole system. If complex mixtures are separated using liquid chromatography (LC) prior to being ionised and analysed by tandem mass spectrometry the setup is referred to as liquid chromatography electrospray ionisation tandem mass spectrometry (LC/ESI-MS/MS).

The first quadrupole is programmed to select only ions of interest, filtering out all other ions. The selected precursor ion then travels into a second quadrupole (or collision cell) where it fragments on contact with an inert gas such as argon to give multiple product ions. The product ions pass into a third quadrupole that can then select for a specific fragmented product ion.

Figure 1.13. The triple quadrupole system is used to select specific fragments generating from an ionised species.

1.7.3 Mass spectrometry in the analysis of bioactive lipid mediators

Several techniques have previously been employed for the analysis of bioactive mediators such as the eicosanoids, hydroxy fatty acids and fatty acid ethanolamides.

These have included immunoassay, thin layer chromatography (TLC) and gas chromatography coupled to mass spectrometry. Whilst each of these methods has particular advantages none is superior to LC/ESI-MS/MS. A major problem with immunoassay is the potential for cross reactivity with other structurally similar compounds. TLC has relatively low sensitivity and gas chromatography requires lengthy derivatisation procedures that can increase the risk of analyte degradation. In comparison, LC/ESI-MS/MS requires no derivatisation step and gives unparalleled sensitivity. Furthermore, LC/ESI-MS/MS is able to identify isomers of any given compound through the use of chiral columns. LC/ESI-MS/MS is thus commonly used for the analysis of biological extracts to aid identification of lipid metabolites including the eicosanoids and hydroxy fatty acids. This technique has been used to identify eicosanoids in brain, liver, plasma, urine and uterine tissues (Masoodi *et al.*, 2008, Masoodi and Nicolaou, 2006, Ulbrich *et al.*, 2009). LC/ESI-MS/MS is also suited to the analysis of fatty acid ethanolamides and has been used to find these compounds in brain tissue (Richardson *et al.*, 2007, Schreiber *et al.*, 2007).

1.8 Aims and objectives

The ageing brain is characterised by increased oxidative stress and membrane changes. This can bring about neurodegeneration leading to impaired cognitive function and neuroinflammation. EPA may act as an antioxidant and may form distinct mediators with vasodilatory, pro- and/or anti-inflammatory properties that could potentially interfere with neuroinflammation, neurodegeneration and the ageing process.

It is recognised that EPA can be metabolically elongated to DPA and that DPA can be retroconverted to EPA. To date there have been no studies to examine the influence of

DPA on eicosanoid and hydroxy fatty acid synthesis in the brain. It is thought that DPA may also incorporate into neural membranes changing the profile of synthesised inflammatory mediators in the brain.

Whilst ageing can bring about neurodegeneration its effect on the synthesis of fatty acid ethanolamides in the brain is still of interest. It is known that fatty acid ethanolamides possess anti-inflammatory properties and are involved with various regulatory processes. However, it is not clear how dietary intake of EPA or DPA may affect the synthesis of fatty acid ethanolamides in the brain.

EPA is known for its cardioprotective actions and the platelet is implicated in the development of the niacin flush which is due to increased secretion of PGD_2 . Treating platelets with EPA could change the profile of secreted eicosanoids and hydroxy fatty acids thereby preventing niacin induced increase in synthesis of PGD_2 .

The main aim of this project has been to investigate aspects of EPA bioactivities by examining whether supplementing the diet with this PUFA will impact the profile of eicosanoids and other bioactive lipid mediators in the brain and platelet. EPA should decrease the presence of AA derived eicosanoids whilst increasing levels of EPA derived eicosanoids in the brain. Supplementing the diet with DPA should mirror the effects of EPA as DPA can be retroconverted to EPA. Furthermore, treating platelets with EPA may modulate levels of PGD_2 secreted in the presence of niacin. It is thought DHA may also change the profile of platelet derived eicosanoids.

This project aimed to test these hypotheses, thus carrying out the following objectives:

1. To develop and validate a sensitive and selective LC/ESI-MS/MS method which would allow for the simultaneous identification and quantitation of the fatty acid ethanolamides of EPA, AA and DHA from brain.
2. To apply this method to examine the profile of these fatty acid ethanolamides in young and aged brain following dietary supplementation with EPA or DPA.
3. Using LC/ESI-MS/MS methods developed in the group to investigate the effect of ageing on the eicosanoid and hydroxy fatty acid profiles of young and aged brain following a diet supplemented with EPA or DPA.
4. Using LC/ESI-MS/MS methods developed in the group to investigate how incubating platelets with EPA or DHA can change the profile of secreted eicosanoids and hydroxy fatty acids.
5. To investigate whether niacin increases platelet PGD₂ production and if EPA can avert this increase.

Chapter 2 : Materials and Methods

2.1 Materials

Prostaglandin D₁ (PGD₁), Prostaglandin E₁ (PGE₁), Prostaglandin F_{1 α} (PGF_{1 α}), 6 keto-Prostaglandin F_{1 α} (6-keto-PGF_{1 α}), Prostaglandin B₂ (PGB₂), Prostaglandin D₂ (PGD₂), Prostaglandin E₂ (PGE₂), Prostaglandin F_{2 α} (PGF_{2 α}), Prostaglandin J₂ (PGJ₂), Δ^{12} -Prostaglandin J₂ (Δ^{12} -PGJ₂), 15-deoxy- $\Delta^{12,14}$ -Prostaglandin J₂ (15-deoxy- $\Delta^{12,14}$ -PGJ₂), Prostaglandin D₃ (PGD₃), Prostaglandin E₃ (PGE₃), Prostaglandin F_{3 α} (PGF_{3 α}), Thromboxane B₂ (TXB₂), Thromboxane B₃ (TXB₃), 8-*iso*-PGE₂, 8-*iso*-PGF_{2 α} , 8-*iso*-15-keto PGE₂, 8-*iso*-15-keto PGF_{2 α} , 13,14-dihydro PGE₁, 13,14-dihydro PGF_{1 α} , 13,14-dihydro PGF_{2 α} , 13,14-dihydro-15-keto PGE₁, 13,14-dihydro-15-keto PGF_{1 α} , 13,14-dihydro-15-keto PGE₂ and 13,14-dihydro-15-keto PGF_{2 α} were purchased from Cayman Chemicals (Ann Arbor, MI, USA). 9-Hydroxy-10*E*,12*Z*-octadecadienoic acid (9-HODE), 13-hydroxy-9*Z*,11*E*-octadecadienoic acid (13-HODE), 5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*,17*Z*-eicosapentaenoic acid (5-HEPE), 18-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*,16*E*-eicosapentaenoic acid (18-HEPE), 9-hydroxy-5*Z*,7*E*,11*Z*,14*Z*,17*Z*-eicosapentaenoic acid (9-HEPE), 8-hydroxy-5*Z*,9*E*,11*Z*,14*Z*,17*Z*-eicosapentaenoic acid (8-HEPE), 15-hydroxy-5*Z*,8*Z*,11*Z*,13*E*,17*Z*-eicosapentaenoic acid (15-HEPE), 12-hydroxy-5*Z*,8*Z*,10*E*,14*Z*,17*Z*-eicosapentaenoic acid (12-HEPE), 5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HETE), 8-hydroxy-5*Z*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid (8-HETE), 11-hydroxy-5*Z*,8*Z*,12*E*,14*Z*-eicosatetraenoic acid (11-HETE), 15-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (15-HETE), 12-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid (12-HETE), 9-hydroxy-5*Z*,7*E*,11*Z*,14*Z*-eicosatetraenoic acid (9-HETE), leukotriene B₄ (LTB₄), 17-hydroxy-4*Z*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-docosahexaenoic acid (17*S*-HDHA), 12*S*-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic-5,6,8,9,11,12,14,15-

d8 acid (12-HETE-*d8*) and Prostaglandin B₂-*d4* (PGB₂-*d4*), were purchased from Cayman Chemicals (Ann Arbor, MI, USA).

Resolvin E1 (RvE1, 5S, 12R, 18R-trihydroxy-6Z, 8E, 10E, 14Z, 16E-eicosapentaenoic acid), resolvin D1 (RvD1, 5S,8R,17S-trihydroxy 4Z,9E,11E,13Z,15E,19Z docosahexaenoic acid) and protectin D1 (PD1, 10R,17S-dihydroxy-docosa-4Z,7Z,11E,15Z,19Z-hexaenoic acid) were kindly provided by Prof. C.N.Serhan, Harvard Medical School, USA.

Docosahexaenylethanolamide (DHA-EA), and eicosapentaenylthanolamide (EPA-EA) were prepared from total organic synthesis by Dr N. Karodia, University of Bradford. Arachidonylethanolamide (A-EA) and AEA-*d8* were purchased from Cayman Chemicals (Ann Arbor, MI, USA).

HPLC-grade chloroform, ethyl acetate, methanol, hexane, ethanol, acetonitrile, hydrochloric acid and formic acid were purchased from Fisher Chemicals, Loughborough, UK. HPLC grade glacial-acetic acid and methyl formate were purchased from Sigma, Dorset, UK.

Eppendorf tubes, disposable pipette tips and disposable 9" and 6" glass Pasteur pipettes were purchased from Fisher Chemicals, Loughborough, UK. 15 ml glass tubes were obtained from VWR International Ltd (Poole, Dorset, UK). 2 ml (12 x 32 mm) autosampler amber glass bottles, 100 µl glass insert vials and 8 mm open screw caps with PTFE 8 mm septa were purchased from Kinesis, Bedfordshire, UK. Solid phase extraction (SPE) cartridges (C18-E 500 mg, 6 ml and Si 100 mg 1 ml) were purchased

from Phenomenex (Macclesfield, UK). Narrow range (2.5-4.5) pH indicator paper strips were purchased from Merck, UK. 10, 50 and 250 µl glass syringes were purchased from SGE, Australia. Nitrogen gas was supplied by BOC gasses, UK.

2.2 Equipment

The Waters Alliance 2695 HPLC pump with a Waters 2690 autosampler coupled to an electrospray (ESI) triple quadrupole Quattro Ultima mass spectrometer with operating software MassLynxTM V4.0 was purchased from Waters, Elstree, UK. The microplate reader was obtained from Labsystems Multiscan (Labsystems Multiscan RC ver 6.0) and operated using Gemini 4.0 software. An SPE vacuum manifold and drying attachment for the manifold were purchased from Phenomenex (Macclesfield, UK). A C18 HPLC column (Luna, 5 µm, 150x2.0 mm), universal SecurityGuard cartridge holder and SecurityGuard cartridges (C18 4 x 2.0 mm) were purchased from Phenomenex (Macclesfield, UK). Dounce mini glass homogeniser (2 ml) was obtained from Wheaton, USA. A polytron Ystral D-79282 blade homogeniser was purchased from Ballrechten-Dottingen, Germany. The vortex was purchased from Hook and Tucker Instruments LTD, UK. Sovrall RT6000B refrigerated centrifuge was purchased from Dupont (UK) LTD, Stevenage, Herts. SS40 waterbath was obtained from Grant Instruments LTD (Cambridge, UK). Cotton Wool was purchased from Robinson Healthcare Limited, Chesterfield.

2.3 Animal tissue

All animals used for this work, their housing, treatment and tissue sample preparation was carried out by Prof M A Lynch's group at the Bio Resources Unit, Trinity College, Dublin.

2.3.1 Housing of animals

All rats used were an inbred strain of male Wistar. Young animals were aged between 2 to 3 months, and aged animals were between 22 to 24 months old. All the animals were supplied by Bantam & Kingman (Hull, UK). Animals were housed in groups of 3 per cage and were maintained under a 12 hr light-dark cycle at the Bio Resources Unit, Trinity College, Dublin. Food and water was available *ad libitum*. Ambient temperature was controlled between 22 and 23°C and animals were maintained under veterinary supervision for the duration of all experiments. All animal experimentation was performed under a licence granted by the Minister for Health and Children (Ireland) under the Cruelty to Animals Act, 1876 and the European Community Directive, 86/609/EC.

2.3.2 Dietary interventions

2.3.2.1 Dietary intervention with EPA for 28 days

Young rats (2-3 months) were randomly assigned to 3 groups (n=8 per group). The control group received normal laboratory chow (Red Mills, Ireland) enriched with monounsaturated fatty acids to ensure isocaloric value. The treatment groups received laboratory chow supplemented with EPA at a) 50mg/rat/day or b) 125mg/rat/day,

(20:5n-3 ethyl eicosapentaenoic acid (>95% pure) with 0.2% dl-tocopherol as an antioxidant; Laxdale, Stirling, UK) for 28 days. Food consumption was measured prior to the beginning of the experimental treatment to determine daily intake. Treatments were prepared freshly and animals were offered their full daily requirement.

2.3.2.2 Dietary intervention with EPA and DPA for 8 weeks

Rats were assigned as either young (2-3 months) or aged (22-24 months) and were randomly divided into 3 subgroups (n=6 per subgroup) within each age bracket. The control group received normal laboratory chow (Red Mills, Ireland) enriched with monounsaturated fatty acids to ensure isocaloric value. The treatment groups received laboratory chow supplemented with either EPA (200mg/kg/day; 20:5n-3 ethyl eicosapentaenoic acid (>95% pure) with 0.2% dl-tocopherol as an antioxidant; Laxdale, Stirling, UK) or DPA (200mg/kg/day; 22:5n-3 ethyl docosapentaenoic acid (>95% pure) with 0.2% dl-tocopherol as an antioxidant; Laxdale, Stirling, UK) for 8 weeks. Food consumption was measured prior to the beginning of the experimental treatment to determine daily intake. Treatments were prepared freshly and animals were offered their full daily requirement.

2.3.3 Preparation of brain tissue

At the end of the dietary manipulation period, rats were anaesthetised by intraperitoneal administration of urethane (1.5 g/kg). The head was positioned in a head holder in a stereotaxic frame (ASI instruments, US). An incision was made at the midline with a scalpel to expose the skull. The periosteum was cleared and electrodes were inserted to assess the ability to sustain long term potentiation (LTP). Rats were then sacrificed by

decapitation and brains were rapidly removed and placed on ice for dissection of the temporal cortex. Cortical tissue was then immediately sliced bi-directionally with the use of a McIlwain tissue chopper (350 x 350 μ m). The tissue was then divided with aliquots placed in empty sterile eppendorf tubes and flash frozen in liquid nitrogen. Tissue was placed in storage at -80°C before being transported to Bradford in dry ice. On arrival in Bradford the samples were immediately stored at -80°C awaiting extraction and analysis.

2.4 Human platelet samples

All human platelet sample treatments were carried out by Dr A Leaver's group, University of Edinburgh. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) from 8 healthy donors and prepared under blood bank conditions with ethical approval.

2.4.1 Preparation of platelet rich and platelet poor plasma

Blood was pooled and centrifuged to obtain platelet rich and corresponding platelet poor plasma. Leucocyte filtration reduced leucocytes to $<5 \times 10^6$ per platelet pack. Platelet rich and platelet poor plasma were stored in separate gas permeable bags (Compostop, F730 1,300 ml platelet container, Fresenius HemoCare, GmbH, Bad Homburg, Germany). 50 ml aliquots of each type of plasma were transferred to docked apheresis gas permeable bags (Complex 400 ml transfer bag, Fresenius, HemoCare GmbH, Bad Homburg, Germany).

2.4.2 Platelet treatment with polyunsaturated fatty acids

EPA, DHA and octanoic acid (sodium salts) were dissolved in ethanol and added to the appropriate plasma bag to a final concentration of 100 μM (50 μl ethanolic solution per bag under sterile conditions). Treatment was added to one bag of platelet rich plasma and a corresponding bag of platelet poor plasma. All bags were incubated under routine blood bank conditions (gentle agitation, 20-24°C) for up to 10 days.

On days 1, 3, 5 and 10, samples (3 ml) were withdrawn under sterile conditions from each treatment bag. The platelet rich samples were centrifuged at 600g for 15 min to pellet the platelets. The supernatant along with the sample from the platelet poor bag were then sent to Bradford in dry ice where they were immediately placed at -80°C awaiting analysis.

2.4.3 Platelet treatment with polyunsaturated fatty acids, indometacin and niacin

EPA was dissolved in 0.1% ethanol and added to platelet rich or platelet poor plasma (50 μl per bag) to give a final concentration of 100 μM . Niacin and/or indometacin were dissolved in ethanol and added to individual bags to give final concentrations of 3 mM and 0.3 mM respectively. The following treatments were performed using platelet rich and platelet poor plasma: a) EPA (100 μM), b) indometacin (0.3 mM), c) niacin (3 mM), d) EPA (100 μM) and indometacin (0.3 mM) and e) EPA (100 μM) and niacin (3 mM). A total of 12 bags of plasma were incubated under routine blood bank conditions (gentle agitation, 20-24°C). Bags were sampled at 30 min, 60 min, 90 min, 120 min, 3 days, 5 days and 8 days. Aliquots (3 ml) were withdrawn under sterile conditions from each treatment bag. The platelet rich samples were centrifuged at 600g for 15 min to

pellet the platelets. The supernatant along with the sample from the platelet poor bag were then sent to Bradford in dry ice where they were immediately placed at -80°C awaiting analysis.

2.5 Analysis of eicosanoids and other polyunsaturated fatty acid derived mediators

2.5.1 Preparation of mediator stock solutions

2.5.1.1 Prostanoids

PGE₁, PGD₁, PGF_{1α}, PGD₂, PGB₂, PGE₂, TXB₂ were purchased as dry powder in individual vials (1 mg each). HPLC-grade ethanol (1 ml) was added to each vial to make a final solution at a concentration of 1 μg/μl. PGJ₂ was supplied as 500 μg in 100 μl ethyl acetate. This solvent was evaporated under a fine stream of nitrogen and 500 μl HPLC grade ethanol was added to the vial to prepare a final solution of PGJ₂ at a concentration of 1 μg/μl. PGD₃, PGE₃, Δ¹²-PGJ₂, 15-deoxy-Δ^{12,14}PGJ₂, 8-iso-15-keto PGF_{2α}, 8-iso-15 keto PGE₂, 8-iso-PGF_{2α}, 8-iso-PGE₂, PGF_{3α}, TXB₃, 13,14-dihydro-15 keto PGF_{2α}, 13,14-dihydro-15 keto PGE₂, 13,14-dihydro-15 keto PGE₁, 6-keto-PGF_{1α}, 13,14-dihydro-15 keto PGF_{1α}, 13,14-dihydro-15 keto PGE₁, 13,14-dihydro PGF_{1α}, 13,14-dihydro PGE₁, 13,14-dihydro PGF_{2α} were purchased as 1 μg/μl solutions. 10 μl of each solution (1μg/μl) was transferred to an amber glass vial and diluted with 990 μl ethanol to give a final concentration of 10ng/μl. 40 μl of each standard (10 ng/μl) was then transferred to a single new amber glass vial and the volume adjusted to 1 ml with ethanol. The final concentration of the composite stock solution was thus 400 pg/μl.

100 μl aliquots of this solution were taken and stored in separate amber glass vials. All of these stock solutions were stored at -20°C .

2.5.1.2 Hydroxy fatty acids

9-HODE, 13-HODE, 5-HEPE, 18-HEPE, 9-HEPE, 8-HEPE, 15-HEPE, 12-HEPE, 5-HETE, 8-HETE, 11-HETE, 15-HETE, 12-HETE, 9-HETE and LTB_4 were purchased as 1 $\mu\text{g}/\mu\text{l}$ solutions. 10 μl of each solution (1 $\mu\text{g}/\mu\text{l}$) was transferred to an amber glass vial and diluted with 990 μl ethanol to give a final solution at a concentration of 10 $\text{ng}/\mu\text{l}$. 17S-HDHA, RvE1, RvD1 and PD1 were already supplied as 10 $\text{ng}/\mu\text{l}$ solutions in ethanol. 40 μl of each standard (10 $\text{ng}/\mu\text{l}$) was then transferred to a single new amber glass vial using a 50 μl syringe and the volume adjusted to 1 ml with ethanol. The final concentration of the composite stock solution was thus 400 $\text{pg}/\mu\text{l}$. 100 μl aliquots of this solution were taken and stored in separate amber glass vials. All of these final composite stock solutions were then stored at -20°C .

2.5.2 Calibration lines

The peak area ratio of each compound relative to the appropriate internal standard was calculated from composite standard solutions at all prepared concentrations and plotted against the concentration of the calibration standards. Calibration lines were plotted according to the least squares linear regression method. Peak area was determined using MassLynxTM software.

2.5.2.1 Prostanoids

Composite prostanoid standard solutions were prepared by mixing and diluting the stock solution (400 pg/ μ l) to give final concentrations of 200 pg/ μ l, 100 pg/ μ l, 50 pg/ μ l, 20 pg/ μ l, 10 pg/ μ l and 4 pg/ μ l, as shown in table 2.1. PGB₂-d₄ (internal standard) was prepared in ethanol (1 ng/ μ l) and 40 μ l added to all composite standards to give a final concentration of 400 pg/ μ l.

Table 2.1. Preparation of prostanoid and hydroxy fatty acid composite standard solutions

Final concentration of composite standard (pg/ μ l)	Volume (μ l)		
	composite stock solution (400 pg/ μ l)	PGB ₂ -d ₄ (IS) (1 ng/ μ l) Or 12-HETE-d ₈ (2 ng/ μ l)	Ethanol
200	50	40	10
100	25	40	35
50	12.5	40	47.5
20	5	40	55
10	2.5	40	57.5
4	1	40	59

2.5.2.2 Hydroxy fatty acids

Hydroxy fatty acid calibration solutions were prepared by mixing and diluting the composite stock solution (400 pg/ μ l) to give final concentrations of 200, 100, 50, 20, 10 and 4 pg/ μ l as shown in table 2.1. 12-HETE-*d*8 (internal standard) was prepared in ethanol as 2 ng/ μ l stock solution and 40 μ l added to all composite standards to give a final concentration of 800 pg/ μ l.

2.5.3 Brain tissue homogenisation and extraction

Ice cold water (1 ml) was added to each ependorph tube containing a slice of brain cortical tissue. The tissue and water were then transferred to a 1 ml Dounce glass mini homogeniser with tight fitting pestle using a glass Pasteur pipette. A further 1 ml of ice cold water was added and the sample homogenised on ice using a total of 40 up and down strokes. The homogenate was transferred to a clean glass tube and a further 1 ml water added such that the final volume of homogenate in the glass tube was 3 ml. 50 μ l of the homogenate was removed from each sample and stored at -20°C in an ependorph tube awaiting protein analysis. Using a 50 μ l glass syringe, 40 μ l of each internal standard (1 ng/ μ l PGB₂-*d*4 in ethanol and 2 ng/ μ l 12 HETE-*d*8) were added to the samples. 530 μ l methanol was then added to each tube to adjust the final concentration of the solution to 15 % (v/v) methanol. The samples were then vortexed for 10 s to ensure thorough mixing and kept on ice in the dark for 30 min to allow protein precipitation. The homogenate was then centrifuged at 3000 rpm for 5 min at 4°C to remove any precipitated proteins. The supernatant was transferred to a clean glass tube using a glass pasteur pipette and stored on ice away from light ready for solid phase extraction.

2.5.4 Plasma sample preparation

Plasma was defrosted gently using hand temperature and a known volume (approximately 250 μ l) transferred to a clean glass tube. Ice cold water was then added to adjust the final volume to 3 ml. 530 μ l Methanol was added to each glass tube to adjust the concentration of the final solution to 15 % methanol (v/v). Using a 50 μ l glass syringe 40 μ l of each internal standard (1 ng/ μ l PGB₂-*d*4 in ethanol and 2 ng/ μ l 12 HETE-*d*8) were added to the glass tubes. The sample was then kept on ice away from light ready for solid phase extraction.

2.5.5 Solid phase extraction of lipid mediators

2.5.5.1 Solid phase preconditioning

Solid phase extraction (SPE) cartridges were conditioned prior to use. The cartridges were attached to a 12 position vacuum manifold and a vacuum of approximately 5 mm Hg applied. Each SPE cartridge was activated by washing firstly with 20 ml methanol and then with 20 ml water to remove the methanol. Throughout the washing procedure a drop-wise flow of solvent was maintained by adjusting the vacuum to avoid channelling effects. Air was not allowed to pass through the cartridges at any point thereby preventing the solid phase from drying out and causing the formation of channels reducing the efficiency of the extraction procedure.

2.5.5.2 Sample extraction

The pH of each sample (plasma or brain homogenate) was adjusted to 3 by adding 0.25 M hydrochloric acid to the sample in a drop-wise manner using a glass Pasteur pipette. The final pH of the homogenate was confirmed using narrow range indicator paper.

The acidified sample was immediately transferred to an activated SPE cartridge. Vacuum was applied such that the passage of homogenate through the cartridge was drop-wise. The cartridge was then washed with a) 20 ml 15% methanol to remove unbound material b) 20 ml water to remove polar molecules c) 10 ml hexane to remove water from the cartridge. At the end of this washing sequence the lipid mediators were eluted from the SPE cartridge with 15 ml methyl formate and collected in clean glass tubes. Methyl formate was then evaporated to dryness in the dark using a fine stream of nitrogen. The remaining residue was dissolved in 100 μ l ethanol and transferred to 100 μ l insert vials using a glass syringe. The vials were then placed in amber glass HPLC vials, sealed with open screw caps and Teflon septa and stored at -20°C in the dark awaiting analysis (up to one week).

2.5.6 LC/ESI-MS/MS analysis of prostanoid and hydroxy fatty acid mediators

2.5.6.1 Liquid chromatography

Prostanoids and hydroxy fatty acids were analysed in two separate chromatographic steps. All analyses were performed on a C18 column (Luna, 5 μm , 150 x 2.0 mm). The injection volume was 5 μ l for standards and 10 μ l for biological extracts. The sample chamber temperature of the autosampler was kept at 8°C . The column was maintained at ambient temperature and the flow rate was maintained at 0.2 ml/min.

2.5.6.2 Separation of prostanoids

Chromatographic separation of prostanoids was achieved using a gradient system composed of two solvents. Solvent A with composition acetonitrile: water: glacial

acetic acid, 45: 55: 0.02 (v/v/v) and solvent B with composition acetonitrile: water: glacial acetic acid, 90: 10: 0.02 (v/v/v) over the following gradient:

Time (min)	Solvent A (%)	Solvent B (%)
0	100	0
8.00	100	0
8.10	50	50
12.00	50	50
12.10	30	70
20.00	30	70
21.00	100	0
30.00	100	0

The total run time was 30 min including a 10 min wash cycle. At the end of each cycle of analysis the column was washed with acetonitrile: water: glacial acetic acid, 50: 50: 0.3 (v/v/v) for 1 hour and then acetonitrile: water, 50: 50 (v/v) overnight. The column was then used for the separation of hydroxy fatty acids.

2.5.6.3 Separation of hydroxy fatty acids

Chromatographic separation of the hydroxy fatty acids was achieved using an isocratic system composed of two solvents (C and D) mixed at a constant ratio of 95:5. Solvent C was methanol: water: glacial acetic acid, 80: 20: 0.02 (v/v/v) and solvent D was acetonitrile: water: glacial acetic acid, 45: 55: 0.02 (v/v/v). The total run time for the assay was 40 min including a 10 min wash. Following hydroxy-fatty acid analysis the

column was washed with methanol: water: glacial acetic acid, 50: 50: 0.3 (v/v/v) for 1 hour and then methanol: water, 50: 50 (v/v) overnight.

2.5.6.4 Multiple reaction monitoring assay

For analysis of both prostanoids and hydroxy fatty acids the instrument was operated in the negative mode. The capillary voltage was set at 3.0 KV; cone voltage: 35eV; RF lens 1: 0.3, aperture: 0.3, RF lens 2: 1.0, source temperature 120°C, desolvation temperature 360°C. The collision energy was optimised for each compound to get maximum sensitivity using argon as a collision gas. Dwell times were 0.2 s with a 0.1 s inter-scan delay. The cone gas flow was 40 L/hr and the desolvation gas flow was 400 L/hr. For optimal results the multiplier was set at 650 V and the collision cell pressure was 6.98×10^{-4} mbar. The MRM cycle time was 3.38 s for the prostanoid assay and 4.92 s for the hydroxy fatty acid assay.

The transitions used for the MRM assay of prostanoids were as follows: PGD₁ *m/z* 353>317, PGE₁ *m/z* 353>317, PGF_{1α} *m/z* 355>311, 6-keto PGF_{1α} *m/z* 369>163, PGB₂ *m/z* 333>175, PGB₂-d₄ *m/z* 337>179, PGD₂ *m/z* 351>271, PGE₂ *m/z* 351>271, PGF_{2α} *m/z* 353>193, PGJ₂ *m/z* 333>271, Δ¹²-PGJ₂ *m/z* 333>271, 15-deoxy-Δ^{12,14} PGJ₂ *m/z* 315>271, PGD₃ *m/z* 349>269, PGE₃ *m/z* 349>269, PGF_{3α} *m/z* 351>193, TXB₂ *m/z* 369>169, TXB₃ *m/z* 367>169, 8-iso-PGE₂ *m/z* 351>315, 8-iso-15-keto PGE₂ *m/z* 349>113, 8-iso-PGF_{2α} *m/z* 353>193, 8-iso-15-keto PGF_{2α} *m/z* 351>315, 13,14-dihydro PGE₁ *m/z* 355>337, 13,14-dihydro-15-keto PGE₁ *m/z* 353>335, 13,14-dihydro-PGF_{1α} *m/z* 357>113, 13,14-dihydro-15-keto PGF_{1α} *m/z* 355>193, 13,14-dihydro-15-keto PGE₂ *m/z* 351>333, 13,14-dihydro PGF_{2α} *m/z* 355>311, 13,14-dihydro-15-keto PGF_{2α} *m/z* 353>113 (Masoodi and Nicolaou, 2006).

The transitions used for the MRM assay of the hydroxy-fatty were as follows: 9-HODE m/z 295>171, 13-HODE m/z 295>195, 5-HEPE m/z 317>115, 18-HEPE m/z 317>133, 9-HEPE m/z 317>149, 8-HEPE m/z 317>155, 15-HEPE m/z 317>175, 12-HEPE m/z 317>179, 5-HETE m/z 319>115, 9-HETE m/z 319>123, 8-HETE m/z 319>155, 11-HETE m/z 319>167, 15-HETE m/z 319>175, 12-HETE m/z 319>179, 12-HETE- $d8$ m/z 328>185, LTB₄ m/z 335>195, 17S-HDHA m/z 343>281, RvE1 m/z 349>195, PD1 m/z 359>206, RvD1 m/z 375>141 (Masoodi *et al.*, 2008).

2.6 Analysis of fatty acid ethanolamides

2.6.1 Preparation of stock solutions

A-EA was purchased as a solution in ethanol at a concentration of 50 $\mu\text{g}/\mu\text{l}$. The solvent was completely evaporated under a gentle stream of nitrogen and the residue reconstituted with 2 ml ethanol to give a final concentration of 2.5 $\mu\text{g}/\mu\text{l}$. A further dilution was made by taking 40 μl of this solution (2.5 $\mu\text{g}/\mu\text{l}$) and adding 960 μl ethanol to give a final concentration of 100 $\text{ng}/\mu\text{l}$. This solution was then further diluted by taking 100 μl (of the 100 $\text{ng}/\mu\text{l}$ solution) and adding 900 μl ethanol to give a final solution at a concentration of 10 $\text{ng}/\mu\text{l}$. AEA- $d8$ was supplied in ethanol at a concentration of 1 $\mu\text{g}/\mu\text{l}$. A dilution was made by taking 10 μl of this solution (1 $\mu\text{g}/\mu\text{l}$) and adding 990 μl ethanol to give a final solution of concentration 10 $\text{ng}/\mu\text{l}$. The 10 $\text{ng}/\mu\text{l}$ solution was then diluted further with ethanol to yield a final solution at concentration 1 $\text{ng}/\mu\text{l}$. Dilutions of EPA-EA and DHA-EA were made by taking 10 μl of these solutions (1 $\mu\text{g}/\mu\text{l}$) and adding 990 μl ethanol to give final solutions of concentration 10 $\text{ng}/\mu\text{l}$.

2.6.2 Calibration lines

A composite solution of the three ethanolamide standards was prepared by pooling 20 μ l of each standard (10 ng/ μ l) and adding 940 μ l ethanol to give a composite solution at a final concentration of 200 pg/ μ l. Dilutions of the composite solution were made as shown by table 2.2.

Table 2.2. Preparation of fatty acid ethanolamide composite standard solutions

Final Concentration of composite standard (pg/ μ l)	Volume (μ l)		
	composite stock standard (200pg/ μ l)	AEA- <i>d8</i> (1ng/ μ l)	Ethanol
120	60	10	30
80	40	10	50
40	20	10	70
20	10	10	80

2.6.3 Limit of detection and limit of quantitation

The limit of detection (LOD) was estimated using a signal to noise ratio (S/N) of 3. The limit of quantitation (LOQ) was determined using a S/N ratio of 10. Peak integrations and S/N calculations were performed using the MassLynxTM V.4.0 software.

2.6.4 Tissue homogenisation and extraction of fatty acid ethanolamides

Method A: Brain tissue (cortical slice) was homogenised in 3 ml acetonitrile using a polytron electric homogeniser and spiked with 10 ng internal standard (10 µl of 1 ng/µl Anandamide-*d8*). Spiked homogenate was left at room temperature in the dark overnight to ensure complete extraction and denaturation of proteins. Pasteur pipettes were packed with cotton wool and washed with 1 ml acetonitrile. Samples were filtered to remove any solid material by applying the homogenate to the washed cotton wool and collecting the eluent. The cotton wool was washed with a further 1 ml acetonitrile and the eluent pooled to ensure complete collection of extract. The filtrate was dried under a fine stream of nitrogen. The flow of nitrogen was adjusted so that at any one time only ripples were formed over the surface of the organic solvent. The dried extract was then reconstituted in 100 µl ethanol and transferred to 100 µl insert vials using a glass syringe. The insert vials were then placed in amber glass HPLC vials, sealed with open screw caps and Teflon septa and stored at -20°C in the dark awaiting analysis.

Method B: Brain tissue (cortical slice) was homogenised in 3 ml ethyl acetate/hexane (9:1, v/v) using a polytron electric homogeniser and left on ice in the dark for 30 min to ensure complete extraction and denaturation of proteins. Each sample was then spiked with 10 ng internal standard (10 µl of 1 ng/µl Anandamide-*d8*). The sample was then washed with 10 % its volume water (300 µl water to wash 3 ml sample in organic solvent) to dissolve polar constituents as follows: water was added to the organic mix and the resulting solution was vortexed for 5 x 1 min. At all times the samples were kept on ice in between vortexing. To ensure phase separation the samples were centrifuged for 10 min (3000 rpm, 4°C). The organic supernatant was then collected and transferred to a clean glass vial. A further 3 ml organic solvent (ethyl

acetate/hexane (9:1, v/v)) was added to the tissue pellet and the washing and centrifugation steps repeated a total of three times to ensure complete extraction. The supernatants from each run were pooled together and stored on ice awaiting drying.

Pooled organic supernatants were dried under a fine stream of nitrogen. The flow of nitrogen was adjusted so that at any one time only ripples were formed over the surface of the organic solvent. The dried extract was then reconstituted in 1 ml chloroform and briefly vortexed ready for clean up by solid phase extraction. The remaining brain pellet from the homogenate was stored at -20°C awaiting protein analysis.

Method C: Brain tissue (cortical slice) was extracted in exactly the same way as in method B but using chloroform/methanol (2:1, v/v) instead of ethyl acetate/hexane.

2.6.5 Solid phase extraction of fatty acid ethanolamides

SPE cartridges (1ml, 100mg silica, Phenomenex Strata) were activated as follows: The cartridges were attached to a 12 position vacuum manifold (Phenomenex) and washed with 5x1 ml chloroform. Vacuum was not applied as gravity was sufficient to ensure a drop-wise flow. At the end of the washing phase the sorbent bed had become translucent confirming the silica was ready for sample application.

The reconstituted brain extracts (in 1 ml chloroform) were applied on an activated SPE cartridge (as described in section 2.6.4 (method B)). It was found that gravity was sufficient to maintain a drop-wise flow. Care was taken not to allow air to pass through the sorbent. The cartridge was then washed with 2x1 ml chloroform to remove any

unwanted organic compounds. Fatty acid ethanolamides were eluted from the cartridge using 4x1 ml 2% (v/v) methanol in chloroform.

The organic solvents were evaporated to dryness in the dark using a fine stream of nitrogen. The resulting residue was dissolved in 100 µl ethanol and transferred to 100 µl insert vials using a glass syringe. The vials were then placed in amber glass HPLC vials, sealed with open screw caps and Teflon septa and stored at -20°C in the dark awaiting analysis (up to one week).

2.6.6 LC/ESI-MS/MS analysis of fatty acid ethanolamides

2.6.6.1 Chromatographic separation of the fatty acid amides

A-EA, DHA-EA and EPA-EA were separated using an isocratic system composed of two solvents (A and B) mixed at a constant ratio of 30:70 (v/v). Solvent A was acetonitrile: water: formic acid, 2: 98: 1 (v/v/v) and solvent B was acetonitrile: water: formic acid, 98:2:1 (v/v/v). Although all analytes were eluted by 15 min, the run time was set at 30 min to wash the column prior to the next sample injection.

Separation was performed on a C18 column (Luna, 5 µm, 150 x 2.0 mm). The injection volume was 5 µl for standards and 10 µl for biological extract. The sample chamber temperature of the autosampler was kept at 8°C. The column was maintained at ambient temperature and the flow rate was maintained at 0.2 ml/min.

2.6.7 ESI-MS/MS optimisation

To optimise the MS and MS/MS conditions required for the most efficient ionisation of each analyte, individual standards at a concentration of 10 ng/ μ l were analysed by direct infusion through a syringe pump (flow rate 10 μ l/min) into the HPLC solvent flow (flow rate 0.2 ml/min). Ions were formed at the positive ionisation mode. The sensitivity of the method was optimised as the cone and capillary voltages were altered in the range of 30-40eV and 2000-4000V respectively. Optimal ionisation was observed when the cone voltage was set at 35eV and the capillary voltage at 35kV.

2.6.7.1 Multiple reaction monitoring assay

Selective fragmentation of the molecular ion $[M+H]^+$ of each analyte was performed in the collision cell applying a collision energy of 10-40 eV. Argon was used as the collision gas. The product ion spectra were recorded with a scan range m/z 50-400. RF lens values were adjusted to attain the best possible ion beam maximising the sensitivity of the instrument. The capillary voltage was set at 35 kV, cone voltage: 35 eV, RF lens 1: 5, Aperture: 0V and RF lens 2: 1.0. The source temperature was set at 120°C and the desolvation temperature was set at 360°C. The collision energy was optimised for each compound to get maximum sensitivity. The MRM assay was set up using the most profound precursor to product ion pairs at the optimal collision energy for each compound.

The transitions and collision energies (CE) used for the MRM assay of the fatty acid amides were as follows: AEA m/z 348>62; CE=14 eV, EPEA m/z 346>62; CE=22 eV, DHEA m/z 372>62; CE=22 eV, AEA-*d*8 m/z 356>63; CE=12eV.

2.6.8 Recovery

The recovery of the fatty acid ethanolamide extraction methodology was assessed using rat brain. Tissue was homogenised as described in section 2.6.4 (method B) and spiked with 10 µl internal standard (1 ng/µl) and 25 µl composite standard (200 pg/µl).

Fatty acid ethanolamides were extracted as described in section 2.6.4 (method B) and cleaned by SPE as described in section 2.6.5. Analysis of the extract was by LC/ESI-MS/MS as shown in sections 2.6.6 and 2.6.7. A set of un-spiked samples were prepared in parallel to the spiked ones, extracted and analysed similarly. At the same time, standards of the same amount as used to spike the biological samples were analysed by LC/ESI-MS/MS directly. The peak areas of the analytes in spiked, non-spiked and directly analysed standards were measured and used to calculate the recovery of the spiked standards using the following equation:

$$\text{Recovery} = \frac{\text{Peak area of spiked} - \text{Peak area of un-spiked}}{\text{Peak area of directly analysed standard}} \times 100$$

2.7 Protein assay

2.7.1 Sample preparation

1. Solid samples (e.g. denatured protein pellet): The pellet was suspended in 3 ml of 1 M NaOH and vortexed to ensure thorough mixing. The suspension was left in a waterbath for up to 1 hour at 40°C to ensure the sample had fully dissolved in the NaOH. 100 µl aliquots of the dissolved sample were taken and mixed with an equal volume of 1 M NaOH. Further dilutions of this solution were made and the amount of protein present determined as described in section 2.7.2.
2. Liquid samples (e.g. plasma): Biological homogenate (50 µl) was mixed with an equal volume of 0.5 M NaOH and vortexed to ensure all material had dissolved. A further 3 dilutions were made from this solution using 0.5 M NaOH.

2.7.2 Protein assay

The Bio-Rad DC protein assay kit (Bio-Rad, USA) was used in accordance with the manufacturer's instructions to determine the amount of protein present in the tissue samples. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. Colour development is primarily due to the amino acids tyrosine and tryptophan and to a lesser extent, cysteine and histidine. Bovine serum albumin (BSA) (30 mg), supplied as a dry powder as part of the kit, was dissolved in 20 ml water to give a final stock solution at a concentration of 1.5 mg/ml. 500 µl aliquots of this solution were stored at -20°C to be used in preparing calibration standards.

Serial dilutions of the BSA standard (1.5 mg/ml) were prepared using 0.5 M NaOH to give a calibration line ranging from 0.25 mg/ml to 1.5 mg/ml as follows:

Final BSA concentration (mg/ml)	Volume (μ l)	
	BSA (1.5 mg/ml)	NaOH (0.5 M)
0.25	25	125
0.5	50	100
0.75	75	75
1.0	100	50

A micropipettor was used to add 5 μ l of BSA standard or biological samples to a 96 well plate. 25 μ l of reagent A (alkaline copper tartrate solution) was added to each of the sample wells. 200 μ l of reagent B (Folin reagent) was added to each of the sample wells. The plate was placed away from light for 15 min to allow the colour to develop ready for absorbance measurement. A microplate reader was used to measure the absorbance of ultraviolet light ($\lambda=650$ nm) in each of the wells. Calibration lines were constructed plotting the absorbance of BSA solutions versus concentration (mg/ml); the lines were calculated using the least squares linear regression method. The concentration of each sample was read off the corresponding calibration line. The protein concentration of each biological sample was estimated as the mean value from each of the dilutions. Each assay was performed in triplicate.

2.8 Statistical analysis

Data were tested for statistical significance using the statistical package SPSS version 14.0. Data were assessed for normality using the Kolmogorov-Smirnov (KS) test together with a visual inspection of the histogram and p-p plot. Parametric data were analysed using a two-tailed independent samples student's *t* test or one way ANOVA with a Bonferroni correction for multiple comparisons. P values <0.05 were considered statistically significant.

**Chapter 3 : Analysis of fatty acid ethanolamides by liquid
chromatography tandem mass spectrometry**

3.1 Introduction

The *N*-acylethanolamides (NAE) are ubiquitous bioactive lipid molecules that are synthesised by neurones and other cells when the need arises. NAE such as anandamide (A-EA) have neuromodulatory actions and prevent neuronal damage by excitotoxicity. NAE have been reported to have analgesic effects and may also induce bradycardia, hypotension and sleep by virtue of their actions on the autonomic nervous system (Di Marzo *et al.*, 1998, Lake *et al.*, 1997, Walker *et al.*, 1999).

The bioactivities of AA, DHA and EPA are well known and the importance of these PUFA to brain function is well described. All of these PUFA can be metabolised to corresponding ethanolamides (Berger *et al.*, 2001). A-EA is known to regulate several physiological processes in the brain through its actions on the cannabinoid receptors and various neurotransmitter systems (Di Marzo *et al.*, 1994, Vogel *et al.*, 1993, Melis *et al.*, 2008). The biological actions of docosahexaenylethanolamide (DHA-EA) and eicosapentaenylethanolamide (EPA-EA) and their activities on the cannabinoid receptors in the brain are not documented. If it is found that levels of these NAE fluctuate with age or diet then this may be another mechanism through which AA, DHA and EPA exert their effects on the brain. Thus, a method is required to assess the presence of these NAE in neural tissue.

Current methods used to quantify the presence of NAE in brain have focussed primarily on A-EA, 2-AG and virodhamine (Hardison *et al.*, 2006, Richardson *et al.*, 2007, Schmid *et al.*, 2000, Schreiber *et al.*, 2007). An attempt has previously been made to detect A-EA, DHA-EA and EPA-EA in the brain and other tissues using an LC/ESI-

MS/MS assay with a methanol based gradient system (Artmann *et al.*, 2008). It was found however that this method resulted in poor chromatographic separation and relatively long retention times. Poor resolution of peaks makes quantification difficult and less reliable. A prolonged run time limits the number of samples that can be analysed in a given time and can adversely impact column life.

In the context of the current study therefore, the aim was to develop a LC/ESI-MS/MS method with high sensitivity and selectivity, which would enable the simultaneous detection and quantification of A-EA, DHA-EA and EPA-EA in brain. The profile of NAE may change with age or dietary intake of PUFA and so a method is required to assess levels of these NAE in brain at any given time.

3.2 Materials

The materials used for this study are listed in section 2.1 whilst the optimised extraction procedure has been described in section 2.6.4 (method B). The final protocol for LC/ESI-MS/MS analysis has been described in section 2.6.6.

3.3 Results

The development of LC/ESI-MS/MS was carried out in three steps as follows:

- a) Optimisation of MS
- b) Optimisation of MS/MS conditions
- c) Setting up of LC/ESI-MS/MS

3.3.1 Optimising MS conditions

The amide group in the NAE molecule easily becomes positively charged making these compounds highly suitable for ESI. The proposed mechanism for the formation of the molecular ion $[M+H]^+$ is shown in Figure 3.1. ESI limits fragmentation at the source and generates mainly singly charged ion species in the positive mode. NAE molecular ions were not detected in the negative ionisation mode (Figure 3.2).

Figure 3.1. Proposed site of ionisation of anandamide (A-EA) in the electrospray source.

Initially, the molecular ion $[M+H]^+$ for each of the individual ethanolamide standards was assessed. Individual standards were directly infused into the HPLC solvent flow to ensure that ionisation was compatible to the conditions of the assay. During infusion several parameters were altered to optimise the signal strength of the molecular ion. Optimum ionisation was achieved for all standards when the capillary voltage was set at 35 kV and the cone voltage at 35 V. The aperture and RF lens values were adjusted to give the maximum signal strength for each compound.

Figure 3.2 shows the molecular ions formed from A-EA in the positive (A) and negative (B) modes. The positively ionised species of anandamide has an m/z value of 348. This species can clearly be seen when the instrument is operated in the positive mode (A).

The negatively ionised species at m/z 346 is absent when the instrument is switched to the negative mode (B). This demonstrates that the anandamide molecule is ionised by the addition of a proton. Thus, NAE ionise best in the positive mode to yield a singly charged ion. The molecular ion $[M+H]^+$ for A-EA is m/z 348. DHA-EA has a mass of 371 giving rise to a molecular ion of m/z 372 (Figure 3.3). EPA-EA has a mass of 345 thereby yielding a molecular ion of m/z 346 (Figure 3.4). A-EA d_8 has a mass of 355 resulting in a molecular ion of m/z 356 (Figure 3.5).

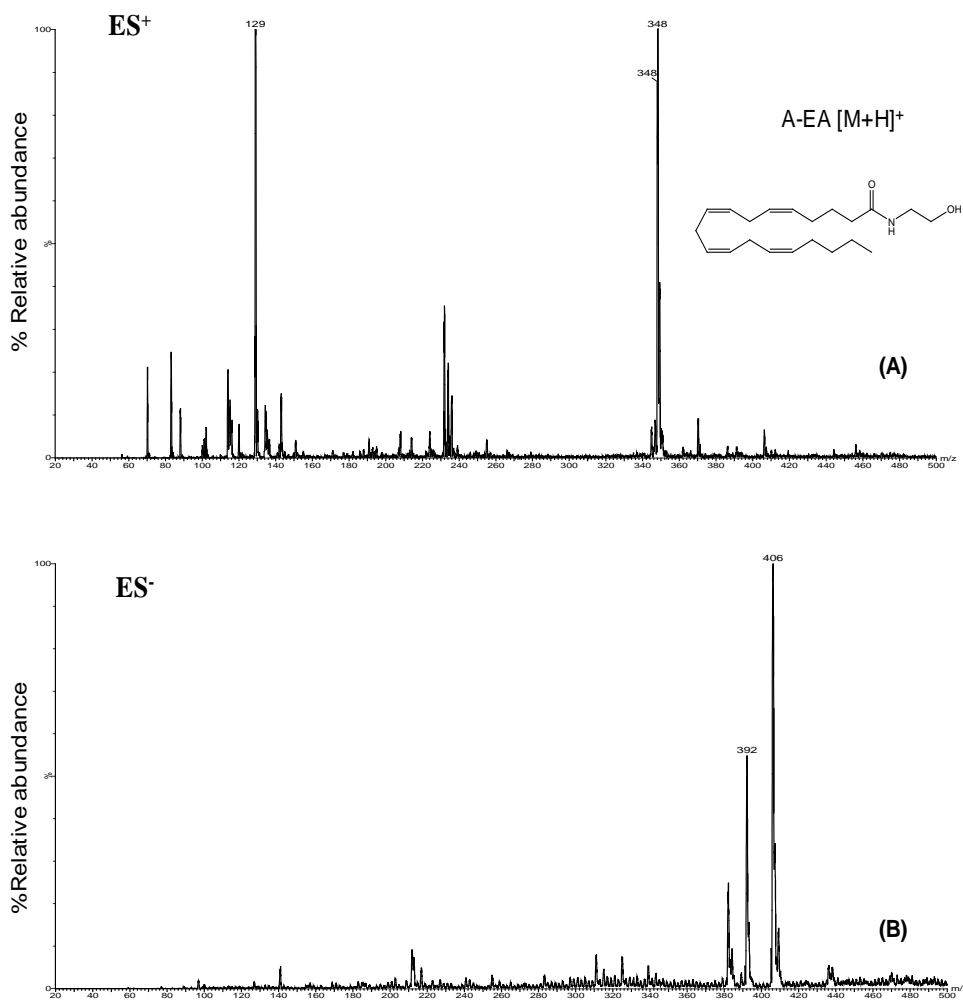


Figure 3.2. Ionisation (ESI-MS) of anandamide (A-EA) in the positive (A) and negative (B) modes.

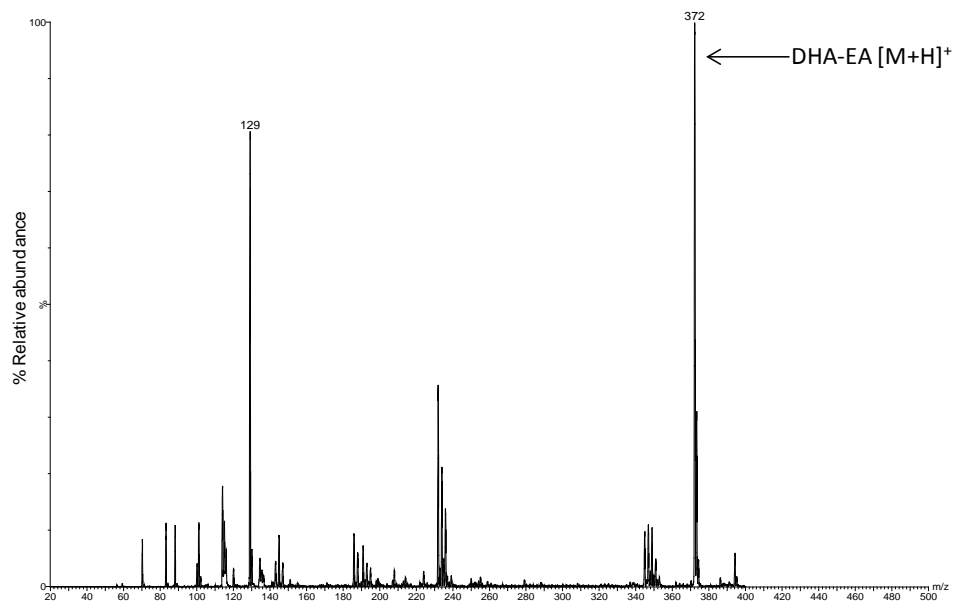


Figure 3.3. Ionisation (ESI-MS) of docosahexaenylethanolamide (DHA-EA) in the positive mode. The molecular ion $[M+H]^+$ is m/z 372.

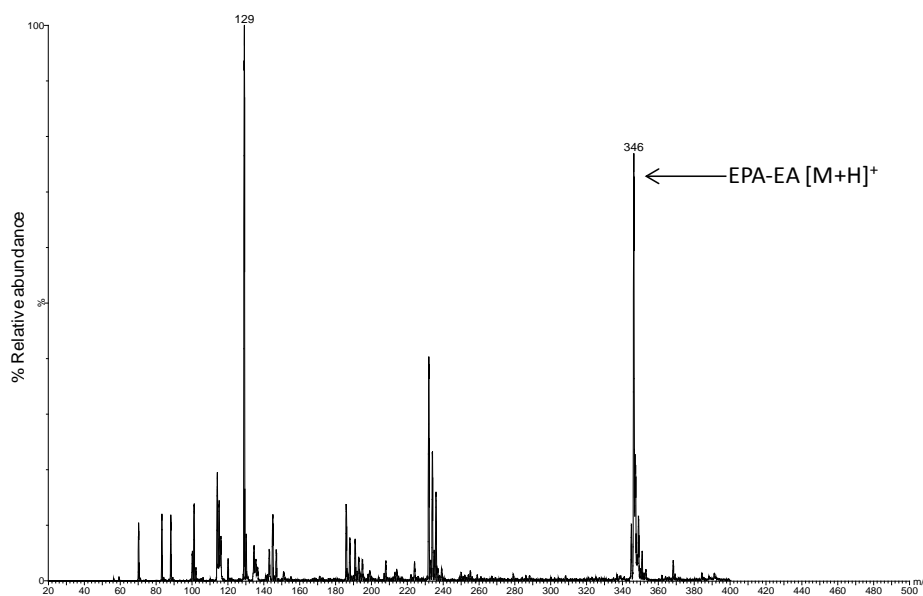


Figure 3.4. Ionisation (ESI-MS) of eicosapentaenylethanolamide (EPA-EA) in the positive mode. The molecular ion $[M+H]^+$ is m/z 346.

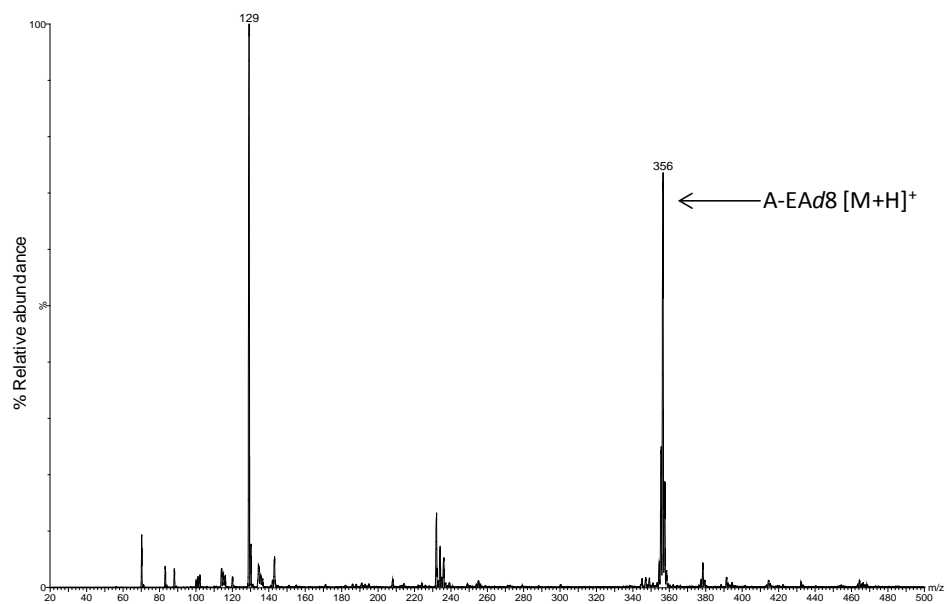


Figure 3.5. Ionisation (ESI-MS) of anandamide-*d*8 (A-EAd8) in the positive mode.

The molecular ion [M+H]⁺ is *m/z* 356.

3.3.2 Optimising the MS/MS assay

Tandem MS (MS/MS) experiments were performed to study the formation of product ions. A general mechanism for the proposed fragmentation of A-EA is outlined in Figure 3.6.

Figure 3.6. Proposed mechanism for the fragmentation of A-EA.

Figure 3.7 (A-C) shows that the principle product ion for DHA-EA, EPA-EA and A-EA has value of m/z 62 corresponding to the loss of the amide group. The deuterated internal standard (Figure 3.7 D) gives a principle product ion with m/z 63 with some ions of m/z 62 and 64 also forming at roughly half the intensity of the ion m/z 63.

It appears that in the case of the internal standard the fragmentation pathway is probably affected by the presence of deuterium atoms. The fragmentation of the internal standard proceeds as before but the presence of deuterium possibly leads to post fragmentation modification. A deuterium atom may replace one (m/z 63) or two (m/z 64) of the hydrogen atoms in the fragment ion increasing the overall mass of the product ion. This phenomenon has been described previously where deuterated molecules gave a higher m/z value than corresponding non-deuterated molecules (Richardson *et al.*, 2007, Weber *et al.*, 2004).

Figures 3.8 to 3.11 show how increasing collision energy affects the fragmentation of individual NAE. At this stage it was necessary find an optimum collision energy value for each molecular ion by balancing the presence of precursor ions with the formation of product ions.

In the case of A-EA Figure 3.8 shows that a collision energy value of 14 eV would give a large signal for the product ion. Similarly Figure 3.9 shows that the optimum collision energy for analysing DHA-EA would be 22eV. From Figures 3.10 and 3.11 the best collision energies for EPA-EA and A-EAd8 were 22eV and 12eV respectively.

A summary of the optimised transitions and collision energies for A-EA, DHA-EA, EPA-EA and AEA-*d8* has been shown in table 3.1. The optimised settings for A-EA and AEA-*d8* are in good agreement with literature (Richardson *et al.*, 2007).

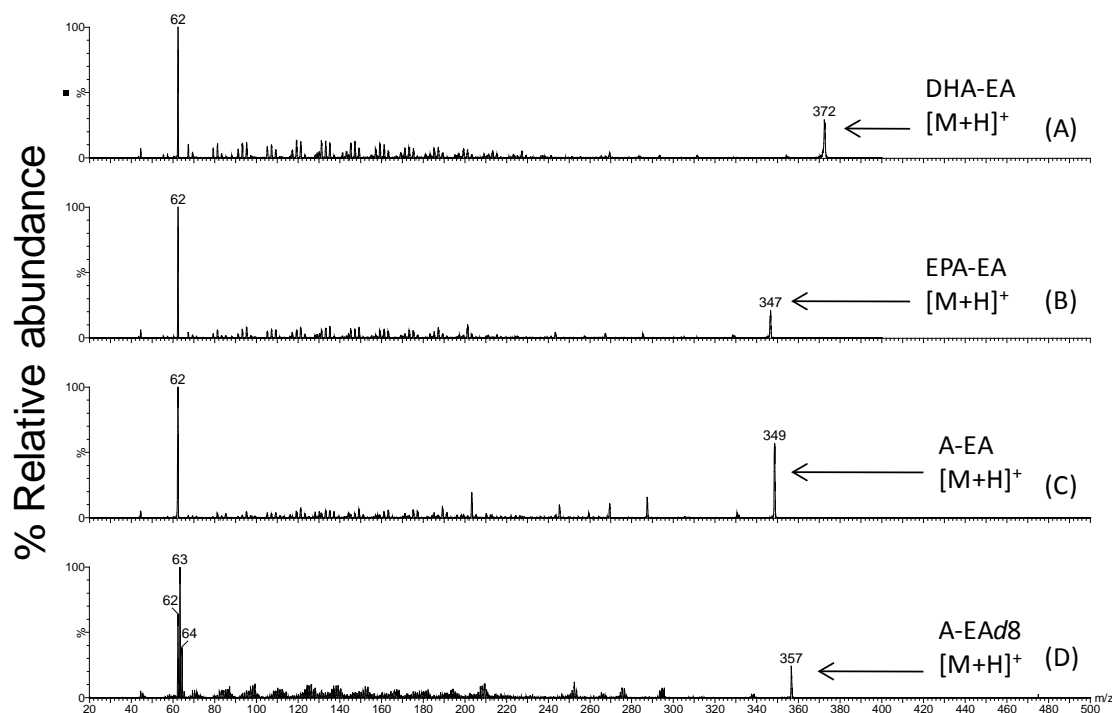


Figure 3.7. Fragmentation (ESI-MS/MS) of docosahexaenylethanolamide (DHA-EA; A), eicosapentasnylethanolamide (EPA-EA; B), anandamide (A-EA; C) and anandamide-*d8* (A-EA-*d8*; D).

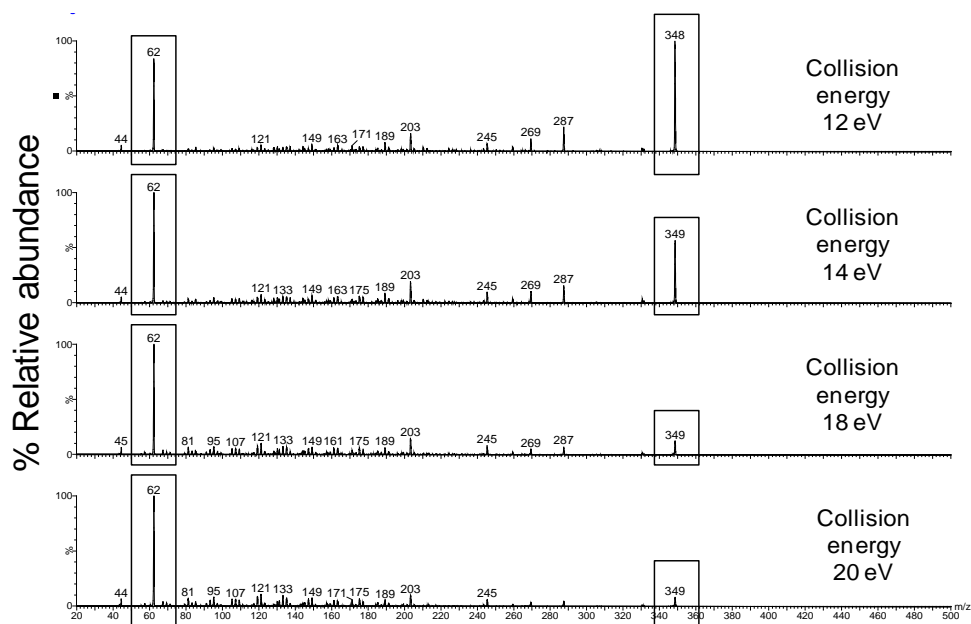


Figure 3.8. The effect of increasing collision energies on the fragmentation of anandamide (A-EA).

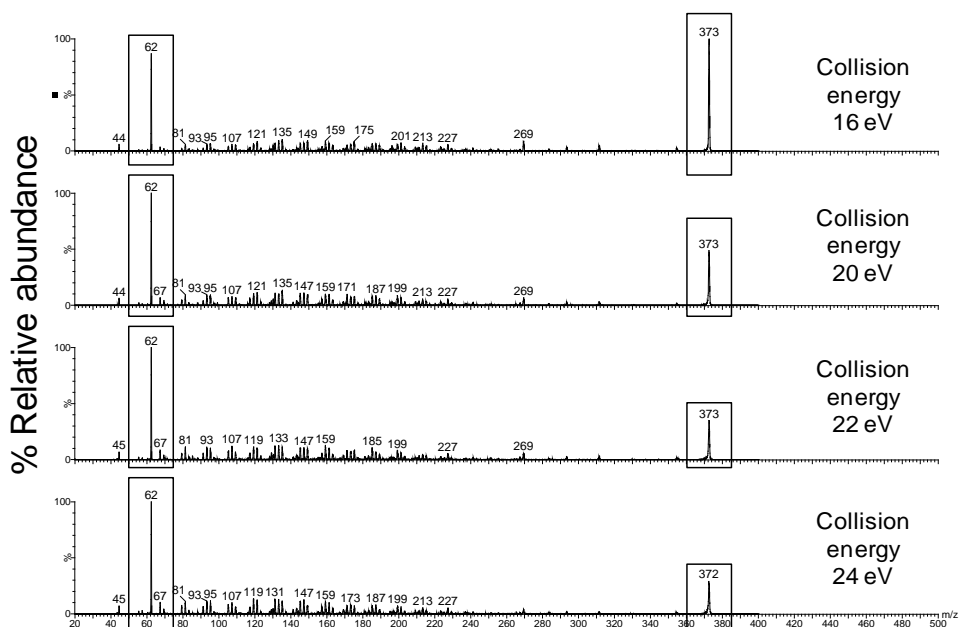


Figure 3.9. The effect of increasing collision energies on the fragmentation of docosahexaenylethanolamide (DHA-EA).

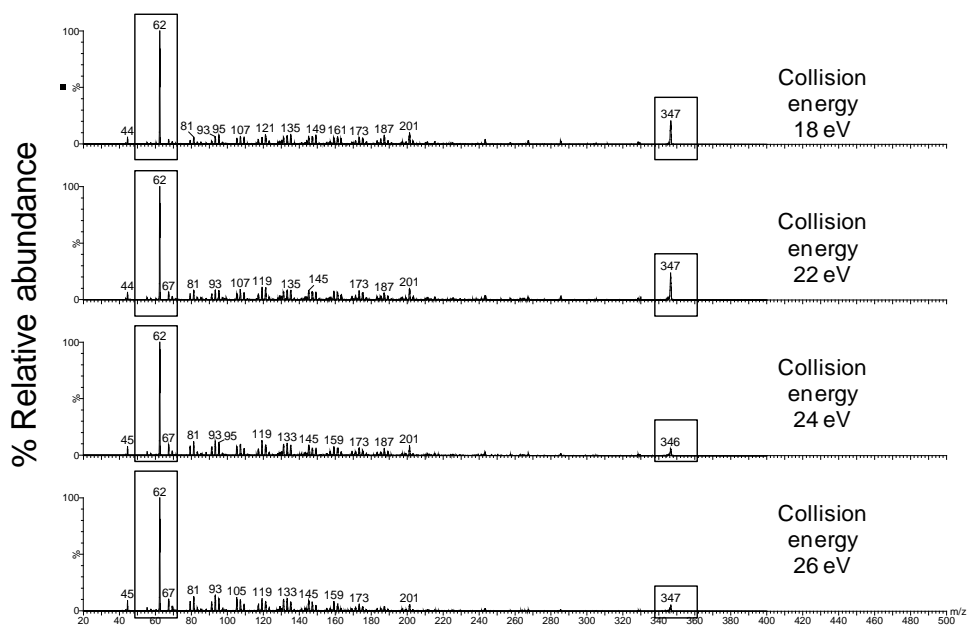


Figure 3.10. The effect of increasing collision energies on the fragmentation of eicosapentaenylethanolamide (EPA-EA).

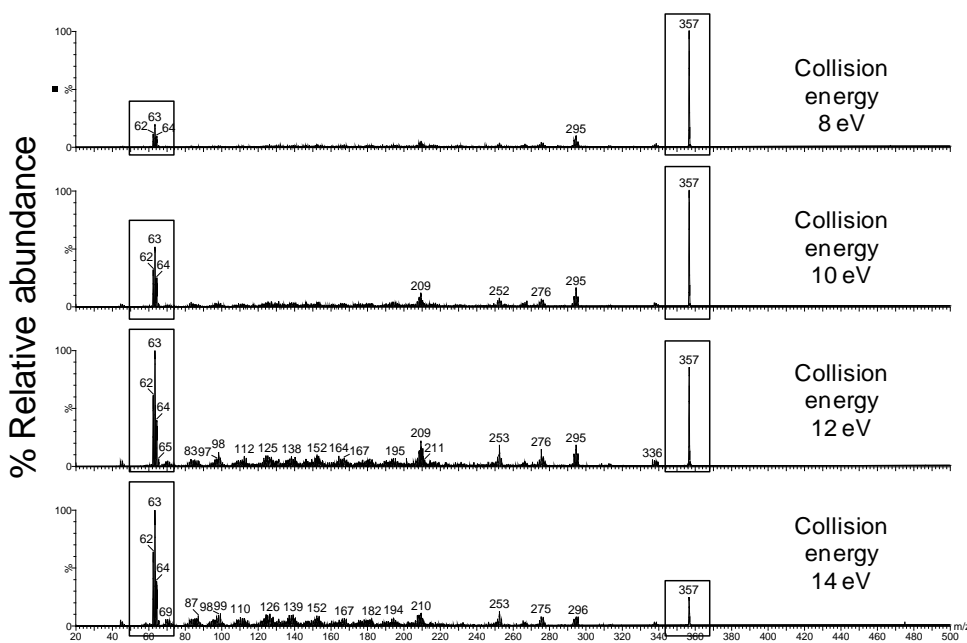


Figure 3.11. The effect of increasing collision energies on the fragmentation of anandamide-*d*8 (A-EAd8) (internal standard).

Table 3.1. Multiple reaction monitoring (MRM) transitions for tandem mass spectrometry (MS/MS) assay of anandamide (A-EA), docosahexaenoylethanolamide (DHA-EA) and eicosapentaenoylethanolamide (EPA-EA).

Compound	MRM (<i>m/z</i>)	Collision energy (eV)
A-EA	348>62	14
DHA-EA	372>62	22
EPA-EA	346>62	22
A-EA _{d8}	356>63	12

3.3.3 Optimising the LC/ESI-MS/MS assay

Acetonitrile has previously been the solvent of choice to elute A-EA and related cannabinoids from the C18 column and so a gradient was initially programmed, as suggested, using this solvent (Richardson *et al.*, 2007, Weber *et al.*, 2004). The suggested gradient system, whilst achieving good separation, prolonged the run time to well over 30 min.

In order to determine the optimum eluting conditions for each compound a shallow linear gradient was programmed going from 0% to 100% acetonitrile over 60 min. Using this system EPA-EA eluted at 45 min corresponding to approximately 60% acetonitrile within the column. DHA-EA eluted around 5 min after EPA-EA and was

closely followed by A-EAd8 and finally A-EA. All the NAE had eluted from the column by 60 min.

In order to bring the elution times forward isocratic runs were programmed composed of solvents A and B where solvent A was acetonitrile: water: formic acid, 2: 98: 1 (v/v/v) and solvent B was acetonitrile: water: formic acid, 98: 2: 1 (v/v/v). Runs were programmed corresponding to 65 %, 70% and 75 % solvent B. Figures 3.12 to 3.14 show how different concentrations of acetonitrile affected the elution of a cocktail of DHA-EA, EPA-EA and A-EA (10 ng of each compound on the column).

Overall the best chromatogram was obtained using an isocratic run at 70% solvent B (Figure 3.13). This gave a relatively short run time (<14 min) without compromising resolution.

3.3.4 Linearity, limits of detection and quantitation

Standard calibration curves were constructed for each compound over the range of 10 to 600 pg on the column. The assay was found to be linear over this range as the response was directly proportional to the analyte concentration as shown by Figure 3.15 (A-C).

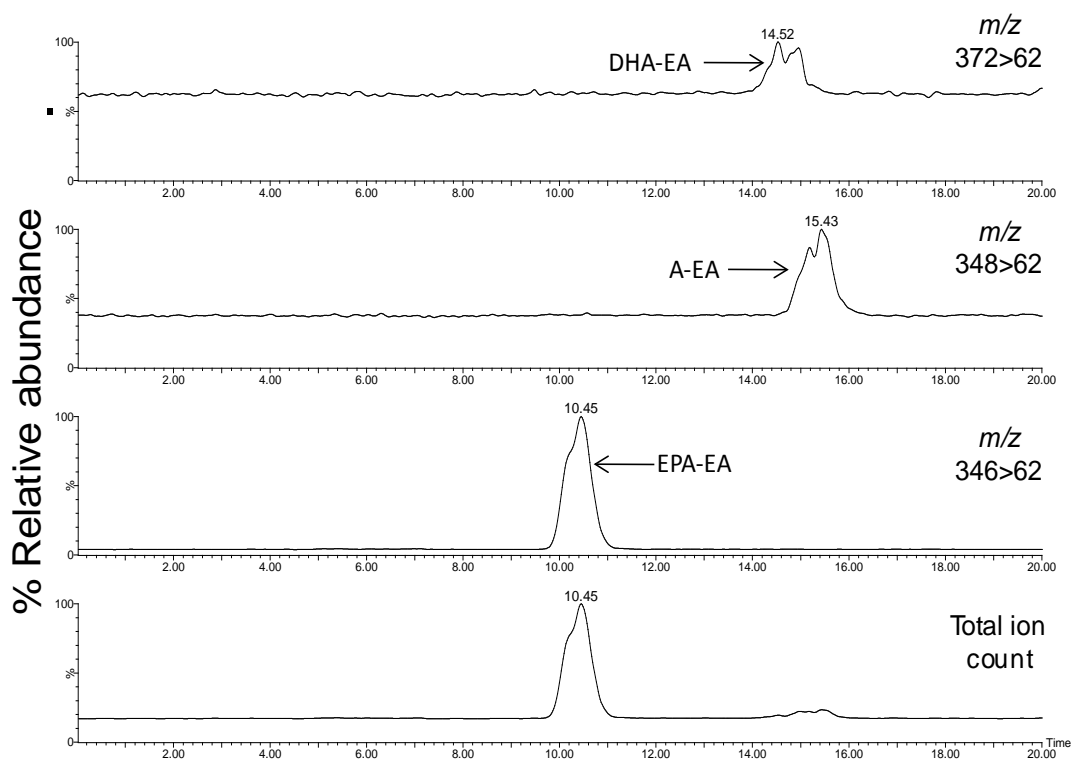


Figure 3.12. LC/ESI-MS/MS analysis of docosahexaenylethanolamide (DHA-EA), anandamide (A-EA) and eicosapentaenylethanolamide (EPA-EA) using 35% solvent A and 65% solvent B (isocratic system). Solvent A was acetonitrile: water: formic acid, 2: 98: 1 (v/v/v) and solvent B was acetonitrile: water: formic acid, 98: 2: 1 (v/v/v).

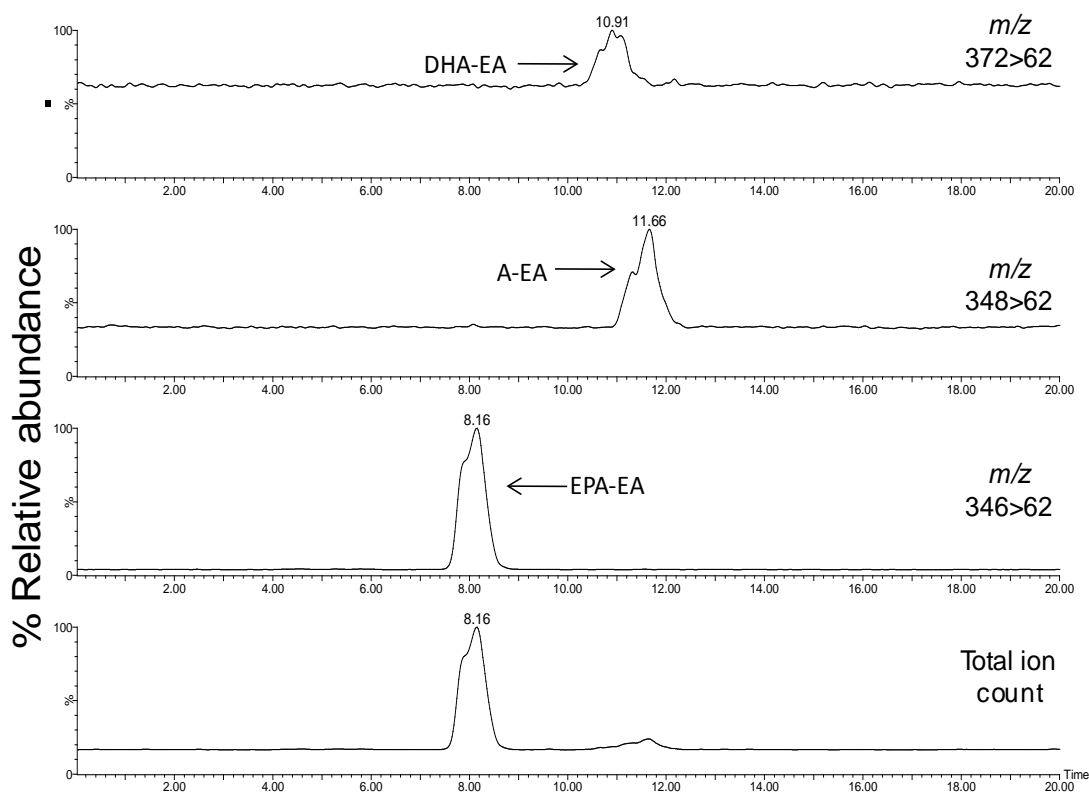


Figure 3.13. LC/ESI-MS/MS analysis of docosahexaenylethanolamide (DHA-EA), anandamide (A-EA) and eicosapentaenylethanolamide (EPA-EA) using 30% solvent A and 70% solvent B (isocratic system). Solvent A was acetonitrile: water: formic acid, 2: 98: 1 (v/v/v) and solvent B was acetonitrile: water: formic acid, 98: 2: 1 (v/v/v).

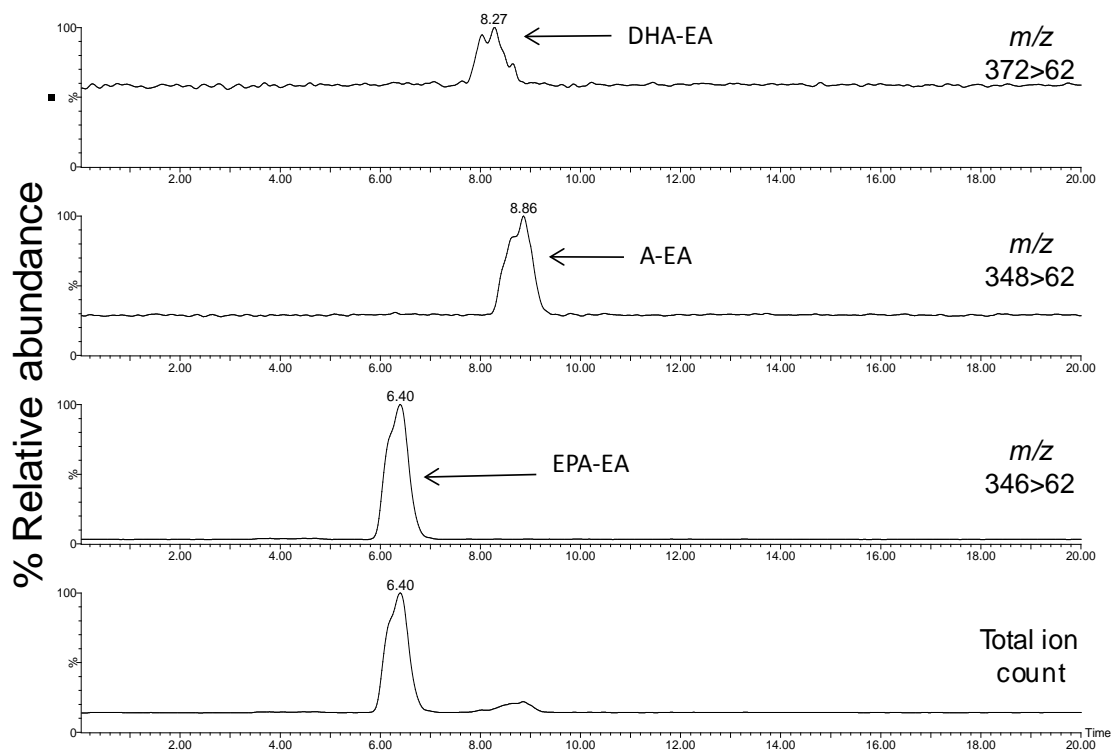


Figure 3.14. LC/ESI-MS/MS analysis of docosahexaenylethanolamide (DHA-EA), anandamide (A-EA) and eicosapentaenylethanolamide (EPA-EA) using 25% solvent A and 75% solvent B (isocratic system). Solvent A was acetonitrile: water: formic acid, 2: 98: 1 (v/v/v) and solvent B was acetonitrile: water: formic acid, 98: 2: 1 (v/v/v).

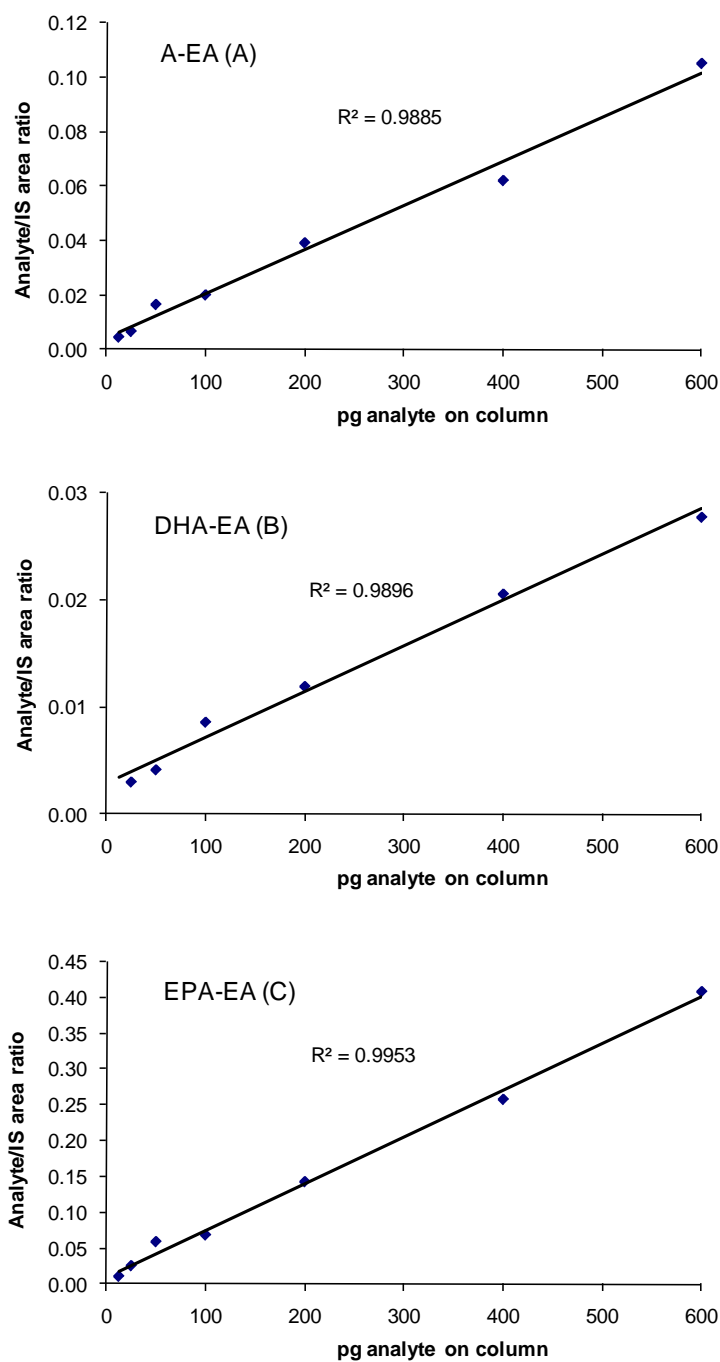


Figure 3.15. Representative calibration lines (10-600 picogram (pg) analyte on column) for (A) arachidonylethanolamide (A-EA), (B) docosahexaenylethanolamide (DHA-EA) and (C) eicosapentaenylethanolamide (EPA-EA).

Calibration lines were calculated by the least-squares linear regression method. This assay was found to be linear over the range 1pg on the column to 500pg on the column (table 3.2). The limit of detection (LOD) was calculated by using a signal to noise (S/N) ratio of 3. The LOD was found to be in the range 0.25-1pg on the column. The limit of quantitation (LOQ) was estimated by using an S/N ratio of 10. The LOQ was found to be in the range 1-5pg on the column. Peak integrations and S/N calculations were performed using the Masslynx software V4.0.

Table 3.2. Linearity, limit of detection (LOD), limit of quantitation (LOQ) of the ESI/LC-MS/MS assay for anandamide (A-EA), docosahexaenoylethanolamide (DHA-EA) and eicosapentaenoylethanolamide (EPA-EA).

Compound	Equation	Correlation Coefficient	LOD (pg)	LOQ (pg)
A-EA	$y=0.0057x-0.0097$	0.986	1	5
DHA-EA	$y=0.0027x-0.0106$	0.990	1	5
EPA-EA	$y=0.0022x+0.0132$	0.995	0.25	1

3.3.5 Extraction of fatty acid ethanolamides from brain tissue

Initially, the brain tissue homogenate was left overnight in 100% acetonitrile to extract all NAE and facilitate protein denaturation as described previously (Weber *et al.*, 2004). Whilst this approach did manage to extract NAE from brain there was co-extraction of

impurities resulting in ion suppression effects. Furthermore, leaving extracts overnight at room temperature increased the probability of degradation.

A second approach was undertaken whereby NAE were extracted with chloroform/methanol (2:1 v/v) as described by other methods (Schreiber *et al.*, 2007) and the extract purified using either C18 or silica SPE cartridges. This approach improved the extraction efficiency and resulted in an improved recovery of NAE.

A third approach involved extracting NAE from brain using an ethyl acetate/hexane (9:1, v/v) based system as described previously (Richardson *et al.*, 2007). The extract was subsequently purified using either C18 or silica SPE cartridges. This method of extraction gave the best recovery values for NAE and cleanest overall chromatograms.

3.3.5.1 Optimisation of solid phase extraction

Biological extracts were initially purified by introducing a C18 SPE cartridge based clean up step as described previously (Richardson *et al.*, 2007, Schmid *et al.*, 2000). Initially, the solvent was evaporated and dried extract was reconstituted in 15% methanol. It was found that most of the analyte was lost in the sample clean up step resulting in poor recoveries. The same SPE protocol was repeated using a modified wash step. In this case cartridges were washed with only 15% methanol alone rather than in addition to water and hexane. The shortened wash step resulted in the co-elution of many impurities that were found to reduce the sensitivity of the assay.

A second approach was undertaken whereby the brain extract was reconstituted in chloroform and SPE performed using a normal phase silica SPE cartridge. Cartridges

were washed with chloroform after application of the extract. NAE were then eluted from the cartridge using 2% methanol in chloroform. The % methanol in the elution solvent was varied in an attempt to optimise recovery. A lower concentration of methanol (1%) resulted in incomplete elution of NAE from the cartridge. A higher concentration of methanol (3%) resulted in co-elution of impurities that reduced the sensitivity of the LC/ESI-MS/MS assay. Acidification of the eluting solvent with formic acid as suggested by previous methods (Richardson *et al.*, 2007) was found to increase co-elution of impurities giving a relatively poor chromatogram. Final method sample clean up was performed using silica based SPE cartridges without addition of acid to the eluting solvent. This optimised recovery values are shown in table 3.3.

3.3.6 Recovery

In order to assess the recovery of the extraction methodologies rat brain was spiked with a cocktail of mixed NAE standards (5 ng/compound) and analysed by LC/ESI-MS/MS. Spiked tissue samples were analysed in parallel with extracts of unspiked tissue samples and the difference in peak-area values was related to the amount of the spike. Recovery was calculated by comparing the peak-area value of the spike to that from the analysis of metabolites that did not undergo extraction which thus represented 100% of the initial concentration. The resulting values show recoveries of each metabolite expressed as a percentage of the initial concentration (table 3.3). The recoveries were found to range from 85 to 127% depending on the analyte. These results are in close agreement with previous literature where recovery of A-EA has been reported to range from 95 to 135% (Richardson *et al.*, 2007).

Table 3.3. Recovery of 5 ng docosahexaenoylethanolamide (DHA-EA), anandamide (A-EA) eicosapentaenoylethanolamide (EPA-EA) and anandamide-*d8* (AEAd8) from spiked brain tissue.

Fatty acid ethanolamide	% recovery
DHA-EA	86 ± 11
A-EA	104 ± 5
EPA-EA	128 ± 10
A-EAd8	95 ± 12

3.3.7 Discussion

The aim of the present study was to develop an extraction and LC/ESI-MS/MS based method for the analysis of A-EA, DHA-EA and EPA-EA from brain tissue. Previous methods have focussed primarily on cannabinoids and other NAE such as oleoylethanolamide or palmitoylethanolamide (Richardson *et al.*, 2007, Schmid *et al.*, 2000, Schreiber *et al.*, 2007). To date there is only one other published method that has previously been employed for the analysis of a range of different cannabinoids and NAE including A-EA, DHA-EA and EPA-EA (Artmann *et al.*, 2008). When this method was attempted it gave long retention times with relatively poor peak shape and separation. This may be due to variation in experimental equipment. Thus, there was a

need to develop a novel method specifically capable of efficiently separating and quantifying A-EA, DHA-EA and EPA-EA from brain tissue.

In addition to developing an LC/ESI-MS/MS based method an attempt was made to improve existing extraction methodologies to improve recovery and reduce co-extraction of impurities. Although previously published methods were available for the extraction of NAE from brain tissue (Kingsley and Marnett, 2003, Richardson *et al.*, 2007) there was no information available for the success of these methods in extracting EPA-EA or DHA-EA. Thus different protocols were tested that involved the use of varying solvent based extraction procedures and different types of SPE cartridges of varying polarity. In the final method NAE were extracted from brain tissue using an ethyl acetate/hexane based system. Extract was reconstituted in chloroform and purified using silica SPE cartridges.

The LC/ESI-MS/MS assay presented in this study can be applied to simultaneously profiling several NAE present within brain tissue with high throughput and sensitivity. The presented method permits comparison analysis of NAE within the same biological sample without requiring any derivatisation step. Comparatively short retention times mean that several samples can be analysed relatively quickly without the need for lengthy wash times. The detection limits for NAE in this study ranged from 0.25 pg on the column to 1 pg on the column and were comparable to those described for previous GC-MS methods (Hardison *et al.*, 2006, Yang *et al.*, 1999). The recovery of the extraction methodology ranged from 86 % to 128 %. This method has been developed specifically to compare EPA and DHA derived NAE with A-EA.

**Chapter 4 : The effects of dietary eicosapentaenoic acid
and docosapentaenoic acid on eicosanoids and hydroxy
fatty acids in young and aged brain cortical tissue**

4.1 Introduction

Ageing has previously been linked to cognitive decline associated with altered glucose metabolism and a change in brain PUFA composition (Delion *et al.*, 1997, Gage *et al.*, 1984, Martin *et al.*, 2002b). It has been shown that ageing is accompanied by altered gene expression of COX and LOX in the brain (Aid and Bosetti, 2007, Manev *et al.*, 2000) and an overall increase in oxidative stress resulting in oxidation of PUFA (Navarro and Boveris, 2004, Yehuda *et al.*, 2002). Ageing has also been associated with neuroinflammation and neurodegenerative disease through the release of inflammatory cytokines (Nolan *et al.*, 2005, Sparkman and Johnson, 2008).

It has been shown that neuroinflammatory changes linked to ageing can be modulated through the use of COX inhibitors (Casolini *et al.*, 2002, Jung *et al.*, 2006). Thus, eicosanoids appear to play a fundamental role in the ageing process and subsequent development of neurodegenerative disease. Altered levels of AA, EPA or DHA coupled to changes in expression of COX or LOX during ageing may affect the synthesis of eicosanoids and other hydroxy fatty acids downstream. Several studies have demonstrated that ageing can significantly change the profile of brain eicosanoids and hydroxy fatty acids (Montine *et al.*, 2002a, Baek *et al.*, 2001, Marmol *et al.*, 1999).

It has been shown that supplementing the diet with EPA can attenuate age related cognitive deficit and increase long term potentiation (LTP) (Lynch *et al.*, 2007, Martin *et al.*, 2002a, Minogue *et al.*, 2007). In particular EPA has been shown to prevent neuroinflammation (Kavanagh *et al.*, 2004, Song and Horrobin, 2004, Song *et al.*, 2007). Elevated levels of oxidative stress are known to reduce LTP but EPA intake has

been shown to effectively reduce oxidative stress thereby preserving brain LTP (Lonergan *et al.*, 2002).

The metabolic fate of dietary *n*-3 DPA is not well understood. It is however known that DPA can be converted to DHA through the Sprecher pathway (Figure 1.1) (Rosenthal *et al.*, 1991). It is also known that DPA can be retroconverted to EPA through β -oxidation (Brossard *et al.*, 1996, Achard *et al.*, 1995). Several studies have shown that both EPA and DPA are inter-converted in vivo giving these two PUFA similar biological actions (Benistant *et al.*, 1996, Innis and Dyer, 2002, Kanayasu-Toyoda *et al.*, 1996). This has led to the development of the hypothesis that DPA exerts its protective actions indirectly through conversion into EPA. Unlike EPA however, it is thought that DPA cannot form eicosanoids. To date there are no studies examining the relationship between DPA intake and eicosanoid or hydroxy fatty acid formation in the brain.

It has been shown that neural tissue contains particularly low levels of EPA and DPA and that dietary supplementation can increase the proportion of these PUFA within the brain whilst simultaneously reducing levels of AA (Kitajka *et al.*, 2002, Philbrick *et al.*, 1987). Thus, it has been shown that supplementing the diet with EPA can change brain PUFA composition. Altering the profile of brain PUFA through EPA intake may affect downstream eicosanoid and hydroxy fatty acid formation thereby influencing the pathology of inflammatory or neurodegenerative processes.

To date there is no consensus on the optimum dose of EPA to elicit changes in brain eicosanoid profile. It has however been shown that rats supplemented with 50 mg ethyl-EPA per day for 4 weeks were able to successfully block inflammatory changes in

the brain (Kavanagh *et al.*, 2004). Other work has shown that 125 mg per day of EPA for 4 weeks significantly attenuated age induced changes in cytokine balance in the brain and preserved LTP (Lynch *et al.*, 2007).

It is therefore hypothesised that a dose of either 50 mg EPA per day or 125 mg EPA per day should stimulate a change in the profile of eicosanoids in rat brain. It is further hypothesised that dietary intake of DPA will have the same effect on brain eicosanoid profile as EPA due to retroconversion. It is hypothesised that ageing will change the profile of eicosanoids and hydroxy fatty acids in the brain and that supplementing the diet with EPA or DPA will modulate this change.

In the present study LC/ESI-MS/MS has been applied to investigate the effect of EPA on brain eicosanoid and hydroxy fatty acid profiles. The effects of ageing on brain eicosanoid and hydroxy fatty acid concentrations will also be investigated and compared with any changes in the profiles that may be affected by supplementing the diet with EPA or *n*-3 DPA.

4.2 Methods

All animal work was carried out by Professor M A Lynch's lab at the Bio Resources Unit, Trinity College, Dublin. (See methods section 2.3 for details). Eicosanoids and hydroxy fatty acids were extracted as described in sections 2.5.3. LC/ESI-MS/MS analysis is described in section 2.5.6.

4.3 Results

4.3.1 The effect of eicosapentaenoic acid on prostanoid production in the brain cortex

Figure 4.1 shows the concentration of prostanoids present in young (aged 2-3 months) rat brain cortical tissue after supplementing the diet with 50mg EPA per day (Figure 4.1(A)) or 125mg EPA per day (Figure 4.1(B)) for 28 days compared to control.

A one way analysis of variance (ANOVA) using post-hoc Bonferroni correction for multiple comparisons was used to check for statistical significance. No statistically significant results were found.

Overall PGD₂ was found to be the most abundant prostanoid in brain cortical tissue (492 pg/mg protein) followed by PGF_{2α} (152 pg/mg protein) and PGE₂ (80pg /mg protein) respectively. There were minor quantities of other prostanoids found but many of these were at or near the limit of quantitation. The prostanoid found to be at the lowest concentration was PGJ₂ (29 pg/mg protein).

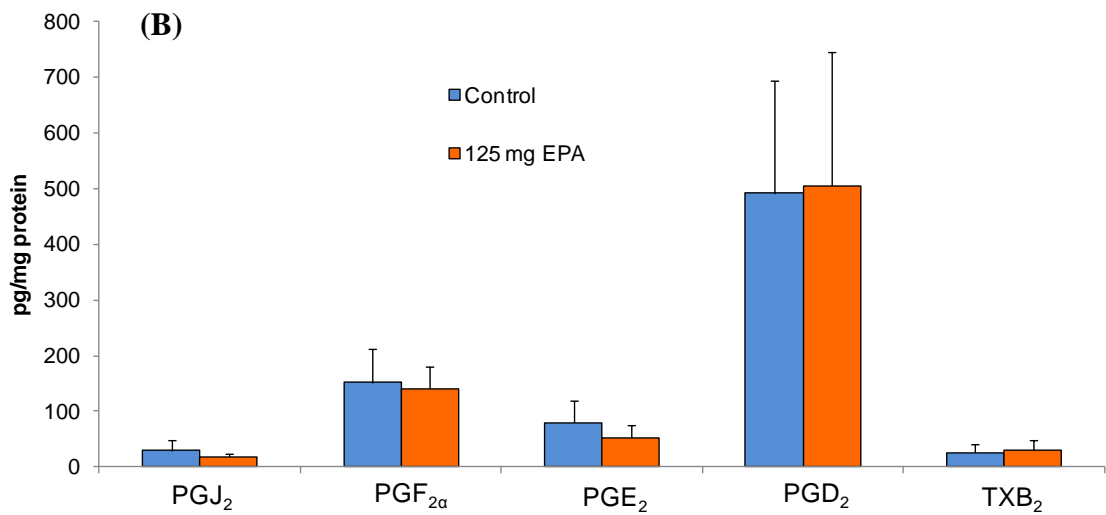
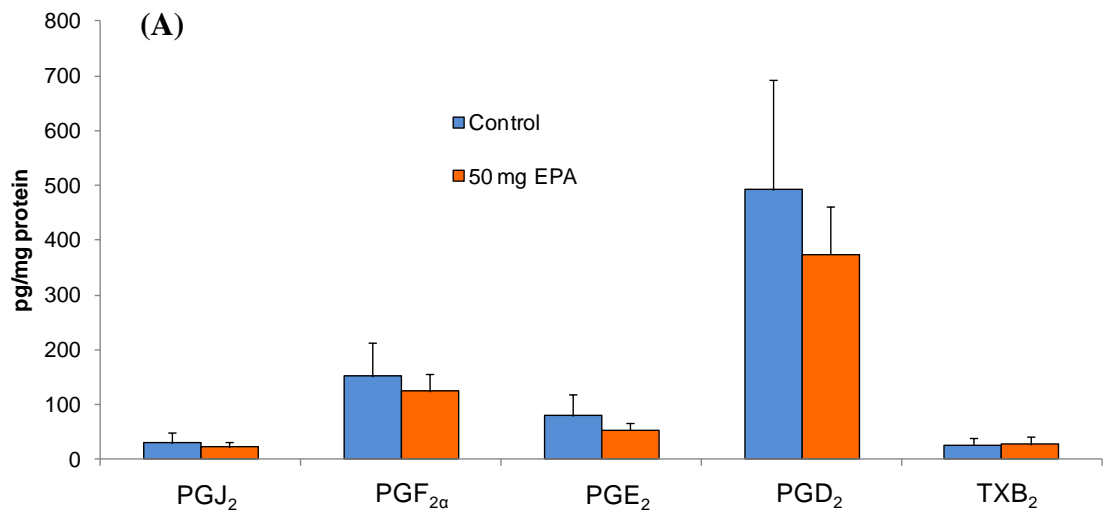


Figure 4.1. The effect of eicosapentaenoic acid (EPA) supplementation for 28 days on prostanoinds in young rat brain cortical tissue; (A) 50 mg EPA/rat/day (B) 125 mg EPA/rat/day. (Results expressed as mean \pm standard deviation; $n=8$ per group).

4.3.2 The effect of eicosapentaenoic acid on hydroxy fatty acid production in the brain cortex

Figure 4.2 shows the concentration of hydroxy fatty acids present in young (aged 2-3 months) rat brain cortical tissue after supplementing the diet with 50mg EPA per day (Figure 4.2(A)) or 125mg EPA per day (Figure 4.2(B)) for 28 days compared to control.

A one way analysis of variance (ANOVA) using post-hoc Bonferroni correction for multiple comparisons was used to check for statistical significance. No statistically significant results were found.

Overall 12-HETE was found to be the most abundant hydroxy fatty acid in brain cortical tissue (410 pg/mg protein) followed by 11-HETE (235 pg/mg protein) and 15-HETE (206 pg /mg protein) respectively. The hydroxy fatty acid found to be present at the lowest concentration was 9-HETE (24 pg/mg protein). Other hydroxy fatty acids that were detected included 13-HODE, 9-HODE, 8-HETE and 5-HETE.

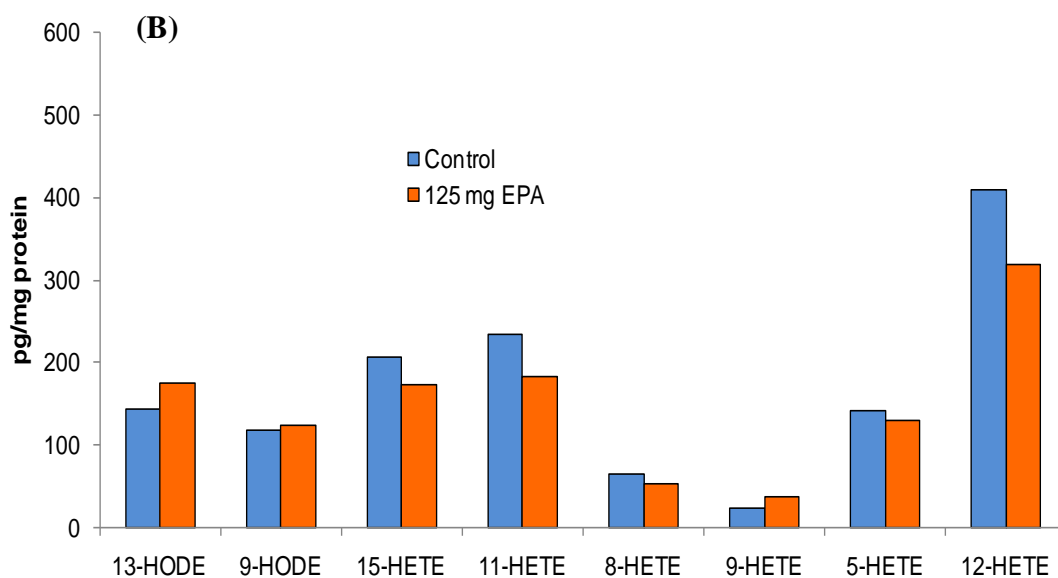
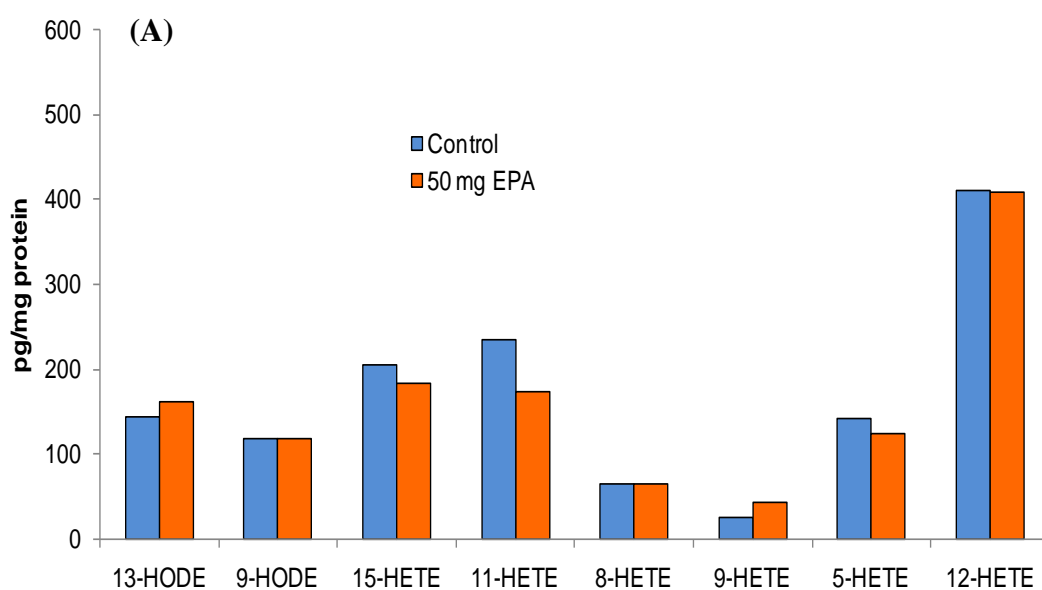


Figure 4.2. The effect of eicosapentaenoic acid (EPA) supplementation for 28 days on hydroxy fatty acids in young rat brain cortical tissue; (A) 50 mg EPA/rat/day (B) 125 mg EPA/rat/day. (Results expressed as mean \pm standard deviation; $n=8$ per group).

4.3.3 The effect of ageing on the profile of prostanoids and hydroxy fatty acids in the brain cortex

Figures 4.3 (A) and 4.3 (B) show the effect of ageing on the prostanoid and hydroxy fatty acid profile of rat brain cortex respectively. Overall there is a significant increase in the proportion of PGD_2 ($p=0.028$; independent sample T-test) and a significant decrease in the proportion of $\text{PGF}_{2\alpha}$ ($p=0.04$; independent sample T-test) in brain cortex during ageing.

Ageing resulted in a significant decrease in the proportion of 11-HETE ($p<0.001$; independent sample T-test) and also 15-HETE ($p=0.005$; independent sample T-test). Ageing significantly increased cortical 12-HETE ($p=0.04$; independent sample T-test). Ageing did not significantly change the proportion of other hydroxy fatty acids in the brain cortex.

The most abundant prostanoid in both young (aged 2-3 months) and aged (aged 22-24 months) cortex was PGD_2 (41 % total prostanoids in young brain and 56 % total prostanoids in aged cortex). Small amounts of PGE_1 (less than 1% total prostanoids in both young and aged cortex) and PGD_1 (less than 1% total prostanoids in both young and aged cortex) were also detected but these were at the limits of detection for this method.

The most abundant hydroxy fatty acid in young tissue was found to be 11-HETE (34 % total hydroxy fatty acids) whereas in aged tissue it was 12-HETE (44 % total hydroxy fatty acids).

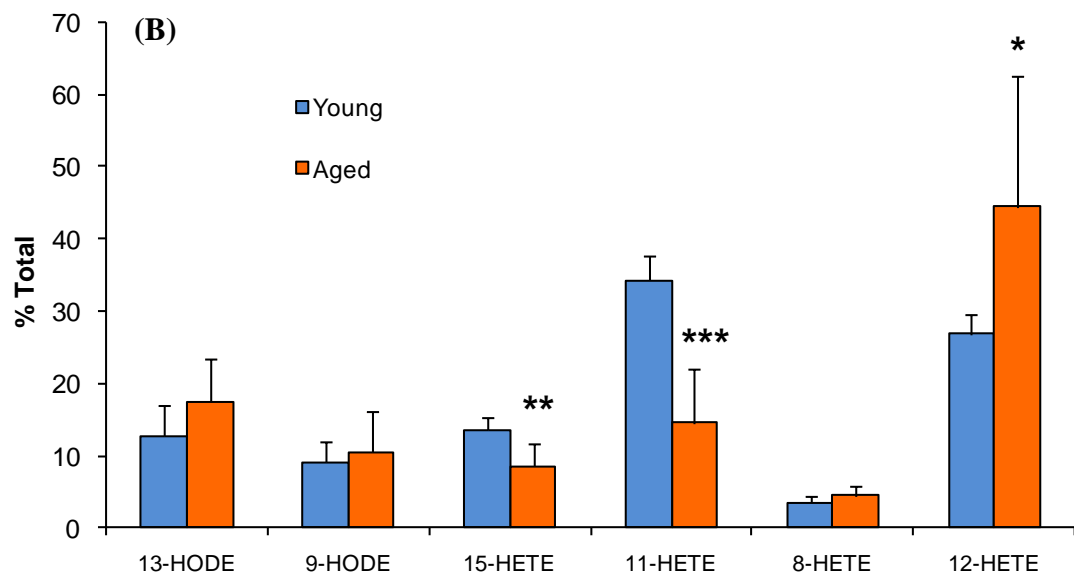
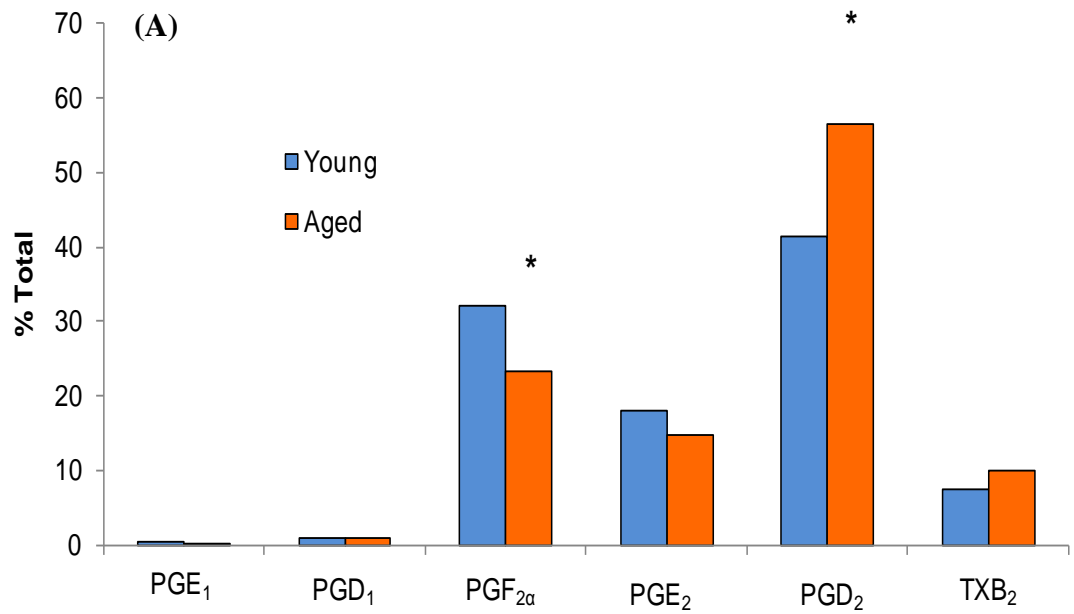


Figure 4.3. The effect of ageing on rat brain prostanooid (A) and hydroxy fatty acid (B) profiles (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (Results expressed as mean \pm standard deviation; $n = 6$ per group).

4.3.4 The effect of eicosapentaenoic acid on the profile of prostanoids and hydroxy fatty acids in the young brain cortex

Figure 4.4 shows the effect of supplementing the diets of young animals with EPA (200 mg/kg/day) for a period of 8 weeks on the prostanoid (A) and hydroxy-fatty acid (B) profiles of rat brain cortex. Supplementing the diets of young animals with EPA did not significantly change the proportions of prostanoids or hydroxy fatty acids in the brain cortex.

4.3.5 The effect of eicosapentaenoic acid on the profile of prostanoids and hydroxy fatty acids in the aged brain cortex

Figure 4.5 shows the effect of supplementing the diets of aged animals with EPA (200 mg/kg/day) for a period of 8 weeks on the prostanoid (A) and hydroxy fatty acid (B) profiles of rat brain cortex. Supplementing the diets of aged animals with EPA significantly reduced the proportion of cortical PGE₂ (p=0.04; ANOVA with Bonferroni correction). EPA did not significantly change the proportions of any other prostanoids or hydroxy fatty acids in the brain cortex.

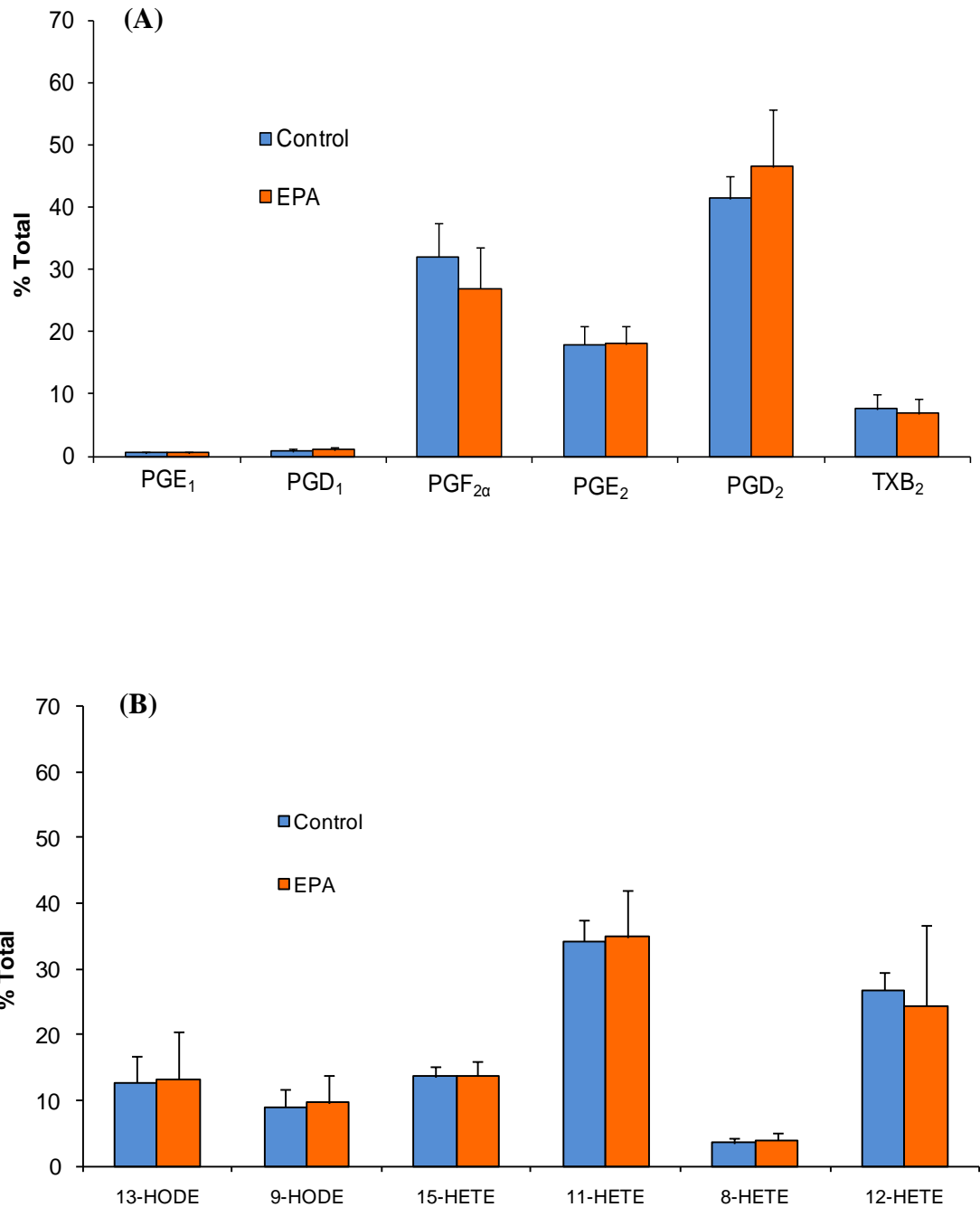


Figure 4.4. Prostanoids (A) and hydroxy fatty acids (B) found in young (aged 2-3 months) rat brain cortical tissue after feeding with 200 mg/kg/day eicosapentaenoic acid (EPA) for 8 weeks compared to control. (Results expressed as mean \pm standard deviation; $n=6$ per group).

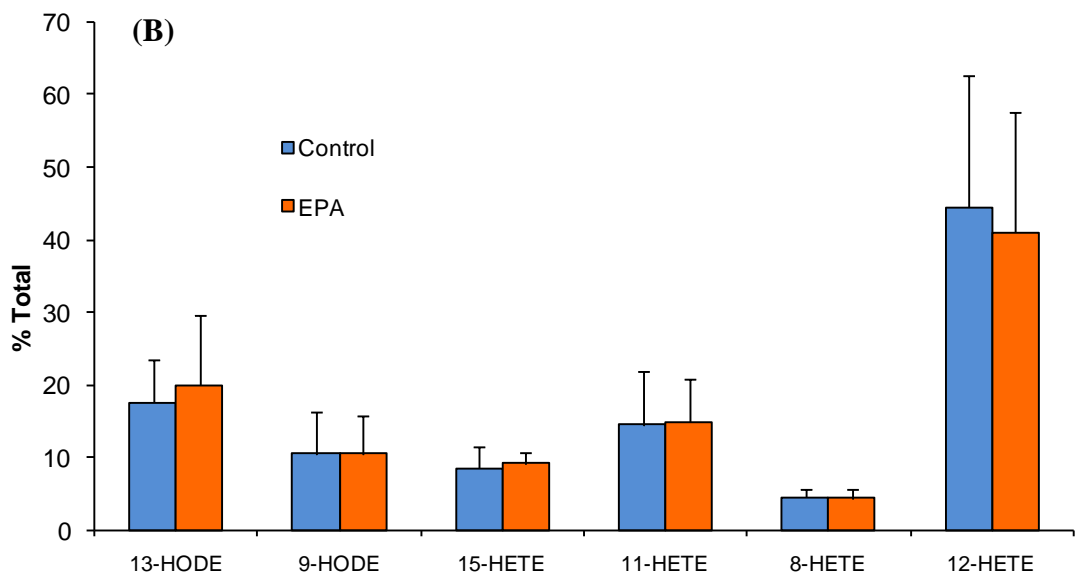
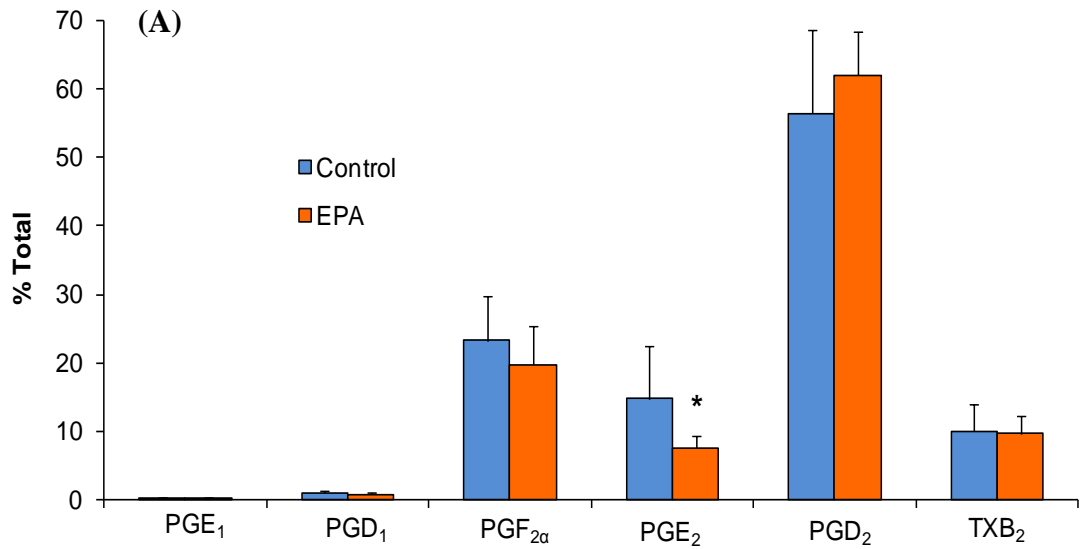


Figure 4.5. Prostanoids (A) and hydroxy fatty acids (B) found in aged (aged 22-24 months) brain cortical tissue after feeding with 200mg/kg/day eicosapentaenoic acid (EPA) for 8 weeks compared to control (* $p < 0.05$). (Results expressed as mean \pm standard deviation; $n=6$ per group).

4.3.6 The effect of docosapentaenoic acid on the profile of prostanoids and hydroxy fatty acids in the young brain cortex

Figure 4.6 shows the effect of supplementing the diets of young animals with DPA (200 mg/kg/day) for a period of 8 weeks on the prostanoid (A) and hydroxy-fatty acid (B) profiles of rat brain cortex. Supplementing the diets of young animals with DPA did not significantly change the proportions of prostanoids but did significantly alter the profile of hydroxy fatty acids in the brain cortex. Levels of 13-HODE and 9-HODE were found to double following DPA supplementation ($p=0.007$ and $p=0.002$ respectively; one way ANOVA with Bonferroi correction). DPA also significantly reduced levels of 15 HETE ($p=0.013$), 11 HETE ($p=0.023$), 8 HETE ($p=0.024$) and 12 HETE ($p=0.008$) in young animals (one way ANOVA in all cases with Bonferroi correction for multiple comparisons).

4.3.7 The effect of docosapentaenoic acid on the profile of prostanoids and hydroxy fatty acids in the aged brain cortex

Figure 4.7 shows the effect of supplementing the diets of aged animals with DPA (200 mg/kg/day) for a period of 8 weeks on the prostanoid (A) and hydroxy-fatty acid (B) profiles of rat brain cortex. Supplementing the diets of aged animals with DPA significantly reduced the proportion of cortical PGE_2 ($p=0.032$; ANOVA with Bonferroni correction for multiple comparisons). DPA did not significantly change the proportions of other prostanoids or hydroxy fatty acids in the brain cortex.

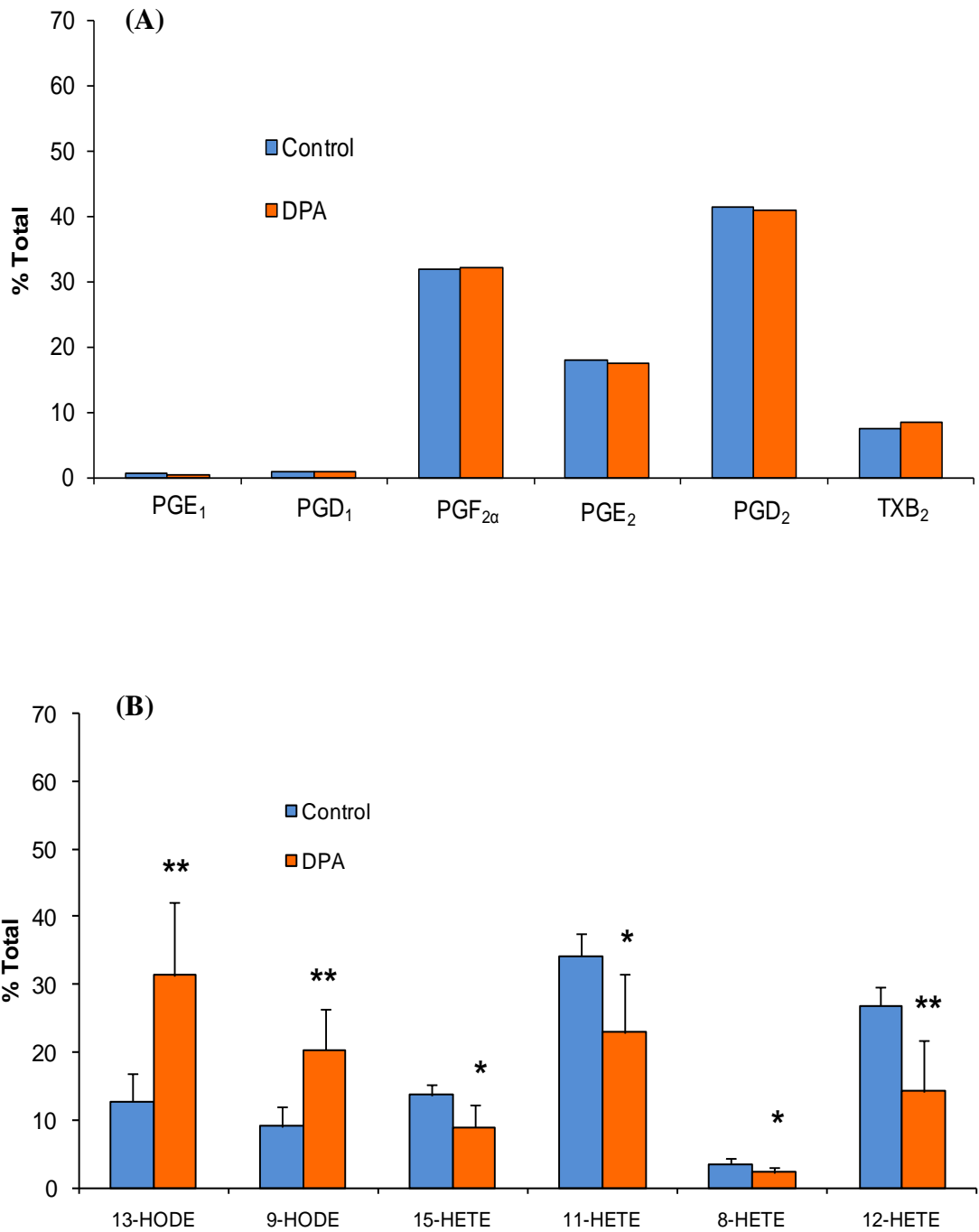


Figure 4.6. Prostanoids (A) and hydroxy fatty acids (B) found in young brain cortical tissue after feeding with 200 mg/kg/day docosapentaenoic acid (DPA) for 8 weeks compared to control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (Results expressed as mean \pm standard deviation; $n = 6$ per group).

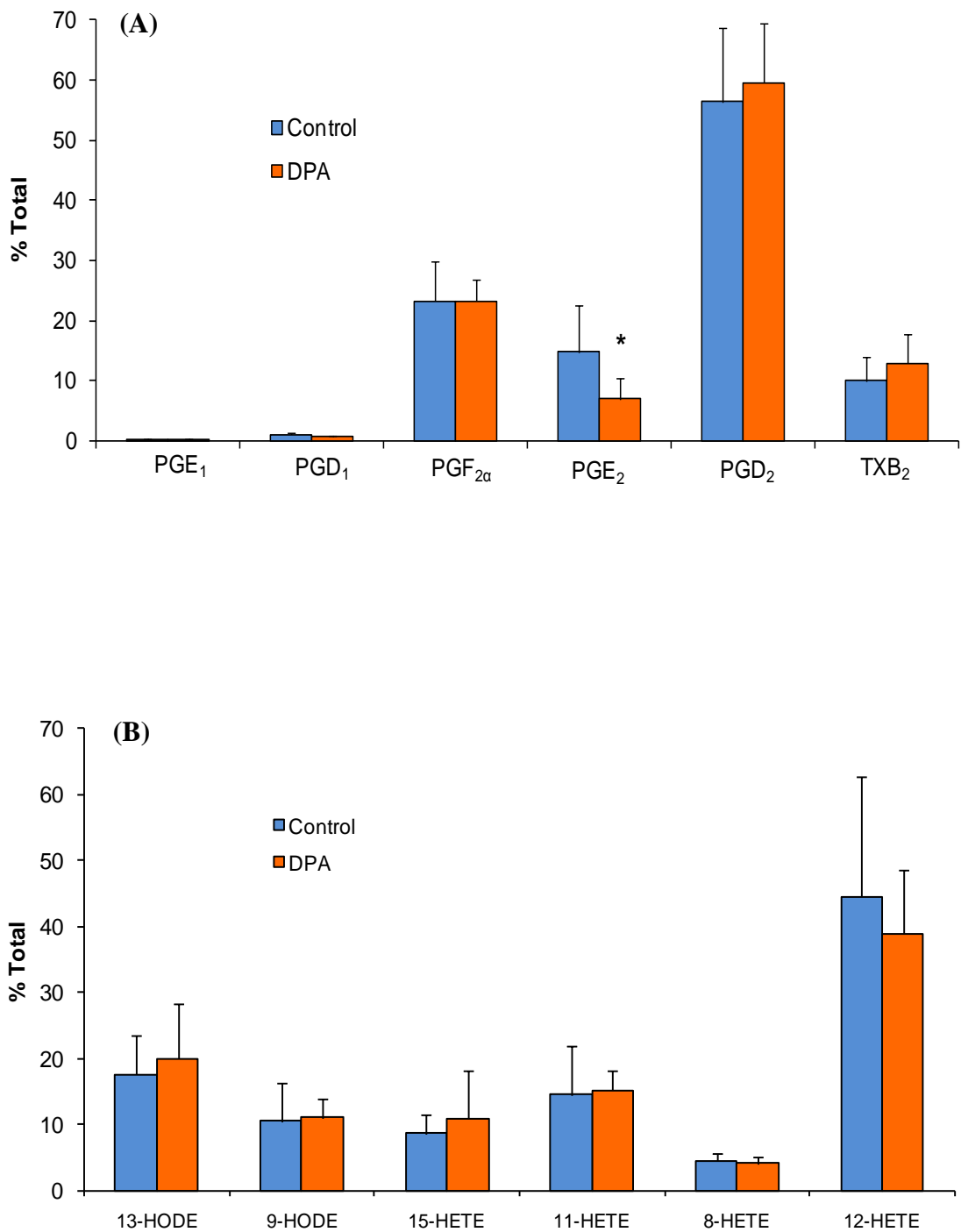


Figure 4.7. Prostanoids (A) and hydroxy fatty acids (B) found in aged brain cortical tissue after feeding with 200 mg/kg/day docosapentaenoic acid (DPA) for 8 weeks compared to control (* $p < 0.05$). (Results expressed as mean \pm standard deviation; $n=6$ per group).

4.4 Discussion

The aim of this study was to investigate changes in the cortical concentration of prostanoids and hydroxy fatty acids caused by ageing. It has been reported that EPA can modulate age related changes in cytokines and other inflammatory mediators. Thus, the effect of dietary supplementation of EPA on young and aged brain cortex was also investigated. Previous work has shown how DPA can be retroconverted to EPA and so indirectly mimic the neuroprotective actions of the latter. Thus, the effects of dietary supplementation of DPA on young and aged brain prostanoid and hydroxy fatty acid profile were also determined.

This study has shown that of the eicosanoids detected, PGD₂ and 12-HETE were the most abundant in rat brain cortex. This finding is in agreement with previous literature where these eicosanoids have been identified as being predominant in cortical tissue (Siren, 1982, Moore *et al.*, 1991, Gerozissis *et al.*, 1983, Adesuyi *et al.*, 1985). This study has also shown that other prostanoids present in the brain cortex included PGE₂, TXB₂, and PGF_{2α}. All of these mediators have previously been found in rat brain tissue (Pham Huu *et al.*, 1987, Weber *et al.*, 1988, Samuelsson, 1964).

To date relatively little work has focussed on the levels of hydroxy fatty acids in the brain. This study has shown that 11-HETE, 8-HETE, 9-HETE, 5-HETE and 15-HETE were all present in brain cortex. Previous work has also identified these mediators in the rat brain (Kim *et al.*, 1991). There have, however, been very few investigations to determine the presence of 9- and 13- HODE in the brain. Most work in identifying HODE has been based on studies using this mediator as a gauge of oxidative stress in the brain (Yoshida *et al.*, 2006, Yoshida *et al.*, 2009). This work has demonstrated the

presence of both 13-HODE and 9-HODE in the brain cortex at levels comparable with HETE (approximately 100 pg/mg protein).

This study has shown that dietary supplementation with EPA at a dose of either 50 mg or 125 mg daily for a period of 4 weeks did not change the profile of prostanoids or hydroxy fatty acids in the brain cortex when compared to control. Although previous work has demonstrated that both doses of EPA can prevent inflammation in the brain through reducing the production of inflammatory cytokines (Kavanagh *et al.*, 2004, Lynch *et al.*, 2007) this study has shown that at these doses EPA did not stimulate a change in eicosanoid profile.

The lack of effect of EPA on the eicosanoid profile of brain cortex during this study may be due to the absence of an appropriate stimulus. During both previous studies where EPA was given at a dose of 50 mg or 125 mg per day animals were injected with an inflammatory bacterial lipopolysaccharide (LPS) which acted as a stimulus (Kavanagh *et al.*, 2004, Lynch *et al.*, 2007). Thus, in the absence of a stimulus the brain conserves the overall profile of prostanoids and hydroxy fatty acids, hence, the apparent lack of effect by EPA supplementation alone. It is envisaged that upon application of a suitable stimulus EPA will be metabolised by the brain to alter the profile of eicosanoids synthesised.

Age is known to be a natural stress upon the brain and is associated with an increased oxidative burden accompanied by an exaggerated neuroinflammatory response (Forster *et al.*, 1996, Godbout *et al.*, 2005, Sparkman and Johnson, 2008). Ageing has also been shown to alter brain phospholipid composition and consequently PUFA profile (Delion

et al., 1997, Mcnamara *et al.*, 2008). Ageing has previously been suggested as a model for neurodegeneration and neuroinflammation (Huuskonen *et al.*, 2005, Zhu *et al.*, 2006) however it has not been used to date to illustrate the effects of EPA on brain eicosanoid profile.

A further study was therefore designed to test the effects of EPA and its elongated metabolite DPA on brain eicosanoid profile. Age was used as a stress stimulus and eicosanoids were profiled in both young and aged animals following dietary supplementation. The dose of PUFA was adjusted according to body weight to ensure bioequivalence and the dosing period was lengthened to 8 weeks. It was found that presenting the data as % total, instead of giving absolute values, reduced spread without affecting the overall profile.

This study showed that ageing led to a significant increase in levels of cortical PGD₂. It is well documented that PGD₂ plays an important role during neuronal development and this prostaglandin has previously been linked to the ageing process (Ueno *et al.*, 1985). According to these studies the level of PGD₂ in rat brain naturally changes as a function of age; increasing initially before eventually decreasing in old age (Mohri *et al.*, 2003, Ueno *et al.*, 1985).

Ageing was also shown in the present work to be accompanied by a decrease in levels of cortical PGF_{2α} (Figure 4.3). This finding is in agreement with previous work where it has been shown that levels of PGF_{2α} decrease with age in comparison with other prostaglandins (Ueno *et al.*, 1985). Whilst levels of cortical PGF_{2α} have been shown to fall as a consequence of ageing other work has demonstrated that the concentration of

this prostaglandin increases significantly as a result of neuronal injury (Egg *et al.*, 1980, Seregi *et al.*, 1985). It has also been shown that the concentration of PGF_{2α} is dependent on many factors as this prostaglandin can be synthesised from either PGE₂, PGD₂ or PGH₂ (Hayashi *et al.*, 1990, Dozier *et al.*, 2008). Thus, levels of PGF_{2α} in the brain can be difficult to interpret owing to the complex interactions of this mediator with other prostaglandins and neuropathology.

The present study has also showed that ageing led to a significant increase in levels of cortical 12-HETE. The role of 12-HETE in the brain is not well understood and the profile of this hydroxy fatty acid during ageing has not to date been undertaken. It has previously been suggested that blood platelet contribution to measured brain 12-HETE concentration is uncertain but highly likely (Adesuyi *et al.*, 1985). Thus levels of 12-HETE may appear artificially elevated due to platelet activation during sacrifice.

Whilst this study has demonstrated an age induced increase in levels of 12-HETE it has also shown that the aged brain contains less 15-HETE and 11-HETE than corresponding younger tissue. Both 15-HETE and 11-HETE have previously been shown to be formed in the brain and from cultured neurones (Ishizaki *et al.*, 1989, Usui *et al.*, 1987). The synthesis of 11-HETE may be dependent upon CYP450 enzymes or could be a product of COX-2 as has been shown previously (Capdevila *et al.*, 2002, Rowlinson *et al.*, 2000). The formation of 15-HETE will depend in part on CYP450 but also on 12-LOX and possibly COX-2. It has been shown that both 12-LOX and COX-2 can generate 15-HETE (Watanabe *et al.*, 1993, Rowlinson *et al.*, 2000). Many of the HETE analogues, including 12 HETE, 15 HETE and 11 HETE are thought to mediate cerebral vasospasm and are therefore described as being highly vasoactive (Hariri *et al.*, 1989).

Supplementing the diets of young rats with EPA at a daily dose of 200mg/kg had no effect on the cortical profile of eicosanoids. This is in accordance with the dose study where neither 50 mg nor 125 mg EPA daily affected the profile of prostanoids or hydroxy fatty acids in the brain.

Supplementing the diets of young rats with *n*-3 DPA at a dose of 200 mg/kg did not affect levels of prostanoids within the brain cortex but did significantly affect levels of hydroxy fatty acids. Overall *n*-3 DPA reduced the proportions of 15-HETE, 11-HETE, 8-HETE and 12-HETE and augmented the formation of 13-HODE and 9-HODE in young brain. Whilst the *n*-3 DPA mediated effects may be of therapeutic value enough is not known about the balance of hydroxy fatty acids to draw conclusions. The fact that *n*-3 DPA is able to alter the profile of hydroxy fatty acids without a stimulus is however intriguing and merits further investigation. The rise in levels of HODE may be indicative of oxidative stress. It is unclear why these animals should be in a state of oxidative stress. Perhaps *n*-3 DPA incorporates itself in cellular membranes thereby liberating LA in the presence of PLA₂ resulting in the formation of HODEs.

Supplementing the diets of aged rats with EPA at a daily dose of 200 mg/kg resulted in a reduction in levels of PGE₂. EPA did not significantly affect the formation of other detected prostanoids and hydroxy fatty acids. There are conflicting reports about the actions of PGE₂ in the brain. Some studies have shown that activation of EP₂ and EP₄ receptors in cultured neurones can help prevent neurodegeneration and that activation of EP₂ receptors in the hippocampus may improve cognition (Yang *et al.*, 2009, Echeverria *et al.*, 2005). Other work has shown that EP₂ receptors are critical during neurodegeneration and that neurotoxicity is attenuated in EP₂ knockout animals (Montine

et al., 2002b). The role of PGE₂ and specifically the EP₂ receptor in neurotoxicity was also demonstrated by later work using neurone cultures and genetic experiments where it was shown that PGE₂ activated the innate immune system resulting in neuroinflammation (Shie *et al.*, 2005). Thus, the statistically significant fall in PGE₂ levels following EPA supplementation could be of therapeutic value in the age model. Lower levels of PGE₂ may attenuate neuroinflammation in the brain thereby modulating neurodegenerative disease.

When aged rats were supplemented with *n*-3 DPA at a daily dose of 200 mg/kg there was a drop in levels of cortical PGE₂ without a significant influence on other prostanoids. Overall *n*-3 DPA appeared to have the same actions as EPA on the prostanoid profile of aged rat brain. *n*-3 DPA did not affect hydroxy fatty acid synthesis in the aged rat brain. The statistically significant effects of *n*-3 DPA noted on hydroxy fatty acids in young brain were no longer observed when this PUFA was given to aged animals.

This study has shown that *n*-3 DPA has an effect on hydroxy fatty acid balance in young rat brains that is not observed in aged brains and is not seen with EPA. Due to lack of previous work with *n*-3 DPA it is not possible to verify these findings without further work. Moreover, the implications of *n*-3 DPA supplementation on young brain and the associated change in hydroxy fatty acid profile are not clear.

This study showed that feeding EPA to both young and aged animals did not result in the synthesis of the series-3 prostanoids or HEPE but did alter the profile of other eicosanoids. This is in agreement with previous work where to date HEPE and series-3

prostanoids have not been found in brain. Since the eicosanoids only act locally there may not be enough EPA incorporated within the membranes of cells to allow significant synthesis of these mediators.

**Chapter 5 : The effects of eicosapentaenoic acid and
docosapentaenoic acid on brain fatty acid ethanolamides
during ageing and following dietary supplementation**

5.1 Introduction

Endogenous anandamide (A-EA) has previously been detected in different regions of the rat brain including the cortex (Bisogno *et al.*, 1999, Felder *et al.*, 1996). Cultured brain neurones have also been shown to release A-EA following stimulation with membrane depolarising agents (Di Marzo *et al.*, 1994). The major route of *N*-acylethanolamine (NAE) biosynthesis in the brain is thought to be via the transacylation phosphodiesterase pathway (Di Marzo *et al.*, 1994, Hansen *et al.*, 2000).

A-EA usually accounts for less than 5% of the total NAE level in rat brain (Artmann *et al.*, 2008, Hansen and Diep, 2009). Palmitoylethanolamide (P-EA), stearoylethanolamide (S-EA) and oleoylethanolamide (O-EA) have been identified as the major NAE species in brain (Artmann *et al.*, 2008, Degn *et al.*, 2007). The pharmacological actions of these NAE is unclear although it is known that like A-EA they are ligands of the TRPV1 receptor (Movahed *et al.*, 2005).

It has been shown that ageing increases expression of *N*-acylphosphatidylethanolamine phospholipase D (NAPE-PLD) (Morishita *et al.*, 2005) possibly leading to elevated level of A-EA. Whilst A-EA is known to protect cortical mouse neurone cultures and prevent neurodegeneration through its action on the CB1 receptor (Kim *et al.*, 2005) it is unclear how other NAE, in particular those derived from *n*-3 PUFA, will affect neurones.

The health benefits of regular consumption of *n*-3 PUFA are well known. These PUFA may influence levels of A-EA and other more common NAE in the brain. Thus, DHA

derived docosahexaenoylethanolamide (DHA-EA) has been found to be more abundant in rat brain than A-EA (Artmann *et al.*, 2008) but EPA derived eicosapentaenoylethanolamide (EPA-EA) has only ever been shown to be present in piglet brain following a modified diet (Berger *et al.*, 2001). It is not known how levels of DHA-EA and EPA-EA change as a result of ageing and whether these changes impact synthesis of A-EA.

In addition to age, diet has also been shown to affect concentrations of NAE in the brain (Artmann *et al.*, 2008, Berger *et al.*, 2001). A major drawback of these dietary studies is that fish oil was used to supply *n*-3 PUFA. Since fish oil is a mixture of several PUFA it is unclear which of these was responsible for any observed changes. This study therefore focuses on the effects of supplementing EPA or *n*-3 DPA individually on levels of A-EA, DHA-EA and EPA-EA in young and aged brain.

It is hypothesised that ageing will lead to increased synthesis of brain NAE possibly due to previously described up regulation of NAPE-PLD. Thus aged brain should express higher levels of A-EA, DHA-EA and EPA-EA compared to young brain. It is further hypothesised that supplementing the diet with EPA will stimulate synthesis of EPA-EA, a previously undetected NAE in rat cortical tissue. Retroconversion of dietary *n*-3 DPA should result in the same effects on brain NAE concentration as EPA. It is thus hypothesised that *n*-3 DPA will also stimulate the formation of EPA-EA.

5.2 Methods

All animal work was carried out by Professor M A Lynch's lab at the Bio Resources Unit, Trinity College, Dublin. (See methods section 2.3 for details). NAE were extracted as described by method B in sections 2.6.4. SPE was performed as described in section 2.6.5. LC-ESI-MS/MS analysis of NAE is described in section 2.6.6.

5.3 Results

5.3.1 The effects of age on brain cortex fatty acid ethanolamides

This study has confirmed that of the three NAE that have been analysed, DHA-EA is the most abundant (Figure 5.1) in rat brain. Young animals included in this study have been shown to express both A-EA and DHA-EA in brain cortical tissue. Ageing has been shown to significantly increase the expression of A-EA ($p = 0.005$; independent sample T-test) as shown by Figures 5.1 and 5.2 (B). Although EPA-EA was not detected in the cortex of young animals this fatty acid ethanolamide is found in tissue from aged animals. Levels of EPA-EA have been shown by this study to significantly increase in the brain cortex as a result of ageing ($p = 0.03$; independent sample T-test) as shown by Figure 5.2 (C). However, as shown in Figures 5.1 and 5.2 (A), the levels of DHA-EA in rat cortex do not significantly change as a result of ageing.

5.3.2 The effects of dietary supplementation of eicosapentaenoic acid on fatty acid ethanolamides in the brain cortex

Young animals that have had their diets supplemented with EPA (200 mg/kg/day) showed no changes in levels of DHA-EA, A-EA or EPA-EA in the brain cortex, as

summarised in Figure 5.3 (A). Aged animals that have had their diets supplemented with EPA at the same dose express significantly more EPA-EA in the brain cortex ($p = 0.014$; ANOVA with Bonferroni correction). EPA dose not significantly change the expression of DHA-EA or A-EA in the aged brain cortex as shown by Figure 5.3 (B).

5.3.3 The effects of dietary supplementation of docosapentaenoic acid on other fatty acid ethanolamides in the brain cortex

This study has shown that supplementing the diets of young animals with *n*-3 DPA (200 mg/kg/day) does not significantly change the levels of A-EA, DHA-EA or EPA-EA in the brain cortex. When *n*-3 DPA was incorporated into the diets of aged animals there was a significant increase in the expression of EPA-EA ($p = 0.013$; ANOVA with Bonferroni correction). *n*-3 DPA did not significantly change the concentrations of A-EA or DHA-EA in aged brain cortex. The relevant data are presented in Figure 5.4.

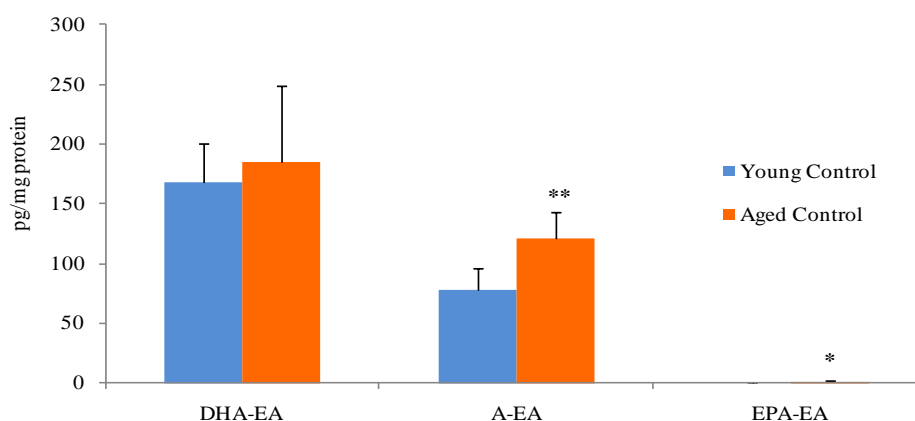


Figure 5.1. The relative abundance of docosahexaenylethanolamide (DHA-EA), anandamide (A-EA) and eicosapentaenylethanolamide (EPA-EA) in young and aged brain cortex (* $p < 0.05$, ** $p < 0.01$). (Results expressed as mean \pm standard deviation; $n=6$ per group).

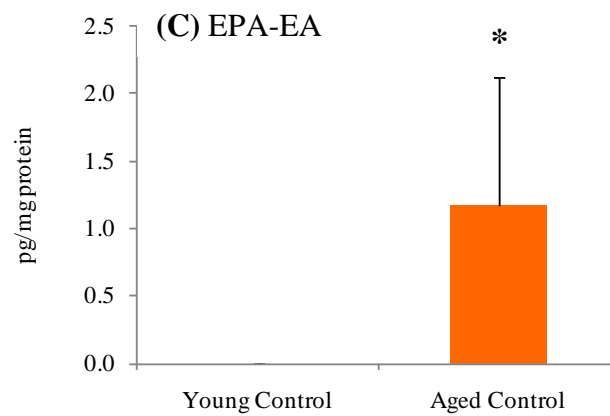
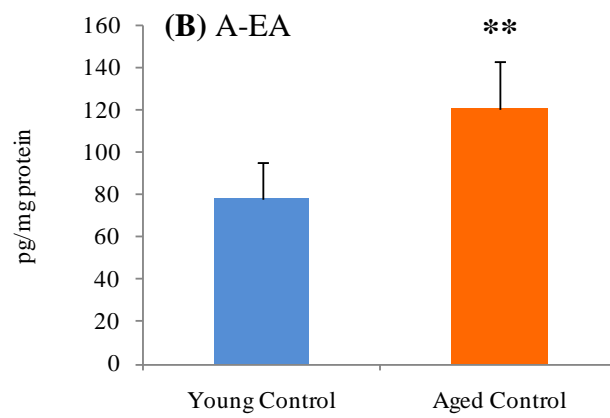
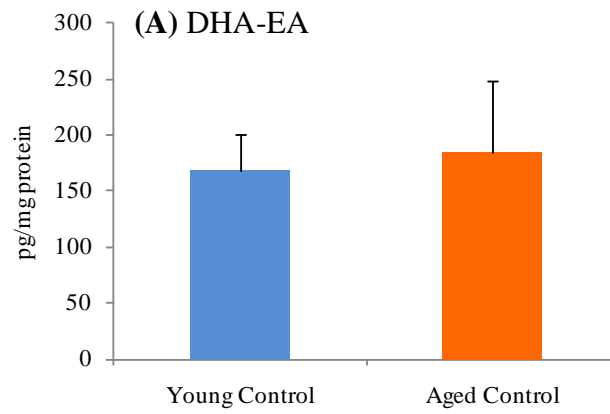


Figure 5.2. The effect of ageing on the profile of (A) docosahexaenoyl ethanolamide, (B) anandamide and (C) eicosapentaenoyl ethanolamide (* $p < 0.05$, ** $p < 0.01$). (Results expressed as mean \pm standard deviation; $n=6$ per group).

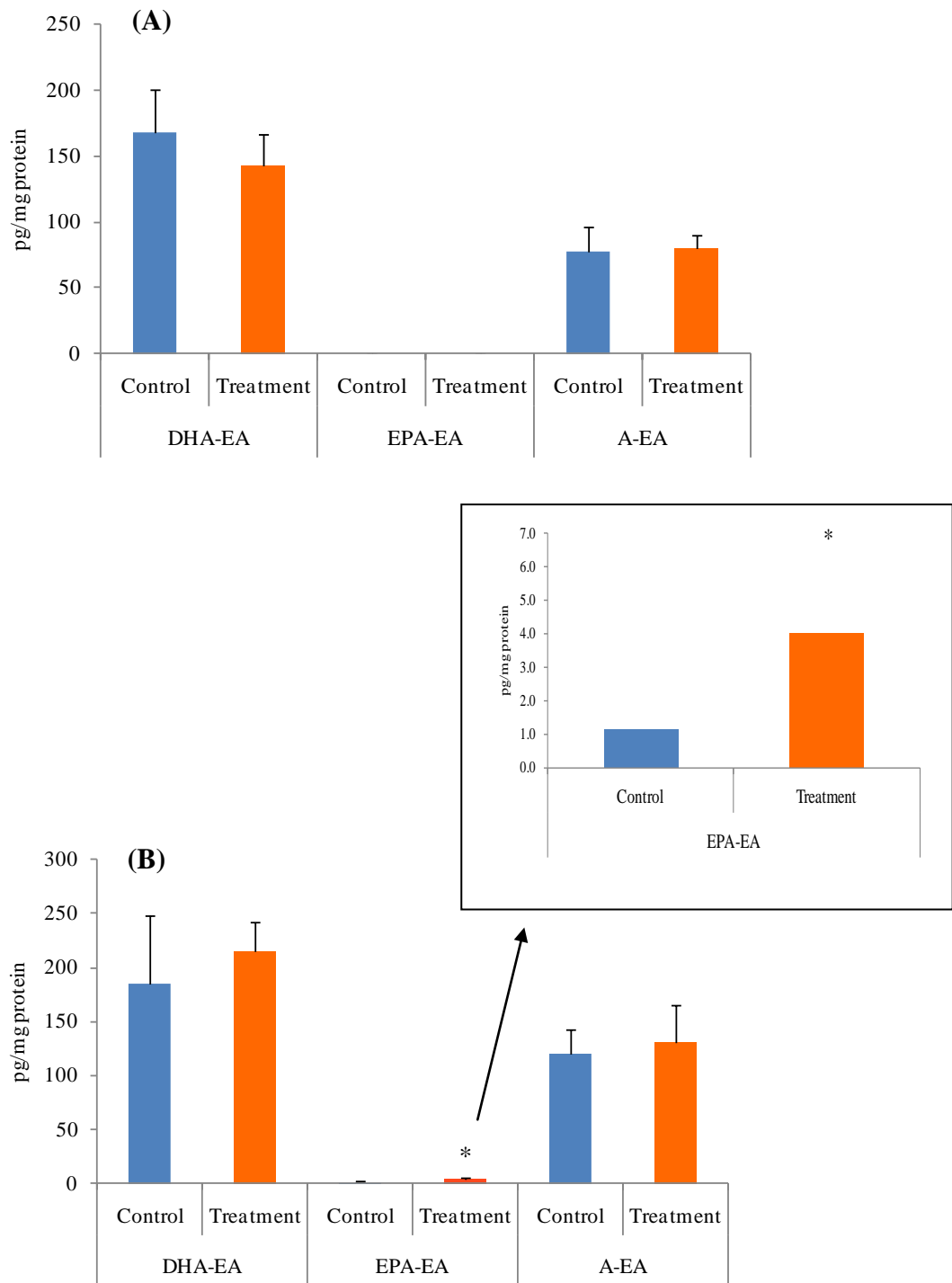


Figure 5.3. The effect of **EPA** intake on the profile of docosahexaenoylethanolamide (DHA-EA), anandamide (A-EA) and eicosapentaenoylethanolamide (EPA-EA) in young (A) and aged (B) brain cortex compared to control (* $p < 0.05$). (Results expressed as mean \pm standard deviation; $n=6$ per group).

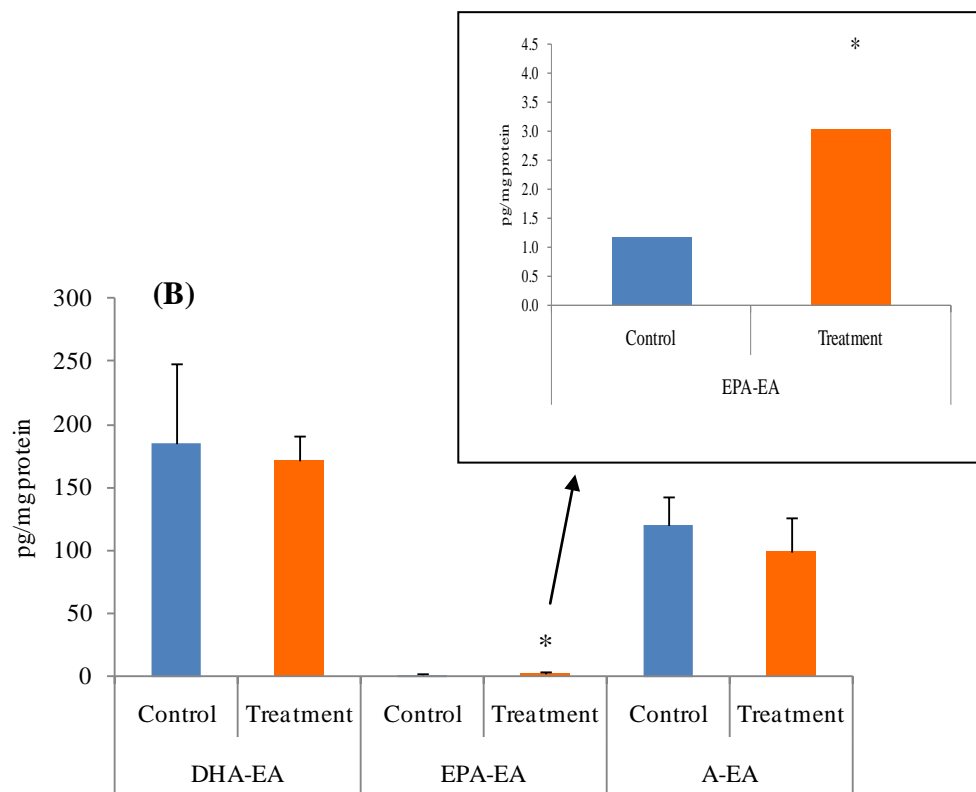
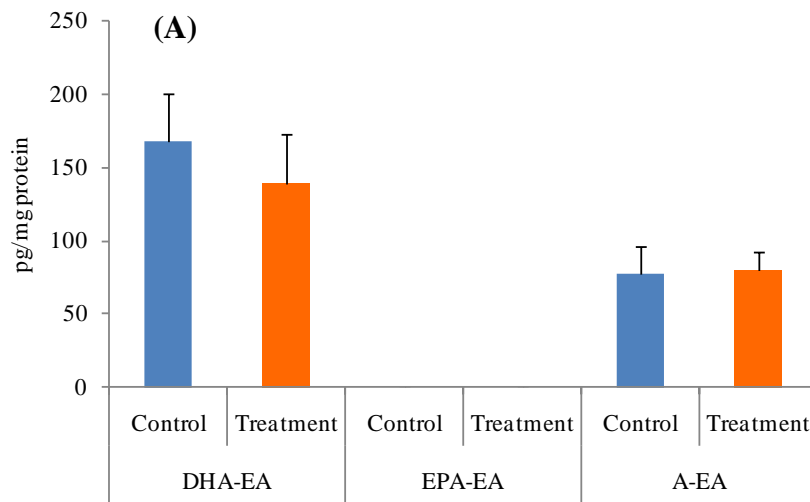


Figure 5.4. The effect of **DPA** intake on the profile of docosahexaenylethanolamide (DHA-EA), anandamide (A-EA) and eicosapentaenylethanolamide (EPA-EA) in young (A) and aged (B) brain cortex compared to control (* $p < 0.05$). (Results expressed as mean \pm standard deviation; $n=6$ per group).

5.4 Discussion

This study was designed to investigate age related changes in the profiles of A-EA, DHA-EA and EPA-EA in rat brain cortex. The effects of supplementing the diet with either EPA or DPA on these profiles were also investigated. Both young and aged animals were fed these PUFA to determine whether a modified diet could modulate any age related changes in the NAE profile of the brain cortex.

This study has shown that ageing alone significantly increases levels of A-EA and EPA-EA in the brain cortex. Ageing does not however significantly change levels of DHA-EA in cortical tissue. It has previously been reported that the ageing process is associated with a natural increase in expression of NAPE-PLD, the precursor of A-EA (Morishita *et al.*, 2005, Moesgaard *et al.*, 2000). The finding that A-EA expression increases with age fits in well with the previously observed higher levels of NAPE-PLD in the ageing rat brain.

To date there are very few studies examining the relationship between ageing and NAE expression in the brain. There are no previous studies that have identified EPA-EA in the aged cortex or described how levels of this ethanolamide may change as a result of the ageing process.

In one previous study it was found that there was no significant difference in the concentration of A-EA in young or aged brain cortex (Maccarrone *et al.*, 2001). A second study by the same group gave similar results with levels of A-EA in the cortex remaining constant throughout the ageing process (Maccarrone *et al.*, 2002b). A third

study also found unchanged levels of A-EA and other NAE in brain tissue from aged animals compared to younger animals (Wang *et al.*, 2003).

Thus it appears as though the significantly increased levels of A-EA observed in the current work is not in agreement with previous studies. This apparent anomaly is probably due to the fact that aged animals from the first two mentioned studies were only 12 months old (Maccarrone *et al.*, 2001, Maccarrone *et al.*, 2002b) and those from the third study only 4 months old (Wang *et al.*, 2003). In the current study aged animals are between 22 to 24 months old. It is therefore feasible that during this current study age related changes will be much more apparent compared to previous work and that increased expression of A-EA may only become significant at a specific time point beyond 12 months of age.

The finding that age alone significantly increased the expression of EPA-EA in the brain cortex is novel. To date EPA-EA has not been found in brain without dietary manipulation. Previous studies have failed to detect EPA in brain tissue from juvenile animals (Suarez *et al.*, 1996, Moriguchi *et al.*, 2004) and only detect a relatively minor quantity of this PUFA in adult brain tissue (Bourre *et al.*, 1993, Marteinsdottir *et al.*, 1998). It therefore appears as though the brain forms EPA during ageing, albeit to a minor extent. This possibly explains why EPA-EA is absent in tissue from young animals but can be detected in tissue from aged animals.

This study has shown that the profile of NAE in the brain is probably linked to the abundance of corresponding PUFA. Thus, of the 3 NAE analysed, DHA-EA is the most abundant, corresponding to the prevalence of DHA in the brain. EPA-EA is the least

abundant as EPA is only present in the brain in minor quantities. Although AA is abundant in brain tissue it is less prevalent than DHA. This may explain why A-EA is found in brain tissue at a lower concentration than DHA-EA but at a higher concentration than EPA-EA. Overall, the relative abundance of A-EA and DHA-EA is in good agreement with previous literature (Artmann *et al.*, 2008, Berger *et al.*, 2001).

This study has shown that supplementing the diet of young animals with either EPA or DPA does not significantly change the profile of A-EA, DHA-EA or EPA-EA in the brain cortex. Supplementing the diet of aged animals with EPA did however significantly increase the level of EPA-EA in the cortex compared to ageing alone and this effect was mimicked by DPA. The fact that EPA can increase levels of EPA-EA in the brain is in agreement with literature where fish oil has previously been found to have this effect (Artmann *et al.*, 2008, Berger *et al.*, 2001).

It has recently been shown that dietary EPA is readily converted in the liver to DHA which is then transported to the brain and elsewhere (Gao *et al.*, 2009). It has also been shown that EPA easily crosses the blood brain barrier at a rate similar to DHA (Ouellet *et al.*, 2009). Unlike DHA, however, EPA has been suggested to be rapidly β oxidised in the brain to other PUFA (Chen *et al.*, 2009). It is well known that the infantile brain undergoes DHA accretion and that this PUFA is essential for neural development (Innis, 2007, Joardar *et al.*, 2006). Thus, younger animals with a developing brain are more likely to metabolise EPA to DHA and other PUFA thereby preventing synthesis of EPA-EA but not significantly affecting the formation of A-EA or DHA-EA as shown by this study.

The metabolic fate of dietary *n*-3 DPA is currently poorly understood. This study has shown that *n*-3 DPA significantly increases formation of EPA-EA in the brain cortex when given to aged animals. This fits in well with the recent observations that *n*-3 DPA is desaturated to DHA and retroconverted in the liver and skeletal muscles to EPA (Kaur *et al.*, 2009). Thus, animals that were given *n*-3 DPA during this study probably have retroconverted it to EPA. This is why supplementing the diet with *n*-3 DPA appears to mimic the effects of supplementing the diet with EPA as far as brain NAE synthesis is concerned.

The biological properties of A-EA are well characterised and include this compounds ability to induce analgesia, hypotension and hypomotility (Calignano *et al.*, 1998, Crawley *et al.*, 1993, Smith *et al.*, 1994). Less well understood are the properties of other NAE and their actions on the brain. It is known that A-EA and other NAE accumulate during neuronal injury and are thought to have a neuroprotective effect (Hansen *et al.*, 2002, Berger *et al.*, 2004). Thus levels of DHA-EA and A-EA have both been found to be dramatically increased following ischaemia, stroke and neurodegenerative disease (Berger *et al.*, 2004, Degn *et al.*, 2007, Hansen *et al.*, 2001).

It is thought that EPA can protect against neuronal damage and it has been proposed that many of its actions are in part mediated through formation of eicosanoids, resolvins and neuroprotectins (Lynch *et al.*, 2007, Serhan *et al.*, 2004). The role of EPA in cognition and in the formation of memory is also well described (Kavanagh *et al.*, 2004, Song and Horrobin, 2004). Ageing is often characterised by neural damage resulting in cognitive decline and this study has shown that both EPA and DPA significantly increase EPA-EA formation in the aged brain. If EPA-EA is neuroprotective then this

could be one way through which EPA acts to prevent neurodegeneration. Thus, EPA appears to have some unique properties when compared to other PUFA and some of its actions on the brain may be mediated through EPA-EA.

This study has for the first time described the formation of EPA-EA in the brain cortex as a result of ageing alone. Furthermore, during this study, rats' diets were supplemented specifically with EPA or DPA. To date all other dietary studies involving *n*-3 PUFA derived ethanolamides have focussed on fish oil, a mixture of PUFA, rather than the individual fatty acids (Artmann *et al.*, 2008, Berger *et al.*, 2001). To date there have been no other dietary studies with *n*-3 DPA examining the relationship between intake of this PUFA and subsequent NAE synthesis in the brain cortex. A-EA and related NAE are known to have neuroprotective properties and are synthesised in the brain following injury. Thus these molecules are of pharmacological importance and could be developed as therapeutic tools in the management of neurodegenerative disease. This study has shown that dietary intake of EPA or DPA significantly increases EPA-EA in the aged brain. To date the pharmacology of EPA-EA remains undescribed although, based on the actions of other NAE, it is thought that this compound may also have neuroprotective properties.

**Chapter 6 : Platelet eicosanoid and hydroxy fatty acid
synthesis following treatment with polyunsaturated fatty
acids and niacin**

6.1 Introduction

Platelets are derived from fragmentation of precursor megakaryocytes and play a fundamental role in hemostasis, leading to the formation of blood clots or thrombi (Hoffbrand *et al.*, 2005). Typically, platelets have a lifespan of up to 10 days after which they undergo aggregation and eventually death (Hartley *et al.*, 2006, Najean *et al.*, 1969). Platelets ubiquitously express platelet type 12 lipoxygenase (hp-12-LOX) and cyclooxygenase-1 (COX-1) through which they synthesise eicosanoids and other hydroxy fatty acids (Hamberg and Samuelsson, 1974). Platelets are essential for smooth bloodflow and can greatly impact microvasculature upon activation. To date platelets have been found to secrete various bioactive lipid mediators, most notably TXA₂ and 12-HETE (Hamberg *et al.*, 1975, Morita *et al.*, 1983) but also PGD₂ and PGE₂ (Lagarde *et al.*, 1979, Oelz *et al.*, 1977).

It has been shown that AA is the most abundant PUFA incorporated within platelet membrane phospholipids (Marcus *et al.*, 1969, Marcus *et al.*, 1962). Other work has shown that the platelet also expresses both EPA and DHA and that the proportion of these PUFA in the membranes increases following a supplemented diet (Von Schacky *et al.*, 1985b, Croset *et al.*, 1992). Thus, platelets are able to incorporate PUFA into their cellular membranes. This can have significant downstream effects with changes in the profile of secreted eicosanoids and hydroxy fatty acids.

Several studies have demonstrated that incubating platelets with *n*-3 PUFA, specifically EPA or DHA, will attenuate aggregation (Croset *et al.*, 1988a, Croset and Lagarde, 1986, Croset *et al.*, 1988b). It is thought that these effects are mediated through eicosanoids that are synthesised locally by the platelet (Gorman *et al.*, 1977, Moncada

and Vane, 1979). Thus, prostanoids such as thromboxane or hydroxy fatty acids such as 15-HETE have been shown to stimulate platelet aggregation (Packham *et al.*, 1987, Setty *et al.*, 1992) whereas PGD₂, 12-HETE and 12-HEPE have been shown to prevent aggregation (Oelz *et al.*, 1977, Takenaga *et al.*, 1986). Other hydroxy fatty acids such as HODE, derived from LA, can also be formed by platelets (Daret *et al.*, 1989).

Nicotinic acid or niacin is a vitamin that is used in the management of hypercholesterolaemia (Altschul *et al.*, 1955, Drexel, 2007, Robinson *et al.*, 2001). Its use for this indication is limited by the fact that it stimulates the release of PGD₂ in the skin which leads to temporary intense vasocutaneous flushing (Morrow *et al.*, 1992b, Warady *et al.*, 1989). In addition to lowering cholesterol niacin has been shown to reduce platelet adhesion and aggregation, stimulate prostacycline formation from the endothelium and reduce inflammation (Swies and Dabrowski, 1984, Walldius and Wahlberg, 1985, Rosenson, 2003). The mechanisms through which niacin exerts these effects are not well understood. However, it is known that eicosanoids are fundamental to aggregation and adhesion and so niacin may be altering the profile of these mediators produced from the platelet.

It is hypothesised that treating platelets with different PUFA will lead to changes in the profile of eicosanoids and hydroxy fatty acids produced. Specifically, EPA should favour the production of disaggregatory prostanoids and hydroxy fatty acids and may actively induce the formation of HEPE and series-3 prostanoids. Whilst DHA cannot undergo COX or LOX metabolism it may alter the profile of PUFA in the platelet membrane and indirectly affect eicosanoid and hydroxy fatty acid synthesis.

As niacin has been shown to inhibit aggregation it is hypothesised that this vitamin may have some effect on platelet eicosanoid synthesis in a manner analogous to EPA. Treating platelets with niacin and EPA could augment the effects of EPA on platelet eicosanoid formation. Thus, the effect of combined EPA and niacin treatment is also of interest.

In the present study LC/ESI-MS/MS has been employed to analyse eicosanoids and hydroxy fatty acids produced from platelets following treatment with EPA and other PUFA. The effect of adding niacin to the platelet incubation medium both with and without EPA is also examined. Indometacin has been used to confirm COX related effects whilst octanoic acid has been used to show that any changes are specifically PUFA related.

6.2 Methods

Plasma and platelet treatments were carried out by Dr Anne Leaver's group at the University of Edinburgh. Experiments were performed with platelet rich plasma (PRP) or platelet poor plasma (PPP) to confirm that any changes in eicosanoid production were due to platelet metabolism and not other factors such as oxidation. Indometacin was used to confirm COX mediated prostanoid formation. Octanoic acid was used in addition to control to confirm any changes were due to the treatment rather than experimental procedure. Incubates were sampled at regular intervals and aliquots collectively sent to Bradford in dry ice for analysis (see section 2.4). Extraction and LC/ESI-MS/MS was carried out as described in section 2.5.

6.3 Results

6.3.1 Production of prostanoids following treatment with fatty acids

6.3.1.1 Time dependent prostanoid production

Figure 6.1 shows the prostanoids detected in PRP and PPP over a period of 10 days. Prostanoid levels appear to remain steady over days 1 to 5 with the exception of PGE₃ and PGD₃ during treatment with EPA. By day 10 all samples showed a large increase in the concentrations of TXB₂ and, to a lesser extent, PGD₂ and PGE₂. Although this study demonstrated that PGE₃ and PGD₃ were the main prostanoids formed following treatment of platelets with EPA, all other experiments confirmed that TXB₂ was the main prostanoid synthesised by platelets. Figure 6.1 shows that prostanoids were formed in both PRP and PPP. With the exception of those platelets treated with EPA, the highest concentration of prostanoids was found in PRP; although by day 10 similar levels of prostanoids were detected in PRP and PPP.

6.3.1.1.1 Time dependent prostanoid production following incubation of platelets with eicosapentaenoic acid

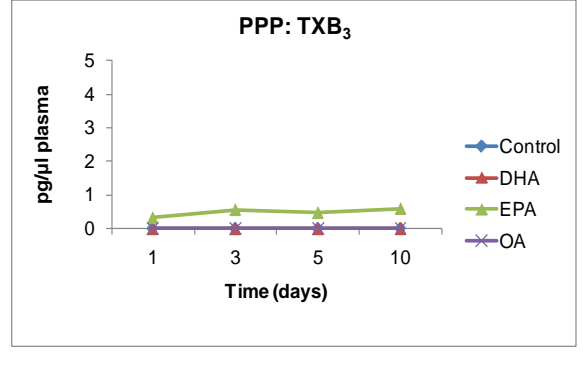
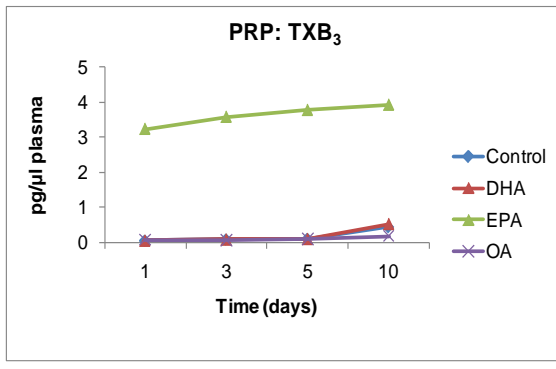
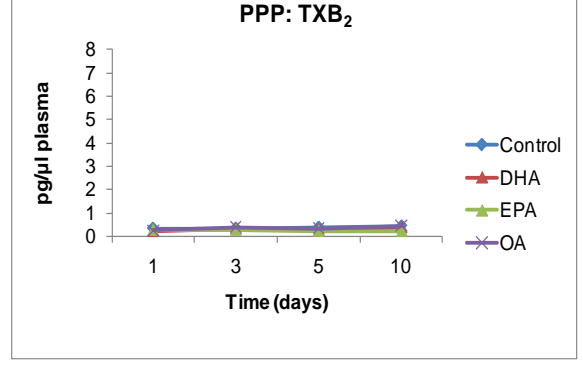
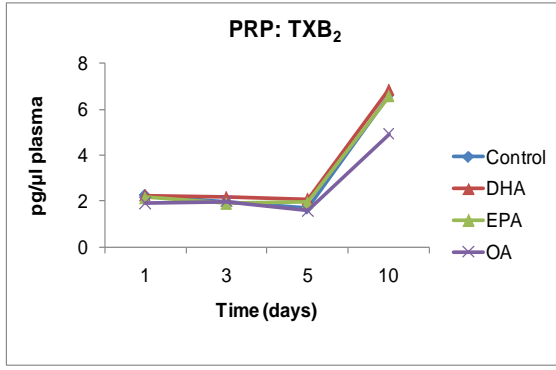
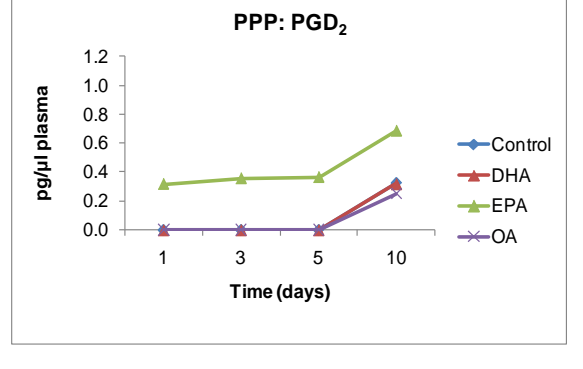
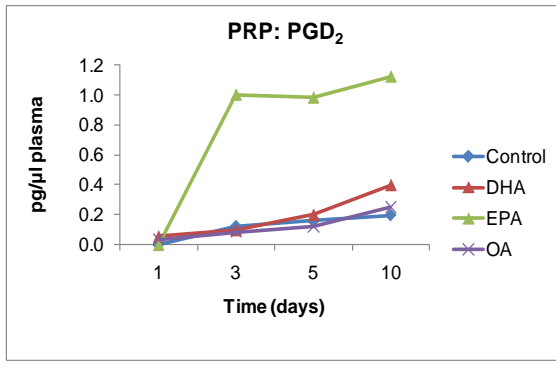
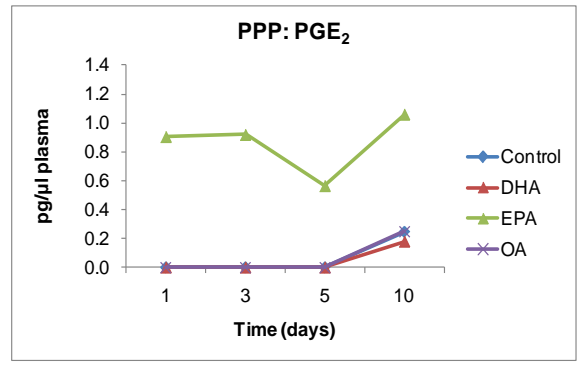
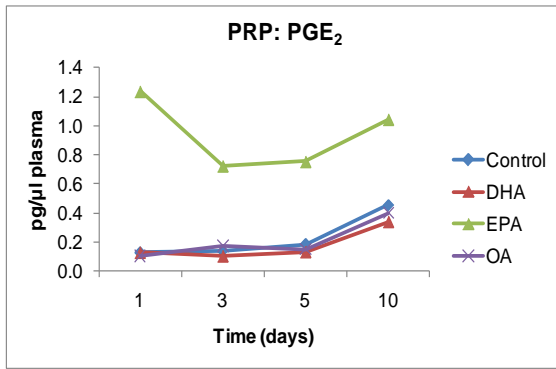
Treating platelets with 100 μ M EPA resulted in the formation of the series-3 prostanoids. Overall EPA stimulated the production of PGE₂, PGD₂, TXB₃, PGD₃ and PGE₃ as shown in Figure 6.1. Levels of these prostanoids were similar in both PRP and PPP; the only exception being TXB₃ where the concentration of this prostanoid was much higher in PRP than PPP. The most abundant prostanoids present in plasma during the treatment of platelets with EPA were PGD₃ and PGE₃. Levels of these prostanoids were initially high at around 20 pg/ μ l following addition of EPA, but subsequently decreased over the 10 day treatment period to around 5 pg/ μ l.

6.3.1.1.2 Time dependent prostanoid production following incubation of platelets with docosahexaenoic acid

Treating platelets with 100 μ M DHA increased PGD₂ formation in PRP compared to control. By day 10 the concentration of PGD₂ in PRP from DHA treated samples was approximately double the level of PGD₂ from the control experiment. Other than changing the level of PGD₂, treating platelets with DHA did not stimulate a change in levels of prostanoids when compared to control. Data shown in Figure 6.1.

6.3.1.1.3 Time dependent prostanoid production following incubation of platelets with octanoic acid

Octanoic acid is a saturated C8 fatty acid that has been used in this study as a second control to confirm whether any observed changes in the levels of mediators are due to experimental procedure or fatty acid treatment. This study has shown that octanoic acid treatment moderately decreased the formation of TXB₂ in PRP (Figure 6.1). This effect is only noticeable by day 10 where there is a large rise in levels of TXB₂ across all treatment groups. Overall, octanoic acid had the same effect on platelet prostanoid production as control. Data shown in Figure 6.1.



(Figure 6.1 continues overleaf...)

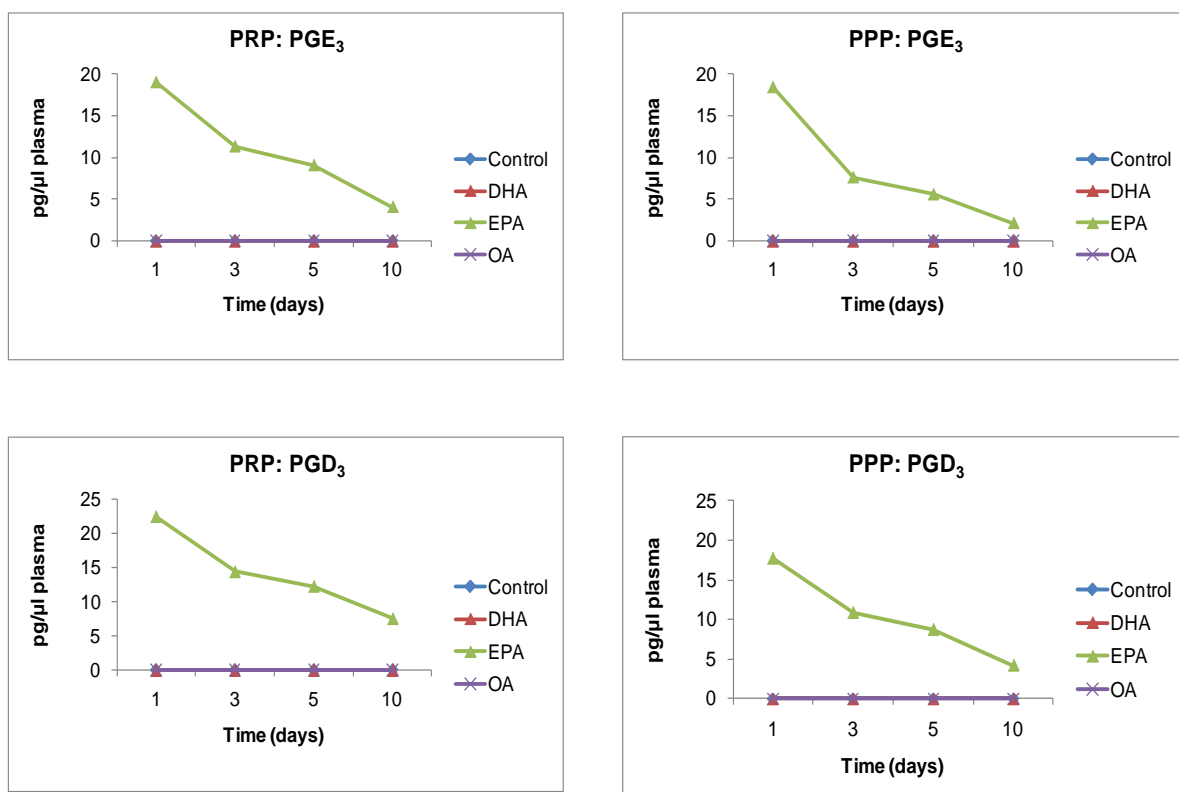


Figure 6.1. The effects of docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and octanoic acid (OA) on prostanoid synthesis in platelet rich (PRP) and platelet poor (PPP) plasma during a 10 day incubation period ($n=1$ for PRP and $n=1$ for PPP). Concentration of respective fatty acids is 100 μM , control is ethanol vehicle.

6.3.2 Production of hydroxy fatty acids following treatment with fatty acids

6.3.2.1 Time dependent hydroxy fatty acid production

Figure 6.2 shows the profile of hydroxy fatty acids detected in PRP and PPP over a period of 10 days. With the exception of those platelets treated with EPA, levels of HETE and HEPE in PRP gradually increased up to day 5 followed by a sharp spike on day 10. Although this study has shown that 18-HEPE and 12-HEPE were the main hydroxy acids formed during treatment of platelets with EPA, all other experiments showed that 12-HETE was the main hydroxy fatty acid synthesised by platelets. The amount of 12-HETE produced across all treatment groups was very similar ranging by day 10 from 496 pg/ μ l to 590 pg/ μ l for PRP. This study has shown that hydroxy fatty acids were formed in both PRP and PPP. There was more HODE formed in PPP than PRP and equal amounts of 15-HETE, 8-HEPE, 9-HEPE and 18-HEPE were detected in PRP and PPP from EPA treatment.

6.3.2.1.1 Time dependent hydroxy fatty acid production following incubation of platelets with eicosapentaenoic acid

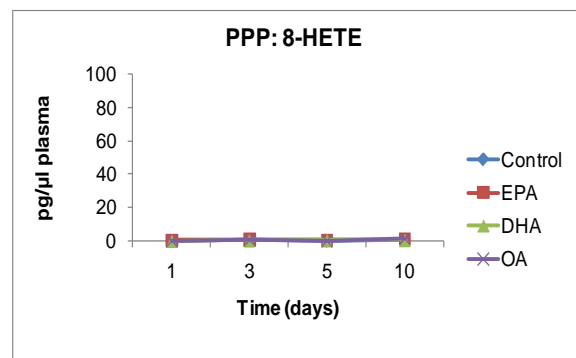
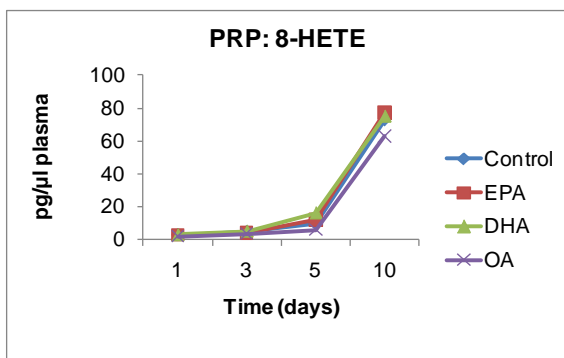
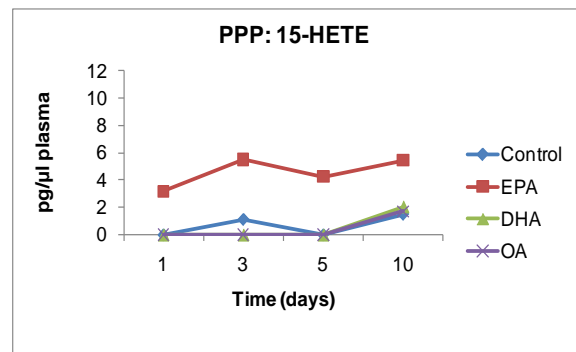
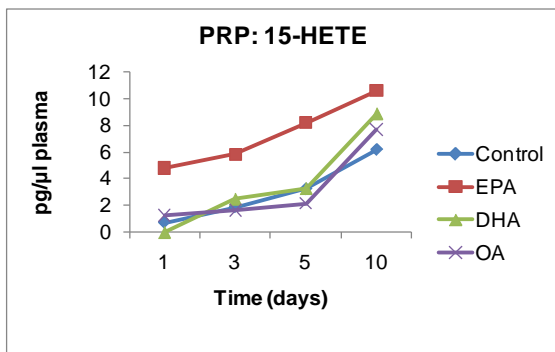
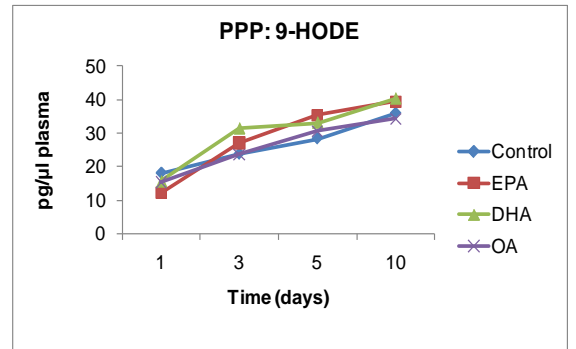
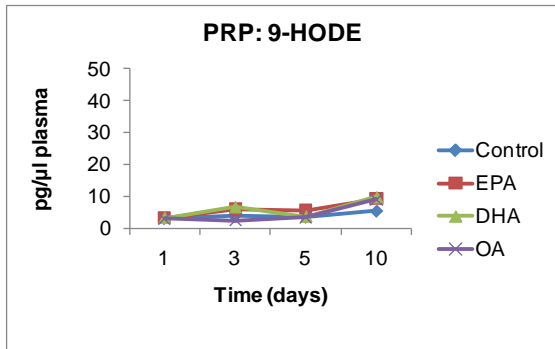
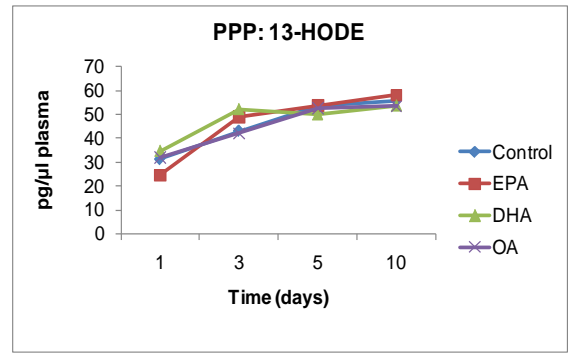
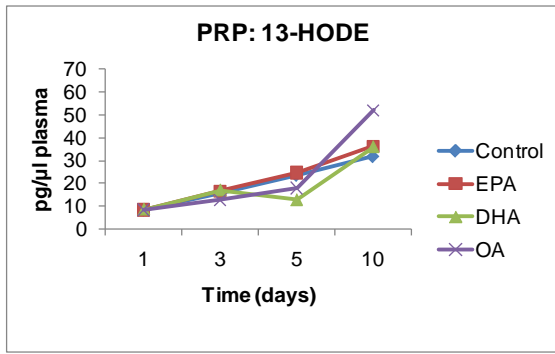
Treating platelets with 100 μ M EPA resulted in the formation of a range of HEPE. Overall, EPA stimulated the synthesis of 15-HETE, 12-HEPE, 8-HEPE, 9-HEPE and 18-HEPE as shown by Figure 6.2. With the exception of 12-HEPE levels of these hydroxy fatty acids were similar in both PRP and PPP. The most abundant hydroxy fatty acids present in plasma following treatment with EPA were 18-HEPE and 12-HEPE. This study showed that levels of HODE and 12-HEPE increased over time but levels of all other HEPE remained constant over the 10 day treatment period with EPA. Data shown in Figure 6.2.

6.3.2.1.2 Time dependent hydroxy fatty acid production following incubation of platelets with docosahexaenoic acid

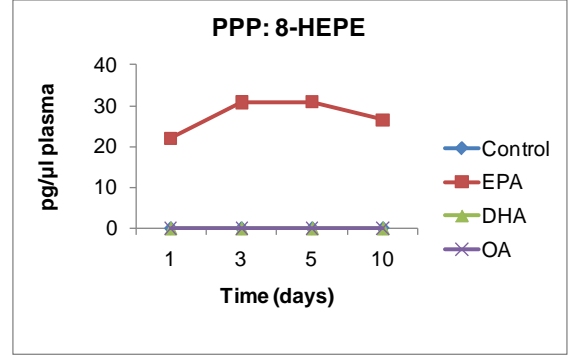
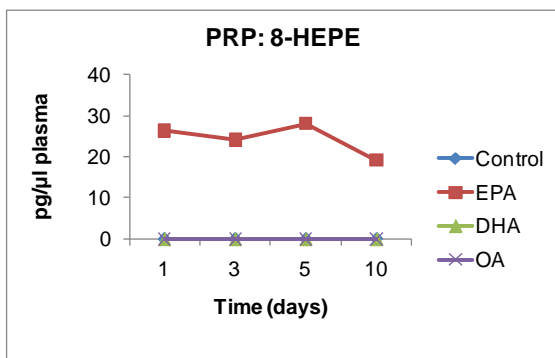
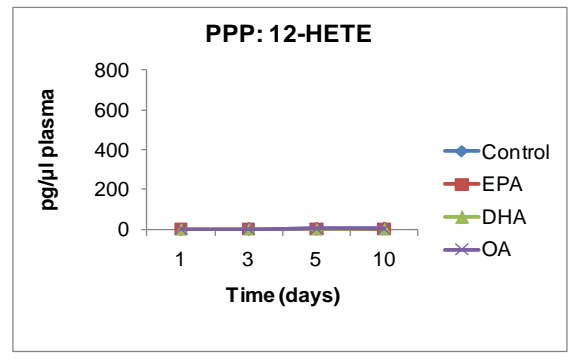
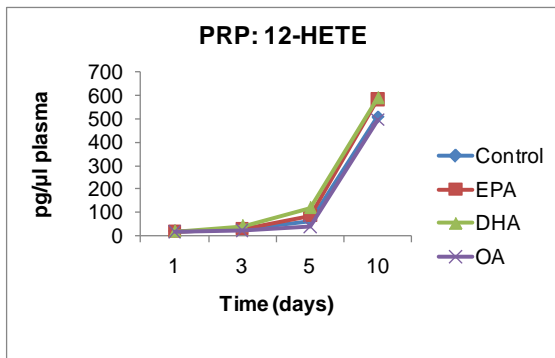
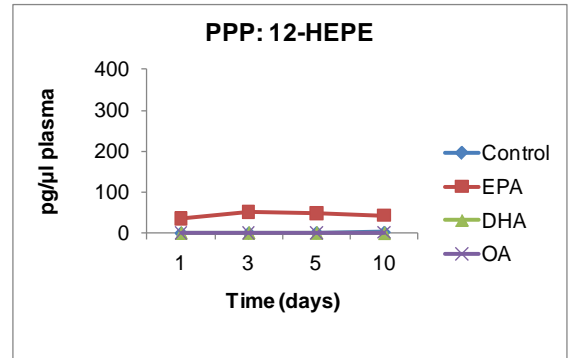
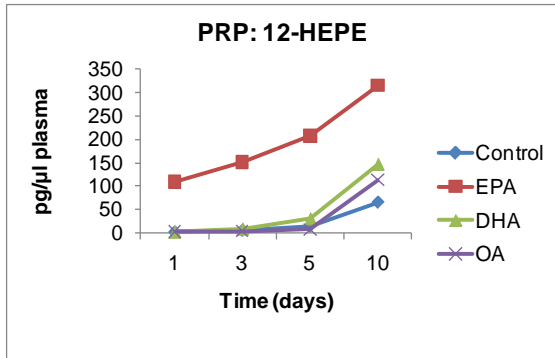
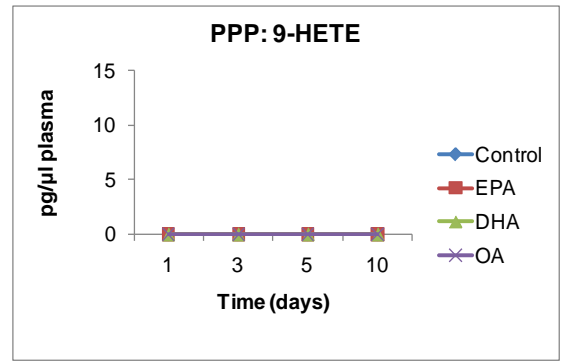
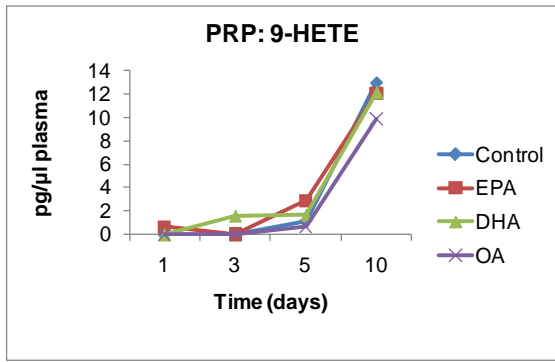
Treating platelets with 100 μ M DHA increased the formation of 12-HEPE in PRP compared to control. By day 10 the concentration of 12-HEPE in PRP from DHA treated samples was approximately double the level of 12-HEPE in control PRP. Data shown in Figure 6.2.

6.3.2.1.3 Time dependent hydroxy fatty acid production following incubation of platelets with octanoic acid

Adding 100 μ M octanoic acid to the incubation medium of platelets did not change the profile of hydroxy fatty acid production from platelets when compared to control. Data shown in Figure 6.2.



(Figure 6.2 continues overleaf...)



(Figure 6.2 continues overleaf...)

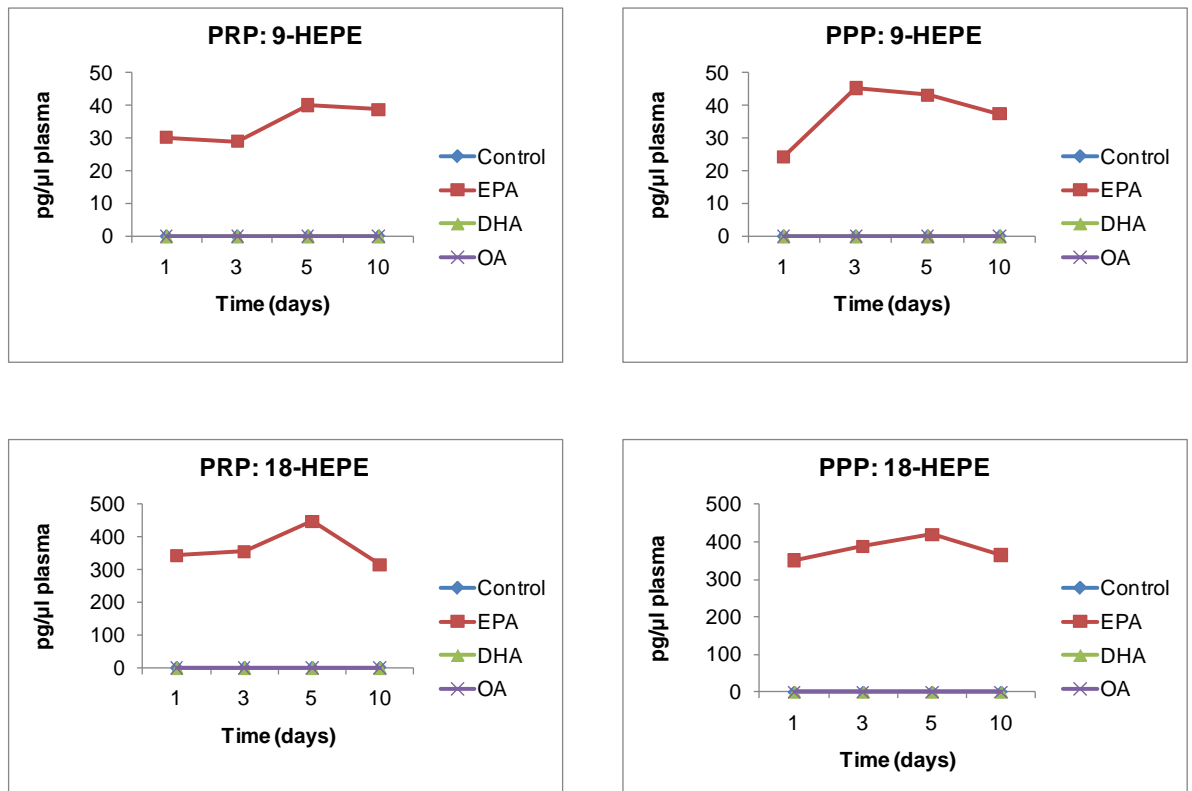


Figure 6.2. The effects of docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and octanoic acid (OA) on hydroxy fatty acid synthesis in platelet rich (PRP) and platelet poor (PPP) plasma during a 10 day incubation period ($n=1$ for PRP and $n=1$ for PPP). Concentration of respective fatty acids is 100 μ M, control is ethanol vehicle.

6.3.3 A study of prostanoid and hydroxy fatty acid production from platelets incubated with eicosapentaenoic acid and niacin

A further study was conducted to investigate the formation of prostanoids and hydroxy fatty acids from platelets immediately after addition of EPA or niacin to the incubation medium. During this study platelets were treated with EPA, niacin or both compounds for a total of 8 days with increased sampling frequency at the start of the study.

6.3.3.1 The effect of eicosapentaenoic acid on platelet prostanoid production

This study has shown that incubating platelets with 100 μ M EPA over a period of 8 days stimulated the synthesis of prostanoids. Specifically, EPA increased the production of PGE₂, PGD₂, TXB₂, TXB₃, PGE₃ and PGD₃ in PRP as shown in Figure 6.3. Levels of PGE₂ and PGD₂ were found to increase in PPP after 3 days whereas levels of PGE₃ and PGD₃ were already relatively high in PPP only 30 min after addition of EPA. All other PPP samples expressed negligible amounts of prostanoid even after 8 days. Overall the concentration of all prostanoids remained relatively constant for up to 120 min after addition of EPA. Between day 5 and day 8 there was a sudden increase in the concentration of all detected prostanoids. In all cases platelets incubated with EPA produced much higher levels of prostanoids than control.

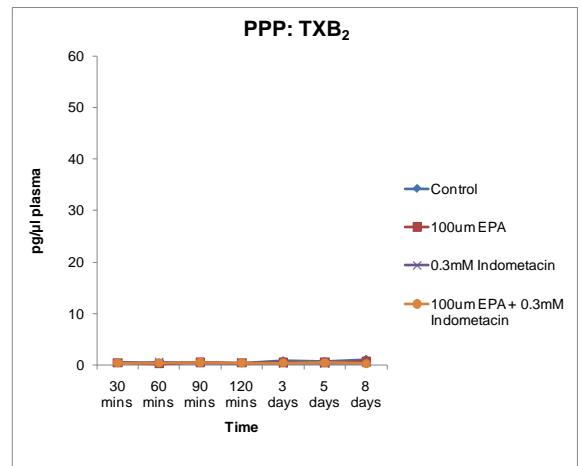
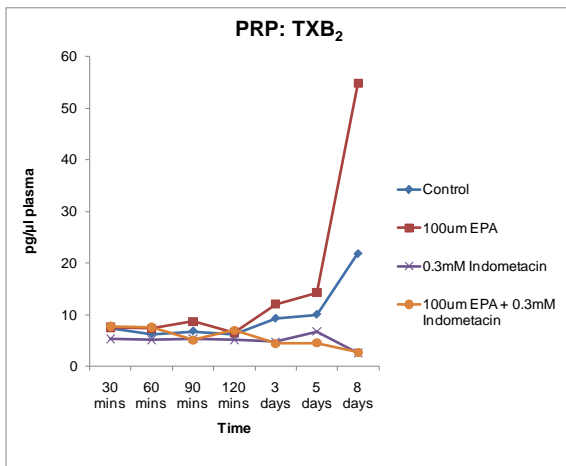
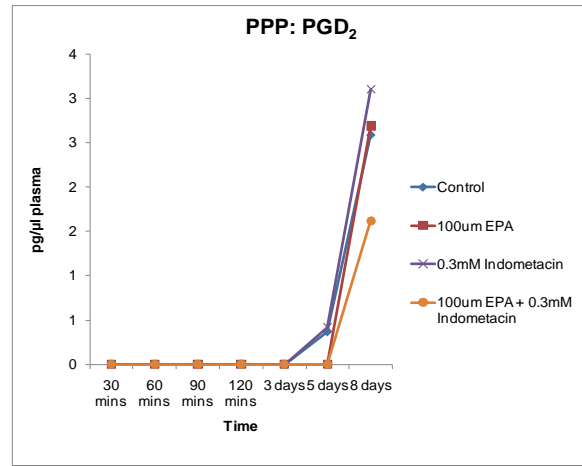
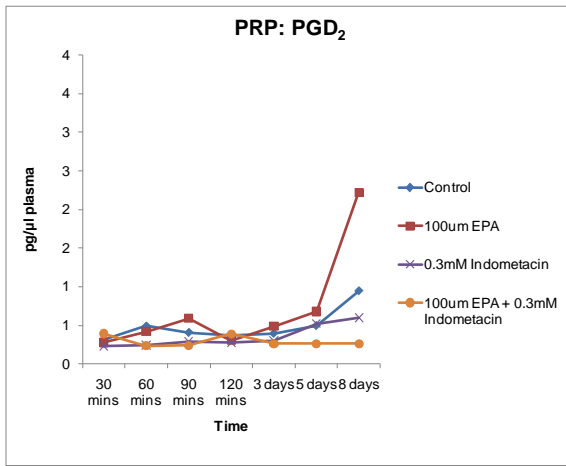
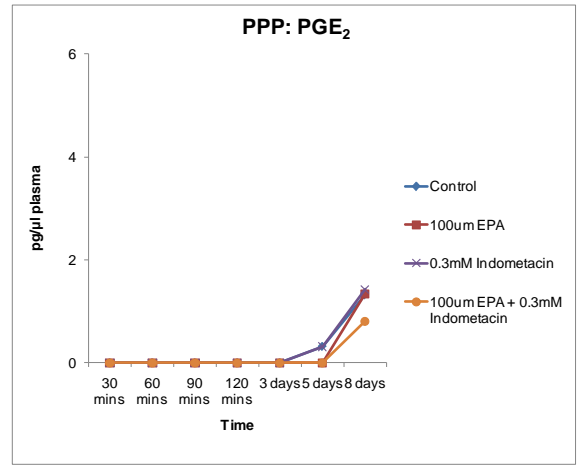
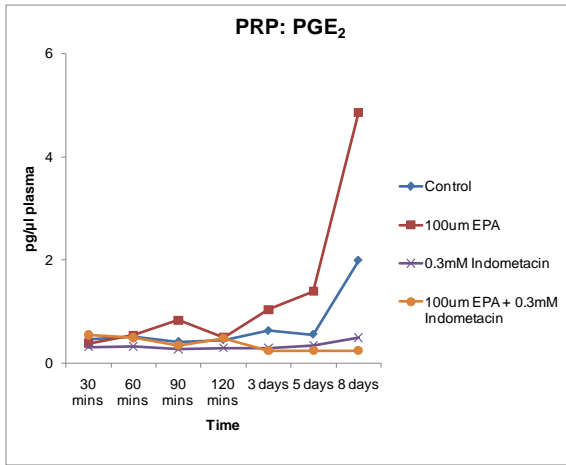
6.3.3.2 The effect of indometacin on platelet prostanoid production

Indometacin has been shown to inhibit prostanoid production in PRP during this study. Levels of prostanoids remain relatively constant for 5 days after addition of 0.3 mM indometacin. Indometacin prevented the spike in prostanoid formation observed in

control samples from day 5 in PRP. As levels of prostanoids appear to gradually rise from day 5 onwards, this indicates incomplete block of COX.

6.3.3.3 Prostanoid production from platelets incubated with eicosapentaenoic acid and indometacin

Whilst EPA alone stimulated prostanoid production in PRP, indometacin has been shown to inhibit prostanoid production as described in Figure 6.3. Platelets treated with both EPA and indometacin only produced relatively small amounts of prostanoids throughout the 8 day incubation period. The only exception to this was in the case of PGE₃ and PGD₃ where levels of these prostanoids from EPA alone and EPA plus indometacin were similar at the start of the study. It was found that levels of PGE₃ and PGD₃ gradually decreased in the presence of indometacin over the 8 day period but continued to increase where platelets were treated with EPA alone.



(Figure 6.3 continues overleaf...)

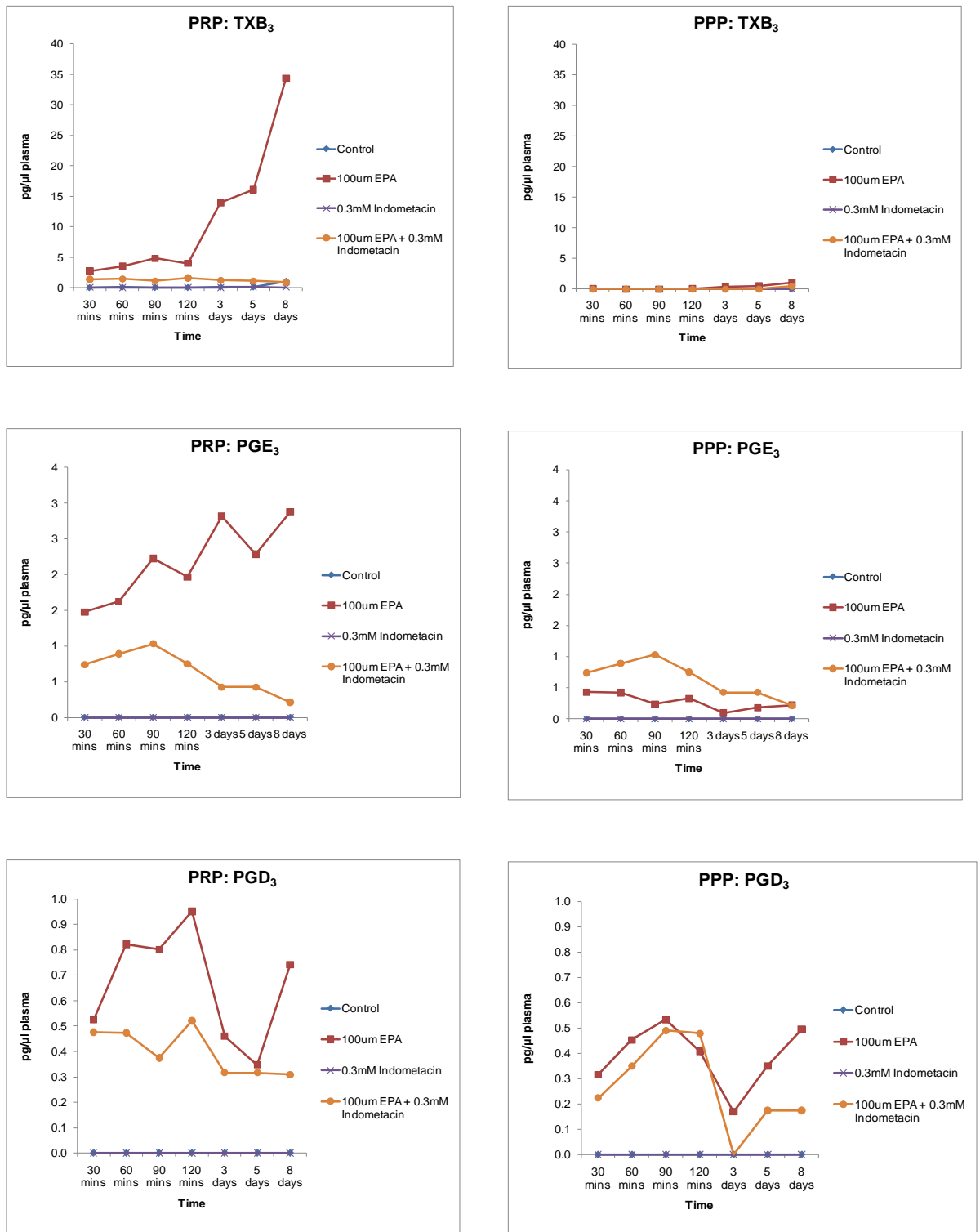


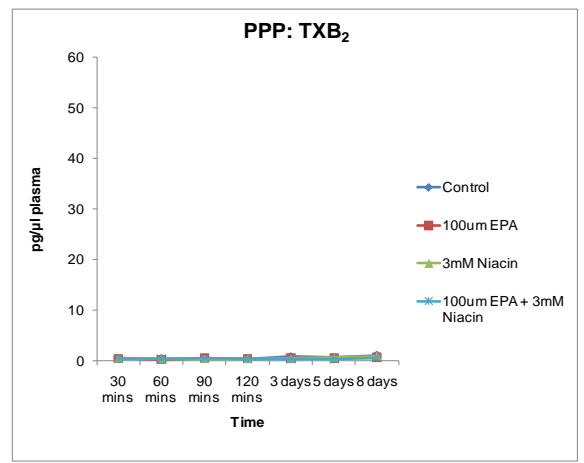
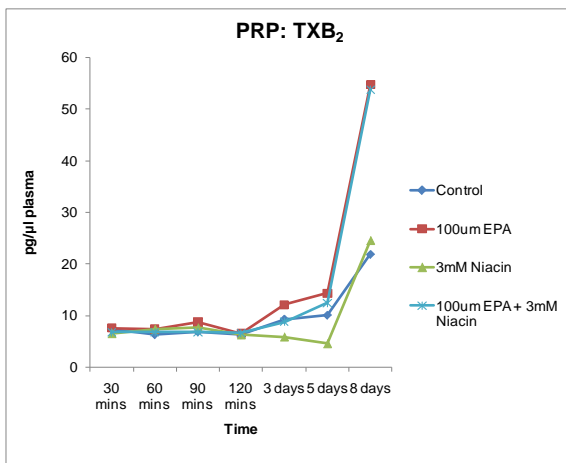
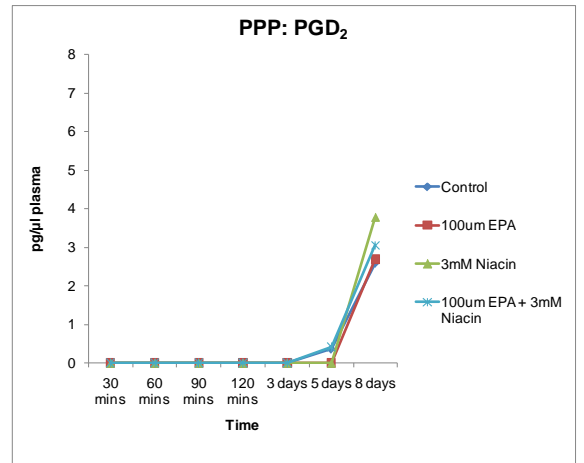
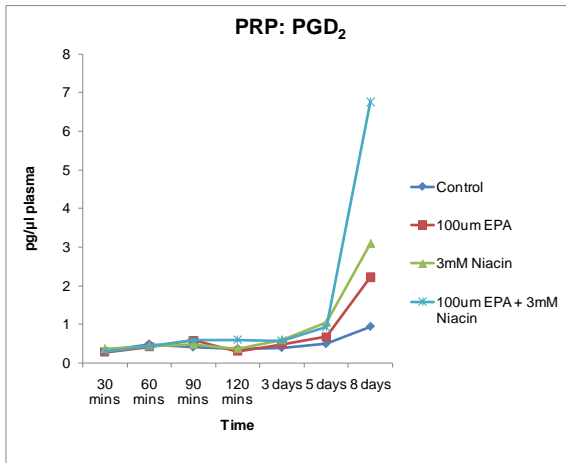
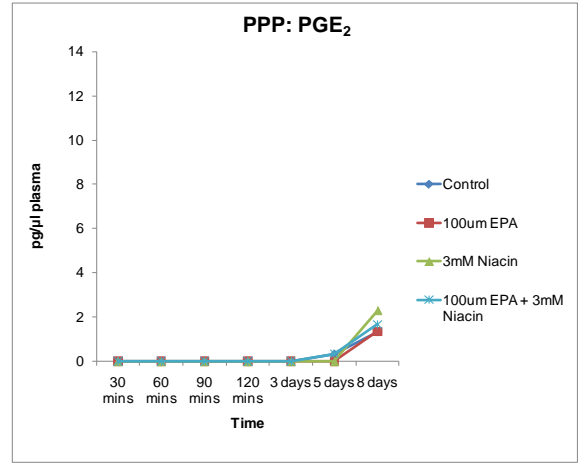
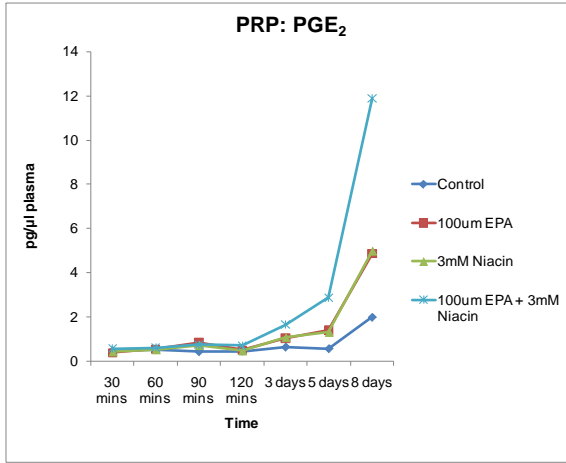
Figure 6.3. The effects of eicosapentaenoic acid (EPA), indometacin alone and EPA plus indometacin on prostanoïd synthesis in platelet rich (PRP) and platelet poor (PPP) plasma during an 8 day incubation period ($n=1$ for PRP and $n=1$ for PPP). EPA and indometacin are 100 µM and 0.3mM respectively, control is ethanol vehicle.

6.3.3.4 The effect of niacin on platelet prostanoid production

Incubating platelets with 3 mM niacin for a period of 8 days stimulated the production of PGE₂ and PGD₂ as shown in Figure 6.4. The effect of niacin on PGE₂ synthesis from PRP is the same as the effect of EPA alone. Niacin stimulated the platelet to produce more PGD₂ than EPA alone or control. Niacin alone did not affect the production of TXB₂ or TXB₃ and did not stimulate release of PGE₃ or PGD₃ from platelets.

6.3.3.5 Prostanoid production from platelets incubated with eicosapentaenoic acid and niacin

Figure 6.4 shows that incubating platelets with 100 μM EPA plus 3 mM niacin stimulated the formation of PGE₂, PGD₂, TXB₂, TXB₃, PGE₃ and PGD₃ in PRP. In each case the combined treatment resulted in greater prostanoid formation than either treatment individually. Thus, it appears as though niacin amplifies the effects of EPA on platelet prostanoid production. The combined treatment also stimulated the formation of PGD₂ in PPP after day 3. As with EPA alone, the combined treatment showed the formation of PGD₃ in PPP.



(Figure 6.4 continues overleaf...)

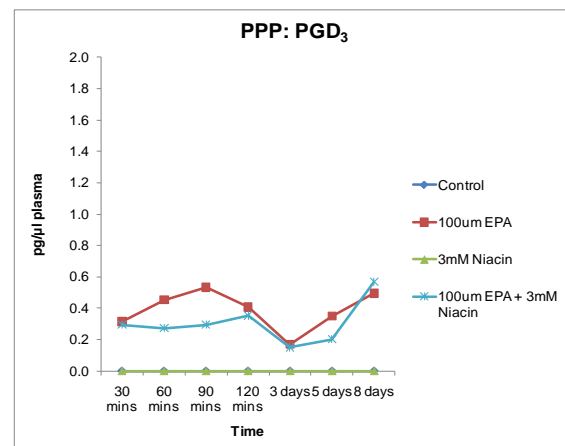
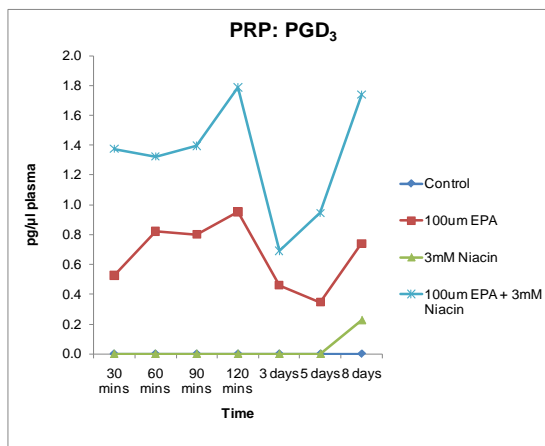
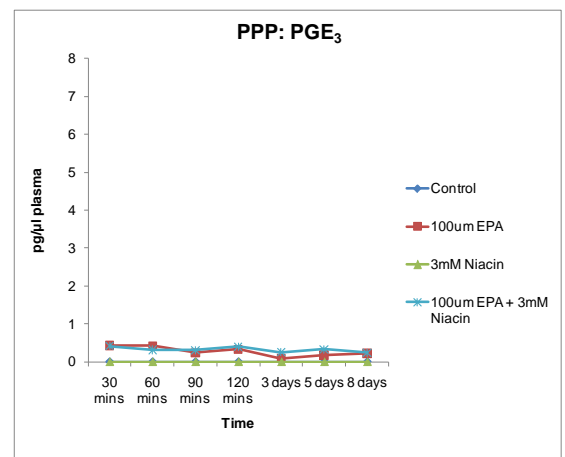
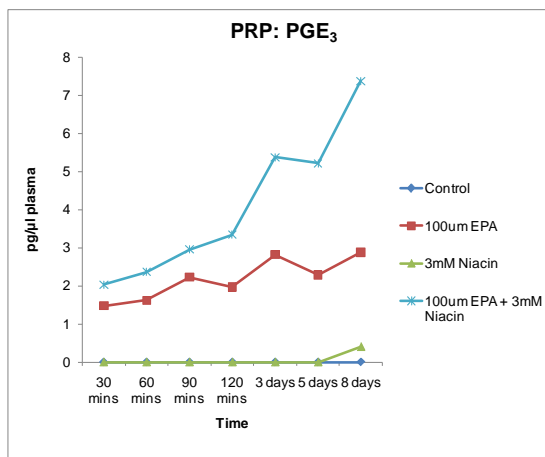
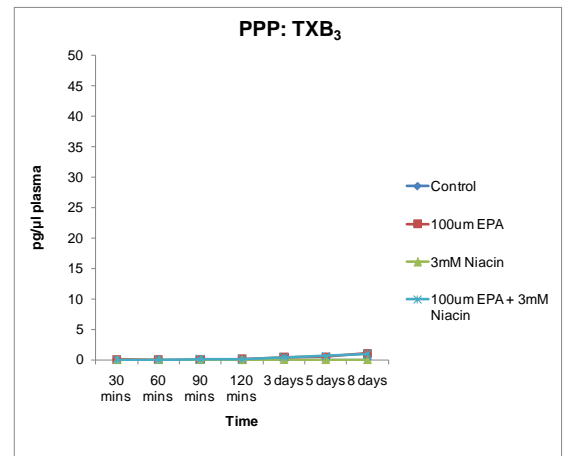
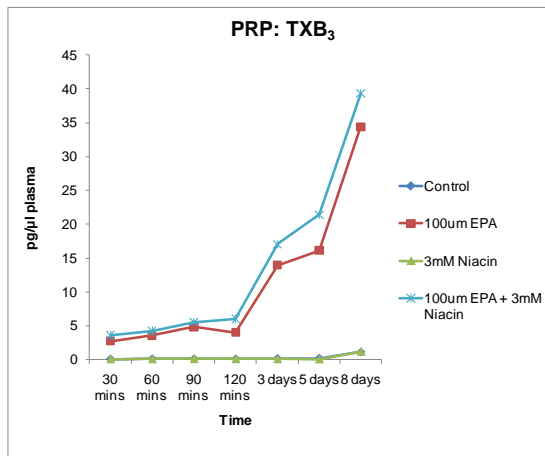


Figure 6.4. The effects of eicosapentaenoic acid (EPA), niacin alone and EPA plus niacin on prostanoid synthesis in platelet rich (PRP) and platelet poor (PPP) plasma following an 8 day incubation period ($n=1$ for PRP and $n=1$ for PPP). Concentrations of EPA and niacin are 100 μM and 3 mM respectively, control is ethanol vehicle.

6.3.3.6 The effect of eicosapentaenoic acid on platelet hydroxy fatty acid production

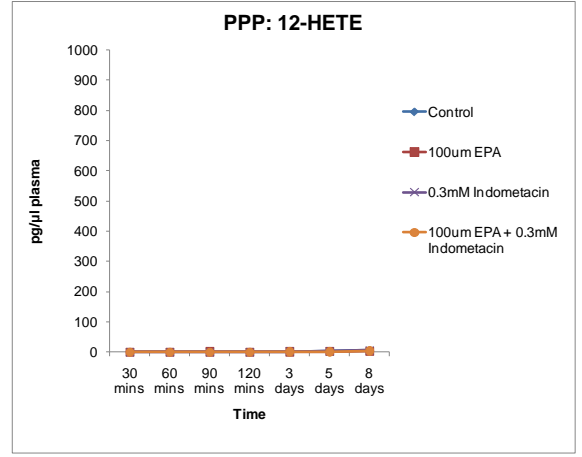
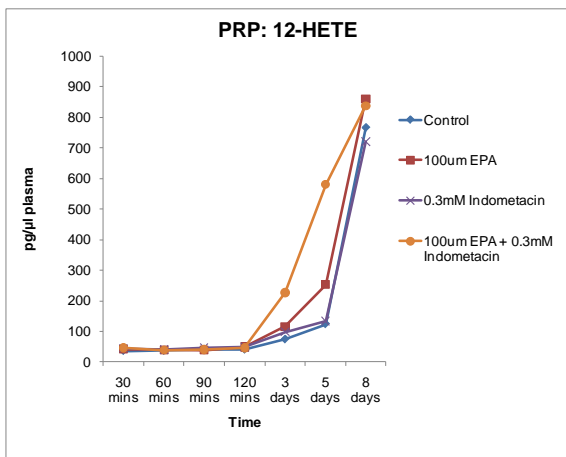
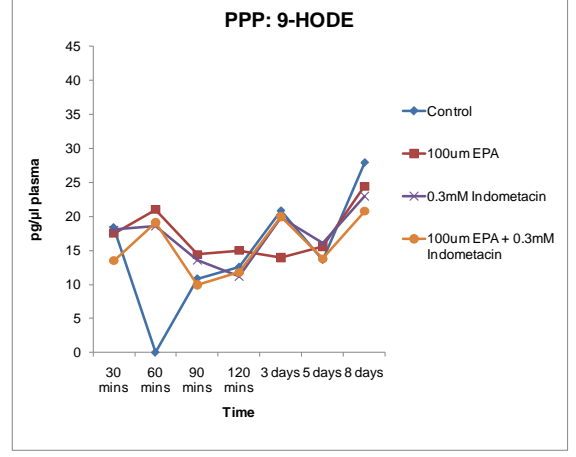
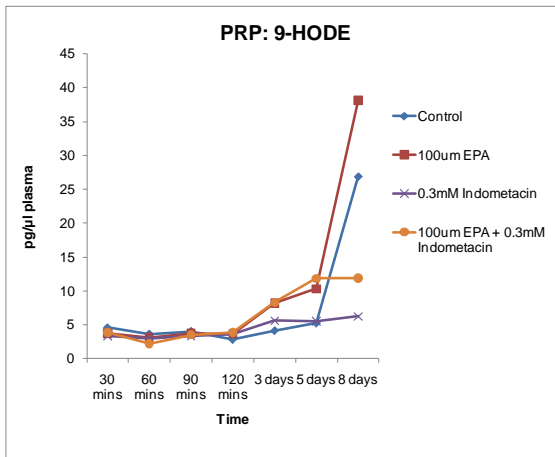
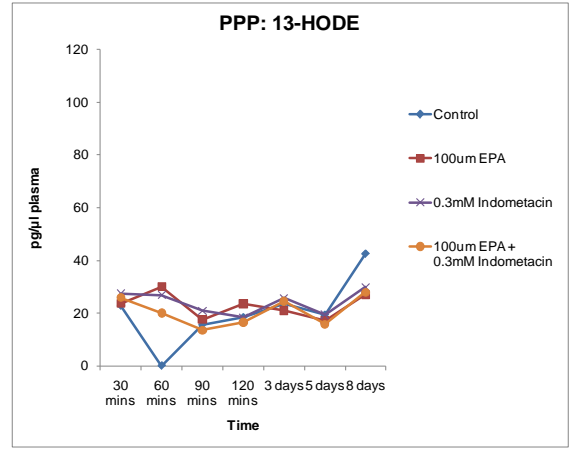
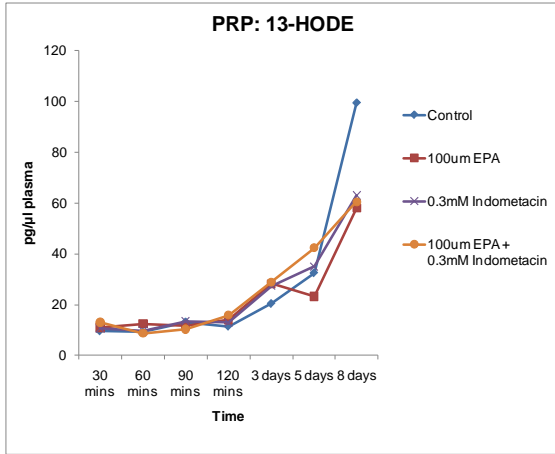
This study has shown that treating platelets with 100 μ M EPA over a period of 8 days stimulated the synthesis of hydroxy fatty acids. Specifically, EPA increased the production of 9-HODE, 8-HEPE and 12-HEPE from PRP when compared to control, as shown in Figure 6.5. The most prominent hydroxy fatty acid found in PRP following treatment with EPA was 12-HEPE. 13-HODE and 9-HODE were formed in both PRP and PPP throughout the treatment period. Whilst 15-HETE and 8-HEPE could be detected in PRP, relatively small amounts of these hydroxy fatty acids could be detected in PPP at around day 5 of treatment. Other hydroxy fatty acids were absent in PPP.

6.3.3.7 The effect of indometacin on platelet hydroxy fatty acid production

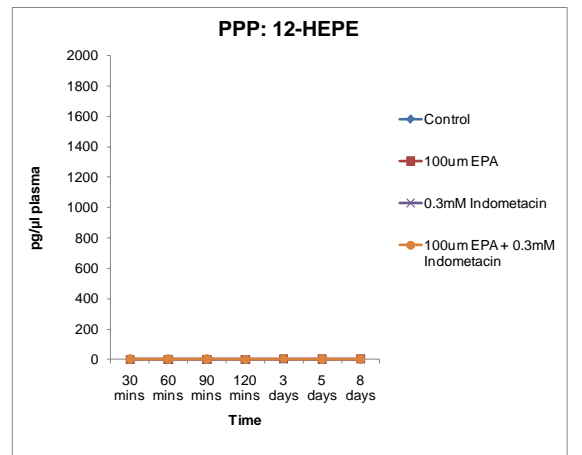
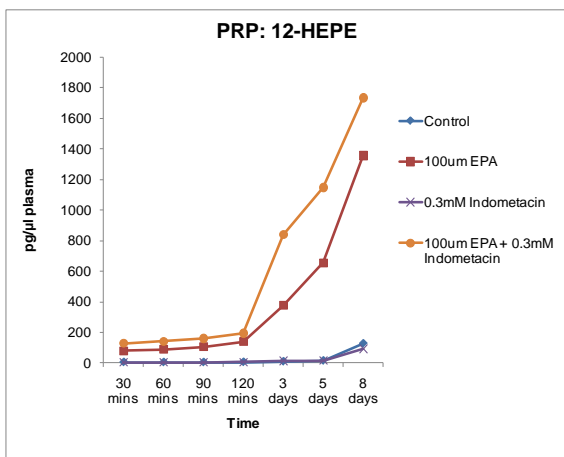
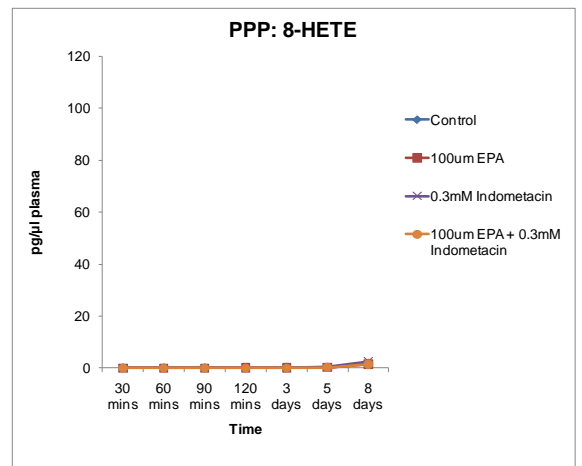
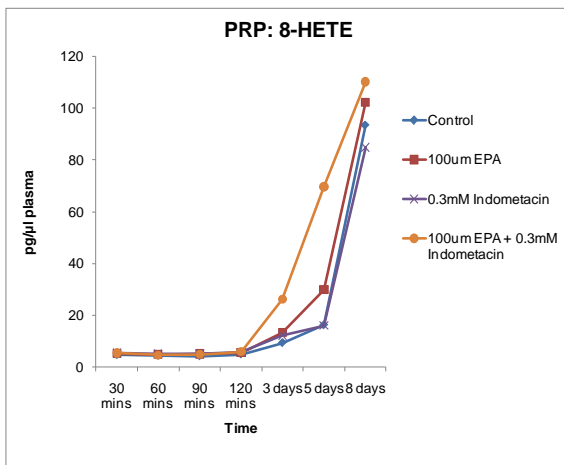
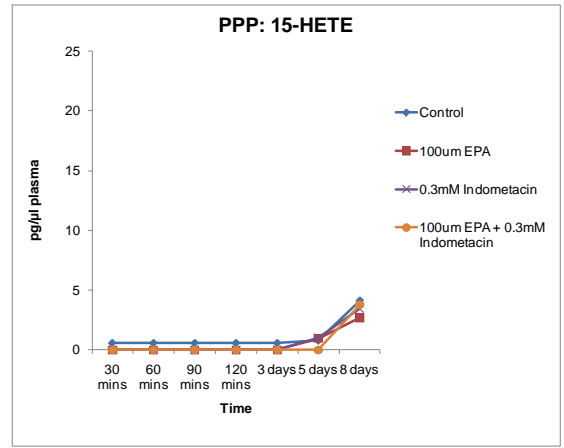
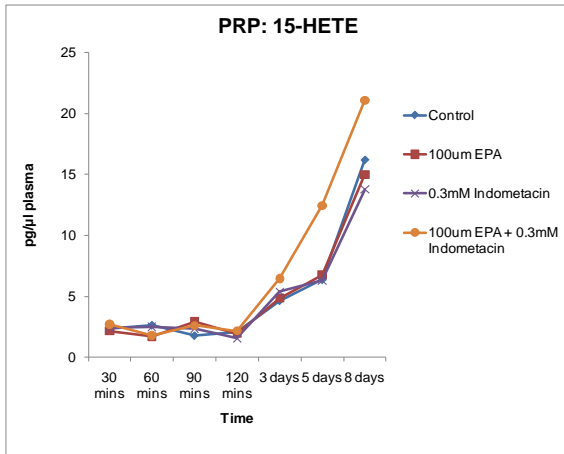
This study has shown that indometacin did not prevent the formation of hydroxy fatty acids in PRP. Levels of hydroxy fatty acids after addition of indometacin continued to increase at the same rate as control. As expected indometacin had no effect on the formation of 15-HETE and 8-HEPE in PPP.

6.3.3.8 Hydroxy fatty acid production from platelets treated with eicosapentaenoic acid and indometacin

Treatment of platelets with EPA and indometacin together had a similar effect on hydroxy fatty acid production as either treatment alone as shown in Figure 6.5.



(Figure 6.5 continues overleaf...)



(Figure 6.5 continues overleaf...)

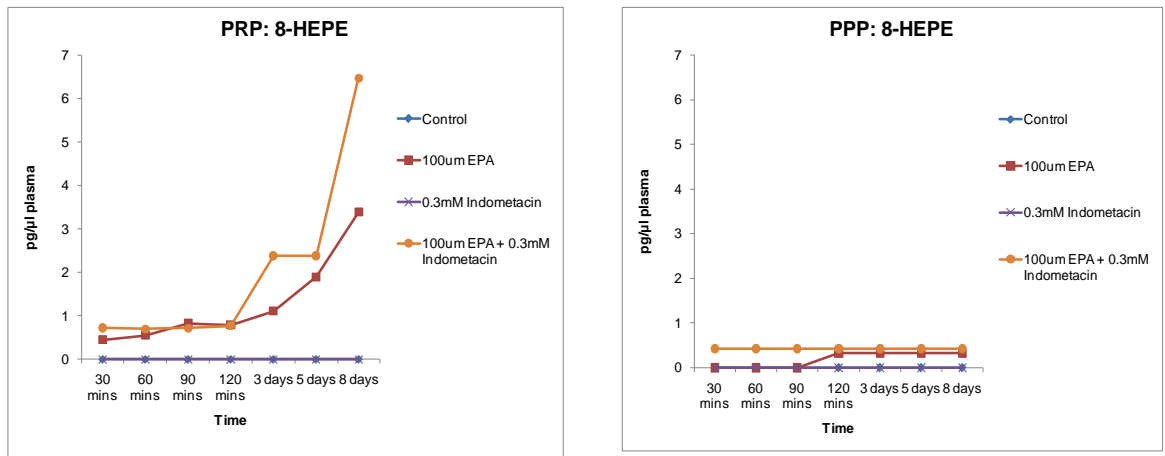


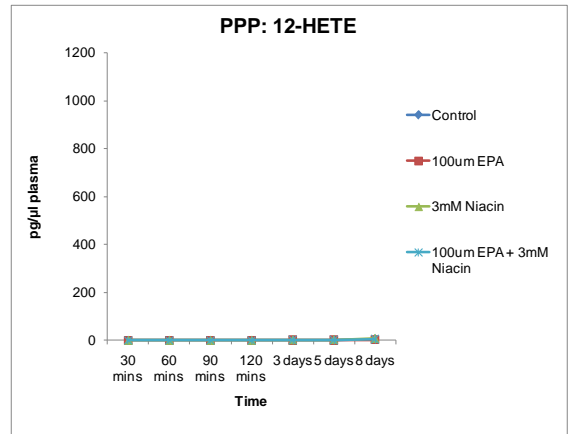
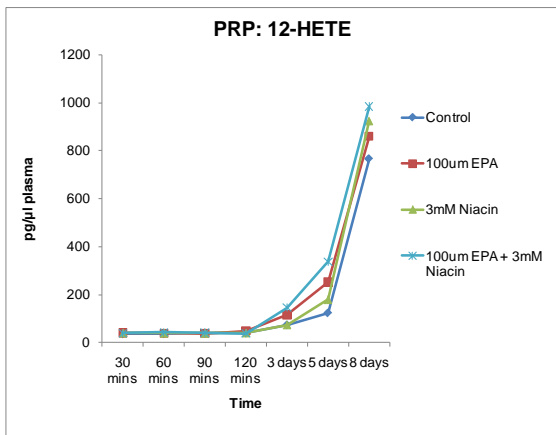
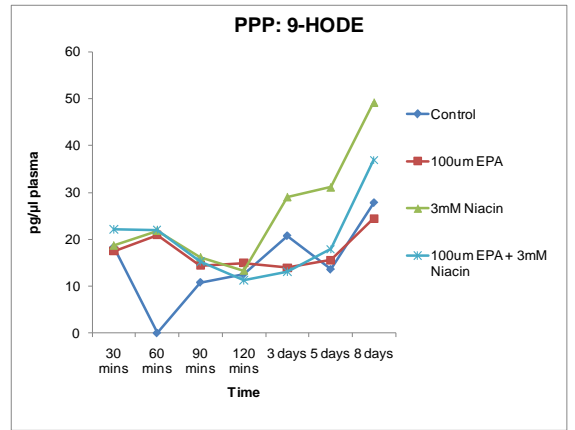
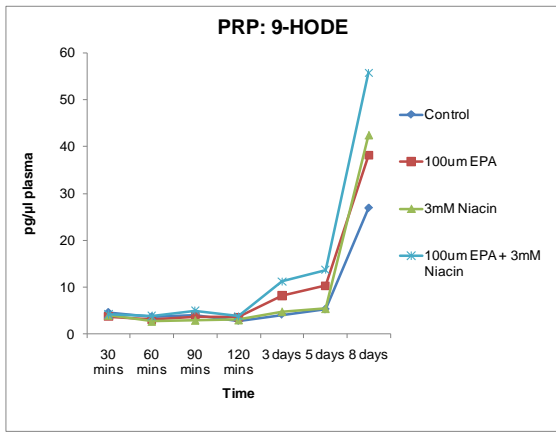
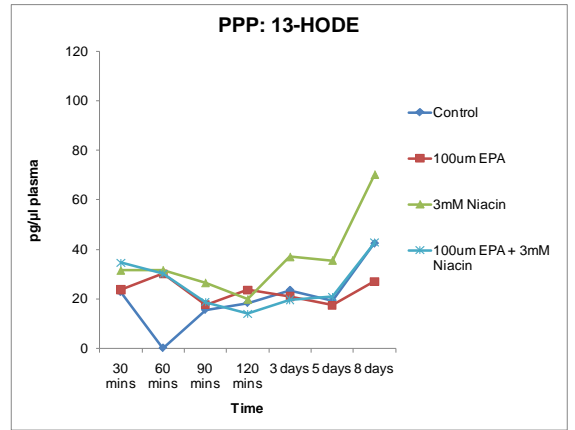
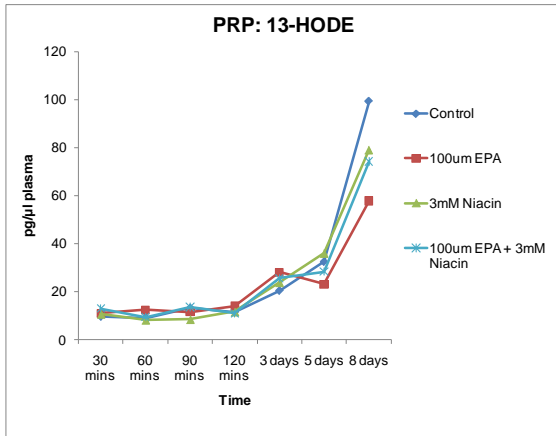
Figure 6.5. The effects of eicosapentaenoic acid (EPA), indometacin alone and EPA plus indometacin on hydroxy fatty acid synthesis in platelet rich (PRP) and platelet poor (PPP) plasma following an 8 day incubation period ($n=1$ for PRP and $n=1$ for PPP). Concentrations of EPA and indometacin are 100 μ M and 0.3 mM respectively, control is ethanol vehicle

6.3.3.9 The effect of niacin on platelet hydroxy fatty acid production

Figure 6.6 shows that treating platelets with 3 mM niacin over a period of 8 days affected platelet hydroxy fatty acid production after day 5. During the initial 5 days of the treatment period the hydroxy fatty acid profile of niacin treated samples was the same as control samples. After day 5 PRP treated with niacin produced a higher concentration of HETE than control. The concentration of HEPE was unaffected by niacin whilst levels of 9-HODE were above control.

6.3.3.10 Hydroxy fatty acid production from platelets treated with eicosapentaenoic acid and niacin

Figure 6.6 shows that treating platelets with 100 μ M EPA plus 3 mM niacin stimulated a rise in levels of 9-HODE, 12-HETE, 15-HETE, 8-HETE and 12-HEPE in PRP. With the exception of 8-HETE the combined treatment resulted in higher concentrations of hydroxy fatty acids than either treatment individually. Thus, it appears as though niacin augments the action of EPA on platelet hydroxy fatty acid production.



(Figure 6.6 continued overleaf...)

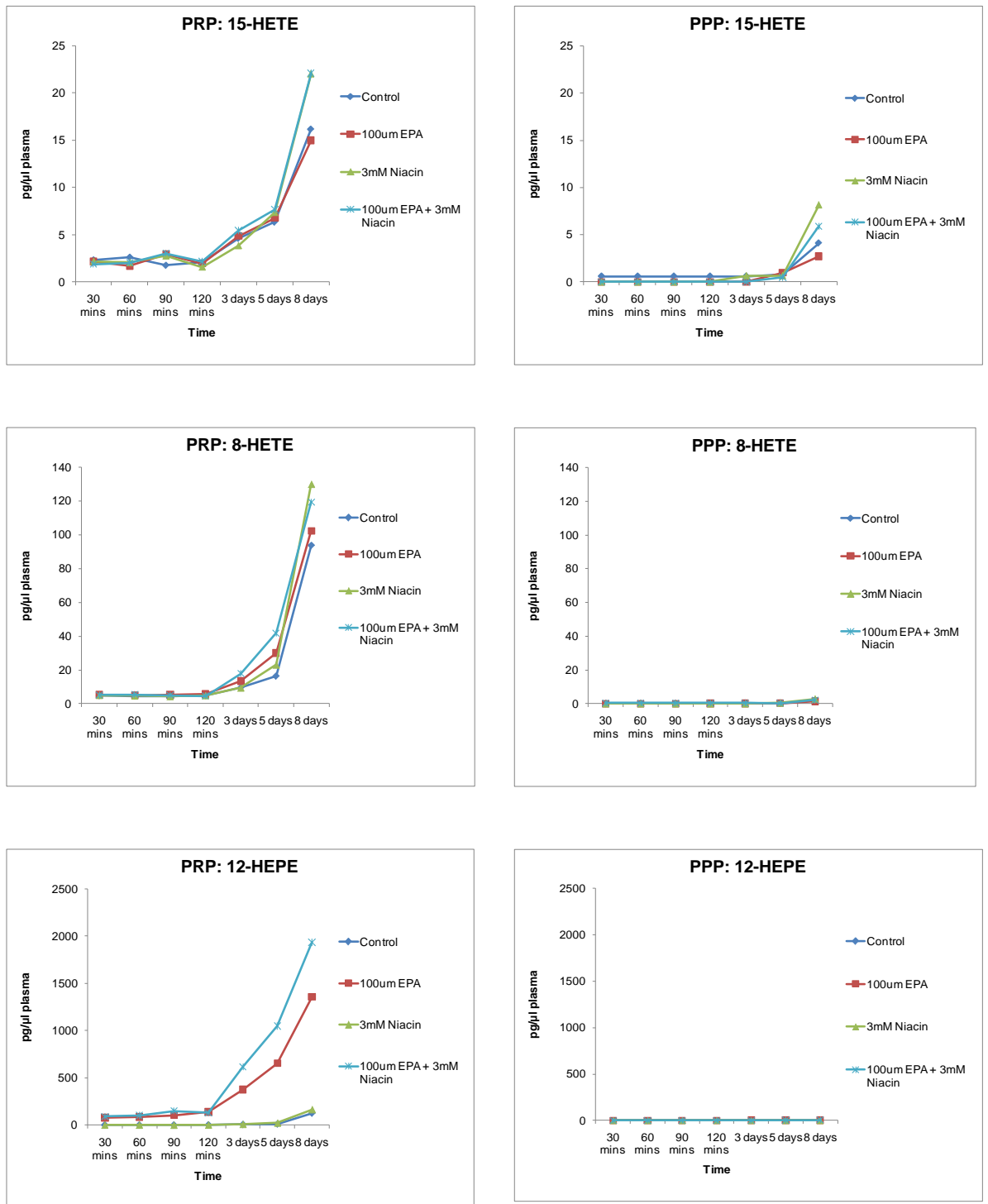


Figure 6.6. The effects of eicosapentaenoic acid (EPA), niacin alone and EPA plus niacin on hydroxy fatty acid synthesis in platelet rich (PRP) and platelet poor (PPP) plasma following an 8 day incubation period ($n=1$ for PRP and $n=1$ for PPP). EPA and niacin are 100 μ M and 3 mM respectively, control is ethanol vehicle

6.4 Discussion

The aim of the current study was to investigate the effects of EPA and DHA on platelet prostanoid and hydroxy fatty acid production in a time dependent manner. The effects of niacin on platelet eicosanoid production were also investigated. Platelets were treated with niacin alone or with niacin and EPA combined. Platelet rich samples were incubated in parallel with platelet poor samples to confirm that any changes were due to platelet metabolism rather than any other mechanism.

Control experiments have shown that out of the mediators analysed, TXB₂ and 12-HETE were the most abundant. This is in agreement with previous work where these mediators have been shown to be formed from platelet COX-1 and hp-12-LOX respectively (Dutilh *et al.*, 1979, Hamberg *et al.*, 1975). In addition to TXB₂ and 12-HETE control experiments have demonstrated that the platelet was able to form prostanoids including PGE₂, PGD₂ and TXB₃. Previous work confirms that the platelet is able to produce minor quantities of PGE₂ and PGD₂ particularly after stimulation with thrombin (Lagarde *et al.*, 1979, Oelz *et al.*, 1977) but the finding in relation to TXB₃ was somewhat surprising.

To date TXB₃ has only been found to be synthesised from platelets in an EPA rich environment (Kramer *et al.*, 1996). Thus, it appears as though EPA was naturally present in the membranes of control platelets. Previous studies have demonstrated that platelets naturally express EPA within their membranes (Croset *et al.*, 1992, Von Schacky *et al.*, 1985b). Further evidence for the presence of EPA in the control samples comes from the finding that 12-HEPE was one of the most abundant hydroxy fatty acids synthesised by platelets, second only to 12-HETE.

Other hydroxy fatty acids formed in PRP during control experiments included the AA metabolites 15-HETE and 8-HETE and the linoleic acid (LA) metabolites 13-HODE and 9-HODE. It has previously been shown that hp-12-LOX, as well as primarily catalysing the formation of 12-HETE and 12-HEPE, also catalyses the formation 8-HETE as well as 13-HODE and 9-HODE (Burger *et al.*, 2000). Platelets are also known to form 15-HETE from AA (Kim *et al.*, 1990, Morita *et al.*, 1990). Both LA and DHA have been found to be naturally occurring within the platelet cell membrane (Renaud *et al.*, 1970, Daret *et al.*, 1989, Marcus *et al.*, 1969). This study has shown that the platelet also formed 9-HETE during control experiments. To date this hydroxy fatty acid has not been found to be a product of platelet metabolism.

This study has shown that control experiments with PPP produced PGE₂, PGD₂ and some 15-HETE from day 5 onwards. This suggests that there were some platelets present in PPP that were forming eicosanoids, some of which were detected from day 5 onwards. Although platelets were the most likely source of these eicosanoids previous work has shown that PGD₂ can be formed through an isomerisation reaction involving albumin present in plasma (Smith *et al.*, 1976, Watanabe *et al.*, 1982). It is suggested that PGH₂, formed within the platelet, can be isomerised to PGD₂ in PPP in the presence of albumin, this would account for the observed elevated concentration of PGD₂ in PPP.

In addition to control experiments platelets were treated with octanoic acid. To date there is no evidence to suggest that octanoic acid affects platelet biochemistry. Incubating platelets with this inactive fatty acid gave a profile of eicosanoids and hydroxy fatty acids identical to control. This shows that any changes associated with

EPA or DHA treatment were related to the PUFA and not their salt or experimental manipulation.

Treating platelets with DHA stimulated the formation of PGD₂, 12-HEPE and 15-HETE when compared to control. It has been shown previously that DHA will actively bind to albumin resulting in a small but significant increase in the formation of PGD₂ as demonstrated by this study (Gaudette and Holub, 1990). It has been suggested that DHA inhibits COX-1 thereby attenuating formation of the prostanoids, particularly TXB₂ and preventing aggregation (Rao *et al.*, 1983). Although levels of PGE₂ are lower in PRP incubated with DHA this study does not support the notion that this PUFA is a COX inhibitor. This study has demonstrated that levels of TXB₂ in PRP following incubation with DHA are the same as control. It appears therefore that the disaggregatory actions of DHA may be partly due to elevated levels of PGD₂ but may also be due to an eicosanoid independent mechanism.

The addition of EPA to the incubation medium resulted in the immediate synthesis of the series-3 prostanoids and HEPE. In particular levels of 12-HEPE and TXB₃ were found to increase several fold when compared to control. This observation is in agreement with previous literature (Taylor *et al.*, 1987, Von Schacky *et al.*, 1985b). There was a sudden presence of PGE₃ and PGD₃ on day 0 which gradually decreased over a 10 day period. Both of these prostaglandins were found in PRP and PPP suggesting that they did not originate from the platelet during this experiment. This change was accompanied by a sudden rise in levels of 8-HEPE, 9-HEPE and 18-HEPE. In all cases levels of these hydroxy fatty acids in PRP were matched by PPP suggesting that they were not formed by platelets in PRP. Only levels of 12-HEPE were higher in

PRP than PPP suggesting that the source of this mediator were platelets. The sudden presence of PGE₃, PGD₃ and 18 HEPE in the platelet incubation medium is previously undescribed. To date there are no studies that show 18-HEPE to be more abundant than 12-HEPE following incubation of platelets with EPA.

This study has effectively demonstrated that EPA changed the eicosanoid and hydroxy fatty acids profiles of incubated platelets. Some of the observed changes were in agreement with literature but the fact that PGD₂, 18-HEPE, 9-HEPE, 8-HEPE and 15-HETE were found in equal measure in PRP and PPP suggests that these eicosanoids did not originate from the platelet. Furthermore, this study showed that many of the effects of EPA, especially in relation to the formation of PGE₃ and PGD₃ appeared within 1 day of incubation. This study demonstrated that by day 10 platelets produced a surge of eicosanoids as they neared the end of their lifespan.

A further study was therefore designed to confirm the EPA related effects observed during this work. Sampling frequency was increased at the start of the incubatory period to explore the immediate formation of PGE₃ and PGD₃. Platelets were incubated for a total of 8 days as it had been demonstrated that by day 10 platelets showed a spike in eicosanoid formation indicating death. Indometacin, a non-selective COX inhibitor, was used to confirm that prostanoid production in the platelet was COX mediated. Platelets were also incubated with niacin to investigate whether this vitamin induced changes in platelet eicosanoid production. Niacin has previously been shown to reduce platelet adhesion and aggregation and reduce inflammation (Swies and Dabrowski, 1984, Walldius and Wahlberg, 1985, Rosenson, 2003).

Treating platelets with EPA during the second study resulted in the formation of the same prostanoids as seen in the previous study. The concentration of prostanoids detected was however much higher in the case of TXB₂ and TXB₃ and much lower in the case of PGE₃ and PGD₃. Furthermore, this experiment showed a time dependent increase in prostanoid production whereas the previous study showed that levels of prostanoid following incubation with EPA either remained constant or decreased. Concentrations of PGE₃ and PGD₃ in PPP during the previous study were especially elevated and found to decrease over time. During the current study levels of these prostaglandins in PPP were much lower and remained relatively constant. This experiment confirmed that the platelet did form PGD₃ and PGE₃ within 30 min of addition of EPA to the incubation medium. The platelet also formed TXB₃ within 30 min of addition of EPA and this was the most abundant prostanoid in PRP. As seen previously, there was a spike in prostanoid formation from day 5 onwards.

The previous study showed 18-HEPE to be the most abundant hydroxy fatty acid present in PRP during incubation with EPA. EPA also stimulated the formation of 9-HETE and 9-HEPE, previously undescribed observations. The current study failed to produce 18-HEPE, 9-HETE or 9-HEPE from PRP following incubation with EPA. Furthermore, as with prostanoids, this study has shown that levels of hydroxy fatty acids gradually increased over the 8 day incubation period whereas in the previous study levels of HEPE were observed to fall with time. Both 12-HEPE and 8-HEPE were formed at much higher concentrations than control within 30 min of addition of EPA. The concentration of HODE appeared to increase in both studies and relatively high levels of HODE were found in both PPP and PRP. Whilst increasing level of both 9-

HODE and 13-HODE in PRP confirms that platelets can form these hydroxy fatty acids their presence in PPP indicates they are also derived from other sources.

Indometacin was found to block the synthesis of all detected prostanoids. Even with the addition of indometacin however, platelets were able to form some PGE₃, PGD₃ and TXB₃ during treatment with EPA. This finding is consistent with the finding that these prostanoids are formed within 30 min of addition of EPA and indometacin does not completely block COX within this timeframe. Thus, it can be seen that levels of PGE₃, PGD₃ and TXB₃ gradually decrease over time following incubation of PRP with EPA and indometacin. Falls in levels of these prostanoids are probably due to their relative instability coupled to the fact that they are no longer being synthesised due to COX inhibition (Karim *et al.*, 1968, Russell *et al.*, 1975). Thus this work has shown that platelet COX is responsible for the formation of prostanoids in PRP.

Indometacin was found to decrease the formation of 13-HODE and 9-HODE but not of any other hydroxy fatty acid. It has previously been reported that both COX and LOX mediate HODE synthesis in the platelet (Daret *et al.*, 1989). This study has shown that by blocking COX through indometacin the platelet can still form relatively lower concentrations of HODE through LOX. The fact that HODE is consistently present in PPP at relatively stable concentrations from 30 min to 8 days suggests that this hydroxy fatty acid is naturally present in circulating blood. This fits in well with the fact that HODE is used as a marker of oxidative stress. Several studies have shown that background levels of HODE are present in plasma that reflect the oxidative burden at any given time and this fits in well with the current study (Niki and Yoshida, 2005, Yoshida *et al.*, 2008a, Yoshida *et al.*, 2008b).

The addition of niacin to the incubation medium of platelets stimulated the formation of both PGE₂ and PGD₂ when compared to control. It is known that PGD₂ prevents platelet aggregation and there are conflicting reports about the aggregatory properties of PGE₂, although most work indicates this prostaglandin as being disaggregatory (Bertele *et al.*, 1984, Kangasaho and Vapaatalo, 1983, Strukova *et al.*, 1985). Niacin did not affect levels of TXB₂ or TXB₃ compared to control. When niacin was added to platelets treated with EPA the effects of EPA were amplified. Thus, the combined treatment increased the formation of PGE₃, PGD₃, TXB₃, PGE₂, PGD₂ and TXB₂ compared to incubation with EPA alone. This study has shown that niacin stimulates the release of disaggregatory prostanoids from the platelet and that niacin in combination with EPA amplifies the effects of EPA alone. It is unclear if the combined treatment would augment or attenuate aggregation based on prostanoid synthesis alone.

Niacin alone only appeared to increase levels of hydroxy fatty acids after 5 days of incubation. This effect was especially apparent for 9-HODE where levels were the same as control up to day 5 but subsequently much higher. Both 12-HETE and 12-HEPE have previously been shown to reduce platelet aggregation (Takenaga *et al.*, 1986). Niacin increased the levels of these hydroxy fatty acids present in PRP. EPA and niacin together particularly increased levels of 12-HEPE compared to any other treatment. This effect was apparent within 120 min of addition of EPA and niacin to the incubation medium. It is not known what the effects of 8-HETE are on platelet aggregation but it is thought that 15-HETE is pro-aggregatory (Setty *et al.*, 1992). Levels of 8-HETE and 15-HETE during platelet incubation with niacin alone and in combination with EPA were raised compared to control.

This study has demonstrated that EPA, DHA and niacin can profoundly change the profile of eicosanoids secreted from platelets over their lifespan. To date there are no other studies that have investigated the effect of niacin on platelet eicosanoid formation. This study has shown that DHA stimulated the production of PGD₂ and 12-HEPE from platelets and this may in part explain how this PUFA prevents aggregation. EPA has been shown to stimulate the formation of several disaggregatory prostanoids and hydroxy fatty acids as well as the aggregatory thromboxanes. EPA is known to prevent platelet aggregation and this study has shown how eicosanoids may be fundamental to that role. Furthermore, this study has demonstrated that EPA can affect eicosanoid formation from the platelet within minutes of addition to the incubation medium. Thus, PGE₃ and PGD₃ are formed within 30 min of addition of EPA.

This study has shown that niacin affects platelet eicosanoid synthesis. Niacin has been shown to duplicate the effects of EPA on platelet eicosanoid secretion. Treating platelets with both EPA and niacin gave an additive effect. EPA did not prevent or reverse niacin induced production of platelet PGD₂. It is unknown whether the overall effect on the platelet of both treatments together would be one of inducing or inhibiting aggregation.

Overall this has been a preliminary investigation into the effects of EPA, DHA and niacin on platelet eicosanoid and hydroxy fatty acid synthesis. Whilst novel trends have been observed further research is required, particularly into the effects of niacin on the platelet, before conclusions can be drawn.

Chapter 7 : Concluding remarks and future work

7.1 Concluding remarks

The low incidence of cardiovascular disease amongst coastal communities initially prompted a series of studies to investigate the therapeutic potential of fish oil (Hirai *et al.*, 1989, Horrobin, 1987). Clinical trials subsequently showed that EPA, a constituent of fish oil, possessed both cardioprotective and neuroprotective properties (Shah *et al.*, 1998, Yokoyama *et al.*, 2007). EPA was shown to be able to form eicosanoids and hydroxy fatty acids through which it could modulate inflammatory processes and regulate platelet aggregation and adhesion. More recently, the discovery of fatty acid ethanolamides offered another pathway through which EPA may influence brain biochemistry. It was found that EPA could be elongated to DPA and it was shown that DPA exhibited many of the actions of EPA. Most of the studies conducted to investigate the protective effects of EPA have involved the use of fish oil, a mixture of several PUFA, rather than EPA in isolation. This has meant that the effects of EPA on the eicosanoid and hydroxy fatty acid profile of brain and the platelet have remained largely unknown. Furthermore, to date there have been very few studies to examine the relationship between EPA or DPA intake and fatty acid ethanolamide formation in the brain.

The eicosanoids collectively form an important class of compounds that are instrumental to proper cell function and survival. They regulate many physiological and pathophysiological processes including amongst others inflammation and thrombus formation. Imbalances within the eicosanoid cascade can precipitate disease ranging from inflammatory conditions such as arthritis to more subtle neurological conditions such as depression and schizophrenia.

In addition to the eicosanoids the fatty acid ethanolamides form a distinct class of mediator the importance of which is only just being realised. Ethanolamides such as anandamide and oleamide have been found to play a fundamental role in neurotransmission regulating diverse processes such as sleep and nociception. EPA and DHA, the most prominent PUFA in the brain, are able to form ethanolamides although the implications of this are currently unknown.

The study of mediators such as eicosanoids or fatty acid ethanolamides is particularly challenging owing to the many feedback loops and labile nature of these compounds. Previous work has shown that many of these mediators are regulated through a number of enzymatic pathways by signalling molecules such as cytokines. Many eicosanoids have been shown to have physiologically opposing actions and often mediate homeostatic processes such as maintaining vascular tone (Mais *et al.*, 1990). The resulting “soup” of eicosanoids is therefore often complex and highly variable. Hence, no one mediator can be examined in isolation, rather, the entire soup of mediators needs to be profiled to obtain a true understanding of how these compounds behave.

The development of novel analytical techniques such as LC/ESI-MS/MS has enabled the profiling of several mediators simultaneously from a single piece of tissue. Thus it has now become possible to profile a range of mediators facilitating a better understanding of how these compounds are expressed at any given time.

Chapter 3 described the development of an assay to extract and analyse fatty ethanolamides by LC/ESI-MS/MS from brain tissue. The developed method is highly sensitive with LOD and LOQ comparable to previous GC-MS based methods. The

extraction procedure was optimised to give a recovery that ranged from 86 % to 128 %. This method reduced retention times giving an overall shorter run time and gave better peak resolution than previous methods. Thus, the developed assay can be applied to the profiling of A-EA, EPA-EA and DHA-EA from neural tissue with an extraction procedure that does not include any derivatisation steps and gives complete recovery.

It is thought that eicosanoid and ethanolamide formation can be stimulated through physical stimulation usually in the form of injury. Thus eicosanoids can be detected at sites of inflammation where they are thought to mediate blood flow and cell signalling to aid recovery. Ageing is characterised by chronic oxidative attack that can manifest itself in the form of cognitive decline. Ageing is thought to alter many physiological parameters within the brain and is a major risk factor for neurodegeneration. Ageing has been proposed as a model for increased risk of neuronal injury. During this study age was used to investigate changes in the eicosanoid and fatty acid ethanolamide profiles within the brain and their relationship to dietary PUFA intake.

In Chapter 4 the LC/ESI-MS/MS assay described in Chapter 3 was used to profile fatty acid ethanolamides in young and aged brain cortex following dietary supplementation with either EPA or *n*-3 DPA for a period of 8 weeks. This study showed that ageing alone increased the cortical concentrations of A-EA and EPA-EA. To date this is the first study to identify and quantify EPA-EA in the rat brain cortex. Both of these findings lend support to the credibility of LC/ESI-MS/MS as a robust and highly sensitive technique. It was found that supplementing young animals with EPA or *n*-3 DPA did not affect the cortical concentrations of EPA-EA, A-EA or DHA-EA but when aged rats were fed these PUFA there was a significant increase in the cortical

concentrations of EPA-EA. Thus, for the first time it was shown that dietary intake of either EPA or DPA in the aged rat directly affected the profile of fatty acid ethanolamides in the cortex with a significant increase in levels of EPA-EA.

The focus of Chapter 5 was to investigate the relationship between ageing and dietary supplementation of EPA or *n*-3 DPA on the prostanoid and hydroxy fatty acid profiles of the brain cortex. Ageing significantly increased the formation of PGD₂ and 12-HETE in the cortex whilst decreasing levels of PGF_{2α}, 11-HETE and 15-HETE. These changes were prevented in aged animals through dietary supplementation with either EPA or *n*-3 DPA. Instead, it was found that both of these PUFA attenuated the formation of PGE₂, a prostaglandin that has been linked to neuroinflammation and eventual neurodegeneration as suggested by some studies (Montine *et al.*, 2002b, Shie *et al.*, 2005). It was found that the profiles of young cortical tissue were unaffected by dietary supplementation with EPA. Intake of *n*-3 DPA did however increase the formation of 13 and 9-HODE whilst decreasing 15-, 11-, 8- and 12-HETE in the young cortex; a previously undescribed finding indicative possibly of oxidative stress.

The action of *n*-3 DPA on young tissue was unexpected especially since these effects have never been observed previously and EPA failed to produce a similar effect. The actions of EPA on young cortex were however consistent with the work on ethanolamides in that this PUFA did not significantly affect levels of eicosanoids in young brain cortex. Further work needs to be undertaken to verify the findings in relation to *n*-3 DPA supplementation and eicosanoid profile of cortical tissue from young animals.

It has recently been demonstrated that ischaemia in the brain stimulates the formation of eicosanoids and A-EA (Bazinet *et al.*, 2005, Farias *et al.*, 2008). Care was taken to remove and freeze brain slices in liquid nitrogen immediately after sacrifice during this study. It is inevitable however that the brain would have undergone some degree of ischaemia following decapitation. Thus, it is possible that during this work ischaemia may have artificially elevated the concentrations of eicosanoids and ethanolamides above basal levels. As all rats were sacrificed in the same manner however any EPA or DPA related effects would still be apparent and the method of sacrifice used in this study would not impact any general trend.

It is well established that platelet aggregation is closely linked to the formation of prostanoids and hydroxy fatty acids including TXB₂ and 12-HETE. Based upon earlier observations and evidence that EPA and DHA attenuate platelet aggregation it was predicted that these PUFA would stimulate a change in platelet eicosanoid formation. It was predicted that incubating platelets with EPA or DHA would attenuate the formation of aggregatory eicosanoids. A preliminary study was conducted in Chapter 6 to investigate the effects of PUFA on platelet eicosanoid and hydroxy fatty acid synthesis. It was shown that DHA did not attenuate the formation of any of the detected eicosanoids or hydroxy fatty acids. Instead DHA was found to stimulate platelet PGD₂, 12-HEPE and 15-HETE production and it is thought that this was in part facilitated by the presence of albumin. EPA stimulated the formation of HEPE and TXB₃ within 30 min of addition to plasma. It is thought that these eicosanoids are less aggregatory than HETE and the series-2 prostanoids. Thus, this study effectively demonstrated that EPA can modulate eicosanoid formation from the platelet and this may in part explain the cardioprotective properties of this PUFA.

A further aim of the work in chapter 6 was to investigate the effect of niacin on platelet eicosanoid and hydroxy fatty acid production. Niacin is a vitamin that also has cholesterol lowering properties. Intake of niacin is associated with unpleasant vasocutaneous flushing which has limited the use of this vitamin in treating hypercholesterolaemia. Niacin has been shown to inhibit platelet aggregation and so it was hypothesised that this vitamin would affect platelet eicosanoid synthesis in a manner analogous to EPA. This preliminary study demonstrated that although niacin could not stimulate the formation of TXB₃ and HEPE like EPA, it did increase levels of PGD₂ and PGE₂. Both of these prostaglandins are thought to attenuate platelet aggregation. Upon incubating platelets with EPA and niacin together it was found that niacin augmented the actions of EPA on platelet eicosanoid synthesis. It was found that EPA could not prevent or reverse niacin stimulated PGD₂ release from the platelet. Hence, this preliminary study has shown that EPA would not attenuate the niacin flush; rather, it would probably intensify the flush. To date this is the first investigation of platelet eicosanoid and hydroxy fatty acid formation following incubation with niacin. Thus it is thought that EPA will significantly impact the micro-circulation of the brain through incorporation in platelets.

7.2 Future work

Whilst this work used ageing as a model for chronic stress in the brain, future work could focus more on acute stress models such as stroke. It is known that EPA can help in the prevention of stroke but it is unclear if EPA has a role to play in recovery from such an injury. DHA and EPA can be metabolised to neuroprotectins and resolvins that together help in the resolution phase of inflammation and have been detected following stroke but were not found in these experiments during ageing (Serhan *et al.*, 2004).

Whilst Chapter 6 focussed on platelets future work should look at the effects of EPA and DHA on whole blood as it is known that resolvins are products of leukocyte metabolism. It is also known that red blood cells actively take up PUFAs from their surroundings. It would also be interesting to find fatty acid ethanolamides as well as resolvins and protectins in blood.

In addition to increasing the synthesis of PGD_2 it is known that niacin stimulates platelets to secrete serotonin which is involved with the flushing side effect (Papaliadis *et al.*, 2008). It would be worthwhile to investigate the effects of EPA on niacin induced platelet serotonin release. The current study has shown that niacin enhanced the effects of EPA on platelet eicosanoid synthesis. It would be interesting to see the concurrent effects of these compounds on brain eicosanoid and fatty acid ethanolamide formation. Thus, a feeding study could be undertaken where animals are supplemented with both EPA and niacin together and individually. Such a study has not been conducted to date.

Due to the possibility of ischaemia during sacrifice all future animal work should involve high energy microwave irradiation of the brain prior to decapitation. It has been reported that this method of sacrifice is much more likely to give a true reflection of basal levels of eicosanoids and fatty acid ethanolamides than decapitation alone (Bazinet *et al.*, 2005, Farias *et al.*, 2008).

Future work should also involve investigations into the biological effects of EPA-EA. It is known that A-EA has a vast biological spectrum and it is envisaged that EPA-EA too could have potent pharmacological actions. Further work should focus more on the

prostamides. These novel mediators are the amide derivatives of the prostaglandins and EPA is thought to form novel prostamides distinct from those formed from AA. Prostamides are commercially available to treat glaucoma and it is thought that the discovery of novel prostamides derived from EPA could bring about new therapeutic applications (Woodward *et al.*, 2008, Woodward *et al.*, 2007).

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