N-3 FATTY ACIDS, EICOSANOIDs AND
CONTROL OF INFLAMMATION

by

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ERRATA

Acknowledgements, page xi: "occasions" not "occasions"


Figure 3.7 on page 78: The symbol for the all-trans isomers of LTB₄ in the Y axis legend should be ....o......

Figure 5.1 on page 95: The chemical structure of panacyl bromide should contain a C in the ester group, not an O.

Figure 7.2 on page 158: The significance of the results depicted in this figure was determined by analysis of variance followed by Newman-Keuls multiple comparison analysis. All experimental groups were significantly different from the control group at the 0.05 significance level.

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SUMMARY

This thesis addresses issues arising from the observation that dietary fish oils favourably modify experimentally-induced inflammation in animals and in human diseases including rheumatoid arthritis (RA). The anti-inflammatory effects described appear related to the presence of n-3 fatty acids in the fish oil, in particular eicosapentaenoic acid (EPA). EPA is a potential substrate for the enzymes 5-lipoxygenase (5-LO) and cyclooxygenase (CO) which are pivotal in the synthesis of lipid mediators of inflammation, known as eicosanoids. Although there have been many studies on the 5-LO metabolites of EPA, little is known about the rate production or activity of the cyclooxygenase metabolite of EPA, prostaglandin E3 (PGE3). The studies undertaken for this thesis required the development of an assay for the measurement of PGE3 and assessment of its biological activity. Possible interactions between conventional drug therapy and dietary fish oil supplements in therapeutic regimens designed to control inflammation were also investigated. Studies were undertaken to assess the mechanism for the putative inhibition of synthesis of leukotriene B4 (LTB4) by the anti-inflammatory agent methotrexate (MTX) since this drug is an effective anti-arthritic agent and the possibility of favourable drug/diet interactions was sought.

Adjuvant-induced arthritis in rats was used as a model of systemic inflammation and polyarthritis in which to investigate the effects of inflammation on the incorporation into leukocytes of dietary n-3 fatty acids. No effect on the rate or level of incorporation of EPA or depletion of arachidonic acid (AA) was seen and further studies were undertaken in normal animals.

The biological activity of PGE3 with regard to oedema formation in mice was examined. Paw swelling was measured 30 minutes after injection of 10 µl PGE2 or PGE3 into the plantar region of the hind paw. Doses investigated ranged from 1 ng - 10 µg. Both PGE2 and PGE3 had substantial oedemogenic activity in this system.

An assay was developed which resolved PGE3 from PGE2. Prostaglandins E1, E2 and E3 were derivatized with p-(9-anthroyloxy)phenacyl bromide (panacyl bromide)
and partly purified by thin layer chromatography (TLC). The PGs were further separated and analysed by reverse phase high pressure liquid chromatography (HPLC) with fluorometric detection. Human, rat and mouse adherent cells were incubated overnight and the culture medium extracted, derivatized and analysed for PG production. PGE$_2$ was detected in supernatants from cells from each species. PGE$_2$ synthesis was reduced following addition of EPA ($5\ \mu$M) to the overnight culture. PGE$_3$ was not detected under these conditions. Studies were also undertaken using adherent cells from rats, mice and humans given dietary fish oil supplements rich in EPA. PGE$_3$ was not detected although the dietary intervention yielded substantial incorporation of EPA into cell membranes and LTB$_5$, a metabolite of EPA, was produced by leukocytes after appropriate stimulation and analysis by HPLC.

These observations suggest that although PGE$_3$ has inflammatory activity comparable to that of PGE$_2$, PGE$_3$ may not be generated in sufficient quantities to play a major role in mediating inflammatory reactions.

Studies were also undertaken to examine the effect of MTX in vitro and ex vivo on the production of the 5-lipoxygenase metabolites of arachidonic acid by rat and human neutrophils. MTX added in vitro to normal rat or human cells was weakly inhibitory and without a convincing dose response relationship. No inhibition of LTB$_4$ production by leukocytes was seen following administration of MTX orally to subjects with RA or by any of three routes of administration investigated in healthy rats (gavage, subcutaneous injection and intraperitoneal injection). The studies thus yield no support for earlier claims that MTX is an inhibitor of 5-LO and the possibility of an additive or synergistic effect of MTX and EPA on 5-LO metabolism was not pursued.
DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I consent to this copy of my thesis being made available for photocopying and loan if accepted for the award of the degree.

SIGNED: Joanna Susan Hawkes

DATE: 5/11/93
ACKNOWLEDGEMENTS

I would like to thank Dr Les Cleland for his supervision, guidance and support throughout the course of this work and for providing me with the opportunity to undertake these studies in his laboratories.

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The staff in the Rheumatology Unit, Royal Adelaide Hospital and the Arthritis Research Laboratory, Hanson Centre for Cancer Research, for their camaraderie and support, particularly Cindy Hall who worked with me throughout most of this undertaking.

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The staff in the RAH/IMVS Medical Illustration Unit, particularly Anna Langen-Zueff and Rob Bryant, and Tony Rice from the Lung Research Laboratory, Hanson Centre for Cancer Research, for help in preparing the illustrations for this thesis.
ABBREVIATIONS

The following abbreviations have been used throughout this thesis.

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>AA</td>
<td>Arachidonic Acid (20:4 n-6)</td>
</tr>
<tr>
<td>ALA</td>
<td>α-Linolenic Acid (18:3 n-3)</td>
</tr>
<tr>
<td>a.u.f.s.</td>
<td>Absorbance Units Full Scale</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund's Adjuvant</td>
</tr>
<tr>
<td>CO</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>c.p.m.</td>
<td>Counts Per Minute</td>
</tr>
<tr>
<td>DGLA</td>
<td>Dihomo-γ-Linolenic Acid (20:3 n-6)</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid (22:6 n-3)</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's Phosphate Buffered Saline</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic Acid</td>
</tr>
<tr>
<td>EFAD</td>
<td>Essential Fatty Acid Deficiency</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic Acid (20:5 n-3)</td>
</tr>
<tr>
<td>ETA</td>
<td>Eicosatetraenoic Acid (20:4 n-3)</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>ETYA</td>
<td>Eicosatetraynoic Acid (20:4 n-6)</td>
</tr>
<tr>
<td>ETrA</td>
<td>Eicosatrienoic Acid (20:3 n-9)</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
</tr>
<tr>
<td>fMLP</td>
<td>Formyl-methionyl-leucyl-phenylalanine</td>
</tr>
<tr>
<td>GLA</td>
<td>γ-Linolenic Acid (18:3 n-6)</td>
</tr>
<tr>
<td>GLC</td>
<td>Gas Liquid Chromatography</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatography-Mass Spectrophotometry</td>
</tr>
<tr>
<td>HETE</td>
<td>Hydroxyeicosatetraenoic Acid</td>
</tr>
<tr>
<td>HEPE</td>
<td>Hydroxyeicosapentaenoic Acid</td>
</tr>
<tr>
<td>HPETE</td>
<td>HydropEROXYEICOSATETRAENOIC Acid</td>
</tr>
</tbody>
</table>
HPEPE  Hydroperoxyeicosapentaenoic Acid
HPLC  High Pressure Liquid Chromatography
IFN  Interferon
Ig  Immunoglobulin
IL  Interleukin
i.p.  Intraperitoneal
LA  Linoleic Acid (18:2 n-6)
LO  Lipooxygenase
LPS  Lipopolysaccharide
LT  Leukotriene
MTX  Methotrexate
NDGA  Nordihydroguaiaretic Acid
NK  Natural Killer Cells
NSAID  Non-Steroidal Anti-Inflammatory Drug
PAB  Panacyl Bromide
PAF  Platelet Activating Factor
PBS  Phosphate Buffered Saline
PEC  Peritoneal Exudate Cells
PG  Prostaglandin
PGI  Prostacyclin
PHA  Phytohaemagglutinin
PLA₂  Phospholipase A₂
PMN  Polymorphonuclear Cells
PUFA  Polyunsaturated Fatty Acid
RA  Rheumatoid Arthritis
Rf  Relativity Factor (mobility of solute relative to mobility of solvent)
RIA  Radioimmunoassay
sd  Standard Deviation
sem  Standard Error of the Mean
SRS-A

THF

TLC

TNF

TX

UV

Slow Reacting Substance of Anaphylaxis

Tetrahydrofuran

Thin Layer Chromatography

Tumour Necrosis Factor

Thromboxane

Ultraviolet

Bq

centigrade

day

centimetre

gram

hour

kilogram

litre

molar

minute

milligram

millilitre

millimetre

nano

pico

second

micro

units

volume for volume

weight for weight
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PUBLICATIONS

Publications in support of this thesis:


During the course of this thesis, various studies were carried out which had no direct relationship with the theme of the thesis. These are listed below.


CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Developments in the field of polyunsaturated fatty acid research and health effects began in the 1950's primarily with studies relating to n-6 fatty acids and their effects on serum cholesterol concentrations in patients with atherosclerosis. The vegetable oils gained notoriety and popularity in the Western world and eventually began to displace other fats in the diet with the emphasis of experimental and clinical work being on control of cardiovascular disease. The "lipid effects" enjoyed the spotlight of research and the contributions of inflammation and thrombosis in the development of heart disease were not thoroughly investigated until the 1970's when the low rates of coronary heart disease and inflammatory disorders in the Greenland Eskimo population were reported. Other epidemiological studies confirmed the findings and showed that despite a high fat diet, fish-eating populations like the Eskimos had less cardiovascular disease. Further clinical and experimental studies emphasized the importance of the marine oils, rich in n-3 fatty acids and eicosapentaenoic acid (EPA) in particular, in altering cell membrane phospholipid fatty acid profiles and modulating a wide range of inflammatory and thrombotic events.

The following sections in this chapter review current knowledge about the metabolism of key fatty acids from both the n-6 and n-3 families of fatty acids leading to the biosynthesis and pharmacological actions of the lipid mediators of inflammation. Particular emphasis is placed on the effects of EPA and its metabolites both in vivo and in vitro with regard to modulating inflammatory responses and disorders.
1.2 PROSTAGLANDINS AND LEUKOTRIENES

1.2.1 Fatty acid chemistry and nomenclature

Fatty acids are the basic chemical building blocks of phospholipids and glycerolipids and are fuel storage molecules. They are made up of a linear chain of carbon atoms. One terminal carbon atom makes up part of the acid section of the molecule, the carboxyl group, and the other is the methyl or omega end. Fatty acids in biological systems usually contain an even number of carbon atoms, typically between 14 and 24, the commonest being the 18 carbon chain. The hydrocarbon chain may be saturated or unsaturated i.e. with double bonds between one or more pairs of carbon atoms. The biophysical properties of fatty acids and of lipids derived from them are markedly dependent on their chain length and on the degree to which they are unsaturated. Unsaturated fatty acids have a lower melting point than saturated fatty acids of the same length. Chain length also affects the melting point. Thus short chain length and unsaturation enhance fluidity of membranes which contain these fatty acids and their derivatives. Typically, the animal fatty acids from storage fats are saturated, long chain fatty acids. Unsaturated fatty acids consist of monounsaturates with one double bond only, and polyunsaturates with two or more double bonds. The position of the first double bond is described by numbering the carbon atoms from the methyl or omega end. Double bonds are usually not found before the third carbon on the chain, are rarely found on the fourth or fifth and are commonly found beginning on the sixth carbon atom. Fatty acids with the first double bond at the third carbon from the methyl terminal are part of the n-3 or omega 3 fatty acid family. Similarly, those with the first double bond at the sixth carbon from the methyl terminal are n-6 or omega 6 fatty acids. Monounsaturates are represented by oleic acid (18:1 n-9) which can be synthesized by all mammals including humans. N-6 and n-3 fatty acids are the "essential fatty acids" because they cannot be made by animals but must be included in the diet. The 18 carbon chain parent fatty acids of the n-6 and n-3 fatty acid series are linoleic acid (LA; 18:2 n-6) and α-linolenic acid (ALA; 18:3 n-3) respectively. These fatty acids are represented diagrammatically in Figure 1.1.
Figure 1.1  Diagrammatic representation of the 18 carbon parent fatty acids of the n-9, n-6 and n-3 families.
OLEIC ACID 18:1 N-9

LINOLEIC ACID 18:2 N-6

ALPHA-LINOLENIC ACID 18:3 N-3

Essential Fatty Acids
Amongst the various fats and oils consumed in the human diet there are certain dominant fatty acids. The saturated fatty acids, stearic acid (18 carbon) and palmitic acid (16 carbon) are commonly found in animal fats. Seed oils (sunflower and corn oil for example) are rich in LA and the leafy green vegetables contain some ALA. The n-6 fatty acids are the dominant family of polyunsaturated fats in the western diet. Fish or marine oils are unique in that they contain relatively large amounts of the n-3 polyunsaturated fatty acids, eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3). Various animal species including man have the ability to further desaturate unsaturated fatty acids but cannot insert double bonds between the omega terminal carbon and the first double bond, wherever that may be.

In addition to modification by desaturation, fatty acids can be elongated by addition of extra pairs of carbon atoms. Thus an 18 carbon two double bond fatty acid with the first double bond at the omega 6 (n-6) carbon (LA) can be elongated and desaturated to a 20 carbon fatty acid with four double bonds (AA) still of the n-6 family. In the same way, using the same enzymic pathways, an n-3 fatty acid starting with 18 carbon atoms and 3 double bonds (ALA) can be converted to a 20 carbon, five double bond fatty acid of the same n-3 family (EPA) and through further elongation and desaturation to a 22 carbon, six double bond fatty acid (DHA). The elongation and desaturation of the essential fatty acids is represented in Figure 1.2. The relative rates of conversion are determined by the relative amounts of n-6 and n-3 substrates since there is competition between the n-3 and n-6 fatty acids for the elongase and desaturase enzymes. LA, ALA and their long chain derivatives are important components of animal cell membranes and in many instances the n-3 fatty acids in particular are distributed selectively in the tissues and among the lipid classes within cell membranes. For example ALA is found predominantly in triglycerides and cholesteryl esters, DHA is found mostly in phospholipids and EPA is found in all three. DHA is one of the most abundant components of brain's structural lipids and is also particularly abundant in the retina, and testis and sperm [Simopoulos, 1991].
Figure 1.2  Essential fatty acid metabolism - desaturation and elongation of n-6 and n-3 to the 20 carbon precursors of the eicosanoids, the lipid mediators of inflammation.
**N-6 Fatty Acids**

- **Linoleic** 18:2 n-6
- **Gamma-Linolenic** 18:3 n-6
- **Dihomogamma-Linolenic** 20:3 n-6
- **Arachidonic** 20:4 n-6

**N-3 Fatty Acids**

- **Alpha-Linolenic** 18:3 n-3
- **Stearidonic** 18:4 n-3
- **Eicosatetraenoic** 20:4 n-3
- **Eicosapentaenoic** 20:5 n-3
1.2.2 Biosynthesis of eicosanoids

It is the 20 carbon polyunsaturated fatty acids, AA and EPA, that are particularly important with regard to inflammation. They are the immediate precursors of the lipid mediators of inflammation known collectively as eicosanoids. The enzyme systems for the metabolism of 20 carbon fatty acids to eicosanoids can use either n-6 or n-3 fatty acid homologues as the substrate which means that these fatty acids will compete with each other for metabolism. There are two principal enzyme pathways involved in eicosanoid synthesis. The cyclooxygenase enzyme complex (CO) produces the ring structure prostaglandins (PG), prostacyclins (PGI) and thromboxanes (TX) and lipoxygenase (LO) produces the straight chain hydroxy acids and the conjugated triene structure leukotrienes (LT).

Eicosanoids are not stored in mammalian tissues and therefore their concentration in tissues is determined largely by their rates of de novo synthesis (as well as their rates of degradation). The concentration of unesterified arachidonic acid in cells in their basal state is very low but there is a comparatively large amount esterified in cell membrane phospholipids [Irvine, 1982]. Unsaturated fatty acids are generally esterified in the 2-acyl position of the glyceryl backbone of the phosphatide molecule. Therefore, the initial and rate limiting step in the biosynthesis of eicosanoids is the availability of free AA released enzymatically following cell receptor activation by antigens and other stimuli. Phospholipase A\textsubscript{2} (PLA\textsubscript{2}) is a key enzyme controlling mobilisation of fatty acids for eicosanoid biosynthesis [Flower and Blackwell, 1976]. The fatty acid residues esterified in the 2-acyl position are liberated by this hydrolytic enzyme. PLA\textsubscript{2} is calcium dependent and thus the intracellular calcium concentration is a controlling factor in arachidonate liberation. Control of PLA\textsubscript{2} activity may also be regulated by conversion of zymogen forms, phospholipid methylation, non-enzymic inhibitor and activator proteins, cyclic AMP and by hormones [reviewed by Van den Bosch, 1980]. Another pathway for arachidonate release involves the activation of phospholipase C which hydrolyses the glyceryl-phosphate bond in the 3-acyl position to liberate a phosphorylated base (i.e. either inositol, choline, ethanolamine or serine) and
a diglyceride. The diacylglycerol can be deacylated, thus liberating AA [Irvine, 1982]. (See Figure 1.3). In addition to the cell's own store of esterified AA, phospholipids, triglycerides and unesterified fatty acids are present in the blood within lipoproteins or bound to albumin. In plasma-rich inflammatory exudates this lipid may serve as another source of rapidly available AA.

**Cyclooxygenase**

Cyclooxygenase (CO) is widely distributed in mammalian cells; only erythrocytes appear to be devoid of the enzyme. CO activity is stimulated by mechanical, chemical or immunological challenge and as inflammation is the response of living tissue to irritation and injury, PG synthesis always occurs in inflamed tissues. However, specific activation of CO is not necessary as the availability of free acid substrate is sufficient for the reaction to occur.

Free AA can be oxidatively metabolised by CO providing the unstable cyclic endoperoxides, PGG2 and PGH2 [Hamberg et al., 1974]. An isomerase transforms the endoperoxides to stable prostaglandins of the E or D series and F-type prostaglandins are formed by a reductive cleavage [Higgs et al., 1981]. PGG2 and PGH2 can also be converted to prostacyclin with a bi-cyclic structure [Moncada et al., 1976] or to a non-prostaglandin structure, thromboxane A2 [Hamberg et al., 1975]. (See Figure 1.4). All of these inflammatory mediators are referred to as belonging to the 2-series prostaglandins.

When humans ingest fish or fish oil, EPA from the diet partially replaces AA in the cell membrane phospholipids. In addition to the actual decrease of available AA, EPA competes with AA for the CO enzyme and may be metabolised to the 3-series prostaglandins, prostacyclins and thromboxanes [Willis, 1981].
Figure 1.3  Possible mechanisms of release of arachidonic acid from cell membrane phospholipid stores.

PE - Phosphatidylethanolamine
PC - Phosphatidylcholine
PI, PIP - Phosphatidylinositol and phosphorylated form
DG - Diacylglycerol
PI, PIP (AA)

Phospholipase C

DG

DG-LIPASE

ARACHIDONIC ACID

PI, PIP (AA)

Phospholipase C

METHYL TRANSFERASES

PE

PC (AA)

Phospholipase A_2

Ca^{++}

EXTERNAL STIMULI

Extracellular Calcium

cell membrane phospholipids

PE

PC (AA)
Figure 1.4  The cyclooxygenase pathway of archidonic acid metabolism.
ARACHIDONIC ACID

\[ \text{Cyclooxygenase} \]

CYCLIC ENOPEROXIDES

\[ \text{PGG}_2 \]

Prostacyclin Synthetase

Prostacyclin

6 Oxo \( F_1 \)

\[ \text{PGF}_{2\alpha} \]

\[ \text{PGD}_2 \]

\[ \text{PGH}_2 \]

\[ \text{Thromboxane Synthetase} \]

Thromboxane \( A_2 \)

\[ \text{Thromboxane B}_2 \]

C17 Hydroxyacid HHT

Malondialdehyde

\[ \text{PGE}_2 \]
Lipoxygenase

An alternative pathway of metabolism for both n-6 and n-3 20 carbon fatty acids is oxidation by lipoxygenase enzymes. The initial reaction with AA leads to the production of unstable hydroperoxy intermediates, the HPETEs. The specific HPETE formed depends on the type of LO present in the cell. For example: 5-LO converts AA to 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) whereas 12-LO converts AA to 12-HPETE. These HPETEs are then converted by peroxidase enzymes to stable monohydroxy acids (HETEs) [Needleman et al., 1986].

The 5-LO system is unique among the LO enzyme systems in that it gives rise to a family of biologically active compounds containing a conjugated triene structure, named leukotrienes [Samuelsson and Hammarström, 1980]. By convention the number of double bonds is indicated by a subscript. For example, when AA (20:4 n-6) is converted to leukotriene B, the original number of double bonds is retained and LTB₄ is formed, whereas EPA (20:5 n-3) forms LTB₅ of the 5-series. It appears that 5-LO is restricted mainly to neutrophils, eosinophils, monocytes, macrophages and mast cells which are all considered to be "inflammatory cells" and this suggests that the 5-LO products are involved in inflammatory responses. By contrast with CO, the 5-LO enzyme pathway has to be activated selectively by a mechanism which involves calcium movement. Various immunological stimuli may activate the enzyme including microorganisms containing bound immunoglobulins, other phagocytic stimuli and multivalent anti-receptor antibodies. Nonimmunologic stimuli include calcium ionophore A23187, fMLP, serum-activated zymosan and lectins. Product formation begins within a few minutes after the stimulus but the process also declines with equal rapidity.

In cells containing 5-LO, the hydroperoxy fatty acid formed from AA may also be enzymatically metabolised to LTA₄ by LTA synthetase [Panossian et al., 1982]. This epoxide of AA is extremely unstable and is further converted to one of four possible products. It can be non-enzymatically hydrolysed to 5(S),12(S) and 5(S), 12(R) all-trans di-hydroxy acids, known as the all-trans isomers of LTB₄ [Borgeat and
Samuelsson, 1979], or enzymatically converted by LTA4 hydrolase to 5(S),12(R) DiHETE, known as LTB4. LTB4 can be metabolised by ω-oxidation to 20-OH LTB4 and further to 20-COOH LTB4 [Hansson et al., 1981]. Alternatively the addition of glutathione to LTA4 by glutathione-S-transferase results in the formation of LTC4 [Rådmark et al., 1980]. The removal of glutamic acid from LTC4 by γ-glutamyl transpeptidase produces LTD4 [Örning et al., 1980], which can be further metabolised by a glycine to the cysteine derivative, LTE4 [Bernström and Hammarström, 1981]. (See Figure 1.5). These peptido-lipid leukotrienes are the components of the bronchoconstricting activity generated during anaphylaxis and are referred to as the "slow reacting substance of anaphylaxis" (SRS-A).

Likewise, EPA is a relatively good substrate for lipoxygenases and is converted readily to the hydroxy-eicosapentaenoic acid (5-HEPE) and the 5-series LTs which have one extra double bond (at the n-3 position) than their n-6 counterparts. These 5-series metabolites differ from the AA metabolites in rates of formation, biologic activities and catabolism.

1.2.3 Pharmacological actions of n-6 eicosanoids

Prostaglandins

The major physiological actions of the PGs are as either smooth muscle contractors [Goldberg and Ramwell, 1975] or relaxants [Oliw et al., 1983] and include effects on blood pressure [Ånggård and Bergström, 1963; Chapnick et al., 1978] and regulation of homeostasis [Moncada et al., 1976; Hamberg et al., 1975].

PGE2 and PGI2 are potent vasodilators which increase blood flow and produce an erythema at sites of injection [Williams, 1979]. These PGs do not have a direct effect on oedema formation but in combination with mediators such as histamine and bradykinin, which increase vascular permeability, PGE2 and PGI2 further enhance plasma exudation in several species [Basran et al., 1982; Williams and Morley, 1973; Moncada et al., 1973]. Similarly, the PGs do not cause overt pain but they sensitize afferent pain nerve endings to the actions of other stimuli and induce a state of
hypersensitivity [Ferreira, 1972]. PGE\textsubscript{2} is also a potent pyrogenic agent and its production is stimulated by the cytokines IL-1, TNF and IL-6 during fever [Dinarello et al., 1991]. PGs protect the gastroduodenal mucosa from damage caused by diverse noxious agents [Wilson, 1991]. This protective effect is due to the stimulation of several factors believed to be important in maintaining normal mucosal integrity, such as mucous synthesis and secretion, mucosal bicarbonate secretion, mucosal blood flow and cellular repair.

There is also evidence that CO products influence leukocyte function. For example, PGD\textsubscript{2}, the major PG product of activated mast cells, is a potent chemokinetic factor with a specificity for eosinophils and PGE\textsubscript{2} has a similar effect at 5 - 25 fold higher concentrations [Goetzl et al., 1979]. PGE\textsubscript{2} also enhances the chemotactic responsiveness of monocytes [McClatchey and Snyderman, 1976]. PGE\textsubscript{1} is chemotactic for rabbit PMNs [Higgs et al., 1975] and PGF\textsubscript{2\alpha} is chemokinetic [Goetzl and Gorman, 1978] and enhances the chemotactic responsiveness of leukocytes to other stimuli [Diaz-Perez et al., 1976]. PGI\textsubscript{2} prevents PMN chemotaxis in vitro [Wekslor et al., 1977] and reduces adherence of human PMNs to nylon fibres or endothelial cells [Boxer et al., 1980]. E-type PGs inhibit the release of lymphokines by activated lymphocytes [Gordon et al., 1976], inhibit the tumoricidal activity of interferon-activated mouse macrophages [Shultz et al., 1978], and suppress murine lymphocyte proliferation [Metzger et al., 1980] and human peripheral blood mononuclear cell proliferation responses particularly after burns and major injury [Grbic et al., 1991]. It was originally suggested that PGE\textsubscript{2} and PGI\textsubscript{2} down-regulate the production of IL-1 by macrophages [Kunkel and Chensue, 1985] implying a model of feedback regulation of IL-1 by PGs. Recent reports indicate that while PGE\textsubscript{2} causes a dose-dependent suppression of IL-1 and TNF release from LPS-stimulated mouse macrophages, it does not affect the level of IL-1\(\alpha\) or IL-1\(\beta\) mRNA accumulation in mouse macrophages [Scales et al., 1989] or expression on IL-1 mRNA in human monocytes [Knudsen et al., 1986], indicating that the regulation of IL-1 is post-transcriptional.
Figure 1.5  The 5-lipoxygenase pathway of arachidonic acid metabolism.
ARACHIDONIC ACID
\[ \xrightarrow{\text{Lipoxygenase}} \]
5-HPETE
\[ \xrightarrow{\text{Peroxidase}} \]
5-HETE
\[ \xrightarrow{\text{Dehydrase}} \]
5,12 DiHETE
5, 6 DiHETE
Leukotriene A\(_4\)
\[ \xrightarrow{\text{Hydrolase}} \]
Leukotriene B\(_4\) (LTB\(_4\))
\[ \xrightarrow{\text{w-oxidation}} \]
20-Hydroxy-LTB\(_4\)
\[ \xrightarrow{\text{w-oxidation}} \]
20-Carboxy-LTB\(_4\)
Leukotriene C\(_4\)
\[ \xrightarrow{\text{gamma-Glutamyltransferase}} \]
Leukotriene D\(_4\)
\[ \xrightarrow{\text{Cysteinyl-glycinase}} \]
Leukotriene E\(_4\)
MonoHETEs

The observation that platelet-derived 12-HETE was a chemotaxin for human PMNs led to the suggestion that lipoxygenase activation was involved in leukocyte migration and hence inflammatory responses [Turner et al., 1975]. Several of the monoHETEs have chemotactic and chemokinetic activity for rabbit and human neutrophils [Palmer et al., 1980] and eosinophils [Goetzl and Gorman, 1978]. Goetzl et al., (1980), showed an order of chemotactic potency indicating that 5-HETE > > 8-HETE = 9-HETE > 11-HETE = 12-HETE > > 15-HETE. 5-HETE and 12-HETE can also stimulate neutrophil degranulation [Stenson and Parker, 1980] and inhibit neutrophil 5-LO thereby inhibiting production of LTB4 and 5-HETE in a form of negative feedback inhibition [Chang et al., 1985a]. The addition of PMNs to platelet suspensions results in a large increase in 12-HETE production which has been shown to be the result of platelet 12-LO utilization of AA released from PMNs [McCulloch et al., 1992]. This demonstrates complex biochemical reactions between the two cell types during eicosanoid production. It has been shown that 12-HETE regulates the surface expression and function of platelet integrin receptors which are mediators of cell-cell and cell-extracellular matrix interactions [Chopra et al., 1991].

MonoHETE effects on other cells include inhibition of the 5-LO of T lymphocytes by 15-HETE and inhibition of platelet PLA2 by 5-, 12- and 15-HETE [Chang et al., 1985b], inhibition of collagen-induced platelet aggregation and platelet CO by 12-HPETE [Siegel et al., 1981] and inhibition of basophil histamine release by 5-HETE [Peters et al., 1982]. Gastric mucosal damage by ethanol stimulates the production of LTC4 and 15-HETE which may mediate reactions such as submucosal venular constriction and microvascular engorgement elicited by other gastric ulcerogens [Peskar, 1991]. Other postulated physiological and pathological roles of the 15-LO products include anti-inflammatory activity in skin, effects on prolactin secretion from pituitary cells and the initiation of the acrosome reaction in mammalian spermatozoa [Ford-Hutchinson, 1991].
Leukotrienes

The slow reacting substance of anaphylaxis (SRS-A) comprises LTC₄, LTD₄ and LTE₄, known particularly for their activities in producing slowly evolving and sustained contractile responses in airway and gastrointestinal smooth muscle preparations. LTs are released during antigen challenge of sensitised guinea-pig or human lung and the major responding cell appears to be the tissue mast cell [Peters et al., 1984]. There exists considerable species variation in the responses of airways to LTs. Very low concentrations of LTD₄ (100 pM/mL) produce detectable contractile responses on smooth muscle strips from guinea pig ilea, trachea and lung parenchyma [Piper, 1983]. LTC₄ and LTE₄ are somewhat less active but are still much more potent than histamine. LTC₄ and LTD₄ are approximately three orders of magnitude more active than histamine or PGF₂α in contracting strips of human bronchus and lung parenchyma [Hedqvist et al., 1980]. LTE₄ is less active but induces sustained contractions which last at least twice as long as those due to LTC₄ and LTD₄ [Samhoun and Piper, 1984]. The LTs have less potent effects in isolated airways of monkey, rabbit, rat and dog [Samhoun and Piper, 1986].

Similarly, the effects of the SRS-A on the vascular changes of inflammation appear to be species specific. LTC₄ and LTD₄ possess vasoconstrictor properties and cause plasma leakage in the skin of guinea pig [Peck et al., 1981] and rat [Ueno et al., 1981]. In humans, release appears to occur through IgE-dependent mechanisms and results in the formation of erythema and a wheal due to increased blood flow without vasoconstriction [Bisgaard et al., 1982]. LTC₄ and LTD₄ may also potentiate the effects of other vasoactive agents. The systemic vascular effects of the SRS-A lead to increased total peripheral, coronary and renovascular resistance with augmented venous permeability and depletion of plasma volume [Badr et al., 1984].

LTB₄ is an extremely potent chemotaxin for both polymorphonuclear leukocytes (neutrophils and eosinophils), monocytes/macrophages and fibroblasts [Dahinden et al., 1984; Bray, 1983]. It is also a potent chemokinetic agent and is able to enhance the random movement of neutrophils, eosinophils, macrophages and lymphocyte enriched
populations [Parker, 1987]. LTB4 also stimulates a number of other important neutrophil functions all of which reflect cell activation. For example, it has the capacity to cause aggregation of PMN suspensions [Ford-Hutchinson et al., 1980], stimulate leukocyte/endothelium adhesion [Hoover et al., 1984; Goldman et al., 1992] and increase vascular permeability [Bray et al., 1981; Goldman et al., 1992]. Degranulation of PMNs has been demonstrated following exposure to LTB4 although this effect appears to be dependent on the presence of cytochalasin B [Rollins et al., 1983]. LTB4 likewise causes PMNs to generate superoxide anions [Serhan et al., 1982]. LTB4 can enhance both degranulation and the oxidative burst stimulated by other agents suggesting a possible synergistic role for LTB4 [Thorsen, 1986]. It has also been reported to increase the number of surface receptors for C3b in PMNs and eosinophils which is likely to be an effect resulting from granule translocation or altered organization of the plasma membrane [Parker, 1987]. LTB4 has been implicated in bone resorption and Ren and Dziak, (1991), recently described inhibition of cell proliferation in normal osteoblastic rat calvaria cells and in two human osteoblast-like cell lines.

Immune responses are linked to inflammatory processes and there is increasing evidence that LTB4 (and other 5-LO products) can modify the activity of a wide range of the cell types involved. LTB4 stimulates IL-1 production by human peripheral blood monocytes [Rola-Pleszczynski and Lemaire, 1985], induces IFN-\(\gamma\) production by human T cells [Rola-Pleszczynski et al., 1987] and is required for IL-2 production [Goodwin, 1986]. On the other hand, LTB4 inhibits T-cell proliferation [Gualde et al., 1985]. This inhibition may be exerted through suppressor T cells as it has been shown that LTB4 augments the proliferative response of CD8 bearing (suppressor phenotype) human peripheral blood T lymphocytes while suppressing that of the CD4 bearing (helper phenotype) cells [Payan et al., 1984; Rola-Plezczynski, 1985]. In addition, LTB4 activates natural killer (NK) cells [Rola-Plezczynski et al., 1983]. Thus, LTB4 could provide a basis for both initial activation against certain microorganisms (through NK cell stimulation), maintenance (through chemotaxis) and regulation (through induction of suppressor cells) of immune reactions.
Eicosanoids in inflammation

CO or CO products have been detected in many human inflammatory conditions including rheumatoid arthritis (RA) [Henderson et al., 1987; Sano et al., 1992], allergic eczema, psoriasis [Barr et al., 1984; Vila et al., 1991], contact dermatitis [Goldyne et al., 1973], ulcerative colitis and gout in addition to many animal models of inflammation [Henderson et al., 1985; Simmons et al., 1983; Sano et al., 1992; Peskar et al., 1991].

Sources of 5-LO products include the "inflammatory cells" and any organ that is undergoing an inflammatory response. Peptido-LTs and LTB4 are found in sputum from patients with cystic fibrosis, bronchitis and asthma [O'Driscoll et al., 1984; Knapp et al., 1992] suggesting that they may contribute to inflammation of the respiratory tract [Dahlén et al., 1991]. LTB4 has been implicated in inflammatory bowel disease [Sharon and Stenson, 1984], gout [Rae et al., 1982] and psoriasis [Ziboh et al., 1984]. Using HPLC, high levels of LTB4 (approximately 150 ng/mL) have been reported in rheumatoid synovial fluids [Klickstein et al., 1980]. Davidson et al., (1983), failed to detect LTB4 using HPLC, however by RIA, concentrations of 0 - 1.5 ng/mL were detected. Belch et al., (1989), measured LTB4 production by peripheral blood PMNs from RA subjects and determined that LTB4 production from a standard number of RA cells is increased compared with those from normal controls suggesting that the high levels of LTB4 detected in RA synovial fluids does not merely reflect an increased PMN population within the fluid. In rabbits with antigen-induced arthritis, an animal model of RA, the synovial fluids contain immunoreactive LTB4 in the early lesion but in the established disease the concentrations of LTB4 are below the limits of detection [Henderson et al., 1985]. In other animal models of inflammation, the peptido-leukotrienes and LTB4 have been measured in exudates obtained from rats after carrageenin-induced inflammation [Peskar et al., 1991] and from stimulated rabbit hearts after myocardial infarction [Mullane et al., 1984]. Studies in our own laboratory have shown greatly increased production of LTB4 by stimulated rat neutrophils following the induction of adjuvant arthritis.
Interactions between eicosanoids

Interactions occur between the products of CO and LO eicosanoid metabolism and the production of one eicosanoid may affect the production of another biologically active lipid from the same or from the alternate pathway. For instance, LTC4 and LTD4 are able to stimulate prostaglandin secretion from macrophages (PGE2, 6-ketoPGF1α and TXB2) and human endothelial cells in culture (PGI2) [Feuerstein et al., 1981; Pologe et al., 1984]. The addition of small amounts of PGE2 greatly enhance the effect of LTB4 on plasma exudation [Bray et al., 1981] and leukocyte chemotaxis [Goetzl et al., 1979]. PGI2 and PGE2 suppress LTB4 production in PMNs [Ham et al., 1983], but LTB4 itself may promote TXB2 production in endothelial cells [Dunham et al., 1984] and PMNs [Puustinen and Uotila, 1984]. Perhaps conversely, the mono-HETEs and di-HETEs have been shown to decrease the production of PGs (in particular PGI2) by porcine coronary artery endothelial cells in culture [Gordon et al., 1991].

1.2.4 Inhibition of eicosanoids

The identification of the eicosanoids as potent mediators of inflammation has inevitably led to the search for ways of modifying their biosynthesis and action. The inhibition can be directed towards altering product activity or reducing product formation. Approaches to reduce the effects of existing LTs and PGs include altered metabolism, inactivation, tissue desensitisation and receptor antagonism. Product formation can be reduced by inhibiting the release of AA or by inhibiting the subsequent metabolism of AA.

PLA2 is an important regulatory enzyme for the entire eicosanoid cascade as it is usually responsible for the liberation of the fatty acid precursors. Glucocorticoids interfere with the generation of eicosanoids by inducing the synthesis of protein inhibitors of PLA2 which cause inhibition of AA release [Blackwell and Flower, 1983]. Many compounds that inhibit PLA2 also appear to inhibit PLC, another enzyme
responsible for the liberation of unsaturated fatty acids from the cell membrane phospholipids. Therefore, the phospholipase inhibitors will block the release of the eicosanoid precursors and effectively reduce the production of both PGs and LTs. The non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the action of cyclooxygenase on AA metabolism by preventing the formation of the cyclic endoperoxides and the subsequent production of the PGs. These agents may act as reversible competitive (substrate) inhibitors (e.g. ibuprofen), or may be time-dependent irreversible inhibitors such as aspirin which chemically alters the active site of the enzyme. Others may have anti-oxidant or radical-trapping properties which reduce enzyme activity by depletion of radical intermediates [Lands, 1981]. In addition to their CO inhibitory properties, some of the NSAIDs appear to also inhibit the action of 5-lipoxygenase on AA. The inhibition of both CO and LO by acetylenic analogues of AA such as eicosatetraynoic acid (ETYA) can be explained by competition with the natural substrate [Higgs and Vane, 1983]. There are other drugs which are more selective for the LO arm of the AA cascade such as nordihydroguaiaretic acid (NDGA) and phenidone but their mechanisms of action are not clear. BW755C is an analogue of phenidone known to be an inhibitor of both CO and LO activity and is equipotent in reducing oedema, PG synthesis and leukocyte accumulation [Salmon et al., 1983]. The anti-inflammatory glucocorticoids reduce PG and LT production by preventing the release of AA from phospholipids, but unfortunately have a broader range of metabolic effects many of which are undesirable.

Another way to reduce the effects in vivo of the inflammatory eicosanoids is through the use of diets rich in n-3 fatty acids, in particular EPA and DHA. This leads to potentially anti-inflammatory biochemical changes, including the inhibition of the pro-inflammatory 4-series LTs with preferential generation of the relatively inactive 5-series LTs, alteration of cytokine production and reduction of PG synthesis.
1.3 N-3 FATTY ACIDS

Epidemiological studies by Bang and Dyerberg in the early 1970's alerted the scientific and medical community to the possible beneficial effects of omega 3 fatty acids, particularly with regard to cardiovascular disease. They reported that the Greenland Eskimos had low rates of coronary heart disease despite their high fat diet [Bang and Dyerberg, 1972]. Eicosapentaenoic acid was implicated in the prevention of heart attacks because of its anti-thrombotic effects, the increase in bleeding time and its effect in lowering serum cholesterol concentrations [Dyerberg et al., 1978; Dyerberg and Bang, 1979]. Following these observations, Kromann and Green, (1980), reported that the Greenland natives also had a low incidence of asthma and the inflammatory skin disorder, psoriasis, although the incidence of arthritis was not recorded. Since these observations were made, n-3 fatty acids have been extensively studied in terms of their anti-inflammatory, hypolipidaemic, anti-thrombotic and vascular effects in relation to arthritis, psoriasis, ulcerative colitis, coronary heart disease, hypertension, asthma, cancer and diabetes and many other pathological conditions [reviewed by Simopoulos, 1991].

1.3.1 Pharmacological actions of n-3 eicosanoids

There is very little EPA in the plasma or cell membranes of humans consuming the usual Western diet which is low in n-3 fatty acids. However, increasing dietary EPA (by increasing dietary fish oil) results in increased cell membrane phospholipid EPA content in humans and animals [Cleland et al., 1988, 1990a]. Increased membrane EPA content results in displacement of n-6 fatty acids, in particular AA [Croft et al., 1988]. Decreased substrate availability for eicosanoid synthesis may result in decreased synthesis of LTB₄ [Terano et al., 1984a; Sperling et al., 1987a; Croft et al., 1988; Cleland et al., 1990b], PGE₂ [Terano et al., 1986; Knapp and FitzGerald, 1989; Ferretti and Flanagan, 1990] and TXA₂/B₂ [Hornstra et al., 1981; Croft et al., 1988; Knapp and FitzGerald, 1989; Terano et al., 1986]. Furthermore, there is
associated generation of varying quantities of the trienoic PGs and the 5-series LTs with unique biological properties of their own which may contribute directly to the anti-inflammatory effects attributed to EPA.

**Thromboxane A₃**

EPA is a poor substrate for cyclooxygenase but appears to have a high binding affinity for platelet cyclooxygenase. Thus, it effectively competes with AA and inhibits, in a dose dependent manner, the conversion of AA to PGH₂ and TXA₂ [Needleman et al., 1979]. Both PGH₂ and TXA₂ aggregate human platelet-rich plasma. Platelets are able to enzymatically convert PGH₃ to TXA₃, however, neither of these 3-series PGs cause platelet aggregation. In addition, PGH₃ undergoes rapid spontaneous degradation to PGD₃ which also inhibits aggregation of human platelets [Whitaker et al., 1979]. Substances that increase platelet cyclic AMP levels inhibit platelet aggregation [Vargaftig and Chignard, 1975] and Needleman et al., (1979), showed that both PGH₃ and TXA₃ increase cyclic AMP whereas PGH₂ and TXA₂ cause little or no change. A dose dependent comparison of the potency of inhibition of platelet aggregation by 3-series prostaglandins showed that PGD₃ was about 10 times more potent than PGH₃ and 100 times more potent than PGE₃ [Needleman et al., 1980]. However, they considered it unlikely that substantial amounts of PGH₃, PGD₃ or PGE₃ would be generated in vivo since EPA is a poor substrate for cyclooxygenase and proposed the principal mechanism of EPA interference with aggregation to be competition with AA. In addition, binding studies have since shown that both EPA, and to an even greater extent DHA, interact with the platelet thromboxane receptor suggesting that receptor antagonism may be an important mechanism by which n-3 fatty acids modulate platelet reactivity in vivo [Swann et al., 1990].

**n-3 Prostacyclin**

The 2-series prostacyclin, PGI₂ and the 3-series prostacyclin, PGI₃, appear to
exert similar effects both on vascular smooth muscle and on platelets [Needleman et al., 1979]. Both compounds are potent vascular relaxants and also inhibit aggregation in human platelet-rich plasma and increase platelet adenylate cyclase activity. Dietary supplementation with fish or fish oil is associated with an increase in PGI₃ production without a concomitant reduction in PGI₂ formation [Hornstra et al., 1990]. In normal platelets, phospholipase activation (occurring for example during adhesion to a damaged blood vessel), releases AA which is enzymatically converted to PGH₂ and TXA₂, thus promoting aggregation. In blood vessels, the release of AA results in PGI₂ production which causes vasodilatation and elevates platelet cyclic AMP thus inhibiting aggregation. The degree of thrombosis may reflect the balance between these two pathways. It is therefore possible that the ingestion of EPA leads to anti-thrombotic conditions by causing a decrease in TXA₂, a potent platelet aggregator and vasoconstrictor, together with an overall increase in prostacyclin by increasing PGI₃ without a decrease in PGI₂, both of these PGs being active vasodilators and inhibitors of platelet aggregation [Kinsella et al., 1990].

Prostaglandin E₃

Many important physiological functions have been attributed to PGE₂, including blood pressure regulation, mediation in events important for fertility and modulation of the immune response. It is a potent vasodilator and hyperalgesic agent, contributing to the pain, oedema and erythema characteristic of the inflammatory response. By contrast, little is known about the relative activity and production of PGE₃ with regard to inflammation. Whitaker et al., (1979), stated that PGE₃ was 10 - 30 times less active than PGE₂ as a contractile agent on nonvascular and vascular smooth muscle but did not show data to support this assertion. By contrast, PGE₃ stimulates rat bone resorption in vitro with a potency similar to that of PGE₂ although EPA was found to be a much less effective precursor for PGE₃ than was AA for PGE₂ [Raisz et al., 1989]. Haynes et al., (1992), examined the effects of PGE₁, PGE₂ and PGE₃ on both the production by mononuclear phagocytes and the action of the inflammatory
cytokines IL-1, TNF and IL-6. Each of these PGs inhibited the mitogenic activity of 
IL-1 for mouse thymocytes, spreading of mouse macrophages on glass, TNF-α & -β 
production by human peripheral blood mononuclear cells and rat macrophages, IL-1 
production by rat and mouse peritoneal macrophages and IFN-γ production by human 
peripheral blood mononuclear cells. They had little effect on the production of IL-1 by 
human monocytes but all enhanced IL-6 production by rat and mouse macrophages and 
human monocytes. The relative potency of the PGs for both inhibitory and stimulatory 
effects was PGE₁ = PGE₂ = or > PGE₃.

Miscellaneous cyclooxygenase products

The observation that PGE₃ and PGD₃ lower intraocular pressure without 
causing ocular inflammation may have significance considering that some surveys have 
demonstrated that Greenland Eskimos have a lower incidence of open-angle glaucoma 
than Caucasians [Kulkarni and Srinivasan, 1989]. Assessment of the protective effect of 
PGF₃α against ethanol-induced gastric mucosal injury in rats, in comparison with 
PGF₂α, indicated that the presence of an additional double bond in the molecule 
markedly reduces the protective effects [Faust et al., 1989].

Lipoxygenase products

Several in vitro studies have demonstrated that although EPA is a poor substrate 
for cyclooxygenase, it is a good substrate for the lipoxygenase enzymes [Jakschik et 
al., 1980; Terano et al., 1984b]. The metabolism of EPA via the lipoxygenase pathway 
to both LTC₅ and LTB₅ was first demonstrated in mouse mastocytoma cells following 
feeding an EPA-rich diet [Hammarström, 1980; Murphy et al., 1981]. Goldman et al., 
(1983), tested the potency of LTB₅ which had been generated from guinea pig 
peritoneal PMNs stimulated in the presence of EPA. They found LTB₅ to be 10 - to 30 
-fold less potent as a human neutrophil chemotaxin than LTB₄, but equipotent as a 
neutrophil degranulating agent. LTB₅, generated from human neutrophils stimulated
with the calcium ionophore A23187 in the presence of EPA, was found to have only 10% of the potency of LTB4 in inducing aggregation of human neutrophils [Prescott, 1984]. In another study, LTB5 biosynthesized from rabbit PMNs was at least 30 times less potent than synthetic LTB4 in causing aggregation of rat neutrophils in vitro, chemokinesis and degranulation of human PMNs in vitro and potentiation of bradykinin-induced plasma exudation in rabbit skin [Terano et al., 1984b].

A comparison between LTs produced in the peritoneal cavity of rats fed diets supplemented with either fish oil or beef tallow showed that LTB5 was 30 - 60 times less potent than LTB4 as a human neutrophil chemotaxin, while LTC5 and LTC4 had equipotent contractile actions on guinea pig pulmonary parenchymal strips and ileal tissues [Leitch et al., 1984].

Studies using a partially purified enzyme showed that EPA is in fact a slightly better substrate for 5-LO than is AA [Jakschik et al., 1980; Ochi et al., 1983]. The EPA-derived metabolites of the 5-LO pathway, LTA5, 5-HPEPE and 5-HEPE, are greater in cells exposed to EPA than the corresponding products, LTA4, 5-HPETE and 5-HETE, from cells incubated with AA, indicating that EPA is indeed the favoured substrate at reactions involving 5-LO and LTA synthetase (Figure 1.6). In fact, the presence of EPA results in inhibition of the production of LTB4 from AA. LTA5 accumulates as it is a less favourable substrate for, but good inhibitor of, LTA hydrolase, resulting in decreased total LTB production [Prescott, 1984]. Nathaniel et al. (1985), also demonstrated that LTA5 inhibits LTA hydrolase using partially purified enzyme studies. However, these observations regarding the relative preferences of cellular enzymes for EPA and AA added exogenously may not accurately reflect the relative metabolism of endogenous EPA and AA by cells in vivo. A close relationship between peritoneal exudate cell membrane phospholipid EPA:AA content and LTB5:LTB4 ratios has been observed over a range of values generated in leukocytes of different rat strains fed either fish oil containing EPA or linseed oil containing ALA, the precursor of EPA [Cleland et al., 1990a], or fed combinations of various vegetable oils and fish oils [James et al., 1991a] and from normal diets with additional dietary EPA [Terano et al., 1984a]. A similar relationship has been observed between the
Figure 1.6  Pathway for leukotriene B synthesis from AA and EPA.

Reactions 1 - 3 are catalysed by the enzymes shown.
EICOSAPENTAENOIC ACID

1. 5-Lipoxygenase

5-HETE ← 5-HPETE

2. LTA Synthetase

→ LTA4

→ LTB4

(4 - series)

3. LTA Hydrolase

→ LTC4

→ LTD4

→ LTE4

ARACHIDONIC ACID

5-HPEPE → 5-HEPE

→ LTA5

→ LTB5

(5 - series)

LTD5

LTE5
relative availabilities of the substrates EPA and AA and leukotriene synthesis by resident macrophages harvested from rats fed corn oil, linseed oil or fish oil diets [Brouard and Pascaud, 1990]. Notwithstanding, the effect of feeding an EPA-rich diet could result in an overall decrease in chemotactic activity because LTB5 has much less biological activity than LTB4 and therefore the synthesis of LTB5 is of little consequence for neutrophil-mediated inflammatory reactions.

In vitro studies show that while LTB4 induces IL-1 - like activity and calcium mobilization in human blood monocytes, LTB5 has no significant effect [Tatsuno et al., 1990]. The differential potency between these LTs in calcium mobilization may also contribute to the differences in activation of neutrophil functions.

1.3.2 Dietary EPA and the cardiovascular system

Populations with high consumption of fish, such as the Eskimos and Japanese, have lower rates of myocardial infarction [Simopoulos, 1991]. The first step in the development of atherosclerosis is a nonspecific injury to endothelium followed by an accumulation of monocytes and macrophages, foam cell formation and platelet aggregation. Deposition of cholesterol in the smooth muscle cells and accumulation of macrophages in the vessel wall eventually leads to plaque formation. Ingestion of n-3 fatty acids may modulate many of the steps in the development of the atherogenic process.

The effects of n-3 fatty acids on serum cholesterol levels are similar to those of other PUFA's in that they lower cholesterol concentrations when they replace saturated fats in the diet. In addition, n-3 fatty acids lower serum triglyceride concentrations and may slightly increase high-density-lipoprotein cholesterol concentrations [Harris, 1989]. The anti-atheromatous actions of n-3 fatty acids are supported by a number of animal studies. In a hyperlipidemic swine model, dietary supplementation with fish oil reduced the development of coronary atherosclerosis [Weiner et al., 1986]. Atherogenesis was inhibited in the aorta, carotid and femoral arteries of primates following dietary fat substitution with n-3 fatty acids [Davis et al., 1987] and it was
also inhibited by fish oils in a rabbit model [Zhu et al., 1988].

Another mechanism of protection which has received considerable attention is the anti-thrombotic action of dietary fish oils. Feeding cod-liver oil to rats leads to a reduction in the formation of platelet TXA₂ and of vascular PGI₂ with a concomitant prolongation in bleeding time and a depression of arterial thrombosis [Hornstra et al., 1981]. Although these authors could not identify the production of TXA₃ and PGI₃ from endogenous precursors in the vessel wall, subsequent studies by Knapp and Salem, (1989), have reported the formation of PGI₃ in the urine of rats fed fish oil without reduced excretion of the corresponding PGI₂ metabolite. Similarly, long term dietary tuna fish oil supplementation resulted in the urinary excretion of PGI₃-M and PGI₂-M, reduction in the urinary level of immunoreactive 2,3-dinor-thromboxane B₂/₃ and a significant reduction in plasma cholesterol (53%) and triacylglycerols (44%) in the marmoset monkey [Abeywardena et al., 1989]. In a canine model of coronary thrombosis, animals fed fish oil showed a reduction in serum TXB and excretion of TXA₂-M with basal PGI₂ formation being unaltered, whereas production of EPA-derived TXA₃ and PGI₃ increased [Braden et al., 1990]. In the animals fed the fish oil, the time to plasminogen activator induced thrombolysis was accelerated but the rate of reocclusion was unaltered. Humans fed a diet rich in sea fish or fish oil were found to have reduced platelet aggregation, reduced formation of immunoreactive TXB₂ by collagen stimulated platelets and an increased bleeding time [Fischer and Weber, 1984]. These changes were accompanied by the excretion of PGI₃-M, the major urinary metabolite of PGI₃, providing evidence for the in vivo formation of PGI₃ in man. The excretion of PGI₂-M was unchanged. In another human trial, volunteers were given dietary supplements of EPA or DHA. Each of these led to the excretion of PGI₃-M but there was considerable interindividual variation in the amount formed [Fischer et al., 1987]. The conversion of EPA into PGI₃ by human umbilical blood vessels has also been demonstrated in vitro [Dyerberg et al., 1981].

Prostaglandins and other eicosanoids affect numerous processes involved in the regulation of blood pressure [Dunn and Gröne, 1985] and there have been numerous studies investigating the link between dietary fats, eicosanoid production and
hypertension. Knapp and FitzGerald, (1989), supplemented the diet of men with mild hypertension with fish oil, safflower oil or a mixture of oils representing the average American diet. They found that blood pressure decreased in the men receiving a high dose of fish oil together with an increase in the synthesis of total PGI due to a marked increase in PGI3 production during the first week of supplementation. As supplementation continued, blood pressure continued to fall but the total prostacyclin metabolite excretion fell also, although the percentage of PGI3, as a proportion of total PGI, remained elevated while supplementation continued. The level of TXA2 metabolites fell and metabolites of TXA3 were detected in the groups receiving fish oil. The formation of PGE2 tended to decrease with fish oil but no PGE3 metabolite was detected. It was concluded that although large doses of n-3 fatty acids can lower blood pressure, an alteration in the synthesis of vasodilator PGs is not the direct mechanism underlying this effect. However, a study in which spontaneously hypertensive rats were given pure EPA as the methyl ester by gavage for 10 days failed to demonstrate a reduction in blood pressure although aortic rings showed increased endothelium-dependent relaxations in response to acetylcholine [Yin et al., 1988].

In summary, many in vitro, ex vivo and in vivo studies on the cardiovascular system of animals and humans indicate that n-3 fatty acids can have beneficial effects on multiple events which contribute or predispose to coronary heart disease.

1.3.3 Dietary EPA and immunity and inflammation

Many experimental studies have provided evidence that incorporation of n-3 fatty acids into tissues may modify immune and inflammatory reactions. The anti-inflammatory effects of fish oil may result from events involving several classes of inflammatory mediators, in particular the lipid mediators, including the eicosanoids, and the peptide mediators or cytokines.
Immunity

The best studied autoimmune model in dietary investigations is the spontaneously developing model of systemic lupus erythematosus (SLE) in New Zealand Black x New Zealand White (NZB x NZW) F1 mice. These mice develop severe proteinuria associated with immune complex glomerulonephritis which leads to premature death [Prickett et al., 1981]. Initially it was demonstrated that essential fatty acid deficiency (EFAD), a condition in which substrates for PG synthesis are reduced, delayed the development of glomerulonephritis in NZB x NZW mice [Hurd et al., 1981]. Prickett et al., (1981), described the ability of a diet enriched in EPA and DHA (fed as menhaden oil) to delay the development of proteinuria and prolong survival in weanling female NZB x NZW mice compared with a control group fed beef tallow. The protective effect was quite dramatic, with the survival rate of mice at 13.5 months of age increasing from 30% to 100%. In addition, levels of circulating anti-DNA antibodies were lower in the menhaden oil fed mice. Further studies demonstrated that the protective effect of marine oil is observed even if dietary treatment was delayed until after onset of immunologic and serologic evidence of the disease [Prickett et al., 1983]. Furthermore, there was no difference in anti-DNA levels between the menhaden oil fed group and the beef tallow fed group suggesting that the protective effect of EPA does not act solely by suppression of anti-DNA antibodies. The effect of the fish oil diet on survival was dependent on the dose of menhaden oil and correlated best with tissue levels of 22:5 n-3 rather than EPA, suggesting that this PUFA may be important in the protection against autoimmune disease [Robinson et al., 1985].

EPA has been reported to inhibit natural killer cell activity of human lymphocytes [Yamashita et al., 1986] and to restore the impaired humoral response evident in mice fed a fat free diet [Wee et al., 1988]. In the latter study, dietary EPA did not affect the activation of B cells and T suppressor cells in the mice fed a fat free diet, suggesting that the immunopotentiation was mediated, at least in part, by enhancement of T helper cell activity. Another effect of dietary EPA on the in vivo induction of humoral immune responses is the induction of 4 to 8 fold greater titres of
IgE and IgG in appropriately challenged fish oil fed rats relative to beef tallow fed rats [Prickett et al., 1982]. These fish oil fed rats also had heightened active cutaneous anaphylaxis and Arthus reactions. Passive inflammatory reactions were not significantly different between the two groups.

**Inflammation**

One of the biochemical changes induced by fish oil which is potentially anti-inflammatory is the inhibition of the 5-LO pathway in neutrophils and monocytes. Supplementation of the diet with fish oil rich in EPA can cause an increase in the EPA content of cellular phospholipids in both human [Cleland et al., 1992; Lee et al., 1985] and animal subjects [Cleland et al., 1990a; Terano et al., 1986; Brouard and Pascaud, 1990]. The effects of changing the *in vivo* balance of eicosanoid precursors are similar to those reported following *in vitro* incorporation of exogenous EPA. The decreased production of LTB₄ by human neutrophils is accompanied by an increase in the generation of LTB₅ from EPA [Lee et al., 1985; Kremer et al., 1987; Payan et al., 1986]. The ingestion of 4 g of purified EPA ethyl ester daily for 8 weeks suppressed significantly the chemotactic responses of PMN leukocytes and augmented the proliferative response of T lymphocytes to an optimal concentration of PHA without consistently altering the distribution of T lymphocytes between helper and cytotoxic subsets [Payan et al., 1986]. Another study investigating the function of human PMNs from normal subjects, who supplemented their diet with 3.2 g of EPA and 2.2 g of DHA daily for 6 weeks, showed that the neutrophil chemotactic response to LTB₄ was inhibited by 70% and the capacity of those neutrophils to adhere to LTB₄-pretreated endothelial cell monolayers was also significantly reduced [Lee et al., 1985].

Animal studies on the effect of dietary supplementation with EPA have yielded similar results to those on human tissues. Supplementation of a normal rat diet with EPA 240 mg/kg per day for 4 weeks caused a significant increase in the formation of LTB₅ and a decrease in the synthesis of LTB₄ by stimulated leukocytes [Terano et al., 1984a]. This was accompanied by an increase in the cellular content of EPA and the
ratio of EPA/AA in leukocyte phospholipids correlated with the LTB5/LTB4 ratio produced. Cleland et al., (1990b), reported similar findings following 4 week periods of feeding fat-free rat chow mixed with fish oil or linseed oil to achieve a final fat level of 10% (w/w, dry wt.). The fish oil was rich in EPA itself and the linseed oil provides the 18 carbon precursor, ALA. There was a strong correlation between the ratio of EPA/AA achieved in the peritoneal exudate cell membrane phospholipids at harvest and the ratio of LTB5/LTB4 produced by these cells following stimulation in vitro. This association was consistent in the five rat strains studied and was independent of the source of EPA (i.e. dietary fish oil or via the dietary precursor, ALA in linseed oil).

Other potentially anti-inflammatory effects induced by dietary fish oil include inhibition of the CO pathway resulting in decreased production of PGE2 and TXA2. The formation of both of these AA metabolites was decreased in mildly hypertensive men receiving either 3 or 15 g of n-3 fatty acids daily for 4 weeks [Knapp and FitzGerald, 1989]. Similarly, PGE2 release by human mononuclear cells stimulated by heat killed Staphylococcus epidermidis decreased following 6 weeks of dietary supplementation with 2.75 g EPA and 1.85 g DHA daily [Endres et al., 1989]. Supplementation of a normal rat chow with 240 mg/kg/day EPA for 4 weeks significantly decreased the concentration of PGE2 and TXB2 in inflammatory exudates derived from subcutaneous implantation of carrageenin impregnated sponges [Terano et al., 1986]. In a slightly different rat model of inflammation, the animals fed the EPA-rich diet were found to have a significant decrease in oedema formation 2 - 6 h after injection of carrageenin into the hind paws. The swelling during this time is considered to be due to the presence of prostaglandins, particularly PGE2 [Terano et al., 1986]. Dietary enrichment with ALA, the 18 carbon precursor of EPA, as 10% linseed oil in fat-free rat chow, also led to a significant decrease in the synthesis of PGE by rat peritoneal macrophages (determined by radioimmunoassay) [Magrum and Johnston, 1983]. No attempt was made to determine whether PGE3 was synthesized or to correct for cross-reactivity in the assay. In contrast, Brouard and Pascaud, (1990), found that whereas supplementation with fish oil led to inhibition of PGE2, TXB2 and 6-ketoPGF1α production by rat resident peritoneal macrophages, linseed oil feeding did
not affect the synthesis of the CO products. However, the EPA content was raised 7-fold in macrophage membrane phospholipids in the fish oil fed group and only 3-fold following linseed oil feeding. Serial blood samples taken from rats fed a fat free synthetic dietary preparation supplemented with 10% by weight of MaxEPA fish oil demonstrated that significant changes in serum fatty acids occur within 48 hours of treatment [Croft et al., 1985]. Serum TXB2 was also depressed after 48 hours and this suppression reached a maximum of 65% after 7 days of dietary treatment. Reduced urinary excretion of PGE2 and 6-keto PGF1α were significant after 7 days of MaxEPA supplementation.

The influence of dietary n-3 fatty acids on the synthesis of inflammatory cytokines has also been investigated. In 1989, Endres et al. reported that the synthesis of IL-1 and TNF by human peripheral blood mononuclear cells stimulated in vitro can be suppressed by prior dietary supplementation with 18 g MaxEPA (containing 153 mg EPA/g and 103 mg DHA/g) daily for 6 weeks. Their results demonstrated that n-3 fatty acids affect the synthesis rather than the release of these cytokines since they measured both cell associated and secreted cytokines. A possible mechanism for decreased IL-1 production is decreased synthesis of LTB4 and generation of the biologically less active metabolite LTB5 derived from EPA. Evidence supporting this mechanism includes the observations that inhibitors of 5-LO can decrease IL-1 synthesis [Dinarello et al., 1984] and TNF synthesis [Schade et al., 1989] and addition of LTB4 can enhance IL-1 [Rolapleszczynski and Lemaire, 1985] and TNF synthesis [Dubois et al., 1989]. A decrease in IL-1 production and release from peripheral blood mononuclear cells obtained from RA patients following ingestion of fish oil supplements for 24 weeks has also been reported [Kremer et al., 1990]. Conconavalin A induced IL-2 production by mononuclear cells from these patients increased from baseline following fish oil supplementation, however, the changes were not statistically significant.

Arthritis - human studies

Rheumatoid arthritis (RA) has many systemic manifestations but is primarily a
disease of the synovial joints. Affected joints are typically swollen and painful and the destruction of cartilage and bone is found in a substantial proportion of patients. The synovial lining is a highly vascularized tissue which, in the rheumatoid patient, becomes heavily infiltrated with chronic inflammatory cells such as lymphocytes, macrophages and dendritic cells [Henderson et al., 1987]. As the inflammatory process develops, neutrophils invade the synovial space where they may release degradative enzymes, toxic free oxygen metabolites and leukotrienes. The signs and symptoms of joint inflammation such as pain, swelling, redness and loss of function represent the cumulative effect of a number of interrelated biochemical and cellular events. IL-1 and TNF increase neutrophil adhesion to endothelium, stimulate chondrocytes to degrade their cartilage matrix through the production and release of lytic enzymes, stimulate chondrocyte and synoviocyte PGE2 production, increase bone resorption and fibroblast proliferation and inhibit synthesis of proteoglycans by articular cartilage [Henderson et al., 1987]. IL-1 and TNF also stimulate the synthesis of adhesion molecules and activate platelets, monocytes and neutrophils [Endres et al., 1989]. The PGE2 produced by the rheumatoid synovial pannus may contribute to the destruction of juxta-articular bone by stimulating bone resorption [Robinson et al., 1982]. PGE2 can cause hyperalgesia and can also synergise with LTB4 to cause vascular leakage leading to oedema and tissue swelling. LTB4 is a potent chemotactic and degranulating agent for neutrophils and platelet activating factor (PAF) may act as a stimulus/response linking second messenger in leukocytes and endothelial cells [Stewart et al., 1990].

Clinical studies have demonstrated that dietary fish oil supplements resulted in a modest decrease of the signs and symptoms of RA patients [Kremer et al., 1985, 1987, 1990; Sperling et al., 1987a; Cleland et al., 1988; van der Tempel et al., 1990]. In these studies, doses of 10 - 20 mL fish oil daily were given to patients who typically had long-standing, established disease and who were receiving a full range of anti-inflammatory and disease-suppressing drugs. In general, subjects were advised to continue their normal diet. All studies demonstrated a beneficial effect in some of the clinical parameters of disease activity.

These clinical studies all monitored compliance by pill counts and serum,
plasma or neutrophil lipid profiles. Significant improvements from baseline were noted in the number of tender joints [Kremer et al., 1985, 1987, 1990; Cleland et al., 1988], duration of morning stiffness [Kremer et al., 1985, 1990; van der Tempel et al., 1990], mean time to onset of fatigue [Kremer et al., 1987], joint swelling index [Sperling et al., 1987a; Kremer et al., 1990; van der Tempel et al., 1990], grip strength [Cleland et al., 1988; Kremer et al., 1990], patient evaluation of pain and physician evaluation of global arthritis activity [Kremer et al., 1990]. The most recent clinical study by Kremer et al., (1990), utilized two different doses of fish oil and continued the dietary intervention for a longer period than that in other studies. In addition to the clinical parameters of disease activity, immunological indices were measured. A decrease in LTB₄ production by stimulated neutrophils has been seen in this and other studies [Kremer et al., 1987, 1990; Sperling et al., 1987a; Cleland et al., 1988; van der Tempel et al., 1990] and is usually associated with the appearance of LTB₅. Production of IgG in vitro in the absence of mitogen stimulation decreased in the fish oil fed groups of patients [Kremer et al., 1990]. The generation of PAF by monocytes decreased [Sperling et al., 1987a], an observation confirmed by studies with monocytes from healthy human volunteers [Sperling et al., 1987b]. The chemotactic response of RA patients' neutrophils to LTB₄ and fMLP appeared to be diminished relative to that reported for healthy volunteers [Sperling et al., 1987a]. However, dietary supplementation with fish oil increased the chemotaxis of neutrophils to both agonists [Sperling et al., 1987a]. Dietary supplementation with fish oil in combination with evening primrose oil (EPO) (or EPO alone) enabled approx. 75% of subjects with mild RA to decrease or stop NSAID treatment [Belch et al., 1988].

**Arthritis - animal studies**

The modulation of inflammatory effects by dietary PUFA can be demonstrated using essential fatty acid deficient (EFAD) rats [Denko, 1976]. EFAD rats showed significantly less adjuvant-induced paw swelling than rats fed a normal diet. Dietary supplementation with marine oils has also been shown to influence the incidence and
severity of animal models of inflammation.

Prickett et al., (1984), showed that Sprague Dawley rats maintained on diets supplemented with fish oil and immunised with native chick type II collagen emulsified in Freund’s adjuvant recorded an increased incidence of arthritis compared with control, beef tallow-fed rats. There was no difference in severity of joint inflammation or delayed-type hypersensitivity to collagen between the two groups, but the mean serum titre of IgG antibodies was lower in the fish oil fed group. Synovial tissue from arthritic rats fed the fish oil released 75 - 80% less PGE₂ during primary culture but this was not accompanied by any detectable production of PGE₃. These results suggest that PGE₂ may exert suppressor influences on the induction of this model of arthritis. In contrast, dietary fish oil decreased susceptibility and severity of collagen arthritis in mice [Leslie et al., 1985]. This was accompanied by an increased percentage of EPA in the phospholipids of macrophages isolated from the fish oil fed mice and a reduction in the amount of PGE₂ and PGI₂ produced following overnight incubation in medium/FCS, relative to macrophages from control, corn oil fed mice. Subsequently, Leslie et al., (1988) determined that dietary fish oil was able to reduce disease severity even if begun after the arthritis was clinically apparent. The results indicated that arthritis severity can be modulated after the onset of collagen induced arthritis by treatment with a fish oil diet, possibly by altering the PG profile of macrophages present at inflammatory sites. The possibility of strain differences in the inflammatory response to dietary intervention with n-3 fatty acids was addressed using a model of adjuvant induced polyarthritis in two rat strains [McColl et al., 1987]. Dietary treatment with fish oil reduced the severity of disease activity in Dark Agouti rats but enhanced disease severity in Hooded Wistar rats.

Overall, the published studies provide evidence for an anti-inflammatory effect of dietary enrichment with EPA or EPA-rich fish oil. A more effective application of this approach requires more information regarding the conditions of background diet against which the beneficial effects of fish oil supplements/EPA can be optimised. In addition, a better understanding of the biochemical effects and cellular sites of the anti-inflammatory effects of n-3 fatty acids is required.
The work described in this thesis arose primarily from the observation that dietary fish oil has been shown to reduce inflammation in human and animal studies. The anti-inflammatory effect described is often modest and appears to be related to the presence of n-3 fatty acids (in particular EPA) in the fish oil and their competition with n-6 fatty acids which are found in abundant quantities in a normal Western diet. Whether the beneficial effect of fish oil can be improved has not been examined in detail. One strategy for increasing the anti-inflammatory effect of fish oil involves enhancing the content of n-3 fatty acids (from fish, certain vegetables and oils) and reducing the content of n-6 fatty acids in the background diet. Other approaches could emerge from a better understanding of the mechanisms of anti-inflammatory actions of n-3 fatty acids. A further important and relatively unexplored approach involves the evaluation and utilization of potentially favourable drug-diet interactions.

The materials and methods used are outlined in Chapter 2, including detail of modifications of existing methods. Section G outlines the final method developed for the separation and detection of PGE₃ and these conditions were used for all experimental work unless stated otherwise.

Many dietary intervention studies are carried out using healthy human volunteers or normal animals. Often, the information gathered from these experiments on the rate and extent of fatty acid incorporation and/or replacement is related to possible beneficial effects on inflammatory diseases. This implies a presumption that fatty acid metabolism is not affected by the pre-existence of inflammation. The first experimental chapter (Chapter 3) describes work investigating the effect of a systemic inflammatory disease induced in rats on incorporation of the n-3 fatty acid, EPA, into cell membrane phospholipids. In addition to fatty acid analyses, several other parameters of inflammation were assessed throughout the course of the adjuvant induced arthritis.

The two main classes of PUFAs are metabolically and functionally distinct and have been shown to have opposing functions in many, but not all, biological systems. It
has become evident that the n-3 fatty acids play an important role in growth and development, platelet-endothelial homeostasis and "control" of inflammation. However, it is important to extend our knowledge of the possible biological effects of dietary fish oil supplementation to enable appropriate dietary recommendations to be formulated. The signs and symptoms of joint inflammation such as pain, swelling, redness and loss of function represent the composite effect of a number of different biochemical and cellular events involving several classes of inflammatory mediators. These include the lipid mediators of inflammation encompassing the prostaglandins and leukotrienes, known as eicosanoids. Although there have been many studies on the activity of the lipoxygenase metabolites of EPA and the vascular effects of some of the cyclooxygenase metabolites, little is known about the activity of PGE3 with regard to inflammation. Experiments to assess the oedemogenic activity of the n-3 metabolite, PGE3, in mice are described in Chapter 4.

The conjugated triene structure of the leukotrienes creates a strong UV absorbance which enables the separate detection of LTB4 and its analogous n-3 eicosanoid, LTB5, by spectrophotometric detection after HPLC. However, studies of the effect of EPA on the production of PGE3 and PGE2 have lagged behind similar studies with leukotrienes possibly due to the difficulty in the separate quantitation of PGE2 and PGE3 which lack the strong UV absorbance associated with conjugated double bonds and which tend to cross react in immunoassays. Although there have been reports of biological effects of PGE3, the separate detection of PGE3 has been attempted rarely and therefore the influence of PGE3 on inflammatory processes is not well understood. Chapter 5 describes the development of an assay for the separate detection of PGE3 following derivatization with panacyle bromide. The method utilizes thin layer chromatography and high pressure liquid chromatography techniques with fluorometric detection.

In Chapter 6, experiments are described in which conditions are developed for the extraction of prostaglandins from biological samples prior to detection by this method. The effects of EPA in vitro and in vivo on the production of PGE2 and PGE3 were investigated.
A potentially anti-inflammatory biochemical change induced by dietary fish oil is the decreased production of LTB$_4$, a 5-LO metabolite of AA. This is explicable in terms of both decreased substrate levels and competitive substrate inhibition. The pathway shown in Figure 1.7 is the oxidation of AA or EPA by 5-LO to LTA followed by further metabolism by LTA hydrolase to the leukotriene B series. Most drug induced inhibition of LTB$_4$ acts via inhibition of 5-LO activity [Ozaki et al., 1986], however, EPA can inhibit LTB$_4$ synthesis via inhibition of a different enzyme - LTA hydrolase [Prescott, 1984]. This raises the potential for additive or synergistic effects with appropriate combinations of fish oil and pharmacological agents. The ideal combination would require a drug which equally or preferentially inhibited the reaction AA $\rightarrow$ LTA$_4$ over the reaction EPA $\rightarrow$ LTA$_5$ [James et al., 1991b]. This would allow LTA$_5$ inhibition of LTA hydrolase to act in addition to the 5-LO inhibition step. It has been demonstrated in vitro that in combination, the anti-rheumatic drug auranofin and EPA have a simple additive effect on inhibition of the 5-LO pathway following A23187 stimulation of human neutrophils [James et al., 1992].

Another drug which has been shown to have important beneficial effects in the treatment of rheumatoid arthritis is the folic acid antagonist, methotrexate (MTX) [Weinblatt et al., 1985]. The reasons for its therapeutic effects in this particular disease are not clearly understood, however Sperling et al., (1990; 1992), have reported a suppression of neutrophil LTB$_4$ synthesis following A23187 stimulation of cells obtained from RA patients following a single oral dose of MTX. The final experimental chapter (Chapter 7), describes the effect of MTX added in vitro or administered in vivo on the production of the 5-LO metabolites of AA by human or rat leukocytes. These experiments were undertaken seeking conditions of inhibition with this agent which could be used for subsequent studies in which methotrexate and EPA supplements could be used in combination.
Figure 1.7 Inhibition of LTB₄ synthesis by pharmacological inhibitors, which affect 5-lipoxygenase activity, and by EPA, which can give rise to LTA hydrolase inhibition.
CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals (brackets indicate common abbreviations)

Acetic acid - filtered analytical grade
   - Ajax Chemicals, Sydney, Australia
Acetonitrile - HPLC grade
   - BDH Ltd., Poole, UK
Albumin, fatty acid free
   - Boehringer Mannheim Australia, Sydney
Alpha tocopheryl acetate (Vitamin E)
   - Henkel Corporation, USA
Bacteriological peptone
   - Oxoid Ltd., Hampshire, UK
Butylated hydroxy anisole (BHA)
   - Sigma Chemical Company, St. Louis, Missouri, USA
Calcium ionophore (A23187)
   - Sigma Chemical Company, St. Louis, Missouri, USA
Chloroform - Analytical grade for TLC, extraction of fatty acids for GLC
   - Ajax Chemicals, Sydney, Australia
   - HPLC grade for extraction of LTs and hydroxy acids
   - BDH Ltd., Poole, UK
Citric acid
   - Sigma Chemical Company, St. Louis, Missouri, USA
Dextran T500
- Pharmacia LKB Biotechnology, Uppsala, Sweden

Dichloromethane
- BDH Ltd., Poole, UK

5(S), 12(R), dihydroxy-eicosatetraenoic acid (LTB₄)
- Merck Frosst, Pointe-Claire Dorval, Canada

5,8,11,14,17-eicosapentaenoic acid (EPA)

5,8,11,14-eicosatetraenoic acid (Arachidonic acid, AA)
- Sigma Chemical Company, St. Louis, Missouri, USA

Ethylenediaminetetra-acetic acid (EDTA)
- Ajax Chemicals, Sydney, Australia

Foetal calf serum (FCS)
- Flow Laboratories, Sydney, Australia

Gentian Violet
- Sigma Chemical Company, St. Louis, Missouri, USA

Gum Tragacanth
- Sigma Chemical Company, St. Louis, Missouri, USA

15(S)-hydroxyeicosatetraenoic acid (15-HETE)

5(S)-hydroxyeicosatetraenoic acid (5-HETE)
- Cayman Chemical Company, Ann Arbor, Michigan, USA

Ibuprofen
- Sigma Chemical Company, St. Louis, Missouri, USA

Indomethacin
- Sigma Chemical Company, St. Louis, Missouri, USA

Interleukin-1β
- Boehringer Mannheim, Indianapolis, Indiana, USA

Lipopolysaccharide (LPS)
- Sigma Chemical Company, St. Louis, Missouri, USA

MaxEPA (Fish oil)
- R.P. Scherer, Melbourne, Australia
Methanol - Analytical grade for TLC, extraction of fatty acids for GLC
- Ajax Chemicals, Sydney, Australia
- HPLC grade for extraction of LTs and hydroxy acids for HPLC
- BDH Ltd., Poole, UK

Methotrexate Injection
- David Bull Laboratories Pty. Ltd., Victoria, Australia

*Mycobacterium tuberculosis* H37 RA
- Difco Laboratories Inc., Michigan, USA
p-(9-anthroyloxy)phenacyl bromide (panacyl bromide, PAB)
- Molecular Probes, Inc., Eugene, Oregon, USA

Percoll
- Pharmacia LKB Biotechnology, Uppsala, Sweden

Prostaglandin A₁ (PGA₁)
Prostaglandin B₁ (PGB₁)
Prostaglandin B₂ (PGB₂)
Prostaglandin E₁ (PGE₁)
Prostaglandin E₂ (PGE₂)
Prostaglandin E₃ (PGE₃)
Prostaglandin F₂α Tromethamine (PGF₂α)
16,16 dimethyl PGE₂
- Cayman Chemical Company, Ann Arbor, Michigan, USA
5,6,8,11,12,14,15(n)-³H prostaglandin E₂ (l³HJPGE₂)
  Specific activity 5.44TBq/mmol
- Amersham Australia Pty. Ltd., Sydney

Sunflower oil
- Flora, Unilever, Adelaide, Australia

Squalane
- Fluka A.G., Buchs, Switzerland

Tetrahydrofuran (THF) - HPLC grade
- BDH Ltd., Poole, UK
Triethylamine
- Sigma Chemical Company, St. Louis, Missouri, USA

Trypan Blue
- Sigma Chemical Company, St. Louis, Missouri, USA

Water (H₂O) - HPLC grade
- BDH Ltd., Poole, UK

Zymosan
- Sigma Chemical Company, St Louis, Missouri, USA
2.1.2 Buffers/Medium

Phosphate Buffered Saline (PBS)

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<tr>
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Dulbecco’s Phosphate Buffered Saline (DPBS)

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RPMI 1640

RPMI 1640 with Hepes, without NaHCO₃
(Flow Laboratories, Irvine, Scotland)

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<tr>
<td>Benzylpenicillin Sodium</td>
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2.1.3 Fat-free rat food

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<tr>
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<td>Cellulose&lt;sup&gt;2&lt;/sup&gt;</td>
<td>6.0</td>
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<tr>
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<tr>
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<tr>
<td>DL-Methionine</td>
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</tr>
<tr>
<td>Vitamin and mineral mix&lt;sup&gt;3&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Calcium phosphate, dibasic</td>
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<tr>
<td>Potassium carbonate</td>
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<td>Limestone</td>
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<sup>1</sup>Bonlac Foods, Melbourne, Australia

<sup>2</sup>Sigma Chemical Company, St. Louis, Missouri, USA

<sup>3</sup>Colborn-Dawes, Wagga, Australia. Vitamin and mineral mix #713 provided the following: (mg/100g) folic acid, 1; niacin, 12; pantothenate, 3; riboflavin, 1.5; thiamine, 0.8; pyridoxine, 1.2; choline chloride, 100; cobalt, 0.09; iodine, 0.1; selenium, 0.01; copper, 1; iron, 10; manganese, 7.5; zinc, 7; magnesium, 5; ethoxyquin, 10; (µg/100g) vit B<sub>12</sub>, 3; biotin, 20; (IU/100g) vit A, 2,200; vit D<sub>3</sub>, 440.
2.2 METHODS

A. Animals and Diets

2.2.1 Rats

Male Hooded Wistar rats, 4 - 8 weeks old, were purchased from the Gilles Plains Animal Resource Centre, Department of Agriculture, Adelaide, Australia. The rats were maintained at the Animal House, Institute of Medical and Veterinary Science, Adelaide. The diurnal cycle was 12 h light / 12 h dark and the animals were allowed commercial rat chow and water *ad libitum* unless stated otherwise.

2.2.2 Mice

Female Swiss albino mice were purchased from the Gilles Plains Animal Resource Centre, Department of Agriculture, Adelaide, Australia. The mice were maintained at the Animal House, Institute of Medical and Veterinary Science, Adelaide. The diurnal cycle was 12 h light / 12 h dark and the animals were allowed commercial mouse pellets and water *ad libitum* unless stated otherwise.

2.2.3 Diets

Fat-free rat food was prepared from basic constituents (See Section 2.1.3). Weighed amounts of fat-free rat food were mixed with oils to achieve 5% oil w/w (dry wt.) in the final mixture. The dietary oils used were sunflower oil or fish oil. Vitamin E was added to the sunflower oil to achieve a final concentration of 1.84 mg/g total oil, the level in MaxEPA fish oil as supplied. A dough was made by adding water; the mixture was pressed into trays, cut into cubes (approx. 1 x 3 x 3 cm), air dried overnight at room temperature and then stored in sealed bags at -20° C. Uneaten food was discarded every evening and fresh (frozen) food supplied.
Human studies were undertaken on healthy volunteers consuming their normal diet. One subject in addition was assessed after four weeks of dietary supplementation with fish oil capsules (MaxEPA) 12 g/day providing EPA 2.4 g/day and DHA 1.4 g/day.

B. Adjuvant-Induced Arthritis

2.2.4 Induction of disease

At 6 - 8 weeks of age the test rats were injected intradermally at the base of the tail with 0.05 mL of heat-killed *Mycobacterium tuberculosis* dispersed in squalane at a final concentration of 10 mg/mL. Control animals comprising rats matched for weight with rats in the corresponding test group were injected similarly at the base of the tail with 0.05 mL normal saline (0.9% NaCl in distilled water).

2.2.5 Joint scores

A disease activity score (total of 0 - 16 points) was determined as follows: for each paw (0 - 4 points) where 0 = no localised articular lesion or swelling, 1 = localised articular lesions or ankle/wrist swelling, 2 = localised articular lesions and ankle/wrist swelling, 3 = moderate generalised ankle/wrist and paw swelling, and 4 = marked generalised ankle/wrist and paw swelling. Assessments were made up to day 15 for both inflamed and control rats as described.

2.2.6 Paw swelling by volume displacement

Glass displacement cylinders (height 14 cm, diameter 3 cm) with an outlet spout at a 45° angle just below the rim were used. The cylinders were filled to the level of the outlet with 1% detergent and with the rat under anaesthesia, each rear paw was lowered into the solution to the level of the ankle. The solution displaced was collected
in pre-weighed containers and weighed. Measurements were taken on groups of 4 rats on each of days 5, 7, 9, 11, 13 and 15 for both inflamed and control rats where stated.

2.2.7 Spleen weight

The spleen of each animal was removed and weighed on the day of sacrifice.

C. Cell Harvesting Procedures

2.2.8 Induction and collection of peritoneal exudate cells (PEC)

Rats were injected intraperitoneally with 10 mL 3% bacteriological peptone in normal saline. After 4 h, animals were anaesthetised with halothane and killed by cervical dislocation. PEC were harvested by lavage of the peritoneal cavity with PBS (2 x 10 mL), added to 4.5% EDTA (3 mL) and centrifuged. The PEC were washed with PBS and the red cells were lysed by 30 s exposure of the cell pellet to hypotonic saline (16 g/L followed by 2 g/L). The PEC were washed again, resuspended in DPBS and counted using a haemocytometer following staining with Gentian Violet. Differential counts indicated that preparations were 80 - 90% neutrophils. Cell viability assessed by Trypan Blue exclusion was > 98%.

2.2.9 Collection of resident peritoneal cells from rats and mice

Resident peritoneal cells were harvested from male Hooded Wistar rats, 8 - 12 weeks old or Swiss mice 8 - 12 weeks old. Following bleeding by cardiac puncture and cervical dislocation, cells were collected by peritoneal lavage with RPMI 1640. Cells were washed by centrifugation and lysed to remove contaminating red blood cells if necessary.
2.2.10 Collection of whole blood and purification of mononuclear cells and neutrophils

Peripheral blood was obtained from healthy human volunteers (for *in vitro* studies) or from subjects with rheumatoid arthritis (for *ex vivo* studies). 20 mL blood was collected into 4 mL EDTA (4.5%) and 4 mL dextran (6% in normal saline) and allowed to settle for 30 - 60 min at 37° C. The plasma was layered over a double Percoll gradient, densities 1.092 g/mL and 1.070 g/mL, and centrifuged at 400 x g for 25 min. The mononuclear cells were harvested from the plasma/Percoll interface and the neutrophils from the junction of the two Percoll layers. The mononuclear cells were washed (2x) in PBS and resuspended in RPMI 1640. The neutrophils were washed with PBS and the red cells were lysed by exposure to hypotonic saline. The cells were then washed (2x) in PBS and resuspended in DPBS or normal saline. Where stated the neutrophils were prepared for fatty acid analysis and leukotriene and hydroxy acid analysis as described in this chapter, Sections D & E.

2.2.11 Adherent cell cultures

Resident peritoneal cells from rats and mice and peripheral blood mononuclear cells from human volunteers were plated at 4 - 5 x 10⁶ cells/35 mm culture dish (Falcon, Becton Dickinson, New Jersey, USA) in RPMI 1640 and incubated for 2 h at 37° C. The non-adherent cells were removed by washing the cell sheet gently (2x) with 2 mL PBS. Adherent cells were maintained in RPMI for short term (4 - 6 h) stimulation experiments or maintained overnight in 4 mL RPMI 1640/1% FCS or RPMI 1640/0.1% fatty acid free albumin. The effects of zymosan, A23187, LPS, LTB₄ and IL-1 and the inhibitors indomethacin and ibuprofen were investigated in preliminary experiments. AA and EPA were added to overnight cultures at specified concentrations. After 24 h the media were removed, acidified with citric acid and extracted with chloroform:methanol (7:3, v/v). A known amount of PGE₁ was added prior to extraction except where stated.
D. GLC Analysis of Phospholipid Fatty Acids

Cell membrane lipids were extracted in chloroform/methanol (2:1, v/v) containing butylated hydroxy anisole (0.005% w/w) according to the method of Bligh and Dyer, (1959). Cells were resuspended in 1.5 mL normal saline and vortexed thoroughly with methanol (2 mL) followed by chloroform (4 mL). Samples were centrifuged at 420 x g for 10 min. The chloroform layer was removed and evaporated in a centrifugal Speed Vac Concentrator (Savant Instruments Inc., Farmingdale, New York, USA). The lipids were resuspended in chloroform/methanol (9:1, v/v) and stored at -20° C prior to gas-liquid chromatographic (GLC) analyses.

Separation of cell membrane phospholipids from the total extracted lipid and GLC analyses of all samples were carried out by Dr. R. Gibson, Department of Paediatrics, Flinders Medical Centre, South Australia using the method described by Cleland et al., (1992). Separation of phospholipids was by thin-layer chromatography (TLC) on silica gel H plates developed in petroleum ether/diethyl ether/acetic acid (90:15:1, v/v/v). The band containing the total phospholipids remained at the origin and was methylated by heating in 1% (v/v) H₂SO₄ in methanol at 70° C for 3 h. The resulting fatty acid methyl esters were extracted and analysed by GLC (Hewlett Packard HP 5880 gas chromatograph; Hewlett Packard, Palo Alto, California, USA). The column was a 50 metre glass column (0.56 mm, I.D.) coated with SP2340 (Supelco Inc.) prepared by Chromolytic Technology Ltd. (Boronia, Victoria, Australia). The esters were separated using a carrier gas (helium) flow of 30 cm/s with a temperature gradient of 120 - 200° C at 5° C/min. The injection-split temperature was 250° C and the flame ionization detector temperature was 300° C. Fatty acid methyl esters were identified against authentic lipid standards supplied by Nuchek Prep (Elyssian, Minnesota, USA). The flame ionization detector was calibrated so that the response obtained for each of the fatty acids between C₁₄-C₂₄ was identical regardless of the number of double bonds.
E. HPLC Analysis of Leukotrienes and 5-Hydroxy Fatty Acids

Cells from each preparation were assayed in quadruplicate. PEC or human neutrophils (1 x 10^6 cells per tube) were pre-incubated at 37° C for 5 min before the addition of A23187 to achieve a final concentration of 0.5 μM in a total incubation volume of 1 mL DPBS. After 5 min at 37° C, the mixture was acidified by the addition of 100 mM citric acid (0.25 mL). 15-Hydroxyeicosatetraenoic acid (15-HETE) (typically 30 μL of a 2 g/mL stock solution), was added as an internal standard. For each experiment, the same amount of 15-HETE was also added to tubes containing standard amounts of LTB4 and 5-HETE. Lipids were extracted with chloroform/methanol (7:3, v/v) and after evaporation of the solvent, the residue was reconstituted in methanol (50 μL). Chromatographic equipment used included two Model 501 pumps, a 710B WISP autoinjector, a Nova-Pak C18 column (3.9 mm x 15 cm) with a precolumn filter (0.5 μm frit) and a precolumn Cartridge Newguard RP-18 (Aquapore 7 μm, 3.2 x 15 mm) from Millipore Waters, Milford, Massachusetts, USA. LTB4, the all-trans isomers of LTB4, 20-OH LTB4, LTB5, the all-trans isomers of LTB5, 5-HETE, 5-HEPE and 15-HETE were resolved by reverse-phase HPLC and quantified by ultraviolet (UV) detection with a SM4000 Programmable Wavelength Detector (Milton Roy, LDC Division, Riviera Beach, Florida, USA).

A gradient was run under the following conditions: 100% mobile phase A (methanol:water:acetic acid, 65:35:0.08, pH 6.2) for 19 min, followed by 100% mobile phase B (methanol:water:acetic acid, 75:25:0.02, pH 5.6) for 21 min, followed by 100% mobile phase A for 10 min to re-establish the initial conditions for the next sample injection. The flow rate was 1 mL/min. Detection of leukotrienes was carried out at 270 nm UV at 0.001 a.u.f.s. (solvent A) and hydroxy fatty acids at 235 nm UV at 0.0005 a.u.f.s. (solvent B). Data were collected using computer software (Delta Chromatography Data Systems, Digital Solutions Pty. Ltd., Queensland, Australia).

Standard curves were constructed from the ratios of the peak heights of LTB4:15-HETE and 5-HETE:15-HETE for the standard amounts of LTB4 and 5-HETE added. The amounts of 5-lipoxygenase products in the extracted biological
samples were estimated from the relevant standard curves. The assumption was made that the all-trans-isomers of LT\textsubscript{B4}, 20-OH LT\textsubscript{B4} and LT\textsubscript{B5} had identical detector responses to LT\textsubscript{B4} standard. Sensitivity of the assay was less than 1 ng (as determined at a signal-to-noise ratio of 2:1) and the coefficient of variation for intra-assay variability for 5-HETE was \(\approx 6\%\) and for LT\textsubscript{B4} was \(\approx 11\%\) [McColl et al., 1986].

\textbf{F. Mouse Paw Swelling Experiments}

Swiss mice were anaesthetised with a mixture of halothane, oxygen and nitrous oxide. The thickness of each hind paw was measured with a dial micrometer (Mitutoyo Corporation, Japan). The inflammatory effects of 1 mM NaHCO\textsubscript{3} in ethanol (EtOH) (1\% or 10\%), histamine alone (0.5 \(\mu\)g - 10 \(\mu\)g), histamine + PGE\textsubscript{2} (1 ng - 10 \(\mu\)g) and PGE\textsubscript{2} alone were assessed in preliminary experiments.

To assess the oedemogenic effects of PGE\textsubscript{3} the plantar region of the left hind paw was injected intradermally with 10 \(\mu\)L of 1 mM NaHCO\textsubscript{3}/1\% EtOH as a control using a glass microlitre syringe (Scientific Glass Engineering Pty. Ltd., Australia). The right hind paw was injected with PGE\textsubscript{2} or PGE\textsubscript{3} in 1 mM NaHCO\textsubscript{3}/1\%EtOH in doses ranging from 1 ng - 10 \(\mu\)g. The total volume injected was 10 \(\mu\)L. Each paw was measured for oedema formation 30 min after injection having first established that swelling was maximal at this time.

\textbf{G. Separation and Detection of PGE}

\textbf{2.2.12 Derivatizing reaction}

Panacyl bromide (PAB) was solubilized in acetonitrile:THF (4:1, v/v). Standard solutions of PGE\textsubscript{1}, PGE\textsubscript{2} and PGE\textsubscript{3} were prepared in distilled ethanol. PGF\textsubscript{2\alpha}, 6-ketoPGF\textsubscript{1\alpha}, TXB\textsubscript{2}, PGA\textsubscript{1}, PGB\textsubscript{2}, PGB\textsubscript{1} and 16,16-dimethylPGE\textsubscript{2} were also investigated during the development of the assay. A small amount of tritium-labelled PGE\textsubscript{2} was added to each reaction to enable localization of the derivatized prostaglandin.
following TLC, calculation of % conjugation and overall quantification of recoveries. Triethylamine 15% was prepared in acetonitrile:THF (4:1, v/v).

The final reaction mixture contained prostaglandin standard (10 ng - 500 ng) (or extracted media from biological samples reconstituted in acetonitrile:THF (4:1,v/v)), [³H]PGE₂ (1 Bq/mL final concentration), panacyl bromide (280 µg/mL final concentration) and triethylamine (15%, 3 µL/mL final concentration) in a final volume of 0.5 mL acetonitrile:THF (4:1, v/v). The reaction was initiated by the addition of the triethylamine and incubated at 37° C for 3 h.

2.2.13 Purification and recovery of sample

Two methods of purification of the conjugated product from the unreacted reagents were assessed.

Thin layer chromatography (TLC)

Reacted samples were evaporated under a stream of nitrogen and reconstituted in 30 µL acetonitrile. The conjugated prostaglandins were separated from the unreacted derivatizing reagents by TLC on 20 cm x 20 cm silica gel 60 plastic plates (Merck, Darmstadt, Germany). The plates were activated prior to use by heating at 100° C for 2 h. Each plate was divided into lanes 1.5 cm wide and each sample applied to the silica 2 cm from the base of the plate. Plates were developed in a solvent bath containing chloroform:methanol (100:7, v/v), dried and photographed under UV light. The location of the panacyl bromide/PGE conjugate was identified by dividing one lane into 1 cm strips and determining radioactivity. The area of interest was eluted overnight in acetonitrile:methanol (1:1, v/v) at room temperature. Silica particles were removed from the eluant by centrifugation at 850 x g for 10 min. The clear supernatant was evaporated to dryness in a Speed Vac Concentrator, reconstituted in 50-100 µL acetonitrile and transferred to HPLC vials for analysis.
Solid phase extraction column chromatography

Bond Elut disposable solid phase extraction columns were obtained from Analytichem International, California, USA. Columns were equilibrated with THF:H₂O (95:5), acetonitrile and dichloromethane (4 - 6 mL each, added sequentially). Reacted samples were applied to the column and eluted with sequential additions of varying combinations of dichloromethane, acetonitrile, methanol, THF and H₂O. Fractions were analysed for content of radioactive tracer.

2.2.14 Separation and detection by HPLC

HPLC was performed with the following components: a Model 501 pump, a 710B WISP autoinjector and a Nova-Pak C18 column (3.9 mm x 150 mm, 4 μm) with a precolumn filter (0.5 μm frit) (Millipore Waters, Milford, Massachusetts, USA). A Model LS-2 Filter Fluorimeter (Perkin-Elmer, Beaconsfield, UK) was used. The excitation wavelength was set at 375 nm and the emission wavelength at 470 nm. Isocratic elution was performed with acetonitrile:water:THF:acetic acid (54:42:4:0.1, v/v/v/v), pH 3.6 (not adjusted) at a flow rate of 1 mL/min. Injection volume ranged from 10 - 25 μL. During the development and verification of the assay 1 min fractions were collected for determination of radioactivity with a Retriever II fraction collector (Isco, Inc., Nebraska, USA). Data were collected using computer software (Delta Chromatography Data Systems, Digital Solutions Pty., Ltd., Queensland, Australia).

H. Methotrexate Experiments

2.2.15 In vitro studies

Peritoneal exudate cells and human neutrophils were incubated at room temperature with methotrexate (MTX) (10 ng/mL - 100 μg/mL) in normal saline for 30 min. Cells (10⁶/mL) were stimulated by the addition of calcium ionophore A23187
(0.5 μM, final concentration) in the presence or absence of EPA (5 μM) at 37°C for a further 5 min. The cell preparations were extracted and analysed for the production of LTB₄, the all-trans isomers of LTB₄ and 5-HETE by HPLC as described in 2.2 (Section E). Incubations were performed in quadruplicate and the production of metabolites by an individual was given by the mean values determined from the replicate assays. Data are expressed as the mean ± sd, ng/10⁶ cells/5 min.

2.2.16 In vivo administration of MTX - rat

Hooded Wistar rats (male) were used in all experiments designed to examine the effect of MTX on LT production by PEC ex vivo. The treatment regimens were as follows:

Subcutaneous

In two experiments of a similar design, 7 rats were divided into two groups. One group received a subcutaneous injection of MTX (0.5 mg/kg bodyweight) (treated, n=4) and the other group received an injection of normal saline (100 μL) (control, n=3). The injections were performed concurrently with (A) or 2 h prior to (B) i.p. injection with 10 mL 3% peptone in normal saline. PEC were harvested 4 h after the peptone injection and stimulated as described.

Intraperitoneal

Six rats were divided into two equal groups. The treatment group was injected i.p. with 3% peptone in 10 mL normal saline containing MTX (1 mg/kg bodyweight) and the control group was injected with 3% peptone in normal saline as described. PEC were harvested from the peritoneal cavity 4 h later. This experiment was repeated three times.
Rats were gavaged daily with MTX (0.5 mg/kg bodyweight) dispersed in Gum Tragacanth (0.05 mg MTX/mL 0.5% Gum Tragacanth) or with Gum Tragacanth (0.5%, 0.5 mL/100 g bodyweight) alone. The effect of oral administration of MTX on LT synthesis by PEC was assessed 24 h after a single dose and 24 h after the last dose in animals treated by gavage for 7 consecutive days. There were 4 rats in each treatment group.

2.2.17 *Ex vivo* studies - human

Blood was obtained from 9 subjects with rheumatoid arthritis. The effects of a single oral dose of MTX were assessed 12 h after administration. Blood samples were taken in the morning on two consecutive days with the MTX dose (typically 10 mg) being taken on the intervening evening. Neutrophils were prepared and stimulated with A23187 as described. LTB₄, the all-trans isomers of LTB₄, 20-OH LTB₄ and 5-HETE were resolved by HPLC and quantified by UV detection as described. Incubations were performed in quadruplicate.

I. Statistical Analyses

2.2.18 Chapter 3

The significance of the results was determined by t-test for analyses of weight gain data, paw swelling as determined by volume displacement, spleen weight and leukotriene production. Differences were considered significant at $p < 0.05$.

Analysis of variance followed by Newman-Keuls multiple comparison analysis [Hays, 1973] was used to identify differences in the measured variables in response to dietary treatments. Differences were considered significant at $p < 0.05$. 

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2.2.19 Chapter 4

The significance of the results was determined by Student's t-test. Since 5 comparisons were made, the family Type I error level (α) was set at 0.01 [Hays, 1973].

2.2.20 Chapter 7

The significance of the results was determined by a t-test for analyses of rat data from ex vivo studies. Differences were considered significant at p < 0.05.

The data from human ex vivo studies were analysed by paired t-test. Results are expressed as the mean ± sd, ng/10^6 cells/5 min.
CHAPTER 3

EFFECT OF INFLAMMATION ON EPA INCORPORATION

3.1 INTRODUCTION

Dietary supplements of fish oils have been shown to have beneficial effects on both the incidence and severity of certain inflammatory diseases in animal models. These effects appear to be mediated, at least in part, by the influence of fatty acids found in fish oils on the relative availability in cell membrane phospholipids of the 20 carbon fatty acids, AA and EPA. These two fatty acids are the immediate precursors of eicosanoid inflammatory mediators of differing biological activity. Although it may be reasonable to predict that the utilisation of the 20 carbon precursors for eicosanoid synthesis will increase during an inflammatory response, the effect of inflammation on the composition of membrane phospholipids of inflammatory cells in relation to these key fatty acids has not been reported.

The main experiment described in this chapter was performed to determine the influence of severe systemic inflammation with polyarthritis on the incorporation of n-3 fatty acids from the diet and associated depletion of n-6 fatty acids in membrane phospholipids of peritoneal exudate cells (PEC) of predominantly neutrophil morphology. Parameters of inflammation following induction of adjuvant disease were examined in parallel with fatty acid analyses. Weight loss (or failure to gain weight) is an expected side effect of severe systemic inflammation as seen with the adjuvant arthritis model. Food intake and rat weight were monitored in the experiments described in this chapter to ensure that the animals were in satisfactory health and that both types of diets were consumed. Joint scores and hind paw swelling by volume displacement were assessed regularly to monitor the progress of the disease and to determine that all inoculated animals were indeed suffering polyarthritis. Changes in appearance and weight of rat spleens are further indices of the presence of a systemic inflammation following injection with adjuvant.
LTB₄ is a potent inflammatory mediator which has been implicated in many human and animal inflammatory conditions. There have been reports of upregulated cyclooxygenase expression [Sano et al., 1992] and PGE₂ secretion by peritoneal macrophages [Johnson et al., 1986a] but the effect of induction of adjuvant arthritis on lipooxygenase activity has not been reported. Further studies were undertaken to extend initial findings with regard to enhanced leukotriene production and increased spleen weight following induction of adjuvant disease.

3.2 EXPERIMENTAL PROTOCOL

Preliminary experiments investigated the effect of inflammation on food intake from day 0 to day 9 after adjuvant injection. In this experiment 24 male Hooded Wistar rats were divided into 3 groups of 8. All rats were fed fat free rat chow supplemented with 5% sunflower oil from day 0 to day 5. From day 5 to day 9 all rats were fed chow supplemented with 5% fish oil. Group A were injected with normal saline and fed ad libitum for the duration of the experimental period. Group B were injected with adjuvant and also fed ad libitum. Group C were injected with normal saline and pair fed with Group B. (Experiment I).

```
<table>
<thead>
<tr>
<th>DAY</th>
<th>0</th>
<th>5</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% Sunflower Oil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% Fish Oil</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
```

↑

Group A - Saline-injected ← ad libitum feeding →
Group B - Adjuvant-injected ← ad libitum feeding →
Group C - Saline-injected ← pair-fed with Group B →
To examine the effect of inflammation on EPA incorporation, the study period following adjuvant injection was extended. In this experiment 48 male Hooded Wistar rats, 4 weeks old, were fed the sunflower oil diet *ad libitum* from 4 to 6 weeks of age. At 6 weeks of age the rats were divided into 12 groups with 4 rats in each group. On day 0, test animals (6 groups of 4 rats each) were injected intradermally with adjuvant. Control animals (6 groups of 4 rats each, comprising rats matched for weight with rats in the corresponding test group) were injected similarly at the base of the tail with normal saline. From day 0 to day 5 all rats were fed the sunflower diet, with the non-inflamed control rats being pair-fed with the corresponding test group. From day 6 until sacrifice all groups were fed the fish oil diet. Assessments of weight gain, joint scores, paw swelling by volume displacement, spleen weight and fatty acid content of PEC were made on each of days 5, 7, 9, 11, 13 and 15 for both test and control rats. (Experiment II).

A further study was undertaken to investigate leukotriene production by PEC and spleen weight immediately following induction of adjuvant disease. Twenty eight male Hooded Wistar rats, 6 weeks of age, were divided into 7 groups of 4 rats each. All rats were fed 5% sunflower oil supplemented rat chow *ad libitum* from day 0 until sacrifice on days 0, 1, 2, 3, 4, 6 and 8. (Experiment III).
3.3 RESULTS

3.3.1 Fatty acid analysis of diets

The dietary oils used in this experiment were sunflower oil and fish oil. Although the food was not examined for lipid oxidation, all diets had the same vitamin E content and were stored at 4° C before use. A portion of each diet was extracted with chloroform/methanol (2:1, v/v) and the fatty acid methyl esters were quantified as described. The dietary fatty acid profiles attained in each diet are shown in Table 3.1.

Table 3.1 Fatty acid content of diets.a

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Dietary Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5% Sunflower</td>
</tr>
<tr>
<td>18:1 n-9</td>
<td>26.6</td>
</tr>
<tr>
<td>18:2 n-6 (LA)</td>
<td>57.7</td>
</tr>
<tr>
<td>20:4 n-6 (AA)</td>
<td>n.d. b</td>
</tr>
<tr>
<td>18:3 n-3 (ALA)</td>
<td>1.6</td>
</tr>
<tr>
<td>20:5 n-3 (EPA)</td>
<td>n.d.</td>
</tr>
<tr>
<td>22:6 n-3 (DHA)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

a Expressed as a percentage of the total fatty acids (w/w).

b n.d. = not detectable; minimum detectable level, 0.1.
3.3.2 Weight gain

The initial study investigated food intake and weight gain from day 0 to day 9 following adjuvant injection (Experiment I). Although there were no clinical indications of inflammation as measured by paw swelling (Table 3.2) or joint scores during this period of investigation, there was a measurable effect of inflammation on food intake and weight gain. The total g/rat/day eaten by Group B (inflamed animals) was $18.5 \pm 0.6$ (mean $\pm$ sd, $n=64$), compared with $23.3 \pm 0.8$ g/rat/day eaten by Group A (control rats). The total weight gain was greater in control rats fed ad libitum (Table 3.2).

The amount of food eaten by inflamed rats fed ad libitum was similar in Experiment II (calculated from day 0 to day 15 after injection of adjuvant) to the amount eaten in Experiment I. Inflamed rats ate $17.1 \pm 2.6$ g/d/rat (mean $\pm$ sd, $n=60$) and increased in weight from a mean of 120 g to 184 g by day 15. Pair-fed control rats increased in weight by the same amount over the experimental period.

3.3.3 Joint scores for adjuvant-injected rats

The initial study covered the period 0 to 9 days after adjuvant injection (Experiment I) and joint scores were zero for all rats on each of the days assessed (0, 5 and 9).

Assessments were made on a greater number of days in Experiment II. Joint scores on days 5, 7 and 9 were zero for all rats. On day 11, 2/4 rats were visibly affected, with scores of 2 and 10 recorded. On day 13 and day 15 all four rats in each test group were affected with joint scores of 1, 6, 9, 11 and 1, 5, 8, 9 respectively. Joint scores for saline-inoculated control rats were zero throughout the experiment.

No joint scores were recorded for Experiment III.
TABLE 3.2 (EXPERIMENT I) Comparison of weight gain and volume displacement by rear paws of inflamed and non-inflamed rats on days 5 and 9 after injection.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Day</th>
<th>Group\textsuperscript{b}</th>
<th>Weight\textsuperscript{c}</th>
<th>Volume Displacement\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>A</td>
<td>202.8 ± 11.4</td>
<td>nd\textsuperscript{e}</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>198.8 ± 17.5</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>204.3 ± 10.4</td>
<td>nd</td>
</tr>
<tr>
<td>5</td>
<td>A</td>
<td>218.3 ± 19.1</td>
<td>1.25 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>198.8 ± 19.6</td>
<td>1.20 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>204.3 ± 13.6</td>
<td>1.17 ± 0.12</td>
</tr>
<tr>
<td>9</td>
<td>A</td>
<td>238.5 ± 3.7</td>
<td>1.32 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>219.5 ± 20.8</td>
<td>1.20 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>216.8 ± 8.9*</td>
<td>1.21 ± 0.10</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Asterisks indicate values that are significantly different from Group A (t-test, p<0.05).

\textsuperscript{b} Group A, control rats fed \textit{ad libitum}; Group B, adjuvant-injected rats fed \textit{ad libitum}; Group C, control rats pair-fed with Group B.

\textsuperscript{c} Weight measured in grams, mean ± sd, Day 0, n=8; Days 5 and 9, n=4.

\textsuperscript{d} Values represent grams of 1\% detergent displaced, mean ± sd, n=8.

\textsuperscript{e} Not done.
3.3.4 Paw swelling

Paw swelling in adjuvant-injected rats, as measured by volume displacement, did not show a significant increase between day 5 and day 9 in the initial study (Table 3.2).

However, paw size in adjuvant-injected rats increased from $0.94 \pm 0.02$ g (mean ± sem) on day 5 to $1.28 \pm 0.07$ on day 15 ($p<0.05$; Figure 3.1). This represents an average increase of 0.34 g in the inflamed rats compared with an increase of 0.13 g over the same period (10 days) in the control rats. The latter increase can be attributed to normal growth.

Paw swelling by volume displacement was not assessed in further studies.

3.3.5 Effect of inflammation on spleen weight

No assessment of spleen weight was made in Experiment I.

By day 5, when the first groups of rats were sacrificed in Experiment II, the spleens of adjuvant-injected rats were already 55% larger (by weight) than corresponding spleens from control rats ($p<0.05$; Figure 3.2). Spleen weight of inflamed rats continued to rise and by day 15 the mean weight in the inflamed rats was $1.83 \pm 0.27$ (g ± sem) compared to a mean of $0.39 \pm 0.01$ in control rats, a relative enlargement of 474%. There was a statistically significant difference in spleen weight between inflamed rats and control rats at all time points measured. Little variance in spleen weight was seen within groups indicating that all adjuvant-injected rats had responded to the adjuvant.

The effect of adjuvant-induced arthritis on spleen weight was also investigated in a separate study on days 0, 1, 2, 3, 4, 6 and 8 (Experiment III). A significant increase in spleen weight was detectable as early as three days after adjuvant injection ($p<0.05$; Figure 3.3).
Figure 3.1 (EXPERIMENT II) Comparison of volume displacement by rear paws of adjuvant-injected rats and control rats (injected with normal saline) on days 5, 7, 9, 11, 13 and 15 after injection. Each point represents the mean ± sem (n=4). Asterisks indicate values that are significantly different from the corresponding control group (t-test, p<0.05).

• Inflamed; ○ Control.
Figure 3.2  (EXPERIMENT II) Comparison of spleen weight of adjuvant-injected rats and control rats (injected with normal saline) on days 5, 7, 9, 11, 13 and 15 after injection. Each point represents the mean ± sem (n=4). Asterisks indicate values that are significantly different from the corresponding control group (t-test, * p<0.05; ** p<0.01).

- Inflamed;  ○ Control.
Figure 3.3  (EXPERIMENT III) Time course of changes in spleen weight of adjuvant-injected rats from day 0 to day 8 following injection. Each point represents the mean ± sem (n=4). Asterisks indicate values that are significantly different from the baseline value at Day 0 (t-test, * p < 0.05; ** p < 0.01).
3.3.6 Fatty acid analysis of peritoneal exudate cells

Data obtained from the fatty acid analyses of PEC harvested from rats in Groups A (control, fed ad libitum), B (adjuvant-injected, fed ad libitum) and C (control, pair-fed with Group B) in Experiment I are shown in Table 3.3.

In Experiment II, the fish oil enriched diet was introduced 5 days after injection with adjuvant or normal saline and this provides the initial point for comparing fatty acid profiles of phospholipids extracted from PEC of systemically inflamed and non-inflamed rats. In PEC membrane phospholipids of inflamed rats the AA level decreased from 26.3\% to 18.6\% in 10 days, accompanied by a rise in EPA from undetectable levels (limit of detection, 0.1\%) to 9.1\% of membrane phospholipids (Figure 3.4). In the non-inflamed rats, the AA level of the membrane phospholipids decreased from 27.5 to 19.9\% and the EPA increased from undetectable levels to 8.7\%. There was no significant difference in the proportion of AA and EPA in PEC phospholipids between inflamed and non-inflamed rats. Similarly, no significant difference was observed between inflamed and non-inflamed rats in the proportion of total n-6 fatty acids and total n-3 fatty acids (Tables 3.4, 3.5). However, the level of 18:2 n-6 was significantly higher (p<0.05) in PEC from inflamed rats than in PEC from the control rats at each time including the pre-fish oil treatment assessment (Figure 3.5). Minor differences in 20:3 n-6 levels were seen, and although not significant on all days, values tended to be higher in the inflamed rats. There was a consistent reduction in the content of longer chain length n-6 fatty acids measured in PEC from inflamed rats compared with that measured in PEC from control rats. The reduction in 22:4 n-6 was statistically significant on days 5, 7, 13 and 15 (p<0.05). 22:5 n-6 was significantly reduced in inflamed rats on all days (p<0.05). Analysis of proportions of n-3 precursors in PEC phospholipids showed a weak trend toward higher levels of 18:3 n-3 and 20:4 n-3 but not EPA, in inflamed compared with non-inflamed rats (Figure 3.6). However, the elevation of fatty acids in inflamed animals compared with control animals was not significant.
TABLE 3.3 (EXPERIMENT I) Fatty acid content of peritoneal exudate cells harvested from inflamed and non-inflamed rats.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Day</th>
<th>Group\textsuperscript{b}</th>
<th>18:2 n-6</th>
<th>20:4 n-6</th>
<th>20:5 n-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>A</td>
<td>10.1 ± 0.3</td>
<td>30.1 ± 0.3</td>
<td>n.d.\textsuperscript{c}</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>12.0 ± 0.3\textsuperscript{***}</td>
<td>27.7 ± 0.3\textsuperscript{***}</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>9.2 ± 0.4\textsuperscript{*}</td>
<td>30.7 ± 0.7</td>
<td>n.d.</td>
</tr>
<tr>
<td>9</td>
<td>A</td>
<td>7.1 ± 0.4</td>
<td>27.4 ± 0.6</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>9.2 ± 1.1\textsuperscript{*}</td>
<td>25.6 ± 1.4</td>
<td>3.0 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>6.3 ± 0.2\textsuperscript{*}</td>
<td>25.0 ± 0.4\textsuperscript{**}</td>
<td>3.9 ± 0.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Results expressed as % of total phospholipid fatty acids and represent the means ± sd of data from 4 rats in each group.

\textsuperscript{b} Group A, control rats fed \textit{ad libitum}; Group B, adjuvant-injected rats fed \textit{ad libitum}; Group C, control rats pair-fed with Group B.

\textsuperscript{c} n.d. = not detectable; minimum detectable level, 0.1.
Figure 3.4  (EXPERIMENT II) Arachidonic acid (AA, 20:4 n-6) and eicosapentaenoic acid (EPA, 20:5 n-3) levels in peritoneal exudate cell membrane phospholipids of adjuvant-injected rats and control rats (injected with normal saline) on days 5, 7, 9, 11, 13 and 15 after injection. Results expressed as % of total phospholipid fatty acids and represent the means ± sd of data from 4 rats in each group.

- Inflamed; ○ Control.
Table 3.4  (EXPERIMENT II) N-6 fatty acid content of peritoneal exudate cells.\(^a\)

<table>
<thead>
<tr>
<th>(DAY)</th>
<th>18:2</th>
<th>20:3</th>
<th>20:4</th>
<th>22:4</th>
<th>22:5</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflamed</td>
<td>13.3 ± 0.4(^b) 0.9 ± 0.0</td>
<td>26.3 ± 0.5(^b) 2.1 ± 0.1(^b)</td>
<td>1.2 ± 0.1(^b)</td>
<td>44.3 ± 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>9.4 ± 0.6  0.8 ± 0.1</td>
<td>27.5 ± 0.5  4.6 ± 0.9</td>
<td>2.2 ± 0.6</td>
<td>44.9 ± 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflamed</td>
<td>10.2 ± 0.9(^b) 0.8 ± 0.1</td>
<td>23.9 ± 1.6  1.3 ± 0.4(^b)</td>
<td>0.8 ± 0.1(^b)</td>
<td>37.3 ± 1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.2 ± 0.2  0.8 ± 0.1</td>
<td>25.8 ± 1.3  2.4 ± 0.6</td>
<td>1.1 ± 0.2</td>
<td>37.3 ± 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflamed</td>
<td>9.0 ± 0.4(^b) 0.8 ± 0.0(^b)</td>
<td>24.5 ± 0.8  0.9 ± 0.2</td>
<td>0.4 ± 0.1(^b)</td>
<td>35.7 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.3 ± 1.6  0.7 ± 0.1</td>
<td>24.2 ± 2.8  0.9 ± 0.2</td>
<td>0.8 ± 0.0</td>
<td>33.5 ± 2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflamed</td>
<td>10.5 ± 0.7(^b) 0.9 ± 0.0(^b)</td>
<td>21.8 ± 0.9  0.7 ± 0.1</td>
<td>0.3 ± 0.1(^b)</td>
<td>34.4 ± 1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.1 ± 1.6  0.7 ± 0.0</td>
<td>22.4 ± 1.7  1.1 ± 0.3</td>
<td>0.7 ± 0.1</td>
<td>32.1 ± 0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflamed</td>
<td>9.0 ± 2.2(^b) 0.8 ± 0.0(^b)</td>
<td>21.4 ± 2.0  0.5 ± 0.0(^b)</td>
<td>0.3 ± 0.0(^b)</td>
<td>32.1 ± 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.9 ± 0.3  0.7 ± 0.0</td>
<td>21.1 ± 1.0  1.0 ± 0.1</td>
<td>0.7 ± 0.3</td>
<td>29.3 ± 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflamed</td>
<td>8.5 ± 1.8(^b) 0.8 ± 0.1</td>
<td>18.6 ± 0.8(^b) 0.4 ± 0.0(^b)</td>
<td>0.3 ± 0.0(^b)</td>
<td>28.6 ± 1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.4 ± 0.2  0.7 ± 0.0</td>
<td>19.9 ± 0.3  0.7 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>27.3 ± 0.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Results expressed as % of total phospholipid fatty acids and represent the means ± sd of data from 4 rats in each group. Limit of detection, 0.1.
Inflamed; injected with adjuvant on day 0, Control; injected with saline on day 0.

\(^b\) p < 0.05, Newman-Keuls Analysis.
Table 3.5  (EXPERIMENT II) N-3 fatty acid content of peritoneal exudate cells.a

<table>
<thead>
<tr>
<th>(DAY)</th>
<th>18:3</th>
<th>20:4</th>
<th>20:5</th>
<th>22:5</th>
<th>22:6</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflamed</td>
<td>0.7 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.1 ± 0.0</td>
<td>n.d.&lt;sup&gt;c&lt;/sup&gt;</td>
<td>n.d.</td>
<td>0.4 ± 0.0</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>Control</td>
<td>0.5 ± 0.5</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.4 ± 0.1</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflamed</td>
<td>0.7 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>n.d.</td>
<td>2.1 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.9 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.5 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>Control</td>
<td>0.5 ± 0.0</td>
<td>n.d.</td>
<td>2.7 ± 0.4</td>
<td>1.5 ± 0.4</td>
<td>1.3 ± 0.1</td>
<td>6.0 ± 0.4</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflamed</td>
<td>0.8 ± 0.1</td>
<td>0.2 ± 0.0</td>
<td>3.7 ± 0.5</td>
<td>1.5 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.5 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.7 ± 0.6</td>
</tr>
<tr>
<td>Control</td>
<td>0.7 ± 0.1</td>
<td>n.d.</td>
<td>3.8 ± 0.5</td>
<td>0.9 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>6.3 ± 0.6</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflamed</td>
<td>0.7 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>4.0 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.6 ± 0.2</td>
<td>2.1 ± 0.1</td>
<td>8.3 ± 0.9</td>
</tr>
<tr>
<td>Control</td>
<td>0.7 ± 0.2</td>
<td>0.3 ± 0.0</td>
<td>5.9 ± 0.2</td>
<td>2.7 ± 0.7</td>
<td>2.0 ± 0.3</td>
<td>11.3 ± 1.0</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflamed</td>
<td>0.9 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.4 ± 0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.8 ± 0.7</td>
<td>2.3 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.3 ± 0.4</td>
<td>12.6 ± 0.4</td>
</tr>
<tr>
<td>Control</td>
<td>0.7 ± 0.1</td>
<td>0.3 ± 0.0</td>
<td>7.0 ± 0.6</td>
<td>3.5 ± 0.4</td>
<td>2.3 ± 0.2</td>
<td>13.8 ± 0.9</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflamed</td>
<td>0.8 ± 0.1</td>
<td>0.5 ± 0.0</td>
<td>9.1 ± 1.2</td>
<td>2.6 ± 0.3</td>
<td>2.7 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.7 ± 1.3</td>
</tr>
<tr>
<td>Control</td>
<td>0.7 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>8.7 ± 0.5</td>
<td>3.3 ± 0.5</td>
<td>2.2 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.2 ± 0.2</td>
</tr>
</tbody>
</table>

a Results expressed as % of total phospholipid fatty acids and represent the means ± sd of data from 4 rats in each group.
Inflamed; injected with adjuvant on day 0, Control; injected with saline on day 0.

b p < 0.05, Newman-Keuls Analysis.

c n.d. = not detectable; minimum detectable level, 0.1.
Figure 3.5  (EXPERIMENT II) Linoleic acid (LA, 18:2 n-6), dihomo-γ-linolenic acid (DGLA, 20:3 n-6) and arachidonic acid (AA, 20:4 n-6) levels in peritoneal exudate cell membrane phospholipids of adjuvant-injected rats and control rats (injected with normal saline) on days 5, 7, 9, 11, 13 and 15 after injection. Results expressed as % of total phospholipid fatty acids and represent the means ± sd of data from four rats in each group. Asterisks indicate values that are significantly different from the corresponding control group (Newman-Keuls analysis, p<0.05).

- Inflamed;  ○  Control.
It can be observed that the levels of DGLA, 20:3 n-6, AA, 20:4 n-6, and LA, 18:2 n-6, decrease over time (days). The graph shows a significant decrease in the percentage of LA, 18:2 n-6, with a peak at around 10 days, followed by a decrease. In contrast, the levels of DGLA, 20:3 n-6, remain relatively stable with a slight increase at 15 days. AA, 20:4 n-6, shows a gradual decrease throughout the time period.

Legend:
- **DGLA, 20:3 n-6**
- **AA, 20:4 n-6**
- **LA, 18:2 n-6**

*Significant change compared to baseline.*
Figure 3.6 (EXPERIMENT II) α-Linolenic acid (ALA, 18:3 n-3), eicosatetraenoic acid (ETA, 20:4 n-3) and eicosapentaenoic acid (EPA 20:5 n-3) levels in peritoneal exudate cell membrane phospholipids of adjuvant-injected rats and control rats (injected with normal saline) on days 5, 7, 9, 11, 13 and 15 after injection. Results expressed as % of total phospholipid fatty acids and represent the means ± sd of data from 4 rats in each group. Asterisks indicate values that are significantly different from the corresponding control group (Newman-Keuls analysis, p < 0.05).

- Inflamed;  ○ Control.
EPA, 20:4 n-3
ALA, 18:3 n-3
ETA, 20:4 n-3

Fatty Acids (%)

Time (days)
3.3.7 Leukotriene production by peritoneal exudate cells

Peritoneal exudate cell numbers were higher from inflamed rats compared with control rats at each day of assessment (Table 3.6).

Leukotriene production by PEC, stimulated by A23187 (0.5 μM) ex vivo, was measured on day 5 and day 9 only in the initial experiment. The results from this experiment are shown in Table 3.7. Both LTB₄ and 5-HETE production by PEC harvested from inflamed rats were elevated compared with LTB₄ and 5-HETE produced by both pair-fed and ad libitum-fed control rats on both days assessed (day 5 and 9). These preliminary results were confirmed in Experiment III. Leukotrienes and 5-HETE were measured on days 0, 1, 2, 3, 4, 6 and 8 following stimulation of PEC from inflamed sunflower oil-fed rats. All lipoxygenase products were elevated by day 1 and continued to rise until day 3 (Figure 3.7). In this experiment the lipoxygenase products had fallen to baseline levels by day 8.
TABLE 3.6 Total peritoneal exudate cells harvested from inflamed and non-inflamed rats.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Day</th>
<th>PEC Number\textsuperscript{b}</th>
<th>Inflamed (Group B)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>75.8 ± 4.3 (Group C)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>92.5 ± 11.1 (Group A)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>40.5 ± 4.5 (Group C)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>81.8 ± 14.3 (Group A)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>183.8 ± 18.8</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>18.8</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>235.0 ± 15.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>15.4</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>92.5 ± 72.1</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>338.3 ± 42.5</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>518.0 ± 140.4</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>240.0 ± 40.9</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>297.8 ± 36.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>117.8 ± 24.3</td>
<td></td>
</tr>
<tr>
<td>62.0 ± 13.4</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>12.8 ± 1.3</td>
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<td></td>
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<tr>
<td>22.8 ± 3.2</td>
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<tr>
<td>80.3 ± 17.1</td>
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<tr>
<td>168.0 ± 26.7</td>
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<td></td>
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</tr>
<tr>
<td>281.0 ± 32.6</td>
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<tr>
<td>328.8 ± 30.9</td>
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<td></td>
<td></td>
<td>62.0 ± 13.4</td>
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<tr>
<td>0</td>
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<td>12.8 ± 1.3</td>
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</tr>
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<td></td>
<td>22.8 ± 3.2</td>
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<tr>
<td>2</td>
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<td>80.3 ± 17.1</td>
<td></td>
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<tr>
<td>3</td>
<td></td>
<td>168.0 ± 26.7</td>
<td></td>
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<tr>
<td>4</td>
<td></td>
<td>281.0 ± 32.6</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>328.8 ± 30.9</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Group A, control rats fed \textit{ad libitum}; Group B, adjuvant-injected rats fed \textit{ad libitum}; Group C, control rats pair-fed with Group B.

\textsuperscript{b} Cell number x 10^6, expressed as mean ± sem, n=4.
### TABLE 3.7. (EXPERIMENT I) Leukotriene and 5-hydroxy fatty acid production by PEC harvested from inflamed and non-inflamed rats.\(^a\)

<table>
<thead>
<tr>
<th>Day</th>
<th>Group(^b)</th>
<th>LTB(_4) (ng/10(^6) cells/5 min.)</th>
<th>5-HETE (ng/10(^6) cells/5 min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>A</td>
<td>16.5 ± 1.6</td>
<td>27.6 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>37.0 ± 3.0*</td>
<td>60.0 ± 2.6*</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>15.0 ± 1.0</td>
<td>29.0 ± 1.7</td>
</tr>
<tr>
<td>9</td>
<td>A</td>
<td>10.3 ± 3.1</td>
<td>17.3 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>24.4 ± 4.1</td>
<td>42.5 ± 7.2</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>8.2 ± 1.2</td>
<td>19.0 ± 2.4</td>
</tr>
</tbody>
</table>

\(^a\) Results expressed as mean ± sem, \(n=4\). Asterisks indicate values that are significantly different from Group A (t-test, \(p<0.05\)).

\(^b\) Group A, control rats fed *ad libitum*; Group B, adjuvant-injected rats fed *ad libitum*; Group C, control rats pair-fed with Group B.
Figure 3.7  (EXPERIMENT III) Effect of adjuvant disease on the synthesis of total 5-lipoxygenase products by rat peritoneal exudate cells. Rats were injected with adjuvant on day 0. Cells were prepared and stimulated with A23187 as described in Chapter 2, C & E. Values represent the mean ± sem of quadruplicate incubations of cells from each of 4 rats per day.

- LTB4;  ○ the all-trans isomers of LTB4;  ■ 5-HETE.
LTB$_4$ and the all-trans isomers (ng/10$^6$ cells/5 min)
3.4 DISCUSSION

It has been shown that the ratios of EPA and AA in rat neutrophil membrane phospholipids correspond closely with the ratios of LTB₅ and LTB₄ produced by stimulated leukocytes [Cleland et al., 1990b]. Since LTB₅ is a weak neutrophil chemotaxin relative to LTB₄ these findings have clear relevance to the use of dietary fish oils to modify inflammatory disease. However, this assumes that the incorporation of EPA from fish oil into leukocyte membranes (and the reciprocal effect on AA content) is not substantially altered in the presence of inflammation. Adjuvant disease causes a severe polyarthritis as well as systemic signs of inflammation including splenic enlargement and reduced weight gain. This model provides an appropriate setting in which to test the effects of inflammation on the response of leukocyte fatty acid composition to dietary fish oil treatment.

Paw swelling as measured by volume displacement corresponded with the joint scores of disease activity. Although there was a trend for paw size to be increased in inflamed rats from day 7, this was not statistically significant until day 13. There was some increase in volume displacement by paws from control rats over the 15 day period which can be attributed to normal growth of the animal. Although disease activity as assessed by joint scores was not evident until day 11 of the study described as Experiment II, there is little doubt that all inoculated rats were affected by the adjuvant injection. As the control rats were pair-fed with the inflamed rats there was no opportunity in this experiment to demonstrate the reduced weight gain characteristic of adjuvant-induced arthritis models. However, it was noted that throughout the experiment the control rats ate all the food that was provided each night. In the preliminary experiment of a similar design, the average daily intake of a group of adjuvant-injected rats was 18.9 ± 0.6 g/d/rat compared with 23.34 ± 0.8 g/d eaten by a matched group of control rats fed ad libitum. As expected, the weight gained by control rats over the 10 day experimental period was greater than that gained by the adjuvant-injected rats over the same period. The findings of similar reduced weight gain in the arthritic and pair-fed rats suggest that the weight loss in arthritic rats is due
to reduced food intake and not due to increased overall metabolic rate.

A further sign of the systemic nature of the disease is the increase in spleen weight. By day 5 after adjuvant injection spleen weight had already increased in the test group compared with spleen weight in the control group. The weight of spleens from inflamed rats continued to rise and was associated with gross enlargement and adhesion to the abdominal wall of the animal. It has been demonstrated that adherent phagocytic cells isolated from the spleens of animals taken at different times after Freund's complete adjuvant (CFA) administration to Lewis rats, exhibit an enhanced ability to secrete both PGE$_2$ and IL-1 in response to LPS [Johnson et al., 1986a]. The increased production of these two inflammatory mediators was apparent from as early as day 3 after CFA injection. The rapid increase in spleen weight following adjuvant injection was also observed in a repeat experiment designed to investigate changes in spleen weight, PEC number and leukotriene production in the first 8 days following induction of adjuvant arthritis (Experiment III).

There were 2 - 3 times more elicited peritoneal exudate cells recovered from arthritic rats compared with the number obtained from normal control rats at times assessed after adjuvant administration. A similar increase in the number of resident peritoneal exudate cells obtained by lavage at day 21 following the injection of CFA has been observed [Bak et al., 1991]. Increased numbers of adherent macrophages derived from resident peritoneal exudates recovered from arthritic rats have also been demonstrated [Johnson et al., 1986a; 1986b]. However, the increase closely paralleled the progression and severity of the arthritic lesion as indicated by leg oedema, not becoming apparent until about day 10 after CFA administration [Johnson et al., 1986a].

The time course of A23187-stimulated leukotriene production indicates that the elicited neutrophil population is already primed, with regard to lipoxygenase activity, 24 hours after the adjuvant injection. All lipoxygenase metabolites measured (LTB$_4$, the all-trans isomers of LTB$_4$ and 5-HETE) increase up to and including day 3, then decline to reach pre-adjuvant levels by day 8. However, results obtained from the initial experiment (Experiment I) indicate that the production of LTB$_4$ and 5-HETE is
still elevated in comparison with that produced by control animals at this time. Data obtained in this experiment indicate that the changes in LTB₄ and 5-HETE are not related to reduced food intake as production by pair-fed non-inflamed rats does not differ from production by control rats fed ad libitum. Presumably, the elevation of lipoxygenase metabolites in inflamed animals is related to the inflammatory disease. Bak et al., (1991), measured LTB₄ production by calcium ionophore (0.3 - 3.0 μM) stimulated resident peritoneal macrophages harvested from rats 21 days after injection of CFA only and were unable to discern any increase compared with control animals. They were also unable to find any difference in secreted PLA₂ activity in the supernatants of stimulated macrophages from adjuvant and normal rats. It is possible that any increased levels of enzyme activity associated with disease progression of adjuvant arthritis might occur earlier during the evolution of the disease. In addition, enhanced secretion of PGE₂ and IL-1 by resident peritoneal macrophages, splenic macrophages and blood monocytes obtained by cardiac puncture are associated with the development of adjuvant-induced arthritis [Johnson et al., 1986a, 1986b; Schenkelaars et al., 1990]. A significant increase in PGE₂ secretion by resident peritoneal macrophages in response to LPS (5 μg/mL) occurred at day 10 after CFA injection, preceding any significant increase in foot swelling [Johnson et al., 1986a]. There was also a significant increase in the number of Ia-positive cells on the synovial surface by day 10 after CFA injection and this continued to increase to day 13 when the increase was associated with synovial membrane thickening and the appearance of infiltrating inflammatory cells. The results presented in this chapter and the findings reported by other workers suggest that biochemical and systemic evidence of inflammation precedes the development of arthritis in this model.

Linoleic acid can be elongated and desaturated to AA, the immediate precursor of the inflammatory mediators LTB₄ and PGE₂. Haataja et al., (1982), found there was no significant difference in serum concentrations of LA or AA between rheumatoid arthritis (RA) patients and control subjects. However, in the present study LA levels were significantly higher in PEC membranes from adjuvant-injected rats. Minor differences in DGLA, the immediate precursor of AA, were seen, and although not
significant at times, values tended to be higher in the inflamed rats. There was no change in DGLA levels with fish oil feeding in either the arthritic or control groups over the period studied. This contrasts with a study of RA patients receiving 18 g MaxEPA/day in whom there was a general depression of DGLA levels in plasma lipid fractions after one month of fish oil feeding [Cleland et al., 1990c]. There has also been evidence of a rise in DGLA levels in plasma and liver phospholipid fractions of normal rats fed increasing amounts of fish oil in combination with decreasing levels of evening primrose oil [Nassar et al., 1986]. There was, however, a reduction in levels of the longer chain fatty acids, 22:4 n-6 and 22:5 n-6. AA is utilized for the production of eicosanoids during inflammation, however, levels of AA are remarkably well preserved in animal species, including man, regardless of inflammatory state or fatty acid deficiencies in the diet. The general depression of long chain n-6 fatty acids seen in the experiment described here, may reflect the utilization of the fatty acids for the maintenance of AA levels in cell membrane phospholipids stores.

In conclusion, the present findings indicate that fatty acid composition of neutrophils in relation to 20 carbon fatty acids is similar in both normal pair-fed and severely inflamed rats during intervention with a fish oil rich diet over a period of 10 days. Steady state was not reached within the study periods but the results suggest that systemic inflammation has little effect on the rate of EPA incorporation or AA depletion in these cells. Therefore, dietary fish oil studies with normal rats can provide suitable data on leukocyte fatty acid profiles for planning dietary studies with inflamed rats which, for ethical reasons, should be limited where possible.
CHAPTER 4

BIOLOGICAL ACTIVITY OF PGE₃ WITH REGARD TO OEDEMA FORMATION IN MICE

4.1 INTRODUCTION

The eicosanoids are a class of lipid mediators in which particular members have different biological activities. In some cases, the n-6 and n-3 counterparts of a certain type of eicosanoid can have substantially different potencies. Because the balance of activities of the particular eicosanoids influences the intensity of processes such as inflammation, it follows that the proportion of eicosanoid production derived from n-6 and n-3 fatty acids will influence inflammation and other relevant homeostatic processes. While the relative production of the n-6 and n-3 leukotrienes in response to different diets [Cleland et al., 1990b; James et al., 1991a] and their biological activities [Lee et al., 1988] have been well characterized and the vascular effects of the 3-series prostacyclin and thromboxane and their activities have been determined, little is known about the activity of the EPA-derived prostaglandin E₃ (PGE₃) with regard to inflammation. PGE₁ and PGE₂ are both derived from n-6 fatty acids and are potent vasodilators which contribute to the oedema and pain characteristic of the inflammatory response [Williams and Morley, 1973; Moncada et al., 1973]. However, PGE₁, PGE₂ and PGI₂ have separate receptors on cells [Dutta-Roy and Sinha, 1985] and although the biological activities of corresponding members of the 1-series and 2-series prostaglandins are in many cases quite similar, they differ significantly in other instances. For example, PGE₁ inhibits aggregation of human platelets in vitro whereas PGE₂ does not, PGE₁ vasodilates bovine coronary and human chorionic plate arteries and PGE₂ constricts these vessels and PGE₁ infusion reduces levels of circulating immune complexes in patients with autoimmune diseases [Zurier, 1990]. Dietary γ-linolenic acid (GLA) can be converted to dihomo-γ-linolenic acid (DGLA) which is the precursor of PGE₁. A GLA-enriched diet suppressed the cellular phase of a
monosodium urate crystal-induced acute inflammation in rats, whereas an EPA-enriched diet suppressed the fluid phase but not the cellular phase of inflammation [Tate et al., 1988]. Since some proposed therapeutic and preventive dietary interventions have sought to maximize the cellular EPA/AA ratio and thus decrease production of n-6 eicosanoids and increase production of n-3 eicosanoids, it was considered important to ascertain the biological activity of PGE3.

Female Swiss mice were used throughout the paw swelling studies. Preliminary experiments established (a) conditions for taking reproducible paw swelling measurements, (b) the effects of the vehicle required for complete dissolution of the prostaglandins to be tested, and (c) the dose range to be investigated.

4.2 RESULTS

4.2.1 Preliminary Experiments

Histamine in 1 mM Na₂CO₃ / 10% EtOH

The effects of histamine alone were assessed in 15 mice in order to establish the assay conditions. A stock solution of histamine was diluted in 1 mM Na₂CO₃/10% ethanol (EtOH). In each mouse, the left hind paw was injected intradermally with 10 μL 1 mM Na₂CO₃/10% EtOH and the right hind paw with 10 μL histamine (0.5, 1.0, 2.5, 5.0 or 10.0 μg total) in this vehicle. Measurements of each hind paw were taken 15 and 30 min after injection. Following injection of vehicle alone paw size was 2.17 ± 0.02 mm (mean ± sem, n=15) at both 15 min and 30 min after injection. Histamine had further oedemogenic activity throughout the time course with maximum activity at 5.0 μg at each time of assessment (Figure 4.1). Measurements were taken at 30 min after injection for all further experiments.
Figure 4.1  Effect of histamine on oedema formation in the rear paws of mice 15 minutes and 30 minutes after injection. Paw swelling in mm represents the difference between the histamine-injected paw and the vehicle-injected control in each mouse. Each point represents the mean ± sem of measurements in 3 animals.

- 30 minutes after injection;  ○  15 minutes after injection.
Histamine + PGE2 in 1 mM Na₂CO₃ / 10% EtOH

A single dose of histamine (2.5 μg) was mixed with doses of PGE2 ranging from 0.001 - 10.0 μg in a final volume of 10 μL. All hind paws were measured prior to injection, then 30 min after injection with either the vehicle (1 mM Na₂CO₃/10% EtOH) or the histamine + PGE2 mixture. The mean thickness of all hind paws before injection of vehicle or histamine + PGE2 was 1.75 ± 0.02 mm (mean ± sem, n=30). Thirty min after injection of vehicle alone paw size had increased to 2.06 ± 0.03 mm (mean ± sem, n=15). Paw swelling induced by the histamine + PGE2 mixtures is shown in Figure 4.2.

Prostaglandin E₂ in 1 mM Na₂CO₃ / 10% EtOH

A stock solution (2 mg/mL) of PGE₂ was diluted in 1 mM Na₂CO₃/10% EtOH to final doses of 0.001, 0.01, 0.1, 1.0 and 10.0 μg/10 μL. In each mouse, the right hind paw was injected with 10 μL of vehicle alone and the left hind paw with 10 μL of a single dose of PGE₂. Each dose was tested in three mice. The results obtained from measurements taken 30 min after injection are shown in Figure 4.2. Paw size 30 min after injection with vehicle alone was 2.01 ± 0.02 mm (mean ± sem, n=15).

A solution of 1% EtOH in 1 mM Na₂CO₃ was found to be adequate for complete dissolution of the prostaglandins of interest and gave consistent results with regard to paw swelling. This solution was chosen as the vehicle for administration of the comparative PG doses.

Histamine + PGE₂ and PGE₂ alone in 1 mM Na₂CO₃ / 1% EtOH

Stock solutions of histamine and PGE₂ were made up in 1 mM Na₂CO₃/1% EtOH. Thirty Swiss mice were used to investigate the inflammatory properties of vehicle alone, PGE₂ mixed with 1 μg histamine and PGE₂ alone (dose range 0.001 - 10.0 μg). All hind paws were measured prior to injection and 30 min after injection.
Figure 4.2 Effect of PGE$_2$ and PGE$_2$ + histamine (2.5 µg) on oedema formation in the rear paws of mice. Paw swelling in mm represents the difference between the histamine/PG-injected paw and the vehicle-injected control in each mouse. Each point represents the mean ± sem of measurements taken 30 minutes after injection in 3 animals.

- Histamine (2.5 µg) + PGE$_2$;  O PGE$_2$ alone.
The mean thickness of all hind paws before injection of vehicle alone or prostaglandins + histamine was 1.83 ± 0.01 mm (mean ± sem, n=60). Thirty min after injection of vehicle alone paw size had increased to 2.2 ± 0.02 mm (mean ± sem, n=30). The inflammatory activity of PGE₂ alone and PGE₂ mixed with 1 μg histamine is illustrated in Figure 4.3.

4.2.2 PGE₃ v PGE₂

Thirty female Swiss mice were divided into two groups. The left hind paw was injected intradermally with 10 μL, 1 mM Na₂CO₃/1% EtOH as a control. The right hind paw was injected with PGE₂ or PGE₃ in this vehicle in doses ranging from 1 ng - 10 μg. The total volume injected was 10 μL. All paws were measured for oedema formation prior to injection and 30 min after injection. The mean thickness of all hind paws before injection of control solution or prostaglandins was 1.82 ± 0.01 mm (mean ± sem, n=60). Thirty min after injection of vehicle alone, paw size had increased to 2.1 ± 0.02 mm (mean ± sem, n=30).

Both PGE₂ and PGE₃ had oedemogenic activity with maximum activity at 1 μg for each prostaglandin (Figure 4.4). At each dose, PGE₃ had less activity than PGE₂ although the differences were not statistically significant at the 0.01 level. The area under the curve for PGE₃ was 67% of that for PGE₂.
Figure 4.3  Effect of PGE$_2$ and PGE$_2$ + histamine (1 $\mu$g) on oedema formation in the rear paws of mice. Paw swelling in mm represents the difference between the PG-injected paw and the vehicle-injected control in each mouse. Each point represents the mean ± sem of measurements taken 30 minutes after injection in 3 animals.

- Histamine (1.0 $\mu$g) + PGE$_2$;  ○ PGE$_2$ alone.
Figure 4.4  Effect of PGE2 and PGE3 on oedema formation in the rear paws of mice. Paw swelling in mm represents the difference between the PG-injected paw and the vehicle-injected control in each mouse. Each point represents the mean ± sem of measurements taken 30 minutes after injection in 3 animals.

○ PGE2;  ● PGE3.
4.3 DISCUSSION

Preliminary experiments compared the inflammatory effects of 10% EtOH with 1% EtOH and the additive effects of histamine and PGE\(_2\) with PGE\(_2\) alone. The two-component hypothesis of acute inflammation postulates that the acute inflammatory response depends on both increased local blood flow and increased microvascular permeability. Histamine acts directly on the vascular endothelium to cause increased vascular permeability and plasma exudation. Prostaglandins of the E series have been shown to cause increased vascular permeability in the skin of rat [Moncada et al., 1973; Crunkhorn and Willis, 1971] and man [Crunkhorn and Willis, 1971; Basran et al., 1982] but have little effect on vascular permeability in guinea pig [Horton, 1963; Williams and Morley, 1973] and rabbit [Williams, 1976]. Prostaglandins appear to exert their inflammatory effects predominantly by increasing vasodilatation rather than increasing vascular permeability. There was a dose-dependent increase in paw swelling in response to increasing amounts of histamine. Response potentiation between prostaglandins and histamine has been described by many workers [Williams and Morley, 1973; Moncada et al., 1973; Williams and Peck, 1977] and it was anticipated that this combination would be required for the comparison between PGE\(_2\) and PGE\(_3\) with regard to oedemogenic activity in the paws of mice. In the preliminary experiments described in this chapter, an increased paw swelling response was observed when the inflammatory mediators PGE\(_2\) and histamine were injected together compared with that measured following injection of PGE\(_2\) alone. However, PGE\(_2\) alone caused measurable dose-related swelling without severe oedema and it was anticipated that this degree of response may be more appropriate for the comparison of the biological activities of two substances. The possible potentiating effects of PGE\(_3\) on histamine or LTB\(_4\) induced oedema formation were planned, but were deferred pending examination of the relevance of PGE\(_3\) effects on inflammation. This was assessed by determining the capacity of macrophages to produce PGE\(_3\) and is discussed further in Chapter 6 of this thesis.
In summary, this study indicated that PGE₃ has substantial oedemogenic activity when injected into the hind paw of mice. Dietary supplements of fish oils rich in EPA influence the relative availability in cell membrane phospholipids of the 20 carbon fatty acids. PGE₃ has been detected using selective ion monitoring in homogenates of renal medullary tissue of rats fed fish oil diets [Ferretti et al., 1981] and metabolites of PGE₃ have been detected by gas chromatography-mass spectrometry in urine of a woman consuming a large dietary fish oil supplement [Ferretti et al., 1988], suggesting that EPA can be metabolized to PGE₃. Dietary fish oil supplements reduce macrophage PGE production in rats as assessed by radioimmunoassay [Terano et al., 1986] but the relative amounts of PGE₂ and PGE₃ could not be determined. Having ascertained that PGE₃ does have inflammatory activity, the significance of PGE₃ in mediating inflammatory events will depend on the amount of PGE₃ produced.
CHAPTER 5

ASSAY DEVELOPMENT FOR THE SEPARATE DETECTION OF PGE3

5.1 INTRODUCTION

The relative production of the n-6 leukotriene, LTB4 and its n-3 counterpart, LTB5, in response to different diets and the relative biological activities of these inflammatory mediators have been well characterized. The triene structure of the leukotrienes creates a strong UV absorbance which allows spectrophotometric detection after HPLC and thereby allows for separation and direct quantitation. By contrast, little is known about the relative production of the n-6 prostaglandin, PGE2, and its n-3 counterpart, PGE3, in response to different proportions of dietary n-3 and n-6 fatty acids in the diet and/or cell membrane phospholipids. Studies of the effect of EPA on the production of PGE3 and the analoguous n-6 product, PGE2, and their biology have lagged behind similar studies with leukotrienes probably due to the difficulty in the separate quantitation of PGE3. Studies to date have generally relied on the use of radioimmunoassays using antisera raised against PGE2 which involve substantial cross-reactivity between PGE2 and PGE3. In vitro studies indicate that EPA is a poor substrate for cyclooxygenase compared with AA but appears to have a high binding affinity and thereby inhibits metabolism of AA [Culp et al., 1979]. However, the conversion of EPA to the antiaggregatory prostaglandin I3 (prostacyclin) and to the relatively inactive thromboxane (TXA3) has been clearly demonstrated in humans [Knapp and FitzGerald, 1989; Fischer and Weber, 1984] and animals [Abeywardena et al., 1989; Knapp and Salem, 1989]. Considerable interest has been shown in the vascular effects of the 3-series prostacyclin and thromboxane and these activities have been determined. In two studies, PGE3 (or its metabolites) has been detected in human urine [Ferretti et al., 1988] and rat renal medullary tissue [Ferretti et al., 1981] by gas chromatography-mass spectrophotometry (GC-MS) with selective ion monitoring. The
following chapter describes the work undertaken to develop an alternative method for the separate detection and quantitation of PGE₃, which would be within the scope of most laboratories to perform. The separation and detection of PGE₁ and PGE₂ by HPLC following derivatization with a fluorescent marker have been described and it was anticipated that this technique could resolve PGE₃ also.

5.2 RESULTS

5.2.1 Derivatizing reaction

p-(9-anthroyloxy)phenacyl bromide (panacyl bromide; PAB) undergoes a rapid reaction with the carboxyl group of prostaglandins in the presence of triethylamine in acetonitrile:tetrahydrofuran (THF) (4:1, v/v) (Figure 5.1). The resultant eicosanoid ester is fluorescent at 365 and 380 nm excitation and emits at 470 - 480 nm.

Initial experiments were based on a method described by Watkins and Peterson, (1982). A further review of the available literature indicated that the molar ratio of PAB:PGE required ranged from 100:1 to 1000:1. The final reaction method described in Chapter 2 (2.2.12) achieves a molar ratio of approximately 200:1 (PAB:PGE). Synthetic PGE₂ was used throughout the assay development experiments unless indicated otherwise.

5.2.2 Purification and recovery of sample

A partial purification before HPLC was required to reduce the amount of free, unreacted panacyl bromide which may interfere with the peaks of interest on HPLC. Two methods of purification prior to HPLC were evaluated - thin layer chromatography (normal phase) and column chromatography (reverse phase).
**Figure 5.1** The reaction sequence leading to the formation of the prostaglandin ester.
Thin layer chromatography

The use of $[^3]$H)PGE$_2$ enabled determination of the chromatographic properties of PGE$_2$ alone and following derivatization of PGE$_2$ with the fluorescent marker, panacyl bromide. Following development of the plate in chloroform:methanol (100:7, v/v), it was air dried and photographed under UV light (Figure 5.2). The plate was highly fluorescent at approximately 0 - 3 cm, 5 - 6 cm and 12 - 15 cm from the origin. The location of these areas of intense fluorescence varied slightly with varying plate developing conditions. The area containing the radioactively labelled prostaglandin was determined by cutting the plastic backed sheet into 1 cm pieces and counting in a liquid scintillation counter. The putative PAB:PGE$_2$ conjugate was well separated from unreacted PGE$_2$ by TLC; Rf values were 0.56 and 0.17 respectively (Figure 5.3). This area containing the PAB:PGE$_2$ conjugate did not correspond with a distinct band of fluorescence. In general, the area chosen for elution and further analysis lay between the highly fluorescent spots at 6 cm and 12 cm, which helped eliminate excess unreacted PAB in the HPLC step of the assay. The proportion of $[^3]$H)PGE$_2$ conjugated was 60-70% of the total added to the initial reaction mixture.

An alternative method of determining the area containing the radioactively labelled conjugate was by thin layer plate scanning (Figure 5.4). This method had the advantage of locating the area of interest without destruction of the plate thus eliminating the need for an extra control reaction with each experiment. However, I was unable to proceed with this method due to difficulty of access to the appropriate equipment.

Several methods of elution were assessed to determine the most efficient recovery of radioactive counts from the silica plate. Conjugated and free $[^3]$H)PGE$_2$ were applied in equal quantities to 5 lanes of a TLC plate and developed. The total counts loaded (conjugated and free $[^3]$H)PGE$_2$) onto each lane was approximately 13000 cpm. Lane 1 was divided into 1 cm squares and each one counted individually to determine the location of the PAB:PGE$_2$ conjugate (11 cm - 13 cm) and the proportion
Figure 5.2  Photograph of fluorescence after the derivatization reaction and thin layer chromatography. The plate was developed in chloroform:methanol (100:7). The initial reaction mixture contained 50 ng of PGE₂. The conjugated PGE₂ migrates approximately 9 - 12 cm from the origin, its position being determined by location of the radioactive tracer.
Figure 5.3 Thin layer chromatography of unreacted tritium-labelled PGE$_2$ ([$^3$H]PGE$_2$) and PAB:[$^3$H]PGE$_2$ ester. Radioactive counts were determined from each cm of the lanes loaded with either unreacted [$^3$H]PGE$_2$ or [$^3$H]PGE$_2$ following conjugation with panacyl bromide.  

---- unreacted [$^3$H]PGE$_2$; ----- PAB:[$^3$H]PGE$_2$ ester.
Figure 5.4 Thin layer plate scanning of a chromatograph of the PAB:[$^3$H]PGE$_2$ ester. The upper portion of the figure shows the trace obtained by thin layer plate scanning. The lower portion of the figure shows the actual counts obtained from each cm of the plate following analysis in a scintillation counter.
of $[^3\text{H}]$PGE$_2$ conjugated. A section from 11 cm - 13 cm was taken from lanes 2 - 5 and each was eluted under different conditions (Table 5.1). Following the elution period the eluants were centrifuged to remove silica particles. The supernatant from each sample was evaporated to dryness, reconstituted and counted to determine recovered radioactivity. In this experiment approximately 40% $[^3\text{H}]$PGE$_2$ was conjugated and therefore, 5200 cpm was the maximum expected from the eluted areas. Recovery efficiency ranged from 50% to 78%, the latter being achieved with an overnight elution with acetonitrile:methanol (1:1, v/v) (Table 5.1). This regimen was used for all subsequent experiments.

<table>
<thead>
<tr>
<th>Sample$^a$</th>
<th>Solvent</th>
<th>Elution Time</th>
<th>Radioactivity Recovered (cpm)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Acetonitrile:Methanol (1:1)</td>
<td>30 minutes</td>
<td>3782</td>
</tr>
<tr>
<td>3</td>
<td>Acetonitrile:Methanol (1:1)</td>
<td>overnight</td>
<td>4076</td>
</tr>
<tr>
<td>4</td>
<td>Acetonitrile (100%)</td>
<td>30 minutes</td>
<td>2624</td>
</tr>
<tr>
<td>5</td>
<td>Acetonitrile (100%)</td>
<td>overnight</td>
<td>3786</td>
</tr>
</tbody>
</table>

$^a$ A band from 11 cm - 13 cm was eluted from replicate lanes (2 - 5) from a TLC plate as described in the text. Sample 1 was counted to determine the area containing the conjugate and the % conjugation.

$^b$ Actual counts recovered out of an expected maximum of 5200 cpm.
Solid phase extraction column chromatography

In addition to developing an efficient TLC method for the purification of unreacted PAB prior to HPLC, several methods utilizing solid phase extraction columns were evaluated. All methods assessed included column equilibration with THF:water (95:5, v/v), acetonitrile and dichloromethane (4 - 5 mL, added sequentially). The reaction sample (0.5 mL) was added to the column before elution under vacuum and collection of the fractions for analysis.

The experiment described below was designed to assess the efficiency of recovery of the conjugated product from the column compared with the recovery following direct application to the TLC plate. Duplicate samples of derivatized PGE2 were applied to two equilibrated columns and treated sequentially with dichloromethane (4 mL) and acetonitrile:methanol (85:15, v/v, 4 mL). Washing with dichloromethane removes the unreacted panacyl bromide and these fractions were discarded. Elution with acetonitrile:methanol removes the conjugated sample from the column. These fractions were evaporated under a stream of nitrogen, reconstituted in methanol and applied to an activated TLC plate. A third identical sample of derivatized PGE2 was applied directly to the TLC plate without passage through the column. Figure 5.5 shows the radioactive counts obtained from each 1 cm fraction of the TLC plate after development in chloroform:methanol (7:3, v/v). There was a considerable loss of conjugated product following this method of column chromatography.

After the development of the HPLC analysis was completed (to be detailed in Section 5.2.3), two other methods of purification by column chromatography were assessed. A sample containing conjugated PGE1 and conjugated PGE2 was divided into 4 aliquots. One aliquot (aliquot 4) was semi-purified by TLC prior to HPLC. The other three aliquots were applied to Bond Elut columns pretreated with THF:water (95:5, v/v), acetonitrile and dichloromethane (4 mL, added sequentially). Column 1 was treated with dichloromethane followed by acetonitrile:methanol (85:15, v/v); column 2 was treated with dichloromethane:methanol (100:1, v/v) and acetonitrile:methanol (85:15, v/v) and column 3 with dichloromethane:acetonitrile (90:10, v/v) and
Figure 5.5  Thin layer chromatography of PAB:[$^3$H]PGE$_2$ ester before and after column chromatography.

The derivatized samples were loaded on to two equilibrated columns and after washing with dichloromethane, the PAB:PGE$_2$ conjugates were eluted with acetonitrile:methanol. The acetonitrile:methanol fractions were applied to the TLC plate and developed together with the third sample.

- PAB:PGE$_2$ applied directly to the TLC plate;
- O &  ■ PAB:PGE$_2$ applied to the TLC plate following solid phase extraction column chromatography.
acetonitrile:methanol (95:5, v/v). All solvent volumes were 4 mL and were labelled Fractions 1A, 1B, 2A, 2B, 3A and 3B respectively. All fractions were evaporated to dryness under a stream of nitrogen, reconstituted with 100 μL acetonitrile and 10 μL was injected directly onto the HPLC column. Traces obtained following HPLC of each of these fractions and the aliquot prepared for HPLC by the TLC method are shown in Figure 5.6. Fractions 1B, 2B and 3B were well purified with regard to excess panacyl bromide and produced clear traces. However, the traces obtained from fractions 1A, 2A and 3A show that there is substantial loss of conjugated product in this purified fraction regardless of which method is used. HPLC of aliquot 4 following semi-purification by TLC only produced a clear trace with a satisfactory recovery of the required product and with the conjugated prostaglandins well separated from interfering fluorescent peaks.

In summary, these experiments show that removal of unreacted panacyl bromide from the derivatizing reaction mixture by column chromatography results in substantially greater loss of the conjugated product than by TLC. Thin layer chromatography was selected as the most appropriate method for the removal of contaminating reactants and was used in the further development and utilization of the assay for the separate detection of PGE3.

5.2.3 High pressure liquid chromatography of standards

Fluorimeter settings and solvent conditions

The fluorimeter was set at 375 nm excitation and 470 nm emission following preliminary findings that the panacyl bromide fluorescent marker had two peaks of excitation (365 and 380 nm) and maximum emission between 470 and 480 nm. The mobile phase was made up of acetonitrile, water, tetrahydrofuran and acetic acid. The relative volumes of water and acetonitrile varied during the development of the assay with attempts to achieve the separate resolution of a conjugated internal standard and the PGE1, PGE2 and PGE3 standards. The usual mixture of solvents used for the
Figure 5.6  HPLC chromatograms of PGE$_2$ and PGE$_1$ following conjugation with panacyl bromide and removal of excess panacyl bromide by column chromatography (aliquots 1 - 3) or thin layer chromatography (aliquot 4).

(a) & (b) Column 1 was treated with dichloromethane (fraction 1A) followed by acetonitrile:methanol (85:15, v/v) (fraction 1B).

(c) & (d) Column 2 was treated with dichloromethane:methanol (100:1, v/v) (fraction 2A) followed by acetonitrile:methanol (85:15, v/v) (fraction 2B).

(e) & (f) Column 3 was treated with dichloromethane:acetonitrile (90:10, v/v) (fraction 3A) followed by acetonitrile:methanol (95:5, v/v) (fraction 3B).

(g) Aliquot 4 was semi-purified by TLC before further separation and detection by HPLC.

HPLC conditions were isocratic elution with acetonitrile:H$_2$O:THF:acetic acid (54:42:4:0.1, v/v/v/v) at a run rate of 1 mL/min.
isocratic elution of conjugated prostaglandin products was acetonitrile:H₂O:THF:acetic acid (54:42:4:0.1, v/v/v/v) unless stated otherwise.

**Isotope effect**

Following the injection of unreacted [³H]PGE₂ there was no fluorescent peak and the radioactivity was apparent in the first 1 - 2 mL fractions collected. Unreacted panacyl bromide eluted with the solvent front. The chromatogram of a standard solution of the fluorescent PGE₂ derivative following HPLC and the radioactive counts of corresponding fractions collected at 1 minute intervals is shown in Figure 5.7. The radioactive PGE₂ conjugate consistently eluted 0.5 - 1.0 min earlier than the unlabelled PGE conjugate. This is indicative of a tritium isotope effect which has been previously reported following the HPLC of vitamin D metabolites [Worth and Retallack, 1988]. The tritiated compound in each case becomes more polar due to bonding changes and retention times may differ from less than 1% to up to 4% depending on the degree and position of tritium substitution and the separation system used. The identity of the non-radioactive PGE conjugate was verified by the peak height response to different amounts of PGE₂ added to the original reaction mixture.

**Separation and detection of PGE₃**

Using the methods described, PGE₁ and PGE₃ have also been successfully conjugated with panacyl bromide. Standard solutions of PGE₁, PGE₂ and PGE₃ were added either separately or together to the reaction mixture and incubated at 37° C for 3 h. The samples were purified by TLC. The area containing the derivatized PGE₂ was assumed to contain the PGE₁ and PGE₃ esters also, as these prostaglandins cannot be separated by TLC under these conditions. PGE₁, PGE₂ and PGE₃ were found to have good resolution following HPLC (Figure 5.8).
Figure 5.7  HPLC chromatogram of PGE\textsubscript{2} following conjugation with panacryl bromide and TLC purification. Unlabelled PGE\textsubscript{2} (20 ng) and [\textsuperscript{3}H]PGE\textsubscript{2} were reacted with panacryl bromide as described in the text. HPLC conditions were isocratic elution with acetonitrile:H\textsubscript{2}O:acetic acid (60:40:0.1, v/v/v) at a run rate of 1 mL/min.

–––  fluorescence units;  ----  cpm.
Figure 5.8  HPLC chromatogram of prostaglandin standards PGE₁, PGE₂ and PGE₃ following conjugation with panacyl bromide and preparative TLC as described in the text. HPLC conditions were isocratic elution with acetonitrile:H₂O:THF:acetic acid (54:42:4:0.1, v/v/v/v) at a run rate of 1 mL/min.
Fluorescence

Retention time (min)

0 10 20 30 40

PGE$_3$ (21.7)
PGE$_2$ (28.0)
PGE$_1$ (30.7)
PGE\(_1\) is the least polar and in this system is separated from PGE\(_2\) by approximately 2 min. PGE\(_3\) has a shorter retention time and is separated from PGE\(_2\) by approximately 6 min. The position of the fluorescent peaks of derivatized PGE\(_1\) and PGE\(_3\) in relation to that of the PGE\(_2\) ester was verified by experiments investigating a range of concentrations of these reagents.

5.2.4 Concentration curves

A linear concentration curve was obtained for each prostaglandin, showing increasing peak height with increasing concentration (Figure 5.9). In this experiment, different amounts of each prostaglandin were mixed together prior to derivatization with panacyl bromide. Following derivatization, TLC and elution from the area of interest, the eluant was evaporated and reconstituted in 100 \(\mu\)L acetonitrile. A proportion of this was analysed for radioactivity and the recovery calculated as a percentage of the known amount added to the initial reaction mixture. The actual amount injected onto the HPLC column was then determined. This calculation assumes the losses of PGE\(_2\) at each step are equivalent for PGE\(_1\) and PGE\(_3\) also. The slope of the concentration curve for each prostaglandin was different, with the assay showing the least sensitivity for the detection of PGE\(_3\). Using the criterion signal-to-noise ratio of 2:1, the lower limit of detection for PGE\(_3\) was determined as \(\approx 2-4\) ng (actual amount injected onto the column).

5.2.5 Internal standards

There are many steps in the procedure including panacyl bromide derivatization, TLC, elution and reconstitution, all of which will involve loss of product to some degree. For this reason it was important to find a standard to include in each reaction to allow quantitative measurements to be made. A number of prostaglandins were analysed by HPLC using this method.
Figure 5.9  Concentration curves of prostaglandin standards PGE\(_1\), PGE\(_2\) and PGE\(_3\) following conjugation with panacyl bromide as described in the text. Amounts shown in ng are actual amounts injected onto the column calculated from the percentage of radiolabelled PGE\(_2\) recovered following derivatization and TLC.

- PGE\(_1\);  ○ PGE\(_2\);  ■ PGE\(_3\).
PGB₁ and 16,16 dimethyl PGE₂ were assessed for possible use as internal standards. Solutions containing 50 ng (10 μL of a 5 μg PG/mL stock solution) or 250 ng (50 μL of a 5 μg PG/mL stock solution) were derivatized and semi-purified by TLC (Figure 5.10). A standard mixture with PGE₂ and [³H]PGE₂ was derivatized and run simultaneously (Lane 3). This lane was counted for radioactivity and the conjugated PGE₂ located 12-13 cm from the origin. A band from 5 cm - 14 cm was eluted from each of the other lanes, evaporated and reconstituted in acetonitrile for analysis by HPLC. No suitable peaks were detected under these standard conditions. The area at the top of the TLC plate (15 - 17 cm) was also eluted and injected onto the HPLC column (Figure 5.11). However, there was an excess of contaminating fluorescent matter which made this material unsuitable for detection and quantification of a standard. The TLC development conditions were changed to further separate material of differing polarities. Reactions were performed to derivatize 100 ng (20 μL of a 5 μg/mL stock solution) and 500 ng (10 μL of a 50 μg/mL stock solution) of PGB₁ and 16,16 dimethyl PGE₂. The products of the reactions were applied to TLC plates and developed in solvent mixtures consisting of chloroform:methanol, 100:2 and 100:4 (v/v) (Figure 5.12). The plate developed in chloroform:methanol (100:2, v/v) was divided into 4 areas (6 - 8 cm, 9 cm, 10 - 12 cm and 13 -14 cm), eluted and analysed by HPLC. The second plate (developed in chloroform:methanol, 100:4, v/v) was also divided into 4 areas (6 - 8 cm, 9 - 10 cm, 11 cm and 12 -14 cm), eluted and analysed by HPLC. No suitable peaks were detected (data not shown). In a separate experiment, increasing concentrations of 16,16 dimethyl PGE₂ were reacted with panacyl bromide. The amounts ranged from 50 ng - 1500 ng. The reacted samples were applied to the TLC plate and developed in chloroform:methanol (100:4, v/v) before elution and separation by HPLC. A fluorescent peak of increasing height with increasing amounts of 16,16 dimethyl PGE₂ was not detected (Figure 5.13).
Figure 5.10 Photograph of fluorescence after the derivatization and thin layer chromatography of standard amounts of PGB₁ (50 & 250 ng), 16,16 dimethyl PGE₂ (50 & 250 ng) and PGE₂ + [³H]PGE₂.

Order of application from left to right was:
Lane 1 - PGB₁ (50 ng)
Lane 2 - PGB₁ (250 ng)
Lane 3 - PGE₂ (50 ng) spiked with [³H]PGE₂
Lane 4 - 16,16 dimethyl PGE₂ (50 ng)
Lane 5 - 16,16 dimethyl PGE₂ (250 ng)

The area containing the PAB:PGE₂ conjugate was located by counting 1 cm fractions from lane 3 and determining radioactivity. The counts were located 12 - 13 cm from the origin. An area from 5 - 14 cm from each of the remaining lanes was eluted and prepared for analysis by HPLC.
Figure 5.11 HPLC chromatogram of the highly fluorescent top area (15 - 17 cm) eluted from Lane 2 (PGB$_1$, 250 ng) of the TLC plate pictured in Fig.5.10. This chromatogram illustrates the contaminating peaks of highly fluorescent material which interfere with peaks of interest. HPLC conditions were isocratic elution with acetonitrile:H$_2$O:acetic acid (60:40:0.1, v/v/v) at a run rate of 1 mL/min.
Figure 5.12 Photograph of fluorescence after the derivatization and thin layer chromatography of standard amounts of PGB₁ (100 & 500 ng) and 16,16 dimethyl PGE₂ (100 & 500 ng).

Order of application from left to right on each plate was:

Lane 1 - PGB₁ (100 ng)
Lane 2 - PGB₁ (500 ng)
Lane 3 - 16,16 dimethyl PGE₂ (100 ng)
Lane 4 - 16,16 dimethyl PGE₂ (500 ng)

(a) Plate 1 was developed in chloroform:methanol, 100:2, v/v, and divided into the areas indicated before elution and further analysis by HPLC.

(b) Plate 2 was developed in chloroform:methanol, 100:4, v/v, and divided into the areas indicated before elution and further analysis by HPLC.
Figure 5.13 HPLC chromatograms of 16,16 dimethyl PGE₂ following conjugation of varying amounts with panacyl bromide. HPLC conditions were isocratic elution with acetonitrile:H₂O:acetic acid (60:40:0.1, v/v/v) at a run rate of 1 mL/min.

(a) 50 ng
(b) 500 ng
(c) 1500 ng
Solutions of PGA₁ and PGB₂ were also derivatized using the standard method. 500 ng (10 μL of a 50 μg/mL stock solution) and 1000ng (20 μL of a 50 μg/mL stock solution) of each synthetic prostaglandin were derivatized and applied to a TLC plate. A wide band was eluted from the TLC plate (developed in chloroform:methanol, 100:7, v/v), avoiding any highly fluorescent areas which would interfere with the peaks of interest. The samples were injected onto the HPLC column under standard conditions (Figure 5.14). No suitable peak was detected under these conditions.

PGF₂α (500 ng) was derivatized alone or mixed with an equal quantity of PGE₂ before derivatization with panacyl bromide. Reactions were semi-purified by TLC in chloroform:methanol (100:7, v/v), eluted overnight and reconstituted in acetonitrile for injection onto the HPLC column. Figure 5.15 shows the chromatogram of an equal mixture of PGF₂α and PGE₂ following derivatization with panacyl bromide. Each prostaglandin was clearly resolved from each other and from any unreacted panacyl bromide and the peak heights indicated equal sensitivity. However, when PGE₁, PGE₂, PGE₃ and PGF₂α were added together, PGE₃ and PGF₂α had indistinguishable retention times under these conditions (Figure 5.16). Mobile phase conditions were changed to alter the retention times. The proportions of tetrahydrofuran and water were increased from 4% and 42% respectively, in increments up to 20% and 52%. Isocratic elution with acetonitrile:water:THF:acetic acid (42:48:10:0.1, v/v/v/v) increased the retention time of PGE₁ to approximately 45 minutes with PGE₂ still clearly resolved from both PGE₁ and PGE₃. However, PGF₂α was still not clearly separated from PGE₃ but appeared as a shoulder on the PGE₃ peak (Figure 5.17).

In summary, I was unable to detect any peaks that were clearly resolved from both free unreacted panacyl bromide and conjugated PGE₁, PGE₂ and PGE₃ following the derivatization of appropriate amounts of PGA₁, PGB₁, PGB₂, 16,16 dimethyl PGE₂ and PGF₂α.
Figure 5.14 HPLC chromatograms of prostaglandin standards PGA₁ and PGB₂ following conjugation with panacyl bromide and preparative TLC as described in the text. HPLC conditions were isocratic elution with acetonitrile:H₂O:acetic acid (60:40:0.1, v/v/v) at a run rate of 1 mL/min.

(a) PGA₁ (1000 ng). TLC fractions 6 - 13 cm from the origin were eluted and pooled prior to analysis by HPLC.

(b) PGB₂ (1000 ng). TLC fractions 7 - 14 cm from the origin were eluted and pooled prior to analysis by HPLC.
Figure 5.15 HPLC chromatogram of prostaglandin standards PGE₂ and PGF₂α following conjugation with panacyl bromide and preparative TLC as described in the text. TLC fractions 8 - 13 cm from the origin were eluted and pooled prior to HPLC. HPLC conditions were isocratic elution with acetonitrile:H₂O:acetic acid (60:40:0.1, v/v/v) at a flow rate of 1 mL/min.
Fluorescence

Retention time (min)

- PGE$_{2\alpha}$ (21.7)
- PGE$_2$ (28.0)
Figure 5.16 HPLC chromatograms of prostaglandin standards following derivatization with panacyl bromide and preparative TLC as described in the text. HPLC conditions were isocratic elution with acetonitrile:H₂O:acetic acid (60:40:0.1, v/v/v) at a flow rate of 1 mL/min.

(a) PGE₁, PGE₂ and PGE₃ mixed prior to derivatization.
(b) Derivatized PGF₂α added to the PGE₁, PGE₂ and PGE₃ mix prior to injection onto the HPLC column.
(a) 

Retention time (min)

Fluorescence

PGE₁ (30.7)  
PGE₂ (28.0)  
PGE₃ (21.7)

(b) 

Retention time (min)

Fluorescence

PGE₁ (30.7)  
PGE₂ (28.0)  
PGE₃ (21.7) / PGF₂₀(21.7)
Figure 5.17 HPLC chromatogram of prostaglandin standards PGE₁, PGE₂, PGE₃ and PGF₂α following derivatization with panacyl bromide and preparative TLC as described in the text. HPLC conditions were isocratic elution with acetonitrile:H₂O:THF:acetic acid (42:48:10:0.1, v/v/v/v) at a flow rate of 1 mL/min.
5.3 DISCUSSION

Panacyl bromide undergoes a rapid reaction with the carboxyl group of prostaglandins in the presence of triethylamine producing an eicosanoid ester fluorescent at 375 nm excitation and 470 nm emission. The removal of the excess unreacted panacyl bromide was necessary as indicated by Watkins and Peterson, (1982), who used this technique to assay PGE$_1$ and PGE$_2$ after column chromatography for the semi-purification prior to HPLC. Other workers have also used a silica gel column method, eluting the column with dichloromethane to remove the unreacted panacyl bromide [Krakauer et al., 1986; Morreal et al., 1985]. Different conditions of column extraction were assessed, however this method led to a considerable loss of conjugated product in addition to the unconjugated panacyl bromide. TLC achieved satisfactory removal of interfering products with a reduced loss of panacyl esters, resulting in a greater signal to noise ratio following HPLC.

The outlined experiments show that the method developed can be used to simultaneously identify and quantify the n-3 prostaglandin PGE$_3$ and its n-6 counterpart PGE$_2$. If one assumes equal losses of each prostaglandin during solvent extraction of the reaction mixture and elution from the TLC plate, the results obtained with standard solutions suggest that there is a difference in the proportion conjugated or sensitivity of fluorescent detection between these prostaglandins. Without the availability of tritium-labelled PGE$_3$ it is not possible to determine the actual recovery of this prostaglandin from the TLC plate compared with that of PGE$_2$. One possible explanation for the discrepancy between final peak heights following HPLC of comparable concentrations of PGE$_2$ and PGE$_3$ is that the stock concentrations were not correct. However, I believe that this is unlikely as similar differences between peak height of comparable amounts of PGE$_2$ and PGE$_1$ were also evident. With regard to sensitivity of detection following HPLC, Krakauer et al., (1986), reported a greater recovery of conjugated PGE$_1$ than conjugated PGE$_2$ from reverse-phase HPLC columns, although other workers do not mention any difference in sensitivity between prostaglandins (PGE$_1$ and PGE$_2$) investigated [Watkins and Peterson, 1982].
Quantitative analysis of PGE2 and PGE3 production by this method can still be achieved by constructing a standard curve and the addition of a known internal standard to each reaction. The criteria for a suitable internal standard were that it must conjugate with panacetyl bromide, resolve from the peaks of interest and be a compound which is not made by the cellular systems to be studied for prostaglandin production. One difficulty in the procedure was the location of the candidate internal standard prostaglandin on the TLC plate without tritium-labelled probes for them. The area on TLC plates suitable for their resolution prior to elution and HPLC was limited to that unoccupied by the strongly fluorescent zones since eluates of these zones led to unmanageable contaminating peaks on the HPLC trace. Having achieved the successful derivatization of PGF2α, and established that it had appropriate resolution characteristics on TLC, I found that this prostaglandin had a similar retention time to that of PGE3. The addition of water to the mobile phase increases the retention times and can separate closely eluting samples. However, an increased retention time also leads to broadening of the peak and increased overall running time. As I was unable to separate satisfactorily PGF2α and PGE3, this prostaglandin was unsuitable for use as an internal standard. The problem was circumvented by using PGE1 as an internal standard since it was not produced in the biological systems which were later investigated (detailed in Chapter 6).

This chapter describes the development of an assay for the separate detection and quantitation of PGE3. Standard PGE3 was successfully conjugated with panacetyl bromide and the resultant ester resolved by HPLC with fluorescent detection. PGE1, PGE2 and PGE3 can be examined individually from the same starting sample. This development has potential uses in the analysis of biological samples, obtained in the presence of exogenous EPA or following dietary fortification with fish oil, with respect to PGE production.
CHAPTER 6

HPLC OF DERIVATIZED PROSTAGLANDINS FROM BIOLOGICAL SAMPLES

6.1 INTRODUCTION

The previous two chapters describe work determining the biological activity of PGE$_3$ with regard to oedema formation in mice (Chapter 4) and the development of an HPLC assay for the separate detection of PGE$_3$ and PGE$_2$ (Chapter 5). Increasing dietary n-3 fatty acid content has been shown to alter eicosanoid production of both the lipoxygenase and the cyclooxygenase pathways. Competition between AA and EPA following dietary supplementation with fish or fish oil leads to a partial replacement of the n-6 fatty acid, AA, with EPA in cell membranes. As a result, ingestion of EPA and DHA leads to a decreased production of LTB$_4$ and an increased production of LTB$_5$, a decrease in TXA$_2$ and an increase in TXA$_3$, an increase in PGI$_3$ without an increase in PGI$_2$, and a decrease in PGE$_2$. This conversion of EPA to TXB$_3$ and PGI$_3$ both ex vivo and in vitro indicates that EPA cannot be considered as simply a cyclooxygenase inhibitor. Recently the metabolites of PGE$_3$ have been detected in the urine and renal medullary tissue of the human or the rat by gas chromatography-mass spectrometry with selective ion monitoring.

This chapter describes the establishment of assay conditions suitable for the detection of PGE$_2$ produced by biological samples and attempts to detect PGE$_3$ synthesized from EPA incorporated into cell membrane phospholipids following dietary supplementation or addition to cell cultures.
6.2 RESULTS

6.2.1 Assessment of culture conditions for the production of PGE₂ by adherent cells

Resident peritoneal cells from rats and mice and peripheral blood mononuclear cells from healthy human volunteers were collected and prepared as described in Chapter 2, 2.2.C. Following a 2 h adherence at 37° C in RPMI, non-adherent cells were removed by washing with PBS. Multiple methods of cell stimulation were investigated in preliminary experiments. Control experiments with human, rat and mouse adherent cells verified that PGE₁ is not produced in measurable quantities under the conditions of these studies, thus enabling PGE₁ to be used as an internal standard where stated.

4 hour incubation

Mouse (or rat) resident peritoneal cells were plated at 5x10⁶ cells/well. Following a 2 h incubation, the non-adherent cells were removed by washing and the remaining cells were incubated overnight in RPMI/10% FCS. At 20 h the adherent cells were washed again in PBS and the culture medium replaced with RPMI only. Replicate wells were incubated for 4 h at 37° C. The culture supernatants were collected, acidified with citric acid and [³H]PGE₂ (1 Bq/mL, final concentration) was added prior to extraction with chloroform:methanol (7:3, v/v). The extracted media were evaporated to dryness, reconstituted with acetonitrile:THF (4:1, v/v) and conjugated with panacyl bromide in the presence of triethylamine. For each experiment, a reaction containing PGE₁, PGE₂ and PGE₃ standards was prepared in tandem with the biological preparation. Following the conjugation reaction, all samples were purified by TLC prior to further analyses by HPLC. The HPLC chromatograms obtained following the injection of 10 μL derivatized PGE₂ standard and 10 μL derivatized products extracted from the culture medium of mouse adherent peritoneal
cells cultured for 4 h are shown in Figure 6.1 (a) & (b) respectively. The peak eluting with a retention time of 24.39 min was identified as conjugated PGE$_2$ by the determination of radioactive counts present in fractions collected at 1 min intervals (Figure 6.1 (a)). There was no corresponding peak of fluorescence in Figure 6.1 (b).

In separate experiments, similar results were obtained following derivatization of products extracted from the culture medium of rat adherent peritoneal cells after a 4 or 6 h incubation with RPMI (Figure 6.2). Conditions were the same as those described for analysis of cultured mouse resident peritoneal cells described above.

4 hour pretreatment with zymosan

Zymosan was boiled in PBS for 30 min and centrifuged 3 times at 200 x g for 7 min each before resuspension in PBS (20 mg/mL) [Bonney et al., 1978]. The stock solution of zymosan was sonicated prior to use in cell cultures. Mouse (or rat) resident peritoneal cells were plated at 5x10$^6$ cells/well. Following a 2 h incubation, the non-adherent cells were removed by washing and the remaining cells were incubated overnight in RPMI/1% FCS. After the overnight incubation and replacement of the supernatant with RPMI, zymosan was added to replicate wells for 4 h at 37° C (100 µg/mL, final concentration). $[^3]$H]PGE$_2$ and a known amount of PGE$_1$ was added to the culture media immediately before acidification and extraction prior to the TLC step. Figure 6.3 shows an HPLC chromatogram obtained following injection of pooled TLC fractions containing derivatized products extracted from the media of mouse adherent peritoneal cell cultures incubated in the presence of zymosan. An injection of standards verified the elution times of derivatized PGE$_2$ and PGE$_1$ as 26.44 min and 28.49 min respectively (data not shown). PGE$_2$ in the cell supernatants was clearly detected (Figure 6.3).

Likewise, PGE$_2$ was detected in media extracted from rat adherent peritoneal cell cultures following incubation with zymosan (Figure 6.4).
Figure 6.1 HPLC chromatograms of (a) PGE2 standard following derivatization with panacyl bromide as described in the text and (b) extract from mouse adherent resident peritoneal cells incubated for 4 h in RPMI/10% FCS. The supernatant was extracted and reacted with panacyl bromide in the same assay as the PGE2 standard. HPLC conditions were isocratic elution with acetonitrile:H2O:acetic acid (60:40:0.1, v/v/v) at a flow rate of 1 mL/min.
Figure 6.2  HPLC chromatogram of extract from rat adherent resident peritoneal cells incubated for 6 h. The supernatant was extracted, reacted with panacyl bromide and purified by TLC as described in the text. The arrow indicates the retention time obtained from a standard solution of conjugated PGE2. HPLC conditions were isocratic elution with acetonitrile:H$_2$O:acetic acid (60:40:0.1), v/v/v) at a flow rate of 1 mL/min.
Figure 6.3  HPLC chromatogram of extract from mouse adherent resident peritoneal cells incubated for 4 h in the presence of zymosan (100 µg/mL). The supernatant was extracted, reacted with panacyl bromide and separated from reactants by TLC as described in the text. PGE₁ was added prior to extraction as an internal standard. Arrows indicate the retention times obtained from standard solutions of conjugated prostaglandin mixtures. HPLC conditions were isocratic elution with acetonitrile:H₂O:THF:acetic acid (54:42:4:0.1, v/v/v/v) at a flow rate of 1 mL/min.
Fluorescence

Retention time (min)

0  10  20  30

PGE_2 (26.4)
PGE_1 (28.5)
Figure 6.4  HPLC chromatogram of extract from rat adherent resident peritoneal cells incubated for 4 h in the presence of zymosan (100 μg/mL). The supernatant was extracted, reacted with panacyl bromide and separated from reactants by TLC as described in the text. PGE$_1$ was added prior to extraction as an internal standard. Arrows indicate the retention times obtained from standard solutions of conjugated prostaglandin mixtures. HPLC conditions were isocratic elution with acetonitrile:H$_2$O:THF:acetic acid (54:42:4:0.1, v/v/v/v) at a flow rate of 1 mL/min.
Fluorescence

Retention time (min)

- PGE₂ (26.2)
- PGE₁ (28.6)
15 minute stimulation with A23187 in the presence of arachidonic acid

Replicate wells of the mouse resident peritoneal cells prepared for pretreatment with zymosan were stimulated simultaneously with 0.5 μM A23187 in the presence of 1 μM arachidonic acid for 15 min at 37°C. The supernatants were removed by aspiration and prepared for analysis of PGE₂ production as described above. The chromatogram obtained from this extracted culture media is shown in Figure 6.5. The cells produced PGE₂ and the derivative eluted with a retention time of 26.4 min, clearly resolved from the internal standard PGE₁, which was added prior to derivatization of the culture supernatant and eluted at 28.5 min. PGE₂ in the cell supernatants was clearly detected (Figure 6.5).

Overnight incubation in RPMI/1% FCS

The cell culture supernatants recovered following overnight incubation of the mouse adherent peritoneal cells described above had PGE₁ added as an internal standard then were extracted, derivatized and analysed by HPLC. PGE₂ was produced in measurable quantities under these conditions (Figure 6.6). Similar chromatograms indicating PGE₂ production were obtained with extracts of culture medium of rat and human adherent cells incubated under the same conditions (rat, Figure 6.7; human, see Fig.6.9 (a), 6.11 (a), 6.12 (a), 6.16 (a)).

Overnight incubation in RPMI/1%FCS in the presence of IL-1β

Recombinant human interleukin 1β (2 U/mL) was added to an overnight incubation of mouse resident peritoneal cells (5x10⁶ cells/well, 2 h adherence in RPMI followed by washing with PBS and resuspension in RPMI/1%FCS as described above). The addition of IL-1β did not significantly alter the production of PGE₂ under these conditions (Figure 6.8).
Figure 6.5  HPLC chromatogram of extract from mouse adherent resident peritoneal cells incubated for 15 min with A23187 (0.5 μM) and arachidonic acid (1 μM). The cells were from the same preparation as described in Fig. 6.3. Derivatization and chromatography conditions were as described in Fig. 6.3. Arrows indicate the retention times obtained from standard solutions of conjugated prostaglandin mixtures.
Figure 6.6  HPLC chromatogram of extract from the same preparation of mouse adherent resident peritoneal cells as described in Fig. 6.3 incubated overnight in RPMI/1%FCS only. The supernatant was extracted, reacted with panaclyl bromide and purified by TLC as described in the text. PGE$_1$ was added prior to extraction as an internal standard. Arrows indicate the retention times obtained from standard solutions of conjugated prostaglandin mixtures. HPLC conditions were isocratic elution with acetonitrile:H$_2$O:THF:acetic acid (54:42:4:0.1, v/v/v/v) at a flow rate of 1 mL/min.
Figure 6.7  HPLC chromatogram of extract from rat adherent resident peritoneal cells incubated overnight in RPMI/1 %FCS only. The supernatant was extracted, reacted with panacyl bromide and purified by TLC as described in the text. PGE₁ was added prior to extraction as an internal standard. Arrows indicate the retention times obtained from standard solutions of conjugated prostaglandin mixtures. HPLC conditions were isocratic elution with acetonitrile:H₂O:THF:acetic acid (54:42:4:0.1, v/v/v/v) at a flow rate of 1 mL/min.
Fluorescence

Retention time (min)

PGE₂ (24.6)
PGE₁ (26.4)
Figure 6.8  HPLC chromatogram of extract from the same preparation of mouse adherent resident peritoneal cells as described in Fig. 6.3 incubated overnight in the presence of IL-1β (2 U/mL). Derivatization and chromatography conditions were as described in Fig. 6.6. Arrows indicate the retention times obtained from standard solutions of conjugated prostaglandin mixtures.
Following these preliminary investigations, the conditions for PG production were as described in the methods section (2.2.11) unless stated otherwise. Briefly, cells (rat, mouse or human) were adhered in RPMI only for 2 h at 37° C. The non-adherent cells were removed by washing with PBS and the remaining adherent cells were maintained overnight in RPMI/1% FCS or RPMI/0.1% fatty acid free albumin in the presence or absence of reagents as specified. After 20 h the culture media were removed, a known amount of PGE1 was added as an internal standard and the medium was acidified prior to extraction, derivatization, preparative TLC and analysis for PG production by HPLC.

6.2.2 Effect of EPA and AA in vitro

Figure 6.9 (a) illustrates the chromatogram obtained following derivatization of products extracted from the culture medium of human adherent mononuclear cells cultured for 24 h in vitro in RPMI/0.1% fatty acid free albumin. The PGE2 derivative elutes with a retention time of 24.0 min, clearly resolved from the internal standard PGE1 added prior to derivatization of the culture supernatant and eluting at 25.8 minutes. The ratio of E2 to the internal standard E1, was 1.3 as determined by peak height in mm. The effect of addition of EPA to human adherent cells is shown in Figure 6.9 (b). A duplicate cell sample was incubated overnight with 5 μM EPA. There was a 50% inhibition of PGE2 production (E2:E1 = 0.7) but no PGE3 was detected.

Comparable results were obtained with addition of EPA in vitro to rat or mouse adherent cells cultured under similar conditions. Figure 6.10 illustrates the chromatograms obtained following derivatization of products extracted from the culture medium of mouse adherent peritoneal cells cultured overnight in RPMI/1%FCS with the addition of (a) EtOH (0.1%), (b) AA 1 μM + EtOH or (c) EPA 5 μM + EtOH. The ratios of E2 to the internal standard E1 were 3.0, 2.9 and 2.0 respectively, indicating that the addition of AA had little effect on the production of PGE2 under these conditions but EPA resulted in inhibition of PGE2. No PGE3 peak was seen.
Figure 6.9  HPLC chromatograms of extract from human peripheral blood adherent cells incubated overnight in the presence or absence of EPA. The supernatant was extracted, reacted with panacyle bromide and purified by TLC as described in the text. PGE₁ was added prior to extraction as an internal standard. Arrows indicate the retention times obtained from standard solutions of conjugated prostaglandin mixtures. HPLC conditions were isocratic elution with acetonitrile:H₂O:THF:acetic acid (54:42:4:0.1, v/v/v/v) at a flow rate of 1 mL/min.
(a) no addition,  (b) EPA (5μM)
Figure 6.10 HPLC chromatograms of extract from mouse adherent resident peritoneal cells incubated overnight in the presence or absence of AA or EPA. PGE₁ was added to the supernatant which was then extracted and reacted with panacyl bromide as described in the text. Reactants were removed by TLC prior to HPLC. Arrows indicate the retention times obtained from standard solutions of conjugated prostaglandin mixtures. HPLC conditions were isocratic elution with acetonitrile:H₂O:THF:acetic acid (54:42:4:0.1, v/v/v/v) at a flow rate of 1 mL/min.

(a) no addition, (b) AA (1 µM), (c) EPA (5 µM)
6.2.3 Effect of other stimulators and inhibitors

In addition to the in vitro experiments with zymosan, A23187 and IL-1β already described, the effects of other proposed stimulants and inhibitors on PGE production were investigated in this system.

Lipopolysaccharide (LPS)

Human peripheral blood adherent mononuclear cells were incubated overnight in RPMI/1%FCS in the presence or absence of LPS (10 μg/mL). The ratio of PGE₂ to the internal standard PGE₁ was 1.2 with or without LPS indicating that the addition of LPS had no effect on the production of PGE₂ under these conditions (Figure 6.11).

Leukotriene B₄ (LTB₄)

Human peripheral blood adherent mononuclear cells were incubated overnight in RPMI/0.1% albumin in the presence or absence of LTB₄ (10⁻¹²M or 10⁻⁸M). At both concentrations investigated the presence of LTB₄ led to a moderate inhibition of PGE₂ production (Figure 6.12). The ratio of PGE₂ to the internal standard PGE₁ following incubation in RPMI/0.1% albumin alone was 1.0. This ratio decreased to 0.7 following the addition of either amount of LTB₄ to the overnight incubation media.

Indomethacin

Figure 6.13 illustrates the chromatogram obtained following derivatization of products extracted from the culture media of mouse adherent cells incubated overnight in the absence or presence of indomethacin (3 μM). The ratios of PGE₂ to the internal standard PGE₁ were 3.0 and 0.7 respectively, which represents a 73% inhibition of PGE₂ production in this experiment. Higher concentrations of indomethacin did not yield any greater inhibitory effect, possibly due to the poor solubility of indomethacin.
in aqueous media. Figure 6.14 illustrates the chromatogram obtained after derivatization of products extracted from culture media following the addition of indomethacin (30 μM) to overnight cultures of human adherent cells. The presence of indomethacin in this experiment resulted in a 65% inhibition of PGE₂ production.

**Ibuprofen**

Ibuprofen (5 μM, 50 μM) also inhibited the production of PGE₂ by mouse resident peritoneal adherent cells under these culture conditions by approximately 75% (Figure 6.15). Inhibition of PGE₂ by ibuprofen was similar at both doses. Figure 6.16 is representative of the chromatograms obtained following the derivatization of products extracted from the culture media of human adherent mononuclear cells cultured overnight in the absence or presence of ibuprofen (50 μM). The ratio of PGE₂ to the internal standard PGE₁ decreased from 1.3 to 0.9 under these conditions.
Figure 6.11 HPLC chromatograms of extract from human peripheral blood adherent cells incubated overnight in the presence or absence of LPS. The supernatant was extracted, reacted with panacyl bromide and subjected to TLC to remove reactants as described in the text. PGE₁ was added prior to extraction as an internal standard. Arrows indicate the retention times obtained from standard solutions of conjugated prostaglandin mixtures. HPLC conditions were isocratic elution with acetonitrile:H₂O:THF:acetic acid (54:42:4:0.1, v/v/v/v) at a flow rate of 1 mL/min.

(a) no addition, (b) LPS (10 μg/mL)
Retention time (min)

(a) Fluorescence

Retention time (min)

(b) Fluorescence
Figure 6.12 HPLC chromatograms of extract from human peripheral blood adherent cells incubated overnight in the presence or absence of LTB₄. The supernatant was extracted, reacted with panacyl bromide and purified by TLC as described in the text. PGE₁ was added prior to extraction as an internal standard. Arrows indicate the retention times obtained from standard solutions of conjugated prostaglandin mixtures. HPLC conditions were isocratic elution with acetonitrile:H₂O:THF:acetic acid (54:42:4:0.1, v/v/v/v) at a flow rate of 1 mL/min.

(a) no addition, (b) LTB₄ (10⁻¹²M), (c) LTB₄ (10⁻⁸M)
Figure 6.13 HPLC chromatograms of extract from mouse adherent resident peritoneal cells incubated overnight in the presence or absence of indomethacin. The supernatant was extracted, reacted with panacyl bromide and purified by TLC as described in the text. PGE$_1$ was added prior to extraction as an internal standard. Arrows indicate the retention times obtained from standard solutions of conjugated prostaglandin mixtures. Run conditions were isocratic elution with acetonitrile:$\text{H}_2\text{O}$:THF:acetic acid (54:42:4:0.1, v/v/v/v) at a flow rate of 1 mL/min.

(a) no addition, (b) indomethacin (3 $\mu$M)
(a) Retention time (min)

(b) Retention time (min)
Figure 6.14 HPLC chromatograms of extract from human peripheral blood adherent cells incubated overnight in the presence or absence of indomethacin. The supernatant was extracted, reacted with panacyl bromide and purified by TLC as described in the text. PGE₅ was added prior to extraction as an internal standard. Arrows indicate the retention times obtained from standard solutions of conjugated prostaglandin mixtures. Run conditions were isocratic elution with acetonitrile:H₂O:THF:acetic acid (54:42:4:0.1, v/v/v/v) at a flow rate of 1 mL/min.

(a) no addition, (b) indomethacin (30 µM)
Figure 6.15 HPLC chromatograms of extract from mouse adherent resident peritoneal cells incubated overnight in the presence or absence of ibuprofen. The supernatant was extracted, reacted with panacyl bromide and purified by TLC as described in the text. PGE₁ was added prior to extraction as an internal standard. Arrows indicate the retention times obtained from standard solutions of conjugated prostaglandin mixtures. HPLC conditions were isocratic elution with acetonitrile:H₂O:THF:acetic acid (54:42:4:0.1, v/v/v/v) at a flow rate of 1 mL/min.

(a) no addition, (b) ibuprofen (5 µM), (c) ibuprofen (50 µM)
Figure 6.16 HPLC chromatograms of extract from human peripheral blood adherent cells incubated overnight in the presence or absence of ibuprofen. The supernatant was extracted, reacted with panacyl bromide and purified by TLC as described in the text. PGE₁ was added prior to extraction as an internal standard. Arrows indicate the retention times obtained from standard solutions of conjugated prostaglandin mixtures. HPLC conditions were isocratic elution with acetonitrile:H₂O:THF:acetic acid (54:42:4:0.1, v/v/v/v) at a flow rate of 1 mL/min.

(a) no addition, (b) ibuprofen (50 μM)
6.2.4 Effect of EPA in vivo

Although PGE3 was not detected under these assay conditions following the in vitro addition of EPA as the free fatty acid, the effects of dietary enrichment with fish oil were investigated. Rats and mice were fed a fish-oil (or sunflower-oil) enriched diet for several weeks prior to overnight culture of adherent resident peritoneal cells in the absence or presence of added EPA (5 μM). While PGE2 was observed, PGE3 was not detected in the culture supernatants following extraction, derivatization, purification by TLC and further purification and analysis by HPLC (Figure 6.17). Likewise, PGE3 was not detected in culture supernatants following overnight incubation of human adherent cells from the subject who was taking fish oil supplements as described in the Methods, Chapter 2, 2.2.3 (Figure 6.18).

6.2.5 Analysis of fatty acids following fish oil feeding

Membrane phospholipid n-3 fatty acid levels of spleen cells from mice fed fish oil for 14 weeks had increased substantially compared with their sunflower oil fed counterparts. The amount of EPA had increased from an undetectable level to 8.8% of total fatty acids and DHA had increased from 1.8% to 11.8%. This increase in n-3 fatty acids was associated with a fall in n-6 fatty acids. AA fell from 23.2% to 7.2% and LA fell from 10.0% to 2.5%. Similar changes in leukocyte cell membrane fatty acids were seen in rats consuming the same diet (see Tables 3.4, 3.5).

The human neutrophils also showed considerable change in EPA content, increasing from undetectable levels to 2.4% after 4 weeks of EPA supplementation.
Figure 6.17 HPLC chromatograms of extract from rat and mouse adherent resident peritoneal cells incubated overnight. Rats and mice were fed a fish-oil enriched diet for several weeks prior to collection of resident peritoneal cells. The supernatant was extracted, reacted with panacyl bromide and purified by TLC as described in the text. PGE_1 was added prior to extraction as an internal standard. Arrows indicate the retention times obtained from standard solutions of conjugated prostaglandin mixtures. HPLC conditions were isocratic elution with acetonitrile:H_2O:THF:acetic acid (54:42:4:0.1, v/v/v/v) at a flow rate of 1 mL/min.

(a) Fish-oil fed rat cells, no addition
(b) Fish-oil fed mouse cells, no addition
(c) Fish-oil fed mouse cells, 5 μM EPA added to overnight culture
Figure 6.18 HPLC chromatogram of extract from human peripheral blood adherent cells incubated overnight in RPMI/0.1% albumin only. Cells were obtained after 4 weeks of dietary supplementation with fish oil capsules (MaxEPA) 12 g/day. The supernatant was extracted, reacted with panacyl bromide and purified by TLC. PGE$_1$ was added prior to extraction as an internal standard. Arrows indicate the retention times obtained from standard solutions of conjugated prostaglandin mixtures. HPLC conditions were isocratic elution with acetonitrile:H$_2$O:THF:acetic acid (54:42:4:0.1, v/v/v/v) at a flow rate of 1 mL/min.
6.2.6 Analysis of lipoxygenase products following fish oil feeding

Following 12 weeks of fish oil feeding, rat resident peritoneal macrophages were stimulated and assayed for leukotriene production. Following stimulation of the cells (2x10⁶ cells, 5 min) with A23187 (0.5 µM) and EPA (1 µM), AA-derived LTB₄ (7.5 ng) and 5-HETE (22.8 ng) and EPA-derived LTB₅ (11.4 ng) and 5-HEPE (96.7 ng) were detected in extracts of the cell suspensions.

Stimulation with A23187 (0.5 µM) of human neutrophils (1x10⁶ cells, 5 min) from the subject taking fish oil supplements generated LTB₅ (4.6 ng) and 5-HEPE (69.5 ng) in addition to LTB₄ (21.3 ng) and 5-HETE (143.5 ng).

These studies show that under the experimental conditions of dietary supplementation with EPA used, metabolism of EPA to 5-LO products by PEC (rat) and neutrophils (human) could be demonstrated.

6.3 DISCUSSION

Dietary EPA appears to be transformed in the body to PGI₃ with little effect on PGI₂ production [Fischer and Weber, 1984; Knapp et al., 1986]. Conversely, many studies on animal and human tissues have shown that EPA reduces synthesis of the other AA-derived cyclooxygenase products TXA₂ and PGE₂ and gives rise to little if any of EPA-derived TXA₃ and PGE₃ [Knapp and FitzGerald, 1989; Needleman et al., 1979; Kelley et al., 1985]. However, the presence of PGE₃ or its metabolite, Δ¹⁷-tetranor-PGE₃, in the urine of humans [Ferretti et al., 1988; Fischer et al., 1988] and urine or renal medullary tissue of rats [Kivits and Nugteren, 1988; Ferretti et al., 1981] after ingestion of large quantities of dietary fish oil has been reported. These studies all used combinations of capillary gas chromatography and selected ion monitoring mass spectrometry for the analysis of PGs in biological samples. Because GC-MS is expensive and requires special instrumentation beyond the scope of most laboratories, I sought to develop an alternative method for the separate detection and quantitation of PGE₃ in biological samples. This chapter describes the investigation of the in vitro
production of prostaglandins by the adherent mononuclear cell population from three
species (rat, mouse and human) after the ingestion of EPA as fish oil or the addition of
exogenous EPA in vitro. Among the immunologically important cells, macrophages are
considered the major producers of prostaglandins [Lewis, 1983], so this cell population
was chosen for investigation. However, under the conditions described here, PGE3 was
not detected in cell supernatants whereas PGE2 production was measurable (under the
same conditions).

PGE2 was not detected in cell supernatants following a 4 - 6 hour incubation of
adherent cells in RPMI without an added stimulus. However, the addition of zymosan
or calcium ionophore A23187 plus AA stimulated the production of PGE2 which was
detected following fluorescent derivatization, TLC to remove reactants and HPLC. The
production of PGE2 was comparable to that detected following an overnight incubation
in RPMI/1% FCS (or RPMI/0.1% fatty acid free albumin) only. Overnight incubation
in media containing only 1% FCS (instead of 10% FCS) did not compromise cell
viability or PG production but enabled the generation of a better signal to noise ratio
chromatographically. Although PGE2 was measurable under these conditions, other
proposed stimulants were investigated in an attempt to maximize the possibility of
detecting PGE3. However, addition of IL-1, LPS and LTB4 did not significantly alter
the production of PGE2 nor stimulated the production of PGE3 to levels detectable by
the method of analysis employed. The cyclooxygenase inhibitors indomethacin and
ibuprofen were added to the overnight cultures to further verify that the presumptive
PGE2 peak was that of a cyclooxygenase metabolite.

Fatty acid analyses of cell samples from rodents and humans who had ingested
fish oil confirmed the displacement of AA with EPA in the phospholipid fraction.
Detailed studies of fatty acid incorporation into rat cell membrane phospholipids
following dietary supplementation with 5% and 10% fish oil have been published
previously by me and my co-workers [Cleland et al., 1990a; James et al., 1991a] and
are also described in Chapter 3 of this thesis. In the mouse feeding experiments, spleen
cells were used as the cell population to be analysed for fatty acid levels of
incorporation. Our previous work with different rat strains has shown that the patterns
of incorporation of n-3 fatty acids into spleen cells and peritoneal exudate cells are similar [Cleland et al., 1990a]. I have assumed that the fatty acid results obtained from the mouse spleen cells reflect broadly the levels of incorporation achieved in the cell type used for the analyses of PGE production.

The leukotriene analyses were carried out to confirm that the level of membrane phospholipid EPA achieved following dietary supplementation with fish oil was sufficient to allow the formation of detectable levels of n-3 lipoxygenase products, LTB5 and 5-HEPE.

Although this work found no evidence of PGE3 release into the media of cultured adherent cells, the results shown for the effects of EPA added in vitro support previous observations that the presence of EPA causes an inhibition of PGE2 production [Ferretti and Flanagan, 1990; Terano et al., 1986]. Ferretti et al., (1981), present evidence that EPA of dietary origin is deacylated from the cellular phospholipids and converted to PGE3 during in vitro incubation of homogenized rat kidney. However, the in vivo conversion of EPA to PGE3 in the urine of rats fed EPA could not be demonstrated. A controlled study of n-3 or n-6 fatty acid supplements in humans demonstrated urinary metabolites of PGI3 and TXB3 by measurement with stable-isotope dilution assays in which capillary gas chromatography-negative chemical ionization-mass spectrometry was used [Knapp and FitzGerald, 1989]. There was a non-significant trend toward reduced production of a PGE2 metabolite with large doses of fish oil but there was no evidence for a metabolite derived from PGE3.

In contrast to these studies, Ferretti et al., (1988), reported finding derivatives of PGE3 in the urine of a female subject who had ingested 10 - 50 g/day of MaxEPA fish oil for 4 years. A PGE3 metabolite has also been detected in the urine of rats after feeding fish oil and although PGE2 was present, measurable PGE3 was not detected [Kivits and Nugteren, 1988]. The greater part of endogenously formed PGs are rapidly excreted as more polar metabolites in the urine therefore the amount of urinary metabolites is often regarded as the best measure of total PG production in the body. However, the amounts present are small and each PG gives rise to a relatively large number of different metabolites depending on the species and the diet amongst other
factors. It has been suggested that the determination of a single metabolite in urine may actually lead to an overestimation of the production of the 3-series PGs. These PGs are relatively poor substrates for certain dehydrogenases and oxygenases and thus PGE$_3$ and PGF$_3\alpha$ are metabolized to a greater extent into their tetranor-PGE and -PGF derivatives than are PGE$_2$ and PGF$_2\alpha$ [Kivits and Nugteren, 1988]. Greater amounts of E and F-type PGs are made in human seminal vesicles than in the rest of the body combined and the effects of dietary fish oil on the amounts of these PGs have been investigated [Knapp, 1990]. A significant reduction in 1-series and 2-series PGs of semen was found following ingestion of 50 mL menhaden oil for 4 weeks. A slight increase in the small amounts of PGE$_3$ normally found in human semen [Oliw et al., 1986] was seen during the supplementation period. The possibility of seminal fluid contamination of urine samples should be considered when assessing data indicating variable urinary prostaglandin levels. This complicating factor has been addressed by using female volunteers when assessing urinary excretion of PGE [Düsing et al., 1990]. In this study, the diet of female volunteers was supplemented with 6 g/day n-3 PUFAs for 6 weeks. Urinary excretion of PGE$_2$ and PGE$_3$ was measured by a radioimmunoassay kit for PGE$_2$ which was known to exhibit significant cross-reactivity with PGE$_3$. Following some correction for this, it was concluded that fish oil supplementation resulted in a decrease in PGE$_2$ production by 26% and a 4 - fold increase in urinary PGE$_3$.

These and other studies have suggested that there is both a tissue difference and a species difference in substrate affinity by cyclooxygenase resulting in disparate conversion of EPA. It has been shown that the peroxide tone can have a dramatic influence on the reactivity of cyclooxygenase [Culp et al., 1979]. EPA is not oxidized by purified CO from sheep vesicular glands in conditions of low peroxide tone in which AA is rapidly oxygenated but when the level of peroxide in incubation mixtures is allowed to rise, CO can react with EPA at one half the rate and one third the extent observed with AA. Thus, the formation of trienoic PG derivatives in a tissue may reflect an abundance of peroxides. Although the conversion of EPA to PGH$_3$ by a ram seminal vesicle preparation was less than that of AA to PGH$_2$, the PGH$_3$ was a good
substrate for both the thromboxane synthetase in blood platelets and the prostacyclin synthetase in blood vessel walls [Smith et al., 1979]. Rates of synthesis of PGI3 differ markedly between in vitro and in vivo conditions suggesting that there could be differences such as changes in peroxide tone, cofactor concentrations and mobilization of endogenous EPA and AA from cellular lipids as well as distinct substrate specificities in different tissues all of which may contribute to different rates of cyclooxygenase activity [Knapp and FitzGerald, 1989]. Species differences may also be important. For example, Whitaker et al., (1979), and Hornstra et al., (1981), were unable to demonstrate conversion of EPA to PGI3 by rat endothelial cells whereas the biosynthesis of PGI3 in man has been clearly demonstrated [Fischer and Weber, 1984]. Similarly, EPA had much less activity in inhibiting TXA2 synthesis in rat platelets than in human cells [Morita et al., 1983]. It is difficult to explain why EPA should be subject to such different rates of metabolism by the cyclooxygenase enzyme complex. It has been suggested that under conditions of high CO activity, such as occurs in vitro, EPA is a poor substrate while if much lower rates of enzyme activity are present, such as in the endothelium in vivo, it is a good precursor for the production of eicosanoids [Knapp, 1990].

In summary, this chapter describes work aimed at identifying the effects of EPA in vitro and in vivo on the relative production of PGE2 and PGE3 by adherent cells from rats, mice and humans. It does not provide evidence that PGE3 is generated in sufficient quantities by leukocytes to play a major role in mediating inflammatory responses by its own actions although it had earlier been found to have oedemogenic activity when injected directly into the mouse hind paw (Chapter 4). The concentration of PGE3 required to produce a substantial oedemogenic effect was 100 ng with maximum oedema produced following injection of 1 μg PGE3. The limit of detection of the assay used to investigate the possible production of PGE3 by cells obtained from rats, mice and humans is 2 - 4 ng which is well below the amount of PGE3 that may be required to mediate an inflammatory response in biological systems. Due to a consistent inability to detect PGE3 produced by leukocytes under the conditions described, further validation of the assay in terms of precision of quantitation of PGE3 was not pursued.
Further work will be needed to clarify the factors regulating substrate preferences of the cyclooxygenase enzyme complex under different conditions and in different tissues before the physiological effects of dietary fish oil can be fully understood.
CHAPTER 7

THE EFFECT OF METHOTREXATE ON LIPOXYGENASE METABOLISM IN NEUTROPHILS

7.1 INTRODUCTION

Experiments are described in the previous chapter demonstrating that PGE₃, derived from EPA, is not a biologically significant agent in the inflammatory eicosanoid cascade. Following this observation, I directed my attention to the effects of EPA on AA metabolism via the lipoxygenase pathway. Some of the anti-rheumatic drugs currently available inhibit LTB₄ synthesis. The mechanism of action of these drugs is usually by inhibition of 5-LO activity, whereas EPA can also inhibit LTB₄ synthesis via inhibition of LTA hydrolase. Therefore, I considered the possibility of additive or synergistic effects between EPA and these drugs with regard to inhibition of LTB₄. Following reports that methotrexate (MTX), a potent anti-rheumatic agent, inhibits LTB₄ synthesis [Sperling et al., 1990, 1992], I commenced a study to examine EPA/MTX interactions in LTB₄ synthesis. The effects of dietary EPA on inflammation and LTB₄ synthesis are documented in Chapter 3 of this thesis. The folic acid antagonist MTX has been shown to be effective in the treatment of rheumatoid arthritis (RA) [Weinblatt et al., 1985] and in the suppression of experimentally-induced arthritis in rodents [Kerwar and Oronsky, 1989]. Studies using rats with adjuvant arthritis have shown that MTX suppresses inflammation and joint destruction as measured by reduction in paw inflammation [Connolly et al., 1988; Jaffee et al., 1989; Welles et al., 1985], increase in final body weight [Connolly et al., 1988; Jaffee et al., 1989] and decreased lymphocyte activating factor activity from splenic leukocytes [Connolly et al., 1988]. Biochemical and cellular changes, which were coincident with the beneficial effects of MTX treatment in this model, included the restoration of elevated T helper / T suppressor cell ratios to control levels [Jaffee et al., 1989], inhibition of macrophage activation as measured by PGE₂ production [Johnson et al., 1988], IL-1
production and surface Ia antigen expression [Johnson et al., 1988; Hu et al., 1988] and restoration of diminished T cell function as measured by responses to mitogenic stimulation [Jaffee et al., 1989; Kourounakis and Kapusta, 1976]. In mice with an induced air pouch, MTX was shown to inhibit neutrophil chemotaxis into the pouch following a phlogistic stimulus [Suarez et al., 1987]. The pharmacokinetics and bioavailability of MTX in human studies have been well documented [Campbell et al., 1985; Brooks et al., 1990] however the mechanism of anti-inflammatory action is less well understood. MTX accumulates intracellularly where it may interfere with purine metabolism, however the physiologic effects of this intracellular accumulation on aspects of the immune response, the production of, or the cellular response to, inflammatory mediators is not known. It has been demonstrated that MTX enhances the release of adenosine, a potent anti-inflammatory substance, from human dermal fibroblasts and umbilical vein endothelial cells and inhibits neutrophil adherence [Cronstein et al., 1991]. In studies of immunologic responses in rheumatoid arthritis, treatment with MTX decreased spontaneous proliferation by mononuclear cells, decreased both the percentage and the absolute number of esterase positive mononuclear cells in peripheral blood [Johnston et al., 1986] and decreased spontaneous IgM rheumatoid factor synthesis by unstimulated mononuclear cells [Olsen et al., 1987]. It has also been reported that the polymorphonuclear leukocytes obtained from subjects with RA following a single 10 mg dose of MTX have a decreased chemotactic migration response to zymosan and fMLP [O'Callaghan et al., 1988], although this was not confirmed in subsequent studies [Sperling et al., 1990, 1992]. In these latter studies, suppression of neutrophil LTB4 synthesis following calcium ionophore stimulation of cells obtained from patients with RA after a single oral dose of MTX was seen. A single intramuscular dose of MTX also suppressed neutrophil LTB4 synthesis in patients with RA [Leroux et al., 1992].

The work described in this chapter was undertaken to investigate the effects of MTX in vitro and ex vivo on the production of the 5-lipoxygenase metabolites of arachidonic acid by rat and human neutrophils. The purpose of the studies was to more clearly define the biochemical effects of MTX on the 5-LO pathway. This was regarded
as a necessary basis for exploring potential synergistic biochemical and clinical effects on 5-LO metabolism of MTX and dietary supplementation with EPA.

7.2 RESULTS

7.2.1 *In vitro* effects of MTX on lipoxygenase metabolism in rat peritoneal exudate cells

*In vitro* incubation of rat PEC with MTX in concentrations ranging from 10 ng - 100 μg/mL, was associated with inhibition of production of 5-LO metabolites. The extent of inhibition was variable with maximum inhibition in individual experiments ranging from 14% - 77%. Figure 7.1 depicts data from a single experiment which illustrates the pattern of the effect of MTX on the production of LTB₄, the all-trans isomers and 5-HETE typically observed. The ω-oxidation product of LTB₄, 20-hydroxy LTB₄ (20-OH LTB₄), was not measurable following stimulation of rat neutrophils under the conditions used.

Effect of MTX and EPA *in vitro*

PEC were incubated in the presence or absence of MTX (50 ng or 100 ng) for 25 min at room temperature. EPA (final concentration 5 μM) or DPBS was added and the cell preparations were incubated for a further 5 min at 37° C prior to stimulation by calcium ionophore A23187 for 5 min followed by extraction and HPLC analyses. Inhibition of LTB₄ was greater in the presence of both MTX and EPA than with either inhibitor alone (Figure 7.2).
Figure 7.1  The effect of MTX on the production of the 5-lipoxygenase metabolites of arachidonic acid by rat peritoneal exudate cells *in vitro*. Each point represents the mean ± sd of quadruplicate incubations.

* significantly different from control values, p < 0.05, t-test.

(a), LTB₄; (b), the all-trans isomers of LTB₄; (c), 5-HETE.

This figure illustrates the results from one of eight independent experiments with concentrations of MTX ranging from 10 ng/mL - 100 µg/mL.
Figure 7.2  The effect of MTX and EPA on the synthesis of LTB₄ by rat PEC in vitro. Percentages represent the percent inhibition compared with the production of LTB₄ by rat PEC incubated with A23187 alone. Values represent the mean ± sd of triplicate incubations.
### 7.2.2 *Ex vivo* effects of MTX on lipoxygenase metabolism in rat peritoneal exudate cells

MTX was administered to rats via three routes, (a) subcutaneous, (b) intraperitoneal and (c) oral.

**A) Subcutaneous**

A single subcutaneous injection of 0.5 mg MTX/kg bodyweight into rats did not cause inhibition of lipoxygenase products following stimulation of cells harvested 4 or 6 h after injection (Table 7.1).

**Table 7.1** Effect of a single subcutaneous injection of MTX (0.5 mg/kg bodyweight) on the production of lipoxygenase metabolites by PEC stimulated *ex vivo* 4 h later (A) and 6 h later (B).\(^a\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LTB(_4)^b</th>
<th>LTB(_4) Isomers</th>
<th>5-HETE</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Saline</td>
<td>11.1 ± 3.4</td>
<td>9.5 ± 1.7</td>
<td>35.3 ± 9.3</td>
</tr>
<tr>
<td>(A) MTX</td>
<td>13.3 ± 2.4</td>
<td>9.3 ± 1.1</td>
<td>43.3 ± 8.6</td>
</tr>
<tr>
<td>(B) Saline</td>
<td>28.8 ± 5.9</td>
<td>20.5 ± 4.7</td>
<td>89.3 ± 21.5</td>
</tr>
<tr>
<td>(B) MTX</td>
<td>38.7 ± 8.4</td>
<td>23.4 ± 2.9</td>
<td>101.1 ± 17.3</td>
</tr>
</tbody>
</table>

\(^a\) PEC were obtained by lavage 4 h after i.p. injection of peptone as described in the Materials and Methods (2.2.C). Subcutaneous injection with MTX was performed concurrently with (A) or 2 h prior to (B) i.p. injection with peptone.

\(^b\) LTB\(_4\), the all-trans isomers of LTB\(_4\) and 5-HETE are expressed as ng/10^6 cells/5 min, mean ± sd, MTX (n=4 rats), normal saline (n=3 rats).
(b) Intraperitoneal

Similarly, there was no inhibition of lipoxygenase products in rat PEC elicited in the presence of i.p. MTX (1 mg/kg bodyweight) (Table 7.2). In one experiment there was a significant increase in LTB₄, the all-trans isomers of LTB₄ and 5-HETE following the administration of MTX (p < 0.01, t-test). However, this finding was not observed in 2 other experiments undertaken using the same conditions.

Table 7.2  Effect of a single intraperitoneal MTX injection (1 mg/kg bodyweight) on the production of lipoxygenase metabolites by rat PEC stimulated ex vivo.ᵃ

<table>
<thead>
<tr>
<th>Expt</th>
<th>Treatment</th>
<th>LTB₄ᵇ</th>
<th>LTB₄ Isomers</th>
<th>5-HETE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Saline</td>
<td>64.2 ± 26.1</td>
<td>30.7 ± 11.5</td>
<td>226.2 ± 78.4</td>
</tr>
<tr>
<td></td>
<td>MTX</td>
<td>59.4 ± 29.2</td>
<td>23.4 ± 7.0</td>
<td>179.5 ± 66.2</td>
</tr>
<tr>
<td>2</td>
<td>Saline</td>
<td>16.6 ± 2.8</td>
<td>12.2 ± 0.5</td>
<td>62.2 ± 14.1</td>
</tr>
<tr>
<td></td>
<td>MTX</td>
<td>38.0 ± 5.7ᶜ</td>
<td>21.2 ± 2.2ᶜ</td>
<td>104.6 ± 6.2ᶜ</td>
</tr>
<tr>
<td>3</td>
<td>Saline</td>
<td>21.1 ± 5.4</td>
<td>15.4 ± 2.0</td>
<td>114.9 ± 28.1</td>
</tr>
<tr>
<td></td>
<td>MTX</td>
<td>19.4 ± 6.2</td>
<td>16.2 ± 1.7</td>
<td>106.2 ± 40.6</td>
</tr>
</tbody>
</table>

ᵃ MTX was mixed with peptone and injected i.p. 4 h prior to harvesting PEC.

ᵇ LTB₄, the all-trans isomers of LTB₄ and 5-HETE are expressed as ng/10⁶ cells/5 min, mean ± sd, n=3 rats.

c indicates significant difference from the control group (t-test, p < 0.01).
There was no significant change in LT production following a single oral dose of MTX (0.5 mg/kg bodyweight) dispersed in Gum Tragacanth (Table 7.3). After gavage feeding for 7 consecutive days, LT production tended to be increased but this was statistically significant for the all-trans isomers of LTB4 only.

Table 7.3  Effect of oral administration of MTX (0.5 mg/kg bodyweight) on the production of lipoxygenase metabolites by rat PEC stimulated *ex vivo*.\(^a\)

<table>
<thead>
<tr>
<th>Duration of Treatment</th>
<th>Treatment</th>
<th>LTB4(^b)</th>
<th>LTB4 Isomers</th>
<th>5-HETE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Day</td>
<td>Control</td>
<td>29.1 ± 13.8</td>
<td>22.6 ± 6.7</td>
<td>84.9 ± 34.5</td>
</tr>
<tr>
<td></td>
<td>MTX</td>
<td>26.0 ± 5.7</td>
<td>18.5 ± 3.7</td>
<td>79.8 ± 11.6</td>
</tr>
<tr>
<td>7 Days</td>
<td>Control</td>
<td>21.9 ± 6.5</td>
<td>17.1 ± 3.3</td>
<td>71.0 ± 15.9</td>
</tr>
<tr>
<td></td>
<td>MTX</td>
<td>30.3 ± 11.1</td>
<td>24.7 ± 5.3(^c)</td>
<td>90.9 ± 27.2</td>
</tr>
</tbody>
</table>

\(^a\) Rats were gavaged daily with Gum Tragacanth (control) or MTX for either one day or seven consecutive days.

\(^b\) LTB4, the all-trans isomers of LTB4 and 5-HETE are expressed as ng/10⁶ cells/5 min, mean ± sd, n=4 rats.

\(^c\) indicates significant difference from the control group (t-test, p<0.05).

7.2.3  *In vitro* effects of MTX on lipoxygenase metabolism in neutrophils from healthy humans

Neutrophils were isolated from healthy human volunteers on three separate occasions and pre-incubated with MTX (30 min) before stimulation with A23187 (0.5 μM, final concentration). In each experiment there was a modest decrease in 5-LO products but there was variation between individuals in the concentration of MTX required for inhibition. Figure 7.3 depicts data from a single experiment which illustrates the type of inhibition curves typically obtained. No clear dose response relationship was observed.
Figure 7.3  The effect of MTX on the production of LTB₄ and the all-trans isomers of LTB₄ by human neutrophils *in vitro*. Each point represents the mean ± sem of quadruplicate incubations.

- • LTB₄;  O the all-trans isomers of LTB₄.

This figure is representative of 3 independent experiments with concentrations of MTX ranging from 100 ng - 100 μg/mL.
LTB$_4$ and the all-trans isomers (ng/10$^6$ cells/5 min)
7.2.4 *Ex vivo* effects of MTX on lipoxygenase metabolism in neutrophils from subjects with rheumatoid arthritis

All nine subjects studied were taking at least one other agent (Table 7.4). Four subjects were commencing MTX therapy (Group A) and five subjects were established on a regimen of weekly oral MTX (Group B). The effect of MTX was assessed 12 h after a single oral dose. Pre-MTX and post-MTX blood samples were taken in the mornings on two consecutive days. Thus, LT production was assessed before and after the subject's first dose of MTX (Group A) or before and after their usual weekly dose of MTX (Group B).

**Table 7.4** Details of methotrexate therapy and concomitant medications in subjects studied.a

<table>
<thead>
<tr>
<th>Case Number</th>
<th>Weekly MTX dose (mg)b</th>
<th>Duration of treatment (months)</th>
<th>Single dose studied (mg)c</th>
<th>Concomitant therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>methylprednisolone</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>auranofin</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>indomethacin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>auranofin</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>auranofin</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>diclofenac</td>
</tr>
<tr>
<td>Group B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>4</td>
<td>7.5</td>
<td>piroxicam</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>13</td>
<td>10</td>
<td>methylprednisolone</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ibuprofen</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>sulphasalazine</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>5</td>
<td>10</td>
<td>naproxen sodium</td>
</tr>
<tr>
<td>8</td>
<td>7.5</td>
<td>12</td>
<td>10</td>
<td>indomethacin</td>
</tr>
<tr>
<td>9</td>
<td>7.5</td>
<td>5</td>
<td>10</td>
<td>diltiazem</td>
</tr>
</tbody>
</table>

a Group A; subjects commencing therapy, Group B; subjects on established therapy.

b Dose most commonly used during established therapy (Group B).

c Blood samples for LT assays were taken 12 h before and 12 h after a single oral dose of MTX.
The data from these studies were analysed by paired t-test. After the MTX dose, an increase in each of the 5-LO metabolites was observed in 8/9 subjects (Figure 7.4). The mean LTB4 production increased from 18.5 ± 7.4 ng/10⁶ cells/5 min to 24.5 ± 7.3 ng/10⁶ cells/5 min (p=0.01). There was also a significant increase in the all-trans isomers (26.9 ± 10.6 to 36.8 ± 9.7 ng/10⁶ cells/5 min; p=0.005). The mean 5-HETE production increased from 91.7 ± 42.9 to 114.3 ± 36.3 ng/10⁶ cells/5 min; however, this increase was not statistically significant (p=0.10). 20-OH LTB4 was measured in 7/9 subjects and as with the other measured 5-LO metabolites, an increase was seen following oral MTX (19.2 ± 8.0 to 24.8 ± 11.3 ng/10⁶ cells/5 min; p=0.02).

The enhancing effect of MTX on 5-LO metabolism was seen in subjects commencing therapy (Group A) in whom significant increases in LTB4 (p=0.01), isomers (p=0.009) and 5-HETE (p=0.005) were seen in post-dose compared to pre-dose values. However, the change in mean values for 5-LO metabolites following stimulation of neutrophils obtained from subjects on established therapy (Group B) was not statistically significant.
Figure 7.4  Production of 5-LO metabolites of arachidonic acid by stimulated human neutrophils *ex vivo* 12 h before (pre-) and 12 h after (post-) a single oral dose of MTX.

- - - - subjects commencing MTX therapy (Group A);

O---O  subjects on established MTX therapy (Group B).

* p=0.02, ** p=0.01, *** p=0.005, paired t-test.
**LTB$_4$**

$\text{ng/10}^6\text{cells/5 min.}$

**Pre-MTX**  **Post-MTX**

**All-trans isomers of LTB$_4$**

**Pre-MTX**  **Post-MTX**

**20-OH LTB$_4$**

$\text{ng/10}^6\text{cells/5 min.}$

**Pre-MTX**  **Post-MTX**

**5-HETE**

$\text{ng/10}^6\text{cells/5 min.}$

**Pre-MTX**  **Post-MTX**

$**$, $***$, $*$
7.3 DISCUSSION

It has been shown that treatment of rats with oral MTX in the range of 0.1 - 1.0 mg/kg/day from the time of adjuvant injection, leads to significant protection against development of polyarthritis [Connolly et al., 1988; Jaffee et al., 1989; Johnson et al., 1988; Hu et al., 1988; Ridge et al., 1988]. It has been reported that no inhibition of 5-LO activity occurs in casein-elicited guinea pig neutrophils following MTX addition in vitro, although the data were not shown [Welles et al., 1985]. In the studies described in this chapter, in vitro incubation with MTX consistently showed a weak inhibitory effect on LT production by calcium ionophore stimulated cells harvested from both rats or humans. However, no clear or reproducible dose response relationship was discernible. MTX is rapidly cleared from the circulation and it has been reported that a single oral dose of 15 g of MTX (< 0.1 mg/kg bodyweight) in rats results in serum levels of 33 ± 8.0 nM after 2 h and < 1 nM at 8 h [Welles et al., 1985]. The concentration range (1 ng/mL - 100 μg/mL) used in the in vitro studies described here is equivalent to approximately 2 nM - 200 μM MTX and encompasses the range of serum levels observed in rats with adjuvant arthritis during oral therapy with MTX at doses resulting in an anti-inflammatory effect [Welles et al., 1985; Ridge et al., 1988]. However, no effect on LT production was observed after in vivo treatment of healthy rats with MTX. In one experiment there was an increase in 5-LO metabolism following stimulation of PEC elicited in the presence of i.p. MTX. However, this finding was not observed in two other replicate experiments. Three routes of administration were assessed and the doses chosen were comparable with those shown to exert effective prophylaxis of the clinical symptoms of adjuvant arthritis models of inflammation. Differences in MTX concentration at the site of 5-LO activity, the degree of polyglutamation of MTX and the time of cell harvesting after MTX administration could all be factors contributing to the lack of inhibition observed following stimulation of PEC collected after in vivo administration of MTX compared with that seen in the in vitro experiments.

Within the cell cytoplasm, MTX is polyglutamated by sequential linkage of
glutamyl residues to the terminal glutamyl moiety of the molecule by the enzyme folypolyglutamate synthetase [Barreuco et al., 1992]. It appears that the longer chain length polyglutamates of MTX are more pharmacologically active than the parent compound and are also more readily retained inside the cell. Cells which carry out the conversion of MTX rapidly and accumulate polyglutamates of two or greater glutamate residues are more susceptible to the cytotoxic effects of MTX because this conversion allows accumulation of MTX within the cell. It is possible that the conditions of in vivo administration or in vitro pre-incubation used in these experiments do not allow for optimal metabolism of MTX with regard to its effects on the 5-lipoxygenase pathway.

In the human ex vivo study, there was typically stimulation rather than inhibition of the production of LTB₄, the all-trans isomers of LTB₄, 20-OH LTB₄ and 5-HETE following a single oral dose of MTX taken by subjects with RA. These results contrast with those of previous studies in which inhibition was observed after an oral dose [Sperling et al., 1990, 1992] and an intra-muscular dose of MTX [Leroux et al., 1992]. Differences in experimental conditions could account for the different findings. For example, under the assay conditions of the previously published studies [Sperling et al., 1990, 1992] 10 times higher concentrations of neutrophils were stimulated with 20 fold higher concentrations of A23187 for 3 - 4 times longer than in the study described in this thesis. The different outcomes raise questions about whether these arbitrarily chosen conditions of stimulation and incubation in vitro accurately reflect conditions at the inflammatory site in vivo. Thus, either inhibition or stimulation of LTB₄ production (or both) could be an artefact of conditions of stimulation in vitro, albeit influenced by prior exposure of neutrophils to MTX in vivo.

A further consideration is the time of blood sampling for assessment of 5-LO metabolism after the MTX dose. Peak plasma concentration levels are reached within 2 h following oral administration of MTX but there is marked interindividual variation in bioavailability [Oguey et al., 1992]. In the studies reporting inhibition of LTB₄ production by human neutrophils, samples were taken 24 h after MTX administration [Sperling et al., 1990, 1992; Leroux et al., 1992]. In this study, blood samples were taken 12 h after MTX administration which may be a significant difference when
assessing the pharmacological activity of MTX. The inhibition of 5-LO activity by weekly oral MTX described by Sperling et al., (1992), appears to be cumulative over a 6 - 8 week period. In my study there was a suggestion that duration of MTX therapy may have an effect since enhancement of 5-LO metabolism by MTX therapy was greater in subjects commencing therapy (Group A). However, the lack of a significant increase in 5-LO metabolites in subjects on established therapy (Group B) could be accounted for by a substantial fall in all metabolites produced by a single cell preparation from one subject. Therefore, due to the relatively small numbers studied in the two groups, it is not possible to infer a change in response related to duration of therapy.

Concomitant therapies of all subjects are shown in Table 7.4. It has been reported that auranofin [James et al., 1992] and sulphasalazine [Horn et al., 1991] are inhibitors of 5-LO and therefore it is unlikely that the presence of these drugs could account for the increased production of 5-LO metabolites described here.

In summary, the in vitro addition of exogenous MTX resulted in a weak suppression of rat and human neutrophil LTB4 and 5-HETE production, but with no clear dose response relationship. The results from administration of MTX to rats by different routes and for different periods did not yield any consistent change in production of the 5-LO metabolites. By contrast, a single oral dose of MTX given to patients with RA resulted in increased production of the 5-LO metabolites of AA at 12 h, particularly in patients commencing therapy. These results do not support the putative inhibitory effect of MTX on 5-LO metabolism. Further studies are required to reconcile differences between this and other published studies. Important questions remain, particularly in relation to the time course of action of MTX, it's distribution and it's state of metabolism within leukocytes and the possible influence of different conditions of cell priming and stimulation.
CHAPTER 8

GENERAL DISCUSSION AND DIRECTIONS

8.1 DISCUSSION AND CONCLUSIONS

Studies in paleolithic nutrition and modern-day hunter-gatherer populations provide information suggesting that man evolved on a diet that was much lower in saturated fats than today’s diet and had a ratio of n-6 to n-3 fatty acids of approximately 1 [Simopoulos, 1991]. From a period as recent as the industrial revolution, food type and consumption patterns have changed rapidly. Modern agricultural techniques have led to significant decreases in the n-3 fatty acid content in domesticated livestock and underpin the widespread use of hydrogenated, n-6-rich vegetable oil spreads. Consequently the ratio of n-6 to n-3 fatty acids in today’s Western diet is closer to 20 - 25:1, suggesting a relative imbalance in the proportions of n-3 and n-6 fatty acids compared with the diet on which humans evolved.

Epidemiological studies, clinical trials and experimental work with animal models have consistently suggested that an increase of dietary n-3 fatty acids in the Western diet would be beneficial. Dietary n-3 enrichment in clinical studies has usually been in the form of fish oil supplements which primarily increase the dietary content of long chain polyunsaturated fatty acids, EPA (20:5 n-3) and DHA (22:6 n-3).

The work described in this thesis was designed to investigate some of the influences, effects and inter-relationships between n-3 fatty acids and the inflammatory process. Therapeutic strategies designed to increase the anti-inflammatory effect of fish oil have not been extended far beyond the stage of simply providing dietary fish oil supplements. For further progress more information is required regarding both the efficiency of incorporation of certain fatty acids from dietary fish oil and the biochemical and cellular sites of their anti-inflammatory action. Typically, studies designed to identify conditions favouring incorporation of different fatty acids into cellular phospholipids or plasma have been carried out on healthy animals or subjects.
with the intention of applying the information to inflammatory systems. In the studies described in Chapter 3 of this thesis I was able to demonstrate that severe systemic inflammation caused by adjuvant-induced arthritis in rats has little effect on the rate or level of EPA incorporation or AA depletion during dietary supplementation with fish oil. Rat adjuvant-induced arthritis is often used as an animal model to mimic aspects of rheumatoid arthritis such as features of joint histopathology and blood changes. These results thus allow confidence that dietary studies undertaken in healthy human subjects should provide a guide to conditions for dietary intervention studies in rheumatoid arthritis and other inflammatory disorders. However, the issue of biochemical and clinical effects of a given dietary treatment strategy (alone or in combination with conventional drug therapy) must ultimately be answered through an appropriately designed study of subjects with the condition in question.

In recent years there has been an increasing awareness that n-6 and n-3 PUFAs are metabolically and functionally distinct and as a consequence, there has been considerable interest in manipulation of dietary n-3 and n-6 ratios. This has been accompanied by the realization that n-3 and n-6 fatty acids and their derived eicosanoids have differing physiological effects in platelet vascular homeostasis and inflammation. There have been detailed studies directed at determining the mechanisms of LTB₄ inhibition by dietary fish oil and the subsequent production of LTB₅. The synthesis of LTB₅ appears to be of little consequence for neutrophil mediated inflammatory responses since it has much less neutrophil chemotactic activity than LTB₄. Similarly, there is some information available on the effects of fish oil on the cyclooxygenase products PGI₂ and TXB₂, and the relative production and biological activities of the corresponding 3-series PGs. In general however, there has been insufficient discrimination between PGE₂ and PGE₃ in examining the effect of fish oil on PGE synthesis. I have presented evidence that PGE₃ has substantial oedemogenic activity when injected into the hind paw of mice (Chapter 4) which indicates the importance of separate quantitation of PGE₂ and PGE₃. Other attempts to detect PGE₃ as a separate entity from PGE₂ have involved the use of gas chromatography-mass spectrophotometry with selective ion monitoring. Chapter 5 describes the successful
development of an assay for the separate detection of PGE3. Standard PGE3 was derivatized with panacyl bromide, separated from reactants on TLC and resolved by HPLC with fluorometric detection. Following the development of the assay using standard prostaglandins, I applied the assay technique to biological samples derived from cultured adherent cells (Chapter 6). Although able to detect biological PGE2, I was unable to demonstrate the production of PGE3 following in vitro or in vivo EPA administration. Although my earlier studies showed that PGE3 and PGE2 have comparable inflammatory activity when injected directly into the mouse paw, the subsequent studies do not provide evidence that PGE3 is generated in sufficient quantities to play a major role in mediating inflammatory responses during cellular interactions. LTB4, an eicosanoid from the 5-lipoxygenase pathway however, is known to play a significant role in promoting and maintaining inflammation through its potent chemotactic, chemokinetic and stimulatory functions. Dietary fish oil leads to the inhibition of the production of LTB4 and therefore it can be proposed that EPA may act in conjunction with pharmacological leukotriene inhibitors to increase their anti-inflammatory effects. Chapter 7 describes experiments designed as a forerunner to assessing possible synergistic or additive effects between methotrexate and EPA with regard to inhibition of 5-LO. MTX was found to be a weak inhibitor of rat and human neutrophil lipoxygenase in vitro. There was no evidence of inhibition following the in vivo administration of MTX to healthy rats or following a single oral dose in RA patients. The findings failed to confirm early claims by Sperling et al., (1990, 1992), that MTX is an inhibitor of LTB4 synthesis and therefore failed to provide support for selection of MTX as an agent to be used with EPA for possible additive or synergistic effects on LTB4 production and anti-inflammatory effects.
8.2 SCOPE FOR FURTHER STUDIES

8.2.1 Dietary studies

The potentially beneficial effects of dietary fish oil appear to be related to the presence of n-3 fatty acids and EPA in particular, and their competition with n-6 fatty acids, the abundance of which greatly exceeds essential requirements in a normal Western diet. It is therefore important to consider factors affecting the absorption and incorporation of EPA into inflammatory effector cells such as neutrophils and mononuclear leukocytes. Oil mixing studies have shown that the combination of fatty acid types is a factor in determining efficiency of incorporation of dietary EPA into leukocyte phospholipids [James et al., 1991a]. In this study, the addition of sunflower oil (rich in n-6 fatty acids, in particular linoleic acid, the 18 carbon precursor of AA) to a rat diet containing fish oil resulted in decreased incorporation of EPA into rat neutrophil membrane phospholipids compared with the incorporation achieved with the same amount of fish oil alone. This was accompanied by an increase in neutrophil LTB4 production relative to the fish oil only diet. Both linseed oil (rich in ALA, the 18 carbon precursor of EPA) and olive oil (rich in monounsaturates) had less EPA-lowering and LTB4-raising effect than sunflower oil and the effect appeared to be related to the respective levels of dietary LA. These results were confirmed in a study with healthy volunteers in which neutrophils were found to contain more EPA when fish oil supplements were taken for 4 weeks with a low LA, olive oil-rich diet, compared with a high LA, sunflower oil-rich diet [Cleland et al., 1992]. Further clinical studies are needed to determine achievable means of maximizing the EPA/AA ratio which, based on data obtained using rats [James et al., 1991a; Cleland et al., 1990b], should enhance inhibition of LTB4 and related anti-inflammatory effects.

Another approach to maximizing inhibition of LTB4 synthesis includes the dietary fortification with the n-9 analogue of both EPA and AA, eicosatrienoic acid (ETrA, 20:3 n-9). In the presence of essential fatty acid deficiency (EFAD), oleic acid (OA, 18:1 n-9) becomes the dominant 18 carbon fatty acid and can be metabolised to
ETrA which accumulates in plasma and cellular phospholipids. ETrA can be converted to LTA3 which is less efficiently metabolised by LTA hydrolase than LTA4 (derived from AA) but can competitively inhibit the production of LTB4 from LTA4. It has been reported that EFAD is associated with reduced inflammation in animal models of inflammation [Bonta et al., 1974, 1977; Denko, 1976] and leads to suppression of LTB4 production by murine neutrophils stimulated in vivo [Lefkowith, 1988; Lefkowith et al., 1990]. Studies undertaken in our laboratory have shown that the neutrophils obtained from a subject with fatty acid changes indicative of severe EFAD demonstrated impaired synthesis of LTB4 when stimulated by A23187 ex vivo [Cleland et al., submitted for publication]. Further investigations into the anti-inflammatory effects of ETrA are hindered by the lack of a high yield biological or synthetic source for its production but the possible advantages of ETrA supplementation over EPA supplementation justify its examination as a possible anti-inflammatory agent of the future.

8.2.2 Drug/diet interactions

The effect of the anti-inflammatory drug methotrexate on the 5-LO pathway was investigated during the course of this thesis. Amid the continuing search for eicosanoid inhibitors, the potential for additive or synergistic effects with appropriate combinations of fish oil and pharmacological agents persists. Other drugs to consider in studies such as these include Zileuton, SK&F86002, bestatin and captopril amongst many others. The potential benefits of complementary EPA and drug actions may be to increase the anti-inflammatory effect of fish oil with lower doses than have been used in clinical trials to date and to decrease anti-rheumatic drug use.

8.2.3 Eicosanoid/cytokine interactions

There is evidence of mutual regulation between the two principal classes of inflammatory mediators - the lipid eicosanoids and the peptide cytokines. Interleukin-1
α (IL-1α), IL-1β and tumour necrosis factor-α (TNF-α) are polypeptide products of macrophages produced in response to an appropriate stimulus such as infection, injury or a specific activating agent. They share a range of physiological functions including the induction of fever, activation of neutrophils, induction of surface antigens on endothelial cells, activation of fibroblasts and enhancement of immune responses [Scales et al., 1989]. Macrophages are also the predominant producers of PGE2 which is known to contribute to the pain, oedema and erythema characteristic of the inflammatory response. PGE2 has been reported to cause a dose-dependent suppression of IL-1 and TNF release from LPS-stimulated macrophages and, conversely, IL-1 or TNF alone will stimulate the release of PGE2 from monocytes and macrophages [Kunkel et al., 1986; Dinarello et al., 1983] suggesting an autoregulatory loop mechanism. Data from Northern blot and nuclear transcription analyses of suitably treated murine macrophages suggests that PGE2 affects TNF-α production at the level of transcription, whereas effects on IL-1α,β activity seem to be post-transcriptional [Scales et al., 1989]. Another eicosanoid commonly found at sites of inflammation is LTB4, produced primarily by neutrophils but also by macrophages and possibly lymphocytes. LTB4 enhances IL-1 production by LPS-stimulated human peripheral blood monocytes [Rola-Pleszczynski and Lemaire, 1985; Tatsuno et al., 1990]. It has also been suggested that lipoxygenase inhibitors are able to suppress the formation of TNF by murine peritoneal macrophages in vitro and in vivo [Schade et al., 1989]. Leukotrienes B4, C4 and D4 are all able to mediate the helper cell requirements for IFN-γ production, a process which appears to usually require IL-2 help [Johnson and Torres, 1984].

In addition to these studies directly examining the inter-relationships between the lipid mediators and the peptide mediators, there is further evidence of possible interactions from dietary studies. It has been reported that decreased production of IL-1α, IL-1β and TNF by stimulated human peripheral blood mononuclear cells was accompanied by a decreased ratio of AA to EPA in the cell membrane phospholipids following dietary supplementation with 18 g MaxEPA/day for 6 weeks [Endres et al. 1989]. Similarly, large reductions in IL-1β and TNF synthesis by mononuclear cells
from post-menopausal woman after dietary supplementation with fish oil have been
demonstrated [Meydani et al., 1991]. The mechanism underlying the suppression of
IL-1 and TNF production following dietary intervention with fish oil is unknown but is
postulated to be related to alterations in AA availability and metabolism.

The implications of an effect of dietary fish oil supplementation on the
production of cytokines coupled with the well documented effects on eicosanoids are
far reaching. Further information is required to understand the possible effects which
alteration of the fatty acid profile may have on cellular events in addition to the
modulation of inflammatory mediators. For example, it is possible that a change in the
EPA/AA ratio may alter integrin expression. Integrins are transmembrane receptors
with specific ligand binding preferences, the expression of which has important
implications in bone metabolism. The integrin subfamilies include the principle
collagen receptor (VLA2), the receptor for VCAM-1 (a cell adhesion molecule found
on cytokine-activated endothelial cells), the fibronectin receptor, which may be
involved in cell differentiation, and the cytoadhesin subfamily [Milam et al., 1991].
IL-1β has been shown to regulate differentially α-subunit integrin mRNA expression in
a human osteosarcoma cell line and to stimulate AA metabolism leading to increased
PGE2 production. However, PGE2 synthesized in response to IL-1β, appears to
function in a negative regulatory loop to counteract the stimulatory effect of IL-1β on
integrin expression. Therefore, in cells in which PG synthesis is markedly stimulated
by IL-1β the final effect may be down-regulation of the expression of certain integrins.
Conversely, if the direct effects of IL-1β predominate, an up-regulation would be
expected [Milam et al., 1991]. Alterations in the abundance or type of integrins on the
cell surface can affect cell-extracellular matrix interactions or cell-cell interactions and
thus affect inflammatory leukocyte infiltration into tissues.

Recent investigations into the protective effect of dietary fortification with n-3
fatty acids suggest that alterations in leukocyte traffic may be a component of the
beneficial effect [Lefkowith et al., 1991]. EFAD, characterized by the depletion of n-6
fatty acids and the appearance of certain n-9 fatty acids, has been shown to decrease the
accumulation of macrophages at a focus of injury [Schreiner et al., 1989]. This
alteration in leukocyte traffic has been considered a result of the decreased production of chemoattractants such as LTB₄ and PAF. However, there is evidence that arachidonate itself may be an important mediator of leukocyte adhesion [Lefkowith et al., 1991]. In this study, inhibition of the major eicosanoid forming pathways of AA metabolism in macrophages did not affect adherence but the inhibition of phospholipase did, an effect that could be overridden by addition of arachidonate. The mechanism may involve an alteration in cell surface adherence molecule function without an effect on surface molecule expression, a possibility which is supported by evidence that cell membrane lipids may play a crucial role in determining integrin binding characteristics [Conforti et al., 1990]. There is also scope for investigations into the effect of n-6/n-3 ratio alterations on membrane fluidity and, consequently, integrin function.

8.2.4 Prophylactic dietary intervention?

Epidemiological studies and controlled therapeutic studies have established that dietary fatty acids can influence inflammatory responses. Consumption of diets rich in n-3 fatty acids and low in n-6 fatty acids has been associated with a low incidence of occlusive vascular disease, asthma and psoriasis. Even dietary fish-oil supplements taken against a background diet high in n-6 fatty acids have a modest but reproducible anti-inflammatory effect on the signs and symptoms of established rheumatoid arthritis. The beneficial effects of fish oils are largely dependent on the final ratio of AA to EPA, these two 20 carbon fatty acids being the immediate precursors of the eicosanoids of the n-6 and n-3 families respectively. N-6 eicosanoids have a range of potent biological activities including pro-inflammatory effects whereas the n-3 eicosanoids appear to be less pro-inflammatory. Understanding the relative production of these mediators and their mechanisms of action will allow the anti-inflammatory effects of dietary fat manipulation to be improved.

Studies to date have examined strategies involving dietary fat manipulations designed to reduce the severity of established inflammatory diseases. An issue which is more difficult to investigate, but potentially more beneficial, is the effect of altering the
ratio of dietary n-6/n-3 fatty acids consumed by a community on the emergence of inflammatory disease. This question could be addressed using epidemiological methods of data acquisition and analysis together with analysis of diets consumed, possibly in response to dietary advice targeted at a particular community or population. Such studies will entail major challenges in design and execution, but may be a necessary step if the health giving potential of dietary fat modification is to be realized fully.
BIBLIOGRAPHY


