NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

METABOLISM OF LEUKOTRIENE B_4 AND OTHER HYDROXYLATED EICOSANOIDS BY THE 12-HYDROXYEICOSANOID DEHYDROGENASE/10,11-REDUCTASE PATHWAY

by

Sandra L. Wainwright

Department of Medicine

Division of Experimental Medicine

McGill University, Montreal

January, 1992

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ISBN 0-315-74936-9

ABSTRACT

Leukotriene B_4 (LTB_4) has previously been reported to be metabolized by porcine polymorphonuclear leukocytes (PMNL) to 10,11-dihydro-LTB_4 and 10,11-dihydro-12-oxo-LTB_4 [Powell, W.S., & Gravelle, F. (1989) *J. Biol. Chem.* **264**, 5364-5369]. The 10,11-dihydro-LTB_4 fraction, prepared as described above, was resolved into two components by normal-phase (NP) high pressure liquid chromatography (HPLC). These products were identified as 10,11-dihydro-LTB_4 and 10,11-dihydro-12-epi-LTB_4 by gas-chromatography mass-spectrometry (GC-MS) and by co-chromatography with authentic chemically synthesized compounds. In the presence of NAD^+, a microsomal 12-hydroxyeicosanoid dehydrogenase converted LTB_4 to 12-oxo-LTB_4, which was identified by GC-MS. This enzyme catalyzes the initial rate-limiting step in the formation of dihydro metabolites of LTB_4. 12-Oxo-LTB_4 was rapidly metabolized to 10,11-dihydro-12-oxo-LTB_4 by a cytosolic 10,11-reductase in the presence of NADH or NADPH. LTB_4 was not converted to any products by this fraction.

12(S)-Hydroxy-5,8,10,14-eicosatetraenoic acid (12(S)-HETE) was metabolized by intact porcine PMNL to 12-oxo-5,8,14-eicosatrienoic acid, 12(R)-hydroxy-5,8,14-eicosatrienoic acid and 12(S)-hydroxy-5,8,14-eicosatrienoic acid, which were identified by GC-MS and nuclear magnetic resonance spectroscopy. The latter two compounds were separated by NP-HPLC following derivatization with methoxy-(trifluoromethyl)phenylacetic acid and were identified by co-chromatography with authentic compounds. 12(S)-HETE was metabolized by 12-hydroxyeicosanoid dehydrogenase, in the presence of NAD^+, to 12-oxo-ETE, identified by its chromatographic and UV spectral properties. 13-Hydroxy-9,11-octadecadienoic acid (13-HODE) was metabolized by porcine PMNL to 13-hydroxy-9-octadecenoic acid and 13-oxo-9-octadecenoic acid which were identified by GC-MS. Specificity studies indicated that the porcine PMNL 12-hydroxyeicosanoid dehydrogenase preferentially oxidizes substrates with a 12-hydroxyl group preceded by at least two conjugated double bonds and followed by a 2-*cis*-octenyl group.

RESUME

Il a été rapporté, auparavant, que la leucotriène B_4 (LTB_4) était métabolisée par les leucocytes polymorphonucléaires du porc (PMNL) en 10,11-dihydro-LTB_4 et en 10,11-dihydro-12-oxo-LTB_4 [Powell, W.S., & Gravelle, F. (1989) *J. Biol. Chem.* **264**, 5364-5369]. La fraction 10,11-dihydro-LTB_4, préparée tel que décrit ci-haut, a été séparée en deux composantes par chromatographie liquide à haute pression (phase normal, NP-HPLC). Ces produits ont été identifiés comme étant les 10,11-dihydro-LTB_4 et 10,11-dihydro-12-epi-LTB_4 par chromatographie gazeuse et spectrographie de masse (GC-MS) ainsi que par co-chromatographie en utilisant les produits authentiques synthétisés chimiquement. En présence de NAD^+, une déshydrogénase 12-hydroxyeicosanoide microsomiale a transformé LTB_4 en 12-oxo-LTB_4, laquelle a été identifiée par GC-MS. Cet enzyme catalyse l'étape initiale limitant la vitesse lors de la formation des métabolites dihydro de la LTB_4. La 12-oxo-LTB_4 a été rapidement métabolisée en 10,11-dihydro-12-oxo-LTB_4 par une 10,11-réductase cytosolique en présence de NADH ou de NADPH. La LTB_4 n'a pas été convertie en aucun produit par cette fraction.

L'acide 12(S)-hydroxy-5,8,10,14-eicosatétraènoique (12(S)-HETE) a été métabolisé par les PMNL intactes du porc intactes en 12-oxo-5,8,10,14-eicosatriénoique, en acide 12(R)-hydroxy-5,8,14-eicosatriénoique et en acide 12(S)-hydroxy-5,8,14-eicosatriénoique, qui ont été identifiés par GC-MS et par spectroscopie de résonnance magnétique nucléaire. Les deux dernières composantes ont été séparées par NP-HPLC après réaction avec l'acide méthoxy-(trifluorométhyl)phénylacétique et ont été identifiées par co-chromatographie en utilisant les produits authentiques. La 12(S)-HETE a été métabolisée par l'enzyme 12-hydroxyeicosanoide déshydrogénase, en présence de NAD^+, en 12-oxo-ETE, identifiées par ses propriétés chromatographiques et de celles du spectre UV. L'acide 13-hydroxy-9,11-octadécadiénoique (13-HODE) a été métabolisé par les PMNL du porc en acide 13-hydroxy-9-octadécénoique et en acide 13-oxo-9-octadécénoique, identifiés par GC-MS. Des études de spécificité ont indiqué que la 12-

hydroxyeicosanoide déshydrogénase des PMNL du porc oxydait de préférence les substrats contenant un groupe 12-hydroxyl précédé d'au moins deux doubles liaisons conjuguées et suivi d'un groupe 2-cis octényl.

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

LIST OF ABBREVIATIONS

g	gram
mg	milligram
μg	microgram
l	liter
ml	milliliter
μl	microliter
mmol	millimole
μmol	micromole
nmol	nanomole
pmol	picomole
M	molar
mM	millimolar
h	hour
s	second
min	minute
x *g*	times gravity
cpm	counts per minute
dpm	disintegrations per minute
Ci	Curie
^{3}H	tritium
^{2}H	deuterium
^{14}C	carbon-14
° C	degrees Celsius
IC_{50}	half maximal inhibitory concentration
t_R	retention time
ODS	octadecylsilyl
HPLC	high-pressure liquid chromatography

NP	normal-phase
RP	reversed-phase
GC-MS	gas chromatography-mass spectrometry
eV	electron volt
m/z	mass to charge ratio
TMS	trimethylsilyl
Me	methyl
NMR	nuclear magnetic resonance
ppm	parts per million
μA	microamp
kV	kilovolt
UV	ultraviolet
V_{max}	maximal velocity
K_m	Michaelis Menton constant
PMNL	polymorphonuclear leukocyte
PUFA	polyunsaturated fatty acid
PG	prostaglandin
PGDH	15-hydroxyprostaglandin dehydrogenase
LT	leukotriene
PGI_2	prostacyclin
TX	thromboxane
HHTrE	12-hydroxy-8,10-heptadecatrienoic acid
MDA	malondialdehyde
Hx	hepoxilin
Lx	lipoxin
DiHETE	dihydroxyeicosatetraenoic acid
EET	epoxyeicosatrienoic acid
FLAP	5-lipoxygenase activating protein
PLA_2	phospholipase A_2

PLAP	phospholipase activating protein
SRS-A	slow-reacting substance of anaphylaxis
IL	interleukin
NSAID	non steroidal anti-inflammatory drug
ETYA	5,8,11,14-eicosatetraynoic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenebis-(oxyethylenenitrilo)tetraacetic acid
MTPA	(R)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid
ATP	adenosine 5'-triphosphate
GTP	guanosine 5'-triphosphate
cDNA	recombinant deoxyribonucleic acid
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
arachidonic acid	5,8,11,14-eicosatetraenoic acid
linoleic acid	9,12-octadecadienoic acid
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
5-HETE	5-hydroxy-6,8,11,14-eicosatetraenoic acid
5-HPETE	5-hydroperoxy-6,8,11,14-eicosatetraenoic acid
12-HETE	12-hydroxy-5,8,10,14-eicosatetraenoic acid
12-HPETE	12-hydroperoxy-5,8,10,14-eicosatetraenoic acid
15-HETE	15-hydroxy-5,8,11,13-eicosatetraenoic acid
15-HPETE	15-hydroperoxy-5,8,11,13-eicosatetraenoic acid
13-HODE	13-hydroxy-9,11-octadecadienoic acid
13-HPODE	13-hydroperoxy-9,11-octadecadienoic acid
9-HODE	9-hydroxy-10,12-octadecadienoic acid
9-HPODE	9-hydroperoxy-10,12-octadecadienoic acid

dh-LTB_4	5(S),12(S)-dihydroxy-6,8,14-eicosatrienoic acid
dh-12e-LTB_4	5(S),12(R)-dihydroxy-6,8,14-eicosatrienoic acid
dho-LTB_4	5(S)-hydroxy-12-oxo-6,8,14-eicosatrienoic acid
12(S)h-20:3	12(S)-hydroxy-5,8,14-eicosatrienoic acid
12(R)h-20:3	12(R)-hydroxy-5,8,14-eicosatrienoic acid

Acknowledgements

I would like to take this opportunity to thank Dr. W.S. Powell for his close supervision and support of the work presented in this thesis. His dedication to research excellence and inovative thinking are qualities I will strive for in my future work as an independent researcher.

I gratefully acknowledge Drs J.R. Falck and P. Yadagiri from the University of Texas Southwestern Medical Center, Dallas, Texas, for their work in producing synthetic standards for this study as well as for the NMR analysis which they performed.

I gratefully acknowledge Dr. A. Brash from the Vanderbilt University Medical School, Nashville, Tennesse, for helpful discussions concerning the synthesis of 12(R)-HETE.

I gratefully acknowledge Drs L. Wolfe and L. Pellerin from the Montreal Neurological Institute, Montreal, Quebec, for sharing their protocol and equipment for preparing monohydroxyeicosanoids by photooxidation.

I would like to thank Francine Gravelle for sharing her technical expertise and for her support and friendship during my time in the laboratory.

Finally, I would like to express my deepest gratitude to my husband Paul, who provided me with unwavering support for my work. His confidence, encouragement and sense of humor proved invaluable on many occasions.

Section I

INTRODUCTION

1. Metabolic pathways which produce hydroxylated polyunsaturated fatty acids

The discovery and characterization of biologically active metabolites of arachidonic acid, which has now spanned six decades of industrious research, has produced many new insights into the regulation of cell function in general, as well as the role of lipid mediators in the body.

There are several enzymes which catalyze the oxygenation of arachidonic acid. Prostaglandin endoperoxide synthase converts arachidonic acid to an endoperoxide intermediate which may be further metabolized by other enzymes, producing prostaglandins, thromboxanes and prostacyclin. There are several lipoxygenase enzymes which catalyze the addition of molecular oxygen to specific carbons of arachidonic acid. The hydroperoxy products are often rapidly reduced to monohydroxy compounds, but in some cases epoxy, epoxyhydroxy and trihydroxy metabolites may also be formed. Of particular importance is an epoxy metabolite of arachidonic acid produced by 5-lipoxygenase, which may be further metabolized to leukotrienes. Cytochrome P-*450* monooxygenases also metabolize arachidonic acid to mono-hydroxy and epoxy compounds. In general, the profile of arachidonic acid metabolites produced by a given cell is determined by the enzymes which are present in that cell type. It should be noted that other polyunsaturated fatty acids (PUFA's), such as 8,11,14-eicosatrienoic acid and 5,8,11,14,17-eicosapentaenoic acid, may also be metabolized by some of these enzymes. The term eicosanoid has been adopted to refer to oxygenated metabolites derived specifically from eicosapolyenoic acids.

1.1. Cyclooxygenase pathway (prostaglandins, thromboxanes & prostacyclin)

1.1.1. Historical perspective

Any discussion of the historical contributions made to the field of eicosanoid research demands a review of the first eicosanoids described and characterized which were the prostaglandins. In 1930, Kurzrok and Lieb unknowingly provided the first description of prostaglandin action. In their quest for a treatment of human sterility, they observed that injection of human semen onto uterine tissue would cause either contraction or relaxation of the uterine muscle (1). Further characterization of this activity came from independent laboratories which investigated both the uterus and other tissues. In 1935, Goldblatt described the pharmacological properties of human seminal plasma (2). Seminal fluid caused an immediate fall in blood pressure when administered intravenously into anesthetized animals. In addition to this depressor effect, seminal fluid also stimulated contraction of both uterine and intestinal muscle strips. At the same time, von Euler reported that an extract from human prostate had similar biological activities, and coined the term "prostaglandin" for the active material (3). Early biochemical investigations characterized "prostaglandin" as a hydroxylated unsaturated fatty acid (4). However, it was not until the early 1960's when the elaborate investigations of Bergström, Samuelsson and coworkers (5,6,7) attributed the activity of "prostaglandin" to a family of compounds. Using the newly developed techniques of gas-chromatography and mass-spectrometry, these researchers determined the structures of prostaglandin (PG) E_1, PGE_2, $PGF_{1\alpha}$, and $PGF_{2\alpha}$ (Figure 1).

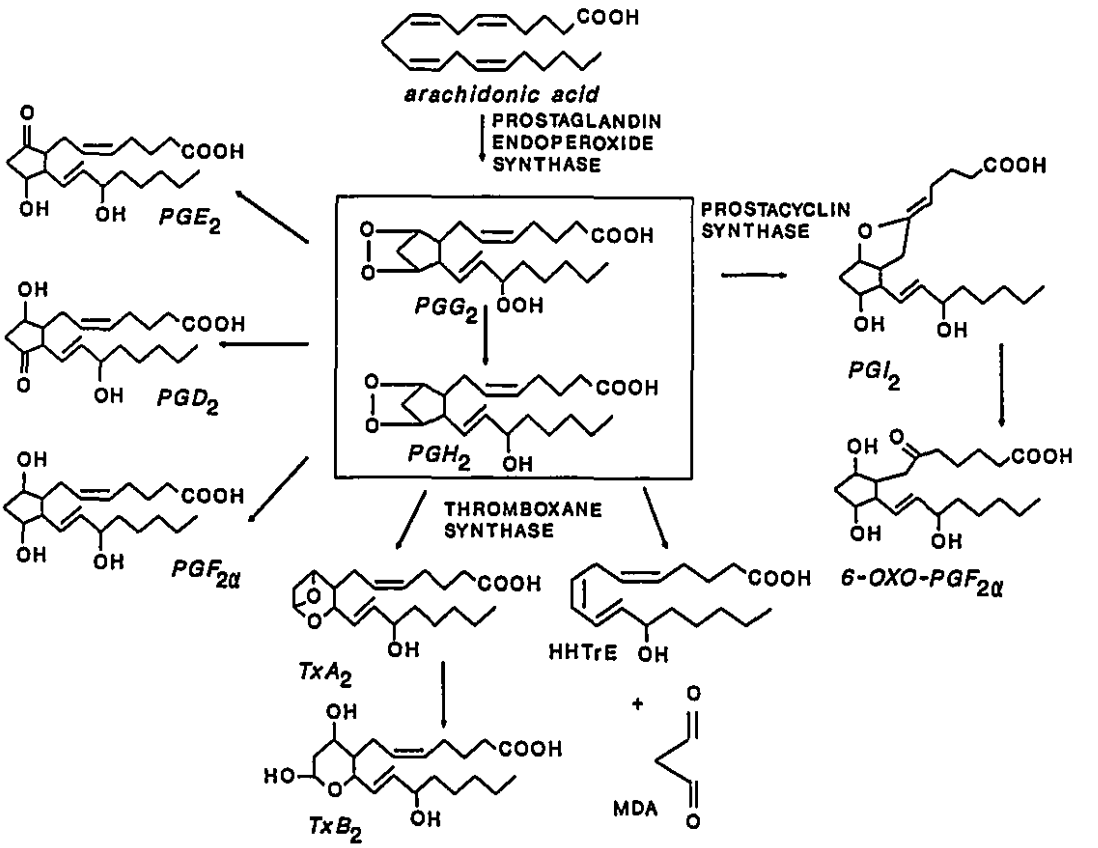

Figure 1. *Arachidonic acid metabolites produced by the cyclooxygenase pathway*

Prostaglandins are all 20-carbon carboxylic acids with a characteristic cyclopentane ring and two aliphatic chains. Substituents of the cyclopentane ring determines the class of prostaglandin, reflected by the letter designation, whereas the numerical subscript indicates the number of double bonds present. The structures of the prostaglandins immediately suggested their origin from fatty acids. This was confirmed in 1964, when arachidonic acid was identified as the precursor for the formation of PGE_2 (8,9).

1.1.2. Prostaglandin endoperoxide synthase

The cyclooxygenase pathway, which results in the formation of prostaglandins, thromboxanes and prostacyclin has been studied extensively and is shown in Figure 1. The first step in this pathway is catalyzed by prostaglandin endoperoxide synthase (Figure 2), which is a microsomal heme enzyme that is ubiquitously distributed in animal tissue (10) and which has two distinct catalytic activities (11). First, prostaglandin endoperoxide synthase exhibits dioxygenase (cyclooxygenase) activity, resulting in the addition of two molecules of oxygen to the fatty acid substrate (12). A proton is first abstracted from C_{13} of arachidonic acid followed by addition of molecular oxygen to C_{11} producing 11-peroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid. The free oxygen of the C_{11} peroxy group is then added to C_9 with a concomitant shift of the electrons in the 8,9-double bond to form a bond between C_8 and C_{12} which is coupled to the addition of a second molecule of oxygen at C_{15}. In the case of arachidonic acid, this results in the production of a bicyclic endoperoxide with a hydroperoxy group in the 15-position termed prostaglandin G_2 (PGG_2) (13,14). Prostaglandin endoperoxide synthase also has peroxidase activity which reduces the C_{15} hydroperoxy group of PGG_2 to a hydroxyl group producing prostaglandin H_2 (PGH_2) (14). The two catalytic activities of prostaglandin endoperoxide synthase reside at separate but interacting sites on the protein (15). Arachidonic acid and 8,11,14-eicosatrienoic acid are equally good substrates for prostaglandin endoperoxide synthase producing PGH_2 and PGH_1 respectively (11,16). 5,8,11,14,17-Eicosapentaenoic acid is also metabolized by prostaglandin endoperoxide synthase to

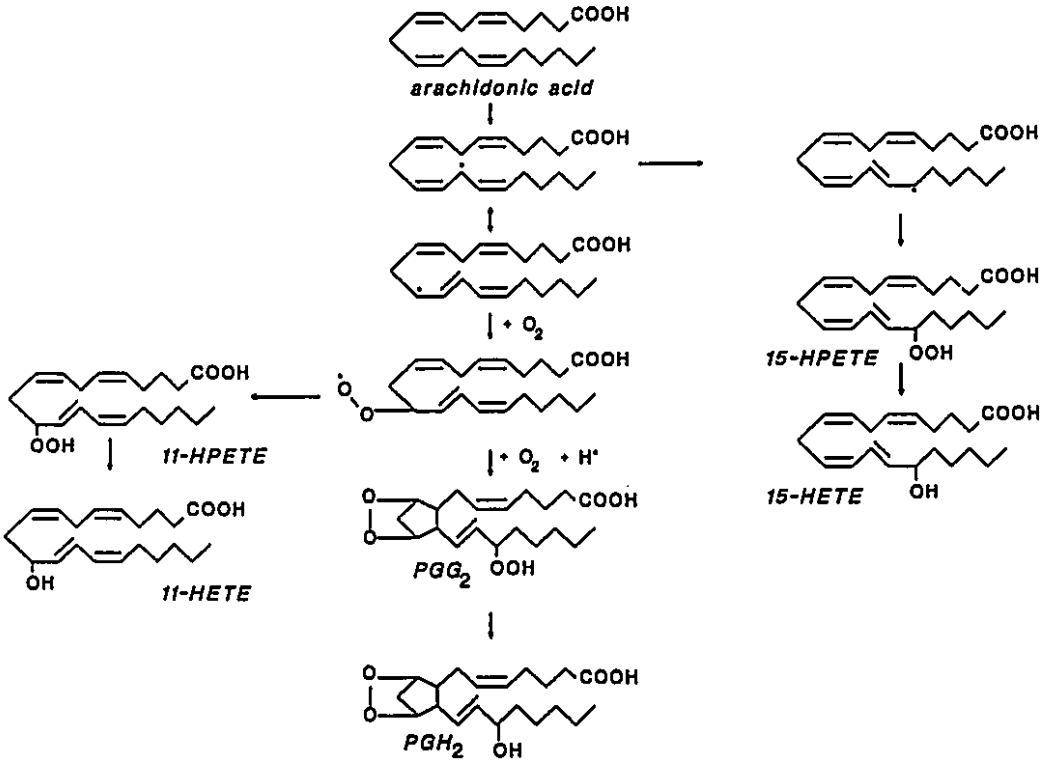

Figure 2. *Scheme of prostaglandin endoperoxide synthase-catalyzed arachidonic acid metabolism*

PGH_3, but the kinetics of this reaction are much slower than for arachidonic acid (17).

Metabolism of arachidonic acid by prostaglandin endoperoxide synthase has also been reported to produced 11(R)-hydroperoxy-5Z,8Z,12E,14Z-eicosatetraenoic acid (11(R)-HPETE) and a mixture of 15(R) and 15(S)-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acids (15-HPETEs) in a 15(R)/15(S) ratio of 2:1 (18). As mentioned above, the initial step for the metabolism of arachidonic acid by prostaglandin

endoperoxide synthase is abstraction of the C_{13} proton followed by addition of molecular oxygen to C_{11} producing 11-peroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid. The 11(S)-peroxy radical, which is the major isomer formed, is efficiently converted to PGG_2, whereas the 11(R) isomer is reported to accumulate and is transformed into 11(R)-HPETE. The initial abstraction of the C_{13} proton may also lead to addition of molecular oxygen to C_{15} producing 15(RS)-HPETE. Prostaglandin endoperoxide synthase can also catalyze the subsequent reduction of 15(RS)-HPETE to 15(RS)-HETE, however 11(R)-HPETE does not appear to be a substrate for this enzyme activity (18). 11(R)-HPETE is reduced to 11(R)-HETE by human platelets, possibly by glutathione-dependent peroxidases.

1.1.3. Thromboxanes and prostacyclin

Following the discovery and characterization of the prostaglandins, arachidonic acid metabolism by the cyclooxygenase pathway in different cell types was examined. In 1974, Samuelsson and coworkers investigated the metabolism of arachidonic acid by human platelets (19). Among the recovered products, PGG_2, PGH_2, PGE_2 and $PGF_{2\alpha}$ were identified, indicating the presence of the cyclooxygenase pathway. However, the two major metabolites produced by the cyclooxygenase pathway of these cells were not classical prostaglandins.

The first major metabolite produced by the cyclooxygenase pathway in platelets was identified as 8-(1-hydroxy-3-oxopropyl)-9,12L-dihydroxy-5,10-heptadecadienoic acid (TxB_2) (19). TxB_2 was reported to be a stable metabolite with no significant

biological activity. Based on the structure of TxB_2, a mechanism was proposed for its formation involving an unstable oxetane-oxane intermediate (TxA_2) (20). The production of TxA_2 by platelets was confirmed indirectly by reacting the unstable intermediate with alcohols (20). However, the actual structure of TxA_2 was not confirmed until 1985, when various TxA_2 isomers were chemically synthesized and compared to platelet-derived TxA_2 (21).

The bicyclic structure of TxA_2 is formed by a rearrangement of the oxygens of PGH_2 (Figure 1). This transformation is catalyzed by thromboxane synthase, which is a microsomal enzyme localized primarily in platelets (22). It is very unstable, having a reported half-life of approximately 32 seconds at 37 °C (20). TxA_2 is a potent stimulator of platelet aggregation and degranulation, and causes contraction of smooth muscle (20,23). TxA_2 is rapidly hydrated to the chemically stable product TxB_2 (21), which is metabolized by two pathways: ß-oxidation, resulting in the production of 2,3,-dinor-TxB_2 (24) and oxidation of the C_{11} hydroxyl group producing 11-dehydro-TxB_2 (25).

The second major platelet cyclooxygenase product was identified as 12-hydroxy-5,8,10-heptadecatrienoic acid (HHTrE) (19). Formation of HHTrE is due to loss of malondialdehyde from the cyclopentane ring of PGH_2 (26) (Figure 1) which is also catalyzed by thromboxane synthase. TxA_2 and HHTrE are formed in equimolar amounts by this enzyme (27). To date, there no major biological function has been attributed to HHTrE.

After the discovery of TxA_2, Moncada and Vane examined the ability of vascular tissue to convert prostaglandin endoperoxides to TxA_2 (28). Following the initial disappointment of finding no TxA_2 produced, these investigators observed the production of a new compound with biological activities opposite to those of TxA_2 (28). This compound was identified as 9-deoxy-6,9α-epoxy-Δ^5-$PGF_{1\alpha}$ (PGI_2) (Figure 1) and was given the trivial name prostacyclin (29). Prostacyclin is a potent inhibitor of platelet aggregation (28), causes vasodilation (30) and has anti-thrombotic properties (31). Like TxA_2, prostacyclin is an unstable compound with a half-life of 2-3 min at 37 °C (32) and is rapidly converted to the less active metabolite 6-keto-$PGF_{1\alpha}$ (29,33).

The opposing activities of platelet-derived TXA_2 and endothelial-derived PGI_2 suggest that the cyclooxygenase pathway is important for maintaining the patency of the vasculature. Imbalances in this antagonism are thought to explain many thrombogenic and atherosclerotic conditions.

1.1.4. Regulation of the cyclooxygenase pathway

One of the most important observations regarding the pharmacological regulation of prostaglandin biosynthesis was the potent inhibitory effect of aspirin and other non-steroidal anti inflammatory drugs (NSAID) on the formation of these substances. In 1971, a series of articles were published describing the inhibitory effects of aspirin on prostaglandin synthesis by guinea pig lung (34), human platelets (35) and dog spleen (36). Aspirin acts as an irreversible inhibitor of

prostaglandin endoperoxide synthase thereby inhibiting the production of prostaglandins, thromboxanes and prostacyclin (37). NSAID were found to inhibit the cyclooxygenase activity of prostaglandin endoperoxide synthase, but to have little effect on the peroxidase activity (38). As a result of this inhibition there is an accumulation of free arachidonic acid which may then be shunted to other metabolic pathways, including the lipoxygenase pathways described below, which are not appreciably affected by these drugs. Aspirin and similar drugs, such as indomethacin and ibuprofen, have been extensively studied as inhibitors of prostaglandin endoperoxide synthase (39) and are often used in studies of the cyclooxygenase pathway.

Acetylenic derivatives of arachidonic acid, which have triple bonds instead of double bonds, also inhibit prostaglandin endoperoxide synthase (40). In particular, 5,8,11,14-eicosatetraynoic acid (ETYA) has been extensively used in studies of eicosanoid metabolism. Addition of ETYA destroys the catalytic site of prostaglandin endoperoxide synthase, resulting in a progressive and irreversible decrease in enzyme activity (41). Unlike the NSAIDs described above, ETYA also inhibits other enzymes involved in the metabolism of arachidonic acid, including lipoxygenases (42)

1.2. Lipoxygenase pathways

As a result of the discovery of prostaglandins, thromboxanes and prostacyclin, and the identification of arachidonic acid as their precursor, arachidonic acid research swelled. This increased interest resulted in studies on the metabolism of arachidonic acid by different tissues and cell types and led to the discovery and characterization of mammalian lipoxygenases. Lipoxygenases in general may be defined as enzymes which incorporate molecular oxygen into cis-polyunsaturated fatty acids producing hydroperoxy compounds. There are three major mammalian lipoxygenases which stereospecifically produce hydroperoxy compounds with *S* configurations. These lipoxygenases may act alone, together, or with other enzymes to produce a plethora of biologically active eicosanoids.

1.2.1. 5-Lipoxygenase pathway (leukotrienes)

5-Lipoxygenase is the initial enzyme in the pathway which results in the conversion of arachidonic acid to leukotrienes (Figure 3). These products are potent proinflammatory agents which are involved in allergic and inflammatory reactions. The discovery of this pathway was a result of the combination of two separate lines of research, namely the metabolism of arachidonic acid by polymorphonuclear leukocytes (PMNL) and the characterization of an unknown mediator of anaphylaxis.

1.2.1.1. Formation of 5(S)-HETE and LTA_4

In 1976, Borgeat et al. reported that arachidonic acid was metabolized by rabbit PMNL to several polar products. The major metabolite first characterized was 5(S)-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5(S)-HETE) (43) . The addition of molecular oxygen at C_5 and the presence of a single pair of conjugated double bonds α to the 5-hydroxyl group of 5-HETE suggested the involvement of a 5-lipoxygenase. 5-Lipoxygenase would act by abstracting a hydrogen from C_7 of arachidonic acid, followed by the addition of molecular oxygen to C_5, producing 5(S)-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5(S)-HPETE). 5(S)-HPETE could then be reduced to give 5(S)-HETE (Figure 3). The subsequent identification of 5-HPETE as an intermediate in this pathway confirmed the involvement of 5-lipoxygenase (44).

In addition to 5(S)-HETE, rabbit PMNL were shown to convert arachidonic acid to a series of dihydroxy eicosanoids which were subsequently identified as 5(S),12(R)-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid (LTB_4) (45), 5(S),12(R)-dihydroxy-6E,8E,10E,14Z-eicosatetraenoic acid (6-*trans*-LTB_4), 5(S),12(S)-dihydroxy-6E,8E,10E,14Z-eicosatetraenoic acid (12-epi-6-*trans*-LTB_4), 5(S),6(R)-dihydroxy-7E,9E,11Z,14Z-eicosatetraenoic acid (5(S),6(R)-DiHETE) and 5(S),6(S)-dihydroxy-7E,9E,11Z,14Z-eicosatetraenoic acid (5(S),6(S)-DiHETE) (46). Examination of the structures of the dihydroxy eicosanoids produced by rabbit PMNL suggested the formation of a 5,6-epoxy intermediate which would be converted to the 5,12- or 5,6-dihydroxy eicosanoids by the addition of water to C_{12} or C_6, respectively.

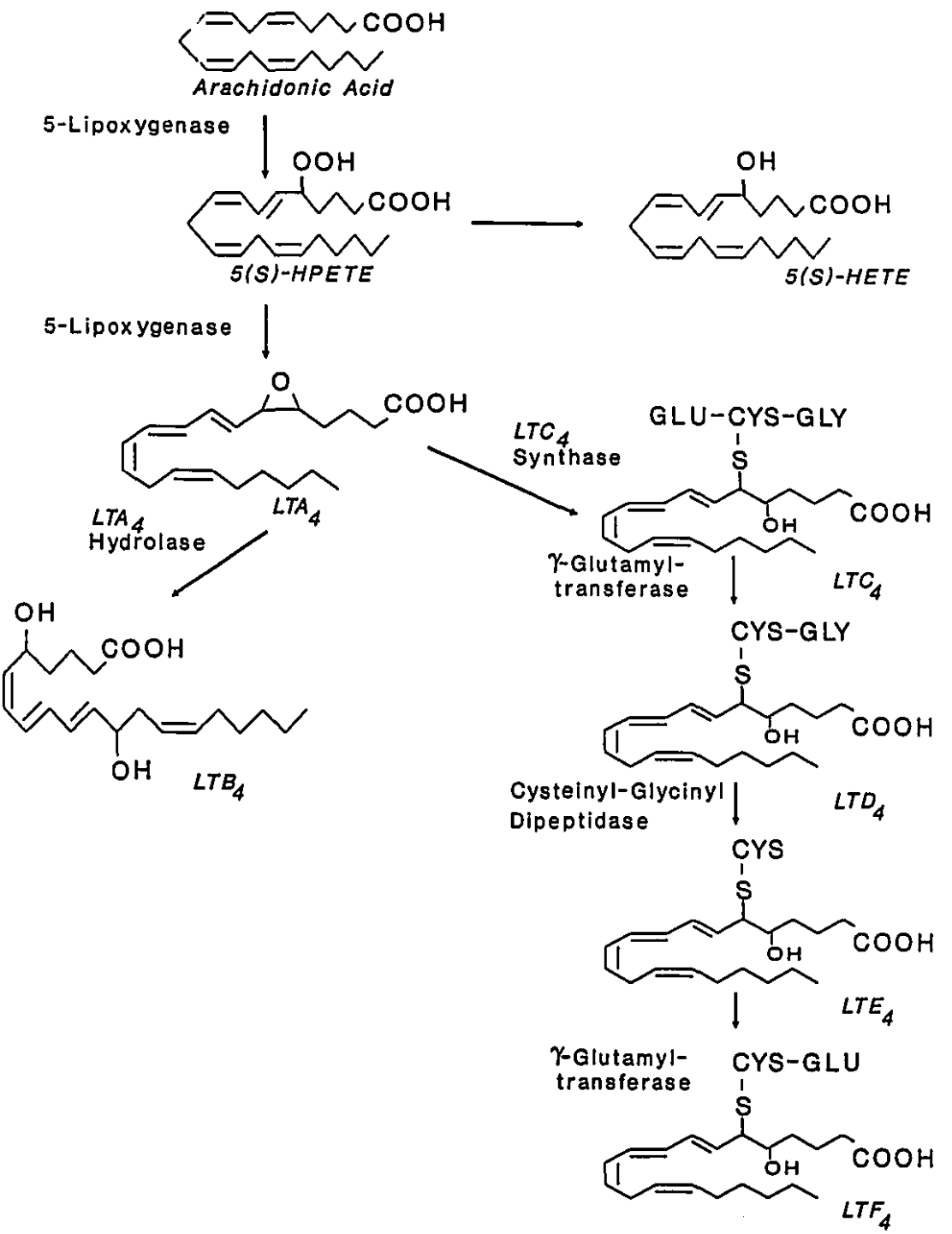

Figure 3. *Arachidonic acid metabolites produced by the 5-lipoxygenase pathway*

Although this intermediate evaded detection due to its innate instability, Borgeat and Samuelsson were able to trap the intermediate through its reaction with various alcohols (47). Using this trapping technique, and subsequent chemical synthesis of various steroisomers of the epoxide (48), the intermediate was identified as 5(S)-5E,6E-oxido-7E,9E,11Z,14Z-eicosatetraenoic acid (LTA_4). LTA_4 is produced by dehydration of 5(S)-HPETE which is also catalyzed by 5-lipoxygenase (49).

1.2.1.2. Formation of LTB_4

LTA_4 may be enzymatically converted to LTB_4 by LTA_4 hydrolase which is also present in PMNL (50). LTA_4 hydrolase, which catalyzes the rate-limiting step for LTB_4 synthesis, is a cytosolic enzyme initially purified from rat PMNL (51), human PMNL (50) and erythrocytes (52). LTA_4 hydrolase has subsequently been detected in mammalian plasma, liver and kidney (53,54,55) as well as in virtually all tissues examined from the guinea pig (56). As well as catalyzing the conversion of LTA_4 to LTB_4, LTA_4 hydrolase may become covalently associated with LTA_4 resulting in irreversible inactivation of the enzyme (52,57), a phenomenon known as suicide inactivation. LTA_4 hydrolase has a strict substrate requirement, metabolizing compounds with a 5,6-oxido-7,9-*trans*-11,14-*cis* configuration (58). LTA_5, which is formed from 5,8,11,14,17-eicosapentaenoic acid, is metabolized by LTA_4 hydrolase to LTB_5 (59). However, metabolism of LTA_5 is less efficient than that of LTA_4. LTA_3, produced from 5,8,11-eicosatrienoic acid, is not a substrate for this enzyme.

Both LTA_3 and LTA_5 have been reported to be irreversible inhibitors of LTA_4 hydrolase (59,60).

The gene for LTA_4 hydrolase was cloned in 1987 from human lung and placenta (61) and in 1989 from human spleen (62). The cDNA revealed an enzyme of 610 amino acids which was found to have no apparent homology with any other known protein (61). Subsequently, Maifroy and coworkers observed the presence of a homologous sequence of amino acids present in LTA_4 hydrolase (63) which was shared with several unrelated Zn^{2+}-metallopeptidase enzymes (64). This family of Zn^{2+}-metallopeptidases includes aminopeptidase M and angiotensin-converting enzyme (63). Bestatin, which is a known inhibitor of angiotensin-converting enzyme and other aminopeptidases, also inhibits LTA_4 hydrolase activity supporting a relationship between these enzymes (65). Although these metallopeptidases do not express any LTA_4 hydrolase activity, LTA_4 hydrolase does have aminopeptidase activity (65), but the relevance of this observation is not yet clear. It is interesting to note that LTA_4 hydrolase is localized in many tissues and cells which do not have 5-lipoxygenase activity, suggesting that this enzyme metabolizes LTA_4 produced by other cells or that its aminopeptidase activity may have a role in these cells. LTA_4 may also undergo non-enzymatic degradation to produce 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, 5(S),6(R)-DiHETE, and 5(S),6(S)-DiHETE (66). Enzymatic formation of 5,6-DiHETE from LTA_4 by mammalian kidney has also been reported (53). In general, products derived from the non-enzymatic hydrolysis of LTA_4 have significantly less biological activity than LTB_4 (67,68).

1.2.1.3. Formation of peptido-leukotrienes

Unlike the 5-lipoxygenase products described above, whose structural identification preceded characterization of biological activity, the peptido-leukotrienes were initially detected on the basis of their biological activities. In 1938, Feldberg and Kellaway observed that the perfusate obtained from a guinea pig lung, after injection of cobra venom, could stimulate a delayed slow contraction of guinea pig ileum (69). The term slow-reacting substance (SRS) was used to describe this activity. In 1940, Kellaway and Trethewie (70) described the production of SRS following antigenic challenge of sensitized tissue. In the aforementioned studies, SRS appeared to be distinct from histamine, which has similar biological activities, based on the kinetics of the induced contraction. However, it was not until 1952 and the availability of potent antihistamine drugs, that Brocklehurst clearly distinguished SRS from histamine and other related compounds and renamed the substance slow reacting substance of anaphylaxis (SRS-A) (71). Interest in SRS-A heightened with the failure of antihistamine drugs to control asthma. SRS-A, which was reported to be produced by asthmatic human lungs *in vitro* (72), was found to have potent bronchoconstrictor activity on human airways (73). SRS-A was therefore considered to be a potentially important mediator of asthma.

Initial characterization of SRS-A revealed it to have a molecular weight of approximately 500 daltons and to contain sulfur (74). However, the small amounts of SRS-A available at the time and its involatile nature made mass spectral analysis impossible. In 1979, Murphy et al. found that mouse mastocytoma cells produced

large amounts of SRS-A which they were able to characterize (75). Both radiolabeled arachidonic acid and cysteine were reported to be incorporated into SRS-A following stimulation of mouse mastocytoma cells with calcium ionophore. To facilitate structural analysis, SRS-A was treated with Raney nickel, which breaks carbon-sulfur bonds and reduces carbon-carbon double bonds. The lipid moiety recovered after this treatment was identified as 5-hydroxyeicosanoic acid by mass spectral analysis. In order to investigate its amino acid component, SRS-A was subjected to acid hydrolysis followed by amino acid analysis. Three amino acids were detected and identified as cysteine, glycine and glutamic acid (76). Further sequence analysis identified the tripeptide as γ-glutamylcysteinylglycine (glutathione). This compound was therefore identified as 5(S)-hydroxyl-6(R)(S)-glutathionyl-7E,9E,11Z,14Z-eicosatetraenoic acid (LTC_4) (Figure 3).

LTC_4 is further metabolized to 5(S)-hydroxy-6(R)(S)-cysteinylglycinyl-7E,9E,11Z,14Z-eicosatetraenoic acid (LTD_4) by γ-glutamyltransferase through the enzymatic removal of the glutamyl residue from LTC_4 (77). In 1981, Bernström, reported the metabolism of LTD_4 to 5(S)-hydroxy-6(R)(S)-cysteinyl-7E,9E,11Z,14Z-eicosatetraenoic acid (LTE_4) by a cysteinyl-glycinyl dipeptidase which removed the glycine residue (78). Readdition of a glutamic acid residue to LTE_4 by γ-glutamyl transpeptidase was found to produce 5(S)-hydroxy-6(R)(S)-cysteinylglutamyl-7E,9E,11Z,14Z-eicosatetraenoic acid (LTF_4) (79) (Figure 3). It is interesting to note that the biological effects first attributed to SRS in the earlier studies described above were found to be due to the combined activities of LTC_4, LTD_4 and LTE_4.

The identification of LTA_4 as the unstable intermediate in the formation of LTB_4 corresponded in time to the first structural description of LTC_4. The striking resemblance of LTA_4 with that of LTC_4, immediately suggested LTA_4 as an intermediate in the formation of peptido-leukotrienes. It was soon established that the addition of glutathione to C_6 of LTA_4 by LTC_4 synthase, results in the formation of LTC_4 (80). Therefore, the 5-lipoxygenase pathway is responsible for the formation of both LTB_4 and the peptido-leukotrienes. However, the preferential production of either LTB_4 or LTC_4 by the 5-lipoxygenase pathway depends on the enzymes present in a given cell. For example, PMNL have LTA_4 hydrolase, and therefore produce LTB_4 from LTA_4, whereas human eosinophils and basophils, which have LTC_4 synthase activity, produce primarily LTC_4 (81,82).

Following the identification of the above compounds, the term leukotriene was introduced by Samuelsson et al. to reflect the leukocyte, where they were first identified, and the conjugated triene chromophore which is characteristic of these compounds (83). The presence of a conjugated triene in leukotrienes results in characteristic UV absorption spectra with three UV maxima at approximately 260, 270, and 280 nm (LTB_4 and related compounds) or 270, 280, 290 nm (peptido-leukotrienes). This characteristic UV absorbance profile often provides initial evidence for identification of leukotrienes, and permits their detection by monitoring UV absorbance of HPLC effluents at high wavelengths where interference from unrelated compounds is minimized.

1.2.1.4. Regulation of 5-lipoxygenase

As the biological importance of the leukotrienes became increasingly evident, studies of the 5-lipoxygenase enzyme intensified with the ultimate goal of pharmacological regulation of the pathway. Initial studies indicated that 5-lipoxygenase was localized in the cytoplasm and showed that it required Ca^{2+} and ATP for full activity (84). More recently, 5-lipoxygenase has been reported to contain iron which is essential for enzymatic activity (85). Inactivation of 5-lipoxygenase by exposure to oxygen permits the release of this otherwise tightly bound iron. In 1988, Rouzer et al. showed that 5-lipoxygenase must translocate from the cytosol to the cell membrane for activity (86). Intensified research of this translocation phenomenon led to the characterization of an 18 kDA membrane protein termed five-lipoxygenase-activating protein (FLAP) (87) which is required for this process and which is essential for 5-lipoxygenase activity. The cDNA of both 5-lipoxygenase (88,89) and FLAP (90), and the characterization of their gene promoter regions have been reported (91,92). Subsequently, a correlation has been shown between the co-expression of 5-lipoxygenase and FLAP and leukotriene synthesis, supporting the requirement of both proteins for enzymatic activity (93).

1.2.2. 12-Lipoxygenase pathway

1.2.2.1. Formation of 12(S)-HETE

In 1974, the first description of a mammalian lipoxygenase was reported by Hamberg and Samuelsson who, while investigating the role of prostanoids in human

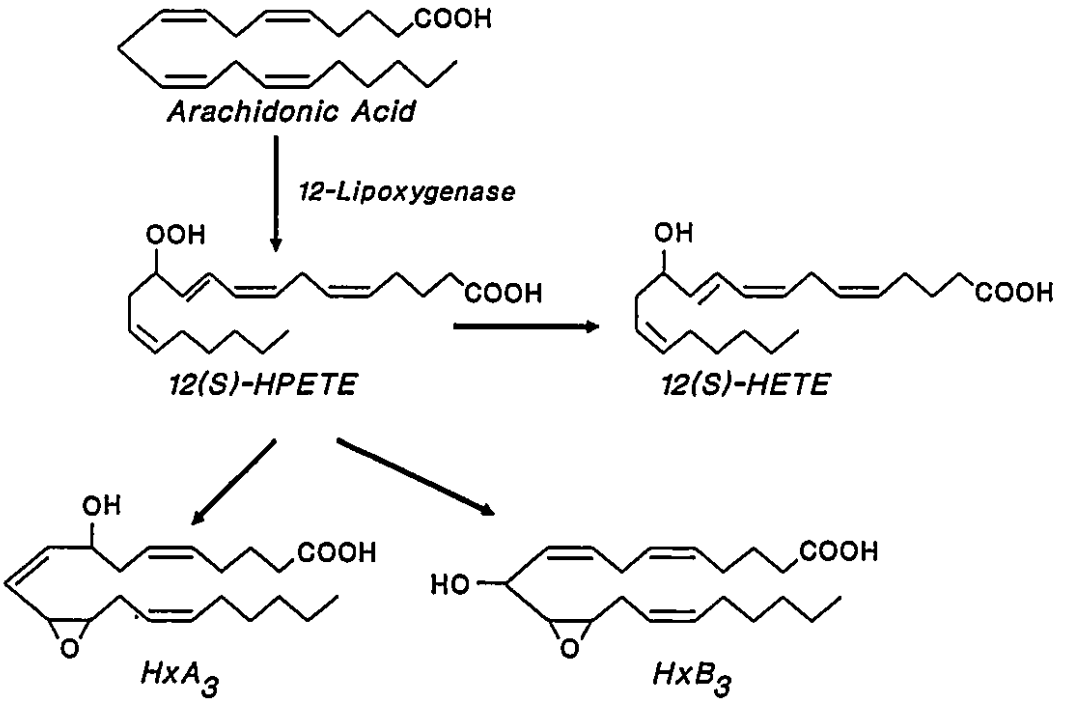

Figure 4. *12-Lipoxygenase-catalyzed metabolism of arachidonic acid*

platelets, examined the metabolites of arachidonic acid produced by these cells (19). The first two metabolites characterized were TXB_2 and HHTrE which are products of the cyclooxygenase pathway as described earlier. However, the major arachidonic acid metabolite produced by human platelets is not a cyclooxygenase product. This compound was identified as 12(S)-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12(S)-HETE) which was shown to be formed by a 12-lipoxygenase (19,94). 12-Lipoxygenase catalyzes the removal of a hydrogen from C_{10} of arachidonic acid which

is coupled to the addition of oxygen to C_{12}, producing 12-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-HPETE). 12-HPETE is subsequently reduced to 12(S)-HETE by peroxidases. The scheme of the 12-lipoxygenase pathway is shown in Figure 4.

Unlike 5-lipoxygenase, 12-lipoxygenase is found in an active form and does not require Ca^{2+}-mobilizing stimuli for activity (95). Since its discovery, the 12-lipoxygenase pathway and the 12-lipoxygenase enzyme itself have been extensively characterized. In the human, this enzyme is found primarily in the platelet with lesser amounts in leukocytes (95). In the pig, which is the model used for this study, the polymorphonuclear leukocyte is the main source of 12-lipoxygenase and 12(S)-HETE is the major product of arachidonic acid metabolism in these cells (96). Different substrate specificities of human platelet 12-lipoxygenase, which preferentially metabolizes eicosapolyenoic acids, and porcine leukocyte 12-lipoxygenase which metabolizes both eicosapolyenoic acids and octadecapolyenoic acids, suggested the occurrence of two types of 12-lipoxygenase (97). A similar divergence in activity was shown for the metabolism of LTA_4. Porcine leukocyte 12-lipoxygenase catalyzes the conversion of LTA_4 to lipoxins, whereas platelet 12-lipoxygenase is relatively inactive (97). Comparison of the cDNA's of porcine leukocyte 12-lipoxygenase (98) and human 12-lipoxygenase cloned from erythroleukemia cells (99) confirmed different forms of the enzyme. Interestingly, there is only 66% homology between the two 12-lipoxygenases even though they have very similar catalytic activities (99).

1.2.2.2. Hepoxilins

In 1978, trihydroxy eicosanoids were found to be produced from the metabolism of arachidonic acid by platelets obtained from man, horse and dog (100). Two trihydroxy compounds were isolated and identified as 8,9,12-trihydroxy-5,10,14-eicosatrienoic acid and 8,11,12-trihydroxy-5,9,14-eicosatrienoic acid. In a similar study the production of trihydroxy eicosanoids by human platelets was reported to involve the 12-lipoxygenase pathway (101). Although unconfirmed in these studies, these trihydroxyeicosanoids were hypothesized to have arisen from the conversion of 12(S)-HPETE to hydroxy-epoxy intermediates followed by the hydrolytic opening of the epoxy group by epoxide hydrolases (100,101). No hydroxy-epoxy compounds were isolated in these studies.

It was not until 1982 that Pace-Asciak, using rat lung tissue deficient in epoxide hydrolases, reported the formation of hydroxy-epoxy metabolites of arachidonic acid which were named hepoxilins (102,103). Two hepoxilins were isolated and characterized as 8-hydroxy-11,12-epoxy-5,9,14-eicosatrienoic acid (HxA_3) and 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid (HxB_3) (Figure 4). Further investigations using rat pancreatic islet cells revealed 12-HPETE as an intermediate in the formation of hepoxilins (103). As the integrity of the system for the formation of hepoxilins from 12-HPETE was maintained after boiling these tissues, formation of hepoxilins appears to be non-enzymatic. The subsequent report of hematin and hemoglobin-catalyzed transformation of 12(S)-HPETE to hepoxilins supports this finding (104).

Hepoxilins are formed by the intramolecular transfer of the hydroxyl moiety from the hydroperoxy group at C_{12} to either C_8 or C_{10}, producing HxA_3 and HxB_3, respectively (103,105). Hepoxilins are metabolized to trihydroxy eicosanoids by an epoxide hydrolase which showed some specificity for epoxyhydroxy fatty acids (106). HxA_3 was also reported to be enzymatically coupled to glutathione in a manner analogous to the formation of peptido-leukotrienes from LTA_4 (107). Glutathione is conjugated to C_{11} of HxA_3 by a glutathione-S-transferase producing a glutathione conjugate (HxA_3-C). The glutamyl residue of HxA_3-C can be removed by the action of γ-glutamyltransferase producing a cysteinyl-glycinyl conjugate termed HxA_3-D (108). Further metabolism of this compound has not been reported.

1.2.3. 15-Lipoxygenase pathway

Mammalian arachidonic acid 15-lipoxygenase activity was first described in rabbit peritoneal PMNL and human PMNL (109), and was later purified and characterized (110). 15-Lipoxygenase abstracts a hydrogen atom from C_{13} of arachidonic acid and adds molecular oxygen to C_{15} producing 15(S)-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15(S)-HPETE). Other cells which are known to contain 15-lipoxygenase include macrophages, eosinophils, endothelial cells, vascular smooth muscle cells, fibroblasts and epithelial cells (95). Like 12-lipoxygenase, 15-lipoxygenase is found in an active form. Although it has been reported that 15-HPETE spontaneously decomposes to 15(S)-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (15(S)-HETE), the possible role of peroxidases is not clear (95).

A distinct 15-lipoxygenase, purified from rabbit reticulocytes (111), has been extensively studied and the human reticulocyte 15-lipoxygenase has been cloned (112). The reticulocyte enzyme has physical and catalytic properties which are distinct from those of the leukocyte 15-lipoxygenase. Unlike the leukocyte enzyme, which specifically oxygenates carbon 15 of arachidonic acid, the reticulocyte 15-lipoxygenase catalyzes the formation of both 15-HPETE and 12-HPETE from arachidonic acid in a ratio of 15:1 (113). Reticulocyte 15-lipoxygenase can also oxygenate phospholipids in biological membranes which may be related to its functional role in the elimination of mitochondria from this cell (113,114).

Human leukocyte 15-lipoxygenase also catalyzes the oxidation of other ω6-PUFA. Linoleic acid (9Z,11Z-octadecadienoic acid), an important dietary fatty acid which is readily incorporated into human leukocyte cellular lipids (115), is oxidized to 13(S)-hydroperoxy-9Z,11E-octadecadienoic acid (13(S)-HPODE) by this enzyme (116). The subsequent reduction of 13-HPODE to 13(S)-hydroxy-9Z,11E-octadecadienoic acid (13(S)-HODE) was reported to involve a glutathione peroxidase (116).

Soybean lipoxygenase, which was the first lipoxygenase to be characterized (117), also metabolizes arachidonic acid and linoleic acid to 15(S)-HPETE and 13(S)-HPODE respectively. Although distinct in structure, soybean lipoxygenase has been extensively used as a model of lipoxygenase activity and is often used as a biosynthetic tool to produce large quantities of 15-HPETE and 13-HPODE.

1.2.4. Other lipoxygenases

As mentioned earlier, lipoxygenases catalyze the addition of molecular oxygen to a 1,4-*cis*,*cis*-pentadiene moiety of polyunsaturated fatty acids to produce hydroperoxy compounds. It is therefore reasonable to suggest that lipoxygenases specific for carbons 8, 9, and 11 of arachidonic acid may also exist in mammalian cells. In 1979, Goetzl and coworkers reported the formation of 8-, 9-, and 11-HETE as minor metabolites of arachidonic acid produced by human neutrophils (118). It was reported that ETYA, an inhibitor of both lipoxygenase and prostaglandin endoperoxide synthase, suppressed the formation of these HETEs. Although, 11-HETE may be produced by prostaglandin endoperoxide synthase, the formation of 8- and 9-HETE by this enzyme would not be expected. However, these monohydroxy eicosanoids could have been formed by non-enzymatic autooxidation of arachidonic acid (119). Formation of 8-, 9-, and 11-HETE could have been catalyzed by free radical intermediates formed during oxidation of arachidonic acid by lipoxygenases or prostaglandin endoperoxide synthase. This could explain the diminished production of these compounds by neutrophils in the presence of ETYA.

More recent studies have reported the enhanced production of 8-HETE by extracts of mouse skin treated with phorbol ester (120,121). This effect was diminished by the addition of lipoxygenase inhibitors, but was not affected by inhibitors of prostaglandin endoperoxide synthase. The chirality of the C_8-hydroxyl group was examined and only the 8(S)-HETE isomer was formed (122). Furthermore, the mechanism for the formation of 8(S)-HETE by this tissue involves a

stereoselective abstraction of the pro*R* hydrogen from C_{10} of the substrate (122). These observations strongly support the involvement of an 8-lipoxygenase.

Historically, lipoxygenases were first isolated from non-mammalian sources. As mentioned earlier, soybean lipoxygenase was the first lipoxygenase identified and characterized (117). Similar lipoxygenase activity is found in peas, potatoes, flaxseed, algae and alfalfa (123,124). Tomato homogenate contains a lipoxygenase of different specificity which catalyzes the production of 9-HODE from linoleic acid (125). Similarly, marine organisms have reported lipoxygenase activities. The gorgonian coral Pseudoplexaura porosa contains a lipoxygenase which stereospecifically converts arachidonic acid to 8(R)-HPETE (126). Eggs obtained from sea urchins (Strongylocentrotus purpuratus) contain a lipoxygenase which catalyzes the conversion of arachidonic acid to 11(R)-HPETE and 12(R)-HPETE (127). In general, these lipoxygenases are quite distinct from mammalian lipoxygenases and therefore are of little value as models of mammalian enzymes. However, the abundance and stereospecific nature of these enzymes makes them very useful for preparing large quantities of a given hydroxylated fatty acid.

1.3. Cytochrome P-*450* monooxygenase pathway

The microsomal cytochrome P-*450* monooxygenases represent another pathway for the metabolism of arachidonic acid. Cytochrome P-*450* enzymes are heme proteins which catalyze the oxidation of many compounds. The catalytic activity of cytochrome P-*450* involves binding and activation of oxygen followed by oxidation of

the substrate (128). Cytochrome P-*450* monooxygenases, in the presence of NADPH and molecular oxygen, catalyze the metabolism of arachidonic acid to 1) monohydroxyeicosatetraenoic acids (HETEs), 2) ω-hydroxylated metabolites of arachidonic acid, and 3) epoxyeicosatrienoic acids (129).

Six HETEs, including 5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE, and 15-HETE, are formed by cytochrome P-*450* monooxygenases. As with lipoxygenase-derived HETEs these compounds have a hydroxyl group followed by a *cis-trans* diene. However, whereas the hydroxyl groups of HETEs derived from mammalian lipoxygenases are all in the *S* configuration, the cytochrome P-*450* monooxygenases show no stereoselectivity and produce both *R* and *S* hydroxyl isomers, with the exception of 12-HETE which has predominantly the *R* configuration at C_{12} (129).

Cytochrome P-*450* monooxygenase were also reported to catalyze the formation of 16,17,18,19, and 20-hydroxyarachidonic acid (130,131,132). Although this activity is generally considered to be catabolic, 20-hydroxy and 19-hydroxy-arachidonic acids have been reported to have biological activities (133,134). Similar ω-hydroxylated products are formed from other long chain fatty acids.

The metabolism of arachidonic acid by cytochrome P-*450* monooxygenase also produces four regioisomeric epoxyeicosatrienoic acids (EETs); 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET (135), which are further metabolized by epoxide hydrolase to dihydroxy eicosanoids. Unlike the hydroxylation reactions described above, epoxidation of arachidonic acid is generally stereospecific, producing >80% (R)(S)-EETs except for 5,6-EET which is produced as a mixture (60/40) of (R)(S) and

(S)(R) isomers (129). Several biological activities have been reported for EETs, however it is unclear if these effects are pharmacological or physiological (129).

Many of the cytochrome P-*450*s in liver and kidney tend to be rather non-specific. These enzymes are thought to have a general role in the metabolism of drugs and other xenobiotics (128). The non-specific nature of these enzymes is an advantage to the organism in a changing environment. Other cytochrome P-*450* enzymes have specific roles in the metabolism of endogenous compounds. A prime example regarding eicosanoids is the conversion of LTB_4 by a specific cytochrome P-*450* in human PMNL to 20-hydroxy-LTB_4 (136) which is the major route of metabolism in these cells. Another cytochrome P-*450*, specific for prostaglandins, catalyzes the formation of 20-hydroxyprostaglandins from prostaglandins in rabbit lung (137).

1.4. Transcellular formation of hydroxylated eicosanoids.

In the past few years, the metabolism of arachidonic acid by the combined enzymatic activities of different lipoxygenases has been recognized as an important route for the production of hydroxylated eicosanoids.

1.4.1. Formation of lipoxins

One of the most recent examples of transcellular metabolism of eicosanoids is the formation of trihydroxytetraene compounds termed lipoxins (lipoxygenase interaction products) (138,139). The two major lipoxins identified are

5(S),6(R),15(S)-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid (LXA_4) and 5(S),14(R),15(S)-trihydroxy-6,10,12-*trans*-8-*cis*-eicosatetraenoic acid (LXB_4). Several naturally occurring isomers of these products have also been identified including 6(S)-11-*trans*-LXA_4, 11-*trans*-LXA_4, 6(S)-LXA_4, 14(S)-8-*trans*-LXB_4 and 8-*trans*-LXB_4. All of the lipoxins identified have a characteristic conjugated tetraene chromophore resulting in a distinctive UV absorbance spectrum with maxima at 287, 301, and 316 nm.

There are two general pathways for the formation of lipoxins: the epoxide (anaerobic) pathway, and the lipoxygenase (aerobic) pathway (140). Both pathways involve the initial metabolism of 15-HETE or 15-HPETE by 5-lipoxygenase to produce 5-hydroperoxy-15-hydro(pero)xy-eicosatetraenoic acid (15-H(P)ETE) (Figure 5). In the epoxide pathway, 5,15-DiHPETE undergoes a dehydration step catalyzed by either 5-lipoxygenase or 12-lipoxygenase generating the tetraene epoxides 15-hydroxy-5(6)-epoxy-7,9,11,13-ETE and 5-hydroxy-14(15)-epoxy-6,8,10,12-ETE respectively (141). This step is analogous to the formation of LTA_4 from 5-HPETE by 5-lipoxygenase as described earlier. The epoxide intermediates could then be converted enzymatically to LXA_4 or LXB_4. The formation of the isomers of LXA_4 and LXB_4 may result from the non-enzymatic hydrolysis of the epoxytetraene intermediates in a manner analogous to the formation of 6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4 from LTA_4, or they may result from the isomerization of LXA_4 and LXB_4.

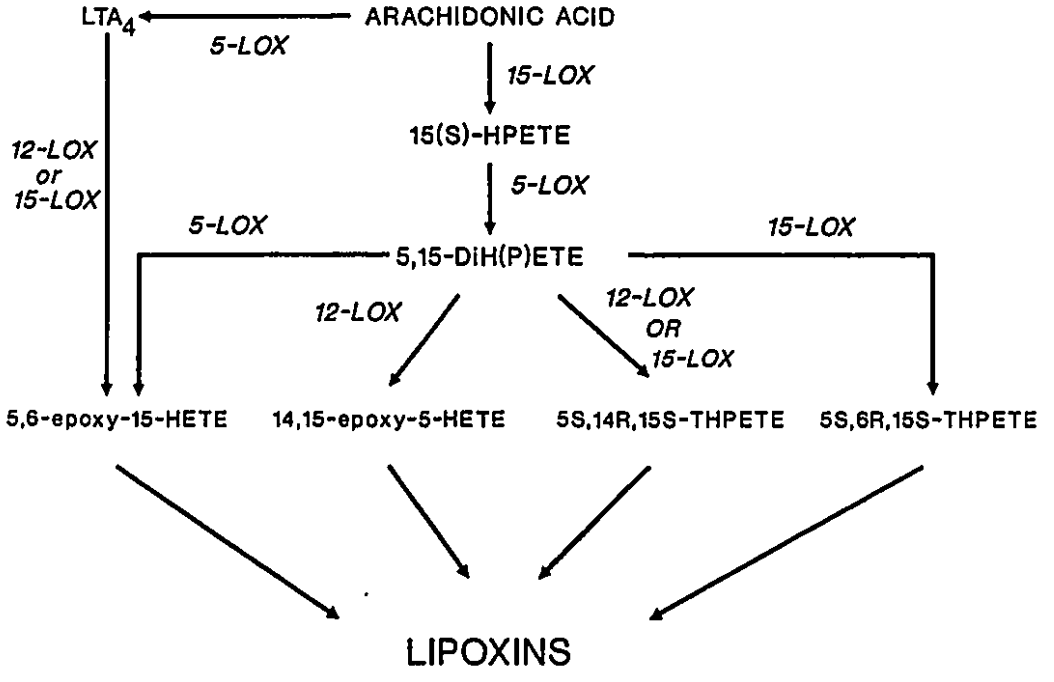

Figure 5. *Scheme for the formation of lipoxins*

The aerobic lipoxygenase pathway for the formation of lipoxins also has 5,15-DiH(P)ETE as an intermediate. In this pathway, a third oxygen is added by a lipoxygenase reaction. Both 12-lipoxygenase and 15-lipoxygenase (soybean lipoxygenase) can abstract a hydrogen from the 10-position of 5,15-DiH(P)ETE and oxygenate either C_{14} or C_6 of 5,15-DiH(P)ETE producing 5(S),14(R),15(S)-trihydroperoxy-ETE and 5(S),6(R),15(S)-trihydroperoxy-ETE. Upon reduction of the hydroperoxy groups to hydroxyl groups, 5(S),6(R),15(S)-11-*cis*-LXA_4 and

5(S),14(R),15(S)-8-*cis*-LXB_4 are produced respectively (140). Recently, 12-lipoxygenase has also been shown to oxygenate C_{15} of LTA_4, which results in the production of LXA_4, LXB_4 and the trans isomers of these compounds (142).

1.4.2. Formation of other dihydroxyeicosanoids

There are several other reports of transcellular metabolism involving oxygenation by two different lipoxygenases to produce DiHETEs. Platelet-derived 12-HETE can be metabolized by activated 5-lipoxygenase of human neutrophils to produce 5(S),12(S)-dihydroxy-6E,8Z,10E,14Z-eicosatetraenoic acid (12-epi-8-*cis*-6-*trans*-LTB_4). Similarly, neutrophil-derived 5(S)-HETE can be metabolized by platelet 12-lipoxygenase to form the same product (143,144,145). Human platelets and porcine leukocytes metabolize arachidonic acid to a mixture of 8,15 and 14,15-DiHETEs as a result of the metabolism of 15-HETE by 12-lipoxygenase (146). Cells which have both 5 and 15-lipoxygenases, such as PMNL, convert arachidonic acid to 5,15-DiHETE by the sequential action of these enzymes (147).

1.4.3. Metabolism of LTA_4

Transcellular metabolism of LTA_4 to other leukotrienes has also been reported. This phenomenon allows for the production of leukotrienes by cells which do not have 5-lipoxygenase. For example, both human platelets and porcine aortic endothelial cells have LTC_4 synthase. Paradoxically, neither of these cell types have 5-lipoxygenase and are therefore unable to produce LTA_4 from arachidonic acid.

However, it has been reported that human platelets and porcine aortic endothelial cells are able to metabolize neutrophil-derived LTA_4 to LTC_4 (148,149). For both of these cell types, the production of LTC_4 is of a magnitude to suggest a physiological relevance (150). Vascular smooth muscle cells (151) and mast cells (152) have also been reported to be able to convert exogenous LTA_4 to LTC_4.

Erythrocytes, which also lack the ability to produce LTA_4, have been reported to contain LTA_4 hydrolase and to convert exogenous LTA_4 to LTB_4 (153). Furthermore, it was found that upon co-incubation of stimulated PMNL with erythrocytes in the presence of arachidonic acid, that the erythrocytes metabolized PMNL-derived LTA_4 to LTB_4.

1.5. Regulation of cellular free arachidonic acid

Of the many eicosanoids described above, none are preformed and stored within the cell. On the contrary, following a stimulus, eicosanoids are synthesized from non-esterified arachidonic acid and then released. In the cell, the majority of arachidonic acid is not free, but is found esterified in cholesterol esters, triglycerides and phospholipids. Early investigations by Flower and Blackwell reported the incorporation of ^{14}C-labeled arachidonic acid into the sn-2 position of phosphatidylcholine by slices of guinea pig spleen (154). Subsequent stimulation of this tissue produced ^{14}C-labeled prostaglandins. This study was followed by several other investigations which showed that phospholipids, in particular

phosphatidylcholine and phosphatidylethanolamine, are the major sources of arachidonic acid for the formation of eicosanoids (155,156).

1.5.1. Phospholipase A_2

Release of arachidonic acid from the sn-2 position of phospholipids is catalyzed by a subset of phospholipases termed phospholipases A_2 (PLA_2), and this action represents the rate-limiting step in the formation of arachidonic acid metabolites. Agents which stimulate the production of prostaglandins, thromboxanes, leukotrienes, etc, are generally thought to do so by modulating PLA_2 activity. The action of PLA_2 also results in the formation of lysophospholipids, including lyso-platelet activating factor, which is the precursor for the formation of platelet activating factor, a potent inflammatory mediator.

PLA_2 activity was first reported in 1903 in cobra venom (157) and was subsequently found in the poisons of other snakes, as well as bees, wasps, and scorpions (158). Pancreas is also a major source of PLA_2 enzymes. More than 40 different PLA_2 enzymes have been characterized from snake venoms and pancreas (159,160,161). Recently, Davidson and Dennis have accumulated all of the available data on these PLA_2 enzymes for comparison (162). In general, these PLA_2 enzymes are approximately 14 kDa in size with multiple cysteines which exist as disulfide pairs. These enzymes are all considered to be secreted and require Ca^{2+} for activity. Although these enzymes specifically cleave the fatty acid from the sn-2

position of phospholipids, no specific preference for arachidonyl-containing phospholipids was seen.

More recently, research in this area has focused on PLA_2 enzymes which favor hydrolysis of phospholipids containing arachidonic acid (163,164,165). One of these PLA_2 enzymes, isolated from a monocytic cell line, has a molecular weight of 110 kDa and cleaves phospholipids with arachidonic acid in the sn-2 position 3-30 fold more rapidly than other related polyunsaturated fatty acids (163). This enzyme is located in the cytosolic fraction of these cells and is referred to as $cPLA_2$ distinguishing it from the secretory PLA_2 enzymes ($sPLA_2$) described earlier. Elevations in intracellular Ca^{2+} concentrations, from levels found in the resting cell to levels found in activated macrophages, resulted in a significant increase in the activity of this $cPLA_2$ (163). Furthermore, these rises in calcium stimulates the translocation of this $cPLA_2$ to membrane vesicles (166). This enzyme was recently cloned and the deduced amino acid sequence showed no homology with $sPLA_2$ (166). Similar $cPLA_2$ with molecular weights of 100 kDa have been reported in rat kidney (167) and human monoblast U937 cells (168). Because of the specificity of these enzymes for arachidonyl-containing phospholipids, $cPLA_2$ enyzmes may play an important role in the regulation of the production of eicosanoids in the cell.

1.5.2. Regulation of phospholipase A_2 activity

As a regulator of the availability of non-esterified arachidonic acid, and therefore the production of arachidonic acid metabolites, PLA_2 is an attractive site for

pharmacological intervention and as such has received a great deal of attention in recent years. Most of that research has focused on endogenous inhibitors and stimulators of PLA_2 activity.

Studies of $sPLA_2$ activity in bee venom, revealed the presence of a $sPLA_2$ stimulatory peptide which was subsequently identified as melittin (169). Melittin had previously been shown to stimulate $sPLA_2$ activity (170) and has been reported to increase eicosanoid synthesis in various types of cells (170,171,172). Mammalian cells were later found to have a similar cellular protein, antigenically related to melittin, which was termed phospholipase-activating protein (PLAP) (173). PLAP is a 28 kDa protein which has recently been cloned by Clark and coworkers from the murine smooth muscle cell line BC3H (173). With the genetic information available, this group showed a correlation between LTD_4-stimulated eicosanoid production and PLAP synthesis by the endothelial cell line CPAE (174). Furthermore, addition of antisense DNA to these cells prevented the increase in arachidonic acid release and eicosanoid formation stimulated by LTD_4, supporting the proposed importance of this compound in the regulation of $sPLA_2$ activity (174).

Inhibitors of PLA_2 have also been reported. Glucocorticoids, such as cortisol and dexamethasone, have been reported to stimulate the production of PLA_2-inhibitory proteins. Depending on the tissue of origin, these proteins were initially referred to as macrocortin (175), lipomodulin (176) and renocortin (177). However, in 1984, the term lipocortin was proposed for these substances (178). Lipocortins were also isolated by other groups based on their Ca^{2+} and actin binding properties, and were

referred to as calpactins (179). There are at least eight different lipocortins which have been identified (180). Lipocortin activity is thought to be regulated by phosphorylation (181). It has been suggested that activation of a cell stimulates the phosphorylation of lipocortins thereby decreasing the inhibitory effects of these proteins on PLA_2. PLA_2 activity then increases resulting in enhanced release of arachidonic acid.

The importance of lipocortins as regulators of PLA_2 activity is not clear. In 1987, Davidson and coworkers provided evidence that lipocortins bind to phospholipids (182). They suggested that the inhibition of PLA_2 by lipocortins was due to a depletion of phospholipid substrate for the enzyme rather than direct interaction with the enzyme itself. Furthermore, there have been several reports of glucocorticoid-mediated inhibition of eicosanoid production and PLA_2 activity which does not involve lipocortin (183,184,185).

1.5.3. Other mechanisms for the release of esterified arachidonic acid

PLA_2-mediated phospholipid hydrolysis is not the only mechanism available in the cell which can liberate esterified arachidonic acid. Arachidonic acid may also be released from phosphatidylinositol by the combined actions of a phosphatidylinositol-specific phospholipase C and diglyceride lipase (186). As about 80% of phosphatidylinositol molecules contain arachidonic acid esterified at the sn-2 position, this may represent an important source of the fatty acid. Arachidonic acid is also hypothesized to be released by the combined actions of phospholipase A_1 and a

lysophospholipase (187). And finally, arachidonic acid may be released by the sequential actions of phospholipase C, diacylglycerol kinase, and a phosphatidic acid-specific phospholipase A_2 (188).

2. Biological activities of hydroxylated PUFA's

2.1. 5-Lipoxygenase products

2.1.1. LTB_4

The human neutrophil is a major site of LTB_4 synthesis and metabolism, and is also the major target for LTB_4 action. LTB_4 promotes the accumulation of leukocytes during inflammation by several mechanisms. LTB_4 is a potent chemotactic agent for neutrophils with optimal activity seen at nanomolar concentrations (189,190). Movement of neutrophils to inflammatory sites is further enhanced by LTB_4-stimulated adhesion of these cells to the vascular endothelium, which is followed by migration through the endothelial cell layer (191). LTB_4 also enhances the inflammatory response by stimulating aggregation (192) and degranulation of leukocytes (193) as well as enhancing superoxide generation (194).

LTB_4 is thought to act on PMNL through LTB_4 receptors coupled to a GTP-dependent regulatory protein (195). There are two classes of LTB_4 receptors on PMNL, high affinity and low affinity (196). Occupancy of the high affinity receptors is believed to mediate an increase in cytosolic Ca^{2+} and a chemotactic response.

Occupancy of the low affinity receptor appears to result in stimulation of the release of lysosomal enzymes associated with degranulation (195).

The effects of LTB_4 on human neutrophils are quite stereospecific, since closely related stereoisomers have markedly lower potencies. 6-*Trans*-LTB_4 and 12-epi-6-*trans*-LTB_4, produced by the non-enzymatic degradation of LTA_4, have similar biological effects on human neutrophils as LTB_4, but with significantly lower potency (197,198). The order of potency for these activities is LTB_4 > 6-*trans*-LTB_4 > 12-epi-*trans*-LTB_4, which is consistent with the relative affinities of these compounds for the LTB_4 high affinity receptors (199). This suggests that 6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4 exert their effects on neutrophils by acting on the LTB_4 receptor with less efficiency than LTB_4.

LTB_4 also modulates the immune response through actions on T and B lymphocytes. LTB_4 enhances the proliferation of suppressor-cytotoxic T-lymphocytes and inhibits the proliferation of helper-T-lymphocytes (200). It has been suggested that LTB_4 acts indirectly by stimulating monocytes to produce prostaglandins and cytokines, which in turn leads to the production of interleukin-1 (201,202,203). LTB_4 has also been reported to augment natural killer (NK) cell activity (204,205). However, this aspect of LTB_4 activity has been questioned by reports from other groups who failed to show a direct effect of LTB_4 on NK cell activity (206). Several B-lymphocytic functions including B-cell activation, IgG and IgM synthesis, and cellular replication are also enhanced by LTB_4 (207), due to an augmentation of the effects of IL-4 and IL-2 on these cells.

2.1.2. Peptido-leukotrienes

As mentioned earlier, peptido-leukotrienes were initially isolated during antigenic challenge of sensitized lung, suggesting a role for these compounds in allergic diseases. The observed *in vivo* formation of peptido-leukotrienes following allergen challenge of allergic patients provided strong evidence for this concept (208).

The major sites of peptido-leukotriene action are the airways where they are thought to act as mediators of allergic asthma (209). LTC_4, and LTD_4 stimulate contraction of the smooth muscle of bronchi (73) and smaller airways of parenchymal tissue (210). Aerosolized LTC_4 and LTD_4, inhaled by normal subjects, are 1000 fold more potent than histamine in compromising airway function (74,211,212). Although the bronchoconstrictor effects of LTE_4 are less than those of LTC_4 and LTD_4, they are reported to be longer-lasting. The mechanism of action of peptido-leukotrienes appears to be species specific. Sensitized guinea pig lung, which is often used as a model of asthma, constricts in response to LTC_4 and LTD_4, partially due to the generation of TxA_2 (213). On the other hand, peptido-leukotrienes appear to act principally by a direct effect on human lung tissue (214).

Peptido-leukotrienes also have effects on microvascular tone and permeability. LTC_4 and LTD_4 are potent vasoconstrictors and have been implicated in the vasoconstriction associated with neonatal pulmonary hypertension (208). There are also some reports of vasodilatory activities of these compounds. LTC_4 and LTD_4 increase microvascular permeability due to a direct effect on endothelial cells and are thought to be involved in the enhanced vasopermeability associated with adult

respiratory distress syndrome (208). Other inflammatory diseases which are thought to involve peptido-leukotrienes include psoriasis, rheumatoid arthritis, gout, and inflammatory bowel disease (208,215).

There is strong evidence for the existence of receptors for peptido-leukotrienes (216). Initial studies comparing the activities of peptido-leukotrienes with synthetically-prepared isomers suggested that their biological effects were receptor-mediated. Subsequent studies utilized synthetic antagonists of peptido-leukotrienes and to date these receptors are defined by their interactions with these inhibitors. Using these techniques, human airway tissue was found to have a receptor which interacts equally well with LTC_4, LTD_4 and LTE_4 (217,218). Guinea pig trachea, which has been extensively studied, has three known receptors, including one LTC_4 receptor (219,220) and two LTD_4 receptors (221). The latter LTD_4 receptors are distinguishable because they interact differently with the various inhibitors (216). Further characterization of peptido-leukotriene receptors will require the isolation and characterization of the proteins themselves.

2.2. 12-Lipoxygenase products

2.2.1. 12-HPETE

12-HPETE, which is primarily produced by human platelets, has been reported to inhibit platelet aggregation (222,223). This is partly due to a reduction in TxA_2 production due to inhibition of prostaglandin endoperoxide synthase. However, direct effects of 12-HPETE on human platelets have also been implicated. 12-HPETE has

been reported to inhibit aggregation of aspirin-treated human platelets (224). This anti-aggregatory effect may in part be due to the reported inhibitory effect of 12-HPETE on intracellular Ca^{2+} mobilization, which is known to accompany platelet activation (224). 12-HPETE has also been reported to stimulate its own production by increasing platelet 12-lipoxygenase activity (225).

The effects of 12-HPETE on other cells has also been investigated. 12-HPETE has been reported to inhibit prostacyclin synthase present in porcine and bovine aortic microsomes (226,227). Decreased production of PGI_2 would be expected to have a thrombogenic effect, but it is unclear if sufficient levels of 12-HPETE are produced *in vivo* to generate a response in the vascular endothelium (227). 12-HPETE has also been reported to be chemotactic for PMNL (228). This chemotactic effect is greater than that seen for 12(S)-HETE, but is significantly less than the chemotactic effects of LTB_4. 12-HPETE has been reported to inhibit renin release from rat kidney (229) and has been implicated as the mediator for the inhibition of renin release by angiotensin II (230). 12(S)-HETE also mediates this effect, but higher concentrations are required. 12-HPETE has also been reported to promote insulin release (231), but this effect may be the result of the subsequent conversion of 12-HPETE to hepoxilins as discussed below (section I.2.2.3).

2.2.2. 12-HETE

Although studies on the biological activity of 12(S)-HETE have not revealed a single major physiological role, this eicosanoid has been shown to have

proinflammatory effects including chemotactic and chemokinetic actions on neutrophils and eosinophils (232). Administration of 12(S)-HETE to human skin *in vivo* results in neutrophilic infiltration (233). However, it should be noted that 12(S)-HETE is 1000 fold less potent as a chemotactic agent than LTB_4 (192). 12(S)-HETE, which is a major lipoxygenase product of macrophage-derived foam cells, is also a potent chemotactic agent for smooth muscle cells (234) suggesting a possible role in atherosclerosis.

Prolonged exposure of bovine aortic endothelial cells to 12(S)-HETE inhibits cyclooxygenase activity thereby significantly decreasing production of prostacyclin (235). As prostacyclin has potent antithrombotic and vasodilatory properties, inhibition of its production could exacerbate conditions of thrombogenesis and atherosclerosis. The effect of 12(S)-HETE on PGI_2 production may have more significance than the inhibitory effect of 12-HPETE described above (section I.2.2.1.), because of the large amounts of 12(S)-HETE which can be produced by platelets and macrophage-derived foam cells. Recently, 12(S)-HETE, whose production by human leukocytes is increased by heat shock treatment, has been reported to induce the formation of heat shock proteins by human leukocytes (236), however the relevance of this observation is not yet clear.

As discussed above, a cytochrome P-*450* monooxygenase converts arachidonic acid stereospecifically to 12(R)-HETE. The inversion of the stereochemistry of the C_{12} hydroxyl group of 12-HETE from the ***S*** to the ***R*** configuration has significant effects on biological activity. In general, 12(R)-HETE is a more potent chemotactic and

chemokinetic agent on human peripheral PMNL than 12(S)-HETE (237), presumably because it interacts more strongly with the high affinity receptor for LTB_4. 12(R)-HETE is also chemotactic for lymphocytes, whereas 12(S)-HETE has negligible activity (238). It should be noted that even though 12(R)-HETE is a more potent chemotactic agent than 12(S)-HETE, it is still less potent than LTB_4 (239). In rat cornea, the opposite relationship was observed, since 12(S)-HETE was reported to be more potent in promoting neutrophil infiltration into this tissue than 12(R)-HETE (240). A recent study showed that 12(R)-HETE, but not 12(S)-HETE, inhibits Na^+,K^+-ATPase in bovine cornea (241).

2.2.3. Hepoxilins

In the short time since the first description of hepoxilins, several biological activities have been reported for these hydroxy-epoxy compounds. Both HxA_3 and HxB_3, which are formed by rat pancreatic islets, act on the islets to enhance glucose-dependent insulin release (103). It had previously been reported that 12(S)-HPETE has a similar effect on islets, which may actually be mediated by its subsequent conversion to hepoxilins. HxA_3-C, which is produced by the conjugation of glutathione to HxA_3, has been reported to be present in rat hippocampus and to have potent hyperpolarizing effects on hippocampal neurons, suggesting a neuromodulatory role for this compound (242).

Hepoxilins also have effects on human neutrophils. HxA_3 stimulates phospholipase C and phospholipase A_2 in human neutrophils resulting in the

production of diacylglycerol and liberation of arachidonic acid respectively (243). Both of these effects appear to be receptor-mediated and are thought to involve a G-protein. HxA_3 also stimulates a rise in intracellular Ca^{+2} in these cells by stimulating both a release of intracellular Ca^{+2} and an influx of extracellular Ca^{+2} (244).

2.3. 15-Lipoxygenase metabolites

2.3.1. 15-HPETE

As was described for 12-HPETE above (section I.2.2.1.), the major biological effect of 15-HPETE is the regulation of arachidonic acid metabolism. 15-HPETE inhibits both 5-lipoxygenase (IC_{50} = 0.95 μM) (245) and 12-lipoxygenase (IC_{50} = 2.5 μM) (246). 15-HPETE also suppresses PGI_2 and TxA_2 formation by inhibiting PGI_2 synthase (226,247) and platelet prostaglandin endoperoxide synthase (246), respectively. Interestingly, 15-HPETE has a paradoxical effect on the release of arachidonic acid from phospholipids. At concentrations which inhibit 12-lipoxygenase and prostaglandin endoperoxide synthase, 15-HPETE (10 μM) stimulates the accumulation of free arachidonic acid in collagen-stimulated human platelets which were pretreated with a dual inhibitor of prostaglandin endoperoxide synthase and lipoxygenase (BW755C) (248). At higher concentrations, 15-HPETE (50 μM) has an inhibitory effect on arachidonic acid release.

Although, 15-HPETE does have inherent biological activities, its most important function may lie in its subsequent conversion to lipoxins. As was discussed in section I.1.4.1., 15-HPETE may be converted to LXA_4, LXB_4 and their various

isomers, by several pathways. Lipoxins have various potent biological effects which are discussed in more detail in section I.2.3.4..

2.3.2. 15-HETE

Although 15-HETE does not appear to have any direct proinflammatory activity, it may have an important role in regulating LTB_4-mediated inflammation. 15-HETE has been reported to inhibit 5-lipoxygenase (IC_{50}= 6 μM) in human neutrophils, resulting in decreased production of LTB_4 (249,250). In addition to inhibiting LTB_4 production, 15-HETE also inhibits LTB_4-induced chemotaxis of human neutrophils with a maximal effect seen at a concentration of 10^{-4} M (251). The high concentrations of 15-HETE required to obtain these results brings into question the relevance of these observations. However, under certain severe inflammatory conditions, such as psoriasis, local concentrations of 15-HETE of up to 4×10^{-5} M have been reported (252). Furthermore, administration of 15-HETE to experimental psoriatic lesions resulted in significant improvements to the affected areas (253). Carrageenan-induced arthritis, which is associated with enhanced production of LTB_4, has also been reported to show improvement following treatment with 15-HETE (254). 15-HETE has also been reported to stimulate its own formation by inhibiting 5-lipoxygenase in human PMNL (255). It appears that a 5-lipoxygenase product may inhibit 15-lipoxygenase, therefore inhibition of 5-lipoxygenase results in increased 15-lipoxygenase activity.

15-HETE has been reported to inhibit platelet 12-lipoxygenase (IC_{50}= 8.2 μM) but has very little effect on platelet prostaglandin endoperoxide synthase (IC_{50}= 135 μM) (256). 15-HPETE is three fold more potent than 15-HETE as a 12-lipoxygenase inhibitor, however 15-HPETE inhibits both platelet 12-lipoxygenase and prostaglandin endoperoxide synthase. Therefore, 15-HETE is a specific inhibitor of platelet 12-lipoxygenase and has been used to investigate the role of 12-lipoxygenase metabolites in platelet function (256).

As early as 1978, 15-HETE, which is the major arachidonic acid metabolite found in human airways, was recognized as a possible mediator of asthmatic responses (257). Following allergen challenge, 15-HETE production is enhanced in patients with atopic asthma (72). Furthermore, administration of aerosolized 15(S)-HETE to human subjects with mild atopic asthma results in augmentation of the early asthmatic response (258). 15-HETE has no inherent bronchoconstrictor activity (259) and, in general, suppresses the formation of peptido-leukotrienes by the 5-lipoxygenase pathway. However, more recently, 15-HETE has been reported to enhance 5-lipoxygenase activity in a mast/basophil cell line (260). As a role for mast cells has been implicated in the generation of the early asthmatic response, the stimulatory effect of 15(S)-HETE on the 5-lipoxygenase of mast cells may mediate the enhanced early response observed.

Reticulocyte 15-lipoxygenase activity has been suggested to be involved in the degradation of reticulocyte mitochondria, an event associated with the maturation of erythrocytes (114). This enzyme oxygenates esterified polyenoic fatty acids within the

lipid bilayer of the organelle. This action may destabilize the membrane, thereby increasing susceptibility to proteolytic degradation (114).

2.3.3. 13-HPETE and 13-HODE

Metabolism of linoleic acid by 15-lipoxygenase produces 13-HPODE, which is subsequently reduced to 13-HODE. One of the most interesting activities attributed to 13-HODE is its property as a vascular chemorepellant. 13-HODE, which is produced by vascular endothelial cells in significant amounts under basal conditions, was found to be a thromboresistant factor thereby reducing the adhesion of platelets to endothelial cells (261). The thromboresistant activity of 13-HODE may in part be attributed to its inhibitory effect on the production of the thrombogenic eicosanoid, TxA_2, by platelets (262). 13-HODE has also been reported to stimulate endothelial cells to produce prostacyclin, which has potent vasodilatory and anti-thrombotic properties (263). However, the chemorepellant effect of 13-HODE has been shown to be independent of prostacyclin production.

2.3.4. Lipoxins

The identification of lipoxins was first reported in 1984. However, in the short intervening time several biological activities have been attributed to these compounds.

Stimulated neutrophils, which contains both 5-lipoxygenase and 15-lipoxygenase, produce lipoxins and therefore the effects of lipoxins on these cells have been examined. LXA_4 has been reported to stimulate superoxide anion

generation by neutrophils, however neither LXA_4 nor LXB_4 stimulate neutrophil aggregation or degranulation (138,139). LXA_4 has been reported to stimulate the formation of prostanoids by human leukocytes (264) and to inhibit the production of LTB_4 by these cells (265). In nanomolar concentrations, LXA_4 is chemotactic for neutrophils, however, since pretreatment of these cells with LXA_4 does not interfere with the chemotactic response to LTB_4, it would appear that the effects of these two compounds are mediated by different mechanisms (266).

A role for lipoxins in lung disease has also been suggested. LXA_4 has been reported to stimulate the contraction of lung parenchymal strips obtained from various species including human tissue (267,268,269). Furthermore, addition of LXA_4 to guinea pig bronchial tissue results in stimulation of capsaicin-sensitive sensory neurons which are involved in the bronchomotor response in this tissue (270). Finally, broncho alveolar lavage fluid, obtained from patients with lung disease, has been reported to contain LXA_4, whereas no lipoxins were found in fluid obtained from normal healthy volunteers (271).

Many other activities have been attributed to lipoxins. Both LXA_4 and LXB_4 are reported to inhibit the cytotoxicity of natural killer cells (272). LXA_4 has also been reported to be a more potent activator of protein kinase C than either DAG or arachidonic acid (273), suggesting a role for lipoxins in the modulation of intracellular signals. LXA_4 also has various reported effects on the kidney, including induced glomerular hyperperfusion, hypertension and hyperfiltration (274).

There is an increasing amount of evidence which suggests that LXA_4 may attenuate some of the actions of peptido-leukotrienes by interacting with peptido-leukotriene receptors. LTD_4 has been reported to reduce the glomerular filtration rate of rat kidney by stimulating contraction of mesangial cells through interactions with specific receptors. LXA_4 has been reported to be a competetive inhibitor of this receptor, thereby attenuating the LTD_4-induced mesangial cell contraction (275). Isomers of LXA_4 were not active in this assay suggesting that the *S,R* orientation of the C_5 and C_6 polar groups is important for interaction with the peptido-leukotriene receptor. LTD_4-treated mesangial cells, co-incubated with neutrophils, were reported to show enhanced neutrophil-mesangial cell adhesion. Although LXA_4 was not reported to stimulate adhesion, preincubation of mesangial cells with LXA_4 attenuated LTD_4-induced neutrophil adhesion to mesangial cells (276). As well as acting as a peptido-leukotriene antagonist, some of the biological activities attributed to LXA_4 may be mediated by peptido-leukotriene receptors. LXA_4 has been reported to stimulate contraction of guinea pig lung strips (277). Pre-treatment of the tissue with peptido-leukotriene antagonists (FPL 55712, L-648,05, LY 17 1883) blocked contractions induced by either LXA_4 or LTC_4. Furthermore, preincubation of this tissue with a high concentration of LXA_4 decreased reactivity to LTC_4, also suggesting that LXA_4 and LTC_4 share the same receptor in guinea pig lung (277).

3. Metabolism of hydroxylated PUFA's

It is evident that hydroxylated PUFA's in general have a wide range of biological effects that must be regulated. One of the most obvious forms of regulation is through the conversion of these compounds to less active metabolites.

3.1. Omega-oxidation

The omega oxidation pathway for the metabolism of LTB_4 and 12-HETE is localized primarily in PMNL in humans and results in oxidation of the omega end of these substances. This is the major pathway for the metabolism of LTB_4 by both unstimulated and activated human PMNL. Omega oxidation of LTB_4 in these cel's is initiated by its conversion to 20-hydroxy-LTB_4 by LTB_4 20-hydroxylase (cytochrome P-450_{LTB}) which is found in the microsomal fraction of PMNL and requires NADPH for activity (278,136). 20-Hydroxy-LTB_4 is further metabolized to 20-oxo-LTB_4 by the same enzyme (279). Conversion of 20-oxo-LTB_4 to 20-carboxy-LTB_4, which is the last step in this pathway, is catalyzed by two distinct enzymes (280). The first enzyme is an NAD^+-dependent aldehyde dehydrogenase, whereas the second enzyme is an NADPH-dependent cytochrome P-*450*, indistinguishable from cytochrome P-450_{LTB}. 12-HETE, produced by human platelets, can be transcellularly metabolized by the omega oxidation pathway in human PMNL to 20-hydroxy (281,282) and 20-carboxy (283) metabolites. These reactions are thought to be catalyzed by the same enzyme(s) involved in the metabolism of LTB_4. The omega-oxidation pathway

metabolizes other dihydroxyeicosanoids less efficiently in the order of LTB_4 > 12-epi-8-*cis*-6-*trans*-LTB_4 > 6-*trans*-LTB_4 > 12-epi-6-*trans*-LTB_4 (136,284).

In general, omega-oxidized metabolites of LTB_4 are considerably less potent than LTB_4 in inducing chemotaxis, aggregation and degranulation of human neutrophils (285,286), which may be a reflection of their decreased ability to bind LTB_4 receptors. In contrast, LTB_4 and 20-hydroxy-LTB_4 were reported to be equipotent in inducing contraction of guinea pig lung parenchymal strips (286). In this tissue, the effect of LTB_4 is not direct, but is mediated by the release of TXA_2 (287).

ω-1 and ω-2 oxidations of LTB_4 have also been reported. LTB_4 is metabolized to 19-hydroxy-LTB_4 by rat PMNL (288), rat mononuclear cells (289) and rat liver microsomes (290), and to 18-hydroxy-LTB_4 by rat PMNL (291). It is clear that LTB_4 19-hydroxylase and LTB_4 20-hydroxylase are different enzymes, but it is not yet known whether the formation of 18-hydroxy-LTB_4 and 19-hydroxy-LTB_4 are catalyzed by distinct enzymes. Omega-oxidation products of LTB_4 subsequently undergo extensive ß-oxidation, the end products being CO_2 and water (292).

15(S)-HETE has also been reported to be converted to 15,20-DiHETE by rabbit lung microsomes (293). The cytochrome P-*450* enzyme which catalyzes this omega hydroxylation is indistinguishable from the cytochrome P-*450* prostaglandin 20-hydroxylase which is known to exist in this tissue (137). 5(S)-HETE and 12(S)-HETE are not substrates for this enzyme (293).

3.2. Beta-oxidation

Metabolism of fatty acids by beta-oxidation is an established pathway for the generation of cellular energy in the form of ATP. Beta-oxidation catalyzes the sequential removal of 2-carbon units from fatty acids, resulting in the production of acetyl CoA. Both mitochondria and peroxisomes have beta-oxidation systems which are distinguishable based on the catalytic enzymes, the reaction mechanisms and the response to various inhibitors (294). Beta-oxidation is also a metabolic pathway for hydroxylated PUFAs.

The first description of lipoxygenase products undergoing beta-oxidation came in 1987. Incubation of 12(S)-HETE with vascular smooth muscle cells produced 8-hydroxyhexadecatrienoic acid through the oxidative removal of four carbons (295). Similar results were also obtained from canine renal tubular MDCK cells (296) and cerebral microvascular endothelium (297). Recently, Mathur et al. have described the beta-oxidation of 12-HETE through 4 cycles, resulting in the production of 4-hydroxy-6-dodecenoic acid by mouse peritoneal macrophages (298). Several intermediates in the formation of the octanor compound were also identified in this study. The inability of mitochondrial beta-oxidation inhibitors to interfere with 12-HETE metabolism, as well as the ability to isolate intermediates of the pathway, suggested the involvement of peroxisomal beta-oxidation. Further evidence that the site of beta-oxidation of 12-HETE is the peroxisomes comes from a comparison of the beta-oxidation of 12-HETE by human skin fibroblasts from normal controls and patients with Zellweger's syndrome. Fibroblasts from patients with Zellweger's

syndrome are devoid of peroxisomes and were unable to convert 12(S)-HETE to any detectable products, whereas normal fibroblasts converted 12(S)-HETE to 8-hydroxy-4,6,10-hexadecatrienoic acid (299).

15(S)-HETE has also been reported to be metabolized through two cycles of beta-oxidation by endothelial cells producing 11-hydroxy-4,7-9-hexadecatrienoic acid (300). T-lymphocytes have also been reported to metabolize 15(S)-HETE. Four cycles of beta-oxidation were completed in these cells as indicated by the production of 7-hydroxy-5-dodecenoic acid and several intermediates (301).

3.3. Dehydrogenase/reductase pathways

The dehydrogenase/reductase pathways represent the third major route for the metabolism of hydroxylated eicosanoids. These pathways produce dihydro metabolites of hydroxylated eicosanoids by reducing one of the conjugated double bonds of the substrate. Reduction of a conjugated double bond results in a hypsochromic shift of approximately 40 nm in the UV absorption spectrum of the product, which is extremely useful for the initial identification of dihydro metabolites.

In 1986, Powell reported the metabolism of 6-*trans*-LTB_4 (λ_{max}, 270 nm) to a less polar metabolite which had a maximal absorbance at 230 nm, and which was identified as 5,12,20-trihydroxyeicosatrienoic acid (302). This metabolite was formed by reduction of a conjugated double bond, migration of the remaining two double bonds, followed by ω-hydroxylation. 12-Epi-6-*trans*-LTB_4 is also metabolized by this pathway producing 5,12-dihydroxy-7,9,14-eicosatrienoic acid (303). Incubation

of 12-epi-6-*trans*-[5,6,8,9,11,12,14,15-^{2}H]LTB_4 with human PMNL resulted in loss of the C_5 deuterium atom in the dihydro product. This suggested the formation of a 5-oxo intermediate as an initial step in the formation of dihydro metabolites by human PMNL (303). Incubation of LTB_4 with human PMNL failed to produce any dihydro metabolites (303). It was hypothesized that this may have been due to the rapid metabolism of LTB_4 by the omega-oxidation pathway of human PMNL.

To determine whether LTB_4 could be metabolized to dihydro products, rat neutrophils, which have only moderate omega-oxidation activity, and porcine PMNL, which have very little omega-oxidation activity, were investigated. Both rat (289) and porcine (304) PMNL metabolized LTB_4 to two less polar products which exhibited maximal UV absorbance at 230 nm. The two metabolites were identified as 5,12-dihydroxy-6,8,14-eicosatrienoic acid (10,11-dihydro-LTB_4) and 5-hydroxy-12-oxo-6,8,14-eicosatrienoic acid (10,11-dihydro-12-oxo-LTB_4), indicating the involvement of a 12-hydroxy dehydrogenase and a 10,11-reductase in this pathway (304,292). The involvement of a 12-hydroxy dehydrogenase in the formation of dihydro metabolites by porcine and rat PMNL distinguishes this dehydrogenase/reductase pathway from that of human PMNL which is thought to involve a 5-hydroxy dehydrogenase.

There are several possible routes for the formation of the 10,11-dihydro metabolites of LTB_4, as shown in Figure 6. 10,11-Dihydro-LTB_4 could be formed by direct reduction of the Δ^{10} double bond of LTB_4 (reaction 1), which could then be converted to 10,11-dihydro-12-oxo-LTB_4 by a 12-hydroxy dehydrogenase enzyme (reaction 2). Alternatively, LTB_4 could first be oxidized to 12-oxo-LTB_4 (reaction 3),

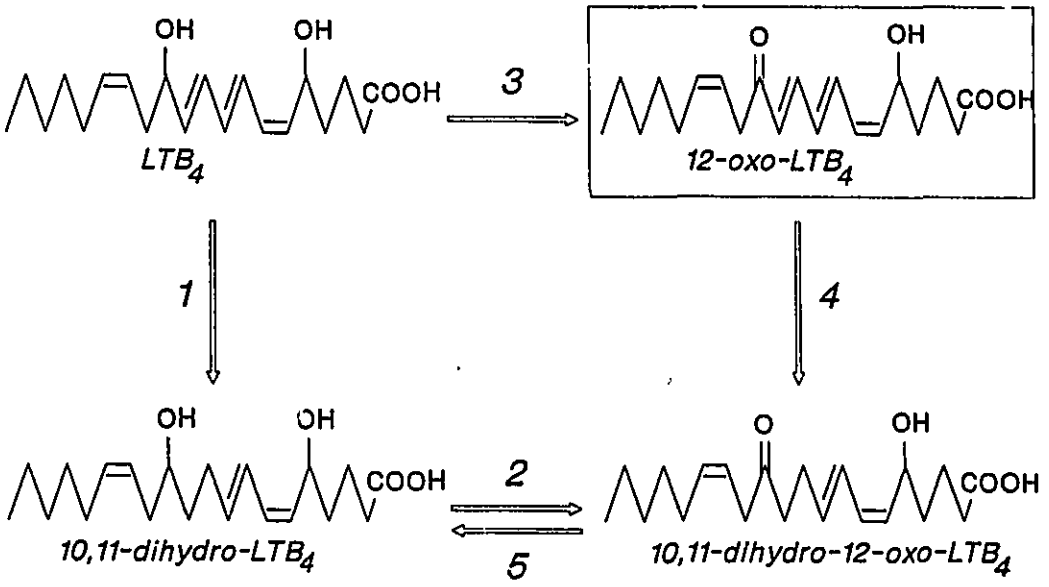

Figure 6. *Possible mechanisms for the formation of dihydro metabolites of LTB_4.* The open arrows indicate possible reactions involved in the pathway. Reactions 1 and 4 would be catalyzed by a 10,11-reductase. Reactions 2 and 3 would be catalyzed by a 12-hydroxy dehydrogenase. Reaction 5 could be catalyzed by either a 12-hydroxy dehydrogenase acting in the reverse direction or by a separate ketoreductase. Prior to this study there was no direct evidence for the formation of 12-oxo-LTB_4.

followed by sequential reductions to 10,11-dihydro-12-oxo-LTB_4 (reaction 4) and 10,11-dihydro-LTB_4 (reaction 5). Powell and Gravelle reported that metabolism of LTB_4, labeled with deuterium at C_{12}, by porcine PMNL produced 10,11-dihydro-LTB_4 with 65% retention of the C_{12} deuterium atom (304). If the formation of a 12-oxo intermediate was involved in the production of 10,11-dihydro-LTB_4, the deuterium atom at C_{12} of LTB_4 would have been removed. Therefore this result suggested that direct reduction of LTB_4 was involved. Although there was no evidence for the formation of 12-oxo-LTB_4, the existence of the other pathway could not be excluded. As mentioned above, formation of 5,12-dihydroxy-7,9,14-eicosatrienoic acid from 12-epi-6-*trans*-LTB_4 by the dehydrogenase/reductase pathway in human PMNL does appear to require oxidation of the C_5 hydroxyl group prior to reduction of the double bond.

4. Polymorphonuclear leukocytes

4.1. Biochemical and physical characteristics

Polymorphonuclear leukocytes (PMNL) are produced in the bone marrow and have a circulating life span of 2-3 days. PMNL are comprised of neutrophils, eosinophils and basophils, but neutrophils account for over 90% of circulating granulocytes. PMNL accumulate at the site of inflammation where their predominant role is phagocytosis and destruction of microorganisms. Mature neutrophils contain a large number of cytoplasmic granules which, based on their contents, are classified

into two populations; the azurophil (primary) and the specific (secondary) granules. Azurophil granules are formed in the early stages of the developing neutrophil. These granules contain acid hydrolases, such as ß-glucuronidase, neutral proteases, cationic proteins, myeloperoxidase and some lysozyme (305). Specific granules are formed later in the development of neutrophils, but mature neutrophils contain 2-3 fold more specific than azurophil granules. Specific granules contain lysozyme, lactoferrin and vitamin B_{12}-binding proteins (305). In the mature neutrophil, the presence of other cytoplasmic organelles, such as rough endoplasmic reticulum and mitochondria, is minimal, and the Golgi complex is reduced in size. These modifications of the cytoplasmic organelles, seen with maturation of the neutrophil, are a reflection of the terminally differentiated state of the mature cell. As already discussed, neutrophils contain 5-lipoxygenase and are the major site of LTB_4 production in the body. Human neutrophils are also the major site for omega-oxidation of LTB_4 and 12-HETE.

4.2. Role in inflammation

Neutrophils, which circulate in the blood under normal conditions, have a major role in the non-specific immune response during the early stages of inflammation (306). In response to an invading organism, neutrophils are stimulated to adhere to the vascular endothelium. Enhanced adhesion of neutrophils to endothelial cells in the area of infected tissues is thought to be mediated by an interaction between leukocyte integrins and endothelial cell adhesion molecules. Cytokine-stimulated endothelial cells express a cell surface glycoprotein termed

endothelial leukocyte adhesion molecule-1 (ELAM-1) which has been reported to be involved in the adhesion of blood neutrophils (307). A second cell adhesion molecule termed intercellular adhesion molecule-1 (ICAM-1) has been reported to contribute to both neutrophil and lymphocyte adhesion (307). These molecules are thought to interact with leukocyte integrins. Stimulated leukocytes have been reported to express three main cell surface integrin molecules, LFA-1, Mac-1 and P 150,95 (308). A receptor-ligand relationship has been reported to exist between ICAM-1 and LFA-1 which may regulate leukocyte migration during inflammation (308). Following adherence, neutrophils undergo diapedesis, a process involving movement through junctional areas between endothelial cells, followed by breaking through the basement membrane. Neutrophils will migrate towards the affected area along a chemotactic gradient generated by chemotaxins produced by the invading microorganism, fragments from the complement cascade, stimulated neutrophils already present, or from other sources such as mast cells, lymphocytes and macrophages. Neutrophils will recognize and phagocytose foreign particles. The resulting phagosome then fuses with cytoplasmic granules, both azurophil and specific, resulting in the formation of a lysosome where digestion of the foreign particles occurs. If the foreign object is too large for phagocytosis, granule contents are released extracellularly. In concert with degranulation, the respiratory burst, which generates oxidants via the hexose-monophosphate shunt, is activated and aids in the killing of microorganisms. Neutrophils die after a short time in exudates and in turn are phagocytosed and digested by macrophages.

4.3. Porcine PMNL

As human blood is often difficult to obtain regularly and in large quantities, blood from other species has been examined, in particular bovine and porcine blood. A study in 1983 (309) compared the morphological and biochemical characteristics of human and porcine PMNL. It was shown that the morphology of porcine PMNL is very similar to that of human PMNL, both containing azurophil and specific granules. Although the degree of enzyme activities did not always correspond, both human and porcine PMNL did express all of the enzyme activities tested.

5. Aims of the present study

The physiological importance of the pathway for the formation of dihydro metabolites of LTB_4 is not clear. This pathway may serve as a mechanism to remove the potent proinflammatory effects of LTB_4. Alternatively, it may produce biologically active metabolites, possibly from substrates other than LTB_4. To gain a further understanding of the relevance of this pathway, it is important to understand the mechanism for the formation of dihydro metabolites, and to identify the products formed from various substrates. Therefore, the general aim of this study was to further characterize the pathway for the formation of dihydro metabolites of eicosanoids.

Although dihydro metabolites of LTB_4 are formed by both porcine and rat PMNL, porcine PMNL were used in this study for several reasons. First, large quantities of viable PMNL could be purified in a reasonable amount of time, allowing

for large scale preparation of metabolites necessary for identification procedures. Secondly, porcine PMNL have minimal interfering omega-oxidation activity, which is present in both human and rat PMNL. Finally, previous experiments from our laboratory revealed the reductase pathway to be the major pathway for the metabolism of LTB_4 by these cells (304).

It is reasonable to suggest that compounds structurally related to LTB_4 may also be metabolized by the reductase pathway. Therefore, the first goal of this study was to examine the metabolism of various hydroxylated PUFA's by porcine PMNL and to characterize the metabolites produced. The strategies employed to attain this goal were as follows:

1. The structural requirements for the formation of dihydro metabolites were identified by correlating the extent of metabolism of various hydroxylated PUFA's by porcine PMNL with their structural features.
2. Dihydro metabolites of substrates other than LTB_4 were identified by GC-MS. In particular the dihydro metabolites of 12(S)-HETE and 13-HODE were examined.

It had previously been shown that porcine PMNL could convert 10,11-dihydro-12-oxo-LTB_4 to 10,11-dihydro-LTB_4. The reduction of the C_{12}-oxo group in this reaction could result in a hydroxyl group with the *R* or the *S* configuration. Therefore, the second major goal of this study was to determine the

configuration of the C_{12}-hydroxyl groups of the dihydro metabolites of LTB_4 and 12(S)-HETE. Two methods for stereochemical analysis were employed:

1. NP-HPLC on a silicic acid column of underivatized dihydro compounds (dihydro-LTB_4).
2. Derivatization of the C_{12}-hydroxyl group with an optically active agent followed by NP-HPLC (12-hydroxy-5,8,14-eicosatrienoic acid).

The final goal of this study was to determine the mechanism for the formation of dihydro metabolites by porcine PMNL. As was mentioned above, the initial step in this pathway could either be direct reduction of the Δ^{10} double bond or oxidation of the C_{12} hydroxyl group to an oxo group. Previous studies of this pathway did not report the formation of 12-oxo-LTB_4, however this product may be labile and undetectable in incubations with whole cells. The strategies employed to determine the mechanism for the formation of dihydro metabolites were as follows:

1. To analyse the kinetics of the metabolism of LTB_4 and the dihydro metabolites of LTB_4 by intact porcine PMNL.
2. To determine the subcellular localization of the enzymes required for the formation of dihydro metabolites by comparing their activities in subcellular fractions with the activities of marker enzymes for various organelles
3. Once isolated, to identify the catalytic activity, substrate specificity, and cofactor requirements of the enzymes of this pathway.

4. To identify any intermediates formed during the metabolism of LTB_4 by subcellular fractions which were undetected using intact cells.

Section II

MATERIALS and METHODS

1. Materials

1.1. Sources of biological samples

Porcine peripheral blood was collected in the presence of EDTA (final concentration 10 mM) from a local abattoir. Human blood (200 ml) was collected from normal volunteers in a blood bag (Miles Inc., Elkhart, IN) containing anti-coagulant (citrate phosphate dextrose adenine solution).

1.2. Chemicals, reagents and solvents

(R)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA), 1,3-dicyclohexyl-carbodiimide, 4-dimethylaminopyridine and Diazald were obtained from Aldrich (Milwaukee, WI). A23187 was obtained from Calbiochem (LaJolla, CA). Unlabeled arachidonic acid, linoleic acid and 5,8,11,14,17-eicosapentaenoic acid were purchased from Nuchek Prep Inc. (Elysian, MN). Unlabeled $PGF_{2\alpha}$ was obtained from the Upjohn Co. (Kalamazoo, MI). [1-^{14}C]Arachidonic acid, [1-^{14}C]linoleic acid, [5,6,8,11,12,14,15-^{3}H]PGE_2 and [5,6,8,9,11,12,14,15-^{3}H]$PGF_{2\alpha}$ were obtained from Du Pont-New England Nuclear (Wilmington, DE). 5,8,11,14-Eicosatetraynoic acid (ETYA) was kindly provided by Dr. J. R. Paulsrud of Hoffman-La Roche. DL-Dithiothreitol, phenylmethylsulfonyl fluoride, o-dianisidine dihydrochloride, phenolphthalein glucuronic acid, PGB_2, NADH and $NADP^+$ were purchased from the Sigma Chemical Co. (St. Louis, MO). Ouabain, adenosine-5'-triphosphate, NAD^+ and NADPH were purchased from Boehringer Mannheim

(St. Laurent, Que.). Dextran T-500 and Ficoll-Paque were purchased from Pharmacia Fine Chemicals (Dorval, Que.). All organic solvents used for HPLC were of HPLC grade and were purchased from Fisher Scientific Co. (Fairlawn, NJ). Formula 963 liquid scintillation fluid was obtained from New England Nuclear Corp. (Montreal, Que.).

2. Preparation of PMNL, platelets and subcellular fractions of PMNL

2.1. PMNL (136)

Peripheral blood was mixed with 6% Dextran T-500 in saline (5:1) which accelerates the sedimentation of red blood cells. After 45 min, the supernatant was removed and centrifuged at 250 x *g* for 10 min to pellet leukocytes. Porcine leukocytes used in preparative experiments were not purified further. Human PMNL and porcine PMNL used for analytical experiments were further purified by layering the crude leukocyte preparation described above over Ficoll-Paque in a ratio of 3:2, followed by centrifugation at 500 x *g* for 30 min at 4 °C. Any remaining red blood cells in either the crude leukocyte or purified PMNL preparations were lysed by incubation with 0.135 M NH_4Cl at 37 °C for 10 min. The cells were washed twice with 0.15 mM NaCl and then resuspended in Dulbecco's phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$.

2.2. Platelets (19)

Human blood was treated with 6% dextran to sediment red blood cells, and leukocytes were removed as described above (section 2.1.). The remaining plasma was centrifuged at 1400 x *g* for 25 min at 4 °C. The pellet, containing the platelets, was washed twice with Tris-saline-EDTA buffer (0.15 M NaCl, 0.15 M Tris, 0.077 M EDTA; pH 7.4) and resuspended in PBS without Ca^{+2} or Mg^{+2}.

2.3. Disruption and fractionation of porcine PMNL

Porcine PMNL were purified as described above and resuspended in Tris-acetate-sucrose buffer (0.01 M Tris-acetate, 0.25 M sucrose; pH 7.4) containing 2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and 1 mM EDTA (187.5 x 10^6 cells/ml). The cells were placed in an ice-water bath and disrupted by sonication (model 4710 Ultrasonic Homogenizer; Sonics & Materials, Danbury, CT) for 2 x 10 sec at a setting of 40 cycles/sec. The disruptate was centrifuged at 1500 x *g* at 4 °C for 10 min to remove unbroken cells and nuclei. The post-nuclear supernatant was centrifuged at 20,000 x *g* at 4 °C for 20 min in a Beckman L-Series Ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA) producing a granule-rich pellet. The 20,000 x *g* supernatant was centrifuged at 150,000 x *g* at 4 °C for 120 min, producing microsomal and cytosolic fractions. Both the granule-rich pellet and the microsomal pellet were resuspended by homogenization with a glass homogenizer and teflon pestle (Potter-Elvehjem) (10 strokes) in Tris-acetate-sucrose buffer

3. Biosynthesis and purification of substrates

3.1. High-pressure liquid chromatography (HPLC) apparatus

Unless stated otherwise, the HPLC system consisted of a Waters solvent delivery system comprised of two model 510 pumps coupled to a model 680 gradient controller and a model 490 UV detector (Waters-Millipore, Bedford, MA) . The radioactivity in column eluates was monitored by a Ramona 5-LS radioactivity detector (Raytest, Fairfield, NJ). Reversed-phase (RP) HPLC was performed on a column (250 x 4.6 mm) of octadecylsilyl (ODS) silica (5 μm Spherisorb ODS-2; Jones Chromatography, Llanbradach, Glam. or Phenomenex, Rancho Palos Verdes, CA) for large scale purification of substrates and their metabolites. RP-HPLC analysis of smaller samples, generated from analytical experiments, was performed on a Novapak C_{18} column (3.9 x 150 mm; Waters-Millipore). Normal-phase (NP) HPLC was performed on a column (300 x 4.6 mm) of silicic acid (5-μm RoSil; Alltech Associates, Inc.; Deerfield, IL).

3.2. Batch extraction procedure (310)

Samples with a volume of 5 ml or more were centrifuged at 400 x g for 10 min at 4 °C to remove cell debris. Methanol or ethanol was added to the supernatant to give a final concentration of 15% followed by acidification to pH 2-3 with 1N HCl. Cartridges of ODS silica (C_{18} Sep-Pak; Waters-Millipore) were treated with methanol (20 ml) and water (20 ml). The sample was then loaded onto a cartridge, which was

then washed with 15% ethanol in water (20 ml), water (20 ml) and petroleum ether (10 ml). A maximum of 60 ml of incubation medium containing MeOH (15%) was loaded onto each cartridge. Metabolites of arachidonic acid and linoleic acid were then eluted with redistilled methyl formate (10 ml; Aldrich).

3.3. Preparation of hydroxyeicosanoids using porcine PMNL

Unlabeled LTB_4, 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, 5(S)-HETE, LTB_5, 12-epi-8-*cis*-6-*trans*-LTB_4 and 12(S)-HETE were all synthesized by incubation of porcine PMNL with arachidonic acid in the presence of A23187 (10 μM) (311,144).

Unlabeled LTB_4, 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, and 5-HETE were prepared by preincubating porcine leukocytes (75 x 10^6 cells/ml) with 10 μM ETYA in PBS for 2 min at 37 °C. Arachidonic acid (final concentration 100 μM) and A23187 (final concentration 10 μM) were then added and the incubation continued for 10 min at 37 °C. Preincubation with ETYA selectively inhibits 12-lipoxygenase thereby allowing 5-lipoxygenase products to predominate. LTB_5 was prepared under identical conditions from 5,8,11,14,17-eicosapentaenoic acid. Unlabeled 12(S)-HETE and 12-epi-8-*cis*-6-*trans*-LTB_4 were prepared by incubating arachidonic acid and A23187 (10 μM) with porcine PMNL in the absence of ETYA. All products released into the supernatants were extracted on C_{18} Sep Pak cartridges. LTB_4, 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, 5-HETE, LTB_5, 12-epi-8-*cis*-6-*trans*-LTB_4 and 12(S)-HETE were purified by RP-HPLC using the conditions described in Table I.

TABLE I

HPLC conditions for purification of hydroxylated polyunsaturated fatty acids

Product	Enzyme Source	HPLC 1[a]	HPLC 2	HPLC 3
LTB_4	Porcine PMNL	GRAD 1 (48)[b]	39% Solv C (35)	4.5% Solv F (35)
6*t*-B_4[c]	Porcine PMNL	GRAD 1 (32)	39% Solv C (35)	
12e-6*t*-B_4	Porcine PMNL	GRAD 1 (38)	39% Solv C (35)	
12e-8*c*-6*t*-B_4	Porcine PMNL	GRAD 1 (48)	39% Solv C (29)	
5-HETE	Porcine PMNL	GRAD 1 (74)	39% Solv C (38)	
LTB_5	Porcine PMNL	GRAD 1 (37)	39% Solv C (30)	
12(S)-HETE	Porcine PMNL	GRAD 1 (71)	49% Solv C (32)	
12(S)-HETE	Human platelets	GRAD 2 (59)	49% Solv C (32)	
HHTrE	Human platelets	GRAD 2 (44)		
15(S)-HETE	Soybean LOX	10% Solv E (29)	49% Solv C (27)	
13(S)-HODE	Soybean LOX	10% Solv E (32)		
9(S)-HODE	Tomato LOX	1.25% Solv F (28)		
12(R)-HETE	Sea urchin eggs	0.5% Solv F (25)	49% Solv C (32)	
dho-B_4	Porcine PMNL	39% Solv C (62)		

[a] The mobile phases for RP-HPLC were prepared from water/acetic acid (100:0.02; Solv A) and either methanol/acetic acid (100:0.02; Solv B) or acetonitrile/acetic acid (100:0.02; Solv C). Gradients generated as follows: Grad 1: 0 min, 59% Solv B; 50 min, 59% Solv B; 55 min, 70% Solv B; 95 min, 84% Solv B, Grad 2: 0 min, 28% Solv C; 60 min, 60% Solv C. All gradient segments were linear. The mobile phases for NP-HPLC were prepared from hexane/acetic acid (100:0.1; Solv D) and either tetrahydrofuran/acetic acid (100:0.1; Solv E) or 2-propanol/acetic acid (100:0.1; Solv F). The flow rate was 2 ml/min in all cases.

[b] Retention times in minutes are shown in brackets.

[c] Abbreviations: 6*t*-B_4, 6-*trans*-LTB_4; 12e-6*t*-LTB_4, 12-epi-6-*trans*-LTB_4; 12e-8*c*-6*t*-B_4, 12-epi-8-*cis*-6-*trans*-LTB_4; dho-B_4, 10,11-dihydro-12-oxo-LTB_4; LOX, lipoxygenase; Solv, solvent.

3.4. Preparation of [1-^{14}C]-labeled-hydroxyeicosanoids produced by human PMNL (109)

[1-^{14}C]-Labeled LTB_4, 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4, 5(S)-HETE, and 12-epi-8-*cis*-6-*trans*-LTB_4 were prepared by incubation of [1-^{14}C]arachidonic acid with human PMNL. [1-^{14}C]Arachidonic acid (3 μCi) was incubated with purified human PMNL (7 ml; 30 x 10^6 cells/ml) for 4 min at 37 °C. The reaction was stopped by addition of methanol (1.5 ml). Products were extracted on a C_{18} Sep Pak cartridge and were purified by HPLC using the conditions outlined in Table I.

3.5. Preparation of [1-^{14}C]-labeled 12(S)-HETE, HHTrE and unlabeled HHTrE using human platelets (19)

[1-^{14}C]Arachidonic acid (3 μCi) or unlabeled arachidonic acid (5 mg) was incubated with platelets (1 x 10^9 cells/ml; 3ml) for 30 min at 37 °C. The reaction was stopped by the addition of methanol (1.5 ml) and the products were extracted using a C_{18} Sep Pak cartridge. 12(S)-HETE and HHTrE were purified by HPLC using the conditions described in Table I.

3.6. Preparation of [1-^{14}C]-labeled and unlabeled 15(S)-HETE and 13(S)-HODE using soybean lipoxygenase (117)

Lyophilized soybean lipoxygenase (1 mg) was dissolved in 7.5 ml 0.1 M sodium borate buffer (pH 9.0). Unlabeled linoleic acid or arachidonic acid (5 mg) was added to 3 ml of the enzyme mixture, whereas [1-^{14}C]linoleic acid or

[1-^{14}C]arachidonic acid (3 μCi) was added to 0.6 ml soybean lipoxygenase. The mixtures were placed in an ice bath and stirred constantly. After 15 min, an amount of enzyme (0.6 or 3 ml) equivalent to that present initially was added and the reaction was allowed to proceed for a further 15 min. The reaction was terminated by addition of ethanol (1.5 ml) and the hydroperoxy products were extracted using a C_{18} Sep-Pak cartridge. The hydroperoxy groups of the products were reduced to hydroxyl groups with $NaBH_4$ (1 mg) in methanol (0.3 ml) for 20 min at 0 °C and 40 min at room temperature. Methanol (0.45 ml) was added and the products were extracted using a C_{18} Sep-Pak cartridge. 13-HODE and 15-HETE were purified by HPLC as described in Table I.

3.7. Preparation of [1-^{14}C]-labeled and unlabeled 9(S)-HODE (125)

A homogenate of a red tomato (skin and seeds excluded) was prepared in 0.1 M sodium acetate buffer (pH 5.5; 1.5 ml per gram tomato). Unlabeled linoleic acid (5 mg) or [1-^{14}C]linoleic acid (2 μCi) was incubated with 20 ml or 5 ml of homogenate, respectively, for 20 min at 25 °C. Oxygen was bubbled through the mixture for 15 second periods every 2 min. The reaction was stopped by the addition of ethanol (0.15 vol) and the 9(S)-hydroperoxy product was extracted using a C_{18} Sep-Pak cartridge. 9(S)-HPODE was reduced to 9(S)-HODE with $NaBH_4$ as described above (section II.3.6.). 9(S)-HODE was purified by HPLC as described in Table I.

3.8. Preparation of 12(R)-HETE (127)

Sea urchins (Strongylocentrutus Purpuratus; Marinus Co., Long Beach, CA) were injected with 0.5 M KCl (5 ml) to induce spawning. Eggs were harvested and washed in Shapiro's artificial seawater (452 mM NaCl, 10 mM KCl, 25 mM $MgCl_2 \cdot 6H_2O$, 17 mM $MgSO_4 \cdot 7H_2O$, 4.4 mM $CaCl_2 \cdot 2H_2O$, 10 mM Tris base: pH 7.8). Following centrifugation at 400 x *g* for 10 min at 4 °C, the eggs were resuspended in 10 vol of 20 mM Tris-HCl containing 5 mM $CaCl_2$ (pH 7.5). The eggs were homogenized on ice with a Tissumizer Mark II homogenizer (Teckmar Inc, Cincinnati, OH) for 4 x 10 sec (13 500 rpm). Arachidonic acid (300 μM) was incubated with the homogenate at 37 °C for 60 min. The incubation was terminated by the addition of MeOH (0.25 vol). The homogenate was centrifuged at 900 x *g* for 10 min to remove cell debris. The supernatant was extracted on C_{18} Sep Pak cartridges as described in section II.3.2., except that the concentration of MeOH in the supernatant and the wash solvent was 25% instead of 15%. Monohydroxy products were separated from fatty acids on an open column of silicic acid (20 g) which was wetted with hexane (75 ml). The sample was loaded onto the column in a mixture of 2-propanol (100 μl), ethyl acetate (500 μl), acetic acid (30 μl) and hexane (30 ml) under nitrogen pressure. The column was washed with 100 ml ethyl acetate/hexane/acetic acid (6:94:0.01) to remove fatty acids, followed by 200 ml ethyl acetate/hexane/acetic acid (20:80:0.1) to elute monohydroxy fatty acids. Under these conditions, 11(R)-HETE and 12(R)-HETE were the major metabolites produced. Small amounts of 11(R)-hydroxyeicosapentaenoic acid (HEPE) and 12(R)-HEPE were

also produced from endogenous 5,8,11,14,17-eicosapentaenoic acid. 12(R)-HETE was purified by HPLC as described in Table I.

The configuration of the 12-hydroxyl group of 12(R)-HETE was confirmed by chiral-phase HPLC on a Chiracel OC column (10 μm; 250 x 4.6 mm; J.T. Baker, Phillipsburg, NJ). The retention time of 12(R)-HETE was compared with the retention time of 12(S)-HETE and a mixture of 12(R)- and 12(S)-HETE prepared by photooxidation of arachidonic acid. The 12-HETE compounds were methylated prior to chiral-phase HPLC. The mobile phase was 2-propanol/hexane (2:98) with a flow rate of 1 ml/min. The retention times of the methyl esters of 12(R)-HETE and 12(S)-HETE were 20 and 22 min, respectively. 12-HETE produced by this procedure was greater than 99% 12(R)-HETE.

3.9. Preparation of 10,11-dihydro-12-oxo-LTB_4 (304)

LTB_4 (2 μM) was incubated with porcine leukocytes (75 x 10^6 cells/ml) for 60 min at 37 °C. The incubation was stopped by the addition of methanol (0.15 vol) and the products were extracted using a C_{18} Sep Pak cartridge. 10,11-Dihydro-12-oxo-LTB_4 was purified by HPLC as described in Table I.

3.10. Preparation of tritiated substrates [*10,11-dihydro-[12-^{3}H]LTB$_4$, 10,11-dihydro-12-epi-[12-^{3}H]LTB$_4$ and [9ß-^{3}H]PGF$_{2\alpha}$*]

3.10.1. Reaction conditions

10,11-Dihydro-[12-^{3}H]LTB$_4$ and 10,11-dihydro-12-epi-[12-^{3}H]LTB$_4$ were prepared by reduction of 10,11-dihydro-12-oxo-LTB$_4$ (25 μg) with sodium [^{3}H]borohydride (15 mCi; 15 Ci/mmol) in methanol (0.15 ml). The mixture was kept at 0 °C for 15 min and at room temperature for a further 45 min followed by extraction using a C_{18} Sep Pak cartridge. [9ß-^{3}H]PGF$_{2\alpha}$ was prepared under identical conditions from PGE$_2$ (100 μg) and sodium [^{3}H]borohydride (15 mCi; 15 Ci/mmol).

3.10.2. Purification of 10,11-dihydro-[12-^{3}H]LTB$_4$ and 10,11-dihydro-12-epi-[12-^{3}H]LTB$_4$

10,11-Dihydro-[12-^{3}H]LTB$_4$ (t_R= 19 min) and 10,11-dihydro-12-epi-[12-^{3}H]LTB$_4$ (t_R= 22 min) were separated by NP-HPLC with a mobile phase of 2-propanol/hexane/acetic acid (4.5:95.5:0.1) under isocratic conditions with a flow rate of 2 ml/min.

3.10.3. Purification [9ß-^{3}H]PGF$_{2\alpha}$

[9ß-^{3}H]PGF$_{2\alpha}$ was purified by RP-HPLC with a linear gradient between acetonitrile/water/acetic acid (30:70:0.02) and acetonitrile/water/acetic acid (40:60:0.02) over 30 min with a flow rate of 1.5 ml/min. The retention times were: [9α-^{3}H]PGF$_{2\beta}$ (t_R= 11 min) and [9ß-^{3}H]PGF$_{2\alpha}$ (t_R= 15 min).

3.11. Synthesis of 12(S)-HETE and 12(R)-HETE by photooxidation of arachidonic acid

To prepare a mixture of 12(S)-HPETE and 12(R)-HPETE, arachidonic acid (20 mg) was dissolved in 0.1% methylene blue in methanol and irradiated with light (illuminator, model 9741-50; Cole Parmer, Chicago, IL) for 60 min at room temperature. The solution was bubbled with oxygen continuously. The reaction products were extracted using a C_{18} Sep Pak cartridge and reduced with $NaBH_4$ as described above (section II.3.6.). Conditions for the purification of 12(R) and 12(S)-HETE were the same as those described for the purification of 12(R)-HETE (Table I).

3.12. Chemical Synthesis of 10,11-dihydro standards (312,313).

10,11-Dihydro-LTB_4 [5(S),12(S)-dihydroxy-6Z,8E,14Z-eicosatrienoic acid], 10,11-dihydro-12-epi-LTB_4 [5(S),12(R)-dihydroxy-6Z,8E,14Z-eicosatrienoic acid], 12(R)-hydroxy-5Z,8Z,14Z-eicosatrienoic acid, and 12(S)-hydroxy-5Z,8Z,14Z-eicosatrienoic acid were chemically synthesized by Drs J.R. Falck and P. Yadagiri at the University of Texas Southwestern Medical Center, Dallas, Texas.

4. Identification of metabolites of hydroxylated PUFA's

4.1. Biosynthesis and purification of metabolites of LTB_4

4.1.1. 10,11-Dihydro-12-epi-LTB_4

LTB_4 (2 μM) was incubated with porcine leukocytes (75 x 10^6 cells/ml) for 60 min at 37 ° C. The incubation was terminated by addition of methanol (0.15 vol) and the products were extracted using a C_{18} Sep Pak cartridge. 10,11-Dihydro-12-epi-LTB_4 was partially purified by RP-HPLC under the same conditions described for 10,11-dihydro-12-oxo-LTB_4 (Table I). 10,11-Dihydro-12-epi-LTB_4 was then separated from 10,11-dihydro-LTB_4 by NP-HPLC as described above (section II.3.10.2.).

4.1.2. 12-Oxo-LTB_4

LTB_4 (4 μM) was incubated with a microsomal fraction from porcine PMNL (approximately 1.5 mg protein/ml) for 40 min at 37 °C. The incubation was terminated by addition of methanol (0.15 vol) followed by immediate extraction on a C_{18} Sep Pak cartridge at neutral pH. 12-Oxo-LTB_4 (t_R= 25 min) was partially purified by RP-HPLC on a Novapak C_{18} column (3.9 x 150 mm; Waters-Millipore) with a mobile phase of acetonitrile/water/acetic acid (38:62:0.02) at a flow rate of 1 ml/min. UV absorbance was monitored with a Waters model 991 photodiode array detector (Waters-Millipore). Further purification of 12-oxo-LTB_4 (t_R= 18.5 min) was achieved by NP-HPLC with a mobile phase of 2-propanol/hexane/acetic acid (4.5:95.5:0.1) at a flow rate of 2 ml/min.

4.2. Biosynthesis and purification of metabolites of 12(S)-HETE [*12-hydroxy-5,8,14-eicosatrienoic acid and 12-oxo-5,8,14-eicosatrienoic acid*]

12(S)-HETE (2 μM) was incubated with porcine leukocytes (75 x 10^6 cells/ml) for 40 min at 37 °C. The incubation was terminated by the addition of ice-cold methanol (0.15 vol) and immediate cooling to -20°C. The supernatant was extracted using a C_{18} Sep Pak cartridge. Products were purified by RP-HPLC with a linear gradient between acetonitrile/water/acetic acid (28:72:0.02) and acetonitrile/water/acetic acid (52:48:0.02) over 60 minutes at a flow rate of 2 ml/min. The major products were 12-hydroxy-5,8,14-eicosatrienoic acid (t_R= 49 min) and 12-oxo-5,8,14-eicosatrienoic acid (t_R= 58 min).

4.3. Biosynthesis and purification of metabolites of 13-HODE [*13-hydroxy-9-octadecenoic acid, 13-oxo-9-octadecenoic acid, 11-hydroxy-7,9-hexadecadienoic acid and 9-hydroxy-5,7-tetradecadienoic acid*]

13-[1-^{14}C]HODE (2 μM) was incubated with porcine leukocytes (75 x 10^6 cells/ml) for 60 min at 37 °C in the presence of 5 μM ETYA (which inhibits ß-oxidation). Metabolites of 13-HODE were purified by RP-HPLC with a mobile phase of acetonitrile/water/acetic acid (55:45:0.02) under isocratic conditions at a flow rate of 2 ml/min. The major products were 13-hydroxy-9-octadecenoic acid (t_R= 57 min) and 13-oxo-9-octadecenoic acid (t_R= 68 min).

Uniformly labelled 13-[1-^{14}C]HODE (2 μM) was also incubated with porcine PMNL (75 x 10^6 cells/ml) in the absence of ETYA for 60 min at 37 °C. Metabolites

were purified by RP-HPLC with a mobile phase consisting of a linear gradient between acetonitrile/water/acetic acid (28:72:0.02) and acetonitrile/water/acetic acid (52:48:0.02) over 60 min at a flow rate of 2 ml/min. The major products were 11-hydroxy-7,9-hexadecadienoic acid (t_R= 40 min) and 9-hydroxy-5,7-tetradecadienoic acid (t_R= 27 min).

4.4. Gas chromatography-mass spectrometry.

Products purified by HPLC were methylated with diazomethane (30 min, 23 °C) unless otherwise indicated. In some cases, products which were suspected to have an oxo group were derivatized by treatment with hydroxylamine HCl (1 mg) in pyridine (0.1 ml) overnight at room temperature. Diethyl ether (2 ml) was then added and the mixture was kept at -20 °C for 30 min. After centrifugation, the diethyl ether was removed and evaporated under a stream of nitrogen. Due to the unstable nature of 12-oxo-LTB_4, this compound was hydrogenated with rhodium on alumina (1 mg; Aldrich) in 0.6 ml MeOH for 3 min at 23 °C. The reagent was subsequently removed by filtering the sample through a small column of silicic acid. All products were then converted to their trimethylsilyl derivatives by treatment with N-methyl-N-trimethylsilyltrifluoroacetamide (30 min, 23 °C).

Electron impact gas-chromatography mass-spectrometry (GC-MS) was carried out on a VG ZAB instrument located in the Biomedical Mass Spectrometry Unit of McGill University. The stationary phase was a column (20 m x 0.32 mm) of DB-1 (J and W Scientific Inc., Folsom, CA).

4.5. Nuclear magnetic resonance spectroscopy

^{1}H-NMR spectroscopy ($CDCl_3$, 500 MHz) was performed by Drs J.R. Falck and P. Yadagiri on a Varian model VXR-500S instrument situated in the Department of Molecular Genetics at the University of Texas Southwestern Medical Center in Dallas, Texas.

4.6. Steric analysis of 10,11-dihydro-12-hydroxyeicosanoids

4.6.1. Resolution and identification of 10,11-dihydro-12-HETE isomers

12-Hydroxy-5,8,14-eicosatrienoic acid was prepared and purified as described in section 4.2.. To enable resolution of the 12(R) and 12(S) isomers, these compounds were first methylated with diazomethane (30 min; 23 °C) and then converted to their MTPA derivatives by treatment with (R)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) (1 mg), 1,3-dicyclohexylcarbodiimide (1 mg) and 4-dimethylaminopyridine (0.1 mg) in 20 μl CCl_4 (10 min, 25 °C). The reaction was terminated by evaporation of the CCl_4 under argon and addition of 50 μl methanol. Separation of the MTPA derivatives from contaminants derived from the reagents was accomplished by RP-HPLC using an Ultremex C_6 column (250 x 4.6 mm; Phenomenex) with a mobile phase of acetonitrile/water (70:30) at a flow rate of 2 ml/min. Experiments with radioactive monohydroxy compounds indicated that the conversion was virtually quantitative and no other radioactive products were detected. Stereoisomers were resolved by NP-HPLC with a mobile phase of 0.08% 2-propanol in hexane at a flow rate of 2 ml/min. The MTPA

derivatives of 12(S)-hydroxy-5,8,14-eicosatrienoic acid (t_R= 23 min) and 12(R)-hydroxy-5,8,14-eicosatrienoic acid (t_R= 25 min) were identified by co-chromatography with MTPA derivatives of authentic standards.

4.6.2. Resolution and identification of 10,11-dihydro-LTB$_4$ isomers

10,11-Dihydro-LTB$_4$ and 10,11-dihydro-12-epi-LTB$_4$ were prepared and purified as described in section II.4.1.1.. 10,11-Dihydro-LTB$_4$ and 10,11-dihydro-12-epi-LTB$_4$ were resolved by NP-HPLC as described in section II.3.10.2. and were identified by co-chromatography with authentic standards.

5. Analysis of metabolites of hydroxylated-PUFA's by HPLC

5.1. Time courses for the production of dihydro metabolites

5.1.1. Time course for the metabolism of 12(S)-HETE

Purified porcine PMNL (20 ml; 5 x 10^7 cells/ml) were incubated at 37 °C with 12(S)-[1-^{14}C]HETE (2 μM; 2 μCi) for various times. The reactions were stopped by addition of ice-cold methanol (0.5 vol) and immediate cooling to -20 °C. After the addition of PGB_2 (200 ng) as internal standard, products were extracted using C_{18} Sep Pak cartridges. The products were purified by RP-HPLC with a mobile phase of acetonitrile/water/acetic acid (55:45:0.02) at a flow rate of 2 ml/min. Material in the peak corresponding to the 10,11-dihydro metabolite of 12(S)-HETE was methylated with diazomethane and derivatized with MTPA. The MTPA derivatives of 12(S)-hydroxy-5,8,14-eicosatrienoic acid and 12(R)-hydroxy-5,8,14-eicosatrienoic acid were

resolved as described above (section II.4.6.1.). The products were quantitated by measuring the radioactivity in fractions collected every 0.5 min by liquid scintillation counting.

5.1.2. Time courses for the metabolism of LTB_4, 10,11-dihydro-LTB_4, 10,11-dihydro-12-epi-LTB_4 and 10,11-dihydro-12-oxo-LTB_4

Porcine PMNL (10 ml; 5 x 10^7 cells/ml) were incubated with LTB_4 (2 μM), 10,11-dihydro-LTB_4 (2 μM), 10,11-dihydro-12-epi-LTB_4 (2 μM) or 10,11-dihydro-12-oxo-LTB_4 (2 μM) at 37 °C for various times. The incubations were terminated by addition of methanol (0.4 vol) followed by cooling to -20 °C. PGB_2 (200 ng) was added to each of the incubation mixtures as an internal standard. The incubation medium was then extracted using a C_{18} Sep Pak cartridge and the products resolved by NP-HPLC as described above (section II.3.10.2.). The amounts of 10,11-dihydro-LTB_4, 10,11-dihydro-12-epi-LTB_4 and 10,11-dihydro-12-oxo-LTB_4 were calculated from the ratios of their peak areas at 235 nm to that of PGB_2, the internal standard, at 280 nm. The extinction coefficients used for the dihydro compounds and PGB_2 were 22 500 and 28 600, respectively.

5.2. Substrate specificity for the formation of dihydro metabolites of hydroxylated PUFA's by intact PMNL

Purified porcine PMNL (50 x 10^6 cells/ml) were incubated in 1 ml PBS at 37 °C with various substrates (2 μM) in the presence of ETYA (5 μM) for 30 min.

The reactions were terminated by addition of 0.6 ml ice cold methanol and immediate cooling to -20 °C. PGB_2 (200 ng) was added to each sample as an internal standard. The samples were then analyzed by precolumn extraction/RP-HPLC (314) using a WISP automatic injector and WAVS automated switching valve (Waters-Millipore). The material retained on the precolumn (C_{18} μ-Bondapak Guard-Pak cartridge; Waters-Millipore) was analyzed on a Novapak C_{18} column (3.9 x 150 mm; Waters-Millipore). The mobile phases for each substrate are shown in Table II. The flow rate was 2 ml/min in all cases. The products were quantitated by measuring radioactivity by liquid scintillation counting in fractions collected every 0.5 minutes with the exception of LTB_5 metabolites which were quantitated by measuring UV absorbance at 235 nm. The extinction coefficient used for dihydro metabolites of LTB_5 was 22 500.

5.3. Metabolism of hydroxylated PUFA's by subcellular fractions from PMNL

Subcellular fractions from porcine PMNL were incubated with various substrates in Tris-acetate-sucrose buffer (1 ml) in the presence of 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$ at 37 °C for various times. The reactions were terminated by addition of 0.6 ml ice cold methanol and immediate cooling to -20 °C. PGB_2 (300 ng) was added to each sample as an internal standard. The samples were then analyzed by precolumn extraction/RP-HPLC as described above (section II.5.2.) except that the entire UV absorbance spectrum of the column eluate was monitored with a model 991 photodiode array detector (Waters-Millipore). The mobile phases for each substrate

TABLE II

RP-HPLC conditions for separation of hydroxylated PUFA's and their dihydro metabolites produced by intact PMNL

Substrates and their dihydro products were resolved by precolumn extraction/RP-HPLC using a WISP automatic injector and a WAVS automated switching valve (Waters-Millipore). The material retained on the precolumn (C_{18} μ-Bondapak Guard-Pak cartridge; Waters-Millipore) was analyzed on a Novapak C_{18} column (3.9 x 150 mm; Waters-Millipore).

Substrate	Initial Condition (0 min)	Final Condition (30 min)
LTB_4	37% solvent B[a]	37% solvent B
12-epi-6-*trans*-LTB_4	37% solvent B	37% solvent B
6-*trans*-LTB_4	37% solvent B	37% solvent B
12-epi-8-*cis*-6-*trans*-LTB_4	37% solvent B	37% solvent B
LTB_5	31% solvent B	31% solvent B
12(S)-HETE	46% solvent B	55% solvent B
5(S)-HETE	46% solvent B	55% solvent B
15(S)-HETE	46% solvent B	55% solvent B
13(S)-HODE	42% solvent B	48% solvent B
9(S)-HODE	42% solvent B	48% solvent B
20-hydroxy-LTB_4	23% solvent B	23% solvent B
PGF_2	26% solvent B	46% solvent B
$PGF_{2\alpha}$	26% solvent B	46% solvent B

[a] Mobile phases were prepared from solvent A (water/acetic acid; 100:0.02) and solvent B (acetonitrile/acetic acid; 100:0.02). All gradients were linear. The flow rate was 2 ml/min.

are shown in Table III. The flow rate was 1 ml/min in all cases. Products were quantitated either by measuring radioactivity by liquid scintillation counting in fractions collected every minute (metabolism of $PGF_{2\alpha}$) or by measuring UV absorbance (metabolism of LTB_4 and 12-HETE). The extinction coefficients used were as follows; LTB_4, (39 500, 280 nm); 12-oxo-LTB_4, (41 000, 318 nm); 12-HETE, (30 500, 235 nm); 12-oxo-ETE, (36 500, 280 nm); PGB_2 (28 600, 280 nm).

Incubations of 12-oxo-LTB_4 with subcellular fractions (5 ml) were performed under identical conditions, except that they were terminated by the addition of 17% MeOH (10 ml), followed immediately by solid phase extraction on C_{18} Sep Pak cartridges at neutral pH. Products were resolved by RP-HPLC on a Novapak C_{18} column (3.9 x 150 mm: Waters-Millipore) with a mobile phase of acetonitrile/water/acetic acid (39:61:0.02) with a flow rate of 1.0 ml/min.

6. Marker enzyme assays

6.1. Myeloperoxidase (315)

Aliquots (100 μl) of subcellular fractions from porcine PMNL were mixed with 1 mM H_2O_2, 0.33 mM o-dianisidine and 0.01 M phosphate buffer (pH= 6), and the rate of increased absorbance at 460 nm monitored (model DU-64; Beckman Instruments Inc., Palo Alto, CA). One unit of activity is equivalent to an increase in absorbance of 0.001/min.

TABLE III

RP-HPLC conditions for separation of hydroxylated PUFA's and their metabolites produced by subcellular fractions from porcine PMNL

Products were resolved by precolumn extraction/RP-HPLC using an Ultra WISP automatic injector and a WAVS automated switching valve (Waters-Millipore). The material retained on the precolumn (C_{18} μ-Bondapak Guard-Pak cartridge; Waters-Millipore) was analyzed on a Novapak C_{18} column (3.9 x 150 mm; Waters-Millipore). The UV absorbance spectrum of the column eluate was monitored with a photodiode array detector (Model 991; Waters-Millipore).

Substrate	Initial Condition (0 min)	Final Condition (30 min)
LTB_4	39% solvent B[a]	39% solvent B
12(S)-HETE/12(R)-HETE	50% solvent B	55% solvent B
$PGF_{2\alpha}$	26% solvent B	46% solvent B

[a]Mobile phases were prepared from solvent A (water/acetic acid; 100:0.02) and solvent B (acetonitrile/acetic acid; 100:0.02). All gradients were linear. The flow rate was 1 ml/min.

6.2. ß-Glucuronidase (316)

Aliquots (200 μl) of subcellular fractions were incubated with 1 mM phenolphthalein-glucuronic acid in 0.1 M acetate buffer (pH 4.6) in a total volume of 1 ml for 6 hr at 37 °C. The reaction was stopped by addition of 2 ml ice-cold glycine buffer (0.2 M glycine and 0.2 M NaCl, pH= 10.4). Phenolphthalein released during the incubation was quantitated by measuring absorbance at 550 nm.

6.3. Ouabain sensitive Na^+-K^+-ATPase (317)

Aliquots (200 μl) of subcellular fractions were incubated with 0.2 mM ATP in 10 mM Tris-HCl containing 5 x 10^{-5} M EGTA (pH 8.6) under the following conditions: A) Mg^{+2}, Na^+, K^+; B) Mg^{+2}, Na^+, K^+, ouabain; C) Mg^{+2}. The final ionic concentrations were: 0.26 mM Mg^{+2}; 100 nM Na^+; 10 mM K^+ and 0.1 mM ouabain. All incubations were for 10 min at 37 °C in a final volume of 1 ml and were terminated by addition of 0.15 ml of 30% trichloroacetic acid and immediately placed on ice. Released phosphate was measured as described in the literature (318), except that the samples were left at 4 °C for 15 hr to allow for color development with minimal ATP hydrolysis. Results were expressed as ouabain inhibitable ATPase activity.

Section III

RESULTS

1. Metabolism of hydroxylated PUFA by intact porcine PMNL

A major goal of this study was to identify metabolites of PUFA's produced by porcine PMNL. This identification included determining the stereochemistry of chiral centers present in these compounds. As there is more than one method available for designating chiral centers, a short synopsis of the method used in this study is given below.

1.1 Stereochemical designation of chiral centers by the Cahn-Ingold-Prelog method (319)

The Cahn-Ingold-Prelog method was employed to designate the configuration of the chiral centers of compounds described in this study. The letters *R* and *S* are used to specify the absolute configurations according to this method.

To ascertain the configuration of a chiral center by this method, each functional group attached to that center is assigned a priority. The priority assigned is determined by the following rules:

1. Atoms of higher atomic number have higher priority than those of lower atomic number.
   ie) I > Br > Cl > F > O > N > C > H
2. A tertiary alkyl substituent has a higher priority than a secondary alkyl group which, in turn, has a higher priority than a primary alkyl group.
   ie) $-C(CH_3)_3 > -CH(CH_3)_2 > -CH_2(CH_3) > -CH_3$

3. When a double or triple bond is encountered between atoms, the atoms involved are considered to be duplicated or triplicated, respectively. Rules 1 and 2 are then applied to determine priorities. ie) -CH=CHR is equivalent to $-C_2H-C_2HR$

4. If two or more of the atoms attached directly to the chiral center are the same, priorities are determined by the second atoms attached. If these atoms are identical, the next atoms are examined sequentially until the first point of difference in attached atoms is reached. At this point, rules 1 and 2 are applied.

The second step to designate a chiral center as *R* or *S* is to orient the tetrahedral molecule so that the functional group with the lowest priority is behind the molecule (Figure 7). The orientation of the remaining three substituents is then observed in order of decreasing priority. A clockwise decreasing order is designated as an *R* (right=rectus) configuration, whereas a counter clockwise decreasing order identifies an *S* (left=sinister) configuration (Figure 7).

Also shown in Figure 7 are configurations of LTB_4 and 10,11-dihydro-LTB_4 about the chiral center at C_{12}. The absence of a Δ^{10} double bond in 10,11-dihydro-LTB_4 results in a different assignment of priorities according to the rules described above. Therefore, the configuration at C_{12} of LTB_4 is designated *R*, whereas the configuration at C_{12} of 10,11-dihydro-LTB_4 is designated *S*, even though the positioning of the hydroxyl group at the assymetric center has not changed.

Another system for determining configurations of chiral centers is the Fischer-Rosanoff method. The letters D and L are used to specify the absolute configurations of chiral centers which are determined by comparison of the unknown compound with

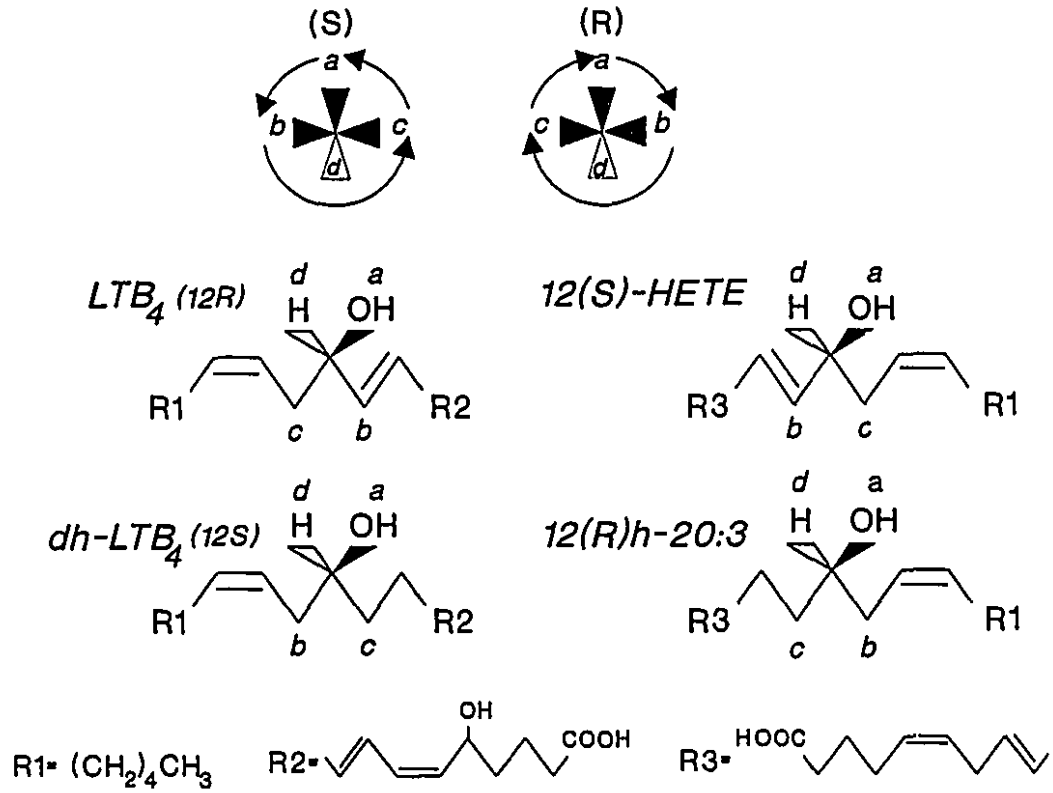

Figure 7. *Stereochemical designations for LTB_4, 12(S)-HETE and their 10,11-dihydro metabolites as determined by the Cahn-Ingold-Prelog method.* Filled wedged lines represent bonds that project above the plane of the page, whereas open wedged lines represent bonds that project below the plane of the page. Abbreviations; dh-LTB_4, 10,11-dihydro-LTB_4; 12(R)h-20:3, 12(R)-hydroxy-5,8,14,-eicosatrienoic acid.

a standard reference compound (dextrorotatory glyceraldehyde). This method is widely accepted for the classification of carbohydrates and amino acids. However, the designation of compounds which are significantly different from the standard molecule, or which have multiple chiral centers, often becomes arbitrary. Therefore,

drawing a three-dimensional structure of chiral compounds identified by the D and L system can be very complicated.

The *R* and *S* designations for chiral centers does cause some confusion concerning the metabolites described in this study. However, this system was chosen because of its simplicity, convenience and universality which enables the reader to draw an accurate three-dimensional structure of the compounds in this study with the aid of a few simple rules.

1.2. Metabolism of LTB_4 by porcine PMNL

When LTB_4 was incubated with porcine PMNL for 60 min at 37 °C and the products analyzed by RP-HPLC, two major metabolites which absorbed at 235 nm but not at 280 nm were observed (Figure 8). These products had previously been identified in our laboratory as 5,12-dihydroxy-6,8,14-eicosatrienoic acid (10,11-dihydro-LTB_4) (t_R= 43 min) and 5-hydroxy-12-oxo-6,8,14-eicosatrienoic acid (10,11-dihydro-12-oxo-LTB_4) (t_R= 61 min) (304). As discussed in the introduction (Figure 6), the stereochemistry of the C_{12}-hydroxyl group of 10,11-dihydro-LTB_4 could have either an *S* or an *R* configuration depending on the mechanism involved. Direct reduction of LTB_4 by a 10,11-reductase would produce 5(S),12(S)-dihydroxy-6,8,14-eicosatrienoic acid. However, if a 12-oxo intermediate is involved in the formation of dihydro products, the 12-oxo group could be reduced to a hydroxyl group with either an *S* or *R* configuration.

1.2.1. Identification of 10,11-dihydro-LTB_4 (product *X*) and 10,11-dihydro-12-epi-LTB_4 (product *Y*)

Since NP-HPLC is usually better than RP-HPLC in resolving diastereoisomers, the material in the 10,11-dihydro-LTB_4 peak obtained after RP-HPLC (Figure 8) was rechromatographed by NP-HPLC. This resulted in the separation of this fraction into two components (products *X* and *Y*) in a ratio of about 1:1.4 (Figure 8, inset).

Analysis of the methyl ester, trimethylsilyl ether derivatives of products *X* and *Y* by GC-MS revealed that they have identical C values (23.4). The mass spectra of products *X* and *Y* were also identical with one another, as well as with the mass spectrum previously reported for 10,11-dihydro-LTB_4 purified by RP-HPLC alone (304). The HPLC and GC-MS results strongly suggest that compounds *X* and *Y* are stereoisomers of 10,11-dihydro-LTB_4, probably with different configurations of the C_{12}-hydroxyl group.

To ascertain the identity of products *X* and *Y*, the chromatographic properties of these compounds were compared with authenic standards. LTB_4 was incubated with porcine PMNL for 5 and 60 min, respectively, and the products were purified by RP-HPLC. The 10,11-dihydro-LTB_4 fraction from each incubation was rechromatographed by NP-HPLC to resolve compounds *X* and *Y*. After 5 min, only compound *X* was recovered (Figure 9A) whereas both products *X* and *Y* were formed after 60 min (Figure 9D). We obtained authentic chemically synthesized 5(S),12(R)-dihydroxy-6,8,14-eicosatrienoic acid (10,11-dihydro-LTB_4) and 5(S),12(R)-dihydroxy-6,8,14-eicosatrienoic acid (10,11-dihydro-12-epi-LTB_4) from Dr. J.R. Falck at the

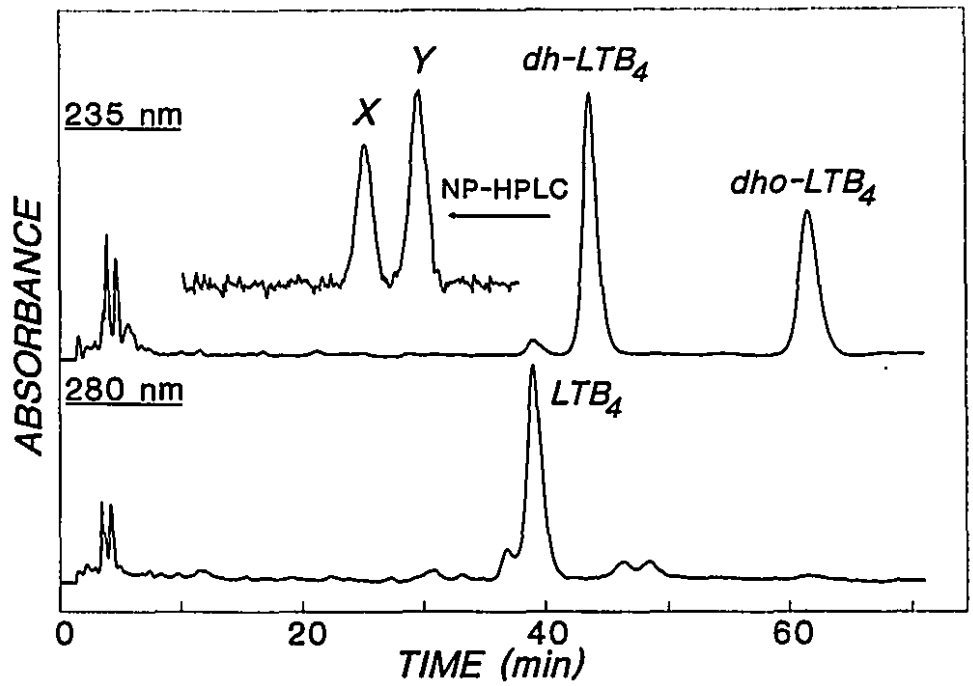

Figure 8. *Reversed-phase HPLC of metabolites of LTB_4 produced by intact porcine PMNL.* LTB_4 (2 μM) was incubated with porcine PMNL (50 x 10^6 cells/ml) for 60 min at 37 °C. The products were extracted on a cartridge of octadecylsilyl silica and analyzed by RP-HPLC on a Novapak C_{18} column (Waters-Millipore) with water/acetonitrile/acetic acid (63:37:0.02) as the mobile phase. The flow rate was 2 ml/min. The product absorbing at 235 nm with a retention time of 43 min was rechromatographed by NP-HPLC (inset) on a silicic acid column (RoSil; Alltech Associates) with a mobile phase of hexane/2-propanol/acetic acid (95:5:0.1) at a flow rate of 2 ml/min. Abbreviations: dh-LTB_4, 10,11-dihydro-LTB_4; dho-LTB_4, 10,11-dihydro-12-oxo-LTB_4.

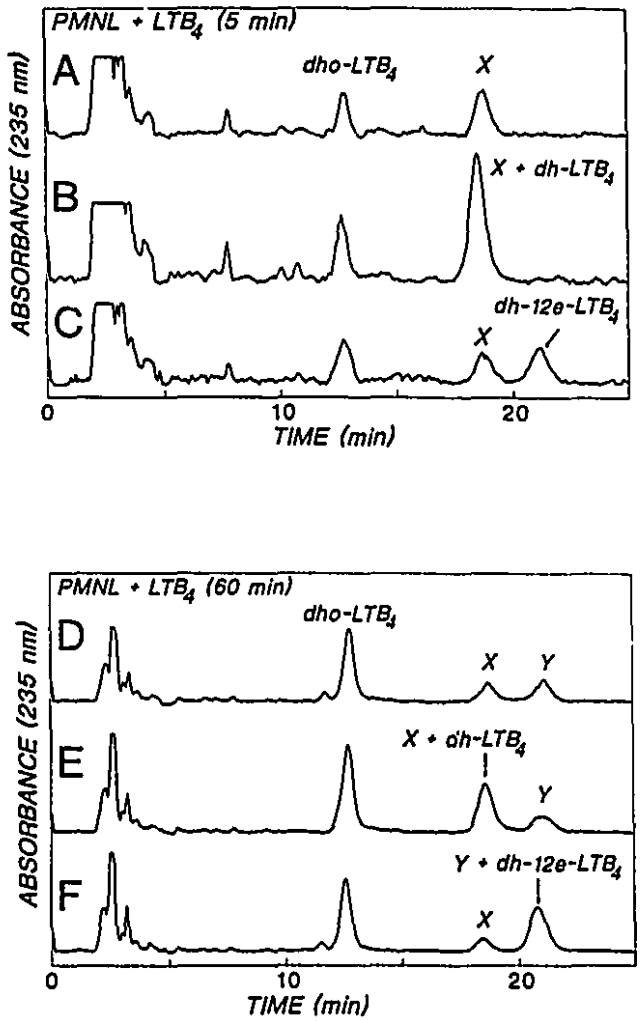

Figure 9. *Normal-phase HPLC of metabolites of LTB_4 formed after 5 min (A-C) and 60 min (D-F).* LTB_4 (2 μM) was incubated with porcine PMNL (50 x 10^6 cells/ml) for either 5 or 60 min. The products were extracted on a cartridge containing octadecylsilyl silica and aliquots were analyzed by NP-HPLC on a silicic acid column (RoSil; Alltech Associates) with hexane/2-propanol/acetic acid (95:5:0.1) as the mobile phase. The flow rate was 2 ml/min. Only products absorbing at 235 nm are shown. (A) and (D) show chromatograms obtained from aliquots of the extract without any additions. The other chromatograms are of aliquots of the extract to which were added 400 ng of either 10,11-dihydro-LTB_4 (B and E) or 10,11-dihydro-12-epi-LTB_4 (C and F). Abbreviations: dh-LTB_4, 10,11-dihydro-LTB_4; dh-12e-LTB_4, 10,11-dihydro-12-epi-LTB_4, dho-LTB_4, 10,11-dihydro-12-oxo-LTB_4.

University of Texas Southwestern Medical Center, Dallas, Texas. Cochromatography of products *X* and *Y* with these standards indicated that product *X* coeluted with 10,11-dihydro-LTB_4 (Figure 9B,E), whereas product *Y* coeluted with 10,11-dihydro-12-epi-LTB_4 (Figure 9C,F). Authentic 10,11-dihydro-LTB_4 and 10,11-dihydro-12-epi-LTB_4 also cochromatographed with products *X* and *Y*, respectively, upon RP-HPLC. However, the two stereoisomers were not separated from one another with either methanol/water/acetic acid (58:42:0.02; t_R= 25 min) or acetonitrile/water/acetic acid (38:62:0.02; t_R= 36 min) as the mobile phase. The results above clearly indicate that product *X* and product *Y* are identical to 5(S),12(S)-dihydroxy-6,8,14-eicosatrienoic acid and 5(S),12(R)-dihydroxy-6,8,14-eicosatrienoic acid respectively.

1.2.2. Time course for the metabolism of LTB_4

The time courses for the formation of 10,11-dihydro-LTB_4, 10-11-dihydro-12-epi-LTB_4 and 10,11-dihydro-12-oxo-LTB_4 from LTB_4 are shown in Figure 10A. 10,11-Dihydro-LTB_4 and 10,11-dihydro-12-oxo-LTB_4 are both formed very rapidly and can easily be detected after 2 min. There is clearly a delay in the formation of 10,11-dihydro-12-epi-LTB_4 and this product could not be detected until 12 min. The concentration of 10,11-dihydro-12-epi-LTB_4 in the incubation mixture gradually increased up to 60 min at the expense of 10,11-dihydro-LTB_4 and 10,11-dihydro-12-oxo-LTB_4. The lag in the formation of 10,11-dihydro-12-epi-LTB_4 from LTB_4 suggests that it is formed from the subsequent metabolism of either 10,11-dihydro-LTB_4 or 10,11-dihydro-12-oxo-LTB_4.

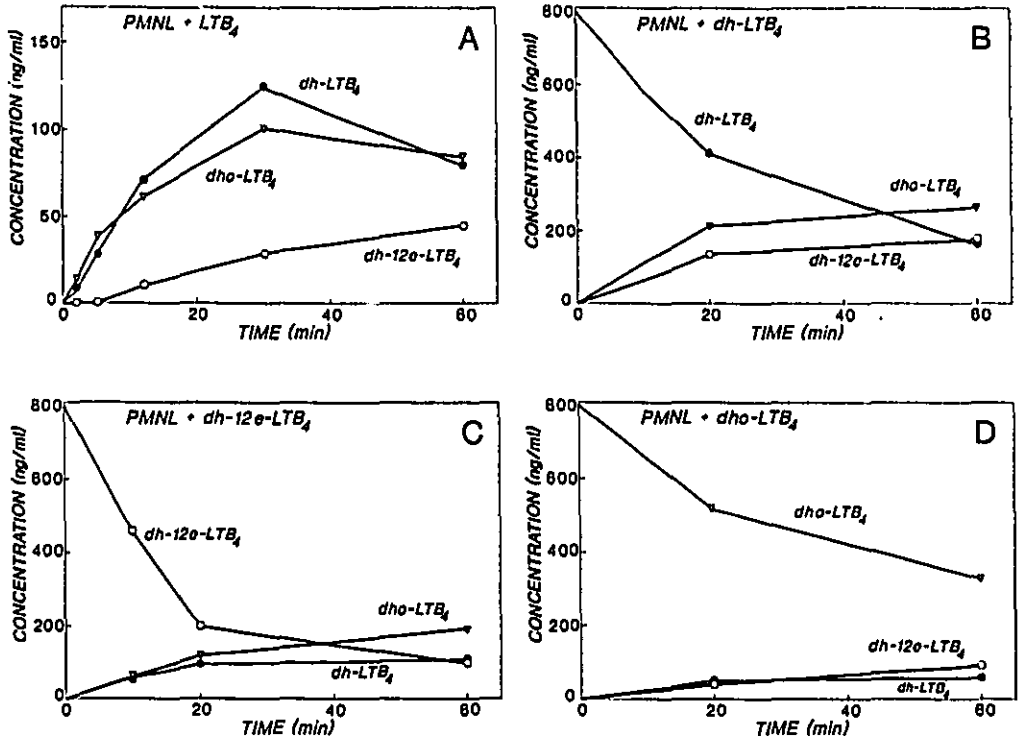

Figure 10. *Time courses for the formation of metabolites of; (A) LTB_4, (B) 10,11-dihydro-LTB_4, (C) 10,11-dihydro-12-epi-LTB_4 and (D) 10,11-dihydro-12-oxo-LTB_4, by porcine PMNL.* Porcine PMNL (50 x 10^6 cells/ml) were incubated with the above substrates (2 μM) for various times at 37 °C. The incubations were terminated by the addition of methanol (0.15 vol) and PGB_2 (430 pmol) was added as an internal standard. Products were extracted on a cartridge of octadecylsilyl silica and were resolved by NP-HPLC as described in the legend to Figure 8. The amounts of 10,11-dihydro-LTB_4 (●), 10,11-dihydro-12-epi-LTB_4 (○) and 10,11-dihydro-12-oxo-LTB_4 (▽) were determined using PGB_2 as an internal standard. Abbreviations: dh-LTB_4, 10,11-dihydro-LTB_4; dh-12e-LTB_4, 10,11-dihydro-12-epi-LTB_4, dho-LTB_4, 10,11-dihydro-12-oxo-LTB_4.

Incubation of 10,11-dihydro-LTB_4 with porcine PMNL and analysis of the reaction products by NP-HPLC indicated that this substrate was converted to both 10,11-dihydro-12-epi-LTB_4 and 10,11-dihydro-12-oxo-LTB_4, with the latter product predominating (Figure 10B). Similar results were obtained when 10,11-dihydro-12-epi-LTB_4 was incubated with porcine PMNL (Figure 10C). As with 10,11-dihydro-LTB_4, the major metabolite after 60 min was 10,11-dihydro-12-oxo-LTB_4. Substantial amounts of 10,11-dihydro-LTB_4 were also formed, indicating that the two 10,11-dihydro isomers are interconvertable. No metabolism of these compounds was observed when the cell suspension was previously placed in a boiling water bath.

The most obvious mechanism for the formation of 10,11-dihydro-12-epi-LTB_4 from LTB_4 would be via reduction of 10,11-dihydro-12-oxo-LTB_4. To determine whether this occurred, 10,11-dihydro-12-oxo-LTB_4 was incubated with porcine PMNL for different times and the products were analyzed by NP-HPLC (Figure 10D). This resulted in the formation of both 10,11-dihydro-LTB_4 and 10,11-dihydro-12-epi-LTB_4 in comparable amounts. No products were detected when 10,11-dihydro-12-oxo-LTB_4 was incubated with a cell suspension which had previously been placed in a boiling water bath. On the basis of these kinetic experiments (Figure 10B,D), 10,11-dihydro-LTB_4 appears to be a considerably better substrate than 10,11-dihydro-12-oxo-LTB_4 for the formation of 10,11-dihydro-12-epi-LTB_4. These results raise the possiblility that the two dihydro-LTB_4 stereoisomers could be directly interconverted. However, the poor metabolism of 10,11-dihydro-12-oxo-LTB_4 could also be explained if it is not transported into the cell as well as 10,11-dihydro-LTB_4.

1.2.3. Metabolism of 10,11-dihydro-[12-^{3}H]LTB$_4$ and 10,11-dihydro-12-epi-[12-^{3}H]LTB$_4$.

To examine further the involvement of 10,11-dihydro-12-oxo-LTB$_4$ in the interconversion of the 10,11-dihydro-LTB$_4$ stereoisomers, 10,11-dihydro-LTB$_4$ and 10,11-dihydro-12-epi-LTB$_4$, labeled with a tritium specifically in the 12-position, were prepared. Interconversion of 10,11-dihydro-LTB$_4$ and 10,11-dihydro-12-epi-LTB$_4$ involving 10,11-dihydro-12-oxo-LTB$_4$ as an intermediate would necessitate abstraction of the C_{12}-tritium atom. Therefore, 10,11-dihydro-[12-^{3}H]LTB$_4$ was incubated with porcine PMNL and the reaction products were analyzed after various times by NP-HPLC (Figure 11). After 60 min, the substrate (t_R= 21 min) was still highly labeled with tritium, whereas the product, 10,11-dihydro-12-epi-LTB$_4$ (t_R= 23 min) was only slightly labeled (Figure 11A). The second product, 10,11-dihydro-12-oxo-LTB$_4$ (t_R= 14 min), did not possess any detectable radioactivity, confirming the presence of tritium almost exclusively at the 12-position of the substrate. Similar results were obtained when 10,11-dihydro-12-epi-[12-^{3}H]LTB$_4$ was incubated with porcine PMNL for 60 min at 37 °C (Figure 11B).

The specific activities of the products formed after different incubation times are shown in Table IV. Insufficient amounts of products were formed after 10 min to determine their specific activities accurately. However, after 20 min the specific activity of 10,11-dihydro-12-epi-LTB$_4$, formed from 10,11-dihydro-[12-^{3}H]LTB$_4$ was only 17% that of the initial substrate. Similar results were obtained when 10,11-dihydro-12-epi-[12-^{3}H]LTB$_4$ was the substrate. The specific activities of both

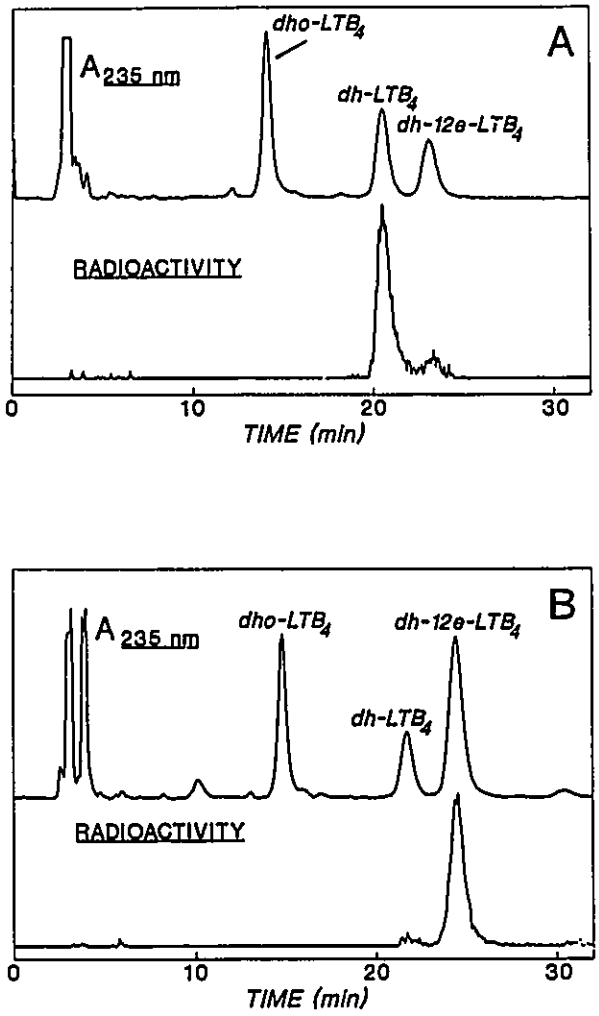

Figure 11. *Normal-phase HPLC of metabolites of 10,11-dihydro-LTB$_4$ (A) and 10,11-dihydro-12-epi-LTB$_4$ (B) labeled with tritium in the 12-position.* Porcine PMNL (5 x 10^8 cells in 10ml) were incubated with 10,11-dihydro-[12-^{3}H]LTB$_4$ (2 μM; 138 000 dpm/incubation) or 10,11-dihydro-12-epi-[12-^{3}H]LTB$_4$ (2 μM; 118 000 dpm/incubation) for 60 min at 37 °C. The products were extracted and purified by NP-HPLC as described in the legend to Figure 8. Abbreviations: dh-LTB$_4$, 10,11-dihydro-LTB$_4$; dh-12e-LTB$_4$, 10,11-dihydro-12-epi-LTB$_4$, dho-LTB$_4$, 10,11-dihydro-12-oxo-LTB$_4$.

TABLE IV

Specific activities of 10,11-dihydro-LTB_4 and 10,11-dihydro-12-epi-LTB_4 after incubation of either 10,11-dihydro-[12-^{3}H]LTB_4 or 10,11-dihydro-12-epi-[12-^{3}H]LTB_4 with porcine PMNL

10,11-Dihydro-[12-^{3}H]LTB_4 (2 μM) and 10,11-dihydro-12-epi-[12-^{3}H]LTB_4 (2 μM) were incubated with porcine PMNL (50 x 10^6 cells/ml) for various times. The products were extracted and analyzed by NP-HPLC as described in the legend to Figure 9.

	Specific Activity (dpm/pmol)	
Time (min)	dh-LTB_4[a]	dh-12e-LTB_4
	dh-LTB_4 --> dh-12e-LTB_4	
0	6.94	
10	6.75	b
20	6.69	1.16
60	7.82	1.23
	dh-12e-LTB_4 --> dh-LTB_4	
0		5.90
10	b	6.57
20	0.80	7.01
60	1.36	9.40

[a] Abbreviations: dh-LTB_4, 10,11-dihydro-LTB_4; dh-12e-LTB_4, 10,11-dihydro-12-epi-LTB_4.

[b] Only small amounts of products were formed after 10 min, and it was not possible to determine their specific activities accurately under the conditions employed.

substrates increased with time and were highest after 60 min which is consistent with the expected isotope effect for loss of the tritium atom in the 12-position.

1.3. Metabolism of 12(S)-HETE by porcine PMNL

Porcine leukocytes were incubated with 12(S)-HETE and the products were analyzed by RP-HPLC as shown in Figure 12. Two less polar radioactive products (*A* and *B*) were formed which did not show any absorbance at 235 nm. The lack of absorbance at 235 nm of products *A* and *B* indicates that they no longer contain conjugated double bonds. The HPLC profile is similar to that observed after incubation of LTB_4 with porcine PMNL (Figure 7), which also shows two less polar peaks absorbing at a lower wavelength than the substrate.

1.3.1. Identification of 12-hydroxy-5,8,14-eicosatrienoic acid (Product *A*).

The mass spectrum of the trimethylsilyl ether, methyl ester derivative of product *A* (Figure 13) exhibited intense ions at m/z 408 (M), 393 (M-15), 377 (M-31), 351 (loss of C_{17}-C_{20}), 318 (M-90), 297 (C_1-C_{12}), 213 (C_{12}-C_{20}), 207 (297-90), 175 (297-(90+32)), 133 (base peak), 129, 121, and 107. This mass spectrum is very similar to that of the corresponding derivative of 12(S)-HETE, except that ions containing the C_1 to C_{12} portion of the molecule, including the molecular ion and the ions at m/z 297 and 207, occur 2 mass units higher than the corresponding ions for 12(S)-HETE. This indicates that one of the double bonds between carbons 1 and 12 of 12(S)-HETE has been reduced and that product *A* is identical to 12-hydroxyeicosatrienoic acid.

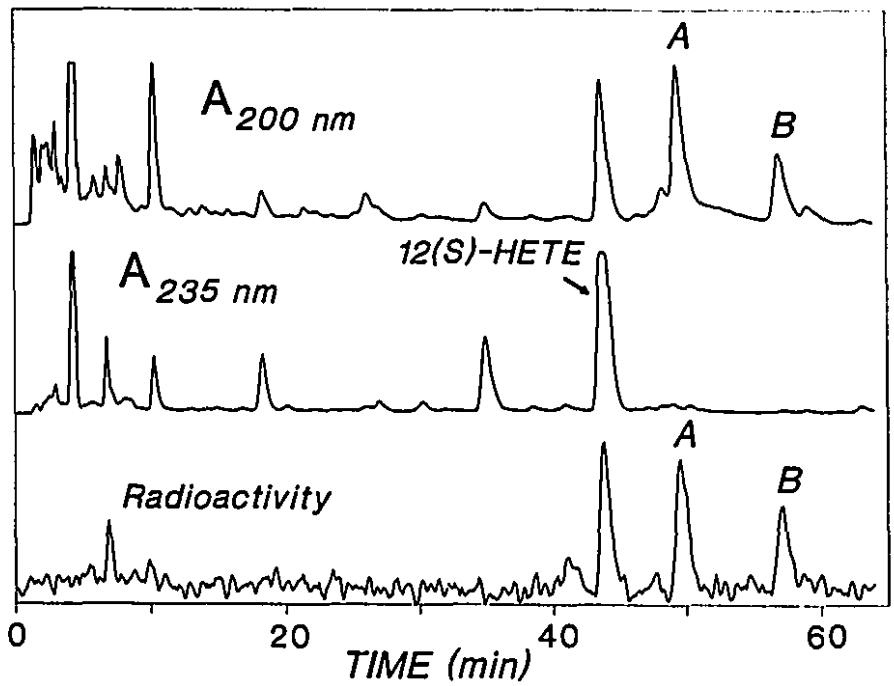

Figure 12. *Reversed-phase HPLC of 12(S)-HETE metabolites produced by intact porcine PMNL.* 12(S)-HETE (2 μM) was incubated with porcine leukocytes (75 x 10^6 cells/ml) at 37 °C for 40 min. Products were extracted on a cartridge of ODS silica and separated by RP-HPLC on a Phenomenex Spherisorb ODS-2 column with water/acetonitrile/acetic acid (55:45:0.02) as the mobile phase. The flow rate was 2 ml/min.

The absence of UV absorbance at 235 nm by 12-hydroxyeicosatrienoic acid indicates that one of the two conjugated double bonds between carbons 8 and 11 of 12(S)-HETE has been reduced. Since the mass spectrum clearly shows that only one double bond has been reduced, it can be assumed that the double bonds present at

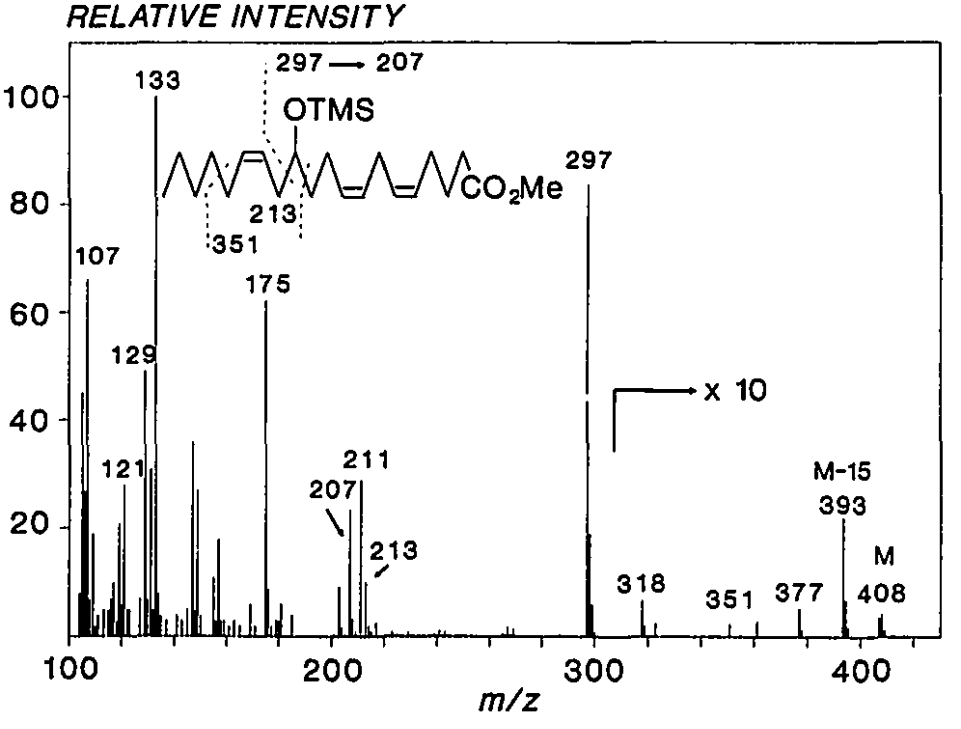

Figure 13. *Mass spectrum of the trimethylsilyl ether, methyl ester derivative of product A formed from 12-HETE.* The mass spectrum was obtained using an ionizing voltage of 70 mV, an accelerating voltage of 6 kV and a trap current of 100 μA. *TMS*, trimethylsilyl.

positions 5 and 14 of 12(S)-HETE are retained in the dihydro metabolite. The remaining double bond, the position of which could not be determined from the mass spectrum, could theoretically reside in one of three locations: (A) C_8-C_9, (B) C_{10}-C_{11} or (C) C_9-C_{10} as shown in Figure 14. To ascertain which of these positions is correct, a sample of 12-hydroxyeicosatrienoic acid was analysed by NMR spectroscopy by Drs

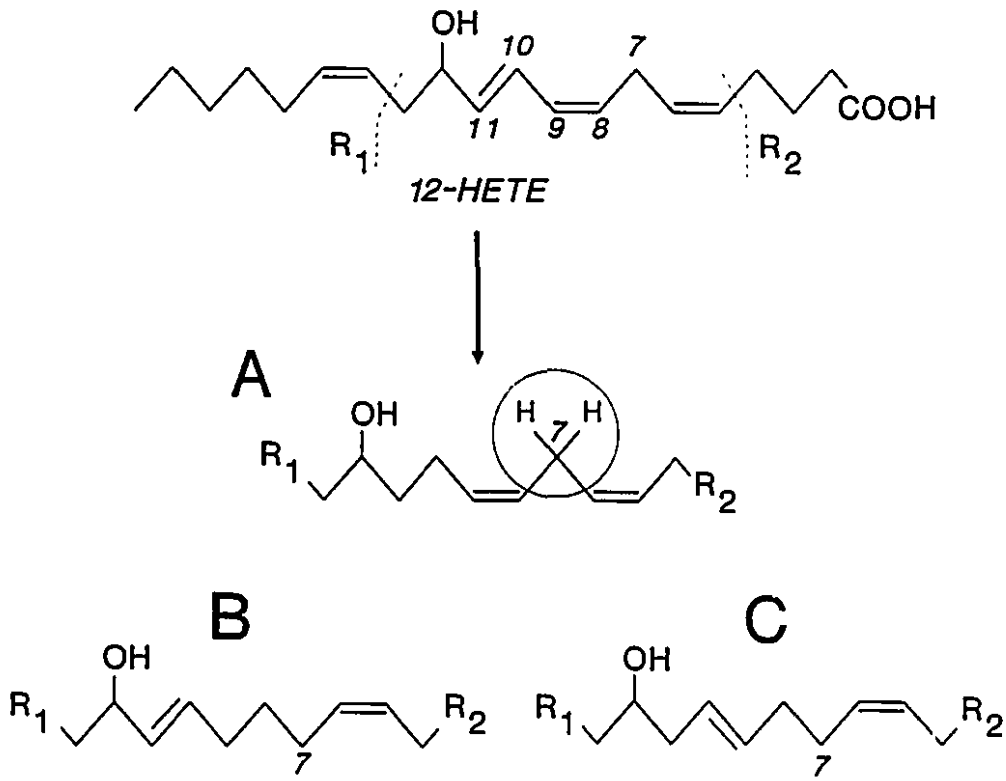

Figure 14. *Possible structures of the dihydro metabolite of 12(S)-HETE (product A).* (A) 12-Hydroxy-5,8,14-eicosatrienoic acid, (B) 12-hydroxy-5,10,14-eicosatrienoic acid, (C) 12-hydroxy-5,9,14-eicosatrienoic acid. The circled methylene group is the one giving rise to the triplet centered at 2.77 ppm in the inset to Figure 15.

Falck and Yadagiri at the University of Texas Southwestern Medical Center, Dallas, Texas (Figure 15). The NMR spectrum shows a triplet centered at 2.77 ppm, characteristic of the bis-allylic methylene group which is present in structure A, but not in either B or C (Figure 14). This clearly indicates that the third double bond of 12-hydroxyeicosatrienoic acid is located between carbons 8 and 9 identifying the

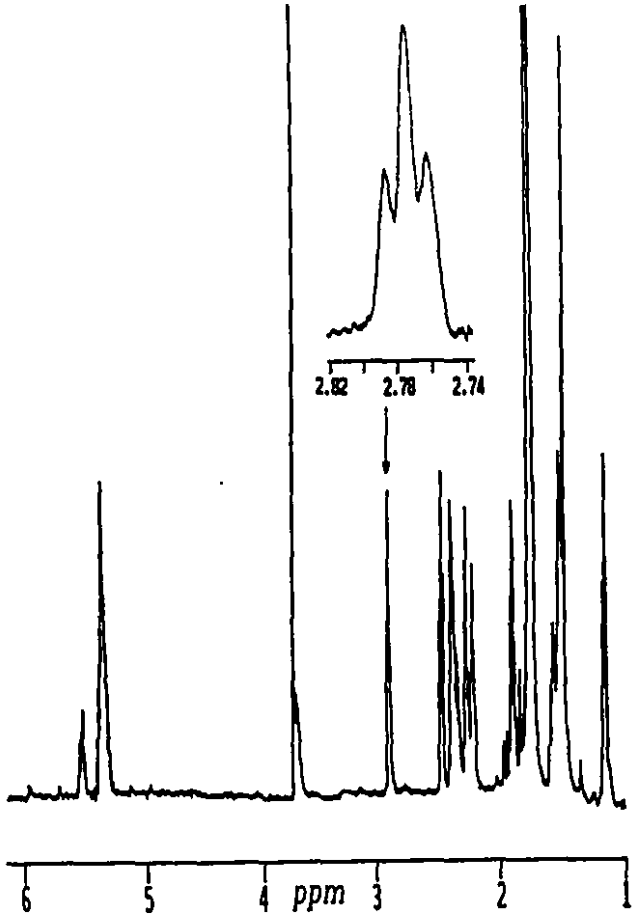

Figure 15. *Nuclear magnetic resonance spectrum of the dihydro metabolite of 12(S)-HETE (product A).* Product A (55 μg) was analysed by ^{1}H NMR spectroscopy ($CDCl_3$, 500 MHz) on a Varian Model VXR-500S instrument by Drs Falck and Yadagiri at the University of Texas Southwestern Medical Center, Dallas, Texas. The inset is an enlargement of the triplet at 2.77 ppm.

compound as 12-hydroxy-5,8,14-eicosatrienoic acid. Moreover, the NMR spectrum is virtually identical to that of authentic chemically-synthesized 12-hydroxy-5,8,14-eicosatrienoic acid. Integration of the relevant peaks revealed that the biological sample was greater than 95% pure with respect to regioisomeric olefins.

1.3.2. Identification of 12-oxo-5,8,14-eicosatrienoic acid (product *B*).

Product ***B*** reacted with hydroxylamine hydrochloride in the presence of pyridine to give an oxime derivative, indicating that it contains an oxo group. The oxime derivative of product ***B*** was converted to its trimethylsilyl ether, ester derivative. The mass spectrum of this compound exhibited intense ions at m/z 479 (M), 464 (M-15), 436 (C_1-C_{17}), 422 (C_1-C_{16}), 368 (C_1-C_{12}), 348 (C_3-C_{20}), 334 (C_4-C_{20}), 320 (C_5-C_{20}), 280 (C_8-C_{20}), 266, 210, 147, and 117 (Figure 16). This mass spectrum indicates that product ***B*** is a dihydro metabolite of 12(S)-HETE with an oxo group at C_{12}. The ion at m/z 280 would suggest that there is a double bond in the 8-position, whereas that at m/z 266 would be more consistent with its presence in the 9-position. However, it is possible that the latter ion could have arisen after a rearrangement of the double bonds, or that there could be a mixture of products which differ from one another in the positions of their double bonds. By analogy with the 10,11-dihydro metabolite of 12(S)-HETE, it would seem more likely that the double bonds are present in the 5, 8, and 14 positions and we would therefore tentatively assign the structure 12-oxo-5,8,14-eicosatrienoic acid to product ***B***.

1.3.3. Stereochemical analysis of 12-hydroxy-5,8,14-eicosatrienoic acid

The mass spectrum of product *A* indicates that it is identical to 12-hydroxy-5,8,14-eicosatrienoic acid. Bovine corneal microsomes have also been shown to convert arachidonic acid to 12(R)-hydroxy-5,8,14-eicosatrienoic acid, however a cytochrome P-*450* pathway not involving 12(S)-HETE was implicated (313). Since

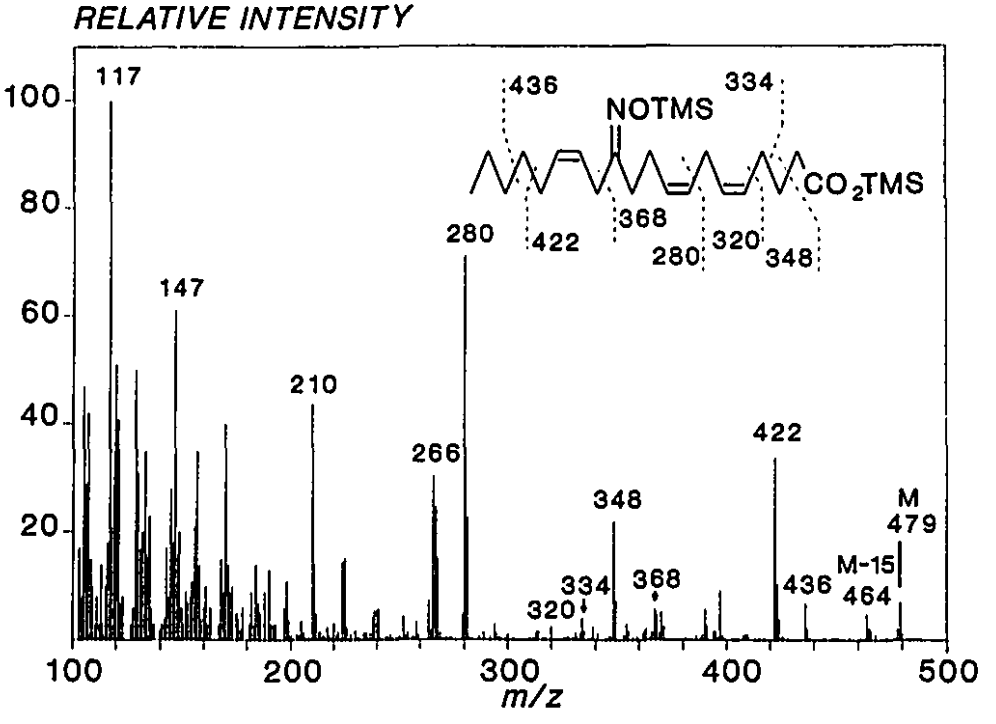

Figure 16. *Mass spectrum of the trimethylsilyl ether oxime derivative of product B formed from 12-HETE.* The mass spectrum was obtained using an ionizing voltage of 70 eV, an accelerating voltage of 6 kV and a trap current of 100 μA. *TMS*, trimethylsilyl.

the 12(R), but not the 12(S), isomer of this compound was reported to have potent proinflammatory properties (237,238), it was of considerable interest to determine the configuration of the 12-hydroxyl group of 12-hydroxy-5,8,14-eicosatrienoic acid generated by porcine PMNL.

12(S)-HETE was incubated with PMNL for 40 min and the products were purified by RP-HPLC as shown in Figure 12. The material in the peak corresponding to 12-hydroxy-5,8,14-eicosatrienoic acid was methylated and converted to its MTPA derivative. After removal of contaminants derived from the reagents by RP-HPLC on a C_6 column, the methyl ester, MTPA derivatives of the 12-hydroxy-5,8,14-eicosatrienoic acid fraction was subjected to steric analysis by NP-HPLC on a silicic acid column (Figure 17). As shown in Figure 17A, 12-hydroxy-5,8,14-eicosatrienoic acid was resolved into two radioactive components which absorbed in the UV region at 200 nm. These two products cochromatographed with the methylated MTPA derivatives of authentic 12(R)-hydroxy-5,8,14-eicosatrienoic acid (t_R= 25 min) and 12(S)-hydroxy-5,8,14-eicosatrienoic acid (t_R= 23 min) which were provided by Dr. J.R. Falck from the University of Texas Southwestern Medical Center, Dallas, Texas (Figure 17B).

The mass spectrum of the unresolved methyl ester, MTPA derivatives of 12-hydroxy-5,8,14-eicosatrienoic acid was identical to that of the corresponding derivative of authentic 12(S)-hydroxy-5,8,14-eicosatrienoic acid. Although these mass spectra were not particularly informative, intense ions were observed at m/z 318 (M-MTPA) and 189 (C_6H_5-$C(CF_3)=^+OCH_3$).

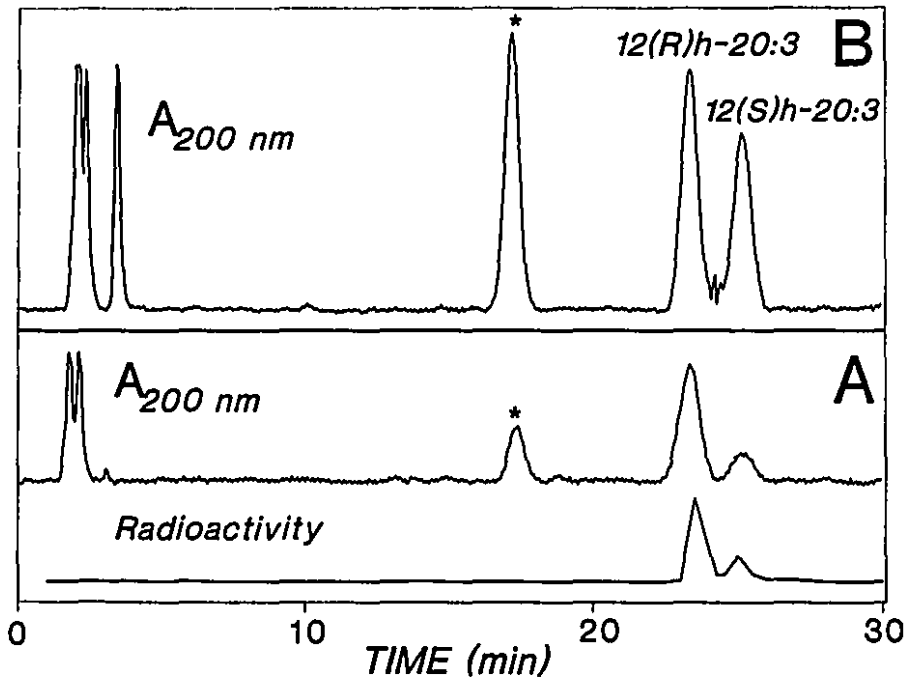

Figure 17. *Normal-phase HPLC of the MTPA derivatives of 12-hydroxy-5,8,14-eicosatrienoic acid.* The 12(R) and 12(S) isomers of the MTPA derivatives were separated by NP-HPLC on a column of silicic acid (RoSil; Alltech Associates). The mobile phase was hexane/isopropanol (99:0.08) at a flow rate of 2 ml/min. (A) NP-HPLC of the methyl ester, MTPA derivatives of 12-hydroxy-5,8,14-eicosatrienoic acid formed after incubation of 12-[1-^{14}C]HETE (2 μM) with purified porcine PMNL (50 x 10^6 cells/ml) for 40 min at 37 °C. The products were extracted on a cartridge of ODS-silica and were purified by RP-HPLC. Dihydro-12-HETE (product *A*) was converted to the MTPA derivative of its methyl ester as described in Materials and Methods. (B) NP-HPLC of a mixture of the methyl ester, MTPA derivatives of chemically-synthesized 12(R)-5,8,14-eicosatrienoic acid (t_R=23 min) and 12(S)-hydroxy-5,8,14-eicosatrienoic acid (t_R=25 min). The peak labeled with an *asterisk* (*) was not radioactive and is presumably a contaminent derived from the derivatization procedure. The abbreviations are: 12(R)h-20:3, 12(R)-hydroxy-5,8,14-eicosatrienoic acid; 12(S)h-20:3, 12(S)-hydroxy-5,8,14-eicosatrienoic acid.

1.3.4. Time course for the formation of 10,11-dihydro metabolites of 12(S)-HETE by porcine PMNL

The time course for the formation of 12(R)-hydroxy-5,8,14-eicosatrienoic acid, 12(S)-hydroxy-5,8,14-eicosatrienoic acid and 12-oxo-5,8,14-eicosatrienoic acid from 12(S)-HETE by porcine PMNL is shown in Figure 18. 12(R)-Hydroxy-5,8,14-eicosatrienoic acid was the predominant product at all time points, reaching a maximum at 20 min and then declining[1]. 12-Oxo-5,8,14-eicosatrienoic acid reached its maximal level by 10 minutes and did not change thereafter. The formation of 12(S)-hydroxy-5,8,14-eicosatrienoic acid exhibited a lag period followed by an increase to 30% of the total 12-hydroxy-5,8,14-eicosatrienoic acid at 40 minutes, suggesting that it is not an initial product but that it may be formed from either 12(R)-hydroxy-5,8,14-eicosatrienoic acid or 12-oxo-5,8,14-eicosatrienoic acid.

The total recovery of the above three 12(S)-HETE metabolites was considerably lower after 40 minutes than after 20 minutes, suggesting that these products may subsequently be metabolized by other pathways such as ß-oxidation. The loss of radioactivity could be substantially lowered by inclusion of ETYA in the incubations (see below), but it was not added in the present experiment to avoid any effects it might have on the ratio of the two stereoisomers of 12-hydroxy-5,8,14-eicosatrienoic acid.

---

[1] Due to the priority rules for assigning *R* and *S* configurations, the configuration of the 12-hydroxyl group of 12(S)-HETE is identical to that of 12(R)-hydroxy-5,8,14-eicosatrienoic acid. Thus, the major product has the same configuration at C_{12} as the precursor (see Figure 7).

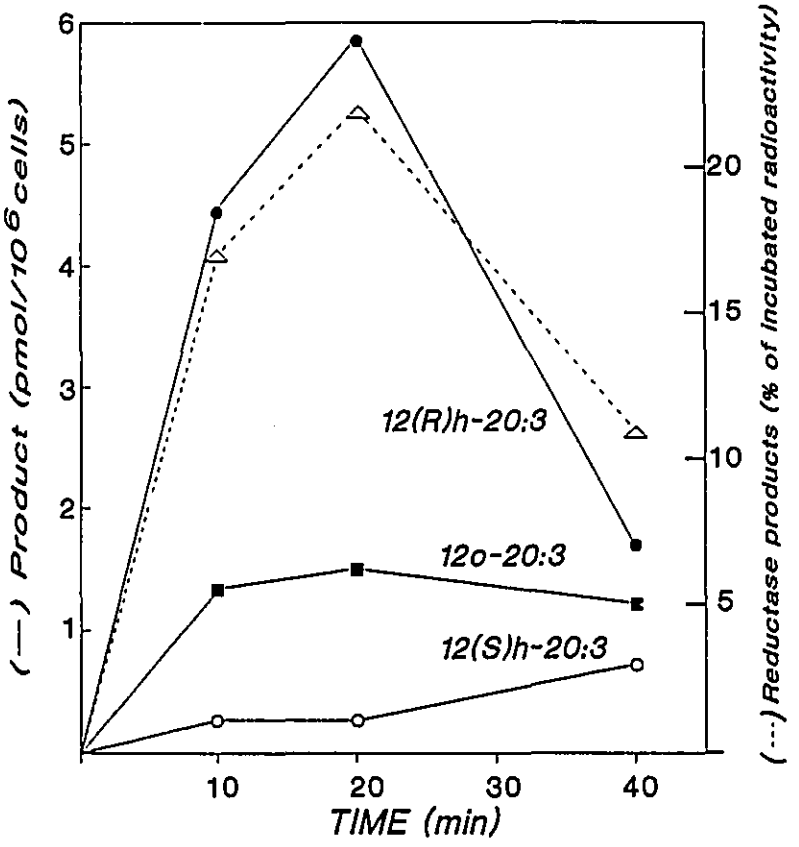

Figure 18. *Time course for the metabolism of 12(S)-HETE by porcine PMNL.* 12-[1-^{14}C]HETE (2 μM) was incubated with purified porcine PMNL (50 x 10^6 cells/ml) for various time periods. 12(R)-Hydroxy-5,8,14-eicosatrienoic acid (12(R)h-20:3;), 12(S)-hydroxy-5,8,14-eicosatrienoic acid (12(S)h-20:3;) and 12-oxo-5,8,14-eicosatrienoic acid (12o-20:3;) were analyzed as described in the legend to Figure 17. Products were quantitated by measurement of radioactivity by liquid scintillation counting in fractions collected every 0.5 minutes. The percentage of incubated 12(S)-HETE recovered as less polar reductase products is also shown ( ).

1.4. Effect of ETYA on the metabolism of monohydroxy PUFA's

The data in Figure 18 indicate that there is a reduction in the total amounts of 10,11-dihydro metabolites of 12(S)-HETE recovered after 4(:in. Preliminary experiments indicated that the loss of dihydro products with time was even more pronounced with other [1-^{14}C]-labeled monohydroxy PUFA's including 15-HETE and 13-HODE. This could largely be attributed to ß-oxidation, since there was a marked reduction in the total amount of radioactivity recovered.

The loss of radioactivity could be reduced by the addition of ETYA. A study of the effect of increasing concentrations of ETYA on the recovery of dihydro metabolites of 13-HODE revealed an optimal concentration of ETYA (5 μM) which did not affect the formation of dihydro and dihydrooxo products, but inhibited their degradation, along with that of the substrate, to other products. Table V shows the percentage recovery of incubated radioactivity by substrates and their dihydro metabolites after incubation of 12-[1-^{14}C]HETE (2 μM), [1-^{14}C]LTB_4 (2 μM) and 13-[1-^{14}C]HODE (2 μM) with porcine PMNL in the presence and absence of ETYA. The recovery of LTB_4 and its reduced metabolites was not appreciably affected by the addition of ETYA, whereas the recovery of 12(S)-HETE and its reduced products was 11% higher in the presence of ETYA. ETYA had a much more pronounced effect on the recovery of 13-HODE and its dihydro metabolites, increasing it to 69% compared to only 9% recovery in the absence of ETYA.

TABLE V

Effect of ETYA on the recoveries of LTB_4, 12-HETE, 13-HODE and their dihydro metabolites

[1-^{14}C]LTB_4 (2 μM) and 12(S)-[1-^{14}C]HETE (2 μM) were incubated with purified porcine PMNL (50 x 10^6 cells/ml) for 10 min at 37 °C with and without 5 μM ETYA. 13-[1-^{14}C]HODE (2 μM) was incubated with purified porcine PMNL (50 x 10^6 cells/ml) for 30 min at 37 °C with and without 10 μM ETYA. Products were resolved as described in Materials and Methods and were quantitated by measurement of radioactivity.

Substrate	Products (pmol/10^7 cells/10 min)		Recovery of
	Reductase (%)[a]	Dihydrooxo	Radioactivity (%)[b]
12 HETE	30 (8)	9	40
12-HETE + ETYA	36 (9)	10	51
LTB_4	75 (19)	32	78
LTB_4 + ETYA	70 (17)	37	82
13-HODE	0 (0)	0	9
13-HODE + ETYA	38 (29)	27	69

[a] The sum of dihydro and dihydrooxo products. The percentages of incubated substrates recovered as less polar reductase products are shown in brackets.

[b] The percentage of incubated radioactivity recovered as the substrate and its less polar dihydro and dihydrooxo products.

1.5. Metabolism of 13-HODE by porcine PMNL in the presence of ETYA

Porcine leukocytes were incubated with 13-HODE in the presence of ETYA (5 μM) and the products were analysed by RP-HPLC (Figure 19). Two less polar radioactive products (*Q* and *R*) were detected. Neither of these compounds absorbed at 235 nm, indicating that the conjugated diene of the substrate is not present in products *Q* and *R*.

1.5.1. Identification of 13-hydroxy-9-octadecenoic acid (Product *Q*)

The mass spectrum of the trimethylsilyl ether, methyl ester derivative of product *Q* showed intense ions at m/z 369 (M-15), 353 (M-31), 337 (M-47), 313 (C_1-C_{13}), 294 (M-90), 262, 199, and 173 (base peak; C_{13}-C_{18}) (Figure 20A). The molecular ion and the ion at m/z 313 (C_1-C_{13}) of this compound are 2 mass units higher than the corresponding ions in the mass spectrum of the trimethylsilyl ether, methyl ester derivative of 13-HODE, indicating that one of the double bonds has been reduced. Although the mass spectrum does not give any information on the location of the remaining double bond, it is probably in the C_9 position by analogy with the corresponding metabolite of 12(S)-HETE. This product is therefore tentatively identified as 13-hydroxy-9-octadecenoic acid (11,12-dihydro-13-HODE).

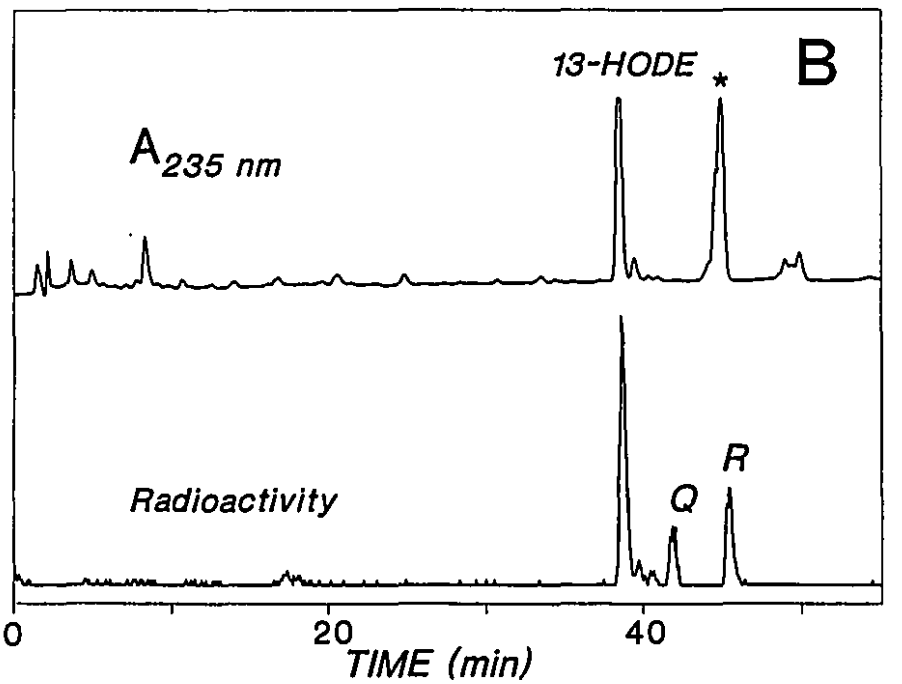

Figure 19. *Reversed-phase HPLC of the metabolites of 13-HODE produced by intact porcine PMNL in the presence of ETYA.* 13-HODE (2 μM) was incubated with porcine leukocytes (75 x 10^6 cells/ml) in the presence of ETYA (5 μM) at 37 °C for 60 min. Products were extracted on a cartridge of ODS-silica and separated on a Phenomenex Spherisorb ODS-2 column with a mobile phase of water/acetonitrile/acetic acid (63:37:0.02) at a flow rate of 2 ml/min. The peak labeled with an *asterisk* (*) and absorbing at 235 nm had a retention time shorter that that of product ***R*** and was not radioactive.

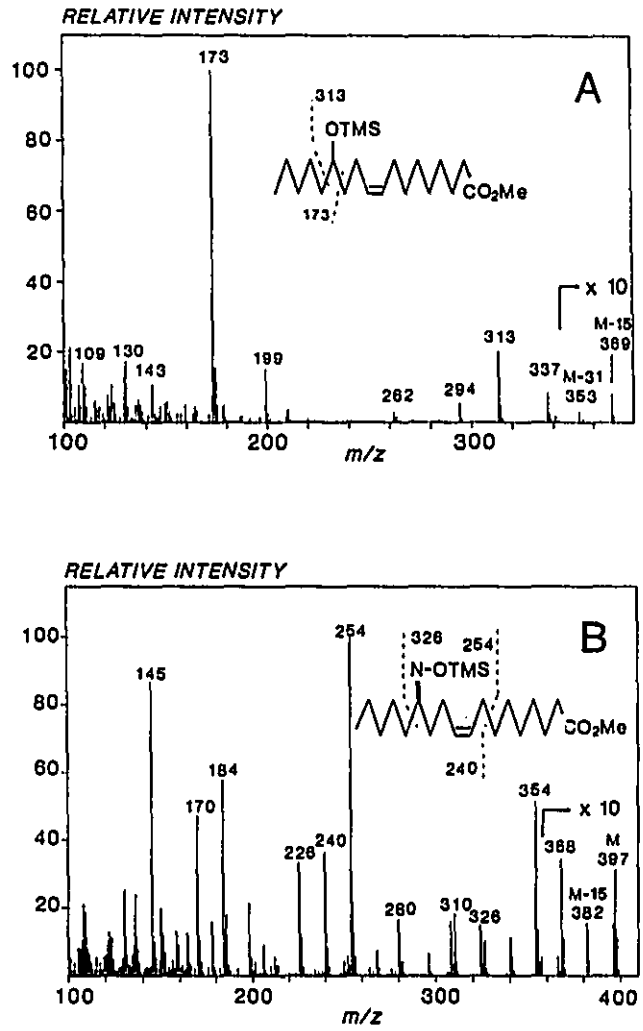

Figure 20. *Mass spectra of (A) the trimethylsilyl ether, methyl ester derivative of product Q and (B) the trimethylsilyl ether, oxime, methyl ester derivative of product R.*

1.5.2. Identification of 13-oxo-9-octadecenoic acid (Product *R*)

Product *R* was found to react with hydroxylamine hydrochloride in the presence of pyridine to give an oxime derivative, indicating the presence of an oxo group. The mass spectrum of the trimethylsilyl ether, oxime derivative of the methyl ester of product *R* showed intense ions at m/z 397 (M), 382 (M-15), 368 (M-29), 366 (M-31), 354 (M-43), 326 (C_1-C_{13}), 310 (C_4-C_{18}), 308, 280, 254 (base peak; C_8-C_{18}), 240 (C_9-C_{18}), 226, 184, 170, and 145 (Figure 20B). This mass spectrum indicates that this metabolite of 13-HODE has an oxo group at C_{13} and a single double bond. The base peak at m/z 254 would strongly suggest that the double bond is in the 9-position and would not be expected if it were in either the 10- or 11- positions. The ion at m/z 240 would support this conclusion, but that at m/z 226 would be more consistent with a double bond in the 10-position. However, as discussed earlier for the dihydrooxo metabolite of 12(S)-HETE, it is possible that the latter ion could have arisen due to a rearrangement of the double bonds or there could be a mixture of dihydrooxo metabolites which differ from one another in the positions of their double bonds. The most likely structure for product *R* is 13-oxo-9-octadecenoic acid (11,12-dihydro-13-oxo-ODE).

1.6. Metabolism of 13-HODE by porcine PMNL in the absence of ETYA

Incubation of 13-[1-^{14}C]HODE with porcine PMNL without ETYA present, results in a significant loss of recovered [1-^{14}C]labeled products (Table V). This observation suggests that, in addition to the dehydrogenase/reductase pathway, porcine

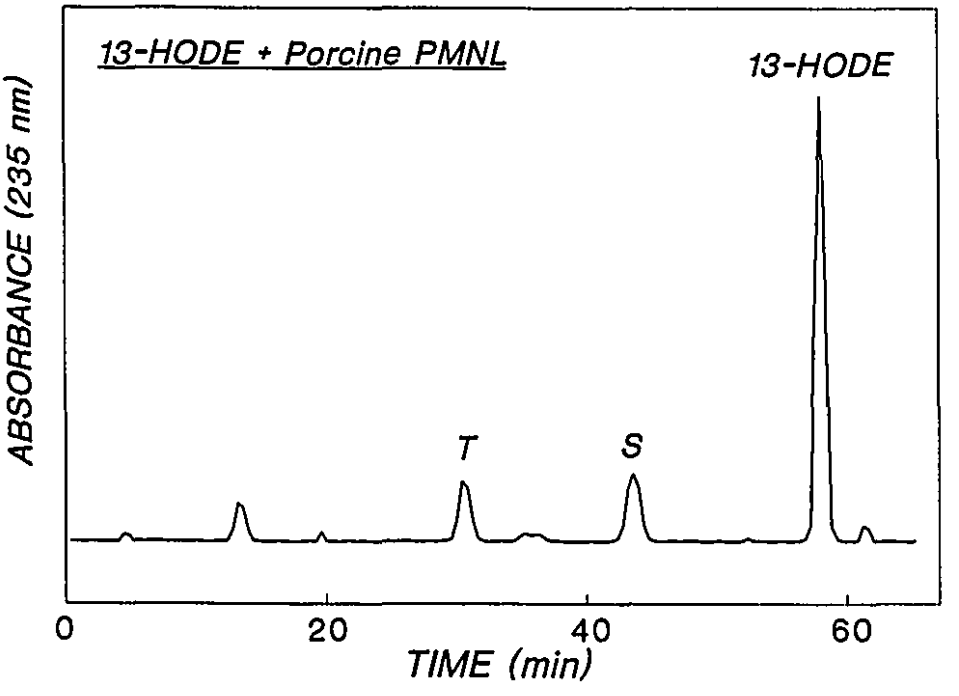

Figure 21. *Reversed-phase HPLC of the metabolites of 13-HODE produced by intact porcine PMNL in the absence of ETYA.* 13-HODE (2 μM) was incubated with porcine leukocytes (75 x 10^6 cells/ml) at 37 °C for 60 min. Products were extracted on a cartridge of ODS-silica and separated on a Phenomenex Spherisorb ODS-2 column with a linear gradient between water/acetonitrile/acetic acid (72:28:0.02) and water/acetonitrile/accetic acid (48:52:0.02) over 60 min at a flow rate of 2 ml/min.

PMNL metabolize 13-HODE by ß-oxidation. The ß-oxidation pathway sequentially removes 2-carbon units from the carboxyl end of the compound which would result in the removal of the ^{14}C in the 1-position of 13-HODE.

In order to investigate the possible production of metabolites produced by ß-oxidation, uniformly labeled 13-[^{14}C]HODE was prepared. The chromatogram obtained by RP-HPLC of the products generated from the incubation of uniformly labeled 13-[^{14}C]HODE with porcine PMNL, in the absence of ETYA, is shown in Figure 21. Two major products, compounds *S* and *T*, eluted prior to the substrate. Both products *S* and *T* absorbed UV light at 235 nm, confirming the integrity of the conjugated diene. These initial observations are consistent with the hypothesis that products S and T are produced from the ß-oxidation of 13-HODE.

1.6.1. Identification of 11-hydroxy-7,9-hexadecadienoic acid (product *S*)

The mass spectrum of the trimethylsilyl ether, methyl ester derivative of product *S* showed intense ions at m/z 354 (M), 339 (M-15), 331, 283 (base peak; C_1-C_{12}), 264 (M-90), 225 (C_7-C_{16}), 143, 130 and 119 (Figure 22A). The mass spectrum indicates that product *S* has 18 carbons, suggesting that it is a product of a single cycle of ß-oxidation of 13-HODE. Mass ions 225 and 283 are consistent with the presence of carbon-carbon double bonds at C_7 and C_9, and a hydroxyl group at C_{11}. Therefore, the most likely structure for product *S* is 11-hydroxy-7,9-hexadecadienoic acid.

1.6.2. Identification of 9-hydroxy-5,7-tetradecadienoic acid (product *T*)

The mass spectrum of the trimethylsilyl ether, methyl ester derivative of product *T* showed intense ions at m/z 326 (M), 311 (M-15), 295 (M-31), 283, 255

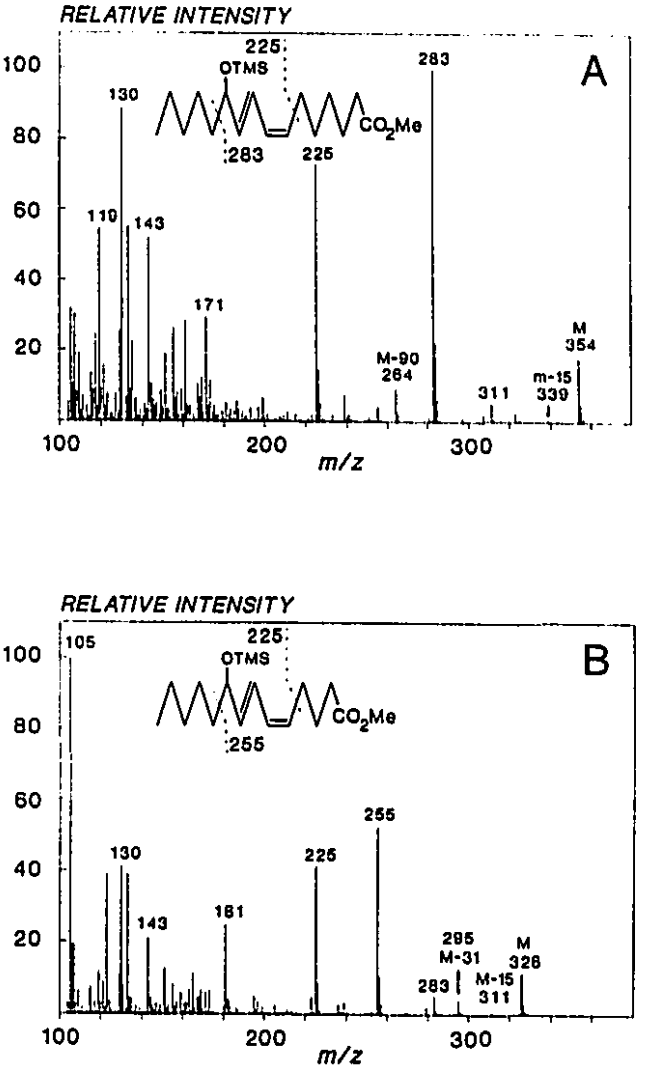

Figure 22. *Mass spectra of the trimethylsilyl ether, methyl ester derivatives of (A) product S and (B) product T.*

(C_1-C_9), 225 (C_5-C_{14}), 181, 130 and 105 (base peak) (Figure 22B). The molecular ion 326 indicates that 4 carbons have been removed from 13-HODE presumably resulting from two cycles of ß-oxidation. The ions at m/z 255 and 225 are consistent with the presence of carbon-carbon double bonds at C_5 and C_7 and a hydroxyl group at C_9. Therefore, the most likely structure for product ***T*** is 9-hydroxy-5,7-tetradecadienoic acid.

1.7. Specificity of the porcine PMNL 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway

As ETYA (5 μM) was found to enhance the recovery of the substrates and their dihydro metabolites, without affecting the formation of the latter, this concentration of ETYA was used in all of the analytical experiments involving intact PMNL described below.

1.7.1. Effect of substrate concentration on the metabolism of LTB_4, 12(S)-HETE and 13-HODE

Porcine PMNL were incubated with various concentrations of [1-^{14}C]LTB_4, 12-[1-^{14}C]HETE and 13-[1-^{14}C]HODE for 6, 6 and 30 min respectively. The products were analyzed by precolumn extraction/RP-HPLC and the apparent K_m and V_{max} values were calculated from Lineweaver-Burk plots (Figure 23A). The apparent K_m values for LTB_4 (0.28 μM) and 12(S)-HETE (0.21 μM) were similar, whereas that for 13-HODE (2.2 μM) was much higher. All three substrates shared an apparent

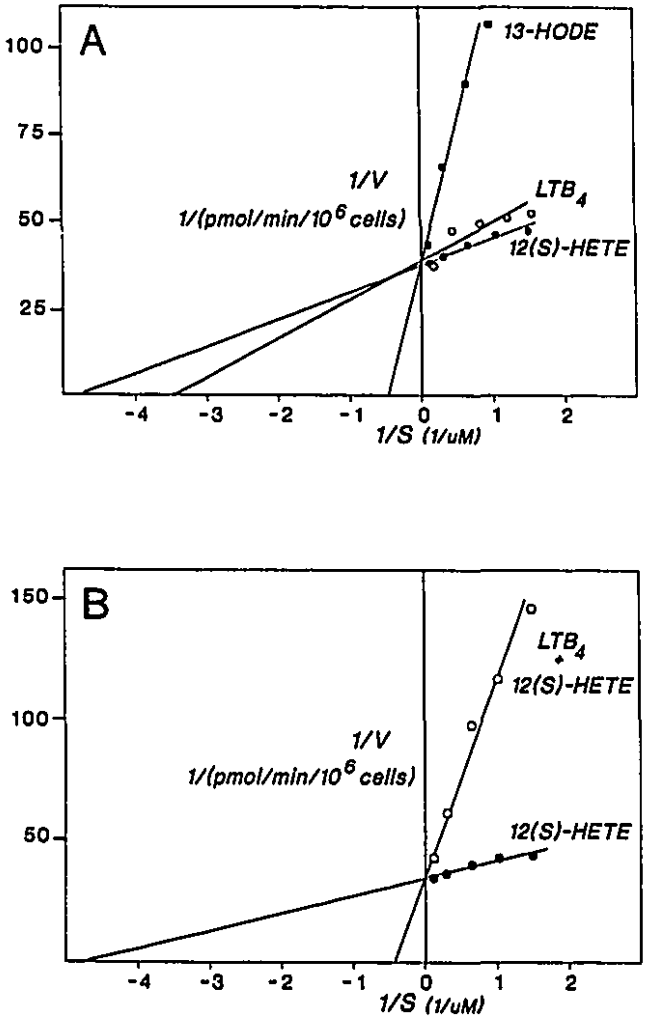

Figure 23. *Lineweaver-Burk plots for the metabolism of LTB_4, 12(S)-HETE and 13(S)-HODE by porcine PMNL.* (A) Various concentrations of [1-^{14}C]LTB_4 (○), 12-[1-^{14}C]HETE (●) and 13-[1-^{14}C]HODE (■) were incubated with porcine PMNL (50 x 10^6 cells/ml) for 6, 6 and 20 minutes, respectively. (B) Various concentrations of 12-[1-^{14}C]HETE were incubated with LTB_4 (2 μM) for 6 min. Products were analysed by precolumn extraction/RP-HPLC and quantitated by measurement of radioactivity. Reductase activity is expressed as the sum of dihydro and dihydrooxo metabolites. Results were corrected for recovery (See Table VI).

V_{max} of approximately 0.029 pmol/min/10^6 cells.

To confirm that 12(S)-HETE and LTB_4 are metabolized by the same enzymes, a competition experiment was performed in which various concentrations of 12-[1-^{14}C]HETE were incubated with PMNL in the presence or absence of a single concentration (2.0 μM) of LTB_4. Lineweaver-Burk analysis revealed that the addition of LTB_4 did not affect the apparent V_{max}, but increased the slope of the line, indicative of competitive inhibition (Figure 23B). Similar results were obtained when 12(S)-HETE (2.0 μM) was incubated with various concentrations of [1-^{14}C]LTB_4.

1.7.2. Metabolism of various hydroxylated PUFA's by porcine PMNL

The substrate specificity of the reductase pathway present in porcine PMNL was investigated using various structurally related compounds derived from arachidonic acid, 5,8,11,14,17-eicosapentaenoic acid and linoleic acid (Table VI). To avoid limitations in the amounts of products formed, due to lack of substrate availability, the incubation conditions employed (2 μM substrate; 10 min) permitted no more than 25% metabolism of the substrate. Although ETYA was included in the incubations, there were significant differences in the percent recovery of the incubated radioactivity, presumably due to differences in ß-oxidation. To correct for these differences, values obtained for the amounts of dihydro and dihydrooxo products recovered were divided by the percentage of recovered radioactivity for each substrate. Both the actual amounts recovered and the corrected values appear in Table VI.

TABLE VI
Specificity of the porcine PMNL dehydrogenase/reductase pathway[a]

Substrate	Products (pmol/10^7 cells/10 min)				Recovery of Radioactivity (%)[d]
	Reductase[b]		Dihydrooxo		
	Corrected	Uncorrected (%)[c]	Corrected	Uncorrected	
LTB_4	85	70 (17)	46	37	82
6-*trans*-LTB_4	85	63 (16)	49	36	77
12e-8*c*-6-*trans*-LTB_4	81	51 (13)	29	18	66
12-epi-6-*trans*-LTB_4	77	60 (15)	22	17	82
12(S)-HETE	72	36 (9)	19	10	51
LTB_5	55	42 (10)	38	29	75
15(S)-HETE	47	23 (6)	36	17	48
13(S)-HODE	47	24 (6)	38	20	51
5(S)-HETE	23	8 (2)	9	3	34
9(S)-HODE	15	10 (2)	13	9	67
20-hydroxy-LTB_4	11	7 (2)	6	4	67
HHTre	10	7 (2)	8	6	76
PGE_2	0	0 (0)	0	0	91
$PGF_{2\alpha}$	0	0 (0)	0	0	90

[a] [1-^{14}C]Labeled substrates (2μM) were incubated with porcine PMNL (50 x 10^6 cells/ml) for 10 min at 37 °C. Products were analyzed by precolumn extraction/RP-HPLC. Products were quantitated by measuring radioactivity by liquid scintillation counting in 0.5 minute column fractions. The amounts of reductase products formed were corrected for recovery by dividing the uncorrected amounts by the % recovery of radioactivity multiplied by 100. Abbreviation: 12e-8*c*-6-*trans*-LTB_4, 12-epi-8-*cis*-6-*trans*-LTB_4.

[b] The sum of dihydro and dihydrooxo products. Products formed by 10,11-reductase and 6,11- reductase pathways were not distinguished from one another.

[c] The values in brackets represent the percentages of incubated substrates which were recovered as less polar reductase products.

[d] The percentage of incubated radioactivity recovered as the substrate and its dihydro and dihydrooxo products was calculated by comparison with control incubations in which the substrate was incubated with buffer alone.

The best substrate for the 10,11-reductase pathway is LTB_4. 6-*Trans*-LTB_4 and 12-epi-6-*trans*-LTB_4 were metabolized to dihydro products at about the same rate as LTB_4. Although the absolute amounts of dihydro metabolites formed from 12-epi-8-*cis*-6-*trans*-LTB_4 and 12(S)-HETE were lower than for LTB_4, after they were corrected for recovery, the amounts were nearly the same as for LTB_4. All of the above eicosanoids share a C_{12} hydroxyl group preceded by two conjugated double bonds and followed by a 2-cis-octenyl group.

LTB_5, which is a metabolite of 5,8,11,14,17-eicosapentaenoic acid, is metabolized to a lesser extent than LTB_4, probably due to the presence of the Δ^{17} double bond in the omega end of the molecule. Introduction of an omega-hydroxyl group, as in 20-hydroxy-LTB_4, nearly eliminates metabolism by the reductase pathway.

Alteration of the position of the hydroxyl group, as in 15-HETE and 5-HETE, reduced the rate of metabolism to approximately 65% and 21%, respectively, that of 12(S)-HETE. HHTrE, which is an endoperoxide-derived arachidonic acid metabolite with a 12-hydroxyl group preceded by two double bonds in the 8 and 10 positions, but followed by a 2-pentenyl group, was metabolized only slightly. This suggests that the distance of the hydroxyl group to the omega end of the molecule is also important for metabolism by this pathway. 13-HODE and 9-HODE, in which the positions of the hydroxyl groups are altered and the carbon chain length reduced, also exhibited decreased metabolism. PGE_2 and $PGF_{2\alpha}$, which, after oxidation of their 15-hydroxyl

groups are metabolized in many tissues by a 13,14-reductase (320), were not converted to any detectable products by porcine PMNL.

It should be noted that a 5-hydroxyeicosanoid dehydrogenase/6,11-reductase pathway also exists in porcine PMNL (370). 6-*Trans*-LTB_4, and 12-epi-6-*trans*-LTB_4 are metabolized by this pathway to 6,11-dihydro and 6,11-dihydro-5-oxo metabolites (370). However, no evidence for the metabolism of LTB_4 or 12(S)-HETE to 6,11-dihydro products by porcine PMNL was found in this study. In this specificity study, we were not able to distinguish between 6,11-dihydro products and 10,11-dihydro products. It is therefore likely that the rates of metabolism of 6-*trans*-LTB_4, and 12-epi-6-*trans*-LTB_4 by the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway are lower than those indicated in Table VI. It is also possible that 5-HETE and 9-HODE, which have a hydroxyl group followed by two conjugated double bonds located on the ω-side of the molecule, could be metabolized principally by the 5-hydroxyeicosanoid dehydrogenase/6,11-reductase pathway.

2. Mechanism for the formation of 10,11-dihydro metabolites by porcine PMNL

As discussed in the introduction (Figure 6), the initial step for the formation of dihydro metabolites of LTB_4 by porcine PMNL could be either reduction of the C_{10}-C_{11} double bond, producing 10,11-dihydro-LTB_4, or oxidation of the C_{12} hydroxyl group producing 12-oxo-LTB_4.

Previous experiments in which LTB_4 and 12(S)-HETE, labeled with deuterium in the 12-position, were incubated with intact porcine PMNL, indicated that the 10,11-dihydro products retained between half and two-thirds of the deuterium in this position (304,321). Since oxidation of the 12-hydroxyl group would result in complete loss of the deuterium from the 12-position, this suggested that PMNL contained a reductase which could reduce the Δ^{10} double bond of the substrate directly, without the requirement for a 12-oxo-intermediate. The partial loss of deuterium from the 12-position could be explained by the subsequent conversion of the initially formed dihydro products to dihydrooxo metabolites, which was shown to be a reversible reaction as discussed above (section III.1.2.2.).

2.1. Tritium isotope effect in the metabolism of 12(S)-[5,6,8,9,11,12,14,15-^{3}H]HETE to dihydro products by porcine PMNL

If the inital step in the formation of dihydro metabolites of LTB_4 and 12(S)-HETE is reduction of the 10,11-double bond, we would predict that the presence of a deuterium or tritium atom in the 12-position would not affect the formation of the 10,11-dihydro metabolite, but would retard the formation of the subsequently formed 10,11-dihydro-12-oxo-metabolite.

To test this hypothesis, we incubated a mixture of 12(S)-[1-^{14}C]HETE and 12(S)-[5,6,8,9,11,12,14,15-^{3}H]HETE with porcine PMNL and analyzed the products by RP-HPLC (Figure 24). Contrary to what we had predicted, the ratio of ^{3}H to ^{14}C in 12-hydroxy-5,8,14-eicosatrienoic acid and 12-oxo-5,8,14-eicosatrienoic acid were

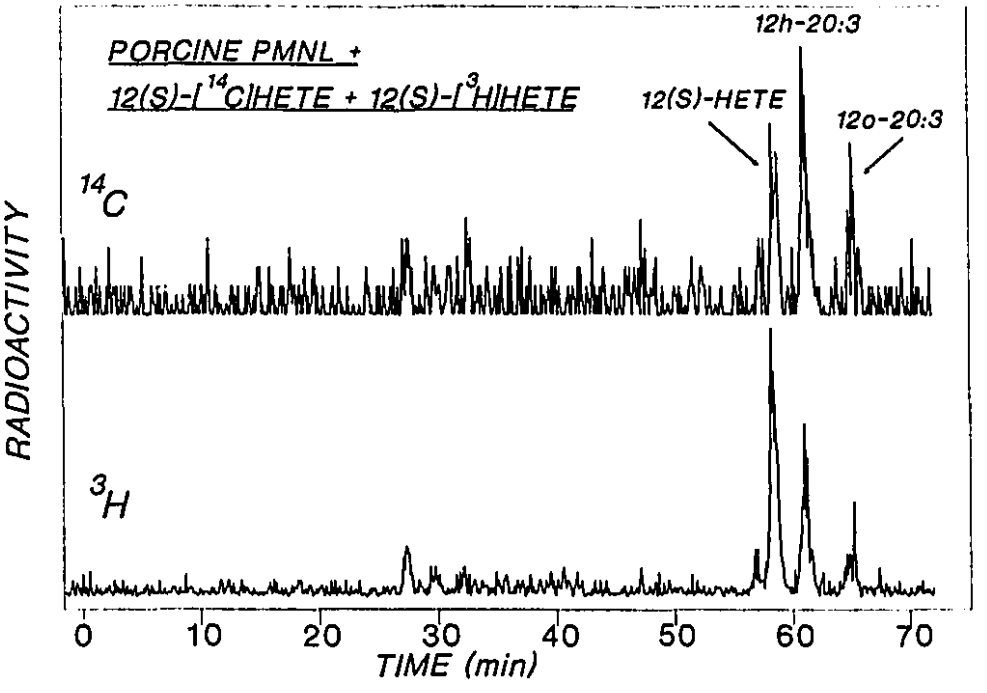

Figure 24. *Reversed-phase HPLC profile of the metabolites of 12(S)-[1-^{14}C]HETE and 12(S)-[5,6,8,9,11,12,14,15-^{3}H]HETE produced by intact porcine PMNL.* A mixture of 12(S)-[1-^{14}C]HETE and 12(S)-[5,6,8,9,11,12,14,15-^{3}H]HETE (2 μM) was incubated with intact porcine PMNL (50 x 10^6 cells/ml) for 30 min at 37 °C. Products were resolved by precolumn extraction/RP-HPLC. Products retained on the precolumn were separated on a Spherisorb ODS-2 column (Jones Chromatography) with a linear gradient between acetonitrile/water/acetic acid (20:80:0.02) and acetonitrile/water/acetic acid (52:48:0.02) over 60 min at a flow rate of 2.0 ml/min. Radioactivity in the column eluate was analyzed using a Ramona-5-LS liquid cell radioactivity detector (Raytest). Abbreviations: 12h-20:3, 12-hydroxy-5,8,14-eicosatrienoic acid; 12o-20:3, 12-oxo-5,8,14-eicosatrienoic acid.

about the same. However, the proportion of 12(S)-[^{3}H]HETE converted to tritiated dihydro products was much lower than the proportion of 12(S)-[^{14}C]HETE converted to the corresponding ^{14}C-labeled products (Figure 24). This marked isotope effect suggests that the rate-limiting step in the formation of dihydro metabolites from 12(S)-HETE is oxidation of the 12-hydroxyl group, which would require cleavage of a carbon-tritium bond.

2.2. Effects of different cofactors on the formation of dihydro metabolites of LTB_4 by a post-nuclear supernatant fraction from PMNL

If LTB_4 and 12(S)-HETE could be reduced directly by a 10,11-reductase, we would expect the reaction to require reduced cofactors such as NADH or NADPH, whereas oxidized cofactors such as NAD^+ or $NADP^+$ would be required if oxidation of the 12-hydroxyl group were the initial step. To determine which cofactors are required for the formation of 10,11-dihydro metabolites of 12-hydroxyeicosanoids, porcine PMNL were disrupted by sonication and the sonicate was centrifuged at 1500 x *g* to remove undisrupted cells and nuclei. LTB_4 was incubated with the 1500 x *g* supernatant fraction for 10 min at 37 °C either in the absence of cofactors or in the presence of NAD^+, $NADP^+$, NADH or NADPH (Table VII). In the presence of NAD^+, large amounts of 10,11-dihydro-LTB_4 and 10,11-dihydro-12-oxo-LTB_4 were formed from LTB_4, whereas only a relatively small amount of 10,11-dihydro-LTB_4 was formed in the presence of NADH and no 10,11-dihydro products were detected in the absence of cofactors or in the presence of either

TABLE VII

Metabolism of LTB_4 by a post-nuclear supernatant fraction from porcine PMNL

LTB_4 (2 μM) was incubated with a 1500 x *g* supernatant fraction from porcine PMNL (6.5 mg protein/ml) for 10 min at 37 °C in a volume of 1 ml. Incubations were terminated by addition of MeOH (0.6 ml) and the products were analyzed by precolumn extraction/RP-HPLC as described in Materials and Methods.

	Products (pmol/min/mg protein)	
Cofactor	10,11-dihydro-LTB_4	10,11-dihydro-12-oxo-LTB_4
None-	-[a]	-
NAD^+	1.40	1.56
$NADP^+$	-	-
NADH	0.53	-
NADPH	-	-

[a] no detectable activity.

$NADP^+$ or NADPH. These results therefore suggest that the initial step in the formation of 10,11-dihydro metabolites by porcine PMNL is oxidation of the 12-hydroxyl group.

2.3. Metabolism of LTB_4 by subcellular fractions from porcine PMNL

To attempt to clarify the individual steps involved in the formation of dihydro metabolites of 12-hydroxyeicosanoids, various subcellular fractions were prepared from sonicates of porcine PMNL. The activities of marker enzymes for azurophil granules (myeloperoxidase and ß-glucuronidase) and plasma membranes (ouabain-sensitive Na^+-K^+-ATPase) were measured in each fraction (Table VIII). Myeloperoxidase and ß-glucuronidase activities were localized primarily in the 20,000 x *g* pellet, indicating that this fraction was enriched in granules. Na^+-K^+ ATPase activity was present in both the granule-rich fraction and the 150,000 x *g* pellet (microsomal fraction). The 150,000 x *g* supernatant (cytosol) contained very little of the above enzyme activities. The activity of a marker enzyme specific for the endoplasmic reticulum was also investigated. Glucose-6-phosphatase activity is reported to be present in the endoplasmic reticulum of various cells (322,323). However, only low levels of activity of this enzyme was seen in the post-nuclear supernatant from porcine PMNL, with the majority of that activity recovered in the cytosolic fraction. It is not clear why this assay failed to detect the endoplasmic reticulum which would be expected to be present primarily in the microsomal fraction. It has been reported that porcine PMNL have very little endoplasmic

TABLE VIII

Marker enzyme activities in subcellular fractions from porcine PMNL

Enzyme activity was measured as described in Materials and Methods. Relative specific activity is calculated by dividing the specific activity in a given fraction by the specific activity in the post-nuclear supernatant.

Subcellular Fraction	Relative Specific Activity		
	Myeloperoxidase	ß-Glucuronidase	Ouabain-Sensitive Na^+-K^+-ATPase
20 000 x *g* Pellet (Granule-rich)	3.10	2.14	5.50
150 000 x *g* Pellet (Microsomes)	1.76	0.70	4.35
150 000 x *g* Supernatant (Cytosol)	- [a]	0.62	n.d.

[a] no detectable activity.

reticulum, therefore it is possible that non-specific phosphatases present in the cell may have diluted out any phosphatase activity specific to the endoplasmic reticulum.

LTB_4 was incubated with each subcellular fraction in the presence of NAD^+ and the products were separated by NP-HPLC which enables the resolution of 10,11-dihydro-LTB_4 and 10,11-dihydro-12-epi-LTB_4 (Figure 25). The 20,000 x *g* pellet did not convert LTB_4 to appreciable amounts of products (data not shown). On the other hand, the metabolism of [1-^{14}C]LTB_4 by the 20,000 x *g* supernatant fraction was very similar to that of intact PMNL and the 1500 x *g* supernatant, in that 10,11-dihydro-LTB_4 and 10,11-dihydro-12-oxo-LTB_4 were the major products (Figure 25A). However, unlike intact PMNL, the 20,000 x *g* supernatant converted this substrate to little or no 10,11-dihydro-12-epi-LTB_4. In contrast to the 20,000 x *g* supernatant, the 150,000 x *g* supernatant (cytosol) exhibited little metabolic activity towards LTB_4, with only a very small amount of 10,11-dihydro-12-oxo-LTB_4 being detected (Figure 25B). Neither were appreciable amounts of dihydro metabolites formed by the microsomal fraction in the presence of NAD^+ (Figure 25C). Instead, this fraction converted LTB_4 to a substance (product *Z*; t_R= 18 min) which absorbed in the UV at 318 nm (Figure 26). Finally, recombination of the microsomal and cytosolic fractions restored the formation of the two dihydro products, but considerably reduced the amount of product *Z* detected (data not shown). These results suggest that the formation of 10,11-dihydro metabolites of LTB_4 requires at least one enzyme present in the microsomal fraction and another enzyme present in the cytosolic fraction.

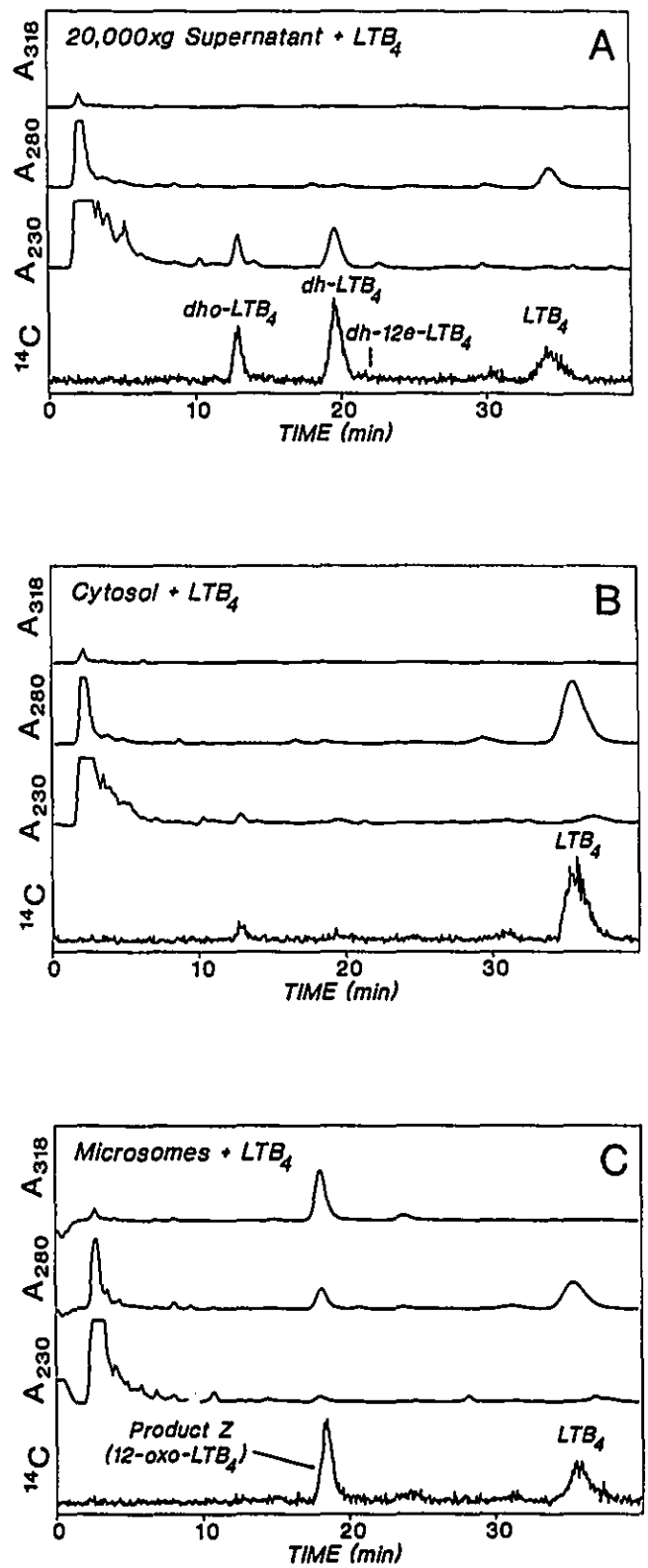
20,000xg Supernatant + LTB4
A
A318
A280
A230
14C
dho-LTB4
dh-LTB4
dh-12e-LTB4
LTB4
0
10
20
30
TIME (min)
Cytosol + LTB4
B
A318
A280
A230
14C
LTB4
0
10
20
30
TIME (min)
Microsomes + LTB4
C
A318
A280
A230
14C
Product Z
(12-oxo-LTB4)
LTB4
0
10
20
30
TIME (min)

Figure 25.

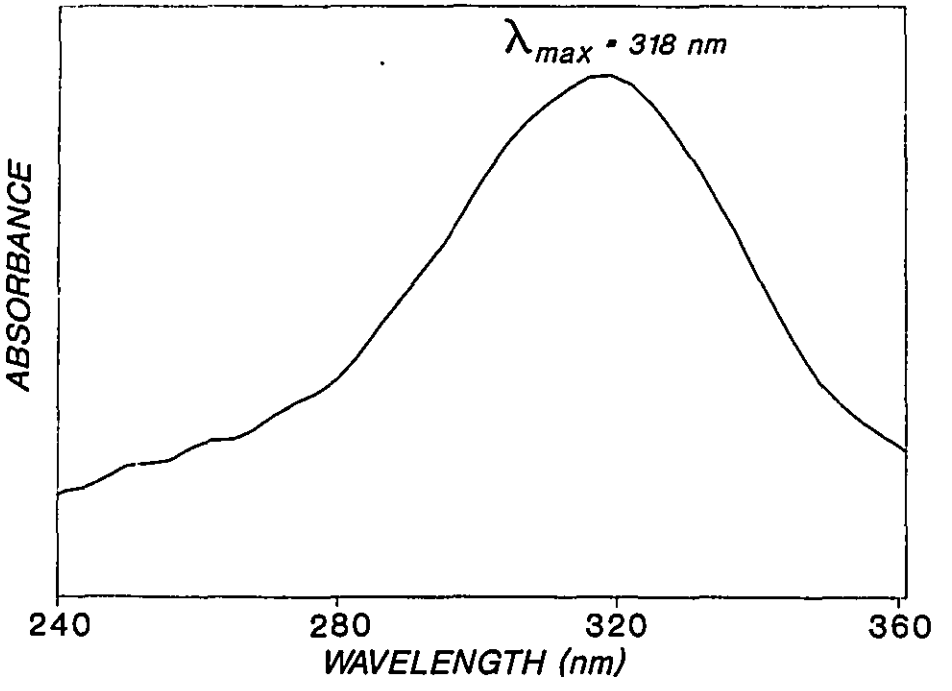

Figure 26. *UV spectrum of product* **Z** *(12-oxo-LTB$_4$).*

The above results describe the metabolism of LTB_4 in the presence of NAD^+. The total amounts of LTB_4 metabolites formed by various fractions in the presence of other cofactors were also determined (Table IX). There was no detectable metabolism of LTB_4 by the granule-rich fraction in the absence or presence of any of the cofactors investigated. The 20,000 x *g* supernatant converted LTB_4 to 10,11-dihydro-LTB_4 and 10,11-dihydro-12-oxo-LTB_4 in the presence of NAD^+, but in its absence or in the presence of NADPH, no metabolites were detected. Only small

TABLE IX

Effects of different cofactors on the metabolism of LTB_4 by subcellular fractions from porcine PMNL.

LTB_4 (2 μM) was incubated with the granule-rich and 20,000 x *g* supernatant fractions for 10 min and the microsomal and cytosolic fractions for 30 min in the presence or absence of cofactors (1 mM). Incubations were terminated by the addition of 0.6 ml MeOH and the products were analyzed by precolumn extraction/RP-HPLC as described in Materials and Methods. The value for products represent the sums of 10,11-dihydro-LTB_4, 10,11-dihydro-12-oxo-LTB_4 and product Z (12-oxo-LTB_4) formed.

	Products (pmol/min/mg protein)				
Subcellular Fraction	NAD^+	$NADP^+$	NADH	NADPH	NONE
Granule	- [a]	-	-	-	-
20,000 x *g* Supernatant	93.3	5.0	14.1	-	-
Microsomes	52.9	29.0	-	-	-
Cytosol	-	-	-	-	-

[a] no detectable activity.

amounts of metabolites were detected in the presence of $NADP^+$ or NADH. The microsomal fraction converted LTB_4 to product *Z* in the presence of NAD^+ and to a lesser extent in the presence of $NADP^+$, but no metabolites were detected in the presence of reduced cofactors. No detectable 10,11-dihydro products were formed by the microsomal fraction in the presence or absence of any of the cofactors tested. LTB_4 was not metabolized to an appreciable extent by the cytosolic fraction in the presence or absence of the above cofactors.

2.4. Identification of 12-oxo-LTB_4 (product *Z*)

The UV absorption spectrum of product *Z* is shown in Figure 26. Unlike LTB_4, which has a typical triene chromophore with absorption maxima at 261, 270, and 282 nm, product *Z* has a single absorbance maximum at 318 nm, indicating the presence of four conjugated double bonds. This suggests that one of the two hydroxyl groups of LTB_4 has been converted to an oxo group.

The mass spectrum of the trimethylsilyl ether, methyl ester derivative of hydrogenated product *Z* (Figure 27) exhibited intense ions at m/z 427(M-1), 413(M-15), 397(M-31), 381(M-47), 327 (C_5-C_{20}), 203 (base peak, C_1 to C_5), 171, 141(C_{12}-C_{20}), 129, and 113. The ions at m/z 203 and 327 confirm the integrity of the C_5 hydroxyl group whereas, the ion at m/z 141 is consistent with a C_{12} oxo group. This mass spectrum is identical to the mass spectrum of the corresponding derivative of 10,11-dihydro-12-oxo-LTB_4 (304), confirming the presence of an oxo group at carbon 12. The mass spectral, UV and chromatographic properties of product *Z* indicate that it is identical to 12-oxo-LTB_4.

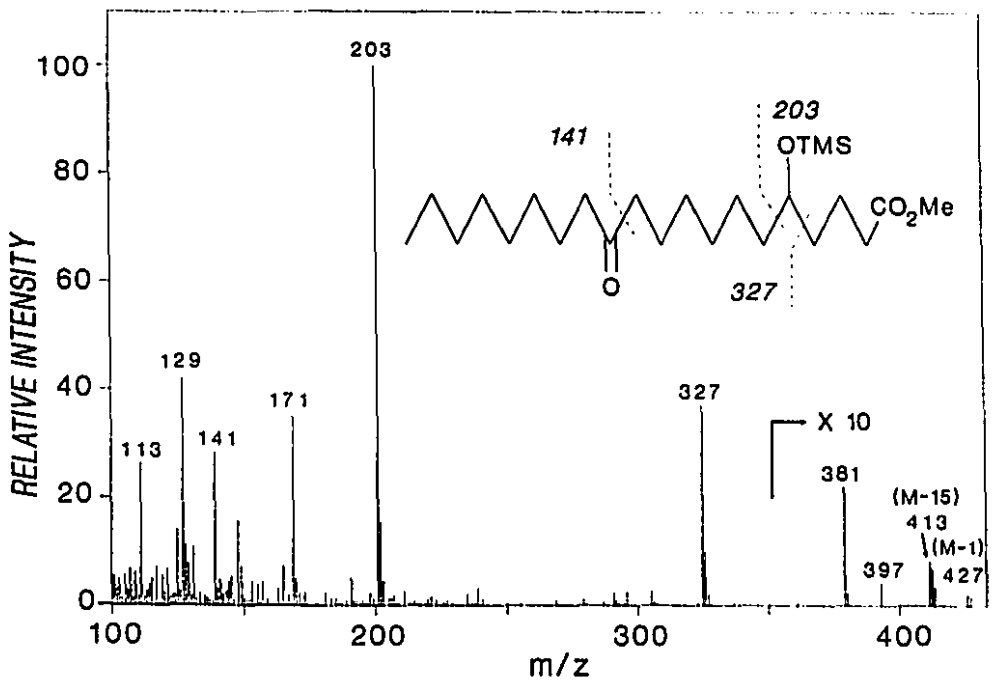

Figure 27. *Mass spectrum of the trimethylsilyl ether, methyl ester derivative of hydrogenated product Z (12-oxo-LTB$_4$).* TMS, trimethylsilyl.

2.5. Metabolism of 12-oxo-LTB$_4$

The results described above suggest that the first step in the formation of 10,11-dihydro metabolites of LTB$_4$ is the formation of 12-oxo-LTB$_4$ through the activity of a microsomal 12-hydroxyeicosanoid-dehydrogenase enzyme. To investigate the next step in the pathway, 12-oxo-LTB$_4$ was incubated with either the microsomal or the cytosolic fraction from porcine PMNL in the presence and absence of NADH and NADPH (Table X). The incubation time used for the microsomal

TABLE X

Metabolism of 12-oxo-LTB_4 by cytosolic and microsomal fractions from porcine PMNL

12-Oxo-LTB_4 (2 μM) was incubated in a final volume of 5 ml with the microsomal fraction (30 min) and the cytosolic fraction (5 min) in the presence or absence of cofactors (1 mM). Incubations were terminated by addition of 17% MeOH (10 ml), followed by immediate extraction on a cartridge of ODS-silica at neutral pH. Products were resolved by RP-HPLC and quantitated by UV absorbance using PGB_2 (300 ng) as an internal standard. 10,11-Dihydro-12-oxo-LTB_4 was the only product formed by the cytosol, whereas the microsomal fraction produced a dihydroxyeicosatetraenoic acid fraction (DiHETE) and 10,11-dihydro-12-oxo-LTB_4 (dho-B_4).

	Product (pmol/10 min/mg protein)					
	NADH		NADPH		NONE	
Subcellular Fraction	dho-B4	DiHETE	dho-B4	DiHETE	dho-B4	DiHETE
Microsomes	2.3	1.4	2.9	0.9	- [a]	-
Cytosol	38.9	-	23.5	-	22.3	-

[a] no detectable activity.

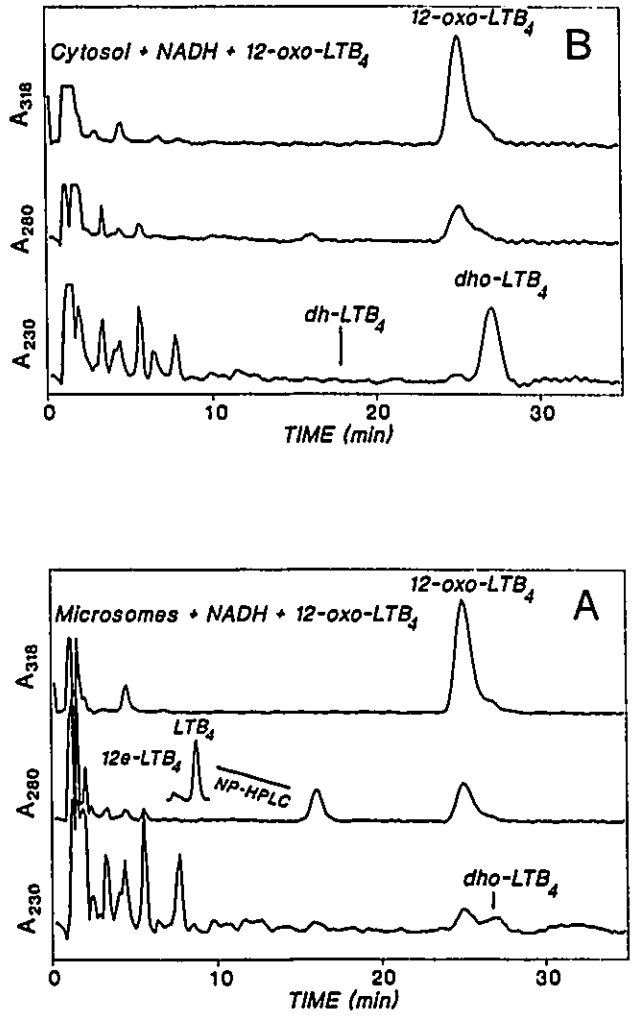

Figure 28. *Reversed-phase HPLC profile of 12-oxo-LTB_4 metabolites produced by A) the microsomal fraction and B) the cytosolic fraction obtained from porcine PMNL.* Porcine PMNL microsomes and cytosol were incubated with 12-oxo-LTB_4 (2 μM) for 30 min and 5 min, respectively, at 37 °C in the presence of 1 mM NADH. The samples were analyzed by precolumn extraction/RP-HPLC. Products retained on the precolumn were separated on a Novapak C_{18} column (Waters-Millipore) with a mobile phase of acetonitrile/water/acetic acid (39:61:0.02). The flow rate was 1 ml/min. The abbreviations are; dh-LTB_4, 10,11-dihydro-LTB_4; dho-LTB_4, 10,11-dihydro-12-oxo-LTB_4; 12e-LTB_4. 12-epi-LTB_4.

fraction (30 min) was much longer than that for the cytosolic fraction (5 min), since the latter was much more active in metabolizing 12-oxo-LTB_4.

12-Oxo-LTB_4 was metabolized by the microsomal fraction in the presence of either NADH or NADPH to two products with retention times and UV spectra identical to those of LTB_4 (t_R= 16.0 min) and 10,11-dihydro-12-oxo-LTB_4 (t_R= 26.5 min) (Figure 28A). Neither of these products was formed in the absence of cofactors. Reduction of 12-oxo-LTB_4 to a DiHETE could give either LTB_4 or 12-epi-LTB_4, which may not be resolved using RP-HPLC with acetonitrile/water/acetic acid as the mobile phase. To examine the stereochemistry of the DiHETE formed from 12-oxo-LTB_4 by PMNL microsomes, this product was rechromatographed by NP-HPLC (Figure 28A inset). The DiHETE peak consisted of one major component (90%) with a retention time identical to that of LTB_4 along with a minor component (10%) with a retention time identical to that of 12-epi-LTB_4, which had been synthesized by reduction of 12-oxo-LTB_4 with sodium borohydride. The formation of LTB_4/12-epi-LTB_4 from 12-oxo-LTB_4 appeared to be favored by NADH whereas the formation of 10,11-dihydro-12-oxo-LTB_4 was greater with NADPH.

The cytosolic fraction of porcine PMNL was much more active than the microsomal fraction in converting 12-oxo-LTB_4 to 10,11-dihydro-12-oxo-LTB_4 in the presence of NADH (Figure 28B). 12-Oxo-LTB_4 was also metabolized to some extent by the cytosolic fraction in the absence of added cofactors, probably due to the presence of endogenous cofactors. Addition of NADPH did not affect the metabolism

of 12-oxo-LTB_4 by the cytosolic fraction appreciably. Neither LTB_4 nor 10,11-dihydro-LTB_4 was detected after incubation of 12-oxo-LTB_4 with the cytosolic fraction (Table X).

2.6. Metabolism of 12(S)-HETE by porcine PMNL microsomes

Earlier in this study (Table VI), it was shown that intact porcine PMNL convert compounds with a C_{12} hydroxyl group preceded by two conjugated double bonds and followed by a 2-*cis*-octenyl group to dihydro products (section III.1.7.2.). LTB_4 and 12(S)-HETE were both good substrates for this pathway. To confirm that 12(S)-HETE is metabolized by the 12-hydroxyeicosanoid dehydrogenase enzyme, 12(S)-HETE was incubated with porcine PMNL microsomes in the presence of NAD^+ and the products resolved by RP-HPLC (Figure 29). A less polar product (t_R= 22.5 min) was produced with a single UV maximum at 278 nm (Figure 29 inset). Based on its chromatographic and UV spectral properties, this metabolite is presumably identical to 12-oxo-5,8,10,14-eicosatetraenoic acid (12-oxo-ETE).

2.7. Time courses for the metabolism of LTB_4, 12(S)-HETE and 12(R)-HETE by porcine PMNL microsomes

12(S)-HETE and LTB_4 have opposite configurations at C_{12}, yet intact cells appear to metabolize both substrates to a similar extent (Table VI). To investigate if the configuration of the C_{12}-hydroxyl group affects metabolism by the 12-hydroxyeicosanoid dehydrogenase, LTB_4, 12(S)-HETE and 12(R)-HETE were

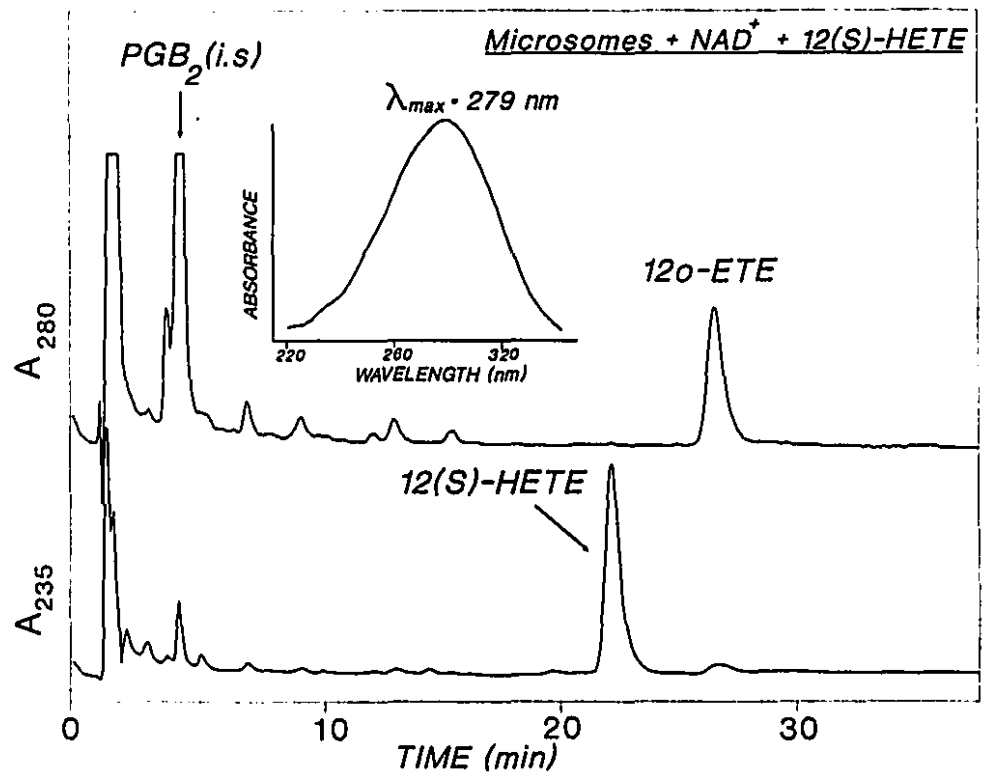

Figure 29. *Reversed-phase-HPLC profile of 12(S)-HETE metabolites produced by the microsomal fraction from porcine PMNL.* 12(S)-HETE (1 μM) was incubated with the microsomal fraction from porcine PMNL for 40 min at 37 °C in the presence of NAD^+ (1 mM). The sample was analyzed by precolumn extraction/RP-HPLC after addition of PGB_2 (300 ng) as an internal standard. Products retained on the precolumn were resolved on a column of octadecylsilyl silica (Novapak C_{18}; Waters-Millipore) with a gradient between acetonitrile/water/acetic acid (50:50:0.02) and acetonitrile/water/acetic acid (55:45:0.02) over 30 min at a flow rate of 1 ml/min. UV absorbance at 280 nm is 4 fold more sensitive than at 235 nm. The inset shows the UV spectrum of the peak eluting with a retention time of 27 min. Abbreviations: i.s., internal standard; 12-oxo-ETE, 12-oxo-5,8,10,14-eicosatetraenoic acid.

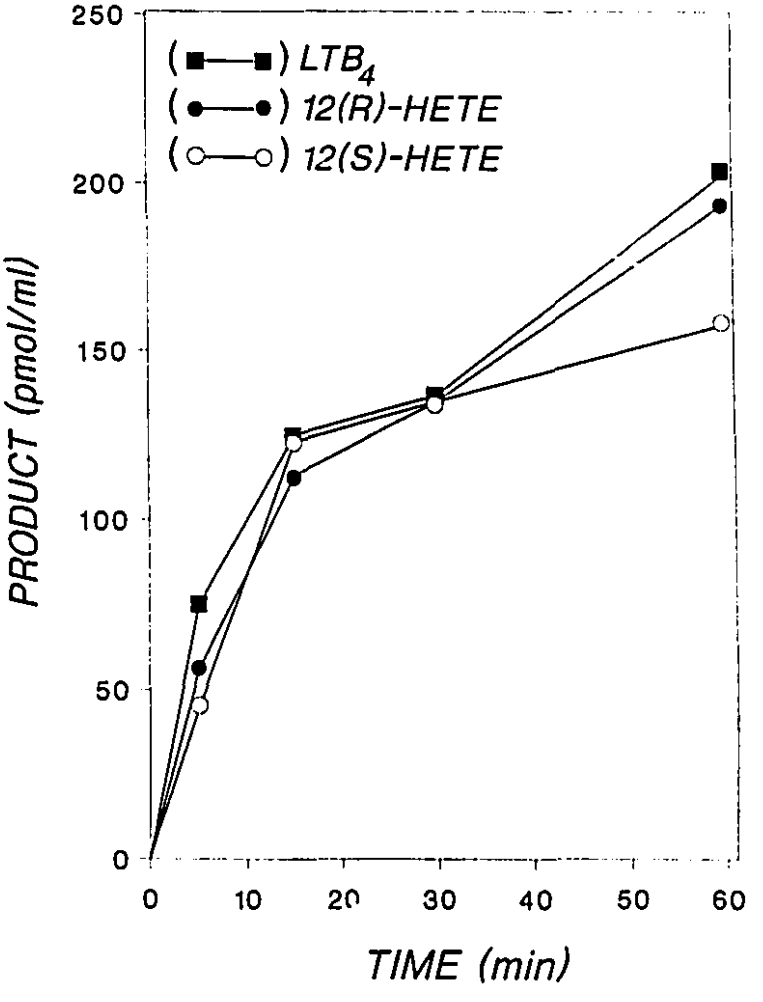

Figure 30. *Time courses for the metabolism of LTB_4, 12(S)-HETE and 12(R)-HETE by the microsomal fraction from porcine PMNL.* LTB_4 (1 μM), 12(S)-HETE (1 μM) and 12(R)-HETE (1 μM) were incubated with the microsomal fraction from porcine PMNL in the presence of 1 mM NAD^+. Products were analyzed by precolumn extraction/RP-HPLC. Products retained on the precolumn were resolved on a Novapak C_{18} column (Waters-Millipore) as described in Materials and Methods and quantitated on the basis of UV absorbance using PGB_2 (300 ng) as an internal standard.

incubated with porcine PMNL microsomes in the presence of NAD^+ for various times (Figure 30). The time courses for the formation of 12-oxo metabolites from all three substrates are quite similar, indicating that the orientation of the 12-hydroxyl group does not appear to affect the metabolism of the substrate by this enzyme. $PGF_{2\alpha}$, which is converted to its 15-oxo metabolite by the cytosolic enzyme 15-hydroxyprostaglandin dehydrogenase (320), was not converted to any detectable products by PMNL microsomes (data not shown).

Section IV

DISCUSSION

Prior to this study, our laboratory reported the presence of a pathway in porcine PMNL which catalyzes the metabolism of LTB_4 to 10,11-dihydro-LTB_4 and 10,11-dihydro-12-oxo-LTB_4 (304). Other eicosanoids, which have a C_{12}-hydroxyl group preceded by two conjugated double bonds and followed by a 2-*cis*-octenyl group, are also metabolized to dihydro products by porcine PMNL. 6-*Trans*-LTB_4, 12-epi-6-*trans*-LTB_4, 12-epi-8-*cis*-6-*trans*-LTB_4 and 12(S)-HETE all fall into this category and were found to be very good substrates for this pathway (Table VI). Based on the results of this study, a scheme for the metabolism of LTB_4 by porcine PMNL is proposed as shown in Figure 31. The individual reactions of this pathway are discussed in more detail below.

1. 12-Hydroxyeicosanoid dehydrogenase

The present study has clearly demonstrated that the initial step in the formation of 10,11-dihydro-LTB_4 metabolites by porcine PMNL is oxidation of the 12-hydroxyl group of LTB_4 by a microsomal 12-hydroxyeicosanoid dehydrogenase producing 12-oxo-LTB_4 (Figure 25). Oxidation of the 12-hydroxyl group also represents the rate-limiting step in this pathway, as a pronounced isotope effect was observed for the saturation of the Δ^{10} double bond when the substrate possessed a tritium atom at C_{12} (Figure 24).

The conversion of LTB_4 to 12-oxo-LTB_4 appears to be reversible since porcine PMNL microsomes converted 12-oxo-LTB_4 to a mixture of LTB_4 and 12-epi-LTB_4 in a ratio of 9:1 (Figure 28). It is not clear if the reduction of 12-oxo-LTB_4 to LTB_4 is

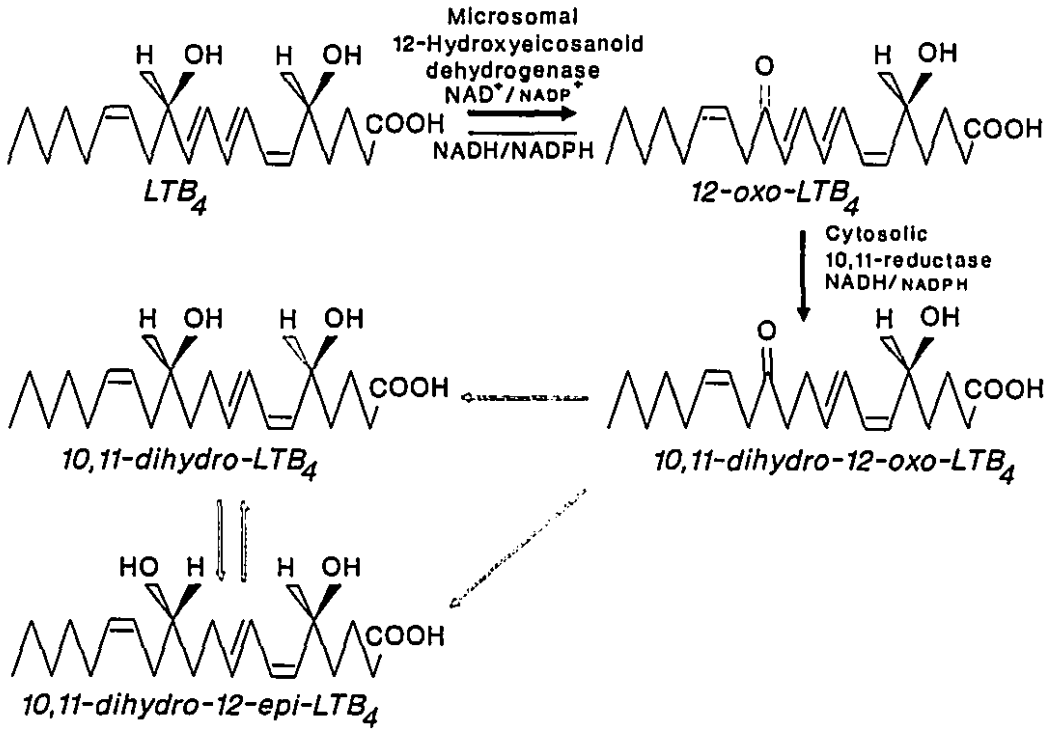

Figure 31. *Proposed scheme for the metabolism of LTB_4 by the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway.* Filled wedges represent bonds that project above the plane of the page, whereas open wedges represent bonds that project below the plane of the page. The solid arrows indicate enzyme reactions characterized in this study, whereas open arrows indicate putative enzyme reactions.

a result of the 12-hydroxyeicosanoid dehydrogenase acting in the reverse direction or if it is catalyzed by a separate ketoreductase present in porcine PMNL microsomes.

2. Previously described dehydrogenases

A number of dehydrogenases, some of which oxidize other hydroxyeicosanoids, have been reported in the literature. Some of the properties of these dehydrogenases are summarized below.

2.1. 15-Hydroxyprostaglandin dehydrogenase

The major route for the metabolism of prostaglandins of the E and F series is the 15-hydroxyprostaglandin dehydrogenase/Δ^{13}-reductase pathway (320). This pathway catalyzes the formation of 15-oxo, 13,14-dihydro-15-oxo and 13-14-dihydro-prostaglandins, analogous to the products formed by the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway described in this study. The first step in the formation of 13,14-dihydro-15-oxo-prostaglandins is oxidation of the C_{15} hydroxyl group to an oxo group by 15-hydroxyprostaglandin dehydrogenase (PGDH) (320). Two types of PGDH have been reported which can be distinguished from one another by their requirement for either NAD^+ (Type I) or $NADP^+$ (Type II) as a cofactor (324,325). Type I PGDH was found to be more specific for prostaglandins than Type II and therefore NAD^+-dependant PGDH is thought to be the key enzyme for inactivation of prostaglandins (326). PGDH activity has been found in many tissues, but is primarily localized in the cytosolic fraction from lung, liver (327) and placenta (328).

As well as oxidizing the ω6 hydroxyl group of prostaglandins, PGDH has also been reported to metabolize certain ω6 mono-hydroxy PUFA's. HHTrE (329),

which is a 17-carbon fatty acid with a C_{12} hydroxyl group, and 15-HETE (330) are metabolized by lung PGDH in the presence of NAD^+ to 12-oxo-5,8,10-heptadecatrienoic acid and 15-oxo-ETE respectively. PGDH does show some selectivity amongst $\omega 6$ hydroxylated fatty acids as 5,15-DiHETE, 8,15-DiHETE and 13-HODE are not good substrates for this enzyme (330).

PGDH does not appear to be responsible for the 12-hydroxyeicosanoid dehydrogenase activity of PMNL for two reasons. First, PGDH is a cytosolic enzyme, whereas 12-hydroxyeicosanoid dehydrogenase was found in the microsomal fraction from porcine PMNL. Secondly, neither intact PMNL nor PMNL microsomes were able to metabolize $PGF_{2\alpha}$ to any detectable products in the presence of NAD^+ (Table VI and section III.2.7.).

2.2. 13-Hydroxy-9,11-octadecadienoic acid dehydrogenase

More recently, Earles et al. have described a 13-hydroxy dehydrogenase present in rat colonic mucosa which catalyzes the conversion of 13-HODE to 13-oxo-ODE (331). The reaction catalyzed by this dehydrogenase is highly specific for NAD^+ and was found to be essentially irreversible. 13-HODE is metabolized by the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway in porcine PMNL to 11,12-dihydro and 11,12-dihydro-13-oxo metabolites, presumably through a 13-oxo-ODE intermediate (Figures 19 & 20). However, the cytosolic localization of the 13-HODE dehydrogenase in colonic mucosa suggests that it is distinct from 12-hydroxyeicosanoid dehydrogenase of porcine PMNL.

2.3. 15-Hydroxyeicosanoid dehydrogenase from mouse liver

15-Hydroxyeicosanoid dehydrogenase activity, distinct from PGDH, has been reported to be present in the microsomal fraction from mouse liver homogenates (332). This enzyme catalyzes the oxidation of 15(S)-HETE to 15-oxo-ETE and requires either NAD^+ or $NADP^+$ as a cofactor. 5,15-DiHETE and 8,15-DiHETE are also metabolized by mouse liver microsomes, but the metabolites were not identified. It is not known if 12-hydroxyeicosanoids are metabolized by this 15-hydroxyeicosanoid dehydrogenase. Therefore, further characterization of this liver enzyme is required to make a clear distinction between these two eicosanoid dehydrogenase activities.

2.4. Other dehydrogenases

Other mammalian dehydrogenases which are capable of oxidizing hydroxylated aliphatic compounds include alcohol dehydrogenases (ADH) and 3α-hydroxysteroid-dehydrogenase.

ADH activity is primarily localized in mammalian liver with lesser amounts found in intestine, kidney, and lung (333,334). There are three major classes of alcohol dehydrogenases distinguishable by physical and catalytic properties. ADH metabolizes primary, secondary and tertiary aliphatic alcohols in the presence of NAD^+ with a preference for unsaturated alcohols over their saturated analogues (335). Fatty acids are potent inhibitors of ADH (336), but it is not clear if long-chain hydroxylated fatty acids, such as the compounds used in this study, are substrates for

this enzyme. As ADH is found in the cytosolic compartment of cells, it is unlikely that this enzyme is responsible for the 12-hydroxyeicosanoid dehydrogenase activity present in porcine PMNL.

3α-Hydroxysteroid dehydrogenase is also a cytosolic enzyme found in mammalian liver tissue. This enzyme is indistinguishable from dihydrodiol-dehydrogenase (337). 3α-Hydroxysteroid dehydrogenase catalyzes the reversible conversion of alcohols to ketones in the presence of either NAD^+ or $NADP^+$ (337,338). The major oxidative activity attributed to 3α-hydroxysteroid dehydrogenase is the oxidation of 3α-hydroxysteroids. Other hydroxylated steroids, with hydroxyl groups in the 3ß, 11ß, 17α, 17ß, 20α, 20ß, 21 and 22 positions, are not appreciably metabolized (338). It is not clear if other hydroxylated aliphatic compounds could be metabolized by 3α-hydroxysteroid dehydrogenase, although arachidonic acid and various prostaglandins have been reported to inhibit its activity (337). The cytosolic localization of this enzyme distinguishes it from the 12-hydroxyeicosanoid dehydrogenase of porcine PMNL.

3. Identification of a cytosolic eicosanoid 10,11-reductase

In this study, we have identified cytosolic 10,11-reductase activity in porcine PMNL which catalyzes the metabolism of 12-oxo-LTB_4 to 10,11-dihydro-12-oxo-LTB_4 in the presence of either NADH or NADPH (Table X). It was previously thought that formation of 10,11-dihydro-LTB_4 by porcine PMNL was the result of direct reduction of the Δ^{10} double bond of LTB_4 by a 10,11-reductase. This

mechanism was hypothesized because intact porcine PMNL converted LTB_4 and 12(S)-HETE, both labeled with deuterium at C_{12}, to dihydro products in which the deuterium was retained to extents of 65% and 50% respectively (304,321). As oxidation of the 12-hydroxyl group to an oxo group would have resulted in the removal of the C_{12} deuterium atom, retention of this deuterium suggested that the Δ^{10} double bond of LTB_4 was reduced directly. However, the present study clearly showed that LTB_4 was not a substrate of the cytosolic 10,11-reductase. It would therefore appear that the partial retention of the C_{12} deuterium atom in the previous study was due to its transfer from C_{12} of the substrate to NAD^+ producing NAD^2H during the formation of the 12-oxo intermediate (Figure 32). In the subsequent reduction of the 10,11-dihydro-12-oxo intermediate to the 10,11-dihydro compound, the deuterium atom of NAD^2H could be returned to the C_{12} position, resulting in partial apparent retention of the C_{12} deuterium.

4. Previously described reductases

There are many examples of the formation of dihydro metabolites of olefinic compounds in the literature. In general, reduction of a carbon-carbon double bond can occur by two mechanisms. The first mechanism requires activation of the double bond prior to reduction. This may be accomplished by oxidation of an adjacent hydroxyl group to an oxo group by a dehydrogenase, followed by reduction of the olefinic double bond of the unsaturated keto intermediate by a reductase. The second mechanism is simply the direct reduction of the double bond by a reductase. The

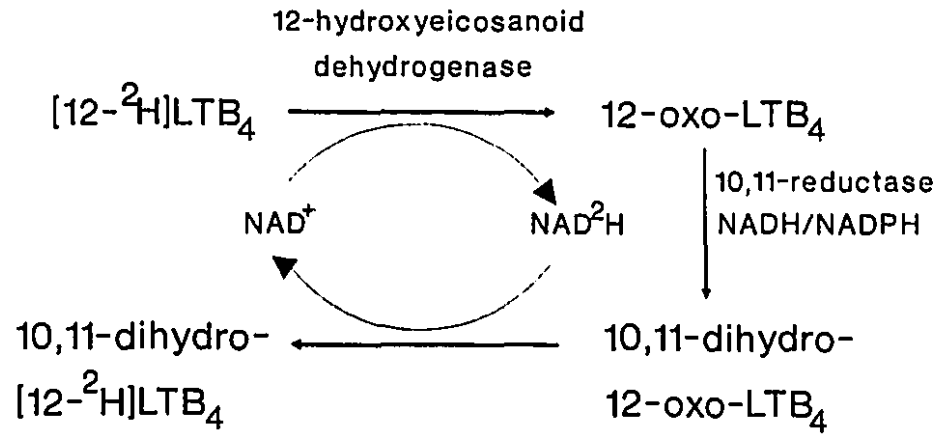

Figure 32. *Scheme for the metabolism of [12-^{2}H]LTB$_4$.*

present study clearly shows that reduction of the Δ^{10} double bond of LTB$_4$, catalyzed by the 10,11-reductase present in the cytosolic fraction from porcine PMNL, requires prior activation of the Δ^{10} double bond by its conjugation with a keto group.

4.1. Reductases that require prior activation of carbon-carbon double bonds

4.1.1. 15-oxoprostaglandin Δ^{13}-reductase

As mentioned above (section IV.2.1.), the major pathway for the metabolism of prostaglandins of the E and F series is the 15-hydroxyprostaglandin dehydrogenase/Δ^{13} reductase pathway. 15-Oxo-prostaglandins, formed by the action PGDH, are further metabolized by Δ^{13}-reductase to 13,14-dihydro-15-oxo metabolites. Reduction of the 13,14-double bond by the Δ^{13}-reductase requires the

presence of an oxo group in the 15-position and will not occur if the substrate has a hydroxyl group in this position (339).

4.1.3. 6,11-Reductase of human PMNL

Human PMNL contain a 6,11-reductase which requires prior oxidation of the substrate by a 5-hydroxyeicosanoid dehydrogenase (303). Human PMNL metabolize 6-*trans*-LTB_4 to 6,11-dihydro-LTB_4 and 6,11-dihydro-5-oxo-LTB_4. Metabolism of 12-epi-6-*trans*-LTB_4, labeled with a deuterium in the 5-position, resulted in the formation of a 6,11-dihydro metabolite which had lost the C_5 deuterium atom (303). This suggests that a 5-oxo intermediate is involved in the formation of this product and suggests that direct reduction of the substrate does not occur.

4.1.3. Other olefin reductases

There are several other examples of reductases which catalyze the reduction of double bonds that are in conjugation with an oxo group. 2-*Trans*-enoyl-CoA reductase, involved in fatty acid chain elongation, catalyzes the reduction of the Δ^2 double bond of 2-*trans*-enoyl-CoA, which is in conjugation with the carbonyl group of the thioester (340). 2,4,-Dienoyl-CoA reductase, which is involved in ß-oxidation of fatty acids, catalyzes the same reaction with 2-*trans*-4-*cis*-enoyl-CoA as the substrate (341). Steroid Δ^4 reductase also catalyzes the reduction of an activated double bond. Biologically active steroids such as progesterone, androgens, estrogens, glucocorticoids and mineralocorticoids all have a C_3-oxo group in conjugation with a

Δ^4 double bond. These Δ^4-3-ketosteroids may be further metabolized by steroid Δ^4-reductase producing 4,5-dihydro-3-keto metabolites (342).

4.2. Reductases that catalyze the direct reduction of carbon-carbon double bonds

There are fewer examples in the literature of direct reduction of carbon-carbon double bonds of olefinic compounds. However, the metabolism of sterols does provide two prominent examples of this phenomenon. 7-Dehydrocholesterol is converted to cholesterol by a Δ^7-reductase which catalyzes the direct reduction of the Δ^7 double bond (343). In a similar manner, Δ^{14}-reductase catalyzes the conversion of $\Delta^{8,14}$-sterols to Δ^8-sterols by the direct reduction of a double bond (344).

5-Enoyl-CoA reductase, which is involved in ß-oxidation of odd-numbered unsaturated fatty acids by rat liver, also provides an example of direct reduction of carbon-carbon double bonds (345). This enzyme catalyzes the direct reduction of the Δ^5 double bond of 5-*cis*-enoyl-CoA intermediates.

5. Possible mechanisms for the formation of 10,11-dihydro-LTB_4 from 10,11-dihydro-12-oxo-LTB_4

This study has clearly demonstrated that the first two steps in the formation of 10,11-dihydro eicosanoids are catalyzed by 12-hydroxyeicosanoid dehydrogenase and 10,11-reductase, respectively. In the case of LTB_4, this results in the formation of 10,11-dihydro-12-oxo-LTB_4. The mechanism for the subsequent formation of 10,11-

dihydro-LTB_4 and 10,11-dihydro-12-epi-LTB_4 is less clear. As discussed above, we have found no evidence for the formation of 10,11-dihydro-LTB_4 by the direct reduction of LTB_4 (section IV.3.). Furthermore, when LTB_4 was incubated with the 20,000 x *g* supernatant from porcine PMNL, only 10,11-dihydro-LTB_4 and not the corresponding 10,11-dihydro-12-epi isomer was detected (Table IX). These observations suggest the involvement of an enzyme which catalyzes the stereospecific reduction of 10,11-dihydro-12-oxo-LTB_4 to 10,11-dihydro-LTB_4. The subcellular locatization of this enzyme activity was not investigated further in this study. However, it was observed that metabolism of 12-oxo-LTB_4 by the 10,11-reductase in the cytosolic fraction of porcine PMNL produced 10,11-dihydro-12-oxo-LTB_4 but no 10,11-dihydro-LTB_4 was detected (Figure 28B). This suggests that the principle site of conversion of 10,11-dihydro-12-oxo-LTB_4 to 10,11-dihydro-LTB_4 may be the microsomal fraction. In agreement with this hypothesis, it was observed that the 12-oxo group of 12-oxo-LTB_4 is stereospecifically reduced to a 12(R)-hydroxyl group by the microsomal fraction in the presence of NADH and NADPH, producing LTB_4 (Figure 28A). Only a small amount of 12-epi-LTB_4 was formed. It is not clear if the microsomal enzyme catalyzing the conversion of 12-oxo-LTB_4 to LTB_4 is also responsible for the reduction of 10,11-dihydro-12-oxo-LTB_4 to 10,11-dihydro-LTB_4.

There are two possible mechanisms for the reduction of 10,11-dihydro-12-oxo-LTB_4 to 10,11-dihydro-LTB_4. First, a ketoreductase may be involved which stereospecifically reduces the C_{12} oxo group to a hydroxyl group. Secondly, 12-hydroxveicosanoid dehydrogenase, which catalyzes the conversion of LTB_4 to

12-oxo-LTB_4, may act in the reverse direction to convert 10,11-dihydro-12-oxo-LTB_4 to 10,11-dihydro-LTB_4.

5.1. Previously described ketoeicosanoid ketoreductases

There are several ketoreductases reported in the literature which catalyze the metabolism of ketoeicosanoids. Most, ketoreductases are located in the cytosolic compartment of cells and stereospecifically reduce carbonyl groups to hydroxyl groups. Human carbonyl reductase, which has been extensively studied, is a cytosolic enzyme that is widely distributed in human tissues (346). This enzyme is reported to non-specifically reduce carbonyl groups, including the carbonyl groups of 9-ketoprostaglandins. An 11-ketoreductase, which stereospecifically reduces PGD_2 to 9α,11ß-PGF_2 has been reported (347). This enzyme was found in the cytosolic fraction of human liver and requires NADPH for activity. Similarly, a 9-ketoreductase, which catalyzes the metabolism of PGE_2 to $PGF_{2\alpha}$, has been reported in rat, monkey and pigeon tissues (348). This enzyme activity was localized primarily in the cytosolic fraction of these tissues, although some activity was also found in microsomal fractions.

Two ketoreductases which catalyze the reduction of 12-oxo-ETE to 12-HETE have been reported. These ketoreductases are particularly relevant to this study as the catalytic activity of these enzymes is similar to that proposed for the reduction of 10,11-dihydro-12-oxo-LTB_4 to 10,11-dihydro-LTB_4. A microsomal fraction from rat liver has been reported to convert 12-oxo-ETE to a mixture of 12(S) and 12(R)-HETE

in a ratio of about 2:1 in the presence of either NADH or NADPH (349). As ketoreductases generally catalyze the stereospecific reduction of carbonyl groups, the formation of both 12(S) and 12(R)-HETE may be due to two separate ketoreductases. Rat peritoneal PMNL microsomes have been reported to specifically reduce 12-oxo-ETE to 12(S)-HETE in the presence of NADH (350). The stereospecific nature of this enzyme and its microsomal location in PMNL suggests that it may be similar to the enzyme which reduces 12-oxo eicosanoids in porcine PMNL.

5.2. Involvement of 12-hydroxyeicosanoid dehydrogenase

The second possible mechanism for the reduction of 10,11-dihydro-12-oxo-LTB_4 is the 12-hydroxyeicosanoid dehydrogenase, acting in the reverse direction. There are many examples in the literature of dehydrogenases which catalyze the reversible conversion of alcohols to carbonyls. We discussed earlier similarities between the PGDH/Δ^{13}-reductase pathway and the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase. Prostaglandins are converted to 15-oxo-prostaglandins and then to 13,14-dihydro-15-oxo-prostaglandins by the sequential activities of PGDH and Δ^{13}-reductase. However, 13,14-dihydro-15-oxo-prostaglandins may be metabolized further to 13,14-dihydro-prostaglandins (320). There is some evidence that this reaction is catalyzed by PGDH acting in reverse (351,320). The dihydro metabolites produced are more active than 15-oxo-prostaglandins, but are still less active than the parent prostaglandins (352). Other

dehydrogenases which catalyze the reversible oxidation of hydroxyl groups include malate dehydrogenase, lactate dehydrogenase, and isocitrate dehydrogenase (353).

The results of the deuterium-labeling experiment discussed in section IV.3. are consistent with the involvement of 12-hydroxyeicosanoid dehydrogenase in the conversion of 10,11-dihydro-12-oxo-LTB_4 to 10,11-dihydro-LTB_4. It was reported that metabolism of LTB_4, labeled with a deuterium at C_{12} produced 10,11-dihydro-LTB_4 which retained 65% of the deuterium label at C_{12} (304). This was presumably due to transfer of the C_{12} deuterium of LTB_4 to NAD^+ during the formation of 12-oxo-LTB_4 producing NAD^2H. The deuterium of NAD^2H could then be transferred back to C_{12} during the reduction of 10,11-dihydro-12-oxo-LTB_4 to 10,11-dihydro-LTB_4 (Figure 32). However, if NAD^2H were produced and liberated into the pool of free cofactors, one would expect that a deuterium from NAD^2H could also be incorporated at C_{10} or C_{11} during the reduction of 12-oxo-LTB_4 to 10,11-dihydro-12-oxo-LTB_4 by 10,11-reductase. No deuterium was observed at these carbons of 10,11-dihydro-LTB_4 (304), suggesting that NAD^2H was not available for the 10,11-reductase. This anomoly could be explained if 12-hydroxyeicosanoid dehydrogenase catalyzes both the oxidation of LTB_4 to 12-oxo-LTB_4 and the reduction of 10,11-dihydro-12-oxo-LTB_4 to 10,11-dihydro-LTB_4. The reversibility of this reaction could allow the cofactors to remain associated with the enzyme. This has been shown to be the case with malate dehydrogenase as illustrated below (Figure 33) (354). As is true for several dehydrogenases, malate dehydrogenase follows an ordered ternary complex mechanism in which NAD^+ binds to the enzyme prior to malate. Malate is

then converted to oxaloacetate which is released. The last step in this pathway, which is the rate-limiting step, is the release of NADH.

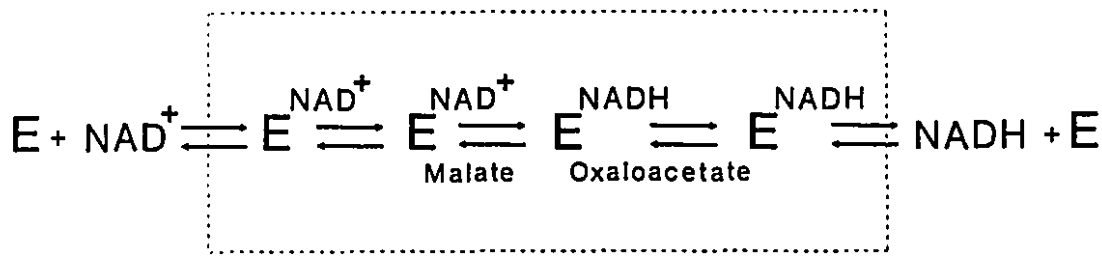

Figure 33. *Enzyme mechanism of malate dehydrogenase.*

As the concentration of malate and oxaloacetate increase, the rate of conversion of the binary complexes (E^{NAD} & E^{NADH}) to ternary complexes increases. The enzyme then becomes "trapped in the box" and neither NAD^+ nor NADH is released into the pool of free cofactors.

The same phenomenon could explain the retention of deuterium at C_{12} of 10,11-dihydro-LTB_4 (304). Conversion of [12-^{2}H]LTB_4 to 12-oxo-LTB_4 by 12-hydroxyeicosanoid dehydrogenase would result in the formation of NAD^2H which could remain associated with the enzyme. 12-Oxo-LTB_4 could then be reduced to 10,11-dihydro-12-oxo-LTB_4 by 10,11-reductase utilizing NADH from the pool of free cofactors. 10,11-Dihydro-12-oxo-LTB_4 could subsequently be reduced to 10,11-dihydro-LTB_4 by 12-hydroxyeicosanoid dehydrogenase which may still be associated with NAD^2H resulting in the transfer of the deuterium back to C_{12}.

Another explanation for the preferential retention of deuterium at C_{12} of 10,11-dihydro-LTB_4 and not at C_{10} or C_{11}, may lie in the distribution of cofactors with the cell. Pyridine nucleotide cofactors do not pass freely through cell and mitochondrial membranes (355). Therefore pyridine nucleotide cofactors within subcellular organelles would not be expected to mix readily with cytosolic cofactors. If 12-hydroxyeicosanoid dehydrogenase is located within the endoplasmic reticulum of porcine PMNL, NAD^2H, produced from the metabolism of [12-^{2}H]LTB_4 to 12-oxo-LTB_4, may not be available for a cytosolic enzyme such as 10,11-reductase. In this instance, sequestered NAD^2H would be available to either 12-hydroxyeicosanoid dehydrogenase or other ketoreductases present in the same subcellular organelle for the reduction of 10,11-dihydro-12-oxo-LTB_4 to 10,11-dihydro-LTB_4. However, it should be noted that we were unable to conclude from this study whether 12-hydroxyeicosanoid dehydrogenase is present in the endoplasmic reticulum or plasma membranes from porcine PMNL.

6. Possible mechanisms for the formation of 10,11-dihydro-12-epi-LTB_4

The mechanism for the formation of 10,11-dihydro-12-epi-LTB_4 by porcine PMNL is less clear. Although we detected substantial amounts of 10,11-dihydro-12-epi-LTB_4 after incubation of LTB_4 with intact porcine PMNL (Figure 8), we did not detect this compound after incubation of LTB_4 with any of the subcellular fractions investigated. The reason for this is not clear, but it may be related to the fact that 10,11-dihydro-12-epi-LTB_4 is formed much more slowly than 10,11-dihydro-

LTB_4 and the incubation times used in the present study may not have been long enough. There are two possible mechanisms for the formation of 10,11-dihydro-12-epi-LTB_4, involving either a ketoreductase or an epimerase.

6.1. Reduction of 10,11-dihydro-12-oxo-LTB_4 by a ketoreductase

One possible mechanism for the formation of 10,11-dihydro-12-epi-LTB_4 is that both 10,11-dihydro-LTB_4 and 10,11-dihydro-12-epi-LTB_4 are formed by the reduction of 10,11-dihydro-12-oxo-LTB_4 by the same enzyme, either 12-hydroxyeicosanoid dehydrogenase or a ketoreductase as discussed above (section IV.5.). However, this would seem unlikely for several reasons. First, studies with intact porcine PMNL indicate that 10,11-dihydro-LTB_4 is formed initially, whereas 10,11-dihydro-12-epi-LTB_4 is formed after a lag period (Figure 10A). Secondly, with the exception of racemases and epimerases, enzymatic reactions are generally stereospecific. One might therefore expect the 12-oxo group of 10,11-dihydro-12-oxo-LTB_4 to be reduced stereospecifically to either a 12(R) or a 12(S)-hydroxyl group.

It would seem much more likely that there are two separate enzymes, each specific for the formation of one of the isomers. The observed lag in the formation of 10,11-dihydro-12-epi-LTB_4 could then be attributed to a difference in the rates of reduction of 10,11-dihydro-12-oxo-LTB_4 by the two enzymes. However, under these conditions, one might expect that the stereochemistry at C_{12} of the initially formed isomer would be the same irrespective of the stereochemistry at C_{12} of the substrate. The results of this study show that this is not true for the metabolism of 12(S)-HETE

and LTB_4. The initially formed 10,11-dihydro metabolite of 12(S)-HETE has the R configuration at C_{12}, whereas the corresponding product formed from LTB_4 has the S configuration at C_{12} (Figures 10A & 18).

6.2. Epimerization of 10,11-dihydro-LTB_4

Another possible mechanism for the formation of 10,11-dihydro-12-epi-LTB_4 that must be considered is the epimerization of 10,11-dihydro-LTB_4. Eicosanoids can undergo chemical epimerization due to the loss of a hydroxyl group and the formation of an intermediate carbonium ion (356). However, the interconversion of 10,11-dihydro-LTB_4 and 10,11-dihydro-12-epi-LTB_4 by intact cells appears to be enzymatically catalyzed, since neither of these reactions occurs if the cell suspensions have previously been placed in a boiling water bath (section III.1.2.2.). Moreover, the formation of a carbonium ion due to the loss of the C_{12} hydroxyl group of 10,11-dihydro-LTB_4 is unlikely, since conversion of 10,11-dihydro-[12-^{3}H]LTB_4 to 10,11-dihydro-12-epi-LTB_4 results in loss of most of the tritium from C_{12} (Figure 11).

Racemases and epimerases generally act by removing a hydrogen atom from the chiral center of the substrate in the form of either H^+ or H^- (357). Removal and addition of a proton can be accomplished due to the presence of two basic groups at the active site of the enzyme, as in the case of proline racemase (358,359), or by a single basic residue as illustrated by mandelate racemase (360). When the chiral center possesses a hydroxyl group, as in nucleotide sugars, a hydride anion may be removed by a tightly bound cofactor (357). For example, NAD^+ is tightly bound at

the active site of UDP-D-glucose 4'-epimerase (361). Catalysis by this enzyme results in abstraction of a hydride anion from the substrate and formation of an oxo intermediate. The hydride anion is subsequently returned to the substrate during reduction of the oxo group producing a product with the opposite stereochemistry at the 4'-carbon (362). However, in some cases, as with D-ribulose-5-phosphate 3-epimerase, the abstracted hydride anion is exchanged with the medium rather than transferred back to the substrate (363).

From the present results it is not possible to conclude which of the above mechanisms is responsible for the production of 10,11-dihydro-12-epi-LTB_4 by the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway.

7. Reductase pathways present in other cells

During the course of this study, other groups reported the formation of dihydro metabolites of eicosanoids by other cells. The cell types involved and their metabolic activities are summarized in Table XI.

7.1. Conversion of LTB_4 to dihydro products by other cells

In 1987, Kaever et al. reported the metabolism of LTB_4 by rat mesangial and fibroblast cells, and mouse macrophages and T-lymphocytes (364). The UV spectrum of the main metabolite produced by these cells had a λ_{max} at 232 nm instead of at 271 nm, indicating the reduction of one of the conjugated double bonds of LTB_4. This metabolite was identified as a 5,12-dihydroxyeicosatrienoic acid (dihydro-LTB_4)

TABLE XI

Cells which have reported eicosanoid reductase activity

Cell Type	Substrate	Products	Reference
Rat			
-mesangial cells	LTB_4	dh-LTB_4[ab]	(364)
-fibroblasts	LTB_4	dh-LTB_4[b]	(364)
-PMNL	LTB_4	dh-LTB_4, dho-LTB_4, dh-12e-LTB_4	(291)
Mouse			
-macrophages	LTB_4	dh-LTB_4[b]	(364)
-T-lymphocytes	LTB_4	dh-LTB_4[b]	(364)
Human			
-mesangial cells	LTB_4	two dh-LTB_4 isomers[b]	(365)
-lung macrophages	LTB_4	dh-LTB_4[b]	(366)
-monocytes	LTB_4	two dihydro metabolites[b]	(367,368)
-chopped lung	LTB_4	dh-LTB_4, dho-LTB_4, dh-12e-LTB_4	(369)
-PMNL	6-*t*-LTB_4 12e-6*t*-LTB_4	6,11-dihydro-metabolites	(303)
Bovine			
-corneal microsomes	20:4	12(R)h-20:3	(313)

[a] Abbreviations: 6*t*-LTB_4, 6-*trans*-LTB_4; 12e-6*t*-LTB_4, 12-epi-6-*trans*-LTB_4; dh-LTB_4, 10,11-dihydro-LTB_4; dho-LTB_4, 10,11-dihydro-12-oxo-LTB_4; dh-12e-LTB_4, 10,11-dihydro-12-epi-LTB_4; 12(R)h-20:3, 12(R)-hydroxy-5,8,14-eicosatrienoic acid.

[b] Positions of remaining double bonds not confirmed.

TABLE XI

Cells which have reported eicosanoid reductase activity

Cell Type	Substrate	Products	Reference
Rat			
-mesangial cells	LTB_4	dh-LTB_4[ab]	(364)
-fibroblasts	LTB_4	dh-LTB_4[b]	(364)
-PMNL	LTB_4	dh-LTB_4, dho-LTB_4, dh-12e-LTB_4	(291)
Mouse			
-macrophages	LTB_4	dh-LTB_4[b]	(364)
-T-lymphocytes	LTB_4	dh-LTB_4[b]	(364)
Human			
-mesangial cells	LTB_4	two dh-LTB_4 isomers[b]	(365)
-lung macrophages	LTB_4	dh-LTB_4[b]	(366)
-monocytes	LTB_4	two dihydro metabolites[b]	(367,368)
-chopped lung	LTB_4	dh-LTB_4, dho-LTB_4, dh-12e-LTB_4	(369)
-PMNL	6-*t*-LTB_4 12e-6*t*-LTB_4	6,11-dihydro-metabolites	(303)
Bovine			
-corneal microsomes	20:4	12(R)h-20:3	(313)

[a] Abbreviations: 6*t*-LTB_4, 6-*trans*-LTB_4; 12e-6*t*-LTB_4, 12-epi-6-*trans*-LTB_4; dh-LTB_4, 10,11-dihydro-LTB_4; dho-LTB_4, 10,11-dihydro-12-oxo-LTB_4; dh-12e-LTB_4, 10,11-dihydro-12-epi-LTB_4; 12(R)h-20:3, 12(R)-hydroxy-5,8,14-eicosatrienoic acid.

[b] Positions of remaining double bonds not confirmed.

based on its UV absorbance spectrum, chromatographic properties and mass spectrum. Further metabolism of dihydro-LTB_4 by the same cells produced several polar metabolites which absorbed in the UV at 232 nm rather than 271 nm, but these products were not characterized further. In 1990, the same group reported that human mesangial cells also converted LTB_4 to dihydro-LTB_4 (365). Rechromatography of the dihydro-LTB_4 fraction under different RP-HPLC conditions resolved two dihydro-LTB_4 isomers which had identical mass spectra. The location of the remaining double bonds of the dihydro-LTB_4 metabolites described in these studies was not determined. However, it is likely that these products are identical to the 10,11-dihydro-LTB_4 isomers produced by porcine PMNL.

Metabolism of LTB_4 to dihydro-LTB_4 by human macrophages and monocytes has also been reported. In 1988, Schönfeld and coworkers reported the metabolism of LTB_4 to a less polar product by human lung macrophages (366). The major metabolite was indistinguishable from the dihydro-LTB_4 metabolite produced by rat fibroblasts, described above, under three different chromatographic conditions. A second less polar metabolite was reported which was only produced by macrophages, but this metabolite was not characterized further. Human monocytes were also reported to convert LTB_4 to two less polar dihydro metabolites (367,368). Both products have a maximal UV absorbance at 232 nm and have similar mass spectra. Again, the positions of the remaining double bonds of these dihydro-LTB_4 metabolites were not determined.

A more recent study has reported the metabolism of LTB_4 to dihydro metabolites by chopped human lung (369). Analysis of the LTB_4 metabolites by RP-HPLC resolved two less polar products. The first metabolite was identified by GC-MS, UV spectroscopy and co-chromatography with authentic standards as 10,11-dihydro-LTB_4. Rechromatography of the 10,11-dihydro-LTB_4 fraction by chiral-phase HPLC revealed the presence of both 10,11-dihydro-LTB_4 and 10,11-dihydro-12-epi-LTB_4. The second LTB_4 metabolite was tentatively identified as 10,11-dihydro-12-oxo-LTB_4 based on the similarity of its chromatographic properties with those of 10,11-dihydro-12-oxo-LTB_4 which was isolated from porcine PMNL in our laboratory (304).

7.2. The 5-hydroxyeicosanoid dehydrogenase/6,11-reductase pathway

As was discussed in the introduction, human PMNL metabolize 6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4 to 6,11-dihydro metabolites by a 5-hydroxyeicosanoid dehydrogenase/6,11-reductase pathway (303). The catalytic mechanism of this pathway is similar to the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway described in this study, involving an initial oxidation of a hydroxyl group followed by reduction of a conjugated double bond (303). However, there are several characteristics of the 5-hydroxyeicosanoid dehydrogenase/6,11-reductase pathway which distinguish it from the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway. First, the two pathways have different substrate specificities. The 5-hydroxyeicosanoid dehydrogenase/6,11-reductase pathway metabolizes compounds

with a 5-hydroxyl group followed by a Δ^6-*trans* double bond (303,370). Compounds which meet these requirements include 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4 and 12-epi-8-*cis*-6-*trans*-LTB_4. We have reported in this study that the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway preferentially metabolizes eicosanoids which have a 12-hydroxyl group preceeded by two conjugated double bonds and followed by a 2-*cis*-octenyl group. Substrates for this pathway include LTB_4, 12-HETE, 6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4 and 12-epi-8-*cis*-6-*trans*-LTB_4. Finally, the presence of these two pathways in PMNL from various species differs. The 5-hydroxyeicosanoid dehydrogenase/6,11-reductase pathway is found in human and porcine PMNL but is absent in rat PMNL, whereas the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway is found in porcine and rat PMNL, but is not present in human PMNL (301,370,291).

7.2.1. Formation of tetrahydro metabolites of 6-*trans* isomers of LTB_4

As mentioned above, the 5-hydroxyeicosanoid dehydrogenase/6,11-reductase pathway is also present in porcine PMNL. A recent study of the metabolism of 6-*trans*-LTB_4 by porcine PMNL revealed the production of several metabolites which were formed by a combination of the 5-hydroxyeicosanoid dehydrogenase/6,11-reductase pathway and the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway (370). 6-*Trans*-LTB_4 was metabolized to 10,11-dihydro and 10,11-dihydro-12-oxo products by the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway, and to 6,11-dihydro and 6,11-dihydro-5-oxo products by the 5-

hydroxyeicosanoid dehydrogenase/6,11-reductase pathway. In addition to these dihydro metabolites, porcine PMNL also converted 6-*trans*-LTB_4 to several metabolites which did not absorb UV light at either 235 nm or 280 nm, indicating the absence of any conjugated double bonds. These metabolites were identified as tetrahydro, 5-oxotetrahydro, and 12-oxotetrahydro metabolites of 6-*trans*-LTB_4, and were presumably formed by the sequential activities of the two pathways. The tetrahydro metabolites of 6-*trans*-LTB_4 have a single double bond between C_5 and C_{12}, but its location was not determined. 12-Epi-6-*trans*-LTB_4, and to a lesser extent 12-epi-8-*cis*-6-*trans*-LTB_4, were also metabolized to tetrahydro products by porcine PMNL.

7.3. Production of 12(R)-5,8,14-eicosatrienoic acid by bovine corneal microsomes

In 1988, Murphy et al. reported the metabolism of arachidonic acid to 12(R)-5,8,14-eicosatrienoic acid by bovine corneal microsomes involving a cytochrome-P-*450* dependent process (313). Two possible pathways for the formation of 12(R)-5,8,14-eicosatrienoic acid were hypothesized. First, cytochrome P-*450* could oxygenate arachidonic acid to 11,12-epoxy-5,8,14-eicosatrienoic acid, which could then undergo an epoxide rearrangement to 12-oxo-5,8,14-eicosatrienoic acid, followed by reduction to 12(R)-hydroxy-5,8,14-eicosatrienoic acid. Alternatively, 12(R)-HETE, formed directly from arachidonic acid by cytochrome P-*450*, could be converted to 12-oxo-5,8,10,14-eicosatetraenoic acid, followed by two stages of reduction; first to 12-oxo-5,8,14-eicosatrienoic acid and then to 12(R)-hydroxy-

5,8,14-eicosatrienoic acid. Both of these reaction pathways would require abstraction of a hydrogen atom from C_{12}, which is consistent with their observation of the formation of only heptadeuterated 12(R)-hydroxy-5,8,14-eicosatrienoic acid from octadeuterated [5,6,8,9,11,12,14,15-^{2}H]arachidonic acid.

The 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway described in this study could account for the conversion of 12(R)-HETE to 12(R)-5,8,14-eicosatrienoic acid by bovine corneal microsomes. We have shown that the rates of metabolism of 12(S)-HETE and 12(R)-HETE to 12-oxo-ETE by microsomal 12-hydroxyeicosanoid dehydrogenase are similar to one another (Figure 31) and that subsequent metabolism of 12-oxo-ETE will produce both 12(R)-hydroxy-5,8,14-eicosatrienoic acid and 12(S)-hydroxy-5,8,14-eicosatrienoic acid. It is not clear whether bovine corneal microsomes also produce 12(S)-hydroxy-5,8,14-eicosatrienoic acid from arachidonic acid. In the study of Murphy et al., the production of 12(R)-hydroxy-5,8,14-eicosatrienoic acid by corneal microsomes was established by comparing the biological activity of the dihydro product with those of authentic chemically-synthesized 12(R)-hydroxy-5,8,14-eicosatrienoic acid and 12(S)-hydroxy-5,8,14-eicosatrienoic acid. It is possible that 12(S)-hydroxy-5,8,14-eicosatrienoic acid was also formed by corneal microsomes, but it would not have been detected by the methods employed, since it is biologically inactive (313,371). As the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway would involve the formation of 12-oxo-ETE, this mechanism would also be consistent with the loss of a

deuterium atom during the formation of the dihydro product from deuterated arachidonic acid as discussed above.

8. Significance of the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway

The 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway may be regarded as either a metabolic pathway for the removal of biologically active eicosanoids or as a mechanism for the formation of biologically active compounds. The presence of this pathway in porcine PMNL, and the reported presence of similar reductase pathways in human monocytes and macrophage, places the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway in close proximity to eicosanoid-producing cells such as platelets, PMNL and the vascular endothelium. It is therefore possible that 12-hydroxyeicosanoids formed by one type of cell could be converted to dihydro metabolites by a different cell type by the phenomenon of transcellular metabolism, thus enhancing the potential significance of the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway *in vivo*.

8.1. Removal of biologically active compounds

Clearly one possible role for the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway of porcine PMNL is the metabolism of LTB_4 to less active products. As mentioned in the introduction, LTB_4 is a potent proinflammatory agent stimulating PMNL chemotaxis, chemokinesis, and adhesion to the vascular

endothelium (189,190,191). The 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway metabolizes LTB_4 to 12-oxo-LTB_4, 10,11-dihydro-12-oxo-LTB_4, 10,11-dihydro-LTB_4 and 10,11-dihydro-12-epi-LTB_4. Although the present study did not examine the biological activities of these metabolites, other groups have examined the proinflammatory effects of dihydro-LTB_4 produced by other cells. Dihydro-LTB_4 produced by rat mesangial cells was reported to have significantly less proinflammatory activity than LTB_4 when assayed for stimulation of human leukocyte chemotaxis, chemokinesis, aggregation, adhesion to endothelium and superoxide anion production (372). Similarly, the two dihydro-LTB_4 isomers produced by human mesangial cells exhibited only marginal chemotactic activity towards human leukocytes at the highest concentration tested (10^{-7} M) (365). Furthermore, 10,11-dihydro-LTB_4 and 10,11-dihydro-12-epi-LTB_4, produced by chopped human lung, were found to be approximately 100 times less potent than LTB_4 in stimulating contraction of guinea-pig lung parenchymal strips, as well as in increasing microvascular permeablility in the hamster cheek pouch (369). Therefore, the reported reduced biological activity of the dihydro-LTB_4 metabolites produced by these cells suggests a role for the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway in removing the potent proinflammatory effects of LTB_4. However, it should be noted that the biological activities of 10,11-dihydro-12-oxo-LTB_4 and 12-oxo-LTB_4 are not known and require further investigation.

8.2. Generation of biologically active compounds

The 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway also catalyzes the formation of biologically active compounds. 12(R)-Hydroxy-5,8,14-eicosatrienoic acid, which can be formed by the metabolism of either 12(S)-HETE or 12(R)-HETE, has the most significant biological activities of all the dihydro metabolites characterized to date. This compound has potent vasodilatory activity and was reported to be 3-4 fold more potent than acetylcholine in generating a vasodilatory response of an isolated rat tail artery preconstricted with phenylephrine (313). 12(R)-Hydroxy-5,8,14-eicosatrienoic acid has also been reported to have angiogenic activity (313), and to be a more potent chemotactic agent for human neutrophils than LTB_4 (371). In contrast to 12(R)-hydroxy-5,8,14-eicosatrienoic acid, 12(S)-hydroxy-5,8,14-eicosatrienoic acid has little proinflammatory activity.

We have also shown that 13-HODE is metabolized by the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway to 13-hydroxy-9-octadecenoic acid and 13-oxo-9-octadecenoic acid, presumably through a 13-oxo-ODE intermediate. 13-Oxo-ODE produced from 13-HODE by a cytosolic dehydrogenase in rat mucosa, has been shown to have mitogenic activity when administered intrarectally in rats (373).

8.3. Enhanced activity of the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway under conditions of acute inflammation

Irrespective of the role of the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway, there is evidence of enhanced metabolism of LTB_4 to 10,11-dihydro products by rat PMNL under conditions of acute inflammation. PMNL obtained from the pleural cavity of rats 4 hrs after injection of carageenan were found to have significantly enhanced production of 10,11-dihydro metabolites of LTB_4 compared to circulating PMNL (291). Similarly, a comparison of the metabolism of arachidonic acid by elicited and circulating rat PMNL, stimulated with the Ca^{+2} ionophore A23187, showed an 8-fold enhanced accumulation of 10,11-dihydro metabolites by elicited cells. This suggests that the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway may be important in conditions of acute inflammation.

8.4. Enzymatic racemization of 12-hydroxyeicosanoids

The stereochemistry of eicosanoids is usually critical for their biological activities. The biological response to LTB_4, for example, is quite specific for the 12(R) configuration. 12-Epi-LTB_4 is only about 5% as potent as LTB_4 as a chemotactic agent for PMNL and has a much higher K_d for the high affinity LTB_4 receptor on PMNL (196). Similarly, the lipoxygenase product 12(S)-HETE possesses considerably less biological activity than 12(R)-HETE. 12(R)-HETE stimulates chemotaxis of neutrophils and lymphocytes and has been implicated in the condition

of psoriasis (237,374). 12(R)-HETE has also been reported to inhibit Na^+,K^+-ATPase in bovine corneal microsomes, whereas 12(S)-HETE is relatively inactive (241). 5(S)-HETE, which is a product of 5-lipoxygenase, has been reported to stereospecifically increase intracellular Ca^{+2} in human PMNL whereas 5(R)-HETE is much less active in this respect (375).

We have shown that the microsomal fraction of porcine PMNL reduces the 12-oxo group of 12-oxo-LTB_4 stereospecifically to a 12(R)-hydroxy group, producing LTB_4, with only a small amount of 12-epi-LTB_4 being formed. We have also shown that 12(S)-HETE is metabolized to 12-oxo-ETE by the 12-hydroxyeicosanoid dehydrogenase. If the microsomal ketoreductase responsible for the conversion of 12-oxo-LTB_4 to LTB_4, expresses the same stereospecificity with 12-oxo-ETE, 12(R)-HETE would be produced. Therefore, depending on the stereospecificity of the ketoreductase, it is theoretically possible that 12(S)-HETE could be converted to 12(R)-HETE through a 12-oxo intermediate. However, we could not obtain any evidence for this interconversion in intact cells, possibly because the cytosolic 10,11-reductase, which showed a much higher activity than the microsomal ketoreductase, would readily convert 12-oxo-eicosanoids to 10,11-dihydro-12-oxo products.

SUMMARY AND CONCLUSIONS

The metabolism of LTB_4 and 12(S)-HETE by porcine PMNL was examined and their metabolites were identified. LTB_4 had previously been reported to be metabolized by porcine PMNL to 10,11-dihydro-LTB_4 and 10,11-dihydro-12-oxo-LTB_4 (301). Further analysis of the 10,11-dihydro-LTB_4 product by NP-HPLC resolved two isomers which were identified as 10,11-dihydro-LTB_4 and 10,11-dihydro-12-epi-LTB_4 by GC-MS and by co-chromatography with authentic standards. The metabolites of 12(S)-HETE produced by porcine PMNL were identified as 12-oxo-5,8,14-eicosatrienoic acid, 12(S)-hydroxy-5,8,14-eicosatrienoic acid and 12-(R)-hydroxy-5,8,14-eicosatrienoic acid by GC-MS and NMR spectroscopy. 12(R)-Hydroxy-5,8,14-eicosatrienoic acid and 12(S)-hydroxy-5,8,14-eicosatrienoic acid co-eluted when analyzed by RP, NP and chiral-phase chromatography. These two isomers were resolved by NP-HPLC following derivatization of their C_{12}-hydroxyl groups with MTPA.

The enzyme kinetics for the metabolism of LTB_4, 12(S)-HETE and 13(S)-HODE, determined by Lineweaver-Burk analysis, suggest that these compounds are metabolized by the same pathway. The apparent K_m values were: LTB_4 = 0.28 μM, 12(S)-HETE = 0.21 μM and 13(S)-HODE = 2.2 μM. All three substrates shared an apparent V_{max} of approximately 0.029 pmol/min/10^6 cells. We also found that LTB_4 could act as a competitive inhibitor of 12(S)-HETE metabolism. This result confirms that LTB_4 and 12(S)-HETE are metabolized by the same enzymes.

The time courses for the metabolism of LTB_4 and 12(S)-HETE are similar. Metabolism of both substrates produces 10,11-dihydro and 10,11-dihydro-12-oxo compounds initially, whereas the formation of 10,11-dihydro-12-epi metabolites follows a lag phase. This suggests that the inverted stereochemistry at C_{12} of 10,11-dihydro-12-epi metabolites is a result of further metabolism of either 10,11-dihydro or 10,11-dihydro-12-oxo products.

The specificity of the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway was examined. It was found that porcine PMNL, preferentially metabolize eicosanoids which have a C_{12}-hydroxyl group preceded by two conjugated double bonds and followed by a 2-*cis*-octenyl group. LTB_4 was the best substrate tested. 6-*Trans*-LTB_4, 12-epi-8-*cis*-6-*trans*-LTB_4, 12-epi-6-*trans*-LTB_4 and 12(S)-HETE were also good substrates for this pathway. Other compounds were metabolized less efficiently in the order of LTB_5 > 15(S)-HETE = 13(S)-HODE > 5(S)-HETE > 9(S)-HODE > 20-hydroxy-LTB_4 > HHTrE.

The mechanism for the formation of dihydro metabolites of LTB_4 was derived from the results of experiments performed with different subcellular fractions from porcine PMNL in the presence of various cofactors. The first step in the metabolism of LTB_4 by porcine PMNL is oxidation of LTB_4 to 12-oxo-LTB_4, catalyzed by a microsomal 12-hydroxyeicosanoid dehydrogenase in the presence of NAD^+ and to a lesser extent $NADP^+$. 12-Oxo-LTB_4 was identified based on its mass spectral, UV and chromatographic properties. Metabolism of substrate labeled with a tritium atom

at C_{12} showed a marked isotope effect, indicating that the initial step, catalyzed by 12-hydroxyeicosanoid dehydrogenase, is rate-limiting.

The stereochemistry of the C_{12}-hydroxyl group of the substrate does not appear to affect metabolism of that substrate by 12-hydroxyeicosanoid dehydrogenase present in porcine PMNL microsomes. Incubation of LTB_4, 12(S)-HETE and 12(R)-HETE with the microsomal fraction, in the presence of NAD^+, produced similar time courses for the formation of their respective 12-oxo-metabolites.

The second step in the metabolism of LTB_4 by porcine PMNL is reduction of 12-oxo-LTB_4 to 10,11-dihydro-12-oxo-LTB_4, which is catalyzed by a cytosolic 10,11-reductase in the presence of either NADH or NADPH. Incubation of LTB_4 with the cytosolic fraction, in the presence of either NADH or NADPH, did not produce any detectable metabolites, indicating that the 10,11-reductase cannot reduce the Δ^{10} double bond of LTB_4 directly.

The pathway for the formation of dihydro metabolites of LTB_4 and 12-HETE in porcine PMNL is clearly distinct from other hydroxyeicosanoid dehydrogenase/reductase pathways, including the 5-hydroxyeicosanoid dehydrogenase/6,11-reductase pathway, which is also present in porcine PMNL, and the 15-hydroxyprostaglandin dehydrogenase/Δ^{13}-reductase pathway. The latter pathway results in the conversion of prostaglandins to 15-oxo, 13,14-dihydro-15-oxo, and 13-14-dihydro metabolilte (317). However, PGE_2 and $PGF_{2\alpha}$ were not metabolized either by intact porcine PMNL or by microsomal fractions from these cells in the presence of NAD^+.

The 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway has been reported in several cell types (Table XI), however the significance of this pathway is not clear. The presence of this pathway in porcine PMNL and human monocytes is consistent with its potential to regulate local concentrations of the proinflammatory agent LTB_4. However, the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway may also play an important role in generating biologically active eicosanoids such as the formation of 12(R)-hydroxy-5,8,14-eicosatrienoic acid from 12(S)-HETE. Clearly future investigations of the biological activities of the metabolites of this pathway will be important to clarify the role the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway *in vivo*.

CLAIMS TO ORIGINAL RESEARCH

1. 10,11-Dihydro-LTB_4, produced from the metabolism of LTB_4 by porcine PMNL, was resolved into two components by NP-HPLC. These compounds were identified as 5(S),12(S)-dihydroxy-6,8,14-eicosatrienoic acid (10,11-dihydro-LTB_4) and 5(S),12(R)-dihydroxy-6,8,14-eicosatrienoic acid (10,11-dihydro-12-epi-LTB_4) by GC-MS and by comparison of their chromatographic properties with those of authentic standards. A time course of the metabolism of LTB_4 by porcine PMNL showed that 10,11-dihydro-12-epi-LTB_4 was formed after a lag phase, presumably due to the subsequent metabolism of 10,11-dihydro-LTB_4 or 10,11-dihydro-12-oxo-LTB_4, which were formed earlier.

2. 10,11-Dihydro-LTB_4 was converted to 10,11-dihydro-12-epi-LTB_4 and 10,11-dihydro-12-oxo-LTB_4 by porcine PMNL, with the latter product predominating. 10,11-Dihydro-12-epi-LTB_4 was converted to 10,11-dihydro-LTB_4 and 10,11-dihydro-12-oxo-LTB_4 by porcine PMNL in a similar manner, indicating that the two 10,11-dihydro stereoisomers of LTB_4 are interconvertible. Incubation of 10,11-dihydro-12-oxo-LTB_4 with porcine PMNL produced 10,11-dihydro-LTB_4 and 10,11-dihydro-12-epi-LTB_4 in comparable amounts.

3. Porcine PMNL metabolize 12(S)-HETE to 12-hydroxy-5,8,14-eicosatrienoic acid and 12-oxo-5,8,14-eicosatrienoic acid, identified by GC-MS and NMR

spectroscopy. Stereochemical analysis of the MTPA derivatives of 12-hydroxy-5,8,14-eicosatrienoic acid revealed the presence of both 12(S)-hydroxy-5,8,14-eicosatrienoic acid and 12(R)-hydroxy-5,8,14-eicosatrienoic acid isomers. A time course for the metabolism of 12(S)-HETE by porcine PMNL showed that 12(S)-hydroxy-5,8,14-eicosatrienoic acid is formed after a lag period, presumably due to the further metabolism of 12(R)-hydroxy-5,8,14-eicosatrienoic acid or 12-oxo-5,8,14-eicosatrienoic acid, which were formed earlier.

4. Incubation of [1-^{14}C]-labeled monohydroxy-PUFA's, including 12-HETE, 15-HETE, 5-HETE and 13-HODE, resulted in a marked reduction in the total amount of radioactivity recovered, presumably a consequence of ß-oxidation. The loss of radioactivity was reduced by the addition of 5 μM ETYA, which did not affect the formation of dihydro and dihydrooxo products.

5. Porcine PMNL metabolize 13(S)-HODE primarily by ß-oxidation. The major metabolites were identified as 9-hydroxy-7,9-hexadecadienoic acid and 7-hydroxy-5,7-tetradecadienoic acid by GC-MS. In the presence of 5 μM ETYA, porcine PMNL metabolized 13(S)-HODE to 13-hydroxy-9-octadecaenoic acid and 13-oxo-9-octadecaenoic acid, identified by GC-MS.

6. The preferred substrate for the 12-hydroxyeicosanoid dehydrogenase/10,11-reductase pathway in porcine PMNL are compounds with a 12-hydroxyl group

preceded by at least two conjugated double bonds and followed by a 2-cis-octenyl group. Structurally related compounds were metabolized in the order LTB_4 = 6-*trans*-LTB_4 > 12-epi-6-*trans*-8-*cis*-LTB_4 > 12-epi-6-*trans*-LTB_4 > 12(S)-HETE > LTB_5 > 15(S)-HETE = 13(S)-HODE >> 5(S)-HETE > 9(S)-HODE > 20-hydroxy-LTB_4 > 12-hydroxy-5,8,10-heptadecatrienoic acid. Prostaglandins E_2 and $F_{2\alpha}$ were not metabolized to any detectable products by these cells.

7. Porcine PMNL metabolized LTB_4, 12(S)-HETE, and 13-HODE to reduced products with apparent K_m values of 0.21, 0.28, and 2.22 μM, respectively as determined from Lineweaver-Burk plots. All three substrates had the same apparent V_{max} of 0.029 pmol min^{-1} $(10^6$ cells$)^{-1}$. Lineweaver-Burk analysis of the data from competition experiments revealed that 12-HETE competetively inhibited the metabolism of LTB_4 and vice versa.

8. The initial step in the formation of 10,11-dihydro metabolites of LTB_4 and 12(S)-HETE was found to be oxidation of the 12-hydroxyl group. This was supported by two lines of experiments: 1) Metabolism of LTB_4 by a post-nuclear supernatant fraction from porcine PMNL required the presence of NAD^+ and 2) A marked isotope effect was observed for the metabolism of 12(S)-HETE labeled with a tritium in the 12-position, suggesting that the initial oxidation of the 12-hydroxyl group is rate-limiting.

9. The 12-hydroxyeicosanoid was found to be located in the microsomal fraction from porcine PMNL, and to require NAD^+ as a cofactor. Porcine PMNL microsomes converted LTB_4 and 12(S)-HETE to 12-oxo-LTB_4 and 12-oxo-ETE, respectively. 12-oxo-LTB_4 was identified by its UV spectral, chromatographic and mass spectral properties, whereas 12-oxo-ETE was identified based on its UV spectral and chromatographic properties. The time courses for the formation of 12-oxo metabolites of LTB_4, 12(S)-HETE and 12(R)-HETE were similar, suggesting the orientation of the 12-hydroxyl group of the substrate does not affect metabolism by this fraction.

10. A cytosolic fraction from porcine PMNL converted 12-oxo-LTB_4 to 10,11-dihydro-12-oxo-LTB_4 in the presence of either NADH or NADPH. LTB_4 was not converted to any detectable products by this fraction, confirming that oxidation of the C_{12}-hydroxyl group of LTB_4 is required prior to reduction of the Δ^{10} double bond.

11 A microsomal fraction from porcine PMNL converted 12-oxo-LTB_4 to 10,11-dihydro-12-oxo-LTB_4 and a mixture of LTB_4 and 12-epi-LTB_4 in a ratio of 9:1 in the presence of either NADH or NADPH. Formation of LTB_4/12-epi-LTB_4 was favored by NADH, whereas the formation of 10,11-dihydro-12-oxo-LTB_4 was greater with NADPH.

12. A facilitated procedure for the preparation of MTPA derivatives of monohydroxy PUFA's was developed. 1,3-Dicyclohexylcarbodiimide was used to

couple MTPA to the hydroxyl group of monohydroxy PUFA's in the presence of 4-(dimethylamino)pyridine and CCl_4. Conversion was virtually quantitative. Separation of the MTPA deri atives from contaminants derived from the reagents was accomplished by RP-HPLC.

Section VII

REFERENCES

1 Kurzrok, R., and Lieb, C.C. (1930) *Proc. Soc. Exp. Biol. Med.* **28**, 268-272

2 Goldblatt, M.W. (1935) *J. Physiol.* **84**, 208-218

3 von Euler, U.S. (1936) *J. Physiol.* **88**, 213-234

4 Bergström, S. (1949) *Prostaglandinets Kemi. Nord. Med.* **42**, 1465-1466

5 Bergström, S., Dressler, F., Ryhage, R., Samuelsson, B., and Sjövall, J. (1962) *Arkiv Kem.* **19**, 563-567

6 Bergström, S., Dressler, F., Krabisch, L., Ryhage, R., and Sjövall, J. (1962) *Arkiv Kem.* **20**, 63-66

7 Bergström, S. (1967) In: *Prostaglandins, Proc. 2nd Nobel Symp. Stockholm, June 1966* (Bergström, S. and Samuelsson, B., eds.) Interscience Publishers, New York, pp 21-30

8 Bergström, S., Danielsson, H., and Samuelsson, B. (1964) *Biochim. Biophys. Acta* **90**, 207-210

9 Van Dorp, D.A., Beerthuis, R.K., Nugteren, D.H., and Vonkeman, H. (1964) *Biochim. Biophys. Acta* **90**, 204-207

10 Christ, E.J., and van Dorp, D.A. (1972) *Biochim. Biophys. Acta* **270**, 537-545

11 Miyamoto, T., Ogino, N., Yamamoto, S., and Hayaishi, O., (1976) *J. Biol. Chem.* **251**, 2629-2636

12 Porter, N.A. (1980) In: *Free Radicals in Biology Vol. IV* (Pryor, W.A., ed.) Academic Press, New York, pp 261-294

13 Hamberg, M., and Samuelsson, B. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 899-903

14 Hamberg, M., Svensson, J., Wakabayashi, T., and Samuelsson, B. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 345-349

15 Marshall, P.J., and Kulmacz, R.J. (1988) *Arch. Biochem. Biophys.* **266**, 162-170

16 Yamamoto, S. (1982) *Meth. Enz.* **86**, 55-60

17 Needleman, P., Raz, A., Minkes, M.S., Ferrendelli, J.A., and Sprecher, H. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 944-948

18 Hecker, M., Ullrich, V., Fischer, C., and Meese, C.O. (1987) *Eur. J. Biochem.* **169**, 113-123

19 Hamberg, M., and Samuelsson, B. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3400-3404

20 Hamberg, M., Svensson, J., Samuelsson, B. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2994-2998

21 Shripad, S., Bhagwat, P.R., Hamann, W., Clark, S., Bunting, S., and Fitzpatrick, F.A. (1985) *Nature* **315**, 511-513

22 Ullrich, V., and Nüsing, R. (1990) *Stroke* **21**, IV134-IV138

23 Svensson, J., Hamberg, M., and Samuelsson, B. (1976) *Acta Physiol. Scand.* **98**, 285-294

24 Roberts II, L.J., Sweetman, B.J., and Oates, J.A. (1981) *J. Biol. Chem.* **256**, 8384-8393

25 Westlund, P., Kumlin, M., Nordenström, A, and Granström, E. (1986) *Prostaglandins* **31**, 413-443

26 Hamberg, M., Svensson, J., and Samuelsson, B. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3824-3828

27 Hecker, M., and Ullrich, V. (1989) *J. Biol. Chem.* **264**, 141-150

28 Moncada, S., Gryglewski, R., Bunting, S., and Vane, J.R. (1976) *Nature* **263**, 663-665

29 Johnson, R.A., Morton, D.R., Kinner, J.H., Gorman, R.R., McGuire, J.C., Sun, F.F., Whittaker, N., Bunting, S., Salmon, J., Moncada, S., and Vane, J.R. (1976) *Prostaglandins* **12**, 915-928

30 Armstrong, J.M., Lattimer, N., Moncada, S., and Vane, J.R. (1978) *Br. J. Pharmacol.* **62**, 125-130

31 Higgs, E.A., Higgs, G.A., Moncada, S., and Vane, J.R. (1978) *Br. J. Pharmacol.* **63**, 535-539

32 Gryglewski, R.J., Bunting, S., Moncada, S., Flower, R.J., and Vane, J.R. (1976) *Prostaglandins* **12**, 685-713

33 Pace-Asciak, C. (1976) *J. Am. Chem. Soc.* **98**, 2348-2349

34 Vane, J.R. (1971) *Nature New Biol.* **231**, 232-235

35 Smith, J.B., and Willis, A.L. (1971) *Nature New Biol.* **231**, 235-237

36 Ferreira, S.H., Moncada, S., and Vane, J.R. (1971) *Nature New Biol.* **231**, 237-239

37 Roth, G.J., Machuga, E.T., and Ozols, J. (1983) *Biochemistry* **22**, 4672-4675

38 Rome, L.H., Lands, W.E.M., Roth, G.J., and Majerus, P.W. (1976) *Prostaglandins* **11**, 23-30

39 Smith, W.L., DeWitt, D.L., Shimokawa, T., Kraemer, S.A., and Meade, E.A. (1990) *Stroke* **21**, IV24-IV28

40 Goetz, J.M., Sprecher, H., Cornwell, D.G., Panganamala, R.V. (1976) *Prostaglandins* **12**, 187-192

41 Tobias, L.D., and Hamilton, J.G. (1979) *Lipids* **14**, 181-193

42 Hammarström, S. (1977) *Biochim. Biophys. Acta* **487**, 517-519

43 Borgeat, P., Hamberg, M., and Samuelsson, B. (1976) *J. Biol. Chem.* **251**, 7816-7820

44 Panossian, A., Hamberg, M., and Samuelsson, B. (1982) *FEBS Lett.* **150**, 511-513

45 Borgeat, P., and Samuelsson, B. (1979) *J. Biol. Chem.* **254**, 2643-2646

46 Borgeat, P., and Samuelsson, B. (1979) *J. Biol. Chem.* **254**, 7865-7869

47 Borgeat, P., and Samuelsson, B. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3213-3217

48 Rådmark, O., Malmsten, C., Samuelsson, B., Clark, D.A., Goto, G., Marfat A., and Corey, E.J. (1980) *Biochem. Biophys. Res. Commun.* **92**, 954-961

49 Rouzer, C.A., Matsumoto, T., and Samuelsson, B. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 857-861

50 Rådmark, O., Shimizu, T., Jörnvall, H., and Samuelsson, B. (1984) *J. Biol. Chem.* **259**, 12339-12345

51 Evans, J.F., Dupuis, P., and Ford-Hutchinson, A.W. (1985) *Biochim. Biophys. Acta* **840**, 43-50

52 McGee, J., and Fitzpatrick, F. (1985) *J. Biol. Chem.* **260**, 12832-12837

53 Medina, J.F., Haeggström, J., Wetterholm, A., Wallin, A., and Rådmark, O. (1987) *Biochem. Biophys. Res. Commun.* **143**, 697-703

54 Haeggström, J., Rådmark, O., and Fitzpatrick, F.A. (1985) *Biochim. Biophys. Acta* **835**, 378-384

55 Fitzpatrick, F., Haeggström, J., Granström, E., and Samuelsson, B. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5425-5429

56 Izumi, T., Shimizu, T., Seyama, Y., Ohishi, N., and Takaku, F. (1986) *Biochem. Biophys. Res. Commun.* **135**, 139-145

57 Orning, L., Jones, D.A., and Fitzpatrick, F.A. (1990) *J. Biol. Chem.* **265**, 14911-14916

58 Ohishi, N., Izumi, T., Minami, M., Kitamura, S., Seyama, Y., Ohkawa, S., Terao, S., Yotsumoto, H., Takaku, F., and Shimizu, T. (1987) *J. Biol. Chem.* **262**, 10200-10205

59 Nathaniel, D.J., Evans, J.F., Leblanc, Y., Léveillé, C., Fitzsimmons, B.J., and Ford-Hutchinson, A.W. (1985) *Biochem. Biophys. Res. Commun.* **131**, 827-835

60 Evans, J.F., Nathaniel, D.J., Zamboni, R.J., and Ford-Hutchinson, A.W. (1985) *J. Biol. Chem.* **260**, 10966-10970

61 Funk, C.D., Rådmark, O., Fu, J.Y., Matsuomo, T., Jörnvall, H., Shimizu, T., and Samuelsson, B. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6677-6681

62 Minami, M., Minami, Y., Ohno, S., Suzuki, K., Ohishi, N., Shimizo, T., and Seyama, Y. (1989) *Adv. Prostaglandin Thromboxane Leukotriene Res.* **19**, 478-483

63 Malfroy, B., Kado-Fong, H., Gros, C., Giros, B., Schwartz, J.C., and Hellmis, R. (1989) *Biochem. Biophys. Res. Commun.* **161**, 236-241

64 Jongeneel, C.V., Bouvier, J., and Bairoch, A. (1989) *FEBS Lett.* **242**, 211-214

65 Orning, L., Krivi, G., and Fitzpatrick, F.A. (1991) *J. Biol. Chem.* **266**, 1375-1378

66 Naccache, P.H., Molski, T.F.P., Becker, E.L., Borgeat, P., Picard, S., Vallerand, P., and Sha'afi, R.I. (1982) *J. Biol. Chem.* **257**, 8608-8611

67 Borgeat, P., and Naccache, P.H. (1990) *Clin. Biochem.* **23**, 459-468

68 Ford-Hutchinson, A.W., Bray, M.A., Cunningham, F.M., Davidson, E.M., and Smith, M.J.H. (1981) *Prostaglandins* **21**, 143-152

69 Feldberg, W., and Kellaway, C.H. (1938) *J. Physiol.* **94**, 187-226

70 Kellaway, C.H., and Trethewie, E.R. (1940) *J. Exp. Physiol.* **30**, 121-145

71 Brocklehurst, W.E. (1960) *J. Physiol.* **151**, 416-435

72 Dahlén, S.-E., Hansson, G., Hedqvist, P., Bjorck, T., Granström, E., and Dahlén, B. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 1712-1716

73 Weiss, J.W., Drazen, J.M., Coles, N., McFadden Jr., E.R., Weller, P.F., Corey, E.J., Lewis, R.A., and Austen, K.F. (1982) *Science* **216**, 196-198

74 Orange, R.P., Murphy, R.C., Karnovsky, M.L., and Austen, K.F. (1973) *J. Immunol.* **110**, 760-770

75 Murphy, R.C., Hammarström, S., and Samuelsson, B. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4275-4279

76 Samuelsson, B., Hammarström, S., Hamberg, M., and Serhan, C.N. (1985) *Adv. Prostaglandin Thromboxane Leukotriene Res.* **14**, 45-71

77 Morris, H.R., Taylor, G.W., Piper, P.J., and Tippins, J.R. (1980) *Nature* **285**, 104-106

78 Bernström, K., and Hammarström, S. (1981) *J. Biol. Chem.* **256**, 9579-9582

79 Anderson, M.E., Allison, R.D., and Meister, A. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1088-1091

80 Hammarström, S., and Samuelsson, B. (1980) *FEBS Lett.* **122**, 83-86

81 Verhagen, J., Bruynzeel, P.L.B., Koedam, J.A., Wassink, G.A., de Boer, M., Terpstra, G.K., Kreukniet, J., Veldink, G.A., and Vliegenthart, J.F.G. (1984) *FEBS Lett.* **168**, 23-28

82 Warner, J.A., Freeland, H.S., MacGlashan Jr., D.W., Lichtenstein, L.M., and Peters, S.P. (1987) *Biochem. Pharmacol.* **36**, 3195-3199

83 Samuelsson, B., Borgeat, P., Hammarström, S., and Murphy, R.C. (1979) *Prostaglandins* **17**, 785-787

84 Rouzer, C.A., and Samuelsson, B. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6040-6044

85 Percival, M.D. (1991) *J. Biol. Chem.* **266**, 10058-10061

86 Rouzer, C.A., and Kargman, S. (1988) *J. Biol. Chem.* **263**, 10980-10988

87 Miller, D.K., Gillard, J.W., Vickers, P.J., Sadowski, S., Léveillé, C., Mancini, J.A., Charleson, P., Dixon, R.A.F., Ford-Hutchinson, A.W., Fortin, R., Gauthier, J.Y., Rodkey, J., Rosen, R., Rouzer, C., Sigal, I.S., Strader, C.D., and Evans, J.F. (1990) *Nature* **343**, 278-281

88 Dixon, R.A.F., Jones, R.E., Diehl, R.E., Bennett, C.D., Kargman, S., and Rouzer, C.A. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 416-420

89 Matsumoto, T., Funk, C.D., Rådmark, O., Höög, J,-O., Jörnvall, H., and Samuelsson, B. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 26-30

90 Dixon, R.A.F., Diehl, R.E., Opas, E., Rands, E., Vickers, P.J., Evans, J.F., Gillard, J.W., and Miller, D.K. (1990) *Nature* **343**, 282-284

91 Hoshiko, S., Rådmark, O., and Samuelsson, B. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9073-9077

92 Kennedy, B.P., Diehl, R.E., Boie, Y., Adam, M., and Dixon, R.A.F. (1991) *J. Biol. Chem.* **266**, 8511-8516

93 Reid, G.K., Kargman, S., Vickers, P.J., Mancini, J.A., Léveillé, C., Ethier, D., Miller, D.K., Gillard, J.W., Dixon, R.A.F., and Evans, J.F. (1990) *J. Biol. Chem.* **265**, 19818-19823

94 Nugteren, D.H. (1975) *Biochim. Biophys. Acta* **386**, 299-307

95 Spector, A.A., Gordon, J.A., and Moore, S.A. (1988) *Prog. Lipid. Res.* **27**, 271-323

96 Yoshimoto, T., Miyamoto, Y., Ochi, K., and Yamamoto, S. (1982) *Biochim. Biophys. Acta* **713**, 638-646

97 Hada, T., Ueda, N., Takahashi, Y., Yamamoto, S. (1991) *Biochim. Biophys. Acta* **1083**, 89-93

98 Yoshimoto, T., Suzuki, H., Yamamoto, S., Takai, T., Yokoyama, C., and Tanabe, T. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2142-2146

99 Funk, C.D., Furci, L., FitzGerald, G.A. (1990) *Proc. Natl. Acad. Sci U.S.A.* **87**, 5638-5642

100 Jones, R.L., Kerry, P.J., Poyser, N.L., Walker, I.C., and Wilson, N.H. (1978) *Prostaglandins* **16**, 583-589

101 Bryant, R.W., and Bailey, J.M. (1979) *Prostaglandins* **17**, 9-18

102 Pace-Asciak, C.R., Mizuno, K., and Yamamoto, S. (1982) *Biochim. Biophys. Acta* **712**, 142-145

103 Pace-Asciak, C.R., (1984) *Prostaglandins Leukotrienes Med.* **16**, 173-180

104 Pace-Asciak, C.R., (1984) *Biochim. Biophys. Acta* **793**, 485-488

105 Pace-Asciak, C.R., (1984) *J. Biol. Chem.* **259**, 8332-8337

106 Pace-Asciak, C.R., and Lee, W.S. (1989) *J. Biol. Chem.* **264**, 9310-9313

107 Laneuville, O., Chang, M., Reddy, C.C., Corey, E.J., and Pace-Asciak, C.R. (1990) *J. Biol. Chem.* **265**, 21415-21418

108 Laneuville, O., Corey, E.J., Couture, R., and Pace-Asciak, C.R. (1991) *Biochim. Biophys. Acta* **1084**, 60-68

109 Borgeat, P., and Samuelsson, B. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2148-2152

110 Narumiya, S., Salmon, J.A., Cottee, F.H., Weatherley, B.C., and Flower, R.J. (1981) *J. Biol. Chem.* **256**, 9583-9592

111 Rapoport, S.M., Schewe, T., Wiesner, R., Halangk, W., Ludwig, P., Janicke-Höhne, M., Tannert, C., Heibsch, C., and Klatt, D. (1979) *Eur. J. Biochem.* **96**, 545-561

112 Sigal, E., Craik, C.S., Highland, E., Grunberger, D., Costello, L.L., Dixon, R.A.F., and Nadel, J.A. (1988) *Biochem. Biophys. Res. Commun.* **157**, 457-464

113 Schewe, T., Halangk, W., Heibsch, C., and Rapoport, S.M. (1975) *FEBS Lett.* **60**, 149-152

114 Kühn, H., and Brash, A.R. (1990) *J. Biol. Chem.* **265**, 1454-1458

115 Cunnane, S.C., Keeling, P.W.N., Thompson, R.P.H., and Crawford, M.A. (1984) *Br. J. Nutrition* **51**, 209-217

116 Reinaud, O., Delaforge, M., Boucher, J.L., Rocchiccioli, F., and Mansuy, D. (1989) *Biochem. Biophys. Res. Commun.* **161**, 883-891

117 Hamberg, M., and Samuelsson, B. (1967) *J. Biol. Chem.* **242**, 5329-5335

118 Goetzl, E.J., and Sun, F.F. (1979) *J. Exp. Med.* **150**, 406-411

119 Grisham, M.B., andd McCord, J.M. (1986) In: *Physiology of Oxygen Radicals* (Taylor, A.E., Matalon, S., and Ward, P.A., eds) Waverly Press Inc., Baltimore, pp 1-18

120 Gschwendt, M., Fürstenberger, G., Kittstein, W., Besemfelder, E., Hull, W.E., Hagedorn, H., Opferkuch, H.J., and Marks, F. (1986) *Carcinogenesis* **7**, 449-455

121 Fischer, S.M., Baldwin, J.K., Jasheway, D.W., Patrick, K.E., and Cameron, G.S. (1988) *Cancer Res.* **48**, 658-664

122 Hughes, M.A., and Brash, A.R. (1991) *Biochim. Biophys. Acta* **1081**, 347-354

123 Taylor, G.W., and Morris, H.R. (1983) *Br. Med. Bull.* **39**, 219-222

124 Shimizu, T., Rådmark, O., Samuelsson, B. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 689-693

125 Matthew, J.A., Chan, H.W.-S., and Galliard, T. (1977) *Lipids* **12**, 324-326

126 Bundy, G.L., Nidy, E.G., Epps, D.E., Mizsak, S.A., and Wnuk, R.J. (1986) *J. Biol. Chem.* **261**, 747-751

127 Hawkins, K.J., and Brash, A.R. (1987) *J. Biol. Chem.* **262**, 7629-7634

128 Guengerich, F.P. (1991) *J. Biol. Chem.* **266**, 10019-10022

129 Fitzpatrick, F.A., and Murphy, R.C. (1989) *Pharmacol. Rev.* **40**, 229-241

130 Falck, J.R., Lumin, S., Blair, I., Dishman, E., Martin, M.V., Waxman, D.J., Guengerich, F.P., and Capdevila, J.H. (1990) *J. Biol. Chem.* **265**, 10244-10249

131 Gonzalez, F.J. (1989) *Pharmacol. Rev.* **40**, 243-288

132 Needleman, P., Turk, J., Jakschik, B.A., Morrison, A.R., and Lefkowith, J.B. (1986) *Ann. Rev. Biochem.* **55**, 69-102

133 Escalante, B., Erlij, D., Falck, J.R., and McGiff, J.C. (1990) *Science* **251**, 799-802

134 Escalante, B., Sessa, W.C., Falck, J.R., Yadagiri, P., and Schwartzman, M.L. (1990) *J. Cardiovasc. Pharmacol.* **16**, 438-443

135 Oliw, E.H., Guengerich, P., and Oates, J.A. (1982) *J. Biol. Chem.* **257**, 3771-3781

136 Powell, W.S. (1984) *J. Biol. Chem.* **259**, 3082-3089

137 Powell, W.S., and Solomon, S. (1978) *J. Biol. Chem.* **253**, 4609-4616

138 Serhan, C.N., Hamberg, M., and Samuelsson, B. (1984) *Biochem. Biophys. Res. Commun.* **118**, 943-949

139 Serhan, C.N., Hamberg, M., and Samuelsson, B. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5335-5339

140 Rokach, J., and Fitzsimmons, B. (1988) *Int. J. Biochem.* **20**, 753-758

141 Yamamoto, S., Ueda, N., Yokoyama, C., Fitzsimmons, B.J., Rokach, J., Oates, J.A., and Brash, A.R. (1988) *Adv. Exp. Med. Biol.* **229**, 15-26

142 Serhan, C.N., and Sheppard, K.A. (1990) *J. Clin. Invest.* **85**, 772-780

143 Lindgren, J.A., Hansson, G., and Samuelsson, B. (1981) *FEBS Lett.* **128**, 329-335

144 Borgeat, P., Picard, S., Vallerand, P., and Sirois, P. (1981) *Prostaglandins Med.* **6**, 557-570

145 Marcus, A.J., Broekman, J., Safier, L.B., Ullman, H.L., and Islam, N. (1982) *Biochem. Biophys. Res. Commun.* **109**, 130-137

146 Maas, R.L., and Brash, A.L. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2884-2888

147 Maas, R.L., Turk, J., Oates, J.A., and Brash, A.R. (1982) *J. Biol. Chem.* **257**, 7056-7067

148 Feinmark, S.J., and Cannon, P.J. (1986) *J. Biol. Chem.* **261**, 16466-16472

149 Maclouf, J.A., and Murphy, R.C. (1988) *J. Biol. Chem.* **263**, 174-181

150 Lagarde, M., Gualde, N., Rigaud, M. (1989) *Biochem. J.* **257**, 313-320

151 Feinmark, S.J., and Cannon, P.J. (1987) *Biochim. Biophys. Acta* **922**, 125-135

152 Dahinden, C.A., Clancy, R.M., Gross, M., Chiller, J.M., and Hugli, T.E. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6632-6636

153 Fitzpatrick, F., Liggett, W., McGee, J., Bunting, S., Morton, D., and Samuelsson, B. (1984) *J. Biol. Chem.* **259**, 11403-11407

154 Flower, R.J., and Blackwell, G.J. (1976) *Biochem. Pharmacol.* **25**, 285-291

155 Chilton, F.H., Ellis, J.M., Olson, S.C., and Wykle, R.L. (1984) *J. Biol. Chem.* **259**, 12014-12019

156 Malone, B., Lee, T.C., and Snyder, F. (1985) *J. Biol. Chem.* **260**, 1531-1534

157 Wittcoff, H. (1951) In: *The Phosphatides*, Reinhold Press, New York pp 99-100

158 Van den Bosh, H. (1980) *Biochem. Biophys. Acta* **604**, 191-246

159 Verheij, H.M., Slotboom, A.J., and de Haas, G.H. (1981) *Rev. Physiol. Biochem. Pharmacol.* **91**, 91-203

160 Dennis, E.A. (1983) In: *The Enzymes* (Boyer, P., ed.) Academic Press, New York, pp 307-353

161 Randolph, A., Sakmar, T.P., and Heinrikson, R.L. (1980) In: *Frontiers in Protein Chemistry* (Liu, T.Y.,Mamiya, C., and Yasunobu, K., eds) Elsevier/North Holland Press, Amsterdam, pp 297-322

162 Davidson, F.F., and Dennis, E.A. (1990) *J. Mol. Evol.* **31**, 228-238

163 Clark, J.D., Milona, N., and Knopf, J.L. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7708-7712

164 Suga, K., Kawasaki, T., Blank, M.L., and Snyder, F. (1990) *J. Biol. Chem.* **265** 12363-12371

165 Leslie, C.C., Voelker, D.R., Channon, J.Y., Wall, M.M., and Zelarney, P.T. (1988) *Biochim. Biophys. Acta* **963**, 476-492

166 Clark, J.D., Lin, L.-L., Kriz, R.W., Ramesha, C.S., Sultzman, L.A., Lin, A.Y., Milona, N., and Knopf, J.L. (1991) *Cell* **65**, 1043-1051

167 Gronich, J.H., Bonventre, J.V., and Nemenoff, R.A. (1990) *J. Biol. Chem.* **271**, 37-43

168 Kramer, R.M., Manetta, J., and Putnam, J.E. (1991) *J. Biol. Chem.* **266**, 5268-5272

169 Habermann, E. (1972) *Science* **177**, 314-322

170 Shier, W.T. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 195-199

171 Hassid, A., and Levine, L. (1977) *Res. Commun. Chem. Pathol. Pharmacol.* **18**, 507-517

172 Johnson, A.R., Revtyak, G., and Campbell, W.B. (1985) *Fed. Proc.* 44, 19-25

173 Clark, M.A., Conway, T.M., Shorr, R.G.L., and Crooke, S.T. (1987) *J. Biol. Chem.* **262**, 4402-4406

174 Clark, M.A., Özgür, L.E., Conway, T.M., Dispoto, J., Crooke, S., and Bomblaski, J.S. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5418-5422

175 Blackwell, G.J., Carnuccio, R., and Persico, P. (1980) *Br. J. Pharmac. Chemother.* **68**, 14-16

176 Hirata, F., Schiffmann, E., Venkatasubramanian, K., Salomon, D., and Axelrod, J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2533-2536

177 Cloix, J.F., Colard, O., Rothut, B., and Russo-Marie, F. (1983) *Br. J. Pharmac. Chemother.* **79**, 313-321

178 Di Rosa, M., Flower, R.J., Hirata, F., Parente, L., and Russo-Marie, F. *Prostaglandins* **28**, 441-442

179 Glenney, J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4258-4262

180 Crompton, M.R., Owens, R.J., Totty, N.F., Moss, S.E., Waterfield, M.D., and Crumpton, M.J. (1988) *EMBO J.* **7**, 21-27

181 Powell, M.A., and Glenney, J.R. (1987) *Biochem. J.* **247**, 321-328

182 Davidson, F.F., Denis, E.A., Powell, M., and Glenney Jr., J.R. (1987) *J. Biol. Chem.* **262**, 1698-1705

183 Piltch, A., Sun, L., Fava, R.A., and Hayashi, J. (1989) *Biochem. J.* **261**, 395-400

184 Hullin, F., Raynal, P., Ragab-Thomas, J.M.F., Fauvel, J., and Chap, H. (1989) *J. Biol. Chem.* **264**, 3506-3513

185 Bienkowski, M.J., Petro, M.A., and Robinson, L.J. (1989) *J. Biol. Chem.* **264**, 6536-6544

186 Majerus, P.W., Neufeld, E.J., and Wilson, D.B. (1984) *Cell* **37**, 701-703

187 Emilsson, A., and Sundler, R. (1984) *J. Biol. Chem.* **259**, 3111-3116

188 Billah, M.M., Lapetina, E.G., and Cuatrecasas, P. (1981) *J. Biol. Chem.* **256**, 5399-5403

189 Ford-Hutchinson, A.W., Bray, M.A., Doig, M.V., Shipley, M.E., and Smith, M.J.H. (1980) *Nature* **286**, 264-265

190 Malmsten, C.L., Palmblad, J., Uden, A.M., Rådmark, O., Engstedt, L., and Samuelsson, B. (1980) *Acta Physiol. Scand.* **110**, 449-451

191 Dahlén, S.-E., Björk, J., Hedqvist, P., Arfors, K.-E. Hammarström, S., Lindgren, J.-A., and Samuelsson, B. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3887-3891

192 Palmer, R.M.J., Stepney, R.J., Higgs, G.A., and Eakins, K.E. (1980) *Prostaglandins* **20**, 411-418

193 Feinmark, S.J., Lindgren, J.A. Claesson, H.-E., Malmsten, C., and Samuelsson, B. (1981) *FEBS Lett.* **136**, 141-144

194 Serhan, C.N., Radin, A., Smolen, J.E., Korchak, H., Samuelsson, B., and Weissman, G. (1982) *Biochem. Biophys. Res. Commun.* **107**, 1006-1012

195 Goldman, D.W., Gifford, L.A., Marotti, T., Koo, C.H., and Goetzl, E.J. (1987) *Fed. Proc.* **46**, 200-203

196 Goldman, D.W., and Goetzl, E.J. (1984) *J. Exp. Med.* **159**, 1027-1041

197 Palmblad, J., Malmsten, C.L., Udén, A.M., Rådmark, O, Engstedt, L., and Samuelsson, B. (1981) *Blood* **58**, 658-661

198 Ringertz, B., Palmblad, J., Rådmark, O., and Malmsten, C. (1982) *FEBS Lett.* **147**,180-182

199 Kreisle, R.A., and Parker, C.W. (1983) *J. Exp. Med.* **157**, 628-641

200 Payan, D.G., Missirian-Bastian, A., and Goetzl, E.J. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3501-3505

201 Rola-Pleszczynski, M., and Lemaire, I. (1985) *J. Immunol.* **135**, 3958-3961

202 Rola-Pleszczynski, M., Chavaillaz, P.A., and Lemaire, I. (1986) *Prostaglandins Leukotrienes Med.* **23**, 207-210

203 Dubois, C.M., Bissonnette, E., and Rola-Pleszczynski, M. (1989) *Am. Rev. Respir. Dis.* **139**, 1257-1264

204 Rola-Pleszczynski, M., Gagnon, L., and Sirois, P. (1983) *Biochem. Biophys. Res. Commun.* **113**, 531-537

205 Gagnon, L., Girard, M., Sullivan, A.K., and Rola-Pleszczynski, M. (1987) *Cell. Immunol.* **110**, 243-252

206 Hall, T.J., and Brostaff, J. (1985) *Immunol. Lett.* **9**, 255-258

207 Yamaoka, K.A., Claésson, H.-E., and Rosén, A. (1989) *J. Immunol.* **143**, 1996-2000

208 Lewis, R.A., Austen, F., and Soberman, R.J. (1991) *New Eng. J. Med.* **323**, 645-655

209 Barnes, N.C., Evans, J., Zakrzewski, J.T., Piper, P.J., and Costello, J.F. (1989) *Agents Actions Suppl.* **28**, 305-312

210 Chagnon, M., Gentile, J., Gladu, M., Sirois, P. (1985) *Lung* **163**, 55-62

211 Barnes, N.C. Piper, P.J., and Costello, J.F. (1984) *Thorax* **39**, 500-504

212 Weiss, J.W., Drazen, J.M., McFadden Jr., E.R., Weller, P., Corey, E.J., Lewis, R.A., and Austen, K.F. (1983) *J. Amer. Med. Ass.* **249**, 2814-2817

213 Piper, P.J., and Samhoun, M.N. (1982) *Br. J. Pharmacol.* 77, 267-275

214 Samhoun, M.N., and Piper, P.J. (1983) *Comparative Actions of Leukotrienes and Other Lipoxygenase Products* (Piper, P.J., ed.) John Wiley, Chichester, pp 161-177

215 Brain, S.D., and Williams T.J. (1990) *Pharmac. Ther.* **46**, 57-66

216 Shaw, A., and Krell, R.D. (1991) *J. Medicinal Chem.* **34**, 1235-1242

217 Buckner, C.K., Krell, R.D., Laravuso, R.B., Coursin, D.B., Bernstein, P.R., Will, J.A. (1986) *J. Pharmacol. Exp. Ther.* **237**, 558-562

218 Snyder, D.W., Giles, R.E., Keith, R.A., Yee, Y.K., Krell, R.D. (1987) *J. Pharmacol. Exp. Ther.* **243**, 548-556

219 Hand, J.M., and Schwalm, S.F. (1987) *Prostaglandins* **33**, 709-716

220 Snyder, D.W., Cho, H.L., Gilmore, J. (1991) *Eur. J. Pharmacol.* **195**, 209-215

221 Fleish, J.H., Rinkema, L.E., and Baker, S.R. (1982) *Life Sciences* **31**, 577-581

222 Hammarström, S., and Falardeau, P. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3691-3695

223 Aharony, D., Smith, J.B., and Silver, M.J. (1982) *Biochim. Biophys. Acta* **718**, 193-200

224 Brüne, B., and Ullrich, V. (1991) *Mol. Pharmacol.* **39**, 671-678

225 Siegel, M.I., McConnell, R.T., Abrahams, S.L., Porter, N.A., and Cuatrecases, P. (1978) *Biochem. Biophys. Res. Commun.* **89**, 1273-1280

226 Salmon, J.A., Smith, D.R., Flower, R.J., Moncada, S., and Vane, J.R. (1978) *Biochim. Biophys. Acta* **523**, 250-262

227 Turk, J., Wyche, A., and Needleman, P. (1980) *Biochem. Biophys. Res. Commun.* **95**, 1628-1634

228 Goetzl, E.J., Hill, H.R., and Gorman, R.R. (1980) *Prostaglandins* **19**, 71-85

229 Antonipillai, I., Nadler, J.L., Robin, E.C., and Horton, R. (1987) *Hypertension* **10**, 61-66

230 Antonipillai, I., Horton, R., Natarajan, R., and Nadler, J. (1989) *Endocrinology* **125**, 2028-2034

231 Metz, S., VanRollins, M., Strife, R., Fujimoto, W., and Robertson, R.P. (1983) *J. Clin. Invest.* **71**, 1191-1205

232 Goetzl, E.J., Woods, J.M., and Gorman, R.R. (1977) *J. Clin. Invest.* **59**, 179-183

233 Woollard, P.M., Cunningham, F.M., Camp, R.D.R., Murphy, G.M., Derm, F.F., Greaves, M.W. (1989) *Prostaglandins* **38**, 465-471

234 Setty, B.N.Y., Graeber, J.E., and Stuart, M.J. (1987) *J. Biol. Chem.* **262**, 17613-17622

235 Hadjiagapiou, C., and Spector, A.A. (1986) *Prostaglandins* **31**, 1135-1144

236 Köller, M., and König, W. (1991) *Biochem. Biophys. Res. Commun.* **184**, 804-809

237 Cunningham, F.M., and Woollard, P.M. (1987) *Prostaglandins* **34**, 71-78

238 Bacon, K.B., Camp, R.D.R., Cunningham, F.M., Woollard, P.M. (1988) *Br. J. Pharmacol.* **95**, 966-974

239 Fretland, D.J., and Djuric, S.W., (1989) *Prostaglandins Leukotrienes Ess. Fatty Acids* **38**, 215-228

240 Wiggins, R.E., Jafri, M.S., and Proia, A.D. (1990) *Prostaglandins* **40**, 131-141

241 Schwartzman, M.L., Balazy, M., Masferrer, J., Abraham, N.G., McGiff, J.C., and Murphy, R.C. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8125-8129

242 Pace-Asciak, C.R., Laneuville, O., Su, W.-G., Corey, E.J., Gurevich, N., Wu, P., and Carlen, P.L. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3037-3041

243 Nigam, S., Nodes, S., Cichon, G., Corey, E.J., and Pace-Asciak, C.R. (1990) *Biochem. Biophys. Res. Commun.* **171**, 944-948

244 Dho, S., Grinstein, S., Corey, E.J., Su, W.-G., and Pace-Asciak, C.R. (1990) *Biochem. J.* **266**, 63-68

245 Vanderhoek, J.Y., Bryant, R.W., and Bailey, J.M. (1982) *Biochem. Pharmacol.* **31**, 3463-3467

246 Vanderhoek, J.Y., Bryant, R.W., and Bailey, J.M. (1980) *J. Biol. Chem.* **255**, 5996-5998

247 Moncada, S., Gryglewski, R.J., Bunting, S., and Vane, J.R. (1976) *Prostaglandins* **12**, 715-737

248 Vedelago, H.R., and Mahadevappa, V.G. (1988) *Biochem. Biophys. Res. Commun.* **150**, 177-184

249 Vanderhoek, J.Y., Bryant, R.W., and Bailey, J.M. (1980) *J. Biol. Chem.* **255**, 10064-10066

250 Camp, R.D.R., and Fincham, N.J. (1985) *Br. J. Pharmacol* **85**, 837-841

251 Ternowitz, T., Fogh, K., and Kragballe, K. (1988) *Skin Pharmacol.* **1**, 93-99

252 Fogh, K., Kiil, J., Herlin, T., Ternowitz, T., and Kragballe, K. (1987) *Arch. Dermatol. Res.* **279**, 504-511

253 Fogh, K., Sogaard, H., Herlin, T., and Kragballe, K. (1988) *J. Am. Acad. Dermatol.* **18**, 279-285

254 Herlin, T., Fogh, K., Hansen, E.S., Andreasen, A., Knudsen, V., Henriksen, T.B., Bunger, C., and Kragballe, K. (1990) *Agents Actions* **29**, 52-53

255 Vanderhoek, J.Y., Karmin, M.T., and Ekborg, S.L. (1985) *J. Biol. Chem.* **260**, 15482-15487

256 Sekiya, F., Takagi, J., Sasaki, K-I., Kawajiri, K., Kobayashi, Y., Sato, F., and Saito, Y. (1990) *Biochim. Biophys. Acta* **1044**, 165-168

257 Adcock, J.J., Garland, L.G., Moncada, S., and Salmon, J.A. (1978) *Prostaglandins* **16**, 163-177

258 Lai, C.K.W., Polosa, R., and Holgate, S.T. (1990) *Am. Rev. Respir. Dis.* **141**, 1423-1427

259 Copas, J.L., Borgeat, P., and Gardiner, P.J. (1982) *Prostaglandins Leukotrienes Med.* **8**, 105-114

260 Vanderhoek, J.Y., Tare, N.S., Bailey, J.M., Goldstein, A.L., and Pluznik, D.H. (1982) *J. Biol. Chem.* **257**, 12191-12195

261 Buchanan, M.R., Haas, T.A., Lagarde, M.,and Guichardant, M. (1985) *J. Biol. Chem.* **260**, 16056-16059

262 Setty, B.N.Y., Berger, M., and Stuart, M.J. (1987) *Biochem. Biophys. Res. Commun.* **148**, 528-533

263 Setty, B.N.Y., Berger, M., and Stuart, M.J. (1987) *Biochem. Biophys. Res. Commun.* **146**, 502-509

264 Conti, P., Reale, M., Trisi, P., Barbacane, R.C., Panara, M.R., Bongrazio, M., and Piatelli, A. (1990) *Agents and Actions* **29**, 50-51

265 Conti, P., Reale, M., Barbacane, R.C., Panara, M.R., and Bongrazio, M. (1991) *Agents Actions* **32**, 85-87

266 Palmblad, J., Gyllenhammar, H., and Ringertz, B. (1988) *Adv. Exp. Med. Biol.* **229**, 137-145

267 Lefer, A.M., Stahl, G.L., Lefer, D.J., Brezinski, M.E., Nicolaou, K.C., Veale, C.A., Abe, Y., and Smith, J.B. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8340-8344

268 Jacques, C.A.J., Spur, B.W., Crea, A.E.G., and Lee, T.H. (1988) *Br. J. Pharmacol.* **95**, 562-568

269 Sigal, E., and Nadel, J.A. (1988) *Am. Rev. Repir. Dis.* **138**, S35-S40

270 Manzini, S., and Meini, S. (1991) *Br. J. Pharmacol.* **103**, 1027-1032

271 Lee, T.H., Crea, A.E.G., Gant, V., Spur, B.W., Marron, B.E., Nicolaou, K.C., Reardon, E., Brezinski, M., and Serhan, C.N. (1990) *Am. Rev. Respir. Dis.* **141**, 1453-1458

272 Ramstedt, U., Ng, J., Wigzell, H., Serhan, C.N., and Samuelsson, B. (1985) *J. Immunol.* **135**, 3434-3438

273 Samuelsson, B., Dahlén, S.-E., Lindgren, J.A., Rouzer, C.A., and Serhan, C.N. (1987) *Science* **237**, 1171-1176

274 Badr, K.F. (1988) *Adv. Exp. Med. Biol.* **229**, 131-135

275 Badr, K.F., DeBoer, D.K., Schwartzberg, M., and Serhan, C.N. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3438-3442

276 Brady, H.R., Persson, U., Ballermann, B.J., Brenner, B.M., and Serhan, C.N. (1990) *Am. J. Physiol.* **259**, F809-F815

277 Dahlén, S.-E., Franzén, L., Raud, J., Serhan, C.N., Westlund, P., Wikström, E., Bjorck, T., Matsuda, H., Webber, S.E., Veale, C.A., Puustinen, T., Haeggström, J., Nicolaou, K.C., and Samuelsson, B. (1988) *Adv. Exp. Med. Biol.* **229**, 107-130

278 Lindgren, J.A., Hansson, G., and Samuelsson, B. (1981) *FEBS Lett.* **128**, 329-335

279 Soberman, R.J., Sutyak, J.P., Okita, R.T., Wendelborn, D.F., Roberts, L.J., Austen, K.F. (1988) *J. Biol. Chem.* **263**, 7996-8002

280 Sumimoto, H., and Minakami, S. (1990) *J. Biol. Chem.* **265**, 4348-4353

281 Wong, P.Y.-K., Westlund, P., Hamberg, M., Granström, E., Chao, P.H.-W., and Samuelsson, B. (1984) *J. Biol. Chem.* **259**, 2683-2686

282 Marcus, A.J., Safier, L.B., Ullman, H.L., Broekman, M.J., Islam, N., Oglesby, T.D., and Gorman, R.R. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 903-907

283 Marcus, A.J., Safier, L.B., Ullman, H.L., Islam, N., Broekman, M.J., Falck, J.R., Fischer, S., and von Schacky, C. (1988) *J. Biol. Chem.* **263**, 2223-2229

284 Soberman, R.J., Okita, R.T., Fitzsimmons, B., Rokach, J., Spur, B., and Austen, F. (1987) *J. Biol. Chem.* **262**, 12421-12427

285 Hansson, G., Lindgren, J.A., Dahlén, S.-E., Hedqvist, P., and Samuelsson, B. (1981) *FEBS Lett.* **130**, 107-112

286 Ford-Hutchinson, A.W., Rackham, A., Zamboni, R., Rokach, J., and Roy, S. (1983) *Prostaglandins* **25**, 29-37

287 Sirois, P., Roy, S., Borgeat, P., Picard, S., and Vallerand, P. (1982) *Prostaglandins Leukotrienes Med.* **8**, 157-170

288 Powell, W.S. (1987) *Biochem. Biophys. Res. Commun.* **145**, 991-998

289 Maas, R.L., Brash, A.R., and Oates, J.A. (1982) *Adv. Prostaglandin Thromboxane Leukotriene Res.* **9**, 29-44

290 Romano, M.C., Eckardt, R.D., Bender, P.E., Leonard, T.B., Straub, K.M., and Newton, J.F. (1987) *J. Biol. Chem.* **262**, 1590-1595

291 Powell, W.S., and Gravelle, F. (1990) *J. Biol. Chem.* **265**, 9131-9139

292 Serafin, W.E., Oates, J.A., and Hubbard, W.C. (1984) *Prostaglandins* **27**, 899-911

293 Okita, R.T., Soberman, R.J., Bergholte, J.M., Masters, B.S.S., Hayes, R., and Murphy, R.C. (1987) *Mol. Pharmacol.* **32**, 706-709

294 Schulz, H. (1991) *Biochim. Biophys. Acta* **1081**, 109-120

295 Hadjiagapiou, C., Sprecher, H., Kaduce, T.L., Figard, P.H., and Spector, A.A. (1987) *Prostaglandins* **34**, 579-589

296 Gordon, J.A., and Spector, A.A. (1987) *Am. J. Physiol.* **253**, C277-C285

297 Moore, S.A., Prokuski, L.J., Figard, P.H., Spector, A.A., and Hart, M.N. (1988) *J. Cell. Physiol.* **137**, 75-85

298 Mathur, S.N., Albright, E., and Field, F.J. (1990) *J. Biol. Chem.* **265**, 21048-21055

299 Gordon, J.A., Figard, P.H., and Spector, A.A. (1990) *J. Clin. Invest.* **85**, 1173-1181

300 Shen, X.Y., Figard, P.H., Kaduce, T.L., and Spector, A.A. (1988) *Biochemistry* **27**, 996-1004

301 Hadjiagapiou, C., Travers, J.B., Fertel, R.H., and Sprecher, H. (1990) *J. Biol. Chem.* **265**, 4369-4373

302 Powell, W.S. (1986) *Biochem. Biophys. Res. Commun.* **136**, 707-712

303 Powell, W.S., and Gravelle, F. (1988) *J. Biol. Chem.* **263**, 2170-2177

304 Powell, W.S., and Gravelle, F. (1989) *J. Biol. Chem.* **264**, 5364-5369

305 Klebanoff, S.J., and Clark, R.A. (1978) In: *The Neutrophil: Function and Clinical Disorders* Elsevier/North-Holland Inc., New York, pp 5-72

306 Weissman, G., Korchak, H.M., Perez, H.D., Smolen, J.E., Goldstein, I.M., and Hoffstein, S.T. (1979) *J. Reticuloendothelial Soc.* **26**, 687-700

307 Bevilacqua, M.P., Stengelin, S., Gimbrone, M.A., and Seed, B. (1989) *Science* **243**, 1160-1165

308 Kishimoto, T.K., Larson, R.S., Corbi, A.L., Dustin, M.L., Staunton, D.E. and Springer, T.A. (1989) *Adv. Immunol.* **46**, 149-182

309 Chibber, R., and Castle, A.G. (1983) *Comp. Biochem. Physiol.* **75B**, 335-340

310 Powell, W.S. (1982) *Methods Enzymol.* **86**, 467-477

311 Powell, W.S. (1983) *Anal. Biochem.* **128**, 93-103

312 Yadagiri, P., Sun, L., Falck, J.R., Karara, A., and Capdevila, J. (1989) *Tetrahedron Lett.* **30**, 429-432

313 Murphy, R.C., Falck, J.R., Lumin, S., Yadagiri, P., Zirrolli, J.A., Balazy, M., Masferrer, J.L., Abraham, N.G., and Schwartzman, M.L. (1988) *J. Biol. Chem.* **263**, 17197-17202

314 Powell, W.S. (1987) *Anal. Biochem.* **164**, 117-131

315 Klebanoff, S.J. (1965) *Endocrinology* **76**, 301-311

316 Brittinger, G., Hirschhorn, R., Douglas, S.D., and Weissmann, G. (1968) *J. Cell Biol.* **37**, 394-411

317 Sha'afi, R.I., Naccache, P., Raible, D., Krepcio, A., Showell, H., and Becker, E.L. (1976) *Biochim. Biophys. Acta* **448**, 638-641

318 Ames, B.N. (1966) *Methods Enzymol.* **8**, 115-118

319 Alworth, W.L. (1972) In: *Stereochemistry and its Application in Biochemistry*, Wiley-Interscience, New York, pp 39-90

320 Bakhle, Y.S. (1983) *Br. Med. Bull.* **39**, 214-218

321 Wainwright, S.L., and Powell, W.S. (1989) In: *New Trends in Lipid Mediators Research vol 3* (Zor, U., Danon, A., eds) Basel, Karger, Switzerland, pp 200-203

322 Nichols, B.A., and Setzer, P.Y. (1981) *J. Histochem. Cytochem.* **29**, 317-320

323 Shio, H., and Lazarow, P.B. (1981) *J. Histochem. Cytochem.* **29**, 1263-1272

324 Hansen, H.S. (1976) *Prostaglandins* **12**, 647-679

325 Ensor, C.M., Yang, J.-Y., Okita, R.T., and Tai, H.-H. (1990) *J. Biol. Chem.* **265**, 14888-14891

326 Chang, D.G.-B., Sun, M., and Tai, H.-H. (1981) *Biochem. Biophys. Res. Commun.* **99**, 745-751

327 Änggård, E., Larsson, C., and Samuelsson, B. (1971) *Acta Physiol. Scand.* **81**, 396-404

328 Braithwaite, S.S., and Jarabak, J. (1975) *J. Biol. Chem.* **250**, 2315-2318

329 Tai, H.-H., Liu, Y., Tai, C.L., Yoden, K. and Shen, R.-F. (1985) *Adv. Prostaglandin Thromboxane Leukotriene Res.* **15**, 159-161

330 Bergholte, J.M., Soberman, R.J., Hayes, R., Murphy, R.C., and Okita, R.T. (1987) *Arch. Biochem. Biophys.* **257**, 444-450

331 Earles, S.M., Bronstein, J.C., Winner, D.L. and Bull, A.W. (1991) *Biochim. Biophys. Acta* **1081**, 174-180

332 Sok, D.-E., Kang, J.B. and Shin, H.D. (1988) *Biochem. Biophys. Res. Commun.* **156**, 524-529

333 Borson, W.F., and Li, T.K. (1980) In: *Enzymatic Basis of Detoxication* (Jakoby, W.B., ed.) Academic Press, New York, pp 231-248,

334 Agarwal, D.P, and Goedde, H.W. (1990) *Pharma. Ther.* **45**, 69-83

335 Pietruszko, R., Crawford, K., and Lester, D. (1973) *Arch. Biochem. Biophys.* **159**, 50-60

336 Winer, A.D., and Theorell, H. (1960) *Acta Chem. Scand.* **14**, 1729-1742

337 Penning, T.M., Mukharji, I., Barrows, S., and Talalay, P. (1984) *Biochem. J.* **222**, 601-611

338 Vogel, K., Bentley, P., Platt, K., -L., and Oesch, F. (1980) *J. Biol. Chem.* **255**, 9621-9625

339 Hamberg, M. and Samuelsson, B. (1971) *J. Biol. Chem.* **246**, 1073-1077

340 Goodridge, A.G. (1985) In: *Biochemistry of Lipids and Membranes* (Vance, D.E., and Vance, J.E., eds) The Benjamin/Cummings Publishing Company, Inc., CA, pp 143-180

341 Schulz, H. (1985) In: *Biochemistry of Lipids and Membranes* (Vance, D.E., and Vance, J.E., eds) The Benjamin/Cummings Publishing Company, Inc., CA, pp 117-142

342 Clark, A.F. (1979) In: *Steroid Biochemistry Vol I* (Hobkirk, R., ed.) CRC Press Inc., Florida, pp 1-21

343 Dempsey, M.E., Seaton, J.D., Schroepfer, G.J. and Trockman, W. (1964) *J. Biol. Chem.* **239**, 1381-1387

344 Paik, Y.K., Trzaskos, J.M., Shafiee, A., and Gaylor, J.L. (1984) *J. Biol. Chem.* **259**, 13413-13423

345 Tserng, K.-Y., and Jin, S.-J. (1990) *J. Biol. Chem.* **266**, 11614-11620

346 Wermuth, B., Bohren, K.M., Heinemann, G., Von Wartburg, J.-P., and Gabbay, K.H. (1988) *J. Biol. Chem.* **263**, 16185-16188

347 Seibert, K., Sheller, J.R. and Roberts II, L.J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 256-260

348 Lee, S.C. and Levine, L. (1975) *J. Biol. Chem.* **250**, 548-552

349 Falgueyret, J.P., Leblanc, Y., Rokach, J. and Riendeau, D. (1988) *Biochem. Biophys. Res. Commun.* **156**, 1083-1089

350 Falgueyret, J.P., Leblanc, Y., and Riendeau, D. (1990) *FEBS Lett.* **262**, 197-200

351 Änggård, E. (1971) *Ann. N.Y. Acad. Sci.* **180**, 200-217

352 Crutchley, K.J. and Piper, P. (1975) *Br. J. Pharmac.* **54**, 397-399

353 Price, N.C. (1989) In: *Fundamentals of Enzymology* Oxford University Press, New York, pp 276-299

354 Price, N.C. (1989) In: *Fundamentals of Enzymology* Oxford University Press, New York, pp 167-168

355 Veech, R.L. (1987) In: *Pyridine Nucleotide Coenzymes* (Dolphin, D., Poulson, R., and Avramovic, O. eds) John Wiley & Sons, New York, pp 79-104

356 Merritt, M.V., Bronson, G.E., Baczynskyi, L., and Boal, J.R. (1980) *J. Am. Chem. Soc.* **102**, 346-351

357 Adams, E. (1976) *Adv. Enzymol. Relat. Areas Mol. Biol.* **44**, 69-138

358 Cardinale, G.J., and Abeles, R.H. (1968) *Biochemistry* **7**, 3970-3978

359 Albery, W.J., and Knowles, J.R. (1986) *Biochemistry* **25**, 2572-2577

360 Whitman, C.P., Hegeman, G.D., Cleland, W.W., and Kenyon, G.L. (1985) *Biochemistry* **24**, 3936-3942

361 Langer, R., and Glaser, L. (1974) *J. Biol. Chem.* **249**, 1126-1132

362 Glaser, L., and Ward, L. (1970) *Biochim. Biophys. Acta* **198**, 613-615

363 McDonough, M. W., and Wood, W.A. (1961) *J. Biol. Chem.* **236**, 1220-1224

364 Kaever, V., Martin, M., Fauler, J., Marx, K.-H., and Resch, K. (1987) *Biochim. Biophys. Acta* **922**, 337-344

365 Kaever, V., Wunder, B., Damerau, G., Zimmer, J., Fauler, K., Wessel, K., Floege, J., Topley, H., Radeke, H., and Resch, K. (1990) *Life Sciences* **46**, 1465-1470

366 Shönfeld, W., Schlüter, R., Hilger, R., and König, W. (1988) *Immunology* **65**, 529-536

367 Fauler, J., Marx, K.-H., Kaever, V., and Frölich, J.C. (1989) *Prostaglandins Leukotrienes Ess. Fatty Acids* **37**, 193-196

368 Schönfeld, W., Kasimir, S., Knöller, J., Jablonski, K., and König, W. (1991) *J. Leukocyte Biol.* **50**, 303-312

369 Kumlin, M., Falck, J.R., Raud, J., Harada, Y., Dahlén, S.-E., and Granström, E. (1990) *Biochem. Biophys. Res. Commun.* **170**, 23-29

370 Powell, W.S., and Gravelle, F. (1990) *Biochim. Biophys. Acta* **1044**, 147-157

371 Rimarachin, J.A., Masferrer, J.L., Gerritsen, M.E., and Schwartzman, M.L. (1989) *FASEB J.* **3**, A913

372 Kaever, V., Damerau, B., Wessel, K., and Resch, K. (1988) *FEBS Lett.* **231**, 385-388

373 Bull, A., and Bronstein, J.C. (1990) *Carcinogenesis* **11**, 1699-1704

374 Woollard, P.M. (1986) *Biochem. Biophys. Res. Commun.* **136**, 169-176

375 Rossi, A.G., Thomas, M.J., and O'Flaherty, J.T. (1988) *FEBS Lett.* **240**, 163-166