Regulation of 5-oxo-ETE Synthesis in Inflammatory Cells

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Zusammenfassung

5-Oxo-ETE (5-oxo-6,8,11,14-eicosatetraenoic acid) ist ein sehr potenter chemo-taktischer Faktor für Granulozyten, der mittels eines selektiven G-Protein-gekoppelten Rezeptors agiert. Dieser potenziell wichtige Entzündungsbotenstoff wird durch Oxidation von dem 5-Lipoxygenaseprodukt 5-HETE (5*S*-Hydroxy-6,8,11,14-eicosatetraensäure) gebildet. Diese Reaktion wird von 5-Hydroxyeisosanoid Dehydrogenase (5h-dh) unter Verwendung von $NADP^+$ als Reduktionspartner katalysiert. Obwohl gezeigt wurde, daß Entzündungszellen und Thrombozyten dieses Enzym expremieren, war wenig über dessen Regulation bekannt. Das Ziel der vorliegenden Studie war es, die der 5-oxo-ETE-Produktion zugrunde liegenden Regulationsmechanismen aufzuklären. Wir gingen diese Aufgabe aus drei Richtungen an, indem wir die Expression von 5h-dh in unbehandelten und differenzierten myeloiden Zelllinien, den Einfluß oxidativen Stresses und Glukose, sowie die Expression von 5h-dh in nicht-myeloiden Zelllinien untersuchten.

Die erste Zielstellung dieser Arbeit war es zu klären, ob die promyeloide Zelllinie HL-60 und die promonozyte Zelllinie U-937 diese 5h-dh enthalten und ob sich deren Aktivität während myeloider Zelldifferenzierung verändert. Um den zellulären Gehalt abzuschätzen, wurden Zellen zunächst mit PMS (Phenazine Methosulfat), das NADPH nicht-enzymatisch in $NADP^+$ umwandelt, vorbehandelt und danach mit dem Substrat 5-HETE inkubiert. Im Vergleich zu Monozyten oder Granoluzyten produzieren undifferenzierte HL-60 und U-937 Zellen vergleichbare Mengen von 5-oxo-ETE. Darüber hinaus verdreifacht eine dreitägige Behandlung von U-937 Zellen mit PMA (Phorbol 12-myristate 13-acetate) die Enzymaktivtät im Vergleich zu der mit Lösungsmittel behandelten Kontrollgruppe. In ähnlicher Weise verdoppelte die Behandlung von HL-60 Zellen mit dh-VitD_3 (1,25-dihydroxy-Vitamin D_3) für den identischen Zeitraum die 5-oxo-ETE-Produktion im Vergleich zur Lösungsmittelkontrolle. Der Einfluß von PMA auf 5h-dh wurde darüber hinaus in der mikrosomalen Fraktion von U-937 Zellen unter Zuhilfenahme der Michaelis-Menten-Kinetik untersucht und mit neutrophilen Mikrosomen verglichen. Nach der Differenzierung dieser Zelllinie mit PMA verdreifachte sich auch die spezifische Enzymaktivität im Vergleich zu Lösungsmittel-behandelten Zellen. Der K_M-Wert ist vergleichbar in U-937 Zellen und Neutrophilen und wird durch die Differenzierung nicht beeinflußt.

Das zweite Hauptanliegen dieser Arbeit war es zu untersuchen, ob die Produktion von 5-oxo-ETE durch oxidativen Stess und Glukose beinflußt wird. Obwohl Leukozyten und Thrombozyten eine hohe mikrosomale 5h-dh-Aktivität besitzen, wandeln unstimulierte Zellen nur wenig 5-HETE in 5-oxo-ETE um. Um dieses Dilemma zu lösen, untersuchten wir die Möglichkeit, daß die Produktion von 5-oxo-ETE durch oxidativen Stress angeregt wird. Wir fanden, daß H_2O_2 und t-butyl Hydroperoxid die Synthese von 5-oxo-ETE in monozytischen U-937 Zellen sehr stark stimulierten. Dieser Effekt hing von dem GSH-Redoxzyklus ab, da er durch Depletion von GSH oder durch Inhibierung der GSH-Reduktase geblockt und mittels Diamid-induzierter Oxidation von GSH zu GSSG simuliert werden kann. Aufgrund seiner Verarbeitung durch den Pentosephosphat Zyklus, der mittels Dehydroepiandrosterone unterbrochen werden kann, inhibierte Glucose den stimulierenden Effekt von H_2O_2. Die Synthese von 5-oxo-ETE wurde durch H_2O_2 auch in aus humanem Blut gewonnen Monozyten, Lymphocyten und Thrombozyten aber nicht in Neutrophilen angeregt. Im Gegensatz zu Monozyten zeigten sich Thrombozyten und Lymphozyten allerdings resistent gegenüber den

inhibierenden Einflüssen von Glukose. T-butyl Hydroperoxid ehöhte auch die Produktion von 5-oxo-ETE nach Zugabe von Ionophore und Arachidonsäure zu mononukleären Blutzellen. Oxidativer Stress agiert möglicherweise durch Depletierung von NADPH, welches zwangsläufig zu $NADP^+$, dem Cofaktor von 5h-dh, umgewandelt wird. Der Pentosephosphat-Zyklus wirkt diesem Mechanismus entgegen, da in ihm unter Verbrauch von Glukose $NADP^+$ wieder in NADPH umwandelt wird.

Der dritte Schwerpunkt dieser Studie galt der Frage ob nicht-myeloide Humanzellen 5h-dh expremieren. Es ist bekannt, daß andere an der Biosynthese von Leukotrienen beteiligte Enzyme (z.B. LTA_4-Hydrolase und LTC_4-Synthase) wesentlich breiter im Körper verteilt sind als 5-Lipoxygenase, die den ersten Schritt der Leukotrien- und 5-oxo-ETE-Biosynthese katalysiert. Diese Ergebnisse veranlaßten uns zu der Vermutung, daß nicht-myeloide Zellen ebenfalls 5h-dh expremieren. Wir nahmen an, daß Zellen, die wichtig für die Leukozytenmigration sind und oxidativem Stress unterliegen, dieses Enzym enthalten. Zunächst überprüften wir mehrere sekundäre Epithelzelllinien auf ihr Vermögen 5-oxo-ETE zu produzieren, indem wir PMS und 5-HETE zugaben. Wir fanden, daß alle untersuchten Epithelzelllinien in der Lage sind, erhebliche Mengen von 5-oxo-ETE zu produzieren. Um abzuklären, daß das für diese Reaktion verantworliche Enzym mit dem zuvor in Leukozyten beschriebenen identisch ist, untersuchten wir diese Reaktion genauer in Zellfraktionen von A549 Zellen, einer Typ-II alveolaren Epithelzelllinie. Drei Indizien lassen vermuten, daß die epithele 5h-dh der myeloiden entspricht: (i) die enzymatische Aktivtät liegt vor allem in der mikrosomalen Fraktion vor, (ii) bei dem Kofaktor handelt es sich um $NADP^+$ und nicht um NAD^+, und (iii) 5*S*-HETE ist das bevorzugte Substrat. Weitere Studien zeigten, daß auch primäre humane Aorta-Endothelzellen 5h-dh expremieren. Die geringe Verfügbarkeit dieser Zellen erlaubte uns allerdings keine weitergehende biochemische Charakterisierung von endotheler 5h-dh. Vergleichbar zu Entzündungszellen wird die Produktion von 5-oxo-ETE auch in Endothel- und in Epithelzellen durch oxidativen Stress angeregt.

Die hier vorgelegten Ergebnisse untermauern die Annahme, daß es sich bei 5-oxo-ETE um einen wichtigen Entzündungsbotenstoff handelt. 5-oxo-ETE wird am Enzündungsort möglicherweise sowohl von Entzündungs- als auch von Gewebezellen gebildet. Oxidativer Stress ist moglicherweise ein wichtiger Mechanismus, um die Produktion von 5-oxo-ETE anzuregen, was schließlich die weitere Infiltration von Granulozyten fördert und somit den Entzündungsprozeß verlängert.

Schlagwörter:

5-oxo-ETE
Oxidativer Stress
Leukozyten
Epithelium
Endothelium
Leukotriene
5-Lipoxygenase
Entzündung

Abstract

5-Oxo-ETE (5-oxo-6,8,11,14-eicosatetraenoic acid) is a highly potent granulocyte chemoattractant that acts through a selective G-protein coupled receptor. This potentially important inflammatory mediator is formed by oxidation of the 5-lipoxygenase product 5-HETE (5S-hydroxy-6,8,11,14-eicosatetraenoic acid) by 5-hydroxyeicosanoid dehydrogenase (5h-dh) with $NADP^+$ as the electron acceptor. Although it had been shown that this enzyme is expressed in inflammatory cells and platelets, little was known about its regulation. The objective of this study was to investigate underlying regulatory mechanisms of 5-oxo-ETE production in human cells. We addressed this matter from three directions by investigating the expression of 5h-dh in undifferentiated myeloid cell and the impact of differentiation, the effects of oxidative stress and glucose on 5-oxo-ETE synthesis by blood cells and myeloid cells, and the expression of 5h-dh in non-myeloid cells.

The first objective of this study was to determine whether the HL-60 promyelocytic cell line and the U-937 monoblastic cell line contain this 5h-dh and if its activity changes during myeloid cell differentiation. To evaluate cellular 5h-dh content, cells were preincubated with PMS (phenazine methosulfate), which converts NADPH to $NADP^+$, the cofactor of this enzyme, followed by the addition of the substrate 5-HETE. Undifferentiated U-937 and HL-60 cells produce similar amounts of 5-oxo-ETE compared to monocytes or neutrophils. Furthermore, incubation of U-937 cells with PMA for 3 days resulted in a 3-fold increase in production of 5-oxo-ETE compared to vehicle treated cells. Similarly, incubation of HL-60 cells with dh-$VitD_3$ for an identical period resulted in a 2-fold increase in 5-oxo-ETE production compared to vehicle treatment. The impact of PMA on 5h-dh was further investigated in the microsomal fraction of U-937 cells and compared to neutrophil microsomes, using the Michaelis-Menten kinetics. After differentiation of this cell line with PMA the specific 5h-dh activity is increased by threefold compared to vehicle-treated cells. The K_M of 5h-dh is similar in U-937 cells and neutrophils and is not affected by differentiation.

The second objective was to investigate whether 5-oxo-ETE production could be regulated by oxidative stress and glucose levels. Although leukocytes and platelets display high microsomal 5h-dh activity, unstimulated intact cells do not convert 5-HETE to appreciable amounts of 5-oxo-ETE. To attempt to resolve this dilemma we explored the possibility that 5-oxo-ETE synthesis could be enhanced by oxidative stress. We found that H_2O_2 and t-butyl hydroperoxide strongly stimulate 5-oxo-ETE formation by U-937 monocytic cells. This was dependent on the GSH redox cycle, as it was blocked by depletion of GSH or inhibition of glutathione reductase and mimicked by oxidation of GSH to the GSSG by diamide. Glucose inhibited the response to H_2O_2 through its metabolism by the pentose phosphate pathway, as its effect was reversed by the glucose-6-phosphate dehydrogenase inhibitor dehydroepiandrosterone. 5-Oxo-ETE synthesis was also strongly stimulated by hydroperoxides in blood monocytes, lymphocytes, and platelets, but not neutrophils. Unlike monocytic cells, lymphocytes and platelets were resistant to the inhibitory effects of glucose. 5-Oxo-ETE synthesis following incubation of peripheral blood mononuclear cells with arachidonic acid and calcium ionophore was also strongly enhanced by t-butyl hydroperoxide. Oxidative stress could act by depleting NADPH, resulting in the formation $NADP^+$, the cofactor for 5h-dh. This is opposed by the pentose phosphate pathway, which converts $NADP^+$ back to NADPH at the expense of glucose.

The third objective was to determine whether non-myeloid human cells express 5h-dh. It has been shown that the distribution of enzymes involved in leukotriene synthesis (LTA_4 hydrolase and LTC_4 synthase) is considerably wider than that of 5-lipoxygenase, which catalyzes the first step in leukotriene and 5-oxo-ETE synthesis. This led us to hypothesize that non-myeloid cells may express 5h-dh activity as well. We speculated that cells that are important for leukocyte trafficking and are subject to oxidative stress in vivo might contain this enzyme. We first screened several secondary epithelial cell lines for their ability to synthesize 5-oxo-ETE by adding 5-HETE and PMS and found that are all are capable of synthesizing substantial amounts of his substance. To clarify whether the enzyme responsible for this activity in epithelial cells is similar to inflammatory cell 5h-dh, we studied the synthesis of 5-oxo-ETE in more detail in subcellular fractions of A549 cells, a lung type II alveolar epithelial cell line. Three lines of evidence suggest that the epithelial 5h-dh and the inflammatory cell 5h-dh are identical: (i) the enzymatic activity is localized in the microsomal fraction, (ii) the cofactor is $NADP^+$ and not NAD^+, and (iii) 5*S*-HETE is the preferred substrate. We also found that primary human aortic endothelial cells express 5h-dh. However, limitations in their availability did not permit extensive investigation of the biochemical characteristics of endothelial 5h-dh. 5-oxo-ETE production by both endothelial and epithelial cells is regulated by oxidative stress in a manner similar to inflammatory cells.

The results of this study support the notion that 5-oxo-ETE is an important inflammatory mediator. At the site of inflammation, it could be produced by both inflammatory cells and structural cells. Oxidative stress could be an important mechanism for stimulating 5-oxo-ETE formation in inflammation, promoting further infiltration of granulocytes thus prolonging inflammation.

Keywords:

5-oxo-ETE
Oxidative Stress
Leukocytes
Epithelium
Endothelium
Leukotrienes
5-Lipoxygenase
Inflammation

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Für meine Eltern.

One does not discover new lands without consenting to lose sight of the shore for a very long time.

Andre Gide (1869-1951)

Abbreviations

List of most frequently used abbreviations. Additional descriptions for enzyme subclasses within the text are explained when introduced. See Appendix for selected eicosanoid structures and most frequently used chemicals.

AA	arachidonic acid; 5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid
ASM	Airway smooth muscle
3-AT	3-Amino-1,2,4-triazole
BCNU	1,2-bis[2-chloroethyl]-1-nitrosourea; carmustine
CAPS	3-(cyclohexylamino)-1-propane sulfonic acid
$cPLA_2$	Cytosolic phospolipase A_2
CYP	Cytochrome P-450
Cys-LTs	Cysteinyl-leukotrienes
DA	Diamide
DHA	4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosahexaenoic acid
DHEA	Dehydroepiandrosterone
dh-$VitD_3$	1,25-dihydroxy-Vitamin D_3
5,15-diHETE	5*S*,15*S*-dihydroxy-6*E*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid
5,20-diHETE	5*S*,20-dihydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid
DMSO	Dimethylsulfoxide
EC_{50}	Effective concentration leading to 50% of the maximal response
EPA	5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-eicosapentaenoic acid
EPO	Eosinophil peroxidase
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
fMLP	*N*-Formylmethionyl-leucyl-phenylalanine
FOG_7	5-oxo-7-glutathionyl-8*Z*,11*Z*,14*Z*-eicosatrienoic acid
G6P-dh	Glucose-6-phosphate dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
GPCR	G protein-coupled receptor
GPx	Glutathione peroxidase
GST	Glutathione-S-transferase

5h-dh	5*S*-hydroxyeicosanoid dehydrogenase
15h-PG-dh	15-hydroxyprostaglandin dehydrogenase
HAEC	Human aorta endothelial cells
HEPPSO	(2-Hydroxyethyl)-piperazine-N'-(2-hydroxypropanesulfonic acid)
HETEs	Hydroxyeicosatetraenoic acids
5-HETE	5*S*-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid
5-HpETE	5*S*-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid
12-HETE	12*S*-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid
15-HETE	15*S*-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid
12-HHTrE	12-hydroxy-5,8,10-Heptadecatrienoic acid
5-HpETE	5*S*-hydroperxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid
13-HODE	13*S*-hydroxy-9*Z*,11*E*-octadecadienoic acid
13-HpODE	13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid
IC_{50}	Concentration leading to 50% of maximal inhibition
IL	Interleukin
JNK	c-jun-NH_2-terminal kinases
MES	2-morpholinoethanesulfonic acid
5-oxo-ETE	5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid
12-oxo-ETE	12-oxo-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid
15-oxo-ETE	15-oxo-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid
5o-ETrE	5-oxo-8*Z*,11*Z*,14*Z*-eicosatrienoic acid
5o-20-HETE	5-oxo-20-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid
5-LO	5-Lipoxygenase
LOOH	Lipid hydroperoxide
LPS	Lipopolysaccharide
LT	Leukotriene
LTA_4	5*S*-*trans*-5,6-oxido-7*E*,9*E*,*11Z*,*14Z*-eicosatetraenoic acid
LTB_4	5*S*,12*R*-dihydroxy-6*Z*,8*E*,10*E*,14*Z*-eicosatetraenoic acid
LTC_4	5*S*-hydroxy-6*R*-(*S*-glutathionyl)-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid
LTC4-S	LTC_4 synthase
LXA_4	Lipoxin A_4

LXB_4	Lipoxin B_4
MAPK	Mitogen activated protein kinase
MAPKK	MAPK kinase
MPO	Myeloperoxidase
NEM	N-Ethylmaleimide
OXE	5-oxo-ETE receptor
PAF	Platelet activating factor
PBMC	Peripheral blood mononuclear cells
$PBS^{+/-}$	Phosphate buffered saline ($^{(+)}$ 2 mM Ca^{++}, 2 mM Mg^{++}; $^{(-)}$ No Ca^{++}/Mg^{++})
PGB_2	Prostaglandin B_2, 9-oxo-15*S*-hydroxy-prosta-5*Z*,8(12),13*E*-trien-1-oic acid
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PMNL	Polymorphonuclear leukocytes
PMS	Phenazine methosulfate
PMSF	Phenylmethanesulfonyl fluoride
PPAR	Peroxisome proliferator-activated receptor
PPP	Pentose-phosphate pathway
Prx	Peroxiredoxin; thioredoxin peroxidases
PTP	Protein tyrosine phosphatase
PUFA	Polyunsaturated fatty acids
RA	Retinoic acid
RP-HPLC	Reverse phase-high pressure lipid chromatography
SA	Sodium azide
SOD	Superoxide Dismutase
tBuOOH	tert-Butyl hydroperoxide
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor-α
Trx	Thioredoxin
TrxR	Thioredoxin reductase

I INTRODUCTION

I.1. Eicosanoids in Inflammation

The focus of this thesis is the production of 5-oxo-ETE by inflammatory and tissue cells and its regulation by oxidative stress. Before describing the body of acquired knowledge on 5-oxo-ETE, oxidative stress and the regulation of 5-lipoxygenase (5-LO) in more detail, a brief overview of eicosanoid production will be presented. As eicosanoids can be formed by the interaction of different cell types, this will be followed by a brief introduction to transcellular metabolism. Finally, the biological effects of eicosanoids on leukocytes will be dissected, with particular emphasis being placed on their potent effects on eosinophil migration.

I.1.1 Formation of Eicosanoids

Almost all mammalian cells except red blood cells produce eicosanoids, the collective name for oxygenation products of arachidonic acid (AA) and other polyunsaturated 20-carbon fatty acids. Eicosanoids are hormone-like, in that they have profound physiological effects at very low concentrations (EC_{50} in the range of 1-10 nM). They act mainly by stimulating 7-transmembrane receptors coupled to various second messenger pathways through G-proteins (GPCRs). The International Union of Pharmacology (IUPHAR) designated the eicosanoid receptors based on their agonist and antagonist selectivity and on the binding affinities of the cloned receptors. The committee receptors of 5-LO derived products are denominated as BLT_1 and BLT_2 (for LTB_4), $cysLT_1$ (for LTD_4), $cysLT_2$ (for LTC_4 and LTD_4), ALX (for lipoxins), and OXE (for 5-oxo-ETE) [1,2]. Unlike hormones, however, eicosanoids are not transported in the blood stream to their site of biological action. Instead, these chemically and biologically unstable substances are metabolized and degraded within minutes or less in the body or *in vitro*, and act as autocrine and paracrine mediators, executing their effects in close proximity to their site of synthesis.

In humans, the most important eicosanoid precursor is arachidonic acid (5,8,11,14-eicosatetraenoic acid). Arachidonate is stored in cell membranes esterified at the S_N2 position of phospholipids. The production of eicosanoids is controlled by arachidonate release from phospholipids, which is facilitated by a phospholipase A_2 (PLA_2). Cytosolic PLA_2 ($cPLA_2$) is the major pathway in the release of AA during inflammatory responses in leukocytes and its activation is crucial for eicosanoid formation [3]. Among the wide family of phospholipases, two secretory PLA_2s, $sPLA_2$-IIA and $sPLA_2$-V have also been implicated in some specific inflammatory processes [3]. In contrast to other mediators such as histamine, which are preformed and stored in granules, eicosanoids are newly synthesized upon cell activation. As shown in Figure I.1, two main pathways exist for arachidonic acid metabolism during inflammation. The cyclooxygenase (COX) pathway forms the prostanoids, including prostaglandins (PGs) D_2, E_2, $F_{2\alpha}$, and I_2 and thromboxane A_2 (TXA_2). The 5-LO pathway leads to the production of leukotrienes (LTs) and 5-oxo-ETE [4]. In contrast to prostaglandins, which can be formed by two isoenzymes (COX-1 and COX-2) in a wide range of cells, 5-LO is largely restricted to white blood cells and mast cells. Various inflammatory and hypersensitivity disorders (such as asthma) are associated with elevated levels of LTs. The different enzymes involved in LT formation as well as the LT receptors are now targets of several drugs in clinical use. There are also other lipoxygenases in humans, including 12-LO

[5], which generates 12-HETE and the Hepoxilins, and 15-LO [6], which produces 15-HETE and the lipoxins. Lipoxins are produced by interaction of these pathways (see below) with 5-LO.

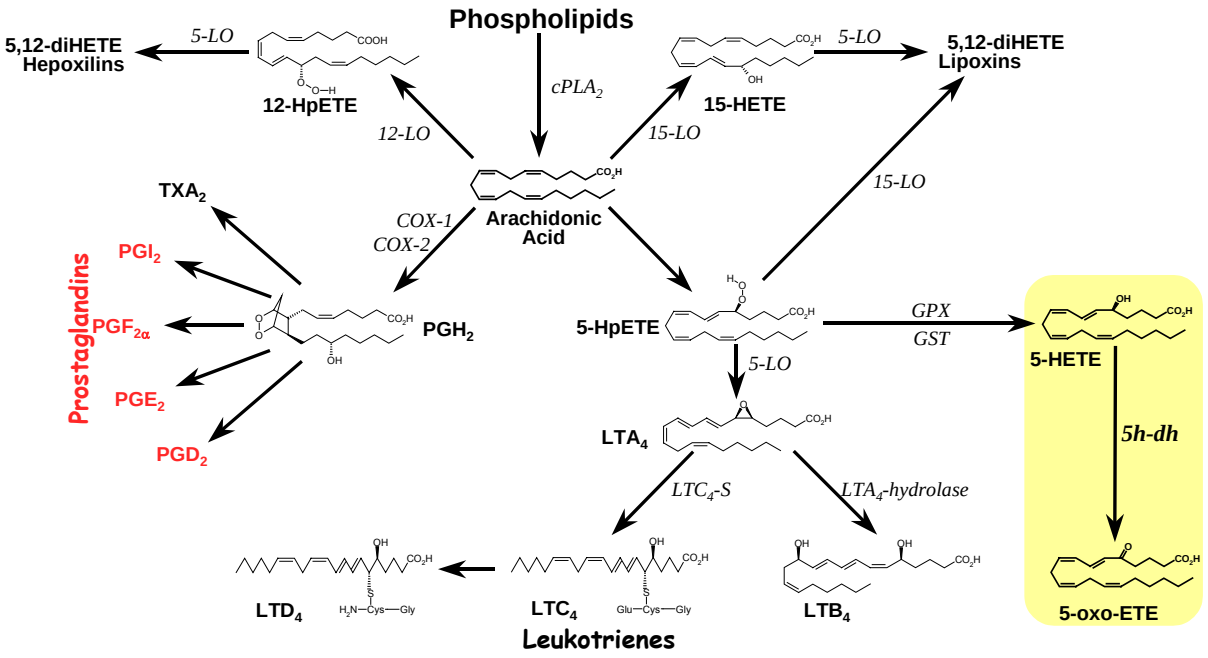


Figure I.1 Predominant pathways of eicosanoid formation in human leukocytes
The formation of eicosanoids during inflammation is initiated by the release of AA from phospholipids by cPLA$_2$. AA is then metabolized by COX, leading to the formation of thromboxanes and prostaglandins, or by 5-LO, which produces LTA$_4$, the precursor of leukotrienes. The 5-LO reaction intermediate 5-HpETE can be reduced by GPx or GST to 5-HETE. 5h-dh, the subject of this study, can convert 5-HETE to 5-oxo-ETE. See text for abbreviations and further information.

LTs result from the catalytic activity of 5-LO, a single protein possessing both dioxygenase activity necessary for the synthesis of 5*S*-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HpETE), and epoxygenase activity leading to leukotriene A$_4$ (LTA$_4$) formation. Depending on the cell type and activation state, LTA$_4$ can be converted by the action of LTA$_4$ hydrolase into LTB$_4$, or it may be conjugated with reduced glutathione by LTC$_4$ synthase (LTC$_4$-S) to produce LTC$_4$, the first member of the cysteinyl-LT family. 5-HpETE, the intermediate of 5-LO can be released from the enzyme. Although it is stable in buffer, it is rapidly reduced in cells by a ubiquitous peroxidase to 5*S*-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HETE). 5-HETE is often the predominant, but relatively inactive metabolite of AA in cells containing 5-LO [7,8]. 5-Hydroxyeicosanoid dehydrogenase (5h-dh) oxidizes and biologically activates 5-HETE to 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-oxo-ETE) utilizing NADP$^+$ as the electron acceptor (see I.2.3 for details).

I.1.2 Transcellular Biosynthesis of Lipoxygenase Products

Transcellular biosynthesis of eicosanoids is a special mode of cell communication whereby the production of eicosanoids by mixed cell populations differs from that expected from each

individual cell type. Intermediates produced in the synthesis of eicosanoids (e.g. PGH_2 and LTA_4) can be released into the extracellular milieu and converted by other cells to biologically active products, a process which is known as intercellular, or transcellular metabolism. Formation of the cysteinyl-LTs (LTC_4, LTD_4, and LTE_4), LTB_4, and lipoxins can all be formed by this mechanism. Transcellular biosynthesis of LTC_4 occurs via transfer of the highly unstable 5-LO product LTA_4 from neutrophils to nearby acceptor cells devoid of 5-LO activity such as platelets or endothelial cells [9]. In humans, LTC_4-S is expressed mainly in eosinophils, basophils, and platelets. In human endothelial cells, microsomal glutathione-S-transferase type-2 (mGST-2) also produces LTC_4 [10]. Transcellular synthesis of cysteinyl-LTs (cys-LTs) also occurs in the liver [11]: LTA_4, synthesized by Kupffer cells, is taken up by hepatocytes, converted into LTC_4, and then released into the extracellular space, acting in a paracrine way on Kupffer and sinusoidal endothelial cells. Thus, hepatocytes cannot directly oxidize arachidonic acid to eicosanoids. In the bloodstream, leukocytes may release LTA_4, which is converted into LTC_4 by endothelial cells. Endothelial and potentially epithelial cells can produce eicosanoids in the form of prostanoids without transcellular metabolism, as they either posses constitutively expressed COX-1 or inducible COX-2 [12,13].

Another important example of transcellular metabolism is the production of lipoxins, which were initially identified as the only inhibitory or anti-inflammatory eicosanoids that act via an agonist role as a "stop signal" by ALX activation [1], by the combined actions of leukocyte 5-LO and either 12-LO, 15-LO, or COX-2. A lipoxygenase product may be released from one cell and then converted by a second lipoxygenase in a different cell type (for review see [14]). Depending on the cellular composition, different routes exist for lipoxin-formation. For example, the abundant platelets contain highly active 12-LO, which can convert neutrophil derived LTA_4 to lipoxin A_4 or lipoxin B_4. 15-LO, present in eosinophils, airway epithelial cells, and IL-4/IL-13 treated monocytes, can also facilitate lipoxin formation [14]. Lipoxins can thus be produced by several pathways involving transcellular biosynthesis in the lung, and increased levels of these substances are found in BAL fluid from subjects with asthma and other lung diseases [15]. In addition to prostaglandins, cyclooxygenase converts AA to small amounts of 11-HETE and 15-HETE. However, when COX-2 is treated with aspirin, unlike COX-1, it remains catalytically active and converts AA to 15*R*-HETE rather than PGH_2 [16]. 15*R*-HETE can then be converted by 5-LO in other cell types to 15*R*-LXA_4 (15-epi-LXA_4), which has the opposite chirality at C_{15} to LXA_4 generated by other mechanisms. For example, coincubation of neutrophils with aspirin-treated endothelial cells or A549 airway epithelial cells in which COX-2 has been induced leads to the formation of "aspirin-triggered lipoxins" [14]. As the receptor for LXA_4 interacts similarly with 15*R*- and 15*S*-LXA_4, the aspirin-triggered 15-epi-LXA_4 has a high biological potency.

I.1.3 Eicosanoids as Mediators in Inflammation

Infections with pathogens or allergic responses trigger an acute inflammatory response in which cells of the immune system move into the affected site [17]. This process is regulated by lipid mediators, cytokines, and chemokines. However, this section will focus on the role of eicosanoids on leukocyte infiltration, with emphasis on the eosinophils, as these cells are the major target for 5-oxo-ETE. A detailed discussion of the role of cytokines and chemokines

would go beyond the scope of this dissertation but an excellent review on this subject by Busse and Lemanske is advisable [18].

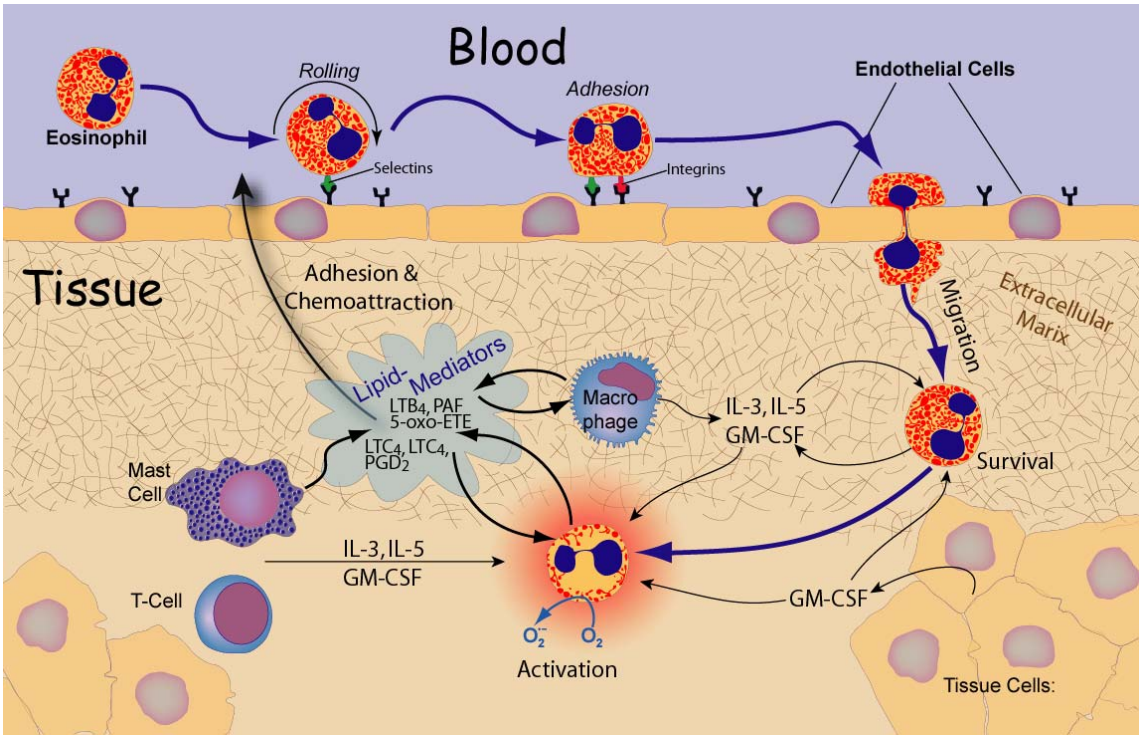


Figure I.2 Role of inflammatory mediators in eosinophil chemotaxis and activation
Peripheral blood eosinophils first roll on and then tightly adhere to the endothelium by interaction of selectins and integrins with the endothelial cell receptors. Following a concentration gradient of chemoattractants, they undergo diapedeisis between endothelial cells and then migrate to the site of inflammation. In the tissue, the life span of the eosinophil may be enhanced by cytokines, i.e. IL-5 and GM-CSF. When the eosinophil is fully activated it releases large amounts of granule proteins, reactive oxygen species such as superoxide ($O_2^{\bullet -}$) and its metabolites, and 5-LO products such as LTC_4, LTD_4, and 5-oxo-ETE. Among other cell types, mast cells and tissue macrophages also produce lipid mediators. Eicosanoids enhance the inflammatory process by stimulating integrin and selectin expression, inducing chemotaxis, stimulate cytokine expression, respiratory burst, and degranulation.

Pronounced tissue eosinophilia is a hallmark of a number of diseases including allergic disorders and parasitic infections [19]. In asthmatic subjects, increased numbers of these cells are found in airways and in bronchoalveolar lavage fluid following allergen challenge [20]. Eosinophils develop in the bone marrow in response to the stimulation of eosinophil progenitor cells by interleukin-5 and other cytokines [19]. Fully mature eosinophils are then released in the blood stream. To participate in inflammatory responses, the eosinophil must migrate from the circulation into the tissue. First, they roll on the blood vessel wall due to loose connections that are established primarily through the interaction of selectins with receptors on endothelial cells for these molecules [19]. When given proper stimuli, tight adhesion of these leukocytes to the endothelium is established, mainly mediated by the binding of integrins to their surface receptors. Eicosanoids can induce shedding of L-selectin from the surface of leukocytes, which is then replaced by other cell surface adhesion molecules such as integrins, thus establishing tighter adhesion [19]. The expression of active integrins on the surface of leukocytes can be induced by certain eicosanoids, enhancing adhesion to the endothelium by exocytosis of

receptor molecules. On exposure to chemoattractant mediators, eosinophils undergo diapedesis between endothelial cells and migrate into the tissues. An internal compass interprets the gradient of an external chemoattractant to orientate the eosinophils polarity in the right direction. Thus, in contrary to bacteria, which swim in a jerky trajectory using a temporal strategy to interpret the gradient, eukaryotes implement a spatial strategy. The accumulation of eosinophils in tissue is further regulated by the generation of survival and activation factors (IL-3, IL-5, and granulocyte–macrophage colony-stimulating factor [GM-CSF]) by T cells, monocytes, and probably mast cells. The indirect effect of 5-oxo-ETE on eosinophil survival by inducing cytokine expression in monocytes is described in more detail in chapter I.2.1.

At the site of inflammation, the leukocytes are activated. In eosinophils, the respiratory burst is induced in order to produce reactive oxygen species (ROS). In addition, eosinophils kill pathogens at the site of inflammation by releasing major basic protein and a matrix composed of eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase (EPO) by degranulation [19]. In chronic inflammatory diseases however, both cytotoxic actions lead to unwanted tissue destruction. Simultaneously, eosinophils start to release inflammatory mediators such as chemokines and eicosanoids, initiating further recruitment of leukocytes to the site of inflammation. Inflammatory mediators such as eicosanoids act at many steps of this complex process. Although *in vitro* experiments often focus on a single mediator, it is important to keep in mind that cells are never exposed to only one specific stimulus but to a variety of endogenous agonists derived from different cell types. Certain cytokines for example may prime cells to respond to another agonist. In this case, the cytokine may not induce the cellular response by itself but enable the cell to respond to a second agonist or enhance the potency of the second agent.

Eicosanoids are involved in tissue migration of leukocytes at all steps. 5-oxo-ETE, the subject of this study, can induce a variety of these responses at several steps of the inflammatory process (described in detail in section I.2.1, below). 5-Oxo-ETE enhances the expression of integrins, is a very potent chemoattractant for eosinophils and other leukocytes, induces GM-CSF release from monocytes, and is capable of terminally activating leukocytes.

Leukotrienes appear to possess additional biological functions. Besides participating in leukocyte tissue migration, cys-LTs are involved in airway smooth muscle (ASM) constriction, explaining at least in part the cause of asthmatic bronchoconstriction [21]. Cys-LTs also appear to have a distinct effect on vascular permeability, which leads to the formation of edema. LTC_4 and LTD_4 have been shown to increase mucus secretion by globlet cells of airway epithelium. Based on these findings LTs were deemed potential targets for anti-asthma drugs, and new LT receptor antagonists have resulted in a better quality of life for at least a subset of asthma patients [21].

I.2. 5-oxo-ETE

I.2.1 Biological Activity of 5-Oxo-ETE

Since the discovery of the pathways for the formation of 5-oxo-ETE in 1992 [22], many biological effects of this compound on myeloid and structural cells have been discovered. 5-oxo-ETE is unique; besides 12-oxo-ETE (12-KETE) it is the only keto-derivative of a HETE

with a higher potency than its parent compound. To date, research has focused on the role of 5-oxo-ETE in inflammatory processes involving leukocyte recruitment and activation. 5-Oxo-ETE shows the most pronounced effects on eosinophils, but also activates neutrophils and monocytes. In this context, 5-oxo-ETE is best known to evoke leukocyte chemotaxis. Additional experiments showed that 5-oxo-ETE is a potent signal for certain non-myeloid cells as well.

Stimulatory Effects of 5-Oxo-ETE on Leukocytes *In Vitro*

Various stages of the inflammatory response can be investigated using different *in vitro* assays. As indicated above, leukocytes leave the blood stream and migrate into the tissue. The necessary steps in this process are rolling and adherence to endothelial cells. 5-oxo-ETE has been shown to induce integrin (e.g. CD11b) expression in neutrophils and eosinophils [23,24] and is able to induce L-selectin shedding [24] as measured by flow cytometry. The Boyden Chamber system has been utilized to study leukocyte migration [25]. In this setting, 5-oxo-ETE proved to be a potent chemoattractant for eosinophils [7], neutrophils [26]. Although 5-oxo-ETE is less potent than LTB_4 as a neutrophil agonist [23,26], it acts via the specific 5-oxo-ETE receptor OXE. 5-oxo-ETE effects are self limiting, suggesting desensitization of OXE, as other neutrophil agonists like LTB_4 or other unrelated agonists do not inhibit its activity [23,26-29]. In contrast, 5-oxo-ETE is a very potent eosinophil chemoattractant [24,30]. Compared to other chemoattractants, it is about 2.5 times more active than platelet activating factor (PAF) and nearly 40 times more active than LTB_4. 5-oxo-ETE also induces a weak chemotactic response in monocytes, but is about 30 times less potent and is 40% less efficacious [31]. 5-oxo-ETE also induces the migration of eosinophils through Matrigel®, which resembles the basement membrane, a thin structure constituted mainly of collagen IV, laminin and proteoglycans [32].

Actin polymerization, a crucial component of chemotaxis, is induced by 5-oxo-ETE in eosinophils [24,33], neutrophils [23,28], and monocytes [31]. Elevation of intracellular Ca^{++} is also an important intracellular signal, but is not as important as actin polymerization for chemotaxis. However, most chemoattractants also induce a rise in intracellular Ca^{++} and its measurement is of great importance for studying receptor pharmacology. 5-Oxo-ETE has been shown to induce Ca^{++} release in eosinophils [7,24,30] and neutrophils [23,29,34,35], but not in monocytes [31]. 5-oxo-ETE possess additional effects on cell signaling, as it induces extracellular signal-regulated kinase (ERK) stimulation [27,36] and phosphatidylinositol-biphosphate 3-kinase [28] in PMNL. Stimulation of ERK, a mitogen activated protein kinase (MAPK), in these cells by 5-oxo-ETE leads to the phosphorylation and subsequent activation of $cPLA_2$ as measured by AA release [27].

5-oxo-ETE Induces GM-CSF Release from Monocytes

5-oxo-ETE may enhance inflammatory responses by inducing GM-CSF release from monocytes. Stamatiou and coworkers recently discovered that 5-oxo-ETE enhances eosinophil survival [37]. Further investigations revealed that this effect is not mediated by direct induction of survival mechanisms in eosinophils, but rather by release of GM-CSF from monocytes [37], which can contaminate eosinophil preparations in small numbers. GM-CSF in turn enhances the life-span of eosinophils [19]. Since GM-CSF is a central cytokine in several immune regulatory mechanisms, 5-oxo-ETE may play a role in additional settings. Moreover it is possible that 5-oxo-ETE could induce other cytokines as well.

Limitations of 5-oxo-ETE responses

5-oxo-ETE shares the effects described above with other chemotactic factors, such as fMLP, IL-8, eotaxin, RANTES, LTB_4, and PAF but is more selective in its responses. Although 5-oxo-ETE is a potent stimulator of many biological effects in the absence of priming agents, by itself it is only a weak inducer of nitric oxide production ($NO^{\bullet}$) [38], PAF synthesis [28], degranulation [27], fMLP receptor induction [28], and priming agent for fMLP induced $O_2^{\cdot -}$ production [27,28] in PMNL. Contrary to 5-oxo-ETE, these biological responses are induced by other chemoattractants such as LTB_4 [27,28,38]. One study showed that 5-oxo-ETE alone failed to induce degranulation in eosinophils [30], while another showed a weak response [39]. 5-oxo-ETE induces $O_2^{\bullet -}$ production in PMNL, but the efficacy is very low ($1/60^{th}$) in comparison to fMLP and only two-fold increases $cPLA_2$ activity in PMNL [40]. This might be surprising, as 5-oxo-ETE stimulates the ERK pathway and induces Ca^{++} influx, two important requirements for $cPLA_2$ activation (see Figure I.8, page 35). However, one has to keep in mind that timing is important. The 5-oxo-ETE induced ERK phosphorylation is transient in comparison to GM-CSF, similar to the increase in intracellular Ca^{++} in comparison to fMLP [26,27]. Therefore the signals induced by 5-oxo-ETE alone may be too transient in order to be translated into a substantial AA release or $O_2^{\bullet -}$ formation.

Synergistic Effects of 5-oxo-ETE and Other Inflammatory Mediators

In vivo, cells are never exposed to a single mediator and the ultimate response would result from an integration of signals. During pulmonary inflammation for example, lipid mediators, interleukins, and chemokines are released from numerous sources. It would seem likely that both classes of chemoattractants, lipid mediators and chemokines, contribute to eosinophil infiltration into the lungs in asthma. Furthermore, it is possible that there could be synergy between lipid mediators and chemokines or interleukins as they act by distinct receptor-mediated mechanisms. As described for the effect of GM-CSF and LT synthesis, one agonist may prime a cell to respond to a second signal without inducing the response by itself. Based on this hypothesis, several studies have been conducted to examine the cross talk of 5-oxo-ETE with other agents. Indeed, 5-oxo-ETE potentiates eosinophil migration in response to platelet activating factor (PAF) at low concentrations [7]. C-C chemokines show synergistic effects with 5-oxo-ETE on human monocytes and appear to prime eosinophils for 5-oxo-ETE responses. 5-oxo-ETE increases monocyte migration in response to monocyte chemotactic protein-1 (MCP-1) and MCP-2 [31]. In the same study, 5-oxo-ETE also lowered the EC_{50} of MCP-1 induced AA release from monocytes. Eotaxin and RANTES increased the potency of 5-oxo-ETE-induced eosinophil chemotaxis at threshold concentrations, at which both chemokines show minimal activity by themselves, shifting the dose-response for 5-oxo-ETE curve to the left [41].

The effects of 5-oxo-ETE can also be enhanced by cytokines, such as IL-5, GM-CSF, and G-CSF. As described above, 5-oxo-ETE and 5-oxo-15-HETE are normally not capable of inducing appreciable superoxide production and degranulation for human PMNL [28], but pretreatment of these cells with GM-CSF or G-CSF renders these otherwise ineffective agents potent inducers of these responses [27,35]. 5-oxo-ETE can induce eosinophil migration through basement membrane components by itself, but addition of IL-5 however enhances the efficacy of this process [42].

Effects of 5-oxo-ETE on Non-myeloid Cells

Besides the widely studied effects of 5-oxo-ETE on inflammatory responses in leukocytes, this eicosanoid also shows biological activity with epithelial cells that may not be signaled through the "classical" 5-oxo-ETE receptor OXE. These alternative signal pathway, a putative 5-HETE receptor and peroxisome proliferator-activated receptors (PPARs), are discussed below. Diarrhea is an important aspect of innate immunity, it serves to wash out bacteria before they cause harm. In the mammalian gut, chloride secretion by epithelial cells is a major determinant of diarrhea. It occurs mainly in the crypt regions of the epithelium. Several inflammatory mediators are released into the lamina propria including kinins [43], and AA derived factors such as cys-LTs [43,44], prostaglandins [45], and the 5-oxo-ETE precursor 5-HETE [46]. In leukocytes 5-HETE and 5-oxo-ETE share the 5-oxo-ETE receptor, which has much lower affinity for 5-HETE [26,47-49]. This suggests that many of the biological effects activated by 5-HETE could be due to its interaction with OXE. This prompted examination of the effects of 5-oxo-ETE on the shrinkage of guinea pig jejunal crypt epithelial cells, an effect already known for 5-HETE [50]. 5-Oxo-ETE caused rapid shrinkage of these cells to a reduced but stable volume. This response was due to induction of Cl^- secretion, since it was prevented by Cl^- and K^+ channel blockers. Furthermore, inhibitors of protein kinase C (PKC) also abolished the volume reduction induced by 5-oxo-ETE, suggesting that this response to 5-oxo-ETE is mediated by PKC. In this setting 5-oxo-ETE showed an extremely high potency, while the previously studied cys-LTs and 5-HETE demonstrated much lower potencies, and LTB_4 showed no activity. In contrast to its effects on crypt cells, 5-oxo-ETE had no effect on the volume of jejunal villus cells, which were still responsive to bradykinin. This suggests that OXE may be selectively expressed in crypt cells in the guinea pig.

Bronchoconstriction is one feature of asthma that greatly affects the patients quality of life. In the acute phase of an allergic response in the lung, mast cells and macrophages induce the release of proinflammatory mediators such as histamine, eicosanoids and ROS [18,20,21]. The cys-LTs have been recognized to be strong inducers of airway smooth muscle contraction (ASM), the underlying mechanism of bronchoconstriction [18,20,21]. Although 5-HETE is only a weak stimulus for guinea pig ASM contraction, it enhances the response to histamine, indicating a regulatory role of this eicosanoids in bronchoconstriction [51,52].

5-oxo-ETE has recently been shown to induce constriction of guinea pig ASM *in vitro* [53]. The inotropic effect of 5-oxo-ETE is presumably not direct, as this response is prostanoid-dependent and its EC_{50} is much higher in comparison to other biological effects of 5-oxo-ETE. 5-oxo-ETE appears to induce the release of prostanoids and acts predominantly through thromboxane A_2 (TXA_2), as TP receptor antagonists inhibit this process. The response of ASM is calcium dependent, as it can be reduced by Ca^{++} depletion. The Rho-kinase inhibitor Y-27632 blocked the tonic responses induced by 5-oxo-ETE, suggesting that 5-oxo-ETE acts in part by sensitizing myofilaments to intracellular free Ca^{2+} [53]. It has yet to be confirmed whether 5-oxo-ETE induces similar responses in human ASM.

5-oxo-ETE enhances the survival of prostate epithelial cells. The effects of fatty acids on these cells have been studied extensively, because epidemiological studies indicate that dietary fat plays a role as a risk factor for the growth and development of prostate cancer. AA and its precursor, linoleic acid (LA), are major components of animal fat and many vegetable oils as part of diets are used in regions where prostate cancer is common. Ghosh and Myers [54]

showed that exogenous arachidonic acid induces mitogenesis in PC3 and LNCaP cells, two commonly used prostate cancer cell lines. The addition of MK886, a 5-LO activating protein (FLAP) antagonist (for FLAP see page 35), inhibits not only the mitogenic effect of arachidonate, but also decreases the growth rate of PC3 cells below control levels. Similar effects were observed with NDGA (nonspecific lipoxygenase inhibitor), AA861 (specific 5-LO inhibitor), but not with ibuprofen (COX inhibitor), baicalein (12-LO inhibitor), or SKF-525A (CYP inhibitor). The authors speculated that this effect is due to inhibition of 5-LO and that leukotrienes or 5-HETE are integral components of PC3 cell mitogenesis. Indeed, addition of 5-HETE and 5-oxo-ETE overcome the inhibitory effects of MK886 induced growth arrest. Furthermore, 5-HETE and 5-oxo-ETE were also able to induce PC3 cell growth while LTB_4, LTC_4, and LTD_4 were not effective. In a later study [55], the same group showed that human prostate cancer cells rapidly undergo apoptosis when they are deprived of arachidonic acid or when the subsequent metabolism of arachidonic acid by the 5-lipoxygenase pathway is interrupted with MK886. This effect was also overcome by the exogenous addition of 5-HETE or 5-oxo-ETE. They surmised that prostate cancer cells form endogenous 5-HETE, as they detected 5-HETE by radioimmunoassay. However, this technique may be questionable, given that 5-HETE posses a rather simple structure, in comparison to cys-LTs for example, so it is possible that it could cross-react to other fatty acids. To examine the presence of 5-LO in these cells, other techniques, such as LC-MS/MS should be employed. The authors did not elaborate whether 5-oxo-ETE and 5-HETE are inter-converted in these prostate epithelial cells.

***In Vivo* Actions of 5-oxo-ETE**

In vivo experiments are essential in order to evaluate the physiological relevance of a mediator. 5-oxo-ETE may be an important mediator of inflammation, because it is a potent stimulator of leukocyte chemotaxis *in vivo*. Similar to LTB_4, 5-oxo-ETE has been shown to induce pulmonary eosinophilia in Brown Norway rats [56] when instilled intratracheally. This effect is not shared by the cys-LTs and can be abolished by monoclonal antibodies to the integrins VLA-4 and LFA-1. 5-oxo-ETE also elicits the infiltration of eosinophils and neutrophils into the skin of human beings *in vivo* after intradermal administration [57]. Macrophage numbers were also increased at the highest concentrations tested, but the numbers of mast cells and lymphocytes were unchanged. Zimpfer and colleagues reported that injection of 5-oxo-ETE into rabbit subcutis causes a severe edema with an inflammatory cell infiltrate resembling an urticarial lesion [58]. These findings indicate that 5-oxo-ETE may play a role in cellular recruitment to the dermis.

I.2.2 5-oxo-ETE Receptor

Leukocyte Based Receptor Studies

It was long speculated that the biological effects of 5-oxo-ETE on leukocytes are mediated through a specific 7-transmembrane G-protein coupled receptor (GPCR), as the effects of 5-oxo-ETE on monocytes, neutrophils and eosinophils are diminished by pertussis toxin [27,31,33,34]. Structure-activity studies indicate that this receptor is highly selective for 5-oxo-ETE, is subject to homologous, but not heterologous desensitization, and is not blocked by selective antagonists of other chemoattractant receptors. Binding studies involving 5-oxo-ETE have been hampered by its metabolism and especially by its esterification into triglycerides (see page 26). O'Flaherty and colleagues circumvented this problem by using the acyl-CoA

synthetase blocker triacsin C to inhibit the first step of esterification. They were then able to demonstrate high specific affinity (K_d, 4 nM) binding sites for this substance [59]. The affinities of related compounds for these binding sites paralleled their biological activities, whereas ligands for other receptors were unable to compete with 5-oxo-[^{3}H]ETE for these sites. 5-oxo-ETE binding to this receptor can be inhibited by excessive amounts of GTP or its analog GTPγS [59]. 5-oxo-ETE also stimulates the binding of GTP to membrane fractions from neutrophils [60]. Both responses are characteristic for GTP/GDP exchange reactions conducted by GPCRs [60].

In leukocytes, the 5-oxo-ETE-receptor signals mainly through G_{i2}. G proteins fall into four sub-families based on the homology of their α-chains: G_i (inhibitory), G_s (stimulatory), G_q, and $G_{12/13}$. There are also 6 β and 12 γ isoforms, but the different βγ combinations are thought to exhibit similar effects on cell signaling in mammals. The α-chains transmit very specific biological responses. As a result, one GPCR can induce different responses in one cell by binding to different Gα subtypes. The different biological effects of agonists are largely due to different interaction patterns of their GPCRs with Gα-chains and the G protein expression levels in the cells. The knowledge of the α-subtype that interacts with a GPCR and its expression state within a cell type allows, within certain limitations, prediction of the biological response to the GPCR agonist *in vitro*. The specific α-subtype that binds to a GPCR can be identified pharmacologically or by using the proper expression systems, but the GPCR sequence has to be known for the latter approach. Pertussis toxin totally inhibits the effects of 5-oxo-ETE on various biological responses in PMNL [23,27,33,34], but does not totally block the effects of other chemoattractants (e.g. C5a, fMLP, PAF and LTB_4). This suggests that OXE has an absolute requirement for G_i proteins, whereas other chemoattractants can also signal through other G protein such as G_q and $G_{12/13}$. Biochemical and pharmacological evidence led to the conclusion that the receptor for 5-oxo-ETE is bound to $G\alpha_{i2}$ in PMNL [27,60], while it was not possible to determine the specific βγ subunits. This finding may explain the limited activation pattern of 5-oxo-ETE in comparison to other chemotactic factors.

Cloning of the 5-oxo-ETE Receptor

The story leading to the cloning of OXE is quite intriguing. On one hand, the purification of putative GPCRs, such as OXE, from leukocytes with standard biochemical techniques is extremely difficult due to low expression levels. On the other hand, vigorous *in silico* searching and cloning of novel genes with sequence motifs characteristic for GPCRs have outpaced the identification of novel endogenous ligands. This led to the accumulation of putative GPCRs, commonly known as orphan receptors, for which the ligands have not been identified. In their effort to identify ligands of proposed eicosanoid orphan receptors, researchers at MerckFrost® always included 5-oxo-ETE in their ligand library (Julie Evans, personal communications) without finding a potential 5-oxo-ETE receptor. In the meantime, Hosoi and colleagues from Tanabe Seiyaku Co. were searching for novel peptide binding GPCRs. They stumbled upon the 5-oxo-ETE receptor by accident. They screened over a thousand molecules, mainly peptides and chemokines (Tesuo Ohnuki, personal communications), before they found 5-oxo-ETE was indeed the specific agonist for clone TG1019 [48]. Simultaneously, researchers at Novartis Pharmaceuticals Co. also came across an orphan GPCR [clone R527] that binds 5-oxo-ETE [47]. The two receptor sequences proved to be identical (99.7%). The latter one differs from the former by a single point mutation (Leu

→ Val) at amino acid 368 and by a 39 amino acid truncation at the C-terminal. Since the results of both studies are remarkably similar, these differences don't seem to affect the biological activity.

The knowledge of the 5-oxo-ETE GPCR sequence enables new approaches to study the physiological role of 5-oxo-ETE by determining the tissue distribution of its receptor. The expression of OXE was examined by PCR and northern blots [47,48]. The mRNA of the gene encoding this receptor is expressed in many tissues excluding the brain. Dot blot analysis showed somewhat strong expression in the liver and the kidneys. Three hybridization bands with molecular lengths of 6.5, 3.3 and 1.8 kb were identified by northern hybridization. The 1.8 kb transcript is expressed in peripheral leukocytes, lungs, placenta, small intestine, spleen, thymus, colon, heart, skeletal muscle, liver, and kidneys. In contrast, the 6.5 and 3.3 kb transcripts are expressed only in liver, kidneys, and, to a smaller degree, in skeletal muscle. The expression pattern of the receptor in leukocytes, as analyzed by real-time PCR, agrees to the functional studies described above. The 1.8 kb transcript is mainly expressed in eosinophils, and to a lesser degree in neutrophils and alveolar macrophages and barely detectable in T cells or differentiated epithelial cells (normalized transcript numbers are 210, 5.8, 1, 0.06, and 0.012, respectively).

Expression of a GPCR in a controlled environment enables direct binding studies of the receptor to G proteins and direct binding studies with potential agonists or antagonists. The 5-oxo-ETE GPCR TG1019 [48] binds to $G\alpha_i$ and to a much smaller extent to $G\alpha_q$, but does not interact with $G\alpha_{sL}$, $G\alpha_{i1}$, or $G\alpha_{qa}$. The EC_{50} for 5-oxo-ETE to induce Ca^{++} via $G\alpha_{16}$-coupling (member of the G_q subfamily) in the expression assay was much higher than the EC_{50} for 5-oxo-ETE to induce the same response in PMNL or the $G\alpha_i$ coupled assay of forskolin-stimulated cAMP inhibition. Forskolin is a potent agonist for adenylate cyclase, the enzyme that inhibited by $G\alpha_i$. These results are supporting the notion that the natural partner of the 5-oxo-ETE GPCR is indeed $G\alpha_i$. The substrate specificity of OXE was confirmed. It does not interact in an agonistic or antagonistic manner with leukotrienes (7 tested), prostaglandins (8 tested), HETEs other than 5-HETE (7 tested), 15-oxo-ETE, thromboxane B_2, or either lipoxin A_4 or B_4. The rank order of the potencies of agonists, as determined in a $G\alpha_i$ mediated GTPγS-binding assay, is 5-oxo-ETE >> 5-HpETE > AA = 5*Z*,8*Z*,11*Z*-eicosatrienoic acid = 5*S*-HETrE = 5(*R*,*S*)-HETE [48]. This result is surprising, since 5-HpETE is known to stimulate Ca^{++} responses in PMNL [61], but the responsible receptor had not been identified. Furthermore, the lack of stereospecificity of the receptor for 5-HETE was unexpected. The stimulatory action of 100 nM 5-oxo-ETE on GTPγS binding can be antagonized by addition of 4*Z*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosahexaenoic acid (DHA), dihomo-γ-linolenic acid, 11Z,14,Z17Z-eicosatrienoic acid, and EPA.

5-oxo-ETE Signals through a Novel 5-HETE GPCR in PC3 Cells

The underlying signal transduction mechanisms of 5-oxo-ETE on PC3 cells were further investigated by O'Flaherty and coworkers [36]. They confirmed that 5-HETE and 5-oxo-ETE stimulate cell proliferation and inhibit MK-886 induced apoptosis in PC3 cells. 5-HETE is more potent than 5-oxo-ETE in this regard and the authors concluded that a novel 5-HETE-specific receptor exists on this cell line is responsible for this effect. Since growth stimulation can only be detected at longer culture times, they investigated the effects of 5-LO products on ERK and Akt phosphorylation. Although 5-oxo-ETE is about 10 times more potent by

inducing ERK1/2 phosphorylation in human PMNL [27], the reverse is seen in PC-3 cells [36]. Here, 5-HETE is 10 times more potent than 5-HETE and more efficacious than 5-oxo-ETE in inducing cell growth and ERK stimulation, whereas 5-oxo-15-HETE is not active at all. These results suggest that these cells posses a novel receptor for 5-HETE that is selective for 5-HETE over 5-oxo-ETE.

PPARα as an Alternative Signal Pathway?

The mechanism of MK886 induced growth arrest and apoptosis remains controversial [62]. In agreement with the studies mentioned above [36,54,55], Ayyub and colleagues demonstrated that the two potent lipoxygenase inhibitors MK886 and BWA4C block proliferation and DNA synthesis in HL-60, K562 and Jurkat cells [62]. However, the effects are only seen at 100-times the concentration needed to inhibit leukotriene generation in whole blood or leukocytes, suggesting that the inhibition of LT synthesis is not responsible for the anti-mitogenic and apoptotic effects of MK886 [62]. Indeed, it was shown recently that MK886 does not induce apoptosis and growth arrest via FLAP. Downregulating FLAP expression with antisense oligonucleotides had no influence on the potency of MK886 in inducing apoptosis [63]. Vice versa, overexpression of FLAP showed no effect either [64]. Based on this, Kehrer and coworkers investigated alternative routes of action for MK886 [65]. Since FLAP, the proposed target of MK886, is a fatty-acid binding protein, it is conceivable that MK886 may affect other such proteins. They speculated that peroxisome-proliferator-activated receptors (PPARs), which have been implicated in apoptosis and are known to bind eicosanoids, represent a target for MK886. Using different expression and cell culture systems, the authors showed in an excellent study that MK886 inhibits PPARα activation [65]. Substantially less inhibition was evident with the β and γ isoforms of PPAR [65] (for review on the PPAR family please see [66]). In opposite to classical receptors, which are highly selective in their choice of agonists, PPARα posses a broad range of stimuli. This is due to the non-specific nature of its binding site that consists of a hydrophobic pocket that closes after binding in a mouse trap mechanism [67]. Due to the broad ligand specificity, it is conceivable that 5-oxo-ETE and 5-HETE may signal through a PPAR-subtype as well.

I.2.3 5-oxo-ETE Synthesis by 5h-dh

Biochemical characterization of 5h-dh

5-Oxo-ETE is synthesized from the 5-LO product 5*S*-HETE by the highly specific $NADP^+$-dependent 5-hydroxyeicosanoid dehydrogenase (5h-dh), and its formation is regulated by the availability of the cofactor. 5h-dh has so far been reported to be present in neutrophils [22], eosinophils [7], monocytes [68], lymphocytes [68], human blood monocyte derived dendritic cells [69], and platelets [70]. In order to determine the subcellular localization, Powell and coworkers used differential centrifugation to separate granules, microsomes, and cytosol [22]. The formation of 5-oxo-ETE from 5-HETE in the presence of $NADP^+$ was highest in the microsomal fraction and negligible in the cytosol. However, significant activity was found in the granule fraction as well. 5h-dh is selective for $NADP^+$ over NAD^+, and is highly substrate specific. It requires the presence of a 5-hydroxyl group in the *S*-configuration followed by a 6-*trans* double bond, as occurring in 5*S*-HETE. For this reason, 5h-dh does not oxidize the closely related 5-LO product LTB_4, which also has a 5*S*-hydroxyl group, but has the 6-trans-double bond in the *cis*-configuration [22]. Similarly, 5*R*-HETE, which has the required 6-

double bond, but possesses the wrong configuration of the 5-hydroxyl group is also a poor substrate. The enzyme prefers the hydroxyl group to be in the 5-position, as HETEs with hydroxyl-groups in the 8-, 9-, 11-, 12-, or 15-positions are not oxidized. However, 5h-dh metabolizes other fatty acids that fulfill the substrate requirements, including the 6-trans isomers of LTB_4 and 5,15-diHETE, although the K_Ms for these fatty acids are higher than that for 5-HETE.

Formation of 5-oxo-ETE in Cells is limited by $NADP^+$ Levels

The availability of the cofactor $NADP^+$ appears to be a critical limiting factor in the ability of cells to synthesize 5-oxo-ETE, because the reaction is reversible and little 5-oxo-ETE is formed from 5-HETE in resting cells. Although human peripheral blood neutrophils possess high levels of microsomal 5h-dh activity. Resting, unstimulated neutrophils however form only small amounts of 5-oxo-ETE from exogenous 5*S*-HETE [22,71]. Instead, they transform 5-HETE to 5,20-diHETE by NADPH dependent ω-oxidation [72]. Preincubation of neutrophils with phorbol 12-myristate 13-acetate (PMA), dramatically increases the ratio of 5-oxo-ETE to 5,20-diHETE from 0.07 to 1.9 [71]. PMA, which bears some structural remembrance of diacylglycerols, stimulates PKC in neutrophils [73]. PKC in turn stimulates superoxide generation by phosphorylating one ($p47^{phox}$) and inducing membrane translocation of two ($p47^{phox}$ and $p67^{phox}$) cytosolic components of the NADPH oxidase complex. The stimulatory effect of PMA on 5-oxo-ETE generation appears to be caused by an inrease in the $NADP^+$/NADPH ratio due to stimulation of NADPH oxidase [74]. Inhibition of NADPH oxidase by mild heating or by diphenylene iodonium completely reverses the effect of PMA. The effect of PMA is not due to superoxide (H_2O_2), as it is not blocked by superoxide dismutase (SOD) or mimicked by addition of xanthine/xanthine oxidase. The hypothesis that 5h-dh activity is regulated by its cofactor levels is further supported by the stimulatory effect of phenazine methosulfate (PMS) on 5-oxo-ETE formation. This substance is known to promote the non-enzymatic conversion of NADPH to $NADP^+$ but does not directly affect 5h-dh [75,76]. These findings are very relevant to the present study on the regulation of 5h-dh activity. The formation of 5-oxo-ETE from 5-HETE (defined here as forward reaction: $NADP^+$ + 5-HETE → NADPH + 5-oxo-ETE) by 5h-dh appears to be a fully reversible reaction as neutrophil microsomes also catalyze the backward reaction (defined as 5-oxo-ETE + NADPH → 5-HETE + $NADP^+$) in the presence of NADPH [22]. In contrast to neutrophils, in platelets it is the reverse reaction that is predominant [70]. However, it is not clear from the literature whether 5h-dh catalyzes both reactions, or whether this is due to a distinct 5-keto-reductase enzyme.

One aim of this study was to examine additional pathways that regulate the synthesis of 5-oxo-ETE from 5-HETE, as not all cells that contain 5h-dh, i.e. platelets and lymphocytes possess functional NADPH oxidase [68,70]. As described in more detail below, oxidative stress leads to the depletion of the NADPH pool. We hypothesized that the formation of 5-oxo-ETE may be enhanced during oxidative stress (I.3.4, page 32) as an alternative pathway to the activation of NADPH oxidase.

Other Hydroxyeicosanoid Dehydrogenases

In addition to 5h-dh, other hydroxyeicosanoid dehydrogenases oxidize HETEs. 15-Hydroxyprostaglandin dehydrogenases (15h-PG-dh), the first such eicosanoid-metabolizing enzymes to be discovered, convert prostanoids and other 15-hydroxy-eicosanoids to their

biologically inactive 15-oxo metabolites [77], whereas 12-hydroxy-eicosanoid dehydrogenases (12h-dh) convert LTB4, 12-HETE and similar compounds to 12-oxoeicosanoids [29] (see also page 24). The biochemistry of 15h-PG-dh has been studied in detail in several settings, while little is known of the biochemistry of 5h-dh. 15h-PG-dh may serve therefore as model for future studies of 5h-dh.

The NAD^+-dependent 15h-PG-dh (type I) is considered the key catabolic enzyme that controls the biological activity of the prostaglandins [77]. This enzyme catalyzes the reversible oxidation of the 15-hydroxyl group of prostaglandins to produce a 15-keto metabolite with greatly reduced biological activity. The pH optimum of human placental 15h-PG-dh type-I for the forward reaction (15-HETE → 15-oxo-ETE) is between 9.0 and 9.5, while that for the rabbit lung enzyme is between 10 and 10.4 [77]. The reverse reaction (15-oxo → 15-HETE) of these enzymes is favored at a pH < 7 [77]. Kinetic analysis has been used to determine the enzymatic mechanism of the human placental enzyme and the porcine kidney enzyme. Based on the results of initial velocity, product inhibition, and dead-end inhibition studies, an ordered Bi-Bi mechanism has been proposed where $NADP^+$ binds first to the enzyme followed by the prostaglandin. Following oxidation of the substrate the 15-keto metabolite is first released followed by NADH. In addition to the NAD^+-dependent 15h-PG-dh, a NADP+-dependent 15h-PG-dh (type II) has also been identified and characterized [78]. The type II enzyme prefers $NADP^+$ as a cofactor but can also use NAD^+ and it appears to have a much broader range of substrates than the type I enzyme. The type II enzyme is identical to general carbonyl reductase and also posses 9-keto reductase activity besides the 15-hydroxy dehydrogenase activity (for more details see [78]).

I.2.4 Alternative Pathways of 5-oxo-ETE Formation

Several 5h-dh-independent pathways have been shown to yield oxo-fatty-acids via free radical-dependent mechanisms. These include heavy metals, heme-proteins, and other biological agents that catalyze peroxidation of unsaturated fatty acids and decomposition of peroxy products (see [35] for references). Researchers in the group of Robert Murphy specifically investigated the 5-HETE-independent formation of 5-oxo-ETE in murine macrophages and human red blood cell membranes.

5-oxo-ETE is formed by an Alternative Pathway in Murine Macrophages.

Zarini and Murphy found that murine peritoneal macrophages convert racemic 5*R*,*S*-HpETE to 5-oxo-ETE [79]. Examining mechanisms for this reaction, it became evident that it was not due to 5h-dh, because (i) the catalytic activity is due to a trypsin- and heat-insensitive substance that is located in the cytosol and microsomes, (ii) the formation of 5-oxo-ETE by this pathway is not cofactor dependent, (iii) racemic 5-HpETE (a mixture of 5*R*,*S*-HpETE) is the substrate, and (iv) 5*S*-HETE is not converted to 5-oxo-ETE by these cells. Based on the lack of stereospecificity, the authors proposed a redox-mechanism for the conversion of racemic 5-HpETE to 5-oxo-ETE, in which an iron-containing factor initially catalyzes the on

e-electron reduction of 5-HpETE to the 5-alkoxy radical intermediate followed by a one-electron oxidation by removal of the C-5 hydrogen atom (Figure I.3). Size exclusion experiments revealed that a 55 kDa protein facilitates this reaction rather than a low molecular weight substance such as inorganic iron or a small molecule like hematin, the oxidized from of

heme. However, this study did not specifically determine whether the microsomal fraction from these cells contains 5h-dh. It has to be established whether mice do express 5h-dh at all and if 5-oxo-ETE can be formed by a similar mechanism in human cells in addition to 5h-dh.

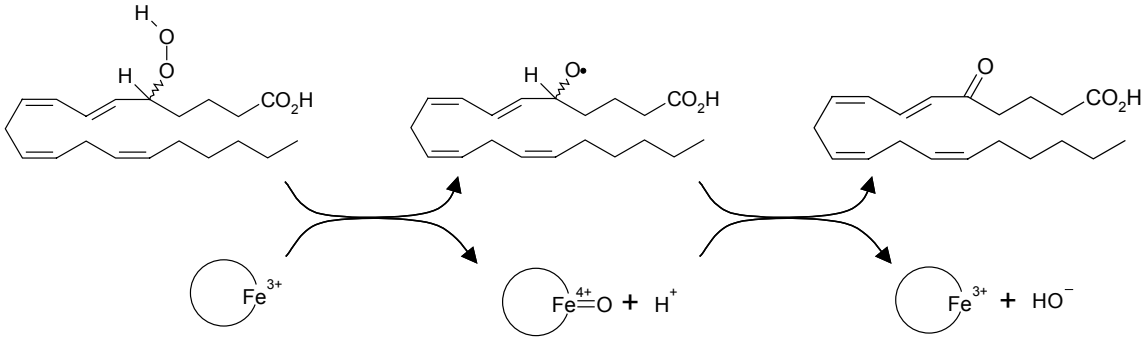


Figure I.3 Proposed mechanism of 5-HpETE to 5-oxo-ETE conversion
Based on this scheme, 5-HpETE is first reduced by an iron-containing factor to the 5-alkoxy radical. In a second oxidation of this radical 5-oxo-ETE is formed as the product [79].

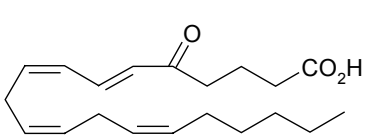
5-oxo-ETE can be formed in Red Blood Cell (RBC) Ghosts by Lipid Peroxidation.

Lipid peroxidation leads to 5-oxo-ETE formation within the plasma membranes of red blood cells (RBC) by a free radical reaction. The oxidation of free arachidonic acid by the iron-containing enzymes of the LO- and the COX-families is carefully controlled. However, the discovery of compounds isomeric to prostaglandins (isoprostanes) led to the hypothesis that peroxides might also be formed from arachidonate by non-enzymatic free radical reactions. Isoprostanes have found significant utility in serving as biomarkers of the initiation of free radical events within pathological states [80]. Hall and Murphy [61] incubated RBC ghosts, which lack the enzymatic and low molecular weight antioxidant defense mechanisms, with high concentrations (10 mM) of the organic hydroperoxide tert-butyl hydroperoxide (tBuOOH). They extracted the glycerophospholipids, hydrolyzed the fatty acids, separated the fatty acids by RP-HPLC, and analyzed the capacity of different fractions to induce a Ca^{++}-response in PMNL. Only after treatment with tBuOOH, were fatty acids found that responded positively in this bioassay. Using LC-MS/MS, the authors detected several oxidized polyunsaturated fatty acids (HpETEs, HETEs, oxo-ETEs, HpODEs, HODEs, oxo-ODEs, and epoxyeicosatrienoic acids (EETs)) [61,81,82], which were esterified to glycerophospholipids. Further analysis revealed that the biological activity was exclusively due to 5-HpETE, 5-HETE, and 5-oxo-ETE. The peroxidation mechanism of RBC ghosts lipids is a typical radical chain reaction [83]. First, Fe^{3+}-catalyzes the formation of a tBuOOH alkoxyl radical, which in turn removes a bis-allylic proton from a polyunsaturated fatty acyl moiety. This initial phospholipid radical then reacts with molecular oxygen to form the lipid hydroperoxide. The reaction leading to the formation of 5-oxo-ETE in this setting is similar to the reaction described above for murine macrophages. RBC ghosts cannot convert 5-HETE to 5-oxo-ETE even if tBuOOH or $NADP^+$ is added to the incubation medium, and 5-oxo-ETE is formed from 5-HpETE under these circumstances [61]. After 90 min incubation 270 pmol 5-oxo-ETE / mg protein were formed from RBC ghosts. Due to the high concentration of tBuOOH, the esterification of the fatty acids, and the absence of antioxidants in this setting it remains to be

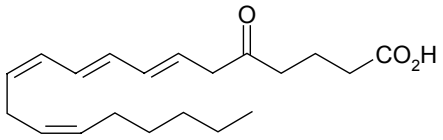
elucidated whether this pathway leads to the formation of free 5-oxo-ETE under physiological conditions.

LTA_4-Hydrolysis Forms a 5-oxo-ETE-Isomer with Biological Activity

In the literature, another compound is also referred to as 5-oxo-ETE. Falgueyret and Riendeau found recently that 5-oxo-(7*E*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acid is a non-enzymatic hydrolysis product of LTA_4 [84]. Unfortunately, they denoted this eicosanoid 5-oxo-ETE as well. As seen in Figure I.4, both analogs have the same molecular mass, but differ in the location and stereochemistry of their double bounds. 5-oxo-(7*E*,9*E*,11*Z*,14*Z*)-eicosatetraenoic acid induces a Ca^{++} influx in neutrophils as does 5-oxo-(6*E*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid. However, the EC_{50} for the degradation product is about 250-times higher than that for the 5h-dh product [34,84]. It is more likely that it signals through the LTB_4 receptor rather than the 5-oxo-ETE receptor, because calcium mobilization by this 5-oxo-ETE isomer was inhibited by the specific LTB_4 receptor antagonist LY223982 and desensitized by pretreatment with LTB_4 [84]. To date it is not clear whether this compound also has chemotactic effects.



5-oxo-(6*E*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid



5-oxo-(7*E*,9*E*,11*Z*.14*Z*)-eicosatetraenoic acid

Figure I.4 Two known isomers of 5-oxo-ETE
5-oxo-(6*E*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid (5-oxo-ETE) is formed by 5h-dh from 5*S*-HETE and stimulates OXE in peripheral blood leukocytes. The isomer 5-oxo-(7*E*,9*E*,11*Z*.14*Z*)-eicosatetraenoic acid is a non-enzymatic hydrolysis product of LTA_4 and activates BLT_1.

I.2.5 Catabolism

Several pathways have been described which metabolize 5-oxo-ETE and may lead to its transformation *in vivo* and these are summarized in Figure I.5.

Reconversion into 5-HETE

As indicated above, the enzymatic conversion of 5*S*-HETE into 5-oxo-ETE is reversible and depends in part on the availability of the cofactor $NADP^+$ [22]. Since healthy cells maintain a high NADPH/$NADP^+$ ratio (see PPP, page 31), under some circumstances the reverse reaction is favored over the forward reaction, as is the case for platelets [70]. Both the forward and the backward reaction are likely to be catalyzed by 5h-dh, as the reaction is reversible in neutrophil microsomes [22], but this has not yet been clearly demonstrated and it is possible that a distinct 5-ketoreductase exists. More details of the 5h-dh-mediated reaction have to be known to answer this question. The binding affinities for substrates (5-HETE and 5-oxo-ETE) and cofactors ($NADP^+$ and NADPH) to 5h-dh have yet to be measured. The standard free energy change ($\Delta G°$) and thus the equilibrium constant are also unknown.

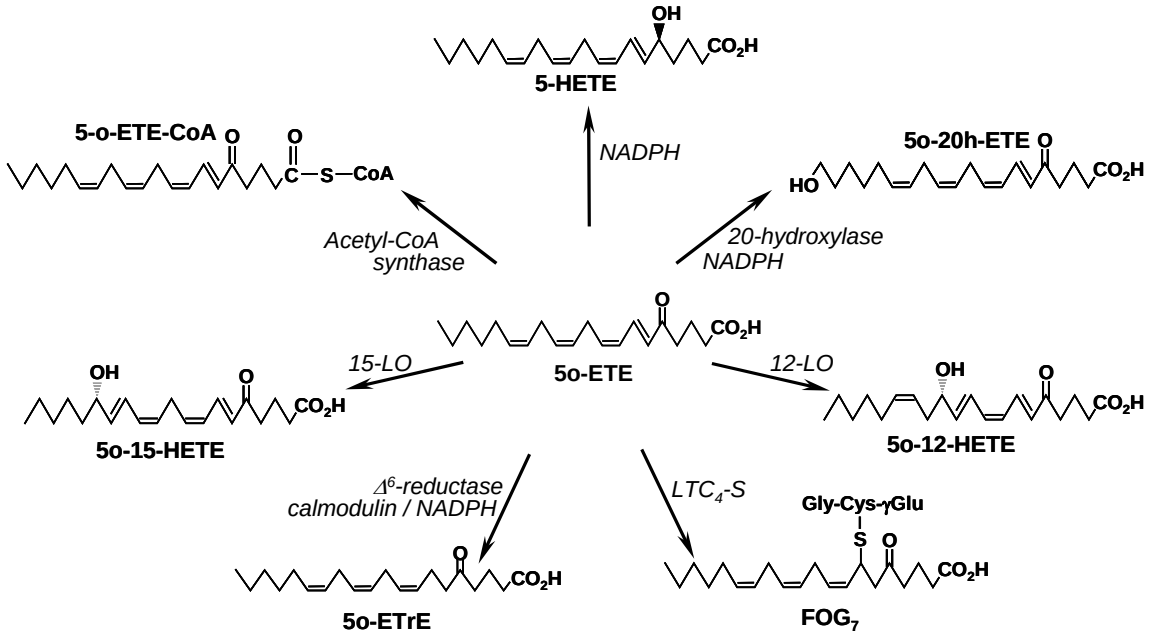


Figure I.5 Catabolic pathways of 5-oxo-ETE metabolism
To date seven pathways have been described by which 5-oxo-ETE is metabolized.

ω-Oxidation of 5-Oxo-ETE

In human neutrophils, 5-oxo-ETE and its precursor 5-HETE are metabolized by the NADPH-dependent LTB_4 hydroxylase, a member of the CYP4F family. In humans, LTB_4 can be hydroxylated by two subtypes of this family, CYP4F2 and CYP4F3A [85]. CYP4F3A expression is restricted to myeloid cells, while CYP4F2 is expressed in various tissues except myeloid cells [85]. In human neutrophils, 5-HETE and 5-oxo-ETE are metabolized by LTB_4 hydroxylase CYP4F3 (Figure I.5) [34,72,86]. Recombinant CYP4F3A has a preference for the 6*Z*,8*E*,10*E*-triene-bond configuration found in LTB_4 and for the chirality of 5*S*-and 12*R*-hydroxyl groups [87]. CYP4F3A has dramatically higher K_M- and lower k_{cat}-values for 5-HETE and 6-*trans*-LTB_4, and presumably for 5-oxo-ETE, than for LTB_4. The enzymatic activity of CYP4F3 depends on cytochrome b and the cofactor NADPH as the electron donor. However, due to the wide tissue distribution of cytochrome b, only the expression of CYP4F3A is limiting for LTB_4 inactivation by this pathway. The expression of CYP4F3A is confined to myeloid cells, especially granulocytes. Its expression is coordinated with that of myeloid differentiation markers such as CD11b and also increases concomitantly with MPO during the maturation of bone marrow cells [88]. There are also species-dependent differences. Mouse macrophages and rat neutrophils, which lack the 20-hydroxylase enzyme, convert 5-oxo-ETE and/or LTB_4 to18- and 19-hydroxylated products [89,90].

Conversion of 5-oxo-ETE by 12-LO

In contrast to unstimulated platelets, which reduce 5-oxo-ETE to 5-HETE, the main pathway for the metabolism of 5-oxo-ETE by activated platelets is conversion to 5-oxo-12*S*-HETE and its 8-transisomer by 12-LO (Figure I.5) [70]. Platelets can also convert 6-trans isomers of LTB_4 to similar products by the action of 5h-dh [70]. Although neither 5-oxo-12-HETE nor its 8-trans isomer posses significant agonist activity, both substances seem to have some antagonist activity, because they were able to inhibit 5-oxo-ETE-induced calcium

mobilization. In the case of the 8-cis derivative, the calcium response to 5-oxo-ETE was virtually abolished at low μmolar concentrations [70].

Glutathionylation of 5-oxo-ETE to FOG_7

Recently, two novel glutathione adducts of 5-oxo-ETE have been identified. Bowers and colleagues identified FOG_7 (Figure I.5), a glutathione adduct of 5-oxo-ETE after incubation of this substance with murine peritoneal macrophage [91]. FOG_7 is biologically active but its effects are more limited than those of 5-oxo-ETE. While FOG_7 induces actin polymerization in neutrophils and eosinophils, as does 5-oxo-ETE, in contrast to the later compound it does not increase the cytosolic Ca^{++} concentration in these leukocytes. In murine macrophages FOG_7 is formed in a 1,4-Michael addition reaction, probably catalyzed by the microsomal LTC_4-S [92]. Recombinant human LTC_4-S also catalyzes this reaction and human platelets too convert 5-oxo-ETE into FOG_7. Surprisingly, the K_M of LTC_4-S for 5-oxo-ETE is similar to that for LTA_4, which had previously been the only recognized substrate of this enzyme. The same study revealed that this is not the only glutathione adduct formed within murine macrophages [92]. At least one of the cytosolic GSTs leads to the formation of another, biological inactive glutathione adduct from 5-oxo-ETE. Since this compound cannot be separated from FOG_7 by RP-HPLC, and shows a similar MS spectrum, it is a stereoisomer of FOG_7 [92].

Inactivation of 5-Oxo-ETE by an Olefin-Reductase

Many eicosanoids are metabolized in the sequence of dehydrogenases, reductases, and keto-reductases (Figure I.6). The first stage is the oxidation of a hydroxyl groups by a $NADP^+$- or NAD^+-dependent dehydrogenase to the corresponding keto- (oxo-) group. The products are then further metabolized by reduction of an adjacent double bond by an olefin reductase in the presence of NADH or NADPH. This may be followed by the reduction of the keto-group by a keto-reductase or by the original dehydrogenase. This process has been described for several prostaglandins, which are first oxidized to their biologically inactive 15-oxo metabolites and then reduced to dihydro-15-oxo-PGs, which can in turn be further reduced to dihydro-PGs ([93] and references within). Similar reactions have been demonstrated for lipoxygenase products such as 12-HETE, 15-HETE, and LTB_4. LTB_4 is normally first converted to 12-oxo-LTB_4 by a NAD^+-dependent dehydrogenase in PMNL and then further metabolized by a NADH-dependent Δ^{10}-reductase and subsequently reduced to the corresponding dihydro compound by a keto-reductase.

PMNL convert 6-trans isomers of LTB_4, which are formed non-enzymatically from LTA_4, but not LTB_4 itself to 6-dihydro-metabolites [94,95]. It was proposed that a novel dehydrogenase facilitates the first step of this reaction (Figure I.6). Powell and coworkers [22] then hypothesized that 5-HETE, since its Δ^6 carbon is in the trans-position, is converted in a similar fashion by this pathway. This hypothesis ultimately led to the discovery of 5-oxo-ETE [22]. 5-oxo-ETE resembles a rare exception from the above examples because it is a biologically active intermediate. Berhane and coworkers then showed that a specific NADPH-dependent Δ^6-reductase facilitates the inactivation of 5-oxo-ETE (Figure I.5) [93]. This enzyme is distinct from PG Δ^{13}-reductase and is Ca^{++}- and calmodulin-dependent. The product, 6,7-dihydro-5-oxo-ETE (5o-ETrE) is 1000 times less potent than its precursor in mobilizing Ca^{++} in human neutrophils, a clear sign of biological inactivation.

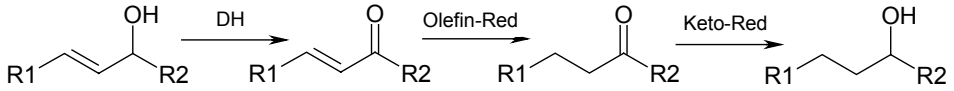


Figure I.6 Common hydroxyeicosanoid metabolism
Many eicosanoids are inactivated by the combined action of a dehydrogenase (DH), leading to the conversion of a hydroxyl to the oxo-group. This is followed by olefin-reductase (Olefin-Red), which reduces an adjacent double bound. The keto-group may be reduced back to a hydroxyl-group by a keto-reductase (Keto-Red). The finding of 6-dihydro metabolites of 6-trans-LTB_4 originally sparked research leading to the discovery of 5h-dh.

LXA_4 and LXB_4 are metabolized (see VII.1, page 107 for structures) in a similar manner by monocytes [96]. First, the hydroxyl-groups at position C-15 (LXA_4) and C-5 (LXB_4) are subject to dehydrogenation followed by subsequent reduction of the adjacent double bounds (Δ^{12} for LXA_4 and Δ^6 for LXB_4) to the corresponding dihydro-compounds. Both intermediates can be further reduced to the dihydro-Lipoxin by the action of a keto-reductase. As the 5-position of LXB_4 is similar to that of 5-HETE, it is possible that LXB_4 is reduced to 5-oxo-LXB_4 by 5h-dh. However, this needs to be further investigated.

Conversion of 5-oxo-ETE by 15-LO

Eosinophils convert 5-oxo-ETE to 5-oxo-15-HETE by 15-LO (Figure I.5) [30]. However this is pathway does not remove its biological activity, as 5-oxo-15-HETE induces similar biological responses as its parent compound in granulocytes. The potency of 5-oxo-15-HETE is lower than that of 5-oxo-ETE, but it is much higher than that of 15-HETE, 15-oxo-5-HETE, or 15-oxo-ETE [7,26-28,30,60].

Esterification into Lipids

During their examination of radioactively labeled AA from human neutrophils, Stenson and Parker discovered that endogenously produced 5-HETE can be esterified into cellular lipids [97]. This is a major pathway for eicosanoid catabolism and is not restricted to 5-HETE, as other fatty acids, among them many lipoxygenase products, are also incorporated into lipids. However, this process is not universally applied to all eicosanoids, as different exogenous lipoxygenases products are differentially distributed among lipid classes in a murine macrophage cell line [98]. In this study, AA was taken up very rapidly, whereas 5-HETE, 12-HETE, and 15-HETE were incorporated more slowly. The final destination of the different HETEs differs from one another and from AA. 5-HETE was esterified to a greater extent than the other HETEs into neutral lipids. 15-HETE was mainly incorporated into phosphatidylinositol, while 5-HETE and 12-HETE were incorporated into all phospholipid classes (PC, PE, PI) tested. In contrast to the HETEs, LTB_4 was not incorporated into cellular lipids over a period of 20 min. The lipid fraction into which a fatty acid would be primarily incorporated is unpredictable, because the final destination of AA and its lipoxygenases products is not only substrate but also tissue specific. Bovine smooth muscle cells, for example, incorporate both 12-HETE and 5-HETE mainly into triglycerides [99]. In opposite to the smooth muscle cells, cultured bovine aortic endothelial cells esterify 5-HETE only in triglycerides whereas 12-HETE was incorporated equally into phospholipids and triglycerides. Both cell types preferentially incorporate AA into phospholipids. The HETEs are esterified in the same way by a standard fatty acid processing pathway: a triacsin C sensitive synthetase forms fatty acid-CoA, which is the substrate for a transacylase to incorporate the fatty acid into lipids [100-102]. The esterification of 5-HETE into lipids by neutrophils is, however,

concentration dependent. At higher substrate concentrations, 5-HETE is mainly converted into ω-oxidation products rather than into lipids [72,86]. In neutrophils the acylation pathway appears to have a high affinity but low capacity for substrate, whereas the ω-oxidation pathway has a high capacity but a low affinity [59].

In their study of the binding of 5-oxo-ETE to neutrophils, O'Flaherty and colleagues discovered that this substance is also incorporated into cellular lipids, thus hampering the establishment of a binding assay [59]. Low amounts (100 pM) of both, radiolabeled 5-oxo-ETE and 5-HETE were taken up by these cells and incorporated primarily into triacyglycerols as detected by thin layer chromatography (TLC). After treatment of the lipid fraction of 5-oxo-ETE-incubated neutrophils with triglycerides lipase only radioactive 5-HETE was recovered. The authors concluded that 5-oxo-ETE was reduced to 5-HETE and then esterified by the process described above [59]. Given the abundance of the Acyl-CoA synthetase it is likely that 5-oxo-ETE is potentially directly esterified into lipids as well, but is not detectable in this setting due to the low 5-oxo-ETE concentration and the reversibility of 5h-dh [22,59]. Indeed, 5-oxo-ETE is esterified into lipids by neutrophil plasma membrane fractions, which lack 5h-dh [59].

I.3. Oxidative Stress

Oxygen is essential to aerobic life but, paradoxically, can be toxic even at the partial atmospheric pressure. Over 95% of the oxygen we breathe undergoes a concerted tetravalent reduction to produce water in a reaction catalyzed by the omnipresent cytochrome oxidase (cytochrome c, oxygen oxidoreductase), which is part of complex IV of the mitochondrial electron transport chain ($O_2 + 4e^- + 4H^+ \rightarrow 2\ H_2O$). Cytochrome oxidase is the terminal electron acceptor in the chain and must hand its reducing equivalents over to oxygen to allow for continued electron transport. In fact, the major function of the respiratory and cardiovascular system is the delivery of oxygen for use in aerobic energy production by oxidative phosphorylation. Thus, the major role of oxygen for all aerobic processes is simply to act as a sink or dumping ground for electrons. This chapter contains a description of major ROS sources, antioxidant mechanisms, some aspects of the role of ROS in cell signaling, and a hypothetical model of ROS effects on 5-oxo-ETE synthesis.

I.3.1 Definition of Oxidative Stress

Before describing the reaction pathways of reactive oxygen species and anti-oxidant defense mechanisms in humans, I want to clarify the term oxidative stress as it is used within this thesis. Youngson describes in a remarkable book chapter [103] how the term stress was introduced into medicine by the Austrian-born Canadian physician Hans Selye. The terms stress and strain originate from the science of engineering and are frequently confused with each other. Stress defines the force that causes a body to deform. Strain is defined as the extent to which a body is deformed when it is subjected to stress. Selye defined stress as the non-specific response of the human body to any demands (fear, distress, sleep-deprivation, etc) made upon it. He had, similar to the author of this dissertation, some difficulties with the English language and admitted later in life that he mistook stress for strain; he meant strain when he developed the concept of stress. This confusion is still present after the term oxidative

stress was introduced in the field of ROS and antioxidant defense mechanisms [104]. Hence, the term oxidative stress is in need of clarification. It is now generally understood that oxidative stress describes the imbalance between ROS and antioxidants in favor of the former [105]. Normally there are sufficient antioxidants present to ensure that the production of small amounts of ROS is inconsequential. Oxidative stress occurs when either antioxidants are diminished or the production of reactive oxygen species is increased. Peroxides, i.e. H_2O_2 and organic peroxides such as tBuOOH or cumin hydroperoxide are commonly used to simulate oxidative stress in vitro by increasing the *stress* on the cell, while glutathione depletion or glucose withdrawal enhance the *strain* on a cell at a given stress level. However, both strategies will lead to *oxidative stress* as defined above.

I.3.2 Sources of Reactive Oxygen Species

Endogenous Sources of Reactive Oxygen Species

Within the body, there are normal metabolic processes that lead to the formation of ROS as byproducts and "professional" superoxide manufacturers. As mentioned above, the mitochondrial electron transport chain leads to the highly coordinated reduction of oxygen. However, due to the special nature of oxygen, this process is not fool proof. Atmospheric oxygen is a biradical with two unpaired electrons ($^{\bullet}O\text{-}O^{\bullet}$) with parallel spins. To overcome its spin restrictions, oxygen accepts electrons one at a time, leading to the initial formation of superoxide (Figure I.7). It is estimated that 1-2% of the electron flux in mitochondria might form superoxide [106]. This makes the electron transfer chain the largest contributor of reactive oxygen species in the body. Additional major sources of superoxide in metabolic processes are the cytochrome P450 (CYP) and the b5 family of oxidoreductases in the endoplasmic reticulum (reviewed in [107]). Lipid hydroperoxides (fatty acid-OOH) are also side products of arachidonic acid metabolizing enzymes cyclooxygenases and lipoxygenases. There are several additional cytosolic and peroxisomal enzymes leading to substantial formation of ROS. For additional information see [107-111] and references within.

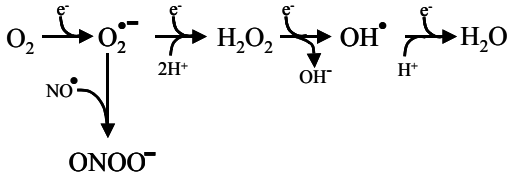


Figure I.7 Reactive Oxygen Species
This figure shows the formation of some reactive oxygen species (ROS). Sequential electron addition to oxygen results in the formation of ROS: Superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$). $O_2^{\bullet-}$ and nitric oxide can react to form peroxynitrite ($ONOO^-$/ONOOH).

At sites of inflammation, "professional" phagocytes, especially neutrophils, but also eosinophils and macrophages, produce very large quantities of ROS to destroy invading microbes. In granulocytes, NADPH oxidase, a multicomponent enzyme which includes the membrane-bound cytochrome b558 consisting of two subunits, $p22^{phox}$ and $gp91^{phox}$ is mainly responsible for superoxide formation [112]. Its activity is absolutely dependent on the presence of three cytosolic factors, $p47^{phox}$, $p67^{phox}$, and rac 2, a ras-related small GTP-binding protein [113]. NADPH oxidase transfers electrons from cytoplasmic NADPH to oxygen, thus generating superoxide: NADPH + 2 O_2 → $NADP^+ + H^+ + 2\ O_2^{\bullet-}$. Given the potential damage that can be inflicted by ROS, it is not surprising that tight control mechanisms have evolved to minimize the risk of collateral tissue damage due to an uncontrolled oxidative burst (for review on granulocyte NADPH oxidase please refer to [114]). Because of its relatively low

antibacterial potency, it is probably not $O_2^{\bullet-}$ itself that kills the microbes. Within the phagosome, $O_2^{\bullet-}$ is converted either spontaneously or by superoxide dismutase (SOD) to H_2O_2. H_2O_2 may then react with $O_2^{\bullet-}$ to generate hydroxyl radicals ($OH^{\bullet}$) and singlet oxygen, both highly reactive and toxic compounds. $O_2^{\bullet-}$ can also react with $NO^{\bullet}$ to yield the highly reactive nitrogen intermediate peroxynitrite ($ONOO^-$). The importance of the NADPH oxidase complex becomes evident in case of chronic granulomatous disease, a rare and typically X-chromosome linked genetic disorder. These patients have a compromised ability to kill bacteria due to disturbed NADPH oxidase complex formation. They suffer from severe and frequent bacterial and fungal infections [114].

MPO and EPO are heme enzymes that convert H_2O_2 and halides (chlorine and bromine) to hypohalous acids. In case of chlorine the product is hypochlorous acid (HOCl): $H_2O_2 + HCl \rightarrow HOCl + H_2O$. HOCl is used in phagocytes to aid the killing of bacteria. MPO and EPO also metabolize H_2O_2 and thiocyanate (SCN^-) to equimolar amounts of $OSCN^-$ (hypothiocyanite) and OCN^- (cyanate) [115], two bacteriostatic and bactericidal compounds. MPO is expressed in neutrophils and monocytes while EPO is exclusively expressed in eosinophils. Because of their large capacity to metabolize H_2O_2, these enzymes can compete with GPx thus lowering the effective concentration of this peroxide when added to cells, without affecting the $NADPH/NADP^+$-ratio. As with many other heme enzymes, MPO, catalase, and EPO are inhibited by sodium azide [116,117].

Exogenous Sources and Inducers of ROS

The separation of endogenous and exogenous sources of ROS is not trivial, as many exogenous sources of ROS are not oxygen radicals by themselves but induce the formation of endogenous ROS or act as catalysts for ROS production. The main direct exogenous sources of ROS are cigarette smoke and pollution. For instance, cigarette smoke inhalation results in increased exposure to both superoxide and hydrogen peroxide. Although the gas phase of cigarette smoke contains some H_2O_2, $O_2^{\bullet-}$, and O_3 , the tar content is more harmful, as it contains a quinone-hydroquinone-semiquinone system that leads to the endogenous formation of superoxide and oxidative stress [118]. CYPs further metabolize some tar contents to their reactive intermediates. Air pollution is another source of environmental ROS since it contains ozone [119]. In addition, particulates in pollution can be taken up by alveolar macrophages or neutrophils, thereby inducing ROS formation through activation of NADPH oxidase [120]. They can also lead to epithelial damage and subsequent apoptosis. In this case, ROS production can be either catalyzed by the metal content [121], as well as being a consequence of apoptosis itself [122].

Ingestion of xenobiotics or alcohol can also be detrimental. Some xenobiotics or their toxic metabolites deplete intracellular GSH pools by more than 90%, thus compromising the liver antioxidant capacity. The analgesic/antipyretic acetaminophen can produce centrilobular hepatic necrosis by this mechanism. It is metabolized by a CYP to N-acetyl-p-benzoquinone imine (NAPQI), which reacts rapidly with GSH [123]. Ethanol can induce a certain CYP (P4502E1, or CYP2E1), which leads to the formation of ROS and may explain the hepatotoxicity of ethanol [123]. As CYP2E1 also metabolizes acetaminophen to NAPQI, alcohol and this pain reliever can act synergistically to induce severe oxidative stress and subsequent liver damage GSH depletion.

I.3.3 Antioxidant defense

Introduction

During the course of evolution, many antioxidant defense strategies have developed leading to redundant systems that include both, enzymes and low molecular weight-molecules. These are present in all cell types and body fluids and collaborate to detoxify ROS and their byproducts. The antioxidants involved in protection from ROS are grouped together into enzymes/molecules that directly scavenge and metabolize ROS (first line of defense), and those that repair ROS-induced damage to cells and tissue.

One common feature of antioxidant biology is that ROS undergo a controlled sequential degradation. The more toxic ROS are scavenged by abundant antioxidant molecules and converted into less aggressive compounds. Subsequently all components of the antioxidant network either decay through dismutation or undergo redox-reactions. As a rule, antioxidants are regenerated at the expense of NADPH, which is mainly provided by the pentose phosphate shunt (PPP), but also by other specific metabolic pathways. This redox recycling of antioxidants markedly increases their biological efficacy by diminishing the need for *de novo* synthesis or large uptake of antioxidant nutrients. Another important feature is the synergistic interaction of the components. They do not only interact in the destruction of the ROS but also protect each other against ROS. For example, SODs protect catalase and glutathione peroxidase (GPx) from inactivation by $O_2^{\bullet-}$.

Non-enzymatic Scavengers

Hydrophilic and hydrophobic enzyme-independent antioxidant scavengers (for review see [124]) are either chemical traps of oxidizing free radicals and activated oxygen species, or physical quenchers of exited singlet oxygen $^1O_2$ (O-O**:**) and triplet states of photosensitizers. The most important hydrophilic scavengers are ascorbate (vitamin C) and glutathione, which are found in the cytosolic, mitochondrial and nuclear aqueous compartments as well as extracellularly. They scavenge oxidizing free radicals by means of one-electron or hydrogen atom transfer. Their intracellular concentrations are typically between 1 and 10 mM, much higher than those of other nucleophilic and reducing biomolecules. Hydrophobic scavengers are found in membranes and lipoproteins and include α-tocopherol (vitamin E), carotenoids, and possibly ubiquinol. They act by either interrupting the propagation step of lipid peroxidation, by destroying peroxyl radicals ($ROO^{\bullet}$), or by blocking the formation of hydroperoxides from $^1O_2$. Through the regeneration of α-tocopherol, ascorbate can also indirectly contribute to the prevention of lipid peroxidation. The kinetics of their scavenging reactions are very fast compared to other biological processes. Free radical products formed by the reaction of ROS with scavengers do not undergo uncontrolled chain reactions, but they decay through dismutaion, recombination or reduction by secondary scavengers. Oxidized and radical derivatives of ascorbate are less aggressive than ROS and are recycled by GSH or thioredoxin related pathways. Dehydroascorbate may be reduced by GSH-dependent or GSH-independent dehydroascorbate reductase and thioredoxin (Trx) [124,125]. Ascorbyl free radicals representing one-electron oxidized ascorbate are recycled by thioredoxin reductase (TrxR) [126].

Enzymatic Defense Mechanisms and Redox State

In humans, two Cu/Zn-SODs are present in the cytosol and mitochondria, whereas Mn-SOD, a remnant of its bacterial ancestor, is present only in the mitochondrial matrix [127]. SODs catalyze the one-electron dismutation of superoxide into hydrogen peroxide and oxygen: $2\ O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$. Although $O_2^{\bullet-}$ undergoes non-enzymatic dismutation at physiological pH in buffer, this reaction is catalyzed by SOD *in vitro*, because the turnover rate of superoxide with SOD is very high ($> 2 \times 10^9\ M^{-1}\ s^{-1}$) over a wide range of pH [128]. The main function of SOD is to inactivate $O_2^{\bullet-}$ before any uncontrolled reactions take place.

In mammals, catalase (H_20_2:H_2O_2 oxidoreductase) is mainly expressed in peroxisomes, but is also present in the cytosol and in very small amounts in the mitochondrial matrix. Catalase is a porphyrin-containing enzyme of high molecular weight which converts H_2O_2 in a two-electron dismutation into water: $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$. Since the K_M of catalase for H_2O_2 is very high (close to 1 mM) [129], the physiological role of catalase in mammalian cells as an antioxidant is not well established. The K_M of catalase for H_2O_2 is higher than the K_M of GPx, which suggests that catalase scavenges H_2O_2 efficiently only at high H_2O_2 concentrations [130]. Even in erythrocytes, which have an unusually high catalase content, H_2O_2 is mainly degraded by the glutathione-redox cycle [131]. Catalase and SOD are dismutases, and therefore do not consume cofactors or require energy and do not rise the redox state of the cell (see below).

The glutathione redox cycle, consisting of GPx and glutathione reductase (GRed) may play a more important role in protecting cells against physiologically relevant oxidants. A wide variety of both, cytosolic and membrane bound glutathione peroxidases (GPx) [132] exist, as well as some isoforms of glutathione-S-transferase [133] reduce H_2O_2 to water or organic peroxides to their corresponding alcohols: $H_2O_2 + 2GSH \rightarrow 2\ H_2O + GSSG$ or $R\text{-}OOH + 2GSH \rightarrow R\text{-}OH + H_2O + GSSG$. Due to the wide substrate spectrum of the GPx (for review on the GPx-family please see [132,133]) and GST families [133,134], the glutathione redox cycle is an integral part of the antioxidant defense mechanism. While the oxidant substrates of the GPxs range from H_2O_2 to organic-hydroperoxides, GSH is the common electron donor for substrate reduction and is oxidized to GSSG. Under most circumstances, H_2O_2 is reduced by selenium-dependent GPx. GSSG is in turn reduced by glutathione reductase (GRed) to GSH, using NADPH as the redox partner.

An additional enzymatic defense mechanism is the thioredoxin system. It consists of the three antioxidant enzymes peroxiredoxin (Prx; thioredoxin peroxidase), thioredoxin (Trx), and Trx reductase (TrxR). Trx represents a ubiquitous family of proteins with oxidoreductase activity of which three distinct variants encoded by separate genes have so far been cloned and characterized in humans (for review on Prx, Trx, TrxR see [126]). Similar to GPx, Prx can directly reduce both H_2O_2 and different alkyl hydroperoxides using Trx^{RED} as the electron donor. TrxR catalyzes the reduction of the active site disulfide in Trx^{OX} using NADPH as cofactor. The addition of peroxides can significantly affect the NAPH/$NADP^+$ ratio in cells through the GSH/GSSG or the Trx^{RED}/Trx^{OX} redox cycles. The importance of the thioredoxin pathway can be distinguished from the GSH redox cycle by use of auranofin, a highly specific TrxR-inhibitor [135], as then only the GSH redox cycle will affect NADPH levels.

ROS affect the cellular redox status of mammalian cells as described above [136]. The term redox state is widely used but often not well defined in biological sciences. Historically, the term "redox state" has been used to describe the ratio of a specific redox couple (e.g. $[NAD^+]/[NADH]$). However, the definition is now normally used to represent the sum of all redox systems in the cell. As the concentrations of GSH and GSSG are higher than those of other redox-couples, their ratio is a good indication of the cellular redox buffer. The overall redox state of a cell is therefore approximated by the half-cell reduction potential and the reducing capacity of GSH and GSSG.

GSH and other antioxidants are maintained in the reduced state within the cytosol by NADPH dependent enzymes. High cytosolic $NADPH/NADP^+$ ratios are maintained by the pentose-phosphate pathway (PPP), which is often considered a member of the second line of the antioxidant defense system. In the oxidative part of the PPP, G6P is oxidized and decarboxylated to ribulose-6-phosphate (Ru5P) with the concomitant reduction of 2 molecules $NADP^+$ to NADPH per cycle. Within the regenerative part of the PPP, excess Ru5P is converted to glycolitic intermediates, fructose-6-phosphate and glyceraldehydes-3-phosphate, which can be recycled to G6P by gluconeogenesis. Thus, including the regenerative part of the PPP, the total oxidation of one molecule glucose via this pathway yields 12 molecules NADPH. In addition to the provision of reducing equivalent for GSSG and Trx^{OX} reduction, NADPH is also consumed by many endergonic reactions, notably the reducing biosynthesis of fatty acids and cholesterol. The activity of the initial enzyme of the PPP, glucose-6-phosphate (G6P) dehydrogenase (G6Pdh), controls the flux through this pathway and thus the rate of NADPH production. *In vivo*, the activity of this enzyme is regulated by the $NADP^+$ concentration. For experimental purposes, the ability of cells to maintain a high $NADPH/NADP^+$ ratio can be modified with dehydroepiandrosterone (DHEA), a commonly used G6Pdh inhibitor, or by glucose depletion [137-139]. The subsequent NADPH depletion will lead to GSSG accumulation during oxidative stress and therefore affect the reductive state of the cell.

I.3.4 Proposed Effect of Oxidative Stress on 5-oxo-ETE Formation

Because of its effect on the $NADPH/NADP^+$-ratio, oxidative stress may affect the formation of 5-oxo-ETE. In the present study, we hypothesized that hydroperoxides could enhance 5-oxo-ETE synthesis through their metabolism by GPx, which would result in the formation of GSSG from GSH and its subsequent NADPH-dependent reduction by GRed. This would provide the $NADP^+$ necessary for the oxidation of 5-HETE to 5-oxo-ETE by 5h-dh. This could serve as an alternative pathway to NADPH oxidase. In addition, lowering NADPH levels would reduce the metabolism of 5-HETE and 5-oxo-ETE by ω-oxidation and the Δ^6-reductase. All the above effects would be opposed by the metabolism of G6P by the PPP, which would reduce the $NADP^+$ generated by GRed and NADPH oxidase.

I.3.5 Oxidative Stress and Cell signaling

In addition to affecting the cellular redox state and $NADP^+$ levels, ROS have important effects on cell signaling. The classical view of ROS effects on cells was that those induce excessive damage to DNA and phospholipids, thus overwhelming the defense mechanisms of

cells and subsequently leading to necrosis or apoptosis. However, there is growing evidence that ROS can mediate physiological responses in cells by interfering with signal cascades. H_2O_2 can inhibit tyrosine protein phosphatases (TPPs) that are critical in determining the phosphorylation state of MAP kinases (MAPKs), which among other kinases are important regulators of eicosanoid formation. For more details on ROS in signal transduction refer to an excellent review by Dröge [140].

Inhibition of Protein Tyrosine Phosphatases (PTPs) by H_2O_2

Phosphorylation of specific protein residues is a very important regulator of enzymatic activities. The phosphorylation state of a protein reflects the balance of the corresponding kinase and phosphatase activities. Exposure to high concentrations of hydrogen peroxide on the order of 1 mM generally leads to increased tyrosine phosphorylation in numerous proteins (for review see [141]). This effect is to some extent, albeit not exclusively, the consequence of the oxidative inhibition of PTPs. The cysteine residue is only a good target for the oxidizing action of H_2O_2 if it is deprotonated and exists in the form of a cysteine thiolate anion ($-S^-$). PTPs contain an essential catalytic cysteine residue in their active sites with a lower pKa (~5.5) than the pKa (~8.0) of other cysteine residues in most proteins [142]. The low pKa makes their thiolate anion especially susceptible to the inhibitory action of H_2O_2. As fewer proteins are phosphorylated under lower H_2O_2 concentrations [141], one has to be cautious not to over-interpret physiological relevance into data gained using high concentrations of peroxides.

Indirect H_2O_2-induced Activation of MAPK pathways

Mitogen-activated protein kinases (MAPK) are involved in the regulation of LT-synthesis and can be activated by H_2O_2. MAPKs consist of three different families, the p38 MAPK, the extracellular signal-regulated kinases (ERK), and the c-jun-NH_2-terminal kinases (JNK). In an excellent study, Lee and Esselman examined the stimulation of those three MAPKs by H_2O_2 in Jurkat cells, a T lymphocyte cell line [143]. In those cells MAPKs are activated by members of the Src family, most likely Lck. T lymphocytes contain three major tyrosine phosphatases, CD45, SHP-1 and HePTP and their activities have been shown to play important roles in T cell activation. H_2O_2 exposure leads to increased phosphorylation of all three MAPKs, which is due to PTP inhibition [143]. In this setting, CD45 is the primary phosphatase controlling Src-family kinases, SHP-1 is accountable for H_2O_2 stimulatory effects on JNK and in part on ERK, and HePTP dephosphorylates p38 and ERK, but has no influence on the activation state of JNK. Further studies revealed that phospholipase C (PLC) and protein kinase C (PKC) are crucial for ERK activation, but do not participate in p38 or JNK regulation. The implications of these findings on LT synthesis are discussed in more detail in chapter I.4.3.

I.4. 5-Lipoxygenase

5-LO is the enzyme responsible for the synthesis of LTs and 5-oxo-ETE. It possesses two distinct enzymatic activities leading to the formation of 5-HpETE, the precursor for 5-HETE and 5-oxo-ETE, and LTA_4, the precursor for leukotrienes. 5-LO is mainly found in cells of the immune system, including neutrophils, eosinophils, macrophages, basophils, B-lymphocytes, and mast cells.

I.4.1 Role and Enzymatic Reaction of 5-Lipoxygenase

5-LO catalyses the incorporation of molecular oxygen into AA (oxygenase activity) to form 5-HpETE and the subsequent formation of the unstable epoxide LTA_4 (LTA_4 synthase activity) [144,145]. The first step is the abstraction of a hydrogen atom of the C_7-position of AA, coupled with the addition of a molecule of oxygen at C_5 to give 5*S*-HpETE. It then catalyzes the removal of a second hydrogen atom from C-10 of 5*S*-HpETE, which is coupled to a cyclization of the 5-hydroperoxy group to give the 5,6-epoxide LTA_4. During this process, considerable amounts of 5*S*-HpETE are release from the enzyme and are reduced by ubiquitous GPx-family members [132,133] and some GSTs [133,134] to the corresponding hydroxy-compound 5*S*-HETE. The ratio of LTA_4 to 5-HETE formed depends on the assay conditions, e.g. the relative concentrations of free AA, membrane association, and the amount of 5-LO [146-150]. The role of 5-LO activating protein (FLAP) in 5-LO metabolism is discussed below.

In the active site of 5-LO, a non-heme iron acts as an electron acceptor and donator during catalysis. Using electron paramagnetic resonance spectroscopy, it was shown that the iron of isolated, inactive enzyme is in the ferrous state (Fe^{2+}) and that treatment with fatty acid hydroperoxides restored the active, ferric (Fe^{3+}) form [151]. Ferric 5-LO then reverts to the ferrous form in the presence of reducing agents. The reaction profile of 5-LO consists of an initiation phase, where 5-LO is converted to the active (ferric) state, a linear propagation phase associated with the highest conversion rate, and an irreversible inactivation phase. Although lipid hydroperoxides are needed to generate active ferric enzyme, oxidants formed during catalysis may be responsible for the rapid inactivation of 5-LO [152,153]. This process is termed suicide inactivation, as reactive reaction intermediates (LTA_4 in the case of 5-LO) covalently bind irreversibly to the enzyme as it has been shown for 15-LO [153,154].

I.4.2 Activation of 5-Lipoxygenase

5-LO activation is a complex process, facilitated by several different mechanisms that synergistically interact with each other. Unlike the cyclooxygenases (COX-1 and COX-2), 5-LO is not active at the basal state within the cell even if sufficient substrate is present. It must be activated by a number of different factors of which a rise in cytosolic Ca^{++} is considered the most important. All lipoxygenases posses two domains, an amino terminal β-barrel structure and a highly conserved carboxy structure consisting predominantly of α-helices and contains the catalytic activity (for review see [155]). It is assumed that Ca^{++} stimulates 5-LO activity by promoting membrane association, a feature of many Ca^{++} binding enzymes, including $cPLA_2$ [156] or PKC [157]. Ca^{++} binds to a specific S2-domain and thereby increases the hydrophobicity of purified 5-LO [158], resulting in the binding of 5-LO to phosphatidyl-choline vesicles [149]. This process is directed by the S2-domain of 5-LO [155]. In intact cells Ca^{++} stimulates 5-LO translocation to the nuclear envelope, where FLAP is located as well [158-161]. With 5-LO, $cPLA_2$ also translocates to the nuclear membrane in response to Ca^{++} stimulation, thus assembling the different parts of the LT-producing machinery at this location [160]. A number of agonists, which activate the PLC→IP_3→Ca^{++} pathway, are therefore good inducers of LT production, as they activate $cPLA_2$ and 5-LO, the two critical steps of this process.

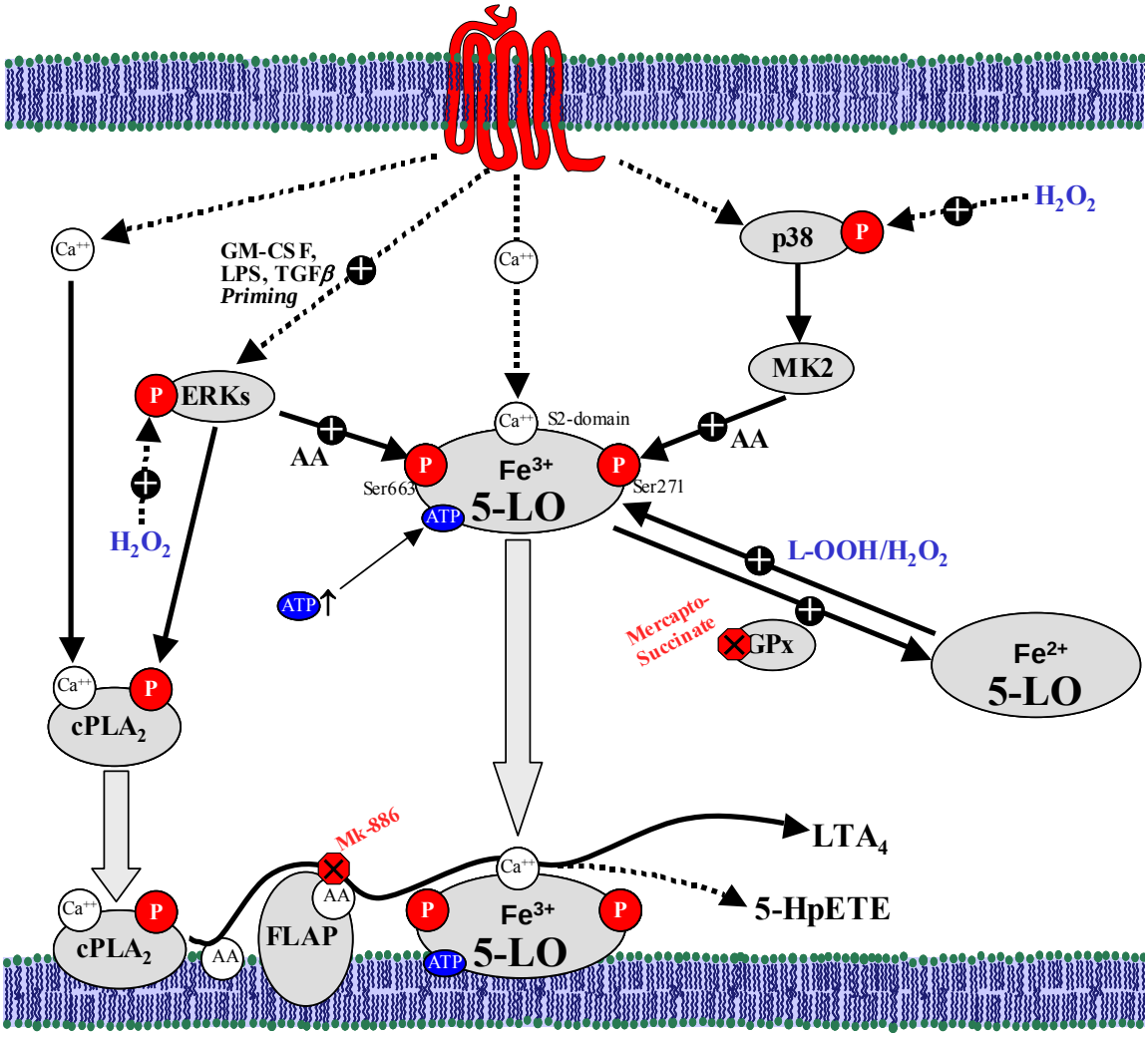


Figure I.8 Activation of 5-lipoxygenase.
The production of leukotrienes can be activated by signals that induce Ca^{++} influx, phosphorylation of 5-LO and $cPLA_2$, or both. Ca^{++} induced binding of these enzymes is required for optimal leukotriene biosynthesis. The oxidation of Fe^{2+} to Fe^{3+} is a requisite for 5-LO enzyme activity. This activation step can be facilitated by lipid hydroperoxides (L-OOH). Lipid hydroperoxides are subject to GPx mediated oxidation. Peroxides such as H_2O_2 can overcome the inhibitory effect of GPx and influencing the redox state of the cell or may promote L-OOH formation from AA via the hydroxyl radical $HO^{\bullet}$. See text for abbreviations and additional details.

Five Lipoxygenase Activating Protein (FLAP)

FLAP is a 18 kDa membrane protein containing 3 membrane spanning regions and is closely related to LTC4-S [162]. It was discovered serendipitously when a potent 5-LO inhibitor (MK-886, Merck-Frosst) was developed. MK-886 inhibits 5-LO activity only in intact cells but does not affect the activity of purified 5-LO. MK-886 also inhibits LTC_4-S, but the potency is much lower [92,162]. With photoaffinity-labeled MK-886, Dixon and colleagues [163] discovered a protein that had a greater affinity for this compound than other proteins of the LT synthesis pathway and demonstrated that this protein is essential for 5-LO activity in whole cells. FLAP was later shown to be located in the nuclear envelope [164], and to bind AA [165] as well as 12-HETE and 15-HETE. FLAP stimulates 5-LO activity by

presenting the substrates AA and 15-HETE to 5-LO, leading to the formation of 5-HETE/LTs [146] and 5,15-diHETE/lipoxins, respectively [165]. FLAP also increases the efficiency of 5-LO mediated conversion of 5-HpETE to LTA_4 [146]. FLAP is considered absolutely necessary for cellular LT synthesis (from endogenous substrate), but it is dispensable for 5-LO activity in cell homogenates [166]. However, there is no absolute requirement for FLAP when cells are stimulated in the presence of exogenous AA [146], and MK886 failed to suppress LT formation under such conditions [167].

Inhibition of 5-LO by Endogenous GPx

Since lipid hydroperoxides or H_2O_2 are required to initiate the enzymatic activity of 5-LO by oxidizing the Fe^{2+} (see above), a negative regulatory role for peroxidases was postulated [168-172]. Indeed, selenium-dependent glutathione peroxidases are potent suppressors of 5-LO activity by reducing lipid hydroperoxides [170-172]. GSH-depleting agents like 1-chloro-2,4-dinitrobenzene have been shown to stimulate 5-LO activity in human neutrophils, a first indication that GPx may play a regulatory role in tonically suppressing 5-LO activity [169].

Jakobsson and colleagues showed that human B lymphocytes do not form 5-LO products when stimulate with Ca^{++} ionophore and AA, even though they posses active 5-LO, as been shown by the formation of LTB_4 and 5-HETE by sonicates from these cells under similar conditions [173]. HL-60 cells and BL41-E95-A cells, a B-lymphocytic cell line, also release only barely detectable amounts of LTs when stimulated with Ca^{++} ionophore and AA [171,172]. It was established that this inhibitory effect depends on serum presence during cell culture. The serum in the medium could be replaced with selenium, which was the first evidence for an involvement of selenium dependent GPx in 5-LO inhibition [171]. It was later shown that in B-lymphocytes and immature myeloid cells, GPx-4 is primarily responsible for the suppression of 5-LO activity [168]. In MonoMac6 cells, a monocytic cell line, 5-LO activity is inhibited by GPx-1 and not GPx-4 [174]. No study exists that compares the amounts of GPx family members in the different leukocyte populations. Total GPx activity measurements however indicated that PMNL contain the lowest activity (1.0 U/g) [175] in comparison to monocytes (3.0 U/g) [175], mononuclear cells (57.2 U/g) [176] and lymphocytes (13.6 U/g) [177]. Attention should be paid to the fact these studies were not conducted following identical protocols and that this may lead to some problems regarding direct comparison of these values. Nevertheless, these findings support the notion that a higher GPx content makes 5-LO activation more difficult. In contrast to this hypothesis, Werz and Steinhilber reported that granulocytic 5-LO is peroxidase-insensitive, and that the high baseline 5-LO activity in neutrophils can not be explained by low GPx activity [171].

Phosphorylation of 5-LO and Direct Stimulation by ATP

Phosphorylation had been shown to stimulate 5-LO activity [178-181]. There are two independent pathways leading to 5-LO phosphorylation: the p38/MK2 pathway, mainly activated by cellular stress, and the ERK pathway, which is mainly stimulated by mitogens. Stimulation of the p38 MAPK leads to the activation of MK2, a MAPK-activated protein (MAPKAP) kinase (MAPKAPK or MK), that in turn phosphorylates Ser271 [182] of 5-LO. In the second pathway stimulation of ERK-2 leads to the phosphorylation of SER663 [183]. Interestingly, the phosphorylation of 5-LO either MK2 or ERK-2 is greatly enhance by addition of AA [183].

A number of conditions prime cells for enhanced LT formation. A priming agent is considered an agent that does not induce the cellular response of interest but rather enhances the response to a second stimulus. Preincubation of neutrophils or whole blood with granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α) or lipopolysaccharide (LPS) for 30 min greatly enhances LT production in response to N-formylmethionyl-leucyl-phenylalanine (fMLP), platelet activating factor (PAF) or ionophore [184-186]. This is due to priming of both $cPLA_2$ and 5-LO. It has been shown that GM-CSF signals through the ERK and fMLP through p38 MAPK [187]. ERK then phosphorylates and activates $cPLA_2$ [187] and 5-LO [183].

It was known early on that 5-LO is stimulated by nucleotides. ATP is the most efficacious, while ADP, AMP, CTP, and UTP are less active. This effect is not due to phosphorylation, since Noguchi et al. showed that γ-S-ATP, a non-hydrolysable ATP analog, is at least as active as ATP itself [188]. The same report showed that ATP is not hydrolyzed by 5-LO itself, indicating a direct stimulatory effect. 5-LO does not have a consensus ATP-binding site, which previously raised questions whether or not this enzyme does indeed bind ATP. However, Zhang and coworkers showed in an excellent study that 5-LO has a single specific nucleotide-binding site that differs from the classic ATP-binding site [189].

I.4.3 Regulation of Leukotriene Synthesis by Reactive Oxygen Species

Hydroperoxides Directly Activate 5-LO by Oxidizing the Catalytic Iron

As mentioned above, activation of resting 5-LO requires the oxidation of the iron at the active site to the ferric state. This switch between Fe^{2+} and Fe^{3+} during the catalytic cycle is crucial for enzyme activity. Among various hydroperoxides, 5-HpETE, 12-HpETE, and 13-HpODE, but not the corresponding hydroxy compounds, have been shown to stimulate 5-LO in homogenates by oxidizing the iron, whereas tBuOOH or cumene hydroperoxide failed in this respect [151,190]. However, fatty acid hydroperoxides are not specifically required for activation of 5-LO, as they can be replaced by H_2O_2 [191].

Indirect Activation of 5-LO by Reactive Oxygen Species

Oxidative stress enhances 5-LO activity by affecting the cellular redox state and potentially by greater MAPK activity. As mentioned above, GPx-1 and GPx-4 and potentially other GPxs or GSTs reduce stimulatory lipid hydroperoxides thus rendering 5-LO inactive by impairing the $Fe^{2+} \rightarrow Fe^{3+}$-transition. It was surmised that agents which inhibit GPx or which change the reductive state within the cell could overcome the inhibitory effects of GPx. In agreement to this hypothesis, 5-LO activity can indeed be enhanced in MonoMac6 cells by addition of the specific GPx-1 inhibitor mercaptosuccinate or the broad GPx inhibitor iodoacetate [192]. Addition of exogenous 13-HpODE also upregulates 5-LO activity in cells through iron oxidation [192]. Analogous results were obtained with other cell types. B-lymphocytes express 5-LO, and considerable amounts of LTs are formed in broken cell preparations [193], whereas stimulation of whole cells with the standard stimulus ionophore plus AA causes only marginal LT production [193]. However, conditions that lead to an elevated redox state, such as depletion of GPx by selenium removal [171], co-addition of H_2O_2 [172] or thiol-reactive agents, such as diamide or N-ethylmaleimide (NEM), that deplete the GSH pool [193] resulted in 5-LO activation and subsequent formation of LTs in response to ionophore plus AA. In

broken cell preparations of BL41-E95-A cells, 5-LO activity was strongly suppressed after reconstitution of GPx activity by addition of millimolar concentrations of thiols [171]. Compared to phagocytes, B-lymphocytes produce only low amounts of endogenous superoxide upon stimulation, and in BL41-E95-A cells superoxide formation was hardly detectable [172]. Intriguingly, ROS generated by exogenous xanthine oxidase or by treatment of cells with antimycin A, which leads to endogenous superoxide release from mitochondria, strongly activated cellular 5-LO in BL41-E95-A cells [172]. Co-addition of catalase, but not of superoxide dismutase abolished these effects, suggesting that hydroperoxides or hydrogen peroxide mediate 5-LO activation, rather than superoxide itself. Further experiments suggest that 5-LO activation is not only restricted to intercellular activation, but transcellular activation as well [172].

As outlined above, ERK and p38 MAPK can phosphorylate and thus activate 5-LO. As oxidative stress activates ERK and p38 MAPK, Werz and colleagues investigated the effects of H_2O_2 on 5-LO activity [180]. They found that H_2O_2 activates p38 MAPK and downstream 5-LO kinases in BL41-E95-A cells. The hypothesis that H_2O_2 acts through p38 MAPK was further supported by the finding that addition of SB203580, a specific p38 inhibitor, reduced H_2O_2-induced 5-LO activity. Thus it is reasonable to hypothesize that pro-oxidative agents may stimulate 5-LO by two ways: (i) promotion of protein phosphorylation resulting in a peroxidase-insensitive 5-LO activity, and by (ii) promoting the formation of Fe^{3+} in the active site of 5-LO. It is further possible that H_2O_2 affects the expression level of 5-LO through its effects on MAPKs. However, there is yet no evidence for such a mechanism for 5-LO induction by H_2O_2.

Inactivation of 5-LO by ROS

With respect to LT synthesis, H_2O_2 is a double-edged sword, as it can stimulate as well as abrogate 5-LO activity. Endogenous H_2O_2 diminishes the formation of LTB_4 by alveolar macrophages and PMNL after stimulation with fMLP or opsonized zymosan in vitro [194]. This effect can be overcome by addition of N-acetylcysteine (NAC), which scavenges endogenous ROS thereby increasing LTB_4 production. LTB_4 synthesis was not affected by SOD, but markedly enhanced by catalase, indicating that H_2O_2 rather than superoxide is responsible for this effect. The inhibitory effect of H_2O_2 appears to be caused by a direct effect on 5-LO and cannot be explained by non-enzymatic oxidation of the product LTB_4. Indeed, concentrations of H_2O_2 similar to those released by phagocytes ($\geq$ 10 μM) inactivate purified 5-LO in the absence of reducing agents [195]. The native (ferrous) enzyme is approximately seven times more sensitive to inactivation by H_2O_2 than the ferric enzyme, suggesting that the mechanism of inactivation possibly involves a Fenton-type reaction. The ferrous enzyme would react with H_2O_2, resulting in the formation of hydroxyl radicals, which are strong oxidants. This reaction can be described as $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$. Although the iron could be in the active state Fe^{3+}, LT synthesis by 5-LO could be blocked by this mechanism in situations where this accompanied by increased H_2O_2 formation (e.g. activation of the respiratory burst) [159].

Effects of Oxidative Stress on AA Levels

Another mechanism by which H_2O_2 could potentially affect the synthesis of 5-LO products is through the availability of the substrate AA. As noted above, H_2O_2 could stimulate $cPLA_2$ phosphorylation through activation of ERK, resulting in increased AA release. H_2O_2 stimulates

free AA levels following stimulation of alveolar macrophages [196], but this effect was due to inhibition of AA esterification into lipid rather than to a direct effect on $cPLA_2$. It was initially proposed that this effect was due to inhibition of arachidonoyl-CoA synthetase [197], but H_2O_2 did not directly inhibit this enzyme in alveolar macrophages, and appeared to act by depleting ATP, which is required for the synthesis of arachidonoyl-CoA [196].

II AIM OF STUDY

> Before ordering a test decide what you will do if it is 1. positive, or 2. negative.
>
> If both answers are the same, don't do the test.
>
> *D. Greenberg (1880-1958)*

In contrast to the biological effects of 5-oxo-ETE, which have been studied in detail, the regulation of 5-oxo-ETE biosynthesis is not well understood. 5-oxo-ETE is formed by the oxidation of 5-HETE. This reaction is catalyzed by 5h-dh, a $NADP^+$-dependent microsomal enzyme that is present in neutrophils, eosinophils, monocytes, lymphocytes, and platelets, and is highly selective for 5-HETE [22,68,70,198]. The aim of this study was to investigate the expression pattern of this enzyme and the regulation of its activity.

II.1. Does Myeloid Differentiation Affect Expression of 5h-dh Activity?

The expression of many enzymes involved in leukotriene synthesis is genetically regulated and confined to specific tissues and differentiation states. 5h-dh has so far been reported to be present in blood leukocytes as well as platelets. It is not known whether 5h-dh is present in myeloid progenitor cells or whether it is induced during their maturation to neutrophils or monocytes. To examine this question we have investigated the synthesis of 5-oxo-ETE from 5-HETE by 5h-dh in HL-60 cells and U-937 [199] cells, which have been widely used in studies on the induction of eicosanoid-forming enzymes [200-209]. The enzyme was examined in whole cells as well as in the microsomal fractions and the effects of various differenting agents were examined. HL-60 cells are a promyelocytic cell line that has been used as a model for granulocyte / monocyte / macrophage differentiation [210]. They can be differentiated into monocyte-like cells by 1,25-dihydroxy-Vitamin D_3 (dh-VitD_3) [211], into macrophage-like cells by phorbol myristate acetate (PMA) [212] and into granulocytic cells by retinoic acid (RA) [213] or dimethyl sulfoxide (DMSO) [214]. U-937 cells, a human promonocytic cell line, are frequently used to study monocyte / macrophage differentiation [215]. This cell line can be differentiated into macrophage-like cells by treatment with PMA [215] or into monocyte-like cells by treatment with dh-VitD_3 [216], RA [217] or DMSO [218]. Unlike HL-60 cells, U-937 cells cannot differentiate into granulocytes [219].

II.2. Does Oxidative Stress Increase 5-oxo-ETE Synthesis?

Despite the presence of very high 5h-dh activity in the microsomal fractions of inflammatory cells, unstimulated intact cells form only very small amounts of 5-oxo-ETE when incubated with 5-HETE. This may be due to limited availability of $NADP^+$, which is present at very low levels in the cytosol, as cells maintain this cofactor in its reduced state (NADPH) as a protective mechanism against oxidative stress [136]. In neutrophils and monocytes, 5-oxo-ETE synthesis is dramatically increased by stimulation of the respiratory burst, which would increase the levels of $NADP^+$ in the cytosol [68,71,74]. While PMNL and monocytes possess NADPH oxidase, which provides the cofactor for 5h-dh when stimulated, lymphocytes and platelets do not express this system. We wished to investigate whether other

systems that may influence the ratio of NADPH to $NADP^+$ could regulate 5-oxo-ETE synthesis in those cells.

We hypothesized that 5-oxo-ETE production is upregulated by oxidative stress and that this is dependent on the glutathione redox pathway facilitates this. H_2O_2, organic peroxides (such as tBuOOH), and lipid peroxides (e.g. 13-HpODE) can affect the cellular redox status, as a major pathway for their inactivation is their GSH-dependent reduction to H_2O by glutathione peroxidases [136]. This results in the formation of GSSG, which is recycled to GSH by glutathione reductase, which concomitantly oxidizes NADPH to $NADP^+$. The latter is in turn reduced back to NADPH by the pentose phosphate pathway (PPP), which uses glucose 6-phosphate as its initial substrate, and is a major defense mechanism against oxidative stress. We reasoned that by increasing $NADP^+$ levels, oxidative stress could be a major regulator of 5-oxo-ETE synthesis. We tested this hypothesis in U-937 cells and then examined the relevance of our findings in regard to different blood cells, including neutrophils, monocytes, lymphocytes, and platelets.

II.3. Can 5-Oxo-ETE by Produced by Structural Cells?

Because of the high expression of 5h-dh in undifferentiated myeloid cell lines (HL-60 and U-937) in the first part of this study, we wondered whether non-myeloid cell types express this enzyme. Under non-pathological conditions, non-myeloid tissue cells contain little or no 5-LO activity, but contain several leukotriene-metabolizing enzymes in a tissue specific distribution pattern. We reasoned that cells that are in close contact to 5-LO-containing leukocytes (e.g. neutrophils and macrophages) and are subject to oxidative stress *in vivo* should be prime suspects to investigate. We therefore investigated whether endothelial as well as epithelial cell lines have the ability to synthesize 5-oxo-ETE.

As our initial experiments confirmed this hypothesis, we wished to characterize the enzyme responsible for this activity to determine whether its properties are similar to those of leukocyte 5h-dh. We also wanted to determine whether the regulation of 5-oxo-ETE synthesis by structural cells is similar to that found in inflammatory cells.

III MATERIAL AND METHODS

III.1. Materials

5-HETE was prepared by total organic synthesis by J. Rokash (Florida Institute of Technology, Melbourne, FL, USA) [220]. 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13-HpODE) and 13*S*-hydroxy-9*Z*,11*E*-octadecadienoic acid (13-HODE) were prepared by oxidation of linoleic acid with soybean lipoxygenase Type 1B. The subsequent was prepared by reduction of 13-HpODE with triphenylphosphine (TPP) [221].

3-Amino-1,2,4-triazole (3-AT), 1,2-bis[2-chloroethyl]-1-nitrosourea (BCNU; carmustine), buthionine sulfoximine (BSO), 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS), dehydroepiandrosterone (DHEA), diamide, dimethyl sulfoxide (DMSO), H_2O_2, N-(2-hydroxyethyl)-piperazine-N'-(2-hydroxypropanesulfonic acid) sodium salt (HEPSSO), 2-morpholinoethanesulfonic acid (MES), N-ethylmaleimide (NEM), tert-butyl hydroperoxide (tBuOOH), phorbol 12-myristate 13-acetate (PMA), phenazine methosulfate (PMS), retinoic acid (RA), and TPP were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Dextran T-500 and Ficoll-Paque were obtained from the Amersham Bioscience Corp (Piscataway, NJ, USA). Auranofin was obtained from the ALEXIS Corporation (Lausen, Switzerland). Phenylmethanesulfonyl fluoride (PMSF), sodium azide (SA), and glucose were purchased from Fisher Scientific (Nepean, Ontario, Canada). RPMI 1640 and other products for cell culture were obtained from Invitrogen (Burlington, Ontario, Canada). 5*S*-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HpETE), arachidonic acid (AA), A23187, and 9-oxo-15*S*-hydroxy-prosta-5*Z*,8(12),13*E*-trien-1-oic acid (PGB_2) were obtained from Cayman Chemicals (Ann Arbor, MI,USA).

III.2. Blood Cell Preparation, Cell Lines, and Culture Conditions

Unless otherwise indicated, cell purifications were done in phosphate-buffered saline (PBS^-; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4 at a pH of 7.4), and incubations in PBS^+ supplemented with calcium (1.8 mM) and magnesium (1 mM) (PBS^+).

III.2.1 U-937 Cells and HL-60 Cells

U-937 and HL-60 cells, obtained from ATCC (Manassas, VA, USA), were cultured in 10% fetal bovine serum (FBS) in modified RPMI 1640 medium containing L-glutamine (2 mM), sodium bicarbonate (1.5 g/L), glucose (4.5 g/L), HEPES (25 mM), and sodium pyruvate (1.0 mM). Cells were maintained at a density between 10^5 and 1.5×10^6 cells/ml. To induce differentiation, cells (2×10^5/ml) were cultured for up to 4 days with vehicle (0.1% DMSO), dh-$VitD_3$ (50 nM), DMSO (1.3%), or RA (300 nM). In case of PMA, cells (10^6 /ml) were incubated with PMA (18 nM) for various times. The higher initial cell concentration was required to ensure sufficient cell numbers, as addition of PMA led to growth arrest. The cells remained in suspension for the duration of all of the above treatments with the exception of the adherent U-937 cells cultured with PMA. In this case, the cells were resuspended by incubation with 2 mM EDTA in PBS^- on ice for 20 min followed by scraping with a rubber policeman.

III.2.2 Epithelial Cell Lines

A-549, BEAS-2B, T84, and HEp-2 cells were obtained from ATCC and cultured in 10% FBS in DMEM/F12 medium containing L-glutamine (2 mM), sodium bicarbonate (1.5 g/L), glucose (2.5 g/L), HEPES (10 mM), and sodium pyruvate (1.0 mM). Cells were sub-cultured before reaching confluence and used before reaching passage 50.

III.2.3 Human Aortic Endothelial Cells (HAEC)

Human aortic endothelial cells (HAEC) were obtained from Clonetics (San Diego, CA, USA) and cultured in endothelial growth medium 2 (EGM-2) (with recommended growth factors, cytokines, and supplements) containing 2% FBS. Cells were subcultured when they reached ~75% confluence at a seeding density of 2,500 to 5,000 cells/cm^2. Cells were used until passage 7.

III.2.4 Preparation of Blood Cells

Neutrophils and peripheral blood mononuclear cells (PBMC) were prepared by treatment of whole blood with Dextran T-500 for 45 min, followed by centrifugation over Ficoll-Paque. Red blood cells (RBC) in the neutrophil-containing pellet were removed by hypotonic lysis [22]. PBMC and neutrophils were then suspended in PBS^-.

Human monocytes were prepared as described in the literature [222] with some modifications. PBMC present at the interface after centrifugation of leukocytes over Ficoll-Paque as described above were washed by centrifugation and resuspended in ice-cold RPMI 1640 (4°C). The washed cells (2×10^7 cells in 10 ml per dish) were then added to Corning tissue culture dishes (Fisher, Nepean, Ontario, Canada) that had been pretreated with 1.5 ml autologous plasma for 20 min at 37°C. After incubation for 30 min at 37°C the loosely adherent lymphocytes were removed with gentle streams of the culture medium from a 10 ml pipette. The lymphocytes were aspirated and this wash-aspiration procedure was repeated twice more with RPMI at 37 ^oC and then with PBS^- at 4° C. The monocyte-enriched adherent cells were detached from the plastic by incubation for 30 min with ice-cold 2 mM EDTA in PBS^- in the cold room, followed by removal with a rubber policeman. This monocyte-enriched fraction (>85% $CD14^+$) is subsequently referred to as monocytes.

For preparation of platelets, whole blood (20 ml) was collected in medium (2.8 ml) containing citric acid (15.5 mM), sodium citrate (90 mM), NaH_2PO_4 (16 mM), dextrose (161 mM), and adenine (2 mM) [70]. After centrifugation at 200 x *g* for 15 min, the supernatant was diluted with an equal volume of medium containing 94 mM citrate and 140 mM dextrose, pH 6.5. After centrifugation at 1000 x *g* for 10 min, the pellet was resuspended in PBS^- to give a platelet concentration of 3×10^8 cells/ml.

III.3. Preparation of Microsomal Fractions

Cells were washed by centrifugation, resuspended in 20 ml PBS^- supplemented with 1 mM PMSF, and disrupted by sonication at a setting of 40 cycles/s (model 4710 ultrasonic homogenizer, Sonics and Materials, Danbury, DC) on ice for 5 × 6 s with 30 s intervals for

cooling [22]. The disruptate was successively centrifuged at 4 °C at 1,500 × g for 10 min, 10,000 × g for 10 min, and 150,000 × g for 80 min and the final pellet was resuspended in PBS^-. Protein was measured with the Bio-Rad *DC* (detergent compatible) protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and adjusted to the indicated concentrations.

III.4. Incubation conditions

III.4.1 5-oxo-ETE Production by Microsomes and Cells in Suspension

Unless otherwise indicated, incubations were performed in PBS^+ in the absence of glucose. Suspensions (1 ml) of HL-60 cells, U-937 cells, lymphocytes, monocytes, PBMC neutrophils (2×10^6 cells/ml), platelets (10^8 cells/ml), or cell microsomes (50 to 500 µg protein/ml) were incubated with 5-HETE (1 µM) for 5 min unless otherwise indicated. To establish the maximal ability of cells to convert 5-HETE to 5-oxo-ETE, cells were preincubated with PMS (100 µM, if not stated otherwise) for 6 min prior to addition of the substrate 5-HETE. PMS stimulated 5-oxo-ETE synthesis by non-enzymatically converting intracellular NADPH to $NADP^+$ (Figure III.1) [75].

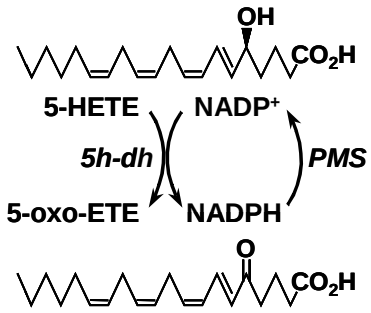


Figure III.1 Measurement of 5h-dh in whole cells. PMS stimulates 5-oxo-ETE synthesis in intact cells through the non-enzymatic conversion of NADPH to $NADP^+$ [75].

To manipulate the effects of oxidative stress, cells or microsomes were preincubated with various substances, none of which affected 5h-dh activity directly, as indicated in Table IV.2, page 55. To study the effect of pH, microsomes were diluted in 50 mM MES (pH 4.0-6.0), 50 mM HEPSSO (ph 6.8 – 9.0), or 50 mM CAPS (pH 9.0 – 11.4).

III.4.2 5-oxo-ETE Production by Adherent Cells

In order to screen epithelial cell lines (A549, BEAS-2B, T84 and HEp-2) for 5h-dh, they were grown in 6-well plates until they reached confluence. For further studies, epithelial cells (A549 and BEAS-2B) were plated approximately 24 h before incubation with 5-HETE in 6-well plates at a cell density of 10^6 cells/well. Prior to the incubations, the culture medium of the epithelial cells was aspirated and the cells were washed once with PBS^+. The incubations were then carried out in 1 ml PBS^+ in the absence of glucose, if not stated otherwise. Endothelial cells were grown to confluence and were treated similarly, except that indicator free Hanks buffer (1 g glucose/ml) was used instead of PBS^+. To estimate the cell number used in each experiments, selected wells were washed once with PBS^- and then trypsinized and counted with a hematocytometer. In our hands 10^6 epithelial cells grew to $1.3\pm0.2 \times 10^6$ and $1.2\pm0.2 \times 10^6$ cells (A549 and BEAS-2B, respectively) within 24h.

III.4.3 Detection of 5-LO activity

To detect 5-LO activity, cells were incubated with AA (20 µM) and the Ca^{++} ionophore A23187 (5 µM) for the indicated times in PBS^{+}. In certain experiments, cells were pretreated for 6 min with PMA (30 nM) or incubated in the presence of tBuOOH (100 µM).

III.5. Eicosanoid Analysis by Precolumn Extraction/(RP)-HPLC

All incubations were terminated by addition of methanol (0.65 mL) and cooling to 0°C. The concentration of methanol in each sample was adjusted to 30% by addition of water and either 13-HODE or PGB_2 were added as internal standards to correct for technical recovery that is not due to metabolism. Alternatively, to detect the more hydrophilic ω-oxidation products of LTB_4, the final MeOH concentration was adjusted to 15%. Eicosanoids were analyzed by precolumn extraction/RP-HPLC [223] using a modified Waters Millenium system (Waters Associates, Milford, MA). The stationary phase was a C_{18} column (150 x 3.9 mm) of octadecylsilyl silica (4 µm particle size Novapak column, Waters). The mobile phase for the detection of 5h-dh activity was a linear gradient composed of solvents A (water/acetic acid (100:0.02)) and B (acetonitrile/acetic acid (100/0.02)) as follows: 0 min: 35% B; 10 min, 90% B. For the detection of 5-LO activity the mobile phase was a linear gradient composed of solvents A (water/acetic acid (100:0.02)), B (acetonitrile/ acetic acid (100/0.02)), and C (methanol/acetic acid (100/0.02)) as follows: 0 min: 65.5% A, 23.7% B, 10.8% C; 30 min, 11.1% A, 37.6% B, 51.3% C. All solvents were of HPLC quality. Products were quantitated by comparing the areas of their peaks of UV absorbance at their λ_{max} with that of the internal standard. The extinction coefficients used were: 5-HETE (235 nm; 27,000), 5-oxo-ETE (280 nm, 20,500), 5-oxo-20-HETE (280 nm, 20,5000), LTB_4 (270 nm, 39,500), PGB_2 (280 nm, 28,680), 13-HODE (235 nm, 23,000), and 13-HpODE (235 nm, 23,000). The identities of the products measured were confirmed by examination of their complete UV spectra.

III.6. Data analysis

For the experiments estimating the metabolism and loss of 5-HETE and 5-oxo-ETE, equal amounts of eicosanoids were incubated in cell-free buffer for 20 min and used as reference. % Remaining LTB_4 was determined as loss of the original added compound. As 5-oxo-ETE and 5-HETE are interconverted in human cells, the sum of 5-oxo-ETE and 5-HETE was compared with the originally added eicosanoid. Since AA has a low extinction coefficient, we used radiolabeled ^{14}C-AA. This was mixed prior to the incubations with unlabeled AA to give a final concentration of 1 µM. AA was analyzed by HPLC and the radioactivity comigrating with cold AA was collected in tubes and analyzed using a β-counter.

EC_{50} and IC_{50} values were estimated by fitting the data to the Hill-equation. In some instances this was done by visual approximation, when the fitting was not appropriate. The results are presented as means ± SE. Statistical significance was always assessed using one-way repeated measures ANOVA followed by the Bonferroni test. A *P* value of less than 0.05 was considered significant. "n" refers to the number of separate independent experiments performed. In the case of blood cells, a different non-asthmatic donor was used for each experiment.

IV RESULTS

IV.1. Effects of Myeloid Cell Differentiation on 5h-dh Activity

IV.1.1 Undifferentiated U-937 and HL-60 cells contain 5h-dh

U-937 and HL-60 cells were treated with vehicle (0.1% DMSO) for 3 days. To determine whether they contain 5h-dh activity, cells (2.5×10^6/ml) were preincubated with 100 µM PMS for 6 min, followed by incubation with 5-HETE (4 µM) for a further 20 min period. Under these circumstances, excessive amounts of substrate were provided while PMS converted NADPH into the cofactor of 5h-dh $NADP^+$ (Figure III.1), thus overcoming the limiting factors of the reaction. Preliminary experiments indicated that this concentration of PMS maximally stimulated 5-oxo-ETE production by these cells (see also Figure IV.10, page 57). The products were then analyzed by reverse-phase-HPLC using PGB_2 as the internal standard. Under these conditions, undifferentiated vehicle-treated U-937 cells synthesized 319 pmol 5-oxo-ETE/10^6 cells (Figure IV.1 A), whereas HL-60 cells synthesized 341 pmol/10^6 cells (Figure IV.1C).

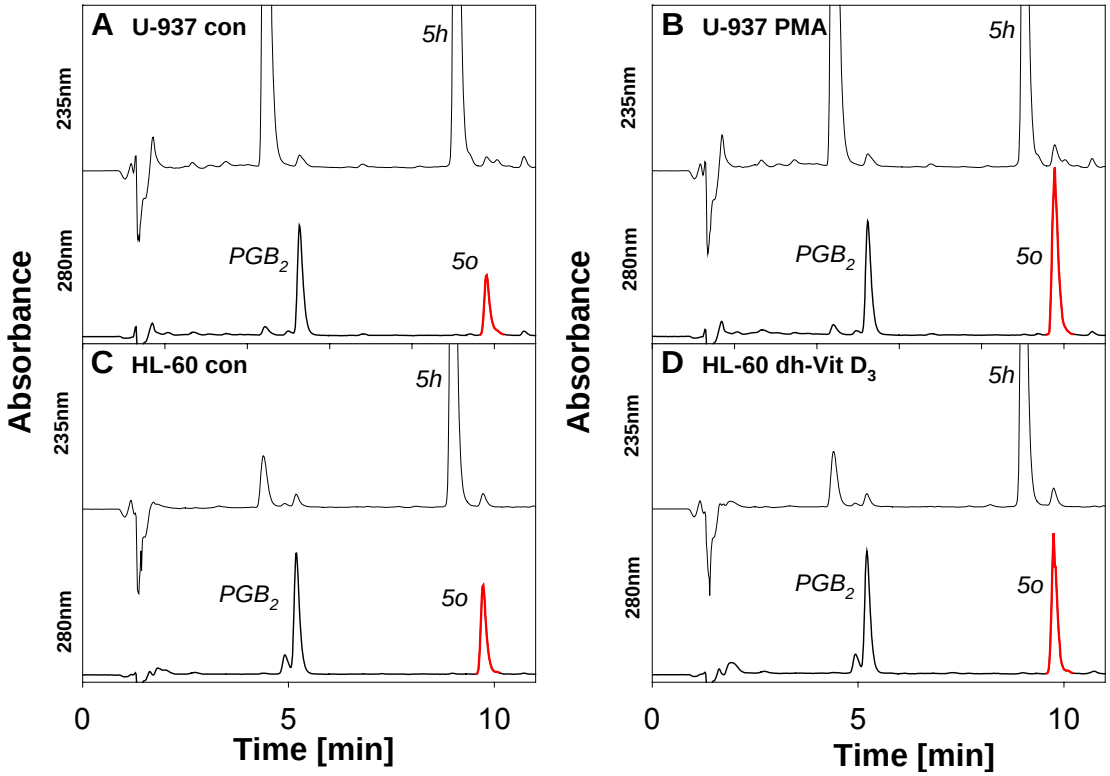


Figure IV.1 Chromatographic analysis of 5-oxo-ETE synthesis by myeloid cell lines
U-937 cells were treated with either vehicle (**A**, 0.1% DMSO) or 18 nM PMA (**B**) for 3 days. Similarly, HL-60 cells were treated with either vehicle (**C**) or 50 nM dh-$VitD_3$ (**D**). Cells (2.5×10^6 in 1 ml) were preincubated with 100 µM PMS, which reduces NADPH non-enzymatically to $NADP^+$, for 6 min prior to incubation with 4 µM 5-HETE for 20 min. The reaction was then terminated by addition of ice-cold MeOH and the amounts of 5-oxo-ETE were analyzed by reversed-phase HPLC using 225 ng PGB_2 as an internal standard as described in Material and Methods.

IV.1.2 Regulation of 5h-dh Activity Expression in U-937 cells

Effects of Differentiating Agents on 5-oxo-ETE Synthesis by U-937 cells

U-937 cells were cultured in the presence of either vehicle or a variety of differentiating agents, including PMA (18 nM), dh-VitD_3 (50 nM), RA (300 nM), and DMSO (1.3%). The cells began to adhere to the surface of the wells after 24 h in the presence of PMA, and were completely adherent by day 2. However, none of the other agents tested induced adherence of U-937 cells under the conditions employed. Treatment of cells with vehicle alone for three days had no effect on their ability to produce 5-oxo-ETE from 5-HETE (Figure IV.2). In contrast, PMA induced a significant ($P < 0.001$) 3-fold increase in 5-oxo-ETE production, from 292 ± 31 to 848 ± 100 pmol/10^6 cells (Figure IV.2). DMSO ($P < 0.01$) and dh-VitD_3 ($P < 0.05$) induced smaller significant increases in 5-oxo-ETE synthesis of approximately 2-fold compared to the vehicle. RA tended to increase 5-oxo-ETE production, but this effect was not statistically significant. For comparison, 5-oxo-ETE synthesis was also assessed in freshly isolated blood monocytes and neutrophils under the identical incubation conditions used for U-937 cells. Undifferentiated U-937 cells produced slightly smaller amounts of 5-oxo-ETE than monocytes (388 ± 15 pmol/10^6 cells; *NS*) and neutrophils (358 ± 150 pmol/10^6 cells; *NS*), whereas PMA-differentiated U-937 cells produced significantly greater amounts.

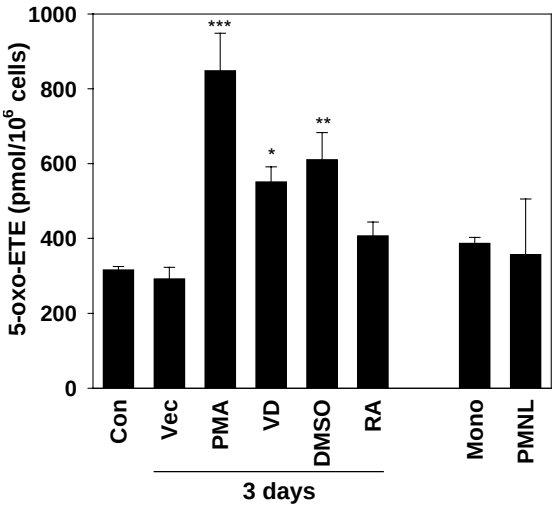


Figure IV.2 Influence of differencing agents on 5h-dh activity in U-U937 cells.
U-937 cells were cultured for 3 days in the presence 18 nM PMA, 50 nM dh-dh-VitD_3 (VD), 0.3 µM all-trans RA or 1.3% DMSO (Vec). The cells were harvested and resuspended in PBS^+ as described in Material and Methods. 2.5×10^6 cells were preincubated with 0.1 mM PMS prior to addition of 4 µM 5-HETE for 20 min. The amounts of 5-oxo-ETE produced by control U-937 cells at day 0 (Con), peripheral blood monocytes (Mono) and neutrophils (PMNL) are shown for comparison. All values are ± SE *, $p \leq 0.05$; **, $p \leq 0.01$; *** $p \leq 0.001$ when compared to vehicle treated cells ($n \geq 4$).

Time Course of PMA Induction of 5-oxo-ETE Synthesis in U-937 cells

As PMA induced the greatest increase in 5-oxo-ETE synthesis, we performed a time course for this response. A significant effect on 5-oxo-ETE synthesis was observed within two days, when the rate of production was double that of control vehicle-treated cells ($P < 0.05$, Figure IV.3A). The response to PMA reached a plateau within three to four days, at which time 5-oxo-ETE was synthesized at about three times the rate of vehicle-treated cells.

Effect of PMA Treatment on Specific 5h-dh Activity in Microsomes

To assess 5h-dh activity in U-937 cells more directly we examined the conversion of 5-HETE to 5-oxo-ETE by microsomal fractions. Microsomes (50 µg protein/ml) from cells treated for four days with either PMA or vehicle were incubated with different concentrations of 5-HETE in the presence of $NADP^+$ (0.1 mM). The amounts of 5-oxo-ETE formed were then

determined by HPLC. Lineweaver-Burk analysis was used to determine the K_M and V_{max} for this reaction. Microsomes from untreated and PMA differentiated cells had similar K_M values of 568 ± 172 nM and 670 ± 182 nM, respectively. PMA treatment resulted in a significant increase in the V_{max} (Figure IV.3 B) from 401 ± 117 to 1289 ± 192 pmol 5-oxo/(min×mg protein) ($P < 0.001$). Table IV.1 shows a comparison of the K_M and V_{max} values for microsomal 5h-dh activity in U-937 cells and peripheral blood neutrophils. The binding affinity of U-937 cell derived 5h-dh tended to be higher than that of neutrophilic 5h-dh (399 ± 137 nM), but the differences were not statistically significant. The V_{max} of neutrophil derived 5h-dh was between that of undifferentiated and PMA-treated U-937 cells (540 ± 295 pmol 5-oxo/(min×mg protein)).

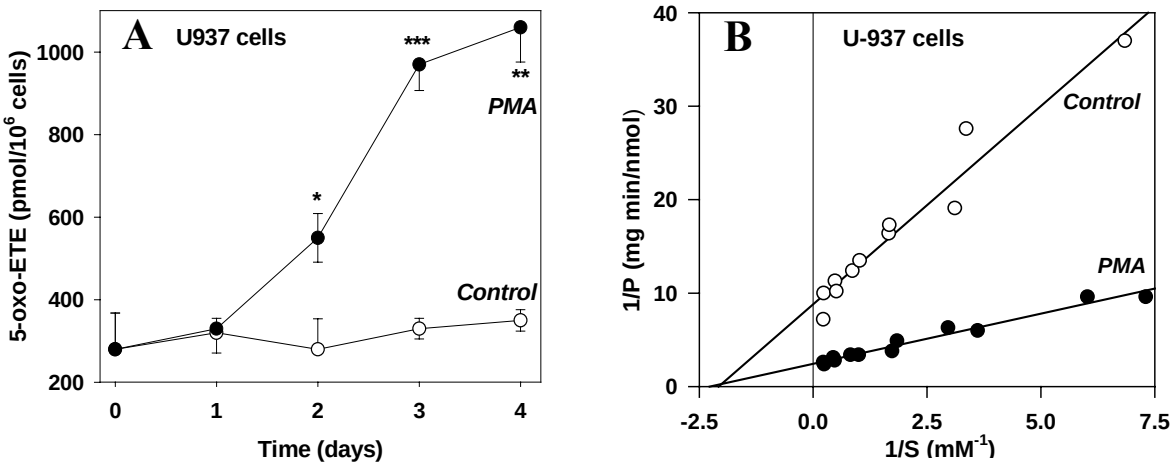


Figure IV.3 Effect of PMA on 5h-dh activity in U-937 cells.
A, U937 cells were treated with either vehicle (Control; ○) or 18 nM PMA (●) for up to four days. At the indicated times the cells were harvested and 5-oxo-ETE formation was determined as described in the legend to Figure IV.1. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ when compared to corresponding vehicle treated cells ($n \geq 4$;). **B,** Lineweaver-Burk Plot. Microsomal fractions obtained from either vehicle-treated (○), or cells differentiated with PMA (18 nM) over 4 days (●), were incubated with 0.1 mM NADP+ and various amounts of 5 -HETE (0.2 – 6 µM) in PBS. After 5 min the reactions were terminated by addition of an equal volume of ice-cold MeOH and the samples were analyzed as described in III.5, page 45.

Table IV.1 Lineweaver-Burk analysis of 5h-dh in neutrophils and U-937 cells
Microsomes (50 µg protein/ml) derived from neutrophils and U-937 cell treated either with vehicle or PMA for 4 days were incubated with increasing amounts of 5-HETE and 100 µM $NADP^+$ for 5 min. The K_M and the V_{max} for 5-oxo-ETE formation were then analyzed by linear regression as shown in Figure IV.3 B. The calculated values for V_{max} and K_M are shown ± SE with the number of experiments in parentheses. The amounts of microsomal protein per cell is shown as well. * V_{max} is significantly (P<0.01) higher in PMA treated U-937 cells than in vehicle control (n).

	K_M (nM)	V_{max} $\left(\frac{\text{pmol}}{\text{min} \times \text{mg protein}}\right)$	Microsomal Protein (µg / 10^6 cells)
U-937 ctrl	568 ± 172	401 ± 117 (5)	37.6 ± 15.8 (4)
U-937 PMA	670 ± 182	1289 ± 192** (5)	33.9 ± 8.4 (4)
Neutrophils	399 ± 137	540 ± 295 (4)	7.0 ± 0.9 (5)

IV.1.3 Examination of 5h-dh in HL-60 cells

Effects of Differentiating Agents on the Synthesis of 5-oxo-ETE by HL-60 cells

The effects of treatment of HL-60 cells with the above differentiating agents for 3 days on 5-oxo-ETE synthesis were investigated after 3 days. The vehicle alone (0.1% DMSO) had no effect on 5-oxo-ETE synthesis by HL-60 cells, which was similar to that observed for both monocytes and neutrophils (Figure IV.4). The only agent that significantly stimulated 5-oxo-ETE synthesis by HL-60 cells was dh-VitD_3, which significantly increased the amount of this eicosanoid from 345 ± 50 to 715 ± 226 pmol 5-oxo/10^6 cells ($P < 0.001$). PMA had a modest effect on 5-oxo-ETE synthesis by these cells, but this was not statistically significant (532 ± 73 pmol 5-oxo/10^6 cells; *NS*). Neither RA nor DMSO had any effect. In contrast to U-937 cells, PMA did not induce adherence of HL-60 cells, but did result in the death of 20-50% of the cells within four days and inhibited cell proliferation within one day.

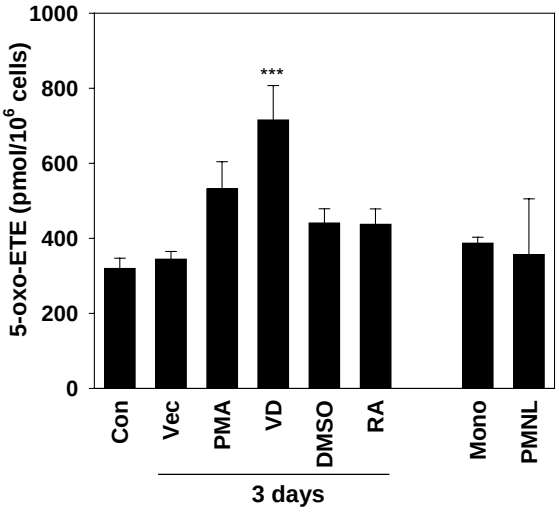


Figure IV.4 Influence of differencing agents on 5h-dh activity in HL-60 cells. HL-60 cells were cultured for 3 days in the presence of vehicle (0.1% DMSO), 18 nM PMA, 50 nM dh-dh-VitD_3 (VD), 0.3 μM all-trans RA, or 1.3% DMSO. The cells were harvested and resuspended in PBS^+ as described in Material and Methods. 2.5×10^6 cells were preincubated with 0.1 mM PMS prior to addition of 4 μM 5-HETE for 20 min. The amounts of 5-oxo-ETE produced by control HL-60 cells at day 0 (Con), peripheral blood monocytes, and neutrophils are shown for comparison. ***, $P \leq 0.001$ when compared to vehicle treated cells ($n \geq 4$).

Previous studies [208,224] showed that maximal neutrophilic differentiation of HL-60 cells with DMSO is reached after extended culture for up to 7 days. In our hands 1.3% DMSO induced considerable loss of viability when cells were cultured in 10% FBS. Over 40% were dead by day 4 as determined by their ability to exclude trypan blue. To investigate the potential effect of DMSO over longer periods, we grew HL-60 cells in 20% FBS in the presence or absence of 1.3% DMSO for up to 6 days [208,224]. After this period, control cells and DMSO-treated cells produced 363 ± 55 and 321 ± 25 pmol 5-oxo-ETE/10^6 cells, respectively (n=3; *NS*).

Time Course for Induction of 5-oxo-ETE Synthesis in HL-60 Cells by dh-VitD_3.

HL-60 cells were cultured in the presence of vehicle or dh-VitD_3 for various times. There were no changes in 5-oxo-ETE synthesis by vehicle-treated cells over a period of four days. In contrast, dh-VitD_3 significantly increased the ability of these cells to synthesize 5-oxo-ETE by day two ($P < 0.05$) (Figure IV.5). Maximal rates of 5-oxo-ETE production were observed after three days of treatment with PMA ($P < 0.01$).

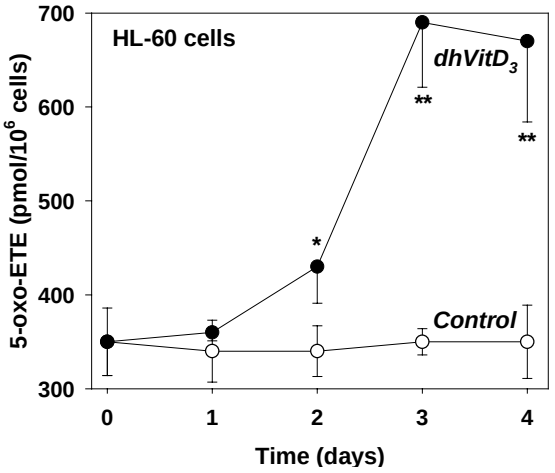


Figure IV.5 Time course of 5h-dh activity after dh-VitD3 differentiation.
HL-60 cells were treated with 50 nM dh-VitD3 (●) or vehicle (○) for up to four days. At the indicated times the cells were harvested as described in material and methods. The cells were then incubated with PMS and 5-HETE and the amount of 5-oxo-ETE produced was analyzed as described above. *, $P \leq 0.05$; **, $P \leq 0.01$ when compared to the corresponding vehicle treatment ($n \geq 3$).

IV.1.4 Interconversion of 5-HETE and 5-oxo-ETE by U-937 Cells

Both, neutrophils and platelets have been shown to convert 5-oxo-ETE and to 5-HETE, potentially due to the activity of 5h-dh, although a distinct keto-reductase has not been excluded [22,70]. We wanted to determine whether the forward (5-HETE → 5-oxo-ETE) or the backward (5-oxo-ETE → 5-HETE) reaction is favored in unstimulated U-937 cells. We incubated 2×10^6 U-937 cells with 1 μM 5-HETE or 5-oxo-ETE for various times and measured the product formation by HPLC. In this case PMS was not added, so that there would be a large excess of cytosolic NADPH over $NADP^+$, thus favoring the reverse reaction. Figure IV.6 A shows the amounts of 5-oxo-ETE and 5-HETE being formed under these conditions. Although the rate of the backward reaction was higher at 3, 6, and 12 min, there was no significant difference in the forward and backward reactions. At 12 min, 24 ± 6 pmol 5-HETE /10^6 cells and 19 ± 1 pmol 5-oxo-ETE/10^6 cells were formed.

We then examined the interconversion of 5-HETE and 5-oxo-ETE more directly. We incubated microsomes from undifferentiated U-937 cells with either 5-oxo-ETE or 5-HETE and the corresponding cofactor (NADPH or $NADP^+$, respectively) for 15 min in 50 mM MES, 50 mM HEPSSO, or 50 mM CAPS (Figure IV.6). The pH ranged from pH 4 - 11.4. For the pH values at which the buffer was changed, separate measurements were made using each of the two buffers. The forward and reverse reactions followed distinct pH dependencies. The pH maximum of the forward reaction was pH 10 (322 pmol 5-oxo-ETE/(min×mg)) and that of the backward reaction was pH 6 (P = 100 pmol 5-HETE/(min×mg)). The conversion rates of the forward and reverse reactions intercepted between pH 6 and 6.8. At the physiological pH of 7.45, the rate of the forward reaction of microsomal 5h-dh is double the rate of the reverse reaction. Both, the forward and the reverse reaction of microsomal 5h-dh are inactivated at extreme pHs (pH ≤ 4.0 and pH ≥ 11.4). $NADP^+$ alone was not capable of oxidize 5-HETE in the absence of microsomes at pH 4, pH 6, pH 7.4, and pH 10.

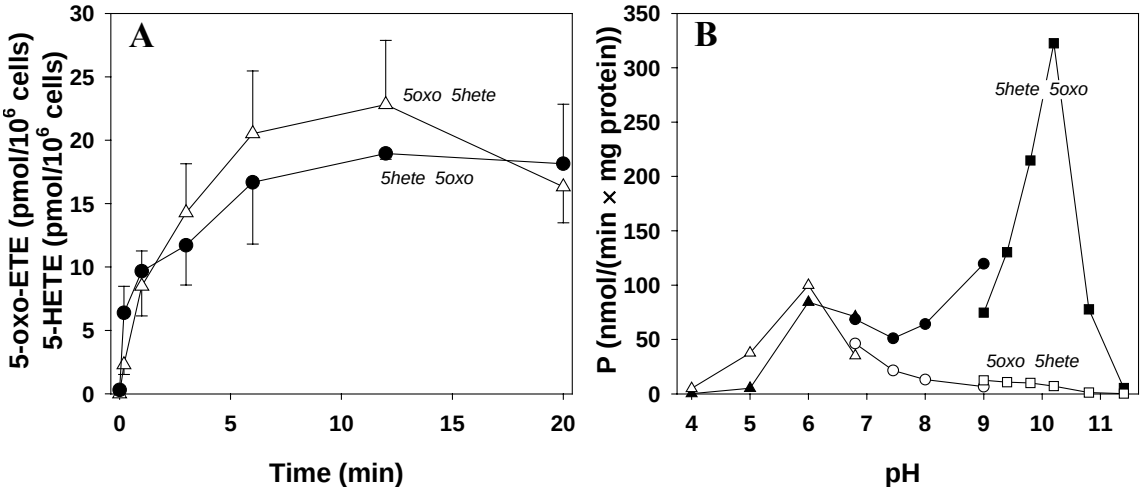


Figure IV.6 Interconversion of 5-oxo-ETE and 5-HETE by U-937 cells
A shows the time course for the formation of 5-oxo-ETE from 1 µM 5-HETE (●), and of 5-HETE from 1 µM 5-oxo-ETE (Δ) following incubation of 2×10^6 U-937 cells in PBS^+ (n=4). **B** shows the pH-dependence of microsomal 5h-dh for the forward and the reverse reaction. 100 µg protein were incubated in 50 mM MES (▲,Δ), HEPSSO (●,○), or CAPS (■,□) for 15 min with either 1 µM 5-HETE and 100 µM $NADP^+$ (filled symbol) or 1 µM 5-oxo-ETE and 100 µM NADPH (open symbols). The results are representative of three such experiments.

IV.1.5 Metabolism of 5-oxo-ETE, 5-HETE, and AA by esterification

We detected a significant loss of 5-oxo-ETE and 5-HETE when they were incubated with U-937 cells. We originally hypothesized that 5-oxo-ETE is converted by LTC_4-S to FOG_7 or by another GST to its stereoisomer [91,225], because U-937 cells contain significant amounts of LTC4-S [201] and have been previously utilized to purify this enzyme [226]. Since FOG_7 does not have a UV chromophore, we conducted experiments with radioactively labeled 5-oxo-ETE. However, initial experiments with radiolabeled 5-oxo-ETE using HPLC protocols, which were optimized to analyze glutathionylated LTs, showed no product peaks other than 5-oxo-ETE or 5-HETE (data not shown).

The absence of radioactive product peaks in the chromatograms sparked a series of experiments to investigate whether 5-oxo-ETE and 5-HETE are esterified to lipids by a CoA-dependent pathway, as the products would not have been detected under the condition we used. Figure IV.7 shows the time course for the recovery of 5-HETE, 5-oxo-ETE, LTB_4 and AA following incubation with U-937 cells. The recoveries of 5-HETE and 5-oxo-ETE were measured as the sum of both eicosanoids, due to the fact they are interconverted by these cells (see Figure IV.6). Recovery of ^{14}C-AA was measured was analyzed by isocratic RP-HPLC. Radioactivity in the fractions collected every minute was detected with a β counter. The recovery of AA and the eicosanoids tested were adjusted to the amounts recovered from cell-free incubations (20 min).

Under our conditions the rate of 5-oxo-ETE loss was significantly larger than the rate of 5-HETE (Figure IV.7) ($P < 0.005$ at 5 min) suggesting that the underlying mechanism prefers 5-oxo-ETE as a substrate over 5-HETE. Although AA had the tendency to be lost more rapidly than 5-oxo-ETE, further experiments are needed to gain statistical significance. In contrast,

LTB_4 was not metabolized in a similar manner. At no time we detected a significant decrease in the amount of LTB_4 compared to cell free incubations.

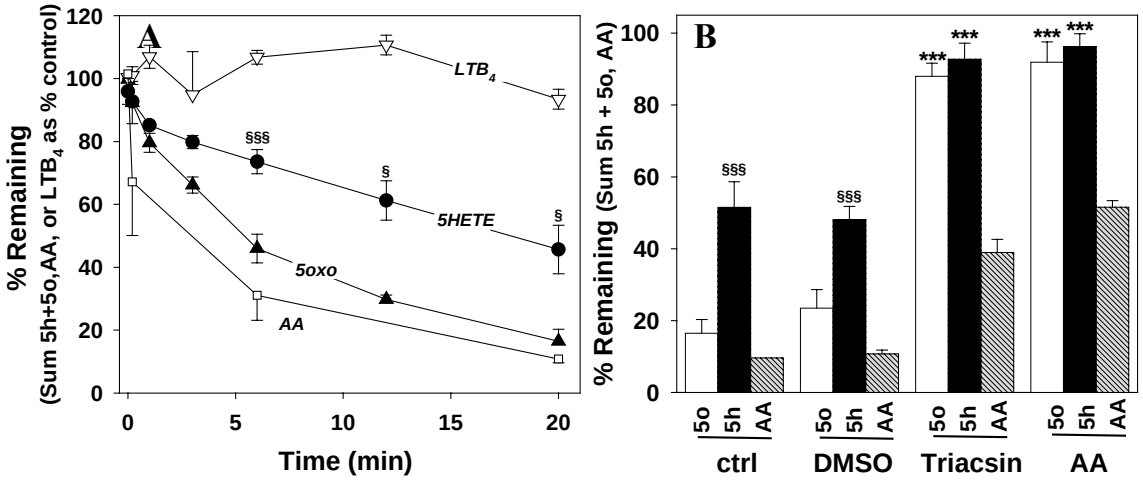


Figure IV.7 Recovery of LTB_4, 5-oxo-ETE, and 5-HETE from U-937 cells.
A 2×10^6 U-937 cells were incubated with 1 µM 5-HETE (●,n=4), 5-oxo-ETE (▲,n=4), LTB_4 (▽,n=4), or AA (□,n=2) for the indicated times. The sum of 5-oxo-ETE and 5-HETE recovered is expressed as the percentage of the original eicosanoid added to buffer in the absence of cells (n=4). LTB_4 and AA are expressed as the amounts of fatty acid recoverd from buffer alone. See Material and Methods for more details. §, $P < 0.05$; §§§, $P < 0.001$ when compared to recovery of 5-oxo-ETE. **B** U-937 cells were treated for 30 min in the absence (ctrl), in the presence of vehicle (DMSO) or Triacsin C (20 µM) prior to addition of substrate. Arachidonic acid (AA, 40 µM) was added at the same time as the substrate: 5-oxo-ETE (1 µM, open bars, n=4), 5-HETE (1µM, solid bars, n=4) or AA (1 µM, striped gray bars, n=2). The incubations were terminated 20 min after substrate addition and the recovery was determined as described above (n=4). §, $P < 0.05$; §§§, $P < 0.001$ when compared to recovery of 5-oxo-ETE; *, $P < 0.05$; **, $P < 0.001$ compared to vehicle treated (DMSO) cells.

Based on the above results we hypothesized that 5-HETE and 5-oxo-ETE are taken up by the cells, bound to Coenzyme A (CoA), and then esterified into either phospholipids or triglycerides, as described for 5-HETE in human neutrophils [59]. To test this hypothesis, we attempted to block the uptake of 5-oxo-ETE and 5-HETE by preincubation of the cells for 30 min with triacsin C, an inhibitor of nonspecific long chain acyl-CoA synthetases and preference for arachidonyl-CoA synthetase *in vitro* [101]. Triacsin C increased the recovery of 5-HETE from 48 ± 4% (vehicle treated cells) to 93 ± 5% and that of 5-oxo-ETE from 23 ± 5% to 88 ± 4% (n=4, $P < 0.001$) after incubation with U-937 cells for 20 min (Figure IV.7). To further substantiate the potential involvement of lipid esterification in the loss of 5-HETE and 5-oxo-ETE we also attempted to block this process by addition of 40µM AA, which should saturate the acylation process. Similar to triacsin C, the addition of AA increased the amount of 5-HETE recovered to 93 ± 5% and that of 5-oxo-ETE to 92 ± 6% (n=4, $P < 0.001$). Analogous results were obtained when AA was replaced by DHA, a 22 carbon ω3-PUFA. The addition of DHA raised the recovery of 5-HETE and 5-oxo-ETE to 102 ± 4% and 95 ± 4%, respectively (n=2). We show here vehicle and untreated control, because initial experiments indicated that DMSO treatment may enhance the recovery of 5-oxo-ETE. However, these differences were not statistically significant.

Preliminary studies were undertaken to compare the recovery of AA with that of 5-oxo-ETE or 5-HETE. AA appears to be taken up more rapidly than 5-oxo-ETE (Figure IV.7A), but more experiments are needed in order to perform statistical analysis. To determine whether AA is metabolized in a similar manner as 5-HETE or 5-oxo-ETE, U-937 cells were pretreated with triacsin C or co-incubated with successive amounts of unlabeled AA. As shown in Figure IV.7B, the treatment with triacsin C and AA had similar effects, enhancing the recovery of AA from 11 ± 1% in vehicle treated cells to 39 ± 4% and 52 ± 2%, respectively.

IV.1.6 5-LO and ω-Oxidation Activity in U-937 and HL-60 cells

5-oxo-ETE and 5-HETE are not Converted by ω-Oxidation in U-937 and HL-60 cells

Neutrophils convert 5-HETE [72] and 5-oxo-ETE [34] to their 20-hydroxy metabolites via LTB_4 20-hydroxylase using NADPH as cofactor and it is not know whether U-937 express CYP4F3 or CYP4F2 (see also page 24). As PMS inhibits this enzyme [74], it could potentially have masked 20-hydroxylase activity in the present studies. However, we were unable to detect any ω-oxidation products following incubation of 5-HETE or 5-oxo-ETE with either intact U-937 or HL-60 cells in the absence of PMS. Differentiation of U-937 cells with PMA did not lead to detectable ω-oxidation of 5-HETE or 5-oxo-ETE. Similarly, differentiation of HL-60 cells with dh-$VitD_3$ for 3 days or with DMSO for up to 6 days did not lead to the formation of detectable ω-oxidation products. Under similar conditions, human peripheral blood neutrophils (2×10^6 PMNL/ml) converted 1 μM 5-HETE within 5 min to 72 ± 6 nM 5,20-diHETE (Figure IV.12, page 59).

U-937 and HL-60 Cells do not contain detectable5-LO Activity

To investigate whether these cell lines are capable of forming 5-oxo-ETE from exogenous AA, we incubated 5×10^6 HL-60 or U-937 cells with 20 μM AA, 5 mM Ca^{++} ionophor A23187 and 30 nM PMA for 20 min and looked for potential 5-LO products (LTB_4, 5-HETE, and 5-oxo-ETE). In our hands, HL-60 and U-937 cells did not have detectable levels of 5-LO activity when cultured under standard conditions (10% FBS in RPMI 1640), in agreement with a previous report [227]. Neither were we able to detect any 5-LO products in U-937 cells differentiated with PMA, or in HL-60 cells treated with dh-$VitD_3$ or DMSO for 3 days. We also examined AA metabolism in HL-60 cells differentiated for five or six days in RPMI 1640 with 20% FBS and 1.3% DMSO. Again, 5-LO products were below the detection limit of our system. In comparison, human PMNL (2×10^6 cells/ml) regularly produced large amounts of 5-LO products (defined as the sum of LTB_4, LTA_4 hydrolysis products, 5-HETE, 5,15-diHETE, 5-oxo-ETE, and the corresponding ω-oxidation products) in the μM-range when stimulated under the same conditions.

For every complex problem, there is a solution that is simple, neat, and wrong.

H. L. Mencken

IV.2. Regulation of 5-oxo-ETE Synthesis by Oxidative Stress

IV.2.1 Oxidative Stress Enhances the Synthesis of 5-Oxo-ETE

H_2O_2 enhances Formation of 5-oxo-ETE from 5-HETE in U-937 Cells

As U-937 cells possess high 5h-dh activity, they were used as a model to investigate the effects of oxidative stress induced on 5-oxo-ETE synthesis. Oxidative stress was induced by treatment with either H_2O_2, tBuOOH, or 13-HpODE. Following incubation of these cells with 5-HETE (1 µM) in the presence or absence of H_2O_2 (100 µM), the amounts of 5-oxo-ETE were measured by HPLC using 13-HODE as an internal standard. In the absence of H_2O_2 very little 5-oxo-ETE was detected (Figure IV.8 A), whereas large amount were formed in its presence (Figure IV.8 B).

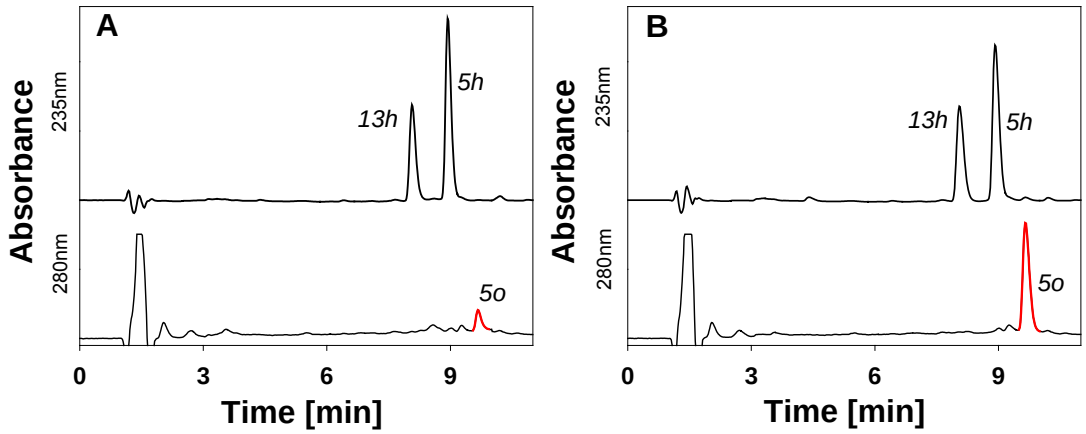


Figure IV.8 Hydrogen peroxide stimulates the formation of 5-oxo-ETE from 5-HETE. Chromatograms of the products formed during incubation of U-937 cells (2 x 10^6 cells/ml) with 5-HETE (1 µM) for 5 min in the presence of either vehicle (**A**) or H_2O_2 (**B**). Precolumn extraction/RP-HPLC was performed as described in Materials and Methods. Abbreviations: 13h, 13-HODE (internal standard); 5h, 5-HETE; 5o, 5-oxo-ETE.

Time Course of H_2O_2 and tBuOOH induced 5-oxo-ETE Synthesis

The time courses for the effects of H_2O_2 (100 µM) and tBuOOH (100 µM) on 5-oxo-ETE formation are shown in Figure IV.9 A. Both hydroperoxides strongly stimulated 5-oxo-ETE synthesis to a similar extent over the first 5 min. However, the amount of 5-oxo-ETE formed in the presence of H_2O_2 subsequently declined, whereas it continued to increase in the presence of tBuOOH and reached maximal levels within 10 to 15 min. To determine whether this transient effect of H_2O_2 could be due to its metabolism, incubations were performed in the presence of sodium azide, which inhibits both catalase and myeloperoxidase [117], two key enzymes involved in H_2O_2 metabolism. Azide did not significantly alter the response to H_2O_2 during the first 5 min, but thereafter strongly enhanced its effect on 5-oxo-ETE synthesis (Figure IV.9 A). Due to the short-lived effect of H_2O_2, all subsequent incubations with 5-HETE were performed for a period of 5 min. No detectable oxidation of 5-HETE occurred when it was incubated with H_2O_2 or tBuOOH in the absence of cells (data not shown).

Table IV.2. Effects of various agents on microsomal 5h-dh activity.
The right column shows the effects of various agents on 5h-dh activity in microsomal fractions (150 µg protein/ml) from U-937 cells, following incubation with 5-HETE (1 µM) and $NADP^+$ (100 µM) for 5 min. The concentrations and preincubation times shown in the left column were used for incubations with both microsomes and intact cells. The amounts of 5-oxo-ETE are expressed as percentages of control values (811± 115 pmol/mg protein) and are the average ± SE of three separate experiments. None of the agents significantly affected 5h-dh activity.

Compound	Action	5-oxo-ETE (% Control)
3-AT (5 mM, 30 min)	Inhibits catalase [228]	106.6 ± 7.9
Auranofin (10 µM, 30 min)	Inhibits thioredoxin reductase [135]	§§
Azide (1 mM, 30 min)	Inhibits heme enzymes (myeloperoxidase, catalase) [117]	110.5 ± 6.3
BCNU (100 µM, 30 min)	Inhibits glutathione and thioredoxin reductases [86]	106.8 ± 4.7
BSO (1 mM, 24 h)	Inhibits γ-glutamylcysteine synthetase (depletes GSH) [229]	§
DHEA (100 µM, 30 min)	Inhibits Gluc-6-P dehydrogenase (Blocks PPP) [137]	114.5 ± 0.2
Diamide (250 µM)	Converts GSH → GSSG [230]	84.8 ± 8.6
DMSO (1%, 30 min)	Vehicle	110.3 ± 5.1
Glucose (1 mg/ml, 30 min)	Gluc → Gluc-6-P → PPP	103.9 ± 7.9
H2O2 (100 µM)	Induces oxidative stress	93.2 ± 1.5
NEM (100 µM, 10 min)	Alkylates SH groups [231]	92.4 ± 12.1
PMA (30 nM, 6 min)	Stimulates PKC & the respiratory burst [232]	102.1 ± 1.1
PMS (100 µM, 6 min)	Converts NADPH → $NADP^+$ [75]	88.2 ± 3.1
tBuOOH (100 µM)	Induces oxidative stress	84.9 ± 5.1

§ Did not affect PMS-stimulated 5-oxo-ETE synthesis in U-937 cells.
§§ Did not affect H_2O_2-stimulated 5-oxo-ETE synthesis in U-937 cells.

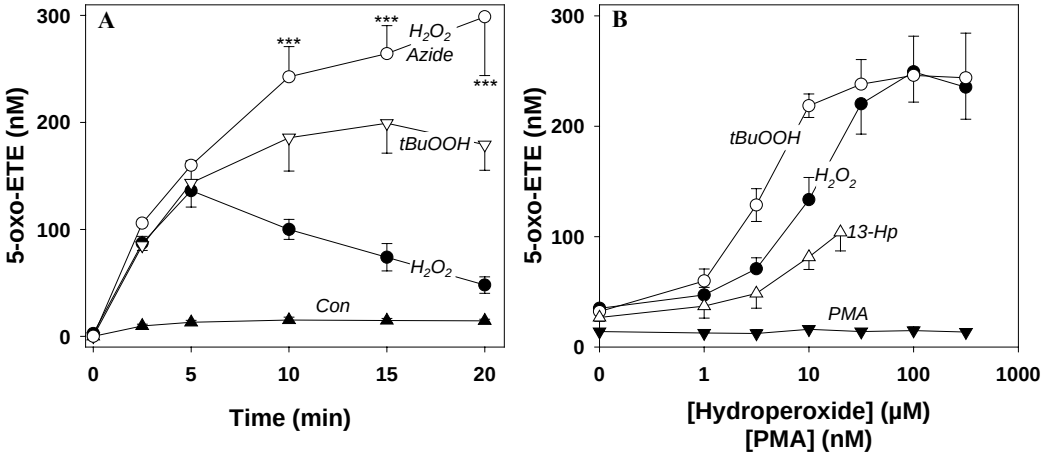


Figure IV.9 Hydroperoxides stimulate the formation of 5-oxo-ETE from 5-HETE.
A: Time courses for the formation of 5-oxo-ETE following incubation of 1 µM 5-HETE with U-937 cells in the presence of vehicle (Control; ▲; n = 4), 100 µM H_2O_2 (•; n = 7), 100 µM H_2O_2 and 1 mM sodium azide (H_2O_2 /Az; ○; n =3), and 100 µM tBuOOH (▽; n = 4). ***, $P < 0.001$ when compared to H_2O_2 without azide. **B:** Concentration-response curves for the effects of H_2O_2 (•; n = 5), tBuOOH (tBu; ▽; n = 3), 13-HpODE (13hp; Δ; n = 3), and PMA (▼; n = 3) on the formation of 5-oxo-ETE following incubation of U-937 cells with 1 µM 5-HETE for 5 min. 5-Oxo-ETE was measured as shown in Figure IV.8A. All values are means ± SE.

The effects of various concentrations of different hydroperoxides on 5-oxo-ETE formation by U-937 cells are shown in Figure IV.9B. tBuOOH (EC_{50}, 3.5 ± 0.9 μM) is the most potent stimulator of 5-oxo-ETE formation, followed by H_2O_2 (EC_{50}, 12.1 ± 2.6 μM). Both hydroperoxides elicited an approximately 7.5-fold increase in 5-oxo-ETE synthesis when compared to controls. The linoleic acid metabolite 13-HpODE also stimulated 5-oxo-ETE formation, but was somewhat less potent. Because of the limited availability of this substance, we were unable to determine the maximal response. In contrast to hydroperoxides, PMA, which is a potent stimulator of 5-oxo-ETE formation by neutrophils [74], had no detectable effect on its formation by U-937 cells after 5 min (Figure IV.9B).

IV.2.2 The GSH Redox Cycle Mediates the Effect of Oxidant Stress on 5-Oxo-ETE Formation

An important means of cellular inactivation of hydroperoxides is their GSH-dependent reduction by glutathione peroxidase, followed by the reduction of the resulting GSSG back to GSH by glutathione reductase, which is accompanied by the formation of $NADP^+$ (for overview see Figure V.1, page 76). To determine whether the stimulatory effect of hydroperoxides on 5-oxo-ETE formation could be mediated by the glutathione redox cycle, we tested the effects of various reagents that affect this cycle.

The glutathione reductase inhibitor BCNU (carmustine) [233] completely blocked the stimulatory effect of H_2O_2 on 5-oxo-ETE formation (Figure IV.10A) and had a similar effect on the response to tBuOOH (data not shown). For comparison, baseline conversion of 5-HETE to 5-oxo-ETE (± SE) in the absence of H_2O_2 or inhibitors is shown by the horizontal lines in Figure IV.10A. The effect of BCNU (30 μM) on the concentration-response to H_2O_2 is shown in Figure IV.10B. In contrast to its strong inhibitory effect on H_2O_2-induced 5-oxo-ETE formation, BCNU had little effect on the response to PMS (Figure IV.10B, inset), which acts by a different mechanism as it non-enzymatically converts intracellular NADPH to NADP+ [76]. Nor did it affect the synthesis of 5-oxo-ETE by microsomal fractions from U-937 cells (Table IV.2). BCNU also inhibits thioredoxin reductase [233], which could also potentially explain its inhibitory effect on the response to H_2O_2. To determine whether thioredoxin redox cycling is involved in this response we investigated the effects of the thioredoxin reductase inhibitor auranofin on H_2O_2-induced 5-oxo-ETE formation. Auranofin, which is a very potent inhibitor of this enzyme (IC_{50}, 4 nM) [135], had no effect on the formation of 5-oxo-ETE in response to H_2O_2 at concentrations as high as 10 μM (Table IV.3). This inhibitor was also without effect on PMS-induced 5-oxo-ETE formation.

To provide further evidence for the role of GSH in response to H_2O_2 we used two approaches to deplete cellular GSH. NEM, which is an extremely efficient alkylator of SH groups, thus preventing the formation of GSSG, completely suppressed H_2O_2-induced 5-oxo-ETE synthesis to levels below baseline (Figure IV.10A). Similar to BCNU, NEM did not have a direct effect on 5h-dh (Table IV.2). The response to H_2O_2 was also inhibited by about 50% following culture of U-937 cells for 24 h in the presence of BSO (Figure IV.10C), which depletes cells of GSH by inhibiting γ-glutamylcysteine synthetase [229]. In contrast, BSO had no effect on the stimulatory effect of PMS on 5-oxo-ETE formation.

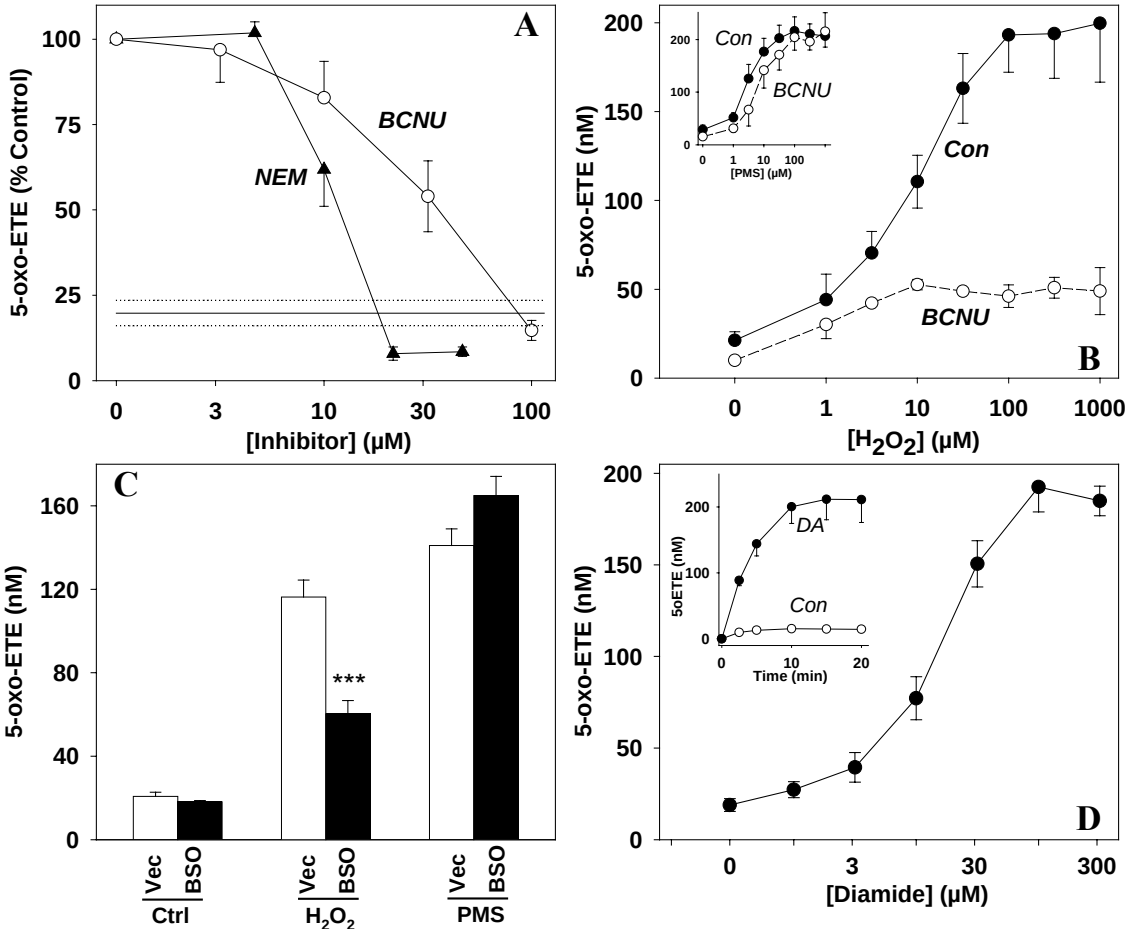


Figure IV.10 The effects of H_2O_2 on 5-oxo-ETE formation are blocked by inhibitors of the GSH redox cycle and stimulated by oxidation of GSH.
A: Effects of different concentrations of BCNU (○; n = 4) and NEM (▲; n = 4) on the amount of 5-oxo-ETE formed during incubation of 5-HETE (1 μM) with U-937 cells in the presence of H_2O_2 (100 μM) for 5 min. The solid and dashed horizontal lines indicate the amount ± SE of 5-oxo-ETE formed from 5-HETE in the absence of H_2O_2 or any other agents. **B:** Concentration-response curves for effects of H_2O_2 on 5-oxo-ETE formation in the presence of either BCNU (○; 30 μM; n = 3) or vehicle (●; Con; n = 3). The inset shows the concentration-response curves for effects of PMS on 5-oxo-ETE formation in the presence of either BCNU (○; 30 μM; n = 3) or vehicle (●; n = 3). **C:** Effects on 5-oxo-ETE synthesis of depletion of GSH in U-937 cells by treatment with vehicle (open bars) or BSO (filled bars) for 24 h (n = 3). All values are means ± SE. ***, $P < 0.001$ when compared to pretreatment with vehicle followed by H_2O_2. **D:** Effect of diamide on 5-oxo-ETE formation. U-937 cells (2 x 10^6 cell/ml) were incubated for 5 min with 5-HETE (1 μM) in the presence or absence of different concentrations of diamide. The inset shows the time courses for the formation of 5-oxo-ETE in the presence (DA; ●) or absence (Con; ○) of diamide (250 μM). All values are means ± SE of 4 separate experiments.

We also examined the effects of diamide, which acts by non-enzymatically oxidizing intracellular GSH to GSSG [230] (Figure IV.10 D). Diamide (EC_{50}, 15.5 ± 2.5 μM) strongly stimulated the formation of 5-oxo-ETE to an extent similar to that observed with H_2O_2. The time course for 5-oxo-ETE synthesis in the presence of diamide, shown in the inset to Figure IV.10D, is very similar to that observed with tBuOOH (Figure IV.9 B), except that the response appears to be somewhat more sustainable. In contrast, diamide had no effect on the conversion of 5-HETE to 5-oxo-ETE by U-937 cell microsomes (Table IV.2).

Table IV.3 Effects of inhibition of thioredoxin reductase on 5-oxo-ETE formation by U-937 cells.
U-937 cells (2 x 10^6 cell/ml) were preincubated with auranofin for 30 min at 37°C followed by incubated for a further 5 min with 5-HETE (1 µM) and H_2O_2 (100 µM). The products were analyzed by precolumn extraction/HPLC as described in Materials and Methods. The results are means ± SE (n = 3).

Treatment	5-Oxo-ETE (pmol/ml)		
	Control	H_2O_2	PMS
Vehicle	23 ± 2	142 ± 9	137 ± 21
Auranofin (1 µM)	21 ± 1	152 ± 11	140 ± 12
Auranofin (10 µM)	20 ± 3	133 ± 6	137 ± 10

IV.2.3 Activation of the Pentose Phosphate Pathway Inhibits 5-Oxo-ETE Formation

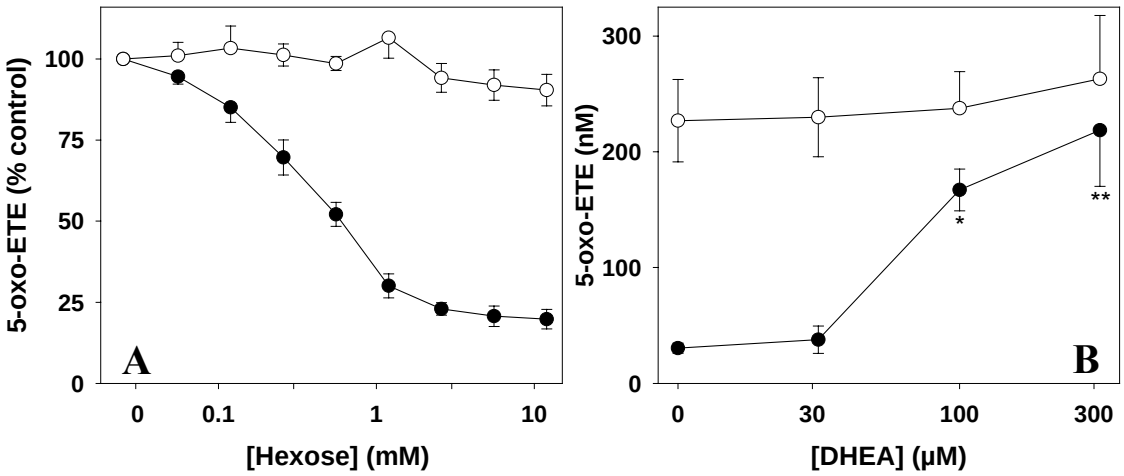


Figure IV.11 Glucose inhibits H_2O_2 induced 5-oxo-ETE formation through the PPP.
A: U-937 cells were incubated with 5-HETE (1 µM) and H_2O_2 (100 µM) for 5 min in PBS^+ containing different concentrations of either glucose (●) or galactose (○). The values are expressed as percentages of the amounts of 5-oxo-ETE formed in control incubations (183 ± 14 pmol/ml) in the absence of any added hexose and are means ± SE of data from 5 separate experiments. **B:** Reversal of the inhibitory effect of glucose by the glucose 6-phosphate dehydrogenase inhibitor DHEA. U-937 cells were preincubated with different concentrations of DHEA in PBS^+ in the presence (●) or absence (○) of glucose (5.6 mM) for 30 min, followed by incubation for a further 5 min with H_2O_2 (100 µM) and 5-HETE (1 µM) (n = 3). *, $P < 0.05$; **, $P < 0.01$ when compared to vehicle in the presence of glucose.

The PPP is the major mechanism whereby cells maintain a high ratio of NADPH to $NADP^+$. To determine whether this pathway could affect the formation of 5-oxo-ETE by reducing $NADP^+$ to NADPH (see Figure V.1, page 76) we investigated the effects of glucose, which is metabolized by the PPP following its phosphorylation to glucose 6-phosphate, compared to other hexose sugars such as galactose, which are not a substrate for this pathway. Glucose (IC_{50}, 0.42 ± 0.08 mM) strongly inhibited the formation of 5-oxo-ETE in the presence of H_2O_2 whereas galactose had no effect (Figure IV.11A). To substantiate the role of the PPP in the inhibitory effect of glucose, we examined the effect of DHEA, which is an inhibitor of glucose 6-phosphate dehydrogenase, the initial enzyme in this pathway [137]. The inhibitory effect of 5.6 mM glucose on H_2O_2-induced 5-oxo-ETE formation was almost completely reversed by

DHEA (EC_{50}, 68 ± 16 μM) (Figure IV.11B). In contrast, DHEA had no effect on H_2O_2-induced 5-oxo-ETE synthesis by U-937 cells in glucose-free medium (Figure IV.11B) and did not affect oxidation of 5-HETE in the presence of microsomal fractions from these cells (Table IV.2).

IV.2.4 Effects of Oxidative Stress on 5-oxo-ETE Synthesis by Peripheral Leukocytes

To determine whether 5-oxo-ETE synthesis by peripheral leukocytes can also be regulated by oxidative stress, we investigated the effects of increasing concentrations of H_2O_2 on its formation by lymphocytes, monocytes, and neutrophils under conditions similar to those employed with U-937 cells. Although baseline synthesis of 5-oxo-ETE by all three types of cells was similar (~15 pmol/ml; Figure IV.12), there were considerable differences in the responses of these cells to H_2O_2. Lymphocytes strongly responded to concentrations of H_2O_2 as low as 1 μM (EC_{50}, 4.1 ± 0.9 μM). H_2O_2 also strongly stimulated 5-oxo-ETE formation by monocytes, but was somewhat less potent (EC_{50}, 28.8 ± 6.9 μM). The maximal response to H_2O_2 was slightly greater for monocytes than for lymphocytes (142 ± 18 vs 108 ± 8 pmol 5-oxo-ETE/ml; $P < 0.05$).

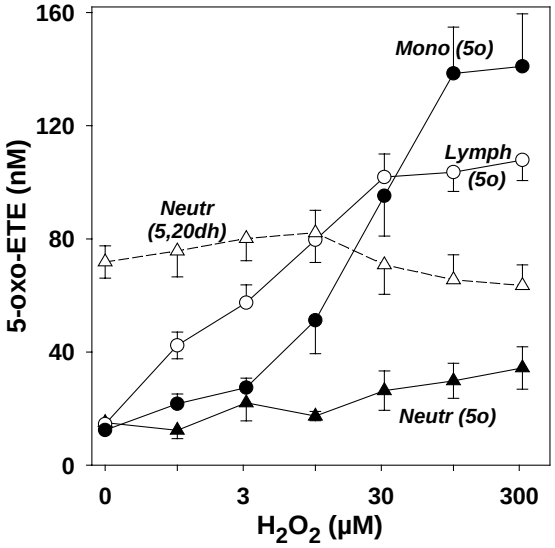


Figure IV.12 Effects of H_2O_2 on 5-oxo-ETE synthesis by leukocytes.
5-HETE (1 μM) was incubated for 5 min with suspensions (2 x 10^6 cells/ml) of lymphocytes (○; Lymph; n = 7), monocytes (●; Mono; n = 5), or neutrophils (▲, D; Neutr; n = 6), prepared from whole blood as described in Materials and Methods, in the presence or absence of different concentrations of H_2O_2. The amounts of 5-oxo-ETE (5o, ▲, ○, ●) and, in the case of neutrophils, 5,20-diHETE (5,20dh; D) were measured by HPLC. All values are means ± SE. The effect of H_2O_2 on 5-oxo-ETE formation was statistically significant at all concentrations tested for lymphocytes ($P < 0.001$), at 10 μM ($P < 0.05$) and higher ($P < 0.001$) for monocytes, and only at a concentration of 316 μM ($P < 0.01$) for neutrophils.

In contrast to monocytes and lymphocytes, H_2O_2 had only a very modest effect on neutrophils, increasing 5-oxo-ETE synthesis from 16 ± 4 pmol/ml in untreated cells to a maximum of 34 ± 8 pmol/ml in cells treated with 316 μM H_2O_2 ($P < 0.01$) (Figure IV.12). Moreover, the dose response curves for the effect of H_2O_2 on neutrophils from individual donors were rather variable (EC_{50}, 201 ± 90 μM). To determine whether the relative unresponsiveness of these cells to H_2O_2 was due to its rapid metabolism by catalase or myeloperoxidase (Figure V.1, page 76), incubations were performed in the presence of either 3-AT (5 mM), which inhibits catalase, or azide (1 mM), which inhibits both enzymes. However, neither of these agents, either alone or in combination, significantly affected the response of neutrophils to H_2O_2 (data not shown). Because they possess high levels of the NADPH-dependent enzyme LTB_4 20-hydroxylase, neutrophils also convert 5-HETE to 5,20-diHETE

[72], and this was the major product under the conditions of our assays, amounting to ~70 pmol/ml (Figure IV.12). However, the formation of this substance was not affected by H_2O_2. Furthermore the amount of 5,20-diHETE formed was not sufficient to deplete the substrate, because under the conditions we used (low cell concentration and short incubation time) only a relatively small proportion (≤ 15%) of the 5-HETE was metabolized. Powell and colleagues had previously shown that 5,20-diHETE could be further metabolized by neutrophils (after a lag period) to 5-oxo-20-HETE [74], but little or none of this product was detected in the present study because of the relatively short 5-min incubation time employed.

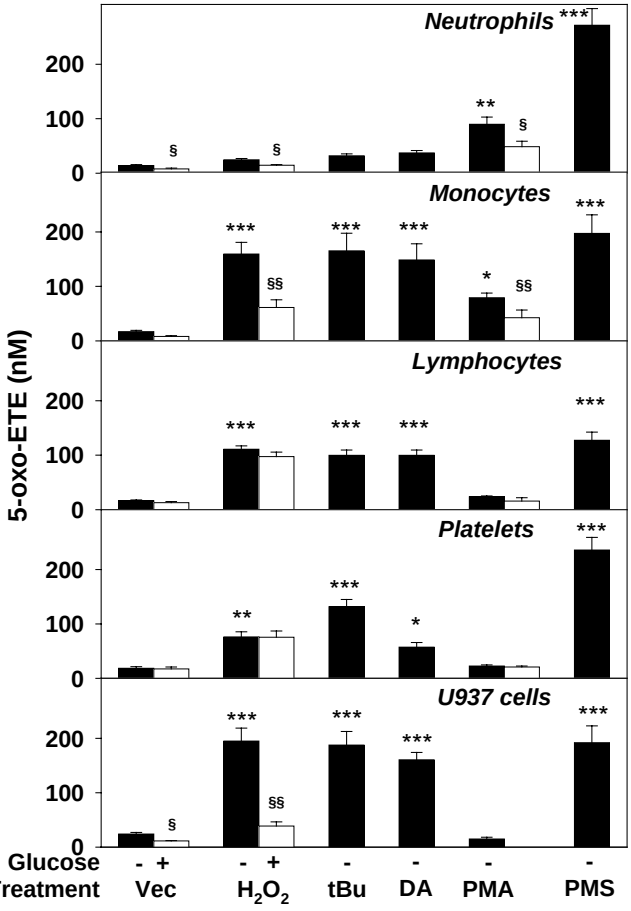


Figure IV.13 Regulation of 5-oxo-ETE synthesis by blood cells.
Neutrophils (**A**), monocytes (**B**), lymphocytes (**C**), platelets (**D**), and U-937 cells (**E**) were incubated for 5 min with 5-HETE (1 μM) in the presence of vehicle (Veh), H_2O_2 (100 μM), tBuOOH (tBu; 100 μM), diamide (DA; 250 μM), PMA (100 nM), or PMS (1 mM for leukocytes and platelets; 100 μM for U-937 cells) in either glucose-free PBS^+ (solid bars) or PBS^+ containing 5.6 mM glucose (open bars). The cell concentrations were 2 x 10^6/ml for leukocytes and 10^8/ml for platelets. The values are means ± SE of data from 6 separate experiments, except for platelets (n = 4). *, P< 0.05; **, P < 0.01; ***, P < 0.001 when compared to vehicle (in the absence of glucose. §, P < 0.05; §§, P < 0.01 when compared to the corresponding treatment in the absence of glucose.

IV.2.5 Cell specific regulation of 5-oxo-ETE Formation by Blood Cells

Powell and co-workers showed previously that PMA strongly stimulates the formation of 5-oxo-ETE by neutrophils by activating NADPH oxidase [74] and the present study indicates that both oxidative stress and the PPP also strongly affect the biosynthesis of this substance. To investigate the abilities of oxidative stress, NADPH oxidase, and the PPP to regulate 5-oxo-ETE synthesis by blood cells, 5-HETE (1 µM) was incubated with neutrophils (Figure IV.13A), monocytes (Figure IV.13B), lymphocytes (Figure IV.13C), and platelets (Figure IV.13D) for 5 min in the presence of either H_2O_2 (100 µM), tBuOOH (100 µM), or PMA (100 nM) with or without 5.6 mM glucose. For comparison, data for U-937 cells obtained under identical conditions are also shown (Figure IV.13E). To estimate the maximal capacities of these cells to produce 5-oxo-ETE, incubations were also performed in the presence of 1 mM PMS, which, in all cases, induced a strong response. H_2O_2 and tBuOOH strongly stimulated 5-oxo-ETE formation by monocytes, lymphocytes, and platelets, but had only very modest effects on neutrophils as noted above. Diamide had effects on 5-oxo-ETE synthesis very similar to H_2O_2 in all of the cell types investigated.

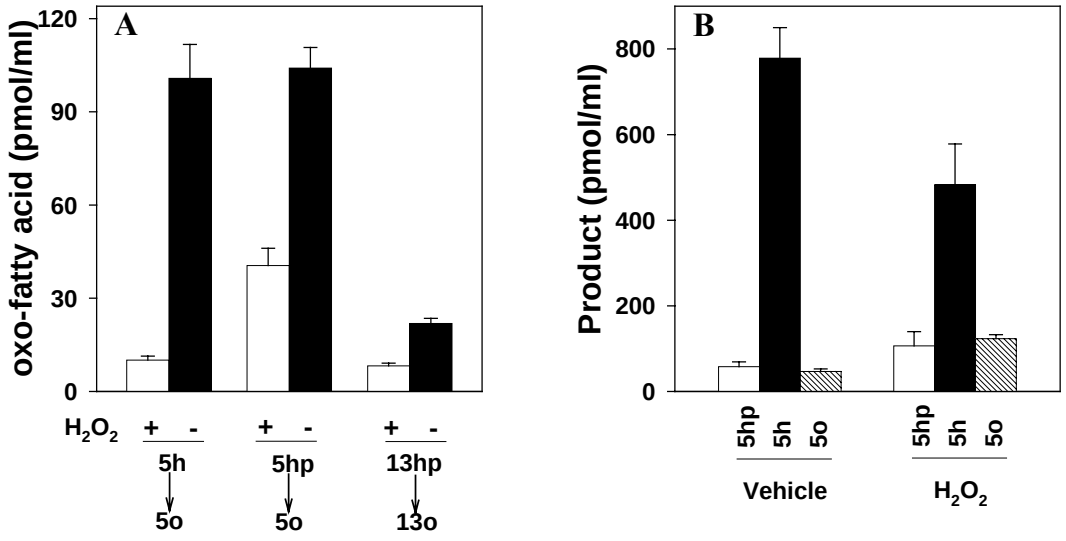


Figure IV.14 Effect of H_2O_2 on the formation of oxoeicosanoids from 5-HETE, 5-HpETE, and 13-HpODE by PBMC.
PBMC (2 x 10^6 cells in 1 ml), prepared by centrifugation of mixed leukocytes over Ficoll-Paque, were incubated with 5-HETE (1 µM), 5-HpETE (1 µM) or 13-HpODE (1 µM) for 5 min at 37 °C and the products were analyzed by RP-HPLC. **A** shows both the amounts of 5-oxo-ETE formed from either 5-HETE (5h → 5o) or 5-HpETE (5hp → 5o) and the amount of 13-oxo-ETE formed from 13-HpODE (13hp → 13o) in the presence or absence of H_2O_2. All incubations were terminated by the addition of triphenylphosphine in 0.4 ml methanol (final concentration, 40 µM) to reduce any unreacted hydroperoxy substrate. **B** shows the amounts of 5-HpETE (5hp), 5-HETE (5h), and 5-oxo-ETE (5o) detected after incubation of 5-HpETE (1 µM) with PBMC (2 x 10^6 cells in 1 ml) for 5 min at 37 °C. In this case triphenylphosphine was omitted to permit estimation of unreacted 5-HpETE. All values are means ± SE (n = 5).

In contrast to H_2O_2, PMA (100 nM) strongly stimulated 5-oxo-ETE formation by neutrophils and monocytes, but did not affect its formation by either lymphocytes or platelets. In neutrophils, PMA inhibited the formation of 5,20-diHETE by 60% ($P < 0.005$), so that in its presence, 5-oxo-ETE was the major 5-HETE metabolite detected (data not shown), in agreement with previous findings by Powell and colleagues [74].

The response to glucose also varied substantially among different cell types. Glucose inhibited both H_2O_2- and PMA- stimulated 5-oxo-ETE formation by neutrophils and monocytes, although not to the extent observed with U-937 cells. In contrast, glucose had no effect on H_2O_2-elicited 5-oxo-ETE synthesis by either lymphocytes or platelets. Moreover glucose did not affect the ω-oxidation of 5-HETE in neutrophils (data not shown).

IV.2.6 Effects of Oxidative Stress On Metabolism of 5-HETE and 5-HpETE by PBMC

5-Oxo-ETE can be synthesized both enzymatically from 5-HETE through the action of 5h-dh [22] as well as by dehydration of 5-HpETE by a heat stable cytosolic factor in mouse macrophages [79]. We examined the effect of H_2O_2 on the formation of 5-oxo-ETE by PBMC from both of these substrates. In the absence of H_2O_2, about four times as much 5-oxo-ETE was formed from 5-HpETE than from 5-HETE following incubation for 5 min at 37°C (Figure IV.14A). H_2O_2 markedly stimulated the formation of 5-oxo-ETE from both substrates, resulting in the formation of approximately equal amounts after 5 min. In contrast, 13-HpODE was only converted to relatively small amounts of 13-oxo-ODE, and although this increased in the presence of H_2O_2, it was oxidized to a much lesser extent than 5-HpETE. In the above experiments, triphenylphosphine was added at the end of the incubation to reduce any remaining hydroperoxy-fatty acid and thereby block any further formation of the corresponding oxo-fatty acid. To determine the extent to which 5-HpETE was reduced to 5-HETE during the incubation, triphenylphosphine was omitted and the products analyzed by HPLC. In control incubations with vehicle, nearly all of the 5-HpETE was converted to 5-HETE (Figure IV.14 6B). However, in the presence of H_2O_2, 5-HETE formation was reduced by about 40% and the amounts of 5-oxo-ETE and unreacted 5-HpETE were greater.

IV.2.7 Effects of Oxidative Stress on Formation of 5-Lipoxygenase Products by PBMC

The experiments described above were performed using either 5-HETE or 5-HpETE as the substrate. To determine whether oxidative stress could also stimulate the formation of 5-oxo-ETE when the substrate is provided by 5-lipoxygenase rather than being added directly, we investigated the effects of tBuOOH on the formation of 5-lipoxygenase products by PBMC stimulated with A23187 (5 μM) in the presence of arachidonic acid (20 μM). Under these conditions, the formation of 5-lipoxygenase products is dependent on the synthesis of 5-HETE and LTA_4 by monocytes, and their conversion to 5-oxo-ETE and LTB_4 by both monocytes and lymphocytes. The major 5-lipoxygenase product formed after stimulation of PBMC with A23187 and arachidonic acid was 5-HETE (Figure IV.15). However, tBuOOH (100 μM), stimulated the formation of 5-oxo-ETE by about 3-fold ($P < 0.01$), and reduced the amount of 5-HETE detected ($P < 0.05$). In contrast, tBuOOH had little effect on the formation of LTB_4 by PBMC.

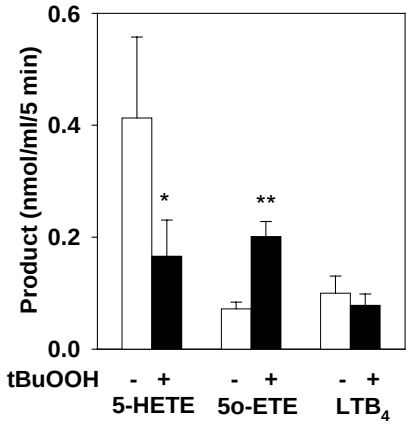


Figure IV.15 Effects of tBuOOH on the synthesis of 5-LO products by PBMC.

PBMC (2 x 10^7 cells/ml), prepared by centrifugation of mixed leukocytes over Ficoll-Paque, were incubated with A23187 (5 μM) and arachidonic acid (20 μM) in the presence (filled bars) or absence (open bars) of tBuOOH (100 μM). The amounts of 5-HETE, 5-oxo-ETE (5o-ETE), and LTB_4 formed after 5 min were determined by HPLC. The values are means ± SE (n = 5). *, $P < 0.05$; **, $P < 0.01$ when compared to control without tBuOOH.

IV.3. Synthesis of 5-oxo-ETE Production by non-myeloid Cells

IV.3.1 Expression and Regulation of 5h-dh in Epithelial Cells

Screening of Epithelial Cell Lines for 5h-dh Activity

To investigate whether the epithelium is capable of producing 5-oxo-ETE from 5-HETE, we screened 4 different epithelial cell lines (HEp-2, T84, A549 and BEAS-2B) for 5h-dh. As with myeloid cells (see Figure IV.2 and Figure IV.4), the cell lines were incubated with 100 µM PMS for 6 min followed by 4 µM 5-HETE for an additional 20 min. Figure IV.16 shows the normalized amounts of 5-oxo-ETE released from the epithelial cell lines tested in comparison to blood neutrophils and monocytes. All epithelial cell lines tested are capable of transforming 5-HETE to 5-oxo-ETE in amounts comparable to blood cells. A549 cells synthezised 390 ± 36 and BEAS-2B cells 197 ± 67 pmol 5-oxo-ETE per 10^6 cells under these circumstances. HEp-2 cells were originally thought to be derived from an epidermoid carcinoma of the larynx, but was later found to have been established via HeLa cell contamination (see ATTC information). Due to our focus on lung diseases and the origin of HEp-2 cells and T84 cells, the latter were derived from a lung metastasis of a colon carcinoma and maintained the original histological characteristics of the colon carcinoma, we continued our studies with A549 cells and BEAS-2B cells.

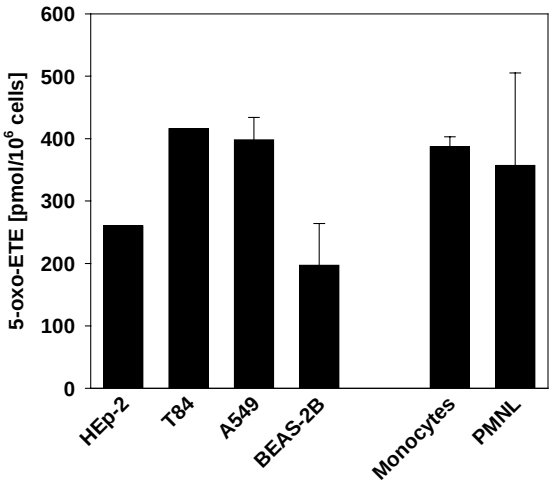


Figure IV.16 Expression of 5h-dh in epithelial Cell lines.
Epithelial cells were cultured to confluence in 6-well plates. Cells were washed twice with PBS^+ and then incubated for 6 min with 100 µM PMS. The cells were then incubated for another 20 min with 4 µM 5-HETE. Parallel cultured cells were detached with trypsin and counted. Results obtained with monocytes and PMNL are shown as comparison. $n \geq 4$, except for T84 and HEp-2cells (n=2).

Characterization of Epithelial 5h-dh

We investigated characteristics of the enzyme in A549 cells responsible for the formation of 5-oxo-ETE in more detail to determine whether it is similar to myeloid cell 5hdh. To examine the substrate specificity, cofactor requirements, and subcellular localization, we incubated cytosol, granules, and microsomes with 4µM 5-HETE, 12-HETE, and 15-HETE respectively, in the presence of 100 µM $NADP^+$ or NAD^+. As shown in Figure IV.17, 5-HETE is metabolized by the microsomal fraction to 1.03 ± 0.32 pmol 5-oxo-ETE/(min×µg protein) in the presence of $NADP^+$. Under similar conditions, but with NAD^+ acting as the electron acceptor, only 0.06 ± 0.02 pmol 5-oxo-ETE/(min×µg protein) were formed. The enzymatic activity to produce 5-oxo-ETE is mainly located in the microsomal fraction, since only half the specific activity is found in the granules and only trace amounts in the cytosol (0.54 ± 0.1 and

0.02 ± 0.01 pmol 5-oxo-ETE/(min×µg protein), respectively). The enzyme responsible for 5-HETE metabolism in the microsomal fraction is highly substrate specific. Only 0.06 ± 0.05 pmol 12-oxo-ETE/(min×µg protein) and 0.03 ± 0.01 pmol 15-oxo-ETE/(min×µg protein) were formed from 12-HETE and 15-HETE, respectively by microsomes in the presence of $NADP^+$. To further investigate the substrate specificity of the epithelial microsomal 5h-dh, we examined the conversion of 5*R*-HETE, the stereoisomer of 5*S*-HETE. 5*R*-HETE is not a good substrate for this enzyme, because only 0.04 ± 0.01 pmol 5-oxo-ETE/(min×µg protein) were formed (Figure IV.17). It is noticeable that more 15-HETE than 5-HETE is oxidized in the cytosolic fraction to the corresponding oxo-ETE (0.08 ± 0.03 pmol 15-oxo-ETE /(min×µg protein)) in the presence of $NADP^+$. We also incubated the cytosolic fraction with $PGF_2\alpha$, which was oxidized to its keto-derivative. The cytosolic 15-dehydrogenase activity of A549 cells is therefore presumably 15h-PG-dh. Under the conditions we employed, the specific activity of microsomal 5h-dh is over 12 times higher than that of cytosolic 15h-PG-dh in A549 cells.

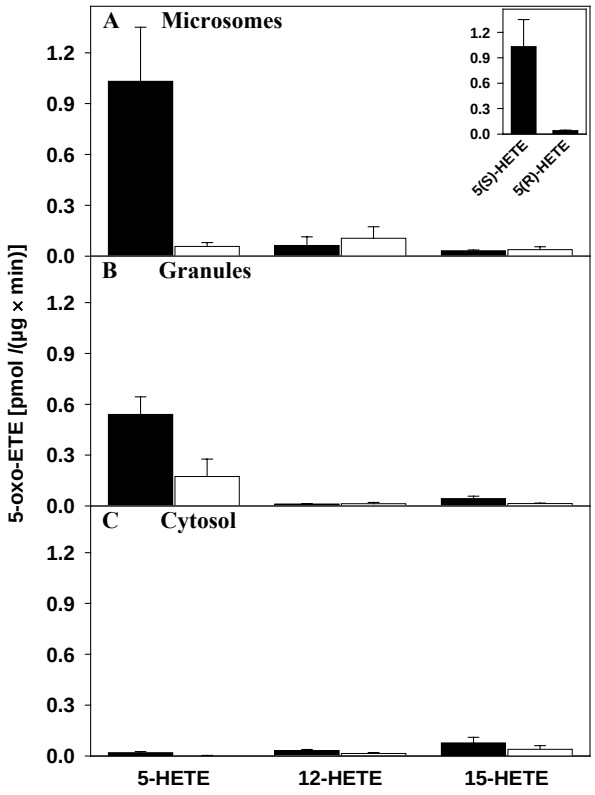


Figure IV.17 Subcellular localization and substrate specificity of 5h-dh in A549 cells. Subcellular fractions of A549 cells were prepared as described in material and methods. The different fractions were incubated with 4 µM 5-HETE, 12-HETE, or 15-HETE for 20 min in the presence of either 100 µM $NADP^+$ (filled bars) or 100 µM NAD^+ (open bars). **A** shows the normalized amounts of the oxo-products formed by microsomes (50 µg protein). The inset shows amounts of 5-oxo-ETE formed from 5*S*-HETE and 5*R*-HETE (n=2). **B** shows the products formed by granules (150 µg protein). In **C** the products formed by the cytosol (400-500 µg protein) are shown. ($n \geq 3$).

To further compare epithelial and leukocyte 5h-dh, we examined the steady state kinetics of their activities using the Lineweaver-Burk plot. Similar to U-937 cells and PMNL, we incubated A549 cell derived microsomes (50 µg protein/ml) with 100 µM $NADP^+$ and various

amounts of 5-HETE for 5min. As shown in Figure IV.18, the reaction follows Michaelis-Menten kinetics. Linear regression of the Lineweaver-Burk plots revealed a V_{max} of 1.6 ±0.5 pmol 5-oxo-ETE /(min×µg) and a K_M of 0.89 ± 0.15 µM (n=5). In comparison, neutrophils and U-937 have a lower V_{max} (0.4 ± 0.12 and 0.5 ± 0.3 pmol 5-oxo-ETE /(min×µg), respectively) and a slightly lower K_M (0.57 ± 0.17 and 0.4 ± 0.14 µM, respectively). For more details see also Table IV.1, page 48.

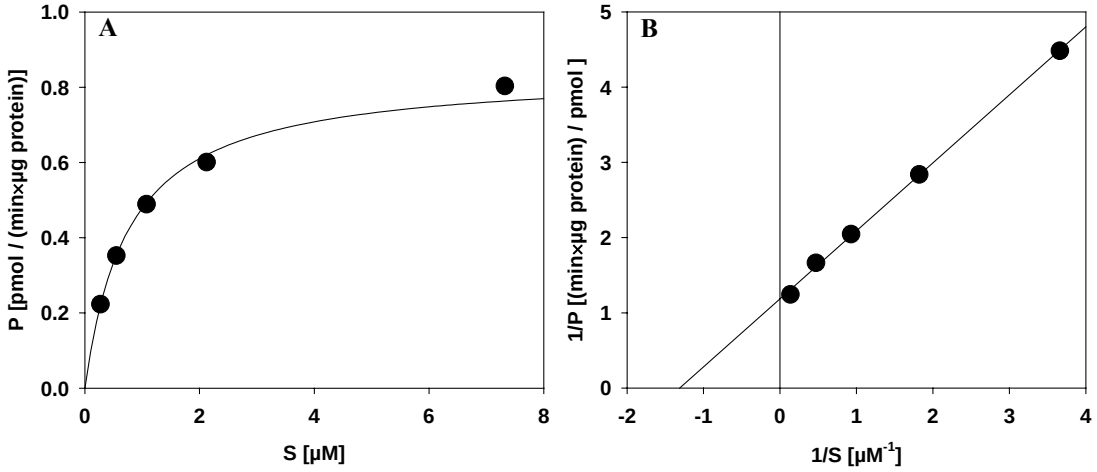


Figure IV.18 Steady State analysis of 5h-dh in A549 cells microsomes.
Microsomal protein (50 µg) derived from A549 cells were incubated with increasing amounts of 5-HETE and 100 µM $NADP^+$ for 5 min. **A:** Specific 5h-dh activity of a representative experiment (n=5) is shown as a function of the substrate concentration. **B:** The corresponding Lineweaver-Burk plot, the double reciprocal presentation, of the same data as in A. Linear regression of all experiments with A549 cell derived microsomes yields a V_{max}=1.6 ± 0.5 pmol/(min × µg protein) and a K_M = 0.89 ± 0.15 µM.

5-oxo-ETE Production in Epithelial Cells is Upregulated by Oxidative Stress

To determine whether 5-oxo-ETE synthesis by epithelial cells can be regulated by oxidative stress, we investigated the effects of increasing concentrations of H_2O_2 and tBuOOH on its formation by A-549 and BEAS-2B cells. To ensure reproducibility, cells were incubated with 4 µM 5-HETE for 10 min in the presence of peroxides. We had to use high concentration of 5-HETE for the experiment, because considerable amounts of both, 5-oxo-ETE and 5-HETE were taken up by epithelial cells presumably into cellular lipids (see also Figure IV.20, page 68). Although baseline synthesis of 5-oxo-ETE by A-549 and BEAS-2B cells was different (36± 7 and 17 ± 2 pmol 5-oxo-ETE/10^6 cells; Figure IV.19 A and C), there were no considerable differences in the relative responses of these cells to H_2O_2 and tBuOOH. A-549 and BEAS-2B cells strongly responded to concentrations of H_2O_2 as low as 10 µM (EC_{50}, 14 ± 4 and 9 ± 3 µM, respectively). Although tBuOOH strongly stimulated 5-oxo-ETE formation by A-549 and BEAS-2B cells, it was somewhat less potent (EC_{50}, 33 ± 12 µM) than H_2O_2 (18 ± 5 µM). tBuOOH appeared to be more efficacious, but these differences were not statistically significant.

To investigate the roles of the GSH redox cycle and the PPP in regulating 5-oxo-ETE synthesis by epithelial cells, 5-HETE (4 µM) was incubated with A-549 cells (Figure IV.19 B) and BEAS-2B cells (Figure IV.19 D) for 10 min in the presence of either H_2O_2 (100 µM) or diamide (250 µM) with or without 5.6 mM glucose. Diamide stimulated 5-oxo-ETE formation

by A-549 cells ($P < 0.001$) to a similar extent as H_2O_2, but appeared to be more efficacious in BEAS-2B cells. Glucose significantly inhibited the 5-oxo-ETE formation stimulated by both H_2O_2 ($P < 0.001$) and diamide ($P < 0.005$) by A-549 cells, although not to the extent observed with U-937 cells. Interestingly, DHEA (100 µM) failed to overcome the inhibitory effect of glucose on H_2O_2-stimulated 5-oxo-ETE formation in A-549. Similar results were obtained when BEAS-2B cells were incubated under identical conditions, but further experiments are needed to gain statistical significance. In contrast to H_2O_2 or diamide, PMA (100 nM) had no effect on 5-oxo-ETE formation by either A549 or BEAS-2B cells (data not shown).

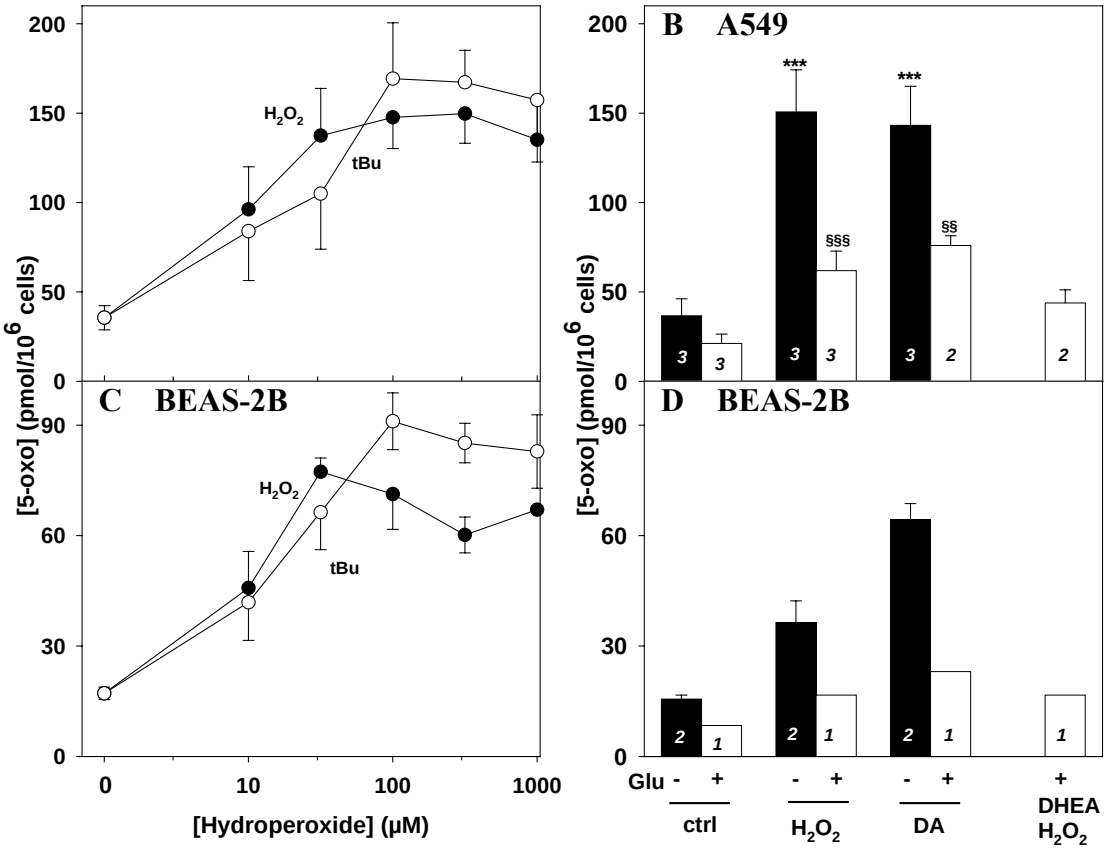


Figure IV.19 Effect of oxidative stress on 5h-dh activity in Epithelial cells.
A-549 (A,B) or BEAS-2B (C,D) cells were plated for 18 h in 6-well plates at a concentration of 0.5×10^6 /ml (10^6/well). A-549 (**A**, n ≥ 3) or BEAS-2B (**C**, n = 3) cells were incubated with 4 µM 5-HETE in the presence or absence of different concentrations of H_2O_2 (●), or tBuOOH (○) for 10 min. A549 (**B**), and BEAS-2B (**D**) cells were incubated for 10 min with 5-HETE (4 µM) in the presence of vehicle (ctrl), H_2O_2 (100 µM), or diamide (DA; 250 µM) in either glucose-free PBS^+ (solid bars) or PBS^+ containing 5.6 mM glucose (open bars). The effect of DHEA (100 µM) on H_2O_2 induced 5-oxo-ETE formation is shown on the right. The amount of 5-oxo-ETE released was normalized to the number of cells counted in parallel (1.3 ± 0.2 and $1.2 \pm 0.2 \times 10^6$ for A-549 and BEAS-2B cells, respectively). ***, $P < 0.001$ when compared to vehicle (in the absence of glucose). §§, $P < 0.005$, §§§, $P < 0.001$ when compared to the corresponding treatment in the absence of glucose (n-values are shown in graph). Note that data shown in **C** and **D** are from different experiments.

Uptake of 5-oxo-ETE and 5-HETE by A549 cells

Preliminary studies showed that unstimulated A-549 cells produce only small amounts of 5-oxo-ETE (< 5 pmol/10^6 cells in 10 min) when incubated with 1 µM 5-HETE. The relative increase in 5-oxo-ETE synthesis in response to H_2O_2, expressed as a percentage of control, was similar at 1 µM and 4 µM substrate concentrations, but the reproducibility and the recovery of the 5-oxo-ETE and 5-HETE were rather poor. We hypothesized that poor recovery is due to a rapid uptake of 5-oxo-ETE or 5-HETE due to incorporation of 5-oxo-ETE and 5-HETE into cellular lipids.

Figure IV.20A shows the recovery of 1 µM 5-oxo-ETE and 1 µM 5-HETE from adherent A549 cells (1.3 ± 0.2× 10^6 cells/well). In contrast to U-937 cells, there is no significant difference in the rate of disappearance of these two eicosanoids during the incubations. After 12 min, less then 50% between the originally added eicosanoids were recovered from the cells. As shown in Figure IV.20B, treatment with vehicle (0.5% DMSO) appeared to lower the recovery of 5-oxo-ETE, but this effect was not significant. Pretreatment with triacsin C enhanced the recovery of 5-oxo-ETE from 28 ± 6% to 65 ± 4% (n=4, P<0.05) and treatment with excess AA (40 µM) had a similar effect (65 ± 6%, n=4, P<0.05). Similar results were obtained with 5-HETE (n ≥ 3), except that the recovery was slightly higher than that of 5-oxo-ETE in the presence of vehicle or agonists (Figure IV.20B). However, these differences were not of statistical significance.

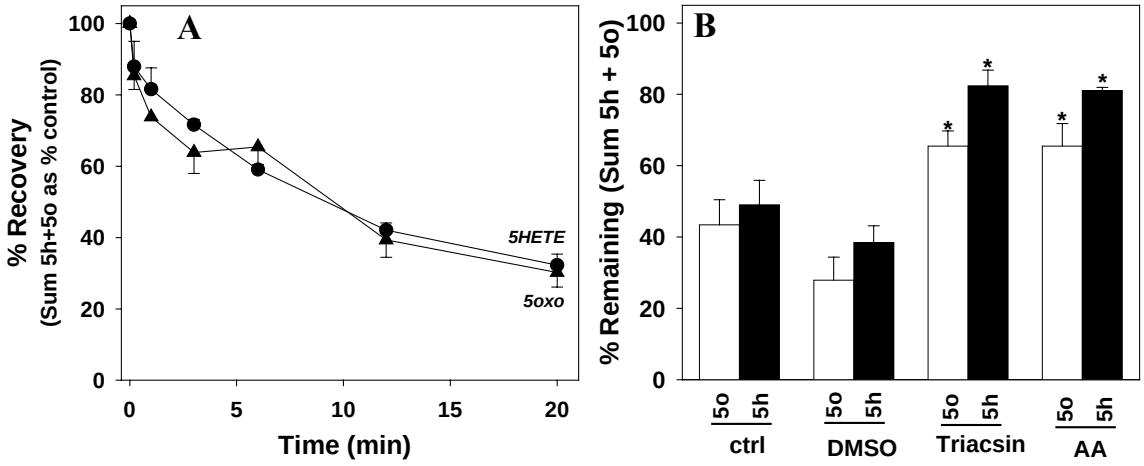


Figure IV.20 Recovery of 5-oxo-ETE and 5-HETE from A549 cells.
A. Time course of the recovery of 5-oxo-ETE and 5-HETE after incubation of adherent A549 cells (1.3 ± 0.2×10^6) with 1µM 5-oxo-ETE (▲,n=3) and 1µM 5-HETE (●,n=3). Cells were incubated for the indicated times in PBS^+. The recovery is expressed as described in Figure IV.7, page 52. **B** A549 cells were treated for 30 min with vehicle (DMSO) or Triacsin C (20 µM). Arachidonic acid (AA, 40 µM) was added at the same time as 5-oxo-ETE (1 µM, open bars, n=3) and 5-HETE (1µM, solid bars, n=3). The incubations were terminated after 20 min and the recoveries were determined as described above. * P < 0.05 compared to vehicle treated cells.

IV.3.2 Synthesis of 5-oxo-ETE by Endothelial Cells

Endothelial cells have been implicated in the formation of cys-LTs through the transcellular conversion of leukocyte-derived LTA_4 at sites of vascular inflammation and injury [234,235]. We hypothesized that endothelial cells, similar to the epithelial cell lines tested, posses 5h-dh

activity and convert 5-HETE to 5-oxo-ETE. To test this hypothesis we used HAEC). We incubated confluent HAEC for 20 min with 1 µM 5-HETE in Hanks buffer. Under these conditions, HAEC produced 24 ± 4 pmol 5-oxo-ETE/10^6 cells. The addition of PMS strongly enhanced the release of this eicosanoid to 360 ±35 pmol /10^6 cells b(Figure IV.22). We had to use Hanks buffer for this set of experiments, because HAEC, as primary cells, cannot be incubated in PBS^+ as they detach after approximately 30 min of culture in PBS^+. This could cause cellular stress and in turn affect the results of the experiment.

We hypothesized that oxidative stress enhances the formation of 5-oxo-ETE by HAEC in a similar fashion as described above for the epithelial cells and leukocytes. Indeed, addition of 316 µM H_2O_2 dramatically enhanced the formation of 5-oxo-ETE in HAEC cells after 5 min to 99 ± 23 compared to 18 ± 8 pmol/10^6 cells in unstimulated cells (Figure IV.21A). In contrast to U-937 cells, this effect was not transient and 5-oxo-ETE levels were maintained for at least 40 min. Diamide induced 5-oxo-ETE formation in a similar fashion as did H_2O_2, thus further substantiating the premise that oxidative stress enhances 5-oxo-ETE formation in HAEC through the GSH redox cycle. Since Hanks buffer contains 5.6 mM glucose, we hypothesized that this could have reduced the response to H_2O_2, because the PPP may inhibit this process by reducing $NADP^+$ to NADPH. To test this hypothesis, we preincubated HAEC for 30 min with 100 µM DHEA to block G6P-dh prior to the addition of H_2O_2- and 5-HETE. As shown in Figure IV.21, DHEA greatly enhanced the effect of H_2O_2 on 5-oxo-ETE formation in HAEC. The time course of 5-oxo-ETE formation did not reach a plateau by 40 min and its rate of formation did not decrease substantially over the period of time. This suggests that H_2O_2 persists much longer in the presence of HAEC compared to the cell lines investigated.

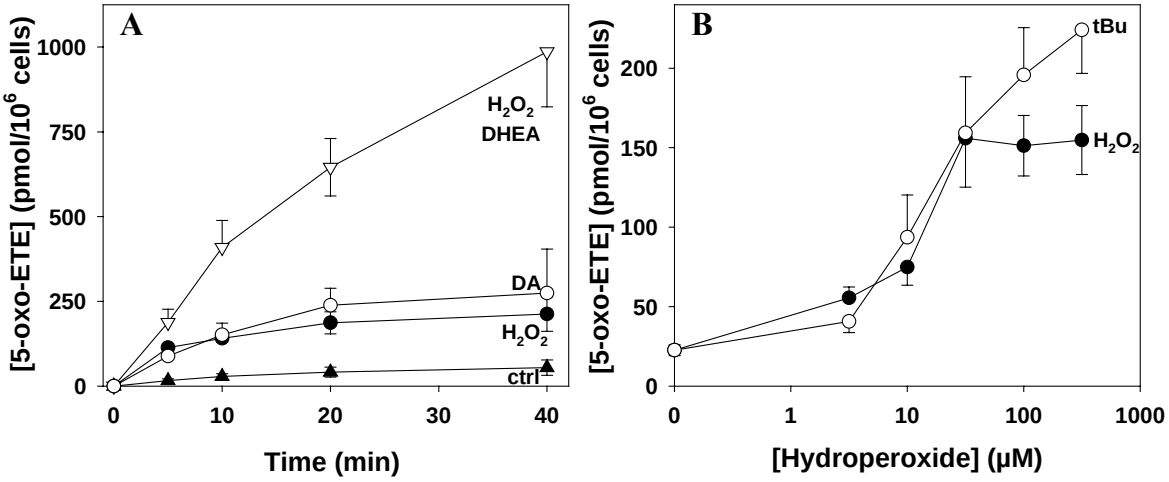


Figure IV.21 Effect of peroxides and diamide on 5h-dh activity in endothelial cells.
A: Time courses for the formation of 5-oxo-ETE following incubation of 1 µM 5-HETE with confluent primary human aorta endothelial cells in the presence of vehicle (Control; ▲; n = 2), 316 µM H_2O_2 (•; n = 3), 316 µM H_2O_2 and 100 µM DHEA (H_2O_2 /DHEA; ∇; n =3), and 250 µM Diamide (○; n = 2). **B:** Concentration-response curves for the effects of H_2O_2 (•; n ≥ 5), and tBuOOH (tBu; ∇; n ≥ 3) on the formation of 5-oxo-ETE following incubation of HAEC with 1 µM 5-HETE for 20 min. Incubations of HAEC were performed in Hanks buffer containing 5.6 mM glucose. All values are means ± SE.

The effects of various concentrations of H_2O_2 and tBuOOH on 5-oxo-ETE formation by HAEC are shown in Figure IV.21B. Both peroxides induced an increase in 5-oxo-ETE synthesis at concentrations as low as 3 µM; however in both cases this increase was only

significant at a concentration of 31.6 µM. tBuOOH (EC_{50}, 22.4 ± 5.0 µM, n=3) was a slightly less potent stimulator (*NS*) of 5-oxo-ETE formation than H_2O_2 (EC_{50}, 14.0 ± 2.7 µM, n=5). TBuOOH was somewhat more efficacious than H_2O_2 in inducing an over 10.5-fold increase in 5-oxo-ETE formation, compared to 7.5-fold for H_2O_2 at concentrations of 316 µM.

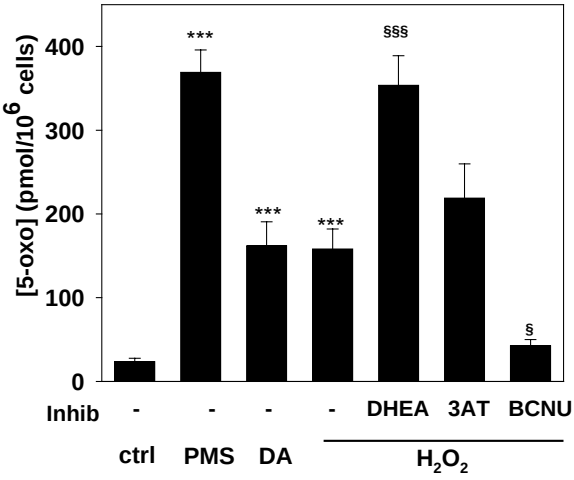


Figure IV.22 Regulation of 5h-dh activity in primary HAEC.
Effects of PMS (100 µM, n=4), diamide (DA, 250 µM, n=5), and H_2O_2 (100 µM, n=6) on the formation of 5-oxo-ETE by HAEC following incubations for 20 min with 1 µM 5-HETE. Prior to addition of 100 µM H_2O_2 and 5-HETE, HAEC were preincubated with DHEA (100 µM, n=5), 3AT (5 mM, n=5), or BCNU (30 µM, n=5). ***, P < 0.001 when compared to control (no H_2O_2 stimulation, n=6); §§§, *P* < 0.001; §, *P* < 0.05 when compared with H_2O_2 alone.

We followed similar approaches as described above to further investigate the regulatory mechanisms of 5-oxo-ETE synthesis in HAEC. Diamide (250 µM, n=5) induced a similar increase in 5-oxo-ETE formation by HAEC as did H_2O_2 (316 µM, n=6), from 24 ± 4 pmol/10^6 cells (vehicle) to 162 ± 29 (DA) and 158 ± 24 pmol 5-oxo-ETE/10^6 cells (H_2O_2). The stimulatory effect of H_2O_2 was almost completely blocked by pretreatment with BCNU to 42 ± 8 pmol/10^6 cells (n = 5, $P < 0.05$). Treatment with 3-AT (5 mM) induced a slight increase of 5-oxo-ETE formation to 219 ± 41 pmol/10^6 cells (*NS*), suggesting that metabolism of H_2O_2 by catalase is not a major factor. As described above, DHEA (354 ± 35 pmol/10^6 cells) significantly enhanced the levels of 5-oxo-ETE synthesized to amounts comparable with those induced by PMS (369 ± 27 pmol/10^6 cells), which non-enzymatically oxidizes NADPH to $NADP^+$.

The great tragedy of science - the slaying of a beautiful hypothesis by an ugly fact.
Thomas Huxley

V DISCUSSION

V.1. Differentiation of Myeloid Cells Enhances 5h-dh Activity

It has previously been shown that circulating leukocytes, including neutrophils, eosinophils, monocytes, and lymphocytes, all possess 5h-dh activity and convert 5-HETE to 5-oxo-ETE [7,22,68]. The objective of the present study was to determine whether cell lines representing pluripotent hematopoietic cells, the precursors of mature blood cells, also have this capability and whether 5h-dh activity could be induced in these cells during differentiation. The availability of a cell line with stable expression of 5h-dh activity would be very useful for studies on the regulation of 5-oxo-ETE biosynthesis. We focused on U-937 cells and HL-60 cells, because they have been frequently used for studies on other components of the eicosanoid pathway and they are of human origin.

We found that both, U-937 cells and HL-60 cells, have high levels of 5h-dh activity, comparable to those found in neutrophils and monocytes. This is the first report of the presence of this enzyme in immature cells. We also found that 5h-dh activity could be induced by differentiation of both cell types towards monocytes or macrophages. The greatest degree of induction was observed when U-937 cells were treated with PMA, which transformed these cells to adherent macrophage-like cells and increased their capacity to synthesize 5-oxo-ETE by 3-fold. Retinoic acid and dh-Vit D_3, which are known to differentiate U-937 cells into monocyte-like cells [216,236], did not cause these cells to become adherent and elicited smaller increases in 5-oxo-ETE formation. In the case of HL-60 cells, only dh-VitD_3, which induces monocytic differentiation, elicited a significant increase in 5-oxo-ETE synthesis. Agents that induced differentiation towards neutrophils (DMSO and retinoic acid) had no effect on 5-oxo-ETE synthesis by these cells.

We evaluated 5h-dh activity by measuring the conversion of 5-HETE to 5-oxo-ETE by intact cells in the presence of PMS. This is a relatively simple assay that avoids the problem of 5-oxo-ETE synthesis being limited by the availability of the 5h-dh cofactor $NADP^+$, which is normally present only at low levels in the cytosol. PMS non-enzymatically oxidizes intracellular NADPH, which is present at relatively high concentrations, to $NADP^+$. Under these circumstances 5h-dh activity should only be limited by the amounts of enzyme and substrate present. In contrast to its effect on 5-oxo-ETE synthesis by intact cells, PMS does not affect 5h-dh activity directly, as we found it has no effect on microsomal enzyme activity (Table IV.2, page 55). The concentration of PMS used in the present study (100 μM) was found to maximally stimulate 5-oxo-ETE formation in myeloid cells. The results obtained using this whole cell assay were validated by examining the effects of differentiation of U-937 cells with PMA on microsomal 5h-dh activity, which revealed a similar 3-fold induction of enzyme activity.

To ensure that our results could not be affected by the presence of other enzymes involved in the biosynthesis and metabolism of 5-HETE and 5-oxo-ETE, we determined whether U-937 cells and HL-60 cells displayed either 5-LO or LTB_4 20-hydroxylase activities. 5-LO is required for the synthesis of 5-HETE, whereas LTB_4 20-hydroxylase is the main pathway for the metabolism of both 5-HETE [72] and 5-oxo-ETE [34] in neutrophils, converting both substances to their 20-hydroxy derivatives. There was no evidence for the formation of

products resulting from either of these enzymes in either of the monocytic cell lines, both before and after differentiation with PMA (U-937 cells), DMSO (HL-60 cells), or dh-$VitD_3$ (HL-60 cells). The absence of 5-LO in U-937 cells is consistent with earlier studies demonstrating that this enzyme is not expressed in these cells due to a high degree of methylation in the promoter region of its gene, even after differentiation towards monocytes [203]. Modest levels of 5-LO activity have previously been reported in DMSO-differentiated HL-60 cells, but it was necessary to use cell concentrations about 10 times higher than those used in the present study and they had to be primed with GM-CSF to demonstrate this [208]. However, we did not use GM-CSF in the present studies. In human neutrophils LTB_4 is hydroxylated by CYP4F3A [85]. In agreement with our data, Mizukami and colleagues showed that LTB_4-hydroxylase is not expressed in HL-60 cells [237]. Differentiation of this cell line with DMSO only lead to a marginal induction of LTB_4 hydroxylase activity in comparison to human peripheral blood neutrophils [237]. Since we used 5-HETE, which is a poorer substrate than LTB_4 [201], we may have failed to detect a low expression of this enzyme under the conditions tested.

HL-60 cells have been employed in studies on the induction of a variety of other enzymes and receptors involved in eicosanoid pathways. The first receptor for a 5-LO product to be cloned took advantage of retinoic acid-induced induction of the BLT1 receptor in these cells [238]. The differentiation pathway-dependent expression of eicosanoid metabolizing enzymes has been studied in HL-60 cells. HL-60 cells undergo neutrophilic differentiation when cultured with DMSO and eosinophilic differentiation when cultured in butyric acid or retinoic acid. In contrast to LTC_4-S, LTA_4 hydrolase, the enzyme responsible for the formation of LTB_4, is induced in HL-60 cells following treatment with DMSO for up to 7 days. In contrast, LTC_4-S, but not LTA_4 hydrolase, is induced by treatment of a subclone of HL-60 cells, which readily undergoes eosinophilic differentiation, with butyric acid [204]. A key enzyme in the biological inactivation of prostaglandins, 15h-PG-dh, is induced by treatment of HL-60 cells with either PMA or DMSO [239]. This enzyme is quite different from 5h-dh, as it resides in the cytosol, requires NAD^+ as a cofactor, and selectively oxidizes the ω6 hydroxyl group of various eicosanoids [205]. Moreover, 15h-PG-dh is induced during neutrophilic differentiation of HL-60 cells with DMSO, which does not significantly affect 5h-dh activity in these cells.

Undifferentiated U-937 cells contain higher levels of LTC_4-S than circulating human monocytes [201], and this enzyme can be upregulated by treatment with DMSO, but not with PMA [201]. This is different from what we observed with 5h-dh in U-937 cells, indicating that different regulatory mechanisms for these two enzymes exist. In our hands, in U-937 cells 5h-dh is induced by PMA and not by DMSO. Since LTC_4-S and 5h-dh have been studied in microsomal fractions from U-937 cells, their activities can be directly compared. The K_M of LTC_4-S for LTA_4 (5.6 μM) [201] is about 10 times higher than that of 5h-dh for 5-HETE, indicating that the latter enzyme has considerably higher affinity for its substrate. The specific activity of 5h-dh in undifferentiated U-937 cell microsomes is about ten times higher than that of LTC_4-S [201]. The regulatory mechanisms of LTC_4-S and 5h-dh activity expression does also considerably differ in HL-60 cells. LTC_4-S is upregulated in HL-60 cells by differentiation with PMA as well as with DMSO. However, after differentiation the level of LTC_4-S activity is only comparable to that of undifferentiated U-937 cells [201]. In contrast, neither PMA nor DMSO induced 5h-dh in HL-60 cells.

Preliminary studies showed that the 5h-dh mediated reaction is highly pH sensitive. The forward reaction is favored above pH 5.5. The kinetics increase dramatically at pH 9.5 with a maximum at pH 10.5 and the forward reaction is favored at the physiological pH 7.4. This is remarkably similar to the reported pH dependence of 15h-PG-dh type I [240]. In opposite to 15h-PG-dh, microsomal 5h-dh is not affected by thiol depleting agents such as NEM (Table IV.2; [240]). Both, the forward as well as the reverse reaction of 5h-dh are inhibited at pH < 4.5 and pH > 11.3.

5-oxo-ETE synthesis by neutrophils in the presence of 100 μM PMS was considerably more variable than by monocytes (Figure IV.2, page 47). This appears to be because, unlike other leukocytes, the induction of 5-oxo-ETE synthesis is not maximal at 100 μM in neutrophils [75]. When we used 1 mM PMS for PMNL to convert NADPH to $NADP^+$, the amounts of 5-oxo-ETE being produced were reproducible and similar to that produced in monocytes stimulated with 100 μM PMS (Figure IV.13, page 60). The concentration for the effect of PMS on 5-oxo-ETE production by monocytes reached the maximum below a concentration of 100 μM PMS (data not shown). We chose to use 100 μM PMS as the standard condition, because PMS tends to overload the extraction column, thus shortening its life-time and causing considerable peak broadening.

In conclusion, both U-937 cells and HL-60 cells possess relatively high levels of 5h-dh, comparable to those found in circulating leukocytes. The activity of this enzyme is markedly increased in U-937 cells differentiated towards macrophages by treatment with PMA, and in HL-60 cells differentiated toward monocytes with dh-$VitD_3$. As these cells are readily maintained in culture they could serve as useful models to examine the regulation of 5-oxo-ETE synthesis from 5-HETE, especially since they lack ω-oxidation activity, which is the major pathway for the metabolism of 5-oxo-ETE in neutrophils. The presence of 5h-dh in undifferentiated immature leukocytes is very interesting, and raises the possibility that 5-oxo-ETE could play a role in cell proliferation or differentiation. In this regard, 5-oxo-ETE has previously been found to stimulate the proliferation of prostate cancer cells and to act as a survival factor following induction of apoptosis with inhibitors of the 5-LO pathway [241]. Furthermore, 5-oxo-ETE has been shown to stimulate ERK-1/2 and Akt [27,36] pathways, which in turn have been implicated in pathways to modulate normal and abnormal hematopoiesis [242].

V.2. Regulation of 5-oxo-ETE Synthesis by Oxidative Stress

Although 5h-dh has been detected in all leukocyte populations and platelets [7,22,68-70], the regulatory mechanisms leading to 5-oxo-ETE formation have only been described in leukocytes and monocytes [68,71]. In these cell types the activation of the NADPH oxidase complex by PMA leads to the formation of superoxide. This process utilizes NADPH as the electron donor leading to the provision of $NADP^+$, the cofactor required by 5h-dh. As this mechanism is not present in platelets and lymphocytes, we wondered how 5-oxo-ETE synthesis may be regulated in these cells. We chose undifferentiated U-937 cells to approach this subject, because they contain levels of 5h-dh comparable to circulating neutrophils and monocytes, but do not convert either 5-oxo-ETE or 5-HETE to ω-oxidation products, as do neutrophils. Furthermore, they lack both 5-LO activity [203] and the NADPH oxidase system [243], which, if present, could complicate the interpretation of the data.

V.2.1 Oxidative Stress Enhances 5-oxo-ETE Synthesis

Both H_2O_2 and tBuOOH strongly stimulated 5-oxo-ETE formation from 5-HETE by U-937 cells, with EC_{50} values in the low μM range. tBuOOH was over 3 times more potent than H_2O_2 and had a longer lasting effect, probably due to its resistance to metabolism. In contrast, the response to H_2O_2 declined after 5 min, presumably due to metabolism by catalase [244] and/or myeloperoxidase [245], both of which are present in U-937 cells, as its effect was prolonged considerably by the addition of azide, which inhibits both of these enzymes. To determine whether fatty acid hydroperoxides have similar effects, we used 13-HpODE, the 13-hydroperoxy metabolite of linoleic acid, as a representative compound. Although it increased 5-oxo-ETE formation substantially, it was clearly not as potent as H_2O_2, suggesting that such compounds may be of less importance than H_2O_2 in regulating 5-oxo-ETE synthesis. However, we cannot rule out the possibility that hydroperoxy metabolites of arachidonic acid, such as 12-HpETE or 5-HpETE, may be more potent than 13-HpODE.

Reactive oxygen species such as H_2O_2 can affect a variety of cellular processes that could potentially affect the synthesis of 5-oxo-ETE. H_2O_2 can act as a second messenger by oxidizing critical SH groups of certain proteins to sulfenic acid (-SOH) residues, resulting in altered activity. H_2O_2 inactivates lymphocyte protein tyrosine phosphatases in this manner, resulting in increased levels of activated intracellular signaling molecules [246]. H_2O_2 can also be converted to highly reactive hydroxyl radicals by the Fenton reaction or to hypochlorous acid by myeloperoxidase, both of which have toxic effects. In addition, hydroperoxides can stimulate lipid peroxidation, which could potentially result in the non-enzymatic formation of 5-oxo-ETE. In this context, incubation of red cell ghosts with a very high concentration (10 mM) of tBuOOH for 90 min induced the formation of substantial amounts of 5-oxo-ETE esterified to phospholipids [61]. However, such a mechanism would not explain the rapid conversion of 5-HETE to 5-oxo-ETE that we observed. Moreover, 5-HETE was not oxidized to 5-oxo-ETE when incubated with H_2O_2 or tBuOOH in the absence of cells (data not shown).

H_2O_2 also affects cellular redox status. Cells protect themselves from oxidative stress by maintaining a highly reducing environment. Hydroperoxides, including H_2O_2, are rapidly reduced by glutathione peroxidase, resulting in the concomitant oxidation of GSH to GSSG (Figure V.1). The GSSG is then reduced back to GSH by glutathione reductase, resulting in the formation of NADP+, the cofactor required by 5h-dh. Alternatively, H_2O_2 can be reduced by peroxiredoxin and thioredoxin in the following sequence [126]:

H_2O_2 → Peroxiredoxin → Thioredoxin → Thioredoxin reductase → NADP+

As it leads to the generation of $NADP^+$, this process could also explain the stimulatory effect of H_2O_2 on 5-oxo-ETE formation. The strong inhibitory effect of BCNU, which blocks both glutathione reductase and thioredoxin reductase, suggests the involvement of at least one of these pathways. This is supported by the inhibitory effect of NEM, which non-selectively alkylates thiol groups, thereby blocking both pathways. In contrast to BCNU, the potent thioredoxin reductase inhibitor auranofin had no effect on 5-oxo-ETE synthesis in response to H_2O_2 at concentrations well above those reported [135] to maximally inhibit this enzyme. This suggests that the effect of H_2O_2 is mediated by the GSH rather than the thioredoxin redox cycle. This is further supported by the finding that depletion of GSH by the γ-glutamylcysteine synthetase inhibitor BSO [247] significantly reduces the response to H_2O_2.

The capacity of cells to reduce hydroperoxides via the GSH redox cycle depends on the availability of NADPH, which is normally maintained at very high levels relative to $NADP^+$ by the PPP [136]. For every molecule of glucose 6-phosphate that enters this pathway, two molecules of NADP+ are reduced to NADPH (Figure V.1, page 76). The increase in $NADP^+$ levels expected following exposure of cells to oxidative stress would thus be tempered by its rapid reduction to NADPH by the PPP. The cytosolic concentration of $NADP^+$ would depend on the relative activities of these two pathways. In the case of U-937 cells, activation of the PPP by addition of the substrate glucose strongly inhibits the effect of H_2O_2 on 5-oxo-ETE synthesis, presumably due to enhanced reduction of the $NADP^+$ generated by glutathione reductase. This inhibitory effect was not shared by galactose, which is not a substrate for the PPP. Furthermore, the effect of glucose could be completely blocked by DHEA, which prevents its metabolism by the PPP by inhibiting the key enzyme G6P-dh (Figure IV.11B) [137].

V.2.2 Effects of Oxidative Stress in Different Leukocyte Populations

Although our initial studies were performed using U-937 cells as a model, our ultimate goal was to examine the regulation of 5-oxo-ETE formation by peripheral blood cells. The effects of oxidative stress on monocytes, lymphocytes, and platelets were very similar to those observed using U-937 cells, with H_2O_2, tBuOOH, and diamide all dramatically increasing 5-oxo-ETE synthesis (Figure IV.13). The only exception was neutrophils, in which 5-oxo-ETE formation was not increased significantly by peroxides or by diamide.

H_2O_2 also stimulated the formation of 5-oxo-ETE from both 5-HETE and 5-HpETE by PBMC. Whereas oxidation of 5-HETE would appear to be solely due to the action of 5h-dh [22], oxidation of 5-HpETE to 5-oxo-ETE could occur by two distinct mechanisms. 5-HpETE could initially undergo peroxidase-induced reduction to 5-HETE, followed by oxidation to 5-oxo-ETE by 5h-dh. Alternatively, it could undergo direct dehydration to 5-oxo-ETE, as reported by Zarini and Murphy to occur in murine macrophages [79]. It is difficult to completely distinguish between these two pathways because of the very rapid peroxidase-induced conversion of 5-HpETE to 5-HETE, which in the case of PBMC was nearly complete by 5 min (Figure IV.14B). H_2O_2 reduced the amount of 5-HETE detected following incubation of 5-HpETE with PBMC. This is probably due to (i) increased metabolism of 5-HETE to 5-oxo-ETE by 5h-dh and (ii) reduced formation of 5-HETE due to competition between H_2O_2 and 5-HpETE for glutathione peroxidases. H_2O_2 could also prolong the lifetime of 13-HpODE in this way, thereby promoting its conversion to 13-oxo-ODE.

Hydroperoxy-fatty acids are known to decompose to their oxo derivatives in the presence of heme proteins [248], but it is unlikely that this process would exhibit a high degree of substrate selectivity. To evaluate the direct conversion of hydroperoxy-fatty acids to oxo-fatty acids we therefore incubated 13-HpODE with PBMC, as 13-HODE, unlike 5-HETE, is not a substrate for 5h-dh [22]. The fact that conversion of 13-HpODE to its oxo derivative proceeds at a much slower rate than the corresponding reaction with 5-HpETE would suggest that 5-oxo-ETE is formed from 5-HpETE by human PBMC primarily through the action of 5h-dh. However, the direct dehydration of 5-HpETE could also make a smaller contribution to the formation of

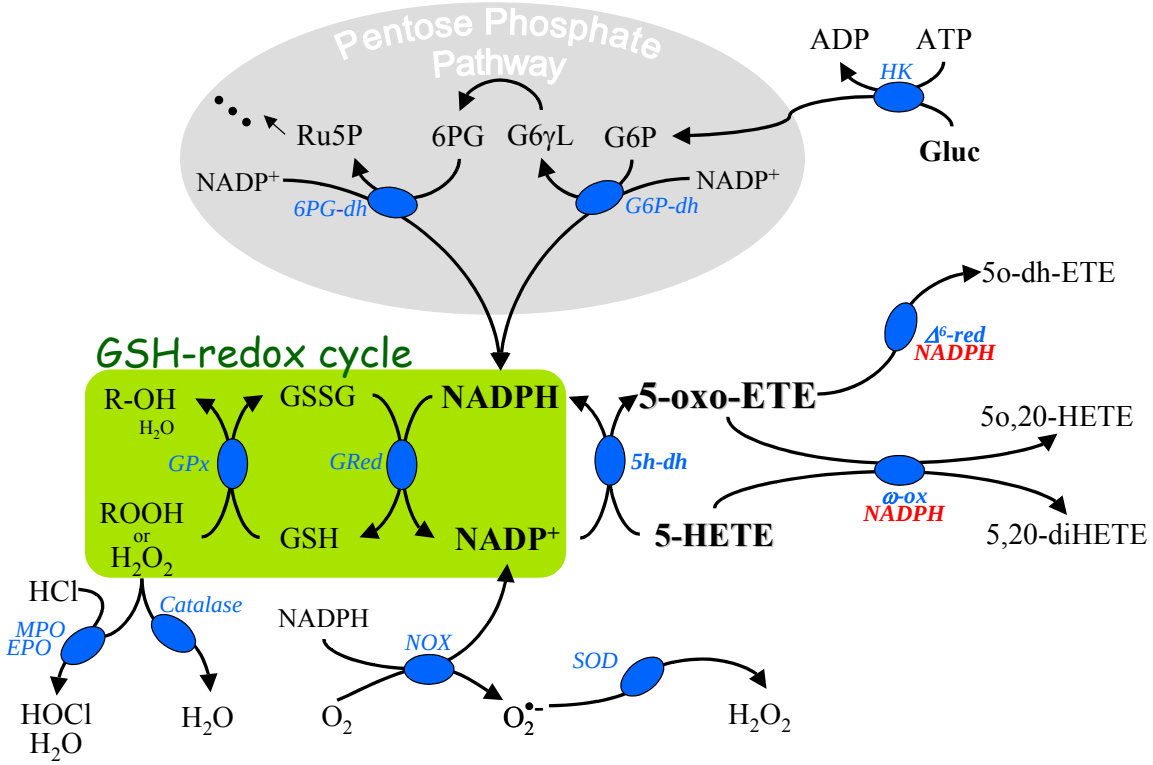


Figure V.1 Regulation of 5-oxo-ETE synthesis by Oxidative Stress
Within the GSH-redox cycle, H_2O_2 or lipid hydroperoxides (LOOH) are reduced by GPx family members to water or their corresponding alcohols using GSH as the electron donor which is oxidized to GSSG. GSSG is in turn recycled at the expense of NADPH by GRed, thus providing the cofactor $NADP^+$ for 5h-dh. In phagocytes, NADPH oxidase (NOX) can also provide significant amounts of $NADP^+$ after respiratory burst induction. The oxidative part of the PPP is reestablishing a low redox state within the cell by reducing $NADP^+$ to NADPH, while oxidizing G6P, which is formed of glucose by hexokinase (HK), to Ru5P. MPO/EPO and catalase also metabolize H_2O_2. Note that two 5-oxo-ETE-metabolizing pathways, Δ^6 –red and ω-ox use NADPH as cofactor.

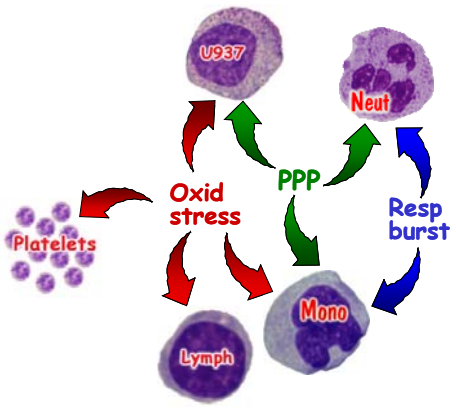


Figure V.2 Principal regulatory pathways of 5-oxo-ETE formation in blood cells and U-937 cells
The induction of oxidative stress upregulates the formation of 5-oxo-ETE in U-937 cells, monocytes (Mc), lymphocytes (Ly), and platelets. The PPP inhibits the formation of 5-oxo-ETE synthesis in neutrophils (Ne), monocytes, and U-937 cells but not in lymphocytes or platelets. The induction of the respiratory stimulates 5-oxo-ETE synthesis only in neutrophils and monocytes but not in the other cell types tested.

5-oxo-ETE. It is interesting that in the absence of H_2O_2, 5-oxo-ETE is formed more efficiently from 5-HpETE than from 5-HETE (Figure IV.14A). This may be due to a stimulatory effect of 5-HpETE on the 5h-dh-catalyzed oxidation of 5-HETE by PBMC. Lymphocytes, the main component of PBMC, are very sensitive to the effects of hydroperoxides, as concentrations of

H2O2 as low as 1 μM dramatically increase 5-oxo-ETE formation (Figure IV.12). This is supported by the results of preliminary experiments that showed that 13-HpODE (1 μM) stimulates the conversion of 5-HETE to 5-oxo-ETE by PBMC from 12.1 ± 1.8 to 30.1 ± 1.8 pmol/ml, respectively (n=2).

Most of the above studies were performed using exogenous 5-HETE as the substrate. We wanted to determine whether oxidative stress could also promote the production of 5-oxo-ETE from 5-HETE provided by the metabolism of arachidonic acid by 5-lipoxygenase. As shown in Figure IV.15, tBuOOH stimulated 5-oxo-ETE formation by PBMC following incubation with arachidonic acid and the calcium ionophore A23187, increasing the ratio of 5-oxo-ETE to 5-HETE from 0.17 to 1.2. This demonstrates that oxidative stress can stimulate 5-oxo-ETE formation not only from exogenous 5-HETE, but also from 5-HETE generated by 5-lipoxygenase. The effect of tBuOOH was specific for 5-oxo-ETE, as it had no effect on the formation of the related 5-LO product LTB_4.

Powell and coworkers previously showed that initiation of the respiratory burst in neutrophils with PMA or opsonized zymosan strongly stimulates 5-oxo-ETE synthesis [74]. However, the present study suggests that this mechanism may be restricted to neutrophils and monocytes because of the highly active NADPH oxidase systems present in these cells. The unresponsiveness of U-937 cells to PMA is presumably due to the lack of a functional NADPH oxidase system [243], confirming the prior conclusion that the effect of PMA is mediated by this pathway [74]. Reduced or absent NADPH oxidase activity in lymphocytes and platelets would also explain their lack of response to PMA.

There were also considerable differences in the responses of different cells to glucose. Glucose was most effective in U-937 cells, inhibiting H_2O_2-elicited 5-oxo-ETE production by 85%. It was somewhat less effective in neutrophils and monocytes, inhibiting the formation of 5-oxo-ETE in response to H_2O_2 and/or PMA by approximately 50%. In contrast, lymphocytes and platelets were resistant to the inhibitory effect of glucose under the conditions employed. Thus, it would appear that the PPP plays a considerably more important role in limiting 5-oxo-ETE formation in neutrophils and monocytes compared to lymphocytes and platelets (Figure V.2). A relatively high PPP activity is important for these cells as they need large amounts of NADPH for ROS generation by NADPH oxidase. Neutrophils are probably the strongest producers of ROS and it is plausible that these cells do not utilize NADPH to defend themselves against oxidant damage through the GSH redox cycle. Instead they use other mechanisms which do not affect the redox state and do not consume NADPH, such as catalase. Furthermore, as they are effector cells, their lifetime at inflammatory sites is limited and cellular damage to them has no further consequences.

The regulation of 5-oxo-ETE synthesis in neutrophils is clearly quite different from that in other types of blood cells. Whereas PMA is a potent stimulus of 5-oxo-ETE formation by these cells, H_2O_2 and tBuOOH have only modest effects. The diminished response of neutrophils to H_2O_2 could potentially be due to their high levels of myeloperoxidase, which is not present in mature monocytes, and catalase, which is much more active in neutrophils than monocytes [175]. However, this would not explain the lack of response to tBuOOH, which is not a substrate for catalase and is a poor substrate for myeloperoxidase [249]. Furthermore, neither the catalase inhibitor 3-AT nor azide, which inhibits both catalase and myeloperoxidase, enhanced the response of neutrophils to H_2O_2. It would seem more likely that the modest

response of neutrophils to hydroperoxides is due to their relatively low GSH levels and glutathione peroxidase activity compared to monocytes [175].

In conclusion, 5-oxo-ETE synthesis is strongly stimulated by oxidative stress in U-937 monocytic cells, blood monocytes, lymphocytes, and platelets, and this effect is mediated by the GSH redox cycle. The respiratory burst plays a significant, but less prominent role in monocytes, but is the major pathway involved in regulation of 5-oxo-ETE synthesis in neutrophils. Withdrawal of glucose also enhances 5-oxo-ETE synthesis in neutrophils and monocytes, but has little effect in lymphocytes and platelets. The regulation of 5-oxo-ETE synthesis by oxidative stress and glucose may be highly relevant in pathological situations accompanied by inflammation. Under such conditions, inflammatory cells are activated and release superoxide, which would be rapidly converted to H_2O_2 by superoxide dismutases. Oxidative stress is associated with a number of such conditions, including asthma [110], ischemia-reperfusion injury [250], and atherosclerosis [251]; this could lead to increased synthesis of 5-oxo-ETE, which could then act to induce the further infiltration of inflammatory cells, leading to prolongation of the inflammatory response. In agreement with this, a recent study reported elevated levels of both 5-oxo-ETE and markers of oxidative stress in lung tissue from patients with pulmonary hypertension, although the nature of this relationship was not investigated [252]. Glucose deprivation, as would occur in ischemia, could also promote the formation of 5-oxo-ETE due to a failure to maintain a high ratio of intracellular NADPH to $NADP^+$. Induction of 5-oxo-ETE synthesis by oxidative stress, low glucose, and the respiratory burst may thus be an important mechanism in sustaining the inflammatory reaction, making 5h-dh and the 5-oxo-ETE receptor attractive targets for the development of novel anti-inflammatory drugs.

V.3. Formation of 5-oxo-ETE in Structural Cells

V.3.1 5h-dh is Present in Epithelial and Endothelial Cells

It has been shown that the epithelium and the endothelium are involved in leukotriene synthesis by means of transcellular metabolism.

Although structural cells, such as epithelial and endothelial contain low levels or no 5-LO,they contain enzymes required to convert leukocyte derived LTA_4 to LTB_4 and LTC_4 [9,10,21,235,253-255]. Epithelial and endothelial cells have furthermore been shown to release inflammatory mediators such as chemokines (IL-8) and prostaglandins (PGE_2) by themselves [12,45,256-259]. We hypothesized that these cells do also contain 5h-dh. We were particularly interested in epithelial and endothelial cells, as they are in constant contact with phagocytes, especially 5-LO-containing neutrophils and macrophages, which may act as a source for 5-HETE in vivo. Under non-pathological circumstances, lung epithelial cells are in close contact to alveolar macrophages. After stimulation of the lung by irritants, such as smoke and allergens, or pathogens like respiratory synovial virus, further leukocytes infiltrate the lung. The first wave consists mainly of neutrophils, which may be replaced by eosinophils if the inflammatory response persists. Endothelial cells are in constant contact with circulating peripheral blood leukocytes and assist in inflammation.

The present study shows for the first time that 5-oxo-ETE can be formed by non-myeloid cells. We found high activity of this enzyme in all epithelial cell lines (A549, BEAS-2B, T84, and HEp-2 cells) investigated as well as in HAEC. On a cellular basis these cells produced similar amounts of 5-oxo-ETE when stimulated with PMS and 5-HETE as do human blood derived monocytes and neutrophils. Several lines of evidence suggest that the enzyme responsible for this activity is identical to leukocyte 5h-dh. First, as described in neutrophils [22] the cofactor required as an electron acceptor for the oxidation of 5-HETE is $NADP^+$ and not NAD^+. Second, the specific activity is mainly located in the microsomal fraction of A549 cells and to a certain extent in the granules, but not in the cytosol. Third, the enzyme is highly specific for a hydroxyl-group in the S-configuration in position 5, since 5*R*-HETE, 12*S*-HETE, and 15*S*-HETE are poor not metabolized to a significant extent. Fourth, the K_M value describing the binding affinity of this enzyme for the substrate 5-HETE is similar to that found in neutrophils and U-937 cells.

V.3.2 Oxidative Stress Enhances 5-oxo-ETE Formation By Epithelial and Endothelial Cells

Oxidative stress enhances the formation of 5-oxo-ETE from 5-HETE in epithelial and endothelial cells as being demonstrated by the stimulatory effects of H_2O_2 or tBuOOH (Figure IV.19 and Figure IV.22). This effect is presumably mediated through the GSH redox cycle, as in leukocytes and platelets, as it can be mimicked by diamide. In HAEC, the stimulatory effect of H_2O_2 on 5-oxo-ETE formation was inhibited by BCNU. HAEC had to be incubated in Hanks buffer, which contains 5.6 mM glucose, because they became detached from the plastic within 30 min if incubated with PBS^+. Even in the presence of glucose, HAEC produced substantial amounts of 5-oxo-ETE. The addition of DHEA further stimulated 5-oxo-ETE synthesis. In contrast to its transient effect on 5-oxo-ETE synthesis in U-937 cells, H_2O_2 had longer lasting effects in HAEC. This difference may be due to the reduced contribution of competing pathways, such as catalase or MPO, which would degrade H_2O_2. This suggests that the major pathway for the metabolism of H_2O_2 in endothelial cells is the glutathione redox-pathway and that the PPP maintains the redox state of the cells.

Whereas oxidative stress had a significant effect on 5-oxo-ETE synthesis in A549 and BEAS-2B cells as in lukocytes, the role of the PPP is less clear in these cells. Similar to U-937 cells, glucose inhibited the stimulatory effect of H_2O_2 in A549 and in BEAS-2B cells. In contrast to U-937 cells and HAEC however, 100 μM DHEA failed to overcome this inhibitory effect in BEAS-2B and A-549 cells. This difference could be possibly due to a different potency of DHEA in inhibiting G6P-dh in epithelial cells, perhaps due to a difference in its cellular uptake or metabolism. It is also possible that other pathways exist that reestablish high NADPH levels in these cells. Further experiments would be required to examine the effect of higher concentrations of DHEA on 5-oxo-ETE production in epithelial cells. If higher DHEA concentrations do not overcome the inhibitory effects of glucose, other pathways would have to be evaluated. For example, $NADP^+$-dependent isocitrate dehydrogenases (ICDH) may play an important role in maintaining a low intracellular redox-state [260]. The ICDHs catalyze the oxidative decarboxylation of isocitrate to 2-oxoglutarate. However, the NAD^+-dependent mitochondrial ICDH is a member of the tricarboxylic acid cycle. The cytosolic and mitochondrial isoenzymes instead require $NADP^+$ and could therefore reestablish high ratios of

NADPH/$NADP^+$ and GSH/GSSG following treatment with H_2O_2. Oxalomalate is a competitive inhibitor of ICDHs and may be useful to examine the involvement of ICDH in the antioxidant pathways in epithelial cells. Although oxalomalate has been shown to enhance 2,20-azobis(2-amidinopropane) hydrochloride-induced lipid peroxidation in U-937 cells [261], I am not aware of a study that examines its effects on H_2O_2 induced oxidative stress.

5-oxo-ETE may act as an autocrine mediator on epithelial cells. It has been shown that 5-oxo-ETE induces the opening of Cl^- channels in crypt jejunal epithelial cells in porcine, suggesting that this eicosanoid is potentially involved in diarrhea [50]. To date it is not clear whether 5-oxo-ETE is expressed in human non-myleoid tissue. Additional experiments are needed in order to answer this question. In the first report of OXE cloning, Hosoi and colleagues did not detect any OXE mRNA in A549 cells [48]. This is however not a final statement, as functional assays are needed to investigate potential biological effects of 5-oxo-ETE on epithelial cells. For example, 5-oxo-ETE diminishes the growth inhibitory effects of MK-886 on human prostate epithelial cells [36,54,55]. It is further possible that 5-oxo-ETE signals through mechanisms independent of OXE. O'Flaherty and colleagues proposed that 5-oxo-ETE signals through a putative 5-HETE receptor in the human prostate cancer cell line PC-3 [36]. It is also possible that 5-oxo-ETE acts, similar to LTB_4, by stimulating PPARs.

V.4. Metabolism and Inactivation of 5-oxo-ETE and 5-HETE

The concentration of 5-oxo-ETE is regulated by its formation as well as by its half-life in tissue. Since it is comparably stable in buffer, its biological half-life depends on its rate of metabolism by resident tissue cells. As indicated above, 5-oxo-ETE catabolism has been so far extensively studied in human neutrophils and murine macrophages, but little is known about other cell types. During our investigations 5-oxo-ETE production by leukocytes and epithelial cells we observed a considerable loss of substrate and product. After 20 min incubations the amounts of 5-HETE and 5-oxo-ETE together accounted for less than 50% of the 5-HETE originally added to the BEAS-2B, A549, HL-60, and U-937 cells. We therefore investigated the underlying mechanisms for the disappearance of these substances.

As we did not detect any UV-absorbing metabolites other than 5-HETE and 5-oxo-ETE, there seemed to be two possibilities for the loss we observed: (i) non-UV absorbing metabolites were formed and (ii) metabolites were formed that were excluded by our precolumn extraction procedure. There are two known pathways for the formation of non-UV absorbing metabolites of 5-oxo-ETE. The first is a metabolism by a Δ^6 reductase to 6,7,-dihydro-5-oxo-ETE, which has been shown to occur in human neutrophils [93] and the second is conjugation of 5-oxo-ETE with GSH to form FOG_7. In both cases the 6-double bound that is the center of the dienone chromophore in 5-oxo-ETE is lost resulting in a loss of maximal absorbance above 200 nm. To circumvent the problem we conducted experiments with ^3H-labeled 5-oxo-ETE. Pilot studies conducted with 5-oxo-[11,12,14,15-^3H]ETE showed no additional radioactive peaks within the HPLC chromatogram. This result strongly argues against the substantial contribution of either of the above pathways to the recovery loss of 5-HETE / 5-oxo-ETE.

Loss of 5-oxo-ETE / 5-HETE through esterification into lipids

We hypothesized that 5-oxo-ETE is directly incorporated into lipids via an acyl-CoA synthetase-, most likely through an arachidonoyl-CoA synthetase-dependent pathway. O'Flaherty and colleagues [59] demonstrated that 5-oxo-ETE can be taken up in neutrophils and esterified into triglycerides. In their setting, using low concentrations of 5-oxo-ETE in the pM range, this eicosanoid was first reduced to 5-HETE by 5h-dh, and then esterified to coenzyme A by a triacsin C sensitive acyl-CoA synthetase. The 5-HETE adduct is presumably then incorporated into triglycerides by a transacylase. We investigated whether the uptake of 5-oxo-ETE is mediated in a similar manner in U-937 cells.

In U937 cells, the main metabolic pathway for 5-oxo-ETE appears to be direct esterification and does not appear to be conversion into 5-HETE. This hypothesis is supported by four lines of evidence: i) the loss of 5-oxo-ETE is more rapid than that of 5-HETE; ii) the kinetics of the forward and he backward reactions in U937 cells are comparable in the absence of any stimulus; iii) this pathway is inhibited by triacsin C; and iv) this pathway is also inhibited by AA or DHA. It seems unlikely that U-937 cells reduce 5-oxo-ETE to 5-HETE prior to further metabolism, since the rate of 5-oxo-ETE loss is more rapid than that of 5-HETE. Furthermore, the amount of 5-oxo-ETE formed in U-937 cells from 5-HETE was similar to that of 5-oxo-ETE formed from 5-HETE, indicating that the kinetics of forward and backward reaction in whole cells are comparable. Triacsin C enhanced the recovery of 5-oxo-ETE, 5-HETE, and AA, presumably by inhibiting the arachidonate-CoA synthetase [59,101]. High amounts of AA or DHA had similar effects, most probably by saturating this pathway and competing with the other substrates. Similarly, AA has been shown to inhibit the uptake of ^{3}H-AA [262] and 15-HETE [102] in a dose dependent manner. We have conducted preliminary experiments to compare the rate of the loss of 5-oxo-ETE and 5-HETE with that of ^{14}C-AA. Initial results suggest that AA may be a better substrate for this pathway than 5-oxo-ETE or 5-HETE in U-937 cells. The more hydrophilic LTB_4 was not taken metabolized by this pathway. This is in agreement with a study from Stenson and coworkers, who examined the incorporation of arachidonic acid and various metabolites into lipids of a murine macrophage cell line [98]. In their setting, LTB_4 was also not incorporated into lipids and AA was incorporated at a higher rate than 5-HETE, 12-HETE, or 15-HETE.

Although our results suggest an involvement of an acyl-CoA synthetase-dependent pathway for the uptake of 5-HETE, 5-oxo-ETE, and AA, we have not yet investigated the lipid class into which these fatty acids are incorporated. There are considerable differences regarding the uptake of HETEs into lipids among cell types as different position isomers may be incorporated into different lipid subclasses (page 26). It is possible that the pathway for 5-oxo-ETE esterification in U-937 cells differs from that in A549 cells. It is notable, that rate of disappearance of 5-oxo-ETE is considerably faster than 5-HETE in U-937 cells, but is the same than that of 5-HETE in A549 cells.

Potential effect of H_2O_2 on fatty acid uptake by esterification

Sporn and colleagues showed [196] that H_2O_2, but not zymosan or A23187, almost completely inhibits the incorporation of AA into lipids of alveolar rat macrophages. H_2O_2 did not directly inhibit arachidonate-CoA synthetase or arachidonate-CoA:lysophosphatide acyltransferase, which catalyze the esterification of AA into phospholipids or stimulate $cPLA_2$ activity. Instead, it markedly depleted these macrophages of ATP, which is the required

cofactor of acyl-CoA synthetase. It was further postulated that ATP depletion is the mechanism whereby H_2O_2 inhibits the acylation of AA.

The incorporation of 5-oxo-ETE into lipids may represent a pathway to limit the biological effects of this inflammatory mediator. It may be postulated that oxidative stress not only enhances the concentration of 5-oxo-ETE by inducing its production, but also potentially by prolonging its half-life at sites of inflammation. In the studies of the effects of oxidative stress on 5-oxo-ETE synthesis from 5-HETE we observed some inhibition of the fatty acid (data not shown). However, a preincubation may be needed to efficiently lower intracellular ATP levels. Oxidative stress could also stimulate the production of 5-oxo-ETE by inhibiting the uptake of AA, resulting in increased availability of its precursor 5-HETE. Further experiments would also be required to address these issues.

V.5. Conclusion

V.5.1 Potential Roles of 5-Oxo-ETE in Diseases

Within the past several years, the research on 5-oxo-ETE made considerable progress. The 5-oxo-ETE receptor was cloned by two groups, allowing further studies regarding its tissue distribution and potentially the regulation of its expression [47,48]. 5-oxo-ETE was recently detected for the first time in human tissue extracts at concentrations possibly higher than those of LTB_4; first evidence that this compound exists at biologically active concentrations in the body [252]. The major finding of the present thesis, including the regulation of 5-oxo-ETE synthesis by oxidative stress and the PPP and the wide tissue distribution of 5h-dh in both immature myeloid and structural cells should open new avenues of research of this inflammatory mediator.

To date it is premature to appraise the physiological role of 5-oxo-ETE, although the biological effects of this eicosanoid suggest that it acts primarily as an inflammatory mediator. To evaluate potential disease areas for the involvement of 5-oxo-ETE, one can focus on areas in which 5-LO plays a pathophysiological role, because 5-oxo-ETE synthesis depends on this enzyme. The 5-LO pathway appears to be a promising pharmacological target in many pathological settings. Funk and Chen reviewed studies based on 5-LO knockout mice in models of inflammation [263]. In six models of inflammation, 5-LO played a detrimental role, which means that the knockout mice fare much better than the wild-type littermates. In only one study (pneumonia), 5-LO-deficient mice exhibited a poorer outcome than wild-type mice, while 5-LO deficiency had no impact in five additional models. Presumably, 5-oxo-ETE, as one mediator of the 5-LO pathway, did not evolve to perform detrimental actions to the host. As 5-LO is mainly expressed in leukocytes and 5-oxo-ETE synthesis is absolutely dependent on this enzyme to provide its precursor, 5-oxo-ETE may protect against infection or invasion of the host against by specific pathogens whose identity has yet to be determined. However, in many pathological settings, such as asthma, host defense mechanisms induce collateral damage to the tissue by inducing the response of very active cells such as eosinophils, which evolved in order to defend the body against parasitic worms and not pollen.

Strategies to Evaluate the Pathophysological Role of 5-oxo-ETE

One can draw an analogy from Koch's postulate, which describes a set of procedures to isolate and identify the causative agent of a particular microbial disease, to determine whether a particular inflammatory mediator is a critical pathological agent. The role of an agent has to be studied on several levels. First, the presence of the agonist or the corresponding receptor needs to be established. Second, the expression of the agonist should correlate with the severity of the disease state and the biological activity of the agonist should reflect the pathological condition. This implies that the presence of the agonist is at least in part responsible for pathological effects rather than a byproduct of the disease. Fourth, when the agent is introduced into a susceptible organism, it must reflect disease conditions. The final proof to establish the pathological role of an agent comes at the bedside; is the inhibition of its production or biological effects beneficial for the medical outcome of the patient?

Based mainly on in vitro assays, the main function of 5-oxo-ETE appears to be as an proinflammatory mediator. Intradermal and intratracheal administration of this eicosanoid cause leukocyte infiltration *in vivo* [56,57]. This response is paralleled in *in vitro* assays in which 5-oxo-ETE proved to be a potent chemoattractant for eosinophils and neutrophils and a weak inducer of monocyte chemotaxis [7,26,31]. Besides chemotaxis, addition of 5-oxo-ETE elicts a variety of inflammatory responses, including AA and ROS release, CD11b upregulation, actin polymerization, and rise of intracellular Ca^{++}. However, due to technical difficulties the pathophysiological role of 5-oxo-ETE has not been established. The study of this agonist in pathophysiological settings has been hampered by the fact that the receptor was not known until recently and there are no OXE receptor antagonists or 5h-dh inhibitors. Furthermore, the detection of 5-oxo-ETE is difficult for technical reasons.

In contrast to 5-oxo-ETE, the involvement of the Cys-LTs and LTB_4 have been studied in many diseases. They are easier to measure than 5-oxo-ETE or 5-HETE, because their complex structure allowed the development of reliable ELISA assays, which are economical and easy to perform. The situation is far more difficult with 5-oxo-ETE. It will prove very difficult to raise a monoclonal antibody against this eicosanoid that possesses a low cross-reactivity with related molecules. Eicosanoids are too small to be used as inducers of antibody production. They have to be coupled to a larger carrier molecule, in order to induce a proper immune response. The ligation to these larger molecules, known as haptenation, is usually accomplished through the formation of an amide bound between the carboxy-terminus of the eicosanoid and amino groups of the carrier. Since the 5-oxo specific region is fairly close to this locus, antibody development may be difficult due to cross-reactivity with other closely related fatty acids.

With ELISA-test presently unavailable, one has to rely on mass-spectrometry to measure 5-oxo-ETE in biological fluids. Powell and coworkers developed a reliable way to detect 5-oxo-ETE by this powerful technology using deuterium-labeled 5-oxo-ETE as an internal standard [264]. This is a promising technology and Bowers and colleagues used this technique to detect 5-oxo-ETE in lung tissue extracts from several patient groups [252]. LC-MS/MS is a very powerful, but also very expensive tool and demands well-trained technicians. For those reasons, it appears unlikely that 5-oxo-ETE will be measured in the near future in large scale in clinical settings. This however is important to estimate the clinical relevance of this compound.

The knowledge of the GPCR for 5-oxo-ETE will allow investigation of the expression patterns of this receptor. It will be important to study potential correlations of OXE expression and pathological conditions such as cardiovascular diseases or asthma (see below).

Does 5-Oxo-ETE Play a Role in Asthma?

The importance of the 5-LO pathway is well established in asthma, and drugs inhibiting LTs have been developed for treating this disease [21]. Montelukast (Singulair®) and Zafirlukast (Accolate®) are $cysLT_1$ receptor antagonists and in clinical use in asthma [21]. LTB_4 is a potent inflammatory mediator, it primarily targets neutrophils and does not appear to play a major role in asthma [21]. For that reason it was justified to focus on the cys-LTs as drug targets in this disease. This strategy, however, does not take the other arm of the 5-LO metabolism into account: 5-HETE and 5-oxo-ETE. 5-HETE was for a long time ignored as it induces only weak biological responses. However, 5-oxo-ETE is a much more potent and efficacious eosinophil chemoattractant than the cys-LTs or LTB_4 and induces eosinophil infiltration in Brown Norway rat lungs after intratracheal administration. It was found recently that 5-oxo-ETE induces contraction of guinea pig ASM cells *in vitro* by inducing thromboxane A_2 release [53,56]. This suggests that 5-LO inhibitors could have some advantage over selective $cysLT_1$ antagonists in treating asthma.

Our finding of high 5h-dh activity in lung epithelial cells may describe a new pathway for the formation of 5-oxo-ETE by transcellular biosynthesis in the lung. 5-HETE may be released from resident macrophages or invading neutrophils and eosinophils and converted by epithelial cells to 5-oxo-ETE. This process could be enhanced by oxidative stress, which is associated with asthma and other allergic diseases [110].

Is 5-Oxo-ETE Involved in the Pathogenesis of Cardiovascular Diseases?

Although cardiovascular diseases are a leading cause of death in the developed world, the mechanism of arterial wall inflammation in atherogenesis are only partially understood. Based on two patient-based studies, 5-LO was recently identified as a potentially important novel target for interrupting the inflammatory cascade in atherosclerosis. Spaenbroek and colleagues found that the expression of 5-LO, but not that of 15-LO, is positively correlated to the disease state [265]. This study shows that in the arterial wall exist inflammatory circuits that expand during the late stage of atherogenesis. Additional support for the role of 5-LO comes from the geneticist Stefansson and his team. Studying a large population in Iceland, they have now identified a mutation in the gene encoding for FLAP that is associated with a two fold greater risk of both heart attacks and strokes [266]. The result of this study was also confirmed in another patient group from the UK [266]. Even though the mutation of the FLAP gene found in the UK was different from that in Iceland, the outcome, a higher rate of LTB_4 synthesis by Ca^{++} ionophor stimulated neutrophils was similar.

Neutrophils and monocytes play important roles in cardiovascular disease. Although 5-oxo-ETE is less potent than LTB_4 in stimulating neutrophils, its effects on these cells are markedly enhanced following priming with GM-CSF [35]. Since 5-oxo-ETE can induce GM-CSF release from monocytes, this could result in amplification of the response of neutrophils to this substance in a paracrine manner [37]. In the narrow space of the vasculature, 5-oxo-ETE could be formed by means of intercellular eicosanoid biosynthesis between leukocytes and the endothelium as been shown for cys-LTs [10]. In this setting 5-HETE may be

released from neutrophils and converted into 5-oxo-ETE by surrounding endothelial cells. Oxidative stress, which appears to be a common feature of cardiovascular disease, may enhance the conversion of 5-HETE to 5-oxo-ETE [105,251], as suggested by our finding that exposure of HAEC to H_2O_2 strongly enhances this process *in vitro* at physiological glucose concentrations.

V.5.2 Prospects for Future Studies

The findings of the present study suggest a variety of avenues for further research, which we were so far unable to explore due to time restraints. Some results have been only briefly discussed above and much preliminary data have been omitted by the author. In some cases further experiments are required to gain statistical significance and other instances more work is necessary to explain the underlying mechanisms.

Mechanism of 5h-dh Catalysis

In unstimulated U-937 cells, the rates of the forward (5-HETE → 5-oxo-ETE) and backward (5-oxo-ETE → 5-HETE) reactions are identical. This differs from platelets, in which the backward reaction is favored [70]. As the NADPH/$NADP^+$ ratio is high in unstimulated cells, which would favor the reverse reaction, it would appear that 5h-dh favors the forward reaction. Direct studies with semi-purified 5h-dh would help to clarify the biochemical properties of this enzyme. Preliminary studies (data not shown) with U-937 cell microsomes demonstrated that the V_{max} of 5h-dh is higher for the forward reaction than for the reverse reaction. However, the K_M of 5h-dh was higher for 5-HETE than for 5-oxo-ETE when microsomes were incubated with similar amounts of $NADP^+$ or NADPH, respectively. Although this finding suggests that 5h-dh may have a higher affinity for 5-oxo-ETE than for 5-HETE, additional studies are needed to confirm this and the protocol needs to be optimized. Further studies are also necessary to examine the underlying reaction mechanism of this enzyme. Many $NADP^+$ or NAD^+-dependent dehydrogenases follow an ordered Bi-Bi reaction mechanism with the nucleotide initiating the binding sequence. To study the K_M values of this enzyme for 5-HETE, it is then sufficient to saturate $NADP^+$ and vary [5-HETE]. To gain more information a wider approach has to be taken, by varying the concentrations of both the substrate and the cofactor [267]. Under these circumstances, it is possible to determine both the reaction mechanism of 5h-dh (sequential Bi-Bi or Ping-Pong Bi-Bi) and the binding affinities of 5h-dh for $NADP^+$ and 5-HETE and possibly for 5-oxo-ETE and NADPH. If these results confirm the higher binding affinity of 5h-dh for 5-oxo-ETE, 5h-dh may be subject to product inhibition.

Subcellular Localization of 5h-dh

5h-dh has been shown to be located primarily in the microsomal fraction. This fraction includes parts of all membrane-containing subcellular fractions of the cell, including primarily the endoplasmatic reticulum, plasma membrane, and Golgi. However, other membranes such as the nuclear envelope and granule membrane can also be included depending on the severity of the cell disruption procedure. To further investigate this issue an alternative disruption procedure would be preferable. For subcellular studies, cells can be destabilized in a hypotonic buffer and then disrupted by nitrogen caviation or with a glass tissue homogenizer. The different membrane fractions are then separated over Percoll or sucrose gradients. Marker proteins have to be measured to evaluate the purity and identity of the derived fractions.

Another procedure to purify specific fractions is based on a modified magnetic antigen cell sorting technology. In this case, monoclonal antibodies against specific subcellular marker proteins are bound to superparamagnetic micro-beads. After binding to the beads the fraction of interest is then retained on separation columns in a high-gradient magnetic field generated by a permanent magnet. By simply rinsing the column with buffer, all the unlabeled fractions are removed and the bound organelles are then eluted by simply removing the separation column from the magnet. In this way fractions of interest can be retained on the column (positive selection), or antibodies against proteins which mark contaminating fractions can be used to retain those impurities (negative selection).

Potential Upregulation of 5-oxo-ETE Synthesis during apoptosis

The formation of 5-oxo-ETE by may be enhanced during apoptosis because of its association with oxidative stress. The molecular events within a cell during apoptosis are often distributed into three phases [268]. The first is a pre-mitochondrial initiation phase, which can be induced by several apoptotic signals. The second is the mitochondrial phase that integrates these signals into one common pathway, which is irreversible and commits the cell to apoptosis. It involves the loss of the inner mitochondrial membrane potential ($\Delta\psi_m$), a dramatic decrease of the NADPH/$NADP^+$-ratio, and GSH oxidation. Only after these events, are downstream caspases activated in the third phase of apoptosis. Although it is artificial, this division provides an attractive framework for the study of apoptosis. Of special interest for us is the second phase, as it leads to the oxidation of NADPH, thus potentially providing the cofactor of 5h-dh needed for the conversion of 5-HETE to 5-oxo-ETE.

Although the decrease of the NADPH/$NADP^+$- ratio is a common feature of early apoptosis, the underlying molecular cause is not well understood. Gendron and colleagues examined the molecular sequence of apoptosis with special focus on the NAD(P)H-levels by auto-fluorescence and HPLC [269]. The results of their study showed that NAD(P)H decreases in parallel to GSH, and is closely related to mitochondrial pore formation and to the loss of $\Delta\psi_m$. The decrease of NADPH is correlated to an increase in $NADP^+$, thus rising the redox state of the cell. As the measurement of $NADP^+$ is more difficult to estimate, due to the absence of a fluorescent group which be detected by FACS, $NADP^+$ is not routinely examined. However, this report lacks a proper explanation for the increase of the redox state within the cell during apoptosis, as $O_2^{\bullet-}$ formation appeared after the decrease of the NAD(P)H levels. Mitochondrial pore formation is known to uncouple the electron chain from the H^+ gradient and subsequently to lead to enhanced formation of $O_2^{\bullet-}$. The authors used hydroethidin to measure $O_2^{\bullet-}$ formation, which is a cell permeable agent that can be oxidized by superoxide to ethidium, a fluorescent compound that is retained within the cell. The authors did not elaborate in their studies whether the GHS redox cycle is a more efficient scavenger than the $O_2^{\bullet-}$ detecting dye hydroethidin. The dye is potentially only oxidized by $O_2^{\bullet-}$ when the redox capacity of GSH and NADPH is diminished.

We initiated experiments to investigate whether apoptosis leads to a higher capacity of 5h-dh-containing cells to produce 5-oxo-ETE. Preliminary experiments with cultured neutrophils suggest that an enhanced percentage of necrotic/apoptotic cells result in a higher conversion of 5-HETE to 5-oxo-ETE. This process can be diminished when lipopolysaccharide or GM-CSF, two compounds which are known to delay the onset of neutrophil apoptosis, are added to the culture medium. We are also planning to induce apoptosis in cell lines and blood

cells with different stressors and apoptosis inducers such as ceramide and αCD95 antibodies. To correlate the molecular events with 5-oxo-ETE formation, we will examine the relationship of its formation with Annexin V staining (e.g. the appearance of phosphatidylserine on the outer membrane leaflet) and the $\Delta\psi_m$ using flow cytometry. We are also setting up protocols to measure the content of purine and adenine nucleotides by HPLC as well as GSH and GSSG by HPLC using a fluorescence detector in conjunction with derivatization of these cofactors to fluorescent analogs.

Determination of 5-oxo-ETE metabolites in U-937 and A549 cells

Although we have strong pharmacological evidence that 5-oxo-ETE and 5-HETE are esterified to CoA, we do not know the lipid class into which these substances are incorporated. It is impossible to predict this from other studies, as there are wide differences in lipid metabolism from one cell type to another. This process is most likely to be substrate dependent, as AA, 5-oxo-ETE and 5-HETE may be handled differently from one another [97-99,102,270]. It is therefore important to identify the final metabolite to further study this pathway. As a first step cells should be labeled with ^{3}H-5-oxo-ETE and the lipid fraction should be analyzed by thin layer chromatography to determine if 5-oxo-ETE is taken up into phospholipids or triglycerides.

Future Co-Culture Experiments

The specific activity of 5h-dh is very high in A549 cells and the basis of our studies with both whole cells and microsomal fractions. Similarly, HAEC also have a high capacity to produce 5-oxo-ETE, presumably via 5h-dh. These findings suggest that the epithelium and the endothelium may be sources for 5-oxo-ETE *in vivo*. As epithelial and endothelial cells lack significant 5-LO activity, we have to investigate whether 5-oxo-ETE can be formed by means of transcellular biosynthesis. To study transcellular metabolism, 5-LO containing phagocytes (monocytes or granulocytes) should be co-stimulated with epithelial or endothelial cells.

Co-culture of neutrophils with epithelial cells does not automatically lead to an enhanced formation of eicosanoids. It has been shown before by Klockmann and coworkers [259] that epithelial derived PGE_2 can diminish LTB_4 release from human neutrophils. Since A549 cells express COX-2 and can produce PGE_2 [13], it possible that A549 cell derived PGE_2 could inhibit neutrophilic 5-LO. For that reason, indomethacin, a non-specific COX inhibitor, should be added to avoid PGE_2 synthesis from A549 cells. Another potential problem is the esterification of AA in A549 cells, which would lower the available substrate concentration for 5-LO. Furthermore, we showed here for the first time that A549 cells have a high capacity to retain 5-oxo-ETE and 5-HETE, which could counteract the stimulatory effects of transcellular metabolism.

> "We have a habit in writing articles published in scientific journals to make the work as finished as possible, to cover up all the tracks, to not worry about the blind alleys or describe how you had the wrong idea at first, and so on. So there isn't any place to publish, in a dignified manner, what you actually did in order to get to do the work."
>
> *Richard Feynman*

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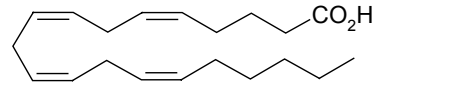
VII APPENDIX

VII.1. Selected Eicosanoids and Fatty Acids



Arachidonic Acid
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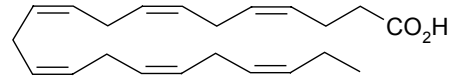

DHA
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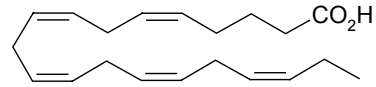
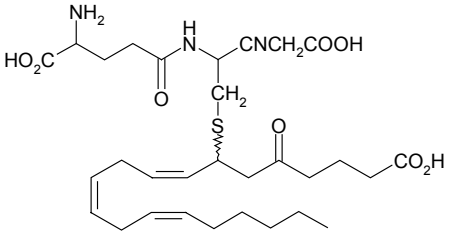
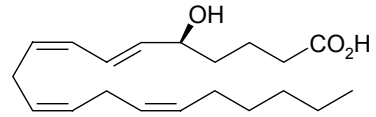


EPA
5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-Eicosapentaenoic acid
Synonyms: Timnodonic acid



FOG_7
5-oxo-7-glutathionyl-8*Z*,11*Z*,14*Z*-eicosatrienoic acid



5-HETE
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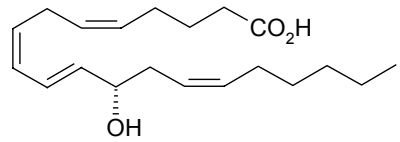

12-HETE
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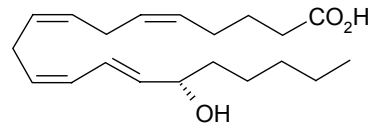
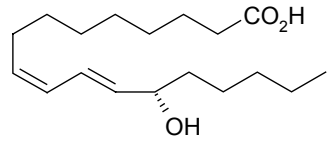


15-HETE
15*S*-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid



13-HODE
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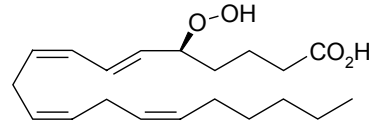
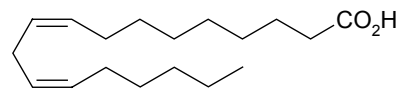
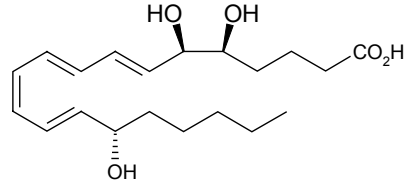


5-HpETE
5*S*-hydroperxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid



Linoleic Acid
9*Z*,12*Z*-octadecadienoic acid



Lipoxin A_4
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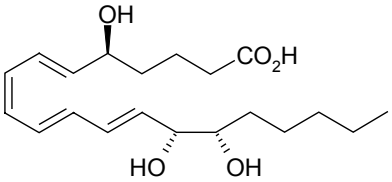

Lipoxin B_4
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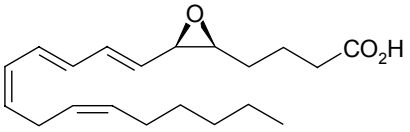

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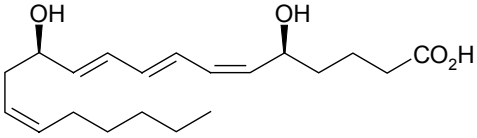

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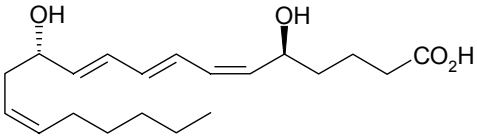

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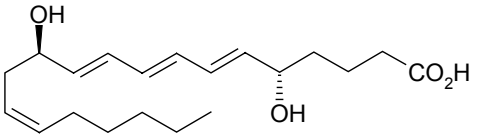

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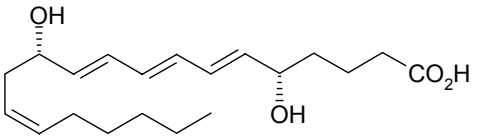

6-trans-12-epi-LTB_4
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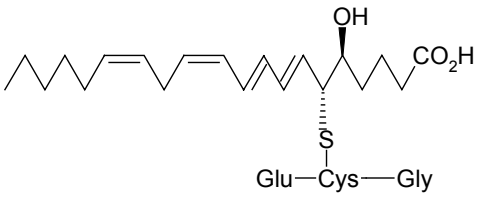
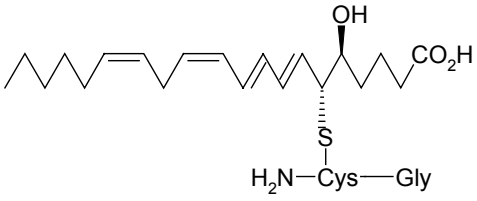
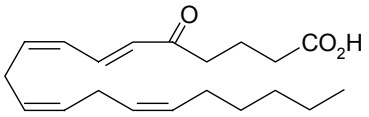


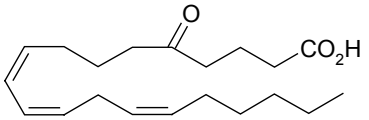
LTC_4
5*S*-hydroxy-6*R*-(S-glutathionyl)-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid



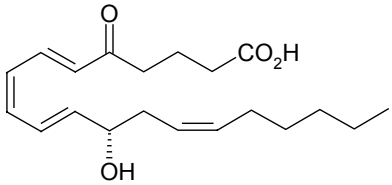
LTD_4
5*S*-hydroxy-6*R*-(S-cysteinylglycinyl)-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid



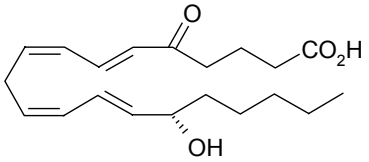
5-oxo-ETE
5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid



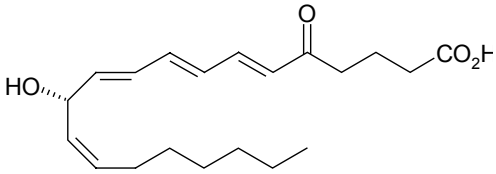
5o-EtrE
5-oxo-8*Z*,11*Z*,14*Z*-eicosatrienoic acid



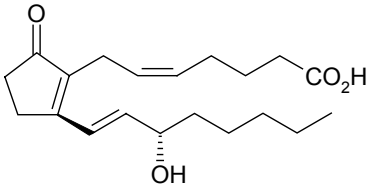
5-oxo-12-HETE
5-oxo-12-hydroxy-6*E*,8*Z*,10*E*,14*Z*-eicosatrienoic acid



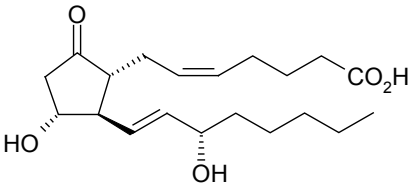
5-oxo-15-HETE
5-oxo-15-hydroxy-6*E*,8*Z*,11*Z*,13*E*-eicosatrienoic acid



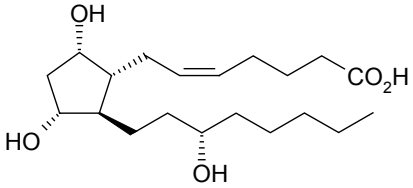
8-trans-5-oxo-12-ETE
5-oxo-12-hydroxy-6*E*,8*E*,10*E*,14*Z*-eicosatrienoic acid



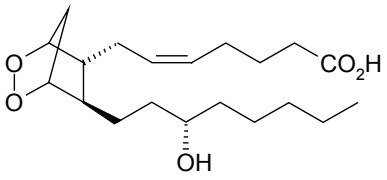
PGB_2
9-oxo-15*S*-hydroxy-prosta-5*Z*,8(12),13*E*-trien-1-oic acid



PGE_2
Prosta-5*Z*,13*E*-dien-11 α,15*S*-dihydroxy-9-oxo-1-oic acid

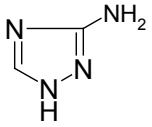


$PGF_2\alpha$
9α,11α,15*S*-trihydroxy-prosta-5Z,13E-dien-1-oic acid

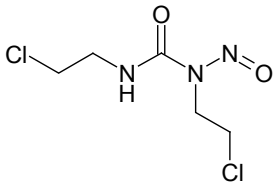


PGH_2
9α,11α-epidioxy-15*S*-hydroxy-prosta-5*Z*,13*E*-dien-1-oic- acid

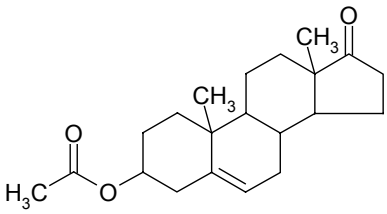
VII.2. Some Frequently Used Chemicals



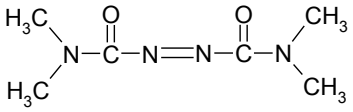
3-AT
3-Amino-1,2,4-triazole



BCNU
1,2-bis[2-chloroethyl]-1-nitrosourea; carmustine

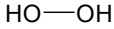


DHEA
Dehydroepiandrosterone

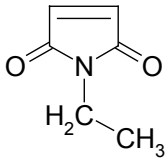


Diamide
N,N,N',N'-Tetramethylazodicarboxamide

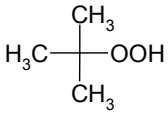
DMSO
dimethylsulfoxide



Hydrogen peroxide, H_2O_2,



NEM
N-Ethylmaleimide



tBuOOH
tert-butyl hydroperoxide

VII.3. Claims to Original Research

The following summary lists research, which was undertaken for the first time over the course of this thesis. To the best knowledge of the author, none of these findings has been presented by other researchers.

- We are the first to demonstrate that undifferentiated myeloid cell lines (U-937 and HL-60) express 5h-dh at levels comparable to human peripheral blood leukocytes and that its activity can be upregulated by differentiation towards monocytes/macrophages.
- We present, for the first time, a novel regulatory pathway by which 5-oxo-ETE synthesis is stimulated. Oxidative stress simulated by hydroperoxide addition dramatically enhances $NADP^+$ levels, the cofactor of 5h-dh, thus enhancing the formation of 5-oxo-ETE from 5-HETE. Using different pharmacological approaches we were able to show that this stimulatory effect is carried into execution by the GSH redox cycle. Oxidative stress dramatically enhances the formation of 5-oxo-ETE from endogenous 5-HETE by all cell types studied except by neutrophils, which proved to be more resistant to this effect. We also found that tBuOOH induces 5-oxo-ETE synthesis from endogenous 5-HETE in AA and Ca^{++} ionophore stimulated PBMC.
- We show here for the first time that glucose diminishes the stimulatory effect of oxidative stress on 5-oxo-ETE synthesis and can affect the baseline conversion of 5-HETE to 5-oxo-ETE in some cell types. This effect is mediated through the PPP by reducing $NADP^+$ to NADPH, thus depleting the cofactor needed for the reduction of 5-HETE to 5-oxo-ETE by 5h-dh.
- This thesis presents for the first time that the epithelial cell lines A549, BEAS-2B, T84, and HEp-2G have the ability convert 5-HETE to 5-oxo-ETE. Further studies with A549 cells revealed that the enzyme catalyzing this reaction appears to be 5h-dh, which had been previously only described in peripheral blood leukocytes and platelets. 5-oxo-ETE synthesis is also stimulated by oxidative stress in epithelial cells and inhibited by glucose. However, to date we were not able to show that the inhibitory effect of glucose is due to the action of the PPP.
- We further demonstrated that human aorta endothelial cells (HAEC) also convert 5-HETE to 5-oxo-ETE. Due to the limited availability of those primary cells, we could not rule out that an enzyme other than myeloid 5h-dh is responsible for this reaction. However, the regulation of 5-oxo-ETE synthesis by oxidative stress appears to be similar to that found in myeloid cells.
- We further examined forward and the backward reaction U-937 cell derived 5h-dh. For the first time we examined the pH-dependence of this catalysis and found the forward reaction to be favored above pH 6.5. There is a dramatic increase in 5-oxo-ETE formation by U-937 microsomes at pH 9, with a maximum at pH 10. The 5h-dh-mediated reaction is inhibited at extreme pHs ($pH \leq 4.0$ and $pH \geq 11.4$).

VII.4. Table of Figures

LIST OF PUBLICATIONS

Peer-Reviewed Publications

K-R. Erlemann, J. Rokach, WS. Powell (2004). Oxidative stress stimulates the synthesis of the eosinophil chemoattractant 5-oxo-6,8,11,14-eicosatetraenoic acid by inflammatory cells. JBC: *Epub ahead of print*.

P. Müller, **K.-R. Erlemann**, K. Müller, J.J. Calvete, E. Töpfer-Petersen, K. Marienfeld, and A. Herrmann (1998) Biophysical characterization of the interaction of bovine seminal plasma protein PDC-109 with phospholipid vesicles. European Biophysical Journal 27: 33-41.

Reviewed Conference Presentations

K-R. Erlemann, J. Rokach, WS. Powell (2003), Keystone Symposium, Lake Tahoe, USA. Cellular Stress Enhances 5-oxo-ETE Synthesis by Myeloid Cells.

K-R. Erlemann, J. Rokach, WS. Powell (2002), 98th International conference of the American Thorax Society, Atlanta, USA. Formation of 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) by epithelial cells.

K-R. Erlemann, J. Rokach, WS. Powell (2001), 97th International conference of the American Thorax Society, San Francisco, USA. Increased formation of 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) during human myeloid cell differentiation.

K-R. Erlemann , S. Lange , J. J. Calvete, E. Töpfer-Petersen, A. Herrmann, P. Müller (1998) 8th International Symposium on Spermatology, Montreal, Canada, Interaction of bovine seminal plasma protein PDC-109 with phospholipid vesicles.

Conference Presentation

K-R. Erlemann (2003), McGill Respiratory Health Axis Meeting, Montreal, Canada. Cellular Stress Enhances 5-oxo-ETE Synthesis by Myeloid Cells.

K-R. Erlemann (2002), McGill Respiratory Health Axis Meeting, Montreal, Canada. Formation of 5-oxo-ETE by epithelial cells – a biochemical approach. *Award for best presentation in graduate student group.*

K-R. Erlemann (2001), McGill Respiratory Health Axis Meeting, Montreal, Canada. Human Myeloid Cell Differentiation increases the Formation of 5-oxo-6,8,11,14-Eicosatetraenoic Acid (5-oxo-ETE).

A. Greube, **K-R. Erlemann**, E. Töpfer-Petersen, P. Manjunath, A. Herrmann, and P. Müller (1999) Jahrestagung der Deutschen Gesellschaft für Biophysik, Ulm, Interaction of bovine seminal plasma protein PDC-109 with membranes.

Montreal, 8. Juli 2004

LEBENSLAUF

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Geburtsort	Korbach
Familienstand	Verheiratet, 1 Kind
Staatsangehörigkeit	Deutsch
Nationalität	Deutsch

Studium und Promotion

Seit 15. März 2000	Promotionsarbeit in den Meakins-Christie Laboratories	Montreal, CA
	Regulation of 5-oxo-ETE Synthesis in inflammatory cells.	
1999	Diplomarbeit	Berlin
	Interaction of the Seminal Plasma Protein BSP-A1/A2 with phospholipids.	
1998	Forschungsaufenthalt in dem *Hôpital Rosement de Masionneuve*	Montreal, CA
1993-1999	Diplomstudium Biophysik, Humboldt-Universität	Berlin

Schulausbildung

1984-1993	Christian Rauch Schule	Bad-Arolsen
1980-1984	MPS Adorf	Diemelsee-Adorf

Montreal, 8. Juli 2004

EIDESTATTLICHE ERKLÄRUNG

Ich erkläre, daß ich diese Arbeit selbständig und nur mit den angegebenen Hilfmitteln angefertigt habe und daß alle Stellen, die dem Wortlaut oder dem Sinne nach anderen Werken entnommen sind, durch Angaben der Quellen als Entlehnung kenntlich gemacht worden sind.

_____

Montreal, 8. Juli 2004

ACKNOWLEDGMENT

I thank Prof. Dr. William Powell for his continuous support and substantial advice. Without his steady support and scientific guidance, the conjunction of oxidative stress and eicosanoid formation would not have been possible. As his extensive knowledge and workload will remain out of reach for me, I will emulate his way of guidance without power demonstrations. His openness to new ideas and the urge to get to bottom of the scientific questions we faced made the research very stimulating. Without the constant supply of 5-HETE from Joshua Rokash, this work would have been literally without substance.

This work would not have been possible without the support of my parents. They always encouraged me to choose my own path, even if it led me to distant shores. I want to show appreciation to Karen for her support and her patience, especially during the final stages of the writing process. My thanks go to Raphaël, who constantly ignored my stress levels, for providing new perspectives and distractions.

I am very grateful to the Meakins-Christie Laboratories for supporting my research and providing opportunities to me to present my results on conferences. The constant exposure to fellows from all around the globe made research a memorable experience. I want to thank Dr. Jim Martin and Dr. Qutayba Hamid for letting me work with them under their roof and keeping me on my toes during many presentations. My specials thanks go to Sylvie, who introduced me to the world of HPLC and always made sure the lab is running fine. Without her assistance during incubations, it would have been impossible to accomplish that many experiments. I want to thank Sandra for her introduction into cell culture and for sharing great times together. Without Sandy, the Meakins would certainly be a different, less connected place. Her constant efforts to ensure the well being of the network took many worries away.

My special thanks goes to Pierre, who is a great person to share not only scientific ideas. He is certainly the only person with whom a Medline search may be entertaining. I am indebted to Guillaume for his great introduction into immunology. Without his anecdotes, cytokines would remain a great mystery to me. I am very glad I met Meri, who certainly changed the social life in the institute. I want to thank Philippe for regularly motivating me to come for a quick run up the mountain.

I would never have reached this point without the scientific education I received at the Humboldt-University. I am especially indebted to Prof. Dr. Lockau for organizing my doctoral thesis defense. I am grateful to Prof. Dr. Herrmann and Dr. Müller for tutoring me during my first steps as a scientist.

My list would certainly not be complete without a special thank-you to my friends outside academia. I would like to thank Donald & Danielle for their presence during my stay in Montreal. They gave me the warmest welcome anybody could imagine while dealing with my slight German accent. My special thanks go to Robert and Matthew. It was a great pleasure to get such company. I would also like to express my gratitude towards Jim for his efforts to improve my English. Finally, yet importantly, I want to express thanks to Klaus for the great discussions we had and the motivation to look beyond boundaries. Without their encouragement during the difficult phases of my project, I would have considered a career change - probably to become a plumber. Setting up and fixing HPLC equipment is certainly a perfect training for this profession.