Characterization of 5-hydroxyeicosanoid dehydrogenase and the regulation of 5-oxo-ETE synthesis

by

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Dedication

This work is dedicated to my parents.

Abstract

Inflammation is associated with numerous diseases including asthma and cancer. 5-Oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE), an arachidonic acid metabolite formed by the 5-lipoxygenase (5-LO) pathway, is a potent chemoattractant for inflammatory cells. 5-LO converts arachidonic acid to 5S-hydroperoxy-ETE, which is reduced to 5S-hydroxy-ETE (5-HETE) by a peroxidase. 5-HETE is then oxidized to 5-oxo-ETE by the microsomal enzyme 5-hydroxyeicosanoid dehydrogenase (5-HEDH), which requires $NADP^+$, and is abundant in most inflammatory cells and at least some structural cells. Although biochemical studies have contributed to our understanding of the regulation of 5-HEDH activity, this enzyme has not been purified nor has its sequence been identified.

Here we report that B cells contain 5-HEDH, and synthesize 5-oxo-ETE from exogenous arachidonic acid in the presence of calcium ionophore. H_2O_2 strongly stimulates 5-oxo-ETE production via activation of the glutathione redox pathway, resulting in rapid increases in GSSG and $NADP^+$. Various cancer cell lines, including PC3 (prostate), A427 (lung), and MCF7 (breast) also possess 5-HEDH activity and H_2O_2 also promotes the rapid synthesis of 5-oxo-ETE in these cells. 5-Oxo-ETE synthesis from 5-HETE by cancer cell lines was stimulated over a longer period of time (24 h) by anti-cancer agents including docosahexaenoic acid, tamoxifen and MK886. Although PC3 cells have little 5-LO activity, coculture experiments revealed that they can utilize neutrophil-derived 5-HETE to synthesize 5-oxo-ETE by transcellular biosynthesis. A similar process could result in the synthesis of 5-oxo-ETE in tumors, which contain large numbers of inflammatory cells. These studies suggest that oxidative stress and cell death, which occur at inflammatory sites and in tumors, could induce the synthesis of 5-oxo-ETE, which could then act to prolong the inflammatory response.

Another goal was to purify and characterize 5-HEDH. We succeeded in partially purifying this enzyme (~20-fold) from microsomal fractions from

neutrophils and monocytic cells by detergent solubilisation followed by DEAE Sepharose and Blue Sepharose chromatography. Tryptic digests of the purified protein fractions were separated by SDS-PAGE and analyzed by mass spectrometry. Several known dehydrogenases were identified, along with one potential candidate with no known substrate: dehydrogenase/reductase SDR family member 7 (DHRS7). Further studies will be required to determine whether this dehydrogenase is identical to 5-HEDH.

Résumé

L'inflammation est associée à de nombreuses maladies dont l'asthme et le cancer. L'acide 5-oxo-6,8,11,14-eicosatetraenoique (5-oxo-ETE), un métabolite de l'acide arachidonique produit par la 5-lipoxygénase (5-LO), est un puissant chimioattractant pour les cellules du système inflammatoire. La 5-LO transforme l'acide arachidonique en 5S-hydroperoxy-ETE, qui est réduit en 5S-hydroxy-ETE (5-HETE) par une peroxydase. Le 5-HETE est oxydé en 5-oxo-ETE par une enzyme microsomale, la 5-hydroxyeicosanoide déshydrogénase (5-HEDH), enzyme dépendante du NADP+ et présente en abondance dans la majorité des cellules inflammatoires ainsi que dans quelques cellules structurales. Bien que les études biochimiques contribuent à une meilleur compréhension de la régulation de la 5-HEDH, cette enzyme n'a pas encore été purifiée ni clonée.

Dans cette étude, nous rapportons que les cellules B possèdent de la 5-HEDH, et produisent du 5-oxo-ETE à partir d'acide arachidonique exogène en présence d'ionophore de calcium. Le H_2O_2 stimule fortement la production de 5-oxo-ETE via l'activation du cycle de réduction du glutathion, provoquant une augmentation rapide du GSSG et du $NADP^+$. Différentes lignées cellulaires cancéreuses, les PC3 (prostate), A427 (poumon), et MCF7 (sein), possèdent de la 5-HEDH et sont capables de synthétiser du 5-oxo-ETE après stimulation par le H_2O_2. Cette synthèse est augmentée suite à une exposition de 24h avec des agents anticancéreux : l'acide docosahenoique, tamoxifène et MK886. Bien que les PC3 n'aient pas beaucoup d'activité 5-LO, des expériences de coculture avec des neutrophiles démontrent qu'elles peuvent utiliser le 5-HETE produit par les neutrophiles pour synthétiser du 5-oxo-ETE par biosynthèse transcellulaire. Un processus similaire peut être à l'origine de la synthèse de 5-oxo-ETE dans les tumeurs, riches en cellules inflammatoires. Cette étude suggère que le stress oxydatif ainsi que la mort cellulaire, présents aux sites inflammatoires et dans les tumeurs, peuvent induire la synthèse du 5-oxo-ETE, qui par lui-même peut prolonger la réponse inflammatoire.

Dans cette étude, nous avons tenté de purifier et de caractériser la 5-HEDH. Nous avons réussi à la purifier partiellement à partir de microsomes, de neutrophiles et de cellules monocytique, solubilisés avec des détergents, suivit par chromatographie avec du DEAE sépharose et du sépharose bleu. Les différentes fractions obtenues ont été digérées par la trypsine, séparées sur gel acrylamide et analysées par spectrométrie de masse. Plusieurs déshydrogénases ont été identifiées, dont la déshydrogénase/réductase membre no. 7 de la famille SDR (DHRS7), qui ne possède pas de substrat connu. D'autres études devront être effectués pour déterminer si cette déshydrogénase correspond à 5-HEDH.

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List of Abbreviations

12-HHT	12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid
12-LO	12-lipoxygenase
13-HODE	13S-hydroxy-9Z,11E-octadecadienoic acid
15-LO	15-lipoxygenase
5-HEDH	5-hydroxyeicosanoid dehydrogenase
5-HETE	5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid
5-HpETE	5S-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid
5-LO	5-lipoxygenase
5-oxo-ETE	5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid
AA	arachidonic acid
APS	ammonium persulfate
ATP	adenosine triphosphate
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea (Carmustine)
BN-PAGE	blue native polyacrylamide gel electrophoresis
BSO	buthionine sulfoximine
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHO	Chinese hamster ovary
$cPLA_2$	cytosolic phospholipase A_2
CYP	cytochrome P-450
DEAE	diethylaminoethylene
DHA	4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid
ECM	extracellular membrane
ERK	extracellular signal-regulated kinase
FABP	fatty acid binding protein
FBS	fetal bovine serum
FLAP	5-lipoxygenase activating protein
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FOG_7	5-oxo-7-glutathionyl-8Z,11Z,14Z-eicosatrienoic acid
G-6-PD	glucose-6-phosphate dehydrogenase

GPCR	G protein-coupled receptor
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GTP	guanosine triphosphate
HA	hydroxylapatite
H_2O_2	hydrogen peroxide
IL-4	interleukin 4
INT	iodonitrotetrazolium
JNK	c-jun N-terminal kinase
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LT	leukotriene
LTA_4	5S-trans-5,6-oxido-7E,9E,11Z,14Z-eicosatetraenoic acid
LTB_4	5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid
LTC_4	5S-hydroxy-6R-(S-glutathionyl)-7E,9E,11Z,14Z-eicosatetraenoic acid
LTD_4	5S-hydroxy-6R-(S-cysteinylglycinyl)-7E,9E,11Z,14Z-eicosatetraenoic acid
mRNA	messenger ribonucleic acid
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
NAD	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NBD	nitroblue tetrazolium
NEM	N-ethylmaleimide
NOX	NADPH oxidase
OXE-R	5-oxo-ETE receptor
PAF	platelet-activating factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PG	prostaglandin

PKA	protein kinase A
PMA	phorbol 12-myristate 13-acetate
PMS	phenazine methosulfate
PMSF	phenylmethanesulfonyl fluoride
PPAR	peroxisome proliferators-activated receptor
PUFA	polyunsaturated fatty acid
RP-HPLC	reversed phase-high performance liquid chromatography
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
TBAH	tetrabutyl ammonium hydroxide
TEMED	N,N,N',N'-tetramethylethylenediamine
TX	thromboxane

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Chapter 1: Introduction and Literature Review

1.1 The immune system and inflammation

The immune system comprises cells designed to fight off infection. Acute inflammation occurs in response to foreign pathogens or tissue injury which trigger the accumulation of immune cells at the site of infection. Innate immune responses involve phagocytes and other cells that release inflammatory mediators including eosinophils, neutrophils, monocytes, macrophages, basophils and dendritic cells. Acquired immune responses become stronger with repeated exposure to antigens and involve T and B lymphocytes.

Several events must occur to result in inflammation, including increased permeability of vascular walls, vasodilation, and migration of leukocytes from the bloodstream to the inflamed tissue [1]. In the tissue, granulocytes (neutrophils, eosinophils, mast cells) release enzymes, reactive oxygen species and other mediators to destroy the invading pathogen. A wide array of activators including cytokines, chemokines and lipid mediators regulate the whole process by differentially activating leukocytes and promoting their directed migration to the affected tissue. In some pathological situations, the inflammatory processes can become uncontrolled, resulting in tissue damage. Asthma is characterized by airflow obstruction, airway inflammation, and bronchial hyperresponsiveness [2].

1.1.1 Eicosanoids in inflammation

Eicosanoids collectively encompass the oxidized metabolic products of 20-carbon polyunsaturated fatty acids (PUFAs), including arachidonic acid (AA), and are synthesized by the majority of human cells. The ω-6 fatty acid, AA, is the major precursor for these bioactive lipid mediators which contribute to inflammatory diseases. Many enzymes are involved in the conversion of AA to its active (or inactive) metabolites; however, there are two major biosynthetic pathways. The first pathway generates prostaglandins (PGs) and thromboxanes (TXs), via the cycloxygenases (COX-1, constitutive, and COX-2, inducible) which catalyze the generation of unstable endoperoxide intermediates. These

intermediates are converted by other enzymes to the final bioactive products. The second major catabolic pathway, involves 5-lipoxygenase (5-LO), which catalyzes the initial reaction in the conversion of arachidonic acid to the leukotrienes (LTs) LTB_4, LTC_4, LTD_4 and LTE_4. 5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HETE) and 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-oxo-ETE) are also products of the 5-LO pathway. Several other lipoxygenases (12-LO and 15-LO) produce additional biologically active metabolites, but are not expressed as widely. Figure 1.1 shows the major synthetic pathways of AA metabolism. Eicosanoids are synthesized and metabolized within seconds or minutes and consequently act locally near where they are produced. These lipid mediators have a multitude of diverse effects on cells under normal conditions as well as in pathological (disease) situations. They exert their effects by activating their respective G-protein coupled receptors (GPCRs). These receptors are designated by the International Union of Pharmacology (IUPHAR) based on their agonist selectivity and binding affinities [3]. LTB_4 activates the BLT1 receptor and weakly activates the BLT2 receptor, which is more sensitive to 12-HHT (12-hydroxy-5Z,8E,10E-heptadecatrienoic acid) [4]. LTD_4 primarily activates the $CysLT_1$ receptor, and LTC_4, and LTD_4 activate the $CysLT_2$ receptor, while LTE_4 is a weak agonist. PGD_2 binds both the DP1 receptor and the DP2 receptor (also known as CRTH2). 5-oxo-ETE and to a lesser extent, 5-HETE, activate the OXE receptor [5]. Recently, the orphan receptor, GPR17 has been determined to bind uracil nucleotides and LTC_4 [6].

1.1.2 Oxidative stress

Cells contain high levels of GSH and NADPH as a means of protecting themselves from reactive oxygen species (ROS) that are generated during mitochondrial respiration and by oxidative enzymes such as NADPH oxidase. Even minor alterations in the redox state of cells can have significant effects. ROS are formed in response to cellular activation by invading pathogens or as by-products of numerous cellular metabolic pathways, including 5-LO. ROS include superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), nitric oxide (NO) and hydroxyl

radicals (·OH) and may act as second messengers activating various signaling pathways. ROS are important for host defence against bacteria, but they may also be damaging if produced in excess. Excess production of ROS, termed oxidative stress, can induce cell death or a change in cellular properties as antioxidant pathways are activated to protect the cell. Cells contain several enzymatic defence systems to remove toxic ROS, including superoxide dismutase (SOD), catalase, thioredoxin and glutathione peroxidase (GPx) [7,8]. Cells also contain nonenzymatic antioxidants including vitamin E, ascorbic acid, and glutathione (GSH) [8].

The pentose phosphate pathway is important for maintaining high intracellular levels of NADPH. When certain types of cells are exposed to excess levels of H_2O_2 or lipid hydroperoxides, GPx catalyzes the reduction of ROOH coupled to oxidation of GSH to its disulfide, GSSG. GSSG is then reduced back to GSH by glutathione reductase (GR) which requires the readily available NADPH as a cofactor. In this reaction, NADPH is oxidized to $NADP^+$. Therefore, stimuli that produce oxidative stress in cells will induce a rapid increase in the levels of $NADP^+$ compared to NADPH. The enzymes, GPx, xanthine oxidase and catalase are also responsible for removing H_2O_2, however xanthine oxidase can also produce superoxide. These antioxidants help to control the intracellular oxidative environment to minimize cellular damage.

1.2 5-Lipoxygenase

There are several lipoxygenase enzymes that catalyze similar reactions but oxidize AA at different positions. 12-LO (present in platelets) and 15-LO (present in leukocytes and reticulocytes) convert AA to 12-HpETE and 15-HpETE, respectively. Peroxidases, including GPx then convert these hydroperoxy products to their corresponding hydroxy compounds (12-HETE and 15-HETE). A major pathway for AA metabolism is via 5-LO, which leads to the formation of the cysteinyl leukotrienes. 5-LO is predominantly expressed in immune cells including neutrophils, eosinophils, macrophages, B lymphocytes, dendritic cells, basophils and mast cells [9,10]. It is also expressed to a lesser

extent in brain [11,12], human keratinocytes [13], and human bronchial epithelial cells [14]. The levels of 5-LO have been determined to be higher in many pathological conditions. For example, 5-LO is present at very low levels in pulmonary artery endothelial cells (PAEC), but its expression is increased in PAEC from patients with pulmonary hypertension [15] and from mice challenged with allergen [16]. In addition, 5-LO has been found to be upregulated in many types of cancer cells [17,18] compared to normal epithelial cells.

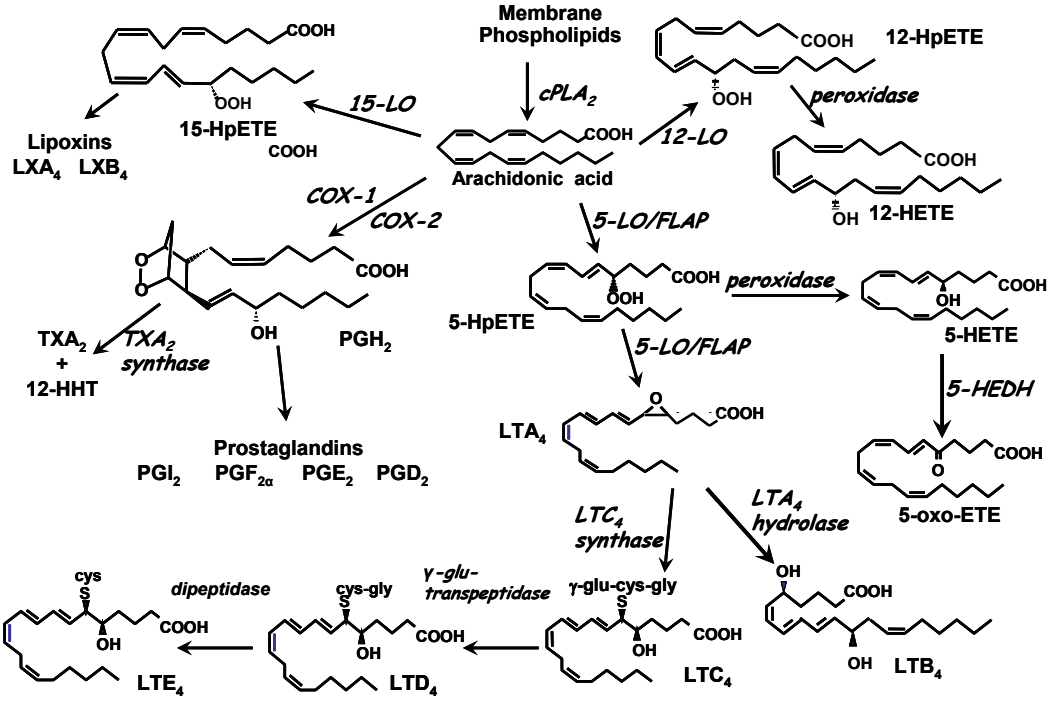


Figure 1.1: Eicosanoid biosynthetic pathways. AA is released from membrane phospholipids by $cPLA_2$ after cellular activation and can then be metabolized by 5-LO, COX and to a lesser extent by 12-LO and 15-LO.

5-Lipoxygenase catalyzes two distinct reactions that form the precursors for leukotrienes. The first reaction involves the removal of hydrogen at the 7-position and addition of oxygen to the 5-position of AA to form the intermediate, 5S-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HpETE). In the second reaction, 5-LO rearranges 5-HpETE to leukotriene A_4 (LTA_4; 5S,6S-oxido-

7E,9E,11Z,14Z-eicosatetraenoic acid). The highly reactive LTA_4 is released from 5-LO and subsequently metabolized by one of two pathways. LTA_4 can be converted to the dihydroxy acid LTB_4 (5*S*,12*R*-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid) by LTA_4 hydrolase or to the glutathione conjugate LTC_4 (5*S*-hydroxy-6*R*-*S*-glutathionyl-7E,9E,11Z,14Z-eicosatetraenoic acid) by LTC_4 synthase. LTC_4 is subsequently converted to LTD_4 (5*S*-hydroxy-6*R*-*S*-cysteinylglycyl-7E,9E,11Z,14Z-eicosatetraenoic acid) by γ–glutamyl transpeptidase, and finally, LTE_4 (5*S*-hydroxy-6*R*-*S*-cysteinyl-7E,9E,11Z,14Z-eicosatetraenoic acid) is synthesized from LTD_4 by dipeptidase. The first 5-LO intermediate, 5-HpETE, can alternatively be released from 5-LO and subsequently reduced to form 5-HETE by peroxidases (such as GPx) [19]. The enzyme, 5-hydroxyeicosanoid dehydrogenase (5-HEDH), catalyzes the conversion of 5-HETE to 5-oxo-ETE utilizing $NADP^+$ as a cofactor.

1.2.1 Regulation of the biosynthesis of 5-LO products

The synthesis of 5-LO products as well as other AA metabolites is tightly controlled due to their diverse biological functions. AA itself can also induce some cellular processes. Therefore, the initial release of AA is highly regulated. To limit the synthesis of these bioactive compounds, resting cells maintain very low levels of free AA. Fatty acids are stored in membrane phospholipids, and AA is esterified to diacyl or ether phospholipids at the sn-2 position. AA is released when agonists, acting via their receptors stimulate the activation of a phospholipase A_2 (PLA_2). These enzymes catalyze the release of fatty acids by hydrolyzing membrane glycerophospholipids at the sn-2 position. This produces a free fatty acid and a lyso phospholipid. Human cells have three main families of structurally distinct PLA_2s including secretory PLA_2 ($sPLA_2$), calcium-independent PLA_2 ($iPLA_2$), and calcium-dependent cytosolic PLA_2 ($cPLA_2$), which have different subcellular localizations and specificities [20,21]. In contrast to other phospholipases, $cPLA_2$ is highly specific for AA, and is the most important phospholipase involved in its release. $cPLA_2$ is localized in the cytosol in resting cells and can be regulated at the transcriptional and post-translational

levels [20]. Agonists that stimulate the release of calcium from intracellular stores activate $cPLA_2$, which then translocates to intracellular membranes. $cPLA_2$ can also be activated by phosphorylation of 2 serine residues by activated kinases [21]. Therefore, agonists that stimulate either the release of calcium from intracellular stores, or that activate kinases may induce the release of AA (Figure 1.2).

After AA is released 5-LO also requires activation before catalyzing its first reaction. In resting cells, 5-LO remains inactive and is located in the cytosol and/or within the nucleus. Similar to $cPLA_2$, agonists that stimulate an increase in intracellular Ca^{2+} levels, activate 5-LO, inducing its translocation to the nuclear membrane [10]. At the nuclear envelope, 5-LO binds to 5-lipoxygenase activating protein (FLAP), an 18 kDa nuclear membrane bound protein [22]. Once bound to the nuclear membrane, 5-LO can interact with FLAP-bound AA that has been released by PLA_2. In intact cells, both 5-LO and FLAP are required for the synthesis of leukotrienes and 5-HETE, whereas in crude cell homogenates, Ca^{2+} and ATP are required for 5-LO activity [23] (Figure 1.2). The redox state of the cell, is also very important for 5-LO activity. 5-LO contains a non-heme iron, and under resting conditions, the iron is in the reduced, ferrous (Fe^{2+}) form. Oxidation of this iron to the ferric state (Fe^{3+}) by a lipid hydroperoxide such as 5-HpETE, is required for activation of 5-LO. 5-LO has 3 separate serine residues that can be phosphorylated by MAPKAPK-2 (mitogen-activated protein kinase-activated protein kinase-2) [24], ERK-2 (extracellular-signal-regulated kinase 2) [25] or cAMP/PKA (protein kinase A) [26]. Phosphorylation of 5-LO by MAPKAPK and ERK-2 results in activation, even in the absence of calcium, whereas phosphorylation by PKA inhibits activity. 5-LO activity can also be regulated by selenium, which is considered an antioxidant, either directly or indirectly. Glutathione peroxidases (GPxs) require selenium and they inhibit 5-LO activity since they reduce lipid hydroperoxides (e.g. 5-HpETE) which are required to oxidize the active site Fe^{2+} to Fe^{3+} [27-29]. Selenium can also directly interact with the 5-LO active site Fe^{2+}, causing irreversible inactivation [30].

Therefore, 5-LO activity is tightly regulated and inducible by stimuli that can activate a variety of signaling pathways.

1.2.2 Transcellular biosynthesis of 5-LO metabolites

The enzymes that convert the initial products formed by 5-LO, into biologically active LTs and 5-oxo-ETE are more widely distributed than 5-LO itself. Red blood cells and various types of structural cells possess little or no 5-LO, but can convert LTA_4 and/or 5-HETE into LTs and 5-oxo-ETE, respectively, because they contain LTA_4 hydrolase, LTC_4 synthase, and/or 5-HEDH. Thus, even though LTA_4 has a very short half-life, it can be exported from donor inflammatory cells and converted to LTB_4 or LTC_4 by acceptor cells [31]. For example, red blood cells express LTA_4–hydrolase and can thereby synthesize LTB_4 in the presence of calcium ionophore-stimulated neutrophils [32]. Platelets also do not express 5-LO, but express large amounts of 12-LO and COX and consequently they synthesize thromboxanes, 12-HpETE and 12-HETE. However, platelets also express LTC_4-synthase and synthesize significant amounts of LTC_4 in the presence of neutrophils activated with fMLP and opsonized zymosan [33]. In contrast, when neutrophils alone are activated in the absence of platelets, LTB_4, but not LTC_4, is synthesized. Similar to platelets, human umbilical vein endothelial cells (HUVEC) do not express 5-LO and only synthesize LTC_4 in the presence of neutrophils [34,35]. AA itself can also be transferred from one cell to another, being released by a donor cell and metabolized by an acceptor cell. Studies have shown that alveolar epithelial cells with incorporated radiolabeled AA, predominantly synthesize prostaglandins [36]. However, when co-incubated with unlabeled alveolar macrophages, radiolabeled LTB_4, and 5-HETE were synthesized [36]. AA has also been shown to be transferred between neutrophils and platelets [37,38]. Recent studies have identified a protein, epithelial fatty acid binding protein (E-FABP), that stabilizes LTA_4 during its cellular release and uptake process [39]. There are five closely related FABPs that differ according to the cell type, which have been shown to increase the half-life of LTA_4 to up to 20 to 30 min compared to only about 30 sec in the absence of binding proteins [31].

FABPs do not bind LTB_4 or 5,6-diHETE since they did not reduce the binding of LTA_4 or AA in competition assays whereas 5-HETE reduced binding by 33% [31,40]. However, FABPs may bind a variety of other fatty acids, which have not been tested yet. The nature and amounts of eicosanoids formed may differ in pathological conditions, such as tumors, since the relative number of different types of cells may alter the profile of bioactive lipid mediators produced. Therefore, understanding the cellular tissue infiltrates in pathological conditions may aid in determining appropriate pharmacological interventions.

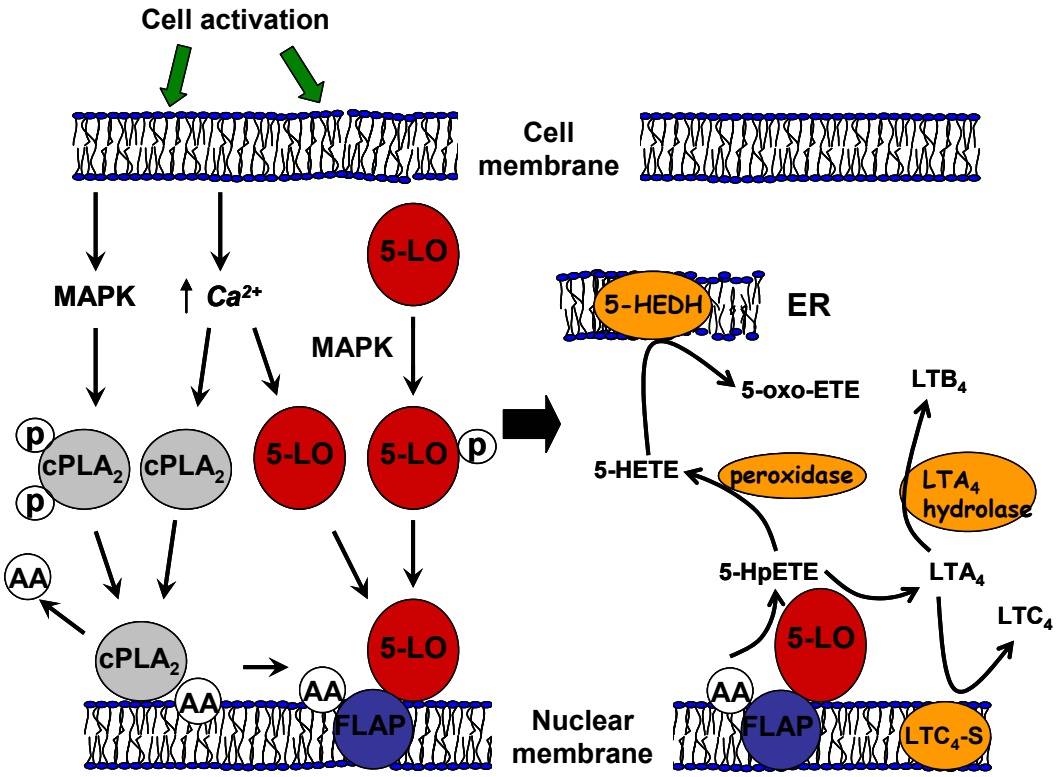


Figure 1.2: Cellular organization of 5-LO activation and leukotriene and 5-oxo-ETE synthesis. Cellular activation stimulated Ca^{2+} mobilization induces translocation of 5-LO and $cPLA_2$ to the nuclear membrane. MAPK can also phosphorylate 5-LO and $cPLA_2$. Activated $cPLA_2$ induces the release of AA which binds FLAP present on the nuclear membrane. AA can then be converted to 5-HpETE and LTA_4 to form 5-HETE or LTC_4 and LTB_4, respectively. 5-HETE can then be converted to 5-oxo-ETE by membrane bound 5-HEDH.

1.2.3 Regulation of 5-LO activity in B cells by oxidative stress

B lymphocytes are an important component of the immune response, and release IgE, which plays a major role in the development of airway inflammation in asthma [2]. Therefore, B cells play an important role in both immune responses and inflammatory processes. It was originally believed that neither T nor B lymphocytes express 5-LO or synthesize leukotrienes [41]. However, Odlander and colleagues discovered that B cells express LTA_4 hydrolase, and can convert LTA_4 to LTB_4 [41-44]. This group subsequently discovered that B lymphocytes do express 5-LO and FLAP, although the levels vary between different B cell lines and B cells of different maturity [45,46]. Conversely, T lymphocytes only express FLAP [45]. These and several other studies also showed that B lymphocytes synthesize 5-LO products (only 5-HETE, LTB_4 and its 6-trans isomers were measured) when incubated with exogenous AA and calcium ionophore, A23187 [43,45-50]. Stimulating B cells with A23187 alone is not sufficient to induce the production of 5-LO metabolites, and products are only synthesized from exogenous AA. This may be explained by the fact that only immature B cells and not mature B cells contain $cPLA_2$ [51]. However, it has not been determined whether immature B cells synthesize 5-LO products when stimulated with A23187 alone.

5-LO activity is very low in intact unstimulated cells, whereas B cell sonicates incubated with exogenous AA and ATP have significantly more activity [45,47]. However, preincubating intact B cells with glutathione depleting reagents such as diamide or n-ethylmaleimide (NEM), results in increased synthesis of 5-LO products [45,48,52-54]. However, there was no correlation between the actual levels of GSH and 5-LO activity [45], suggesting that the antioxidant properties of the intracellular environment normally inhibit 5-LO activity in unstimulated B cells. Several studies determined that altering the redox status, and inducing oxidative stress by adding H_2O_2 to B lymphocytes stimulates the synthesis of 5-LO products [48,54,55]. Fatty acid hydroperoxides, such as 12-HpETE, 13-HpODE and 15-HpETE have a similar effect. In addition to oxidative stress, osmotic stress, chemical stress (sodium arsenite) and inflammatory

cytokines (TNF-α plus IL-1) have also been shown to increase 5-LO activity in B cells [54]. The activation of 5-LO by these stimuli was shown to be mediated by the activation of p38 MAPK [54]. Stimuli that induce the formation of superoxide, such as xanthine oxidase/xanthine [55], also stimulate 5-LO activity. B cells express NADPH oxidase and synthesize superoxide when stimulated with phorbol myristate acetate (PMA) and can convert superoxide to H_2O_2 when activated [56]. Stimulation of surface IgG also stimulates superoxide formation in these cells [56]. However, the NADPH oxidase activity in B cells is less than 5% of the activity found in phagocytes. Antimycin A, which inhibits the electron transport of complex III of the respiratory chain, thereby increasing endogenous superoxide levels, also increases 5-LO activity in B cells [55]. The capability of different B cell lines for synthesizing superoxide and 5-LO metabolites varies greatly [53] and one study [55] did not detect any superoxide in one B cell line. However, ROS may be generated by other cells adjacent to B cells. Phagocytic cells (including neutrophils, monocytes and macrophages) release ROS when activated and co-localize with B cells in vivo [55]. B cells synthesize increased levels of 5-LO products when co-incubated with isolated granulocytes stimulated with PMA to synthesize and release ROS. On the other hand, GPx and selenium, and catalase, which neutralizes H_2O_2, all inhibit H_2O_2-induced 5-LO activity in these cells [55].

The role of B cell-derived 5-LO products is not entirely clear, although the tight regulation of their formation would suggest that they may serve an important function. B cells express the BLT1 receptor and respond to LTB_4 with increased expression of the activation marker CD23 and increased immunoglobulin production [53].

1.2.4 Pathophysiological role of the 5-LO pathway

Many pharmacological (small molecule) inhibitors have been synthesized which have facilitated studies on the physiological role of 5-LO products. MK886 and MK591 (synthesized by Merck and Co.) both inhibit FLAP, whereas Zileuton (synthesized by Abbott Laboratories) inhibits 5-LO directly [57].

Additionally, genetic studies and studies with knockout mice or transgenic mice for the 5-LO pathway enzymes have revealed roles for 5-LO in pathological conditions including atherosclerosis [58,59], the pathogenesis of pulmonary fibrosis [60], the infiltration of neutrophils during acute lung inflammation [61], and myocardial infarction and stroke [62].

1.2.5 Inflammation, 5-LO and cancer

Cancer cells have been shown to induce immune responses and to be linked with inflammation. An understanding of the role of the immune response in cancer would be essential for developing appropriate therapeutic strategies to combat tumor growth or metastasis. Many tumors are caused by viruses or genetic mutations and the immune system responds to viral antigens specific to the tumor cells [63]. However, an understanding of the role of the immune system is hindered since there are many differences between types of cancers. For example, many tumors elicit only minor immune responses, whereas others can suppress an immune response to varying degrees. In addition, the nature of leukocyte infiltrates also varies greatly between types and sizes of tumors [64,65].

Many cancers originate at sites of chronic inflammation or infection [66]. The continual presence of activated inflammatory cells may cause tissue damage and/or DNA damage leading to cancer [66]. Compared to normal cells, tumor cells differentially produce inflammatory mediators activating a strong inflammatory response and recruiting inflammatory cells to the site of the tumor. The tumor microenvironment therefore comprises resident inflammatory cells as well as the growth factors, lipid mediators and other cytokines released by these cells and the tumor cells. Eosinophils and macrophages are commonly present in the cellular infiltrates of tumors of colon and breast [66,67]. As a defence against parasites, eosinophils release the contents of their granules, but their role in host defence against tumors is not clear. Different studies have shown that eosinophils can either function to suppress tumor cell growth [68] or to promote tumor cell proliferation [69]. Some studies have also suggested a positive prognosis of patient survival associated with tumor associated eosinophilia [70-72], but these

are not conclusive. Cormier and colleagues demonstrated that the recruitment of eosinophils into tumors was an early inflammatory response independent of Th2 immune responses [67]. The authors also showed that eosinophils accumulate in tumors throughout their growth, and suggested that the resident tumor cells release eosinophil chemokines thereby promoting cellular infiltration.

Epidemiological studies suggest that a diet high in fat may increase the risk for many types of cancers, including prostate cancer [73]. Studies have revealed that 12-LO and 12-HETE play a role in cancer metastasis [74,75], whereas COX and 5-LO products play a role in the growth of certain cancers [76]. Blocking these pathways with specific inhibitors have been shown to induce cancer cell death, and their efficacy depends on the type of cancer [76]. Specific inhibitors of COX, 12-LO and cytochrome P450 do not block AA-induced prostate cancer cell growth, whereas it is blocked by the general lipoxygenase inhibitor NDGA [77]. Several studies have looked at the role of 5-LO in prostate cancer growth. Normal epithelial cells do not express significant amounts of 5-LO or FLAP, but elevated protein and mRNA levels have been detected in several cancer cell lines. Gupta and colleagues carried out an epidemiological study looking at the expression of 5-LO in patients with prostate cancer [18]. The results revealed that malignant tissue expressed 6-fold and 2.6-fold more 5-LO mRNA and protein, respectively, compared to benign tissue from the same patient [18]. Studies looking at the role of 5-LO in cancer have revealed that 5-LO pathway inhibitors (MK886 and NDGA) induce apoptosis of several types of cancer cells including breast [78,79], prostate [77,80], lung [81], and pancreatic cancer [82]. Several studies have also linked the growth stimulation and survival effects of various factors to their activation of 5-LO. For example, 5-LO was found to mediate the stimulatory effect of insulin–like growth factor 1 (IGF-1) on breast cancer cell growth and survival [78]. In contrast, the 5-LO pathway inhibitors NDGA and MK886 were shown to block IGF-1-induced responses while exogenous 5-HETE and CysLTs enhanced the proliferation of the cancer cells. Furthermore, IGF-1 stimulates a rapid 15-fold increase in 5-HETE levels in breast cancer cells, suggesting a role for the latter compound [78]. Other studies

showed that components of the extracellular matrix (ECM), which interact with cell surface receptors, can also stimulate intracellular responses [83], and that the ECM can promote the survival of pancreatic cancer cells in vitro [82]. This response was found to be mediated by the activation of NADPH oxidase via 5-LO and the subsequent production of ROS. Blocking 5-LO or NADPH oxidase induced apoptosis of these cells [82]. Additionally, several clinical and epidemiological studies have associated selenium, which inhibits 5-LO (as mentioned in Section 1.2.1, pg 6), with a reduced risk of prostate cancer and other types of cancers [84-86]. In vitro, selenium was found to induce apoptosis of prostate cancer cells but not normal prostate epithelial cells [87].

Although many studies have supported a relationship between 5-LO expression and cancer cell growth, there is conflicting evidence for the role of 5-LO as well as other lipoxygenases in apoptosis, since their inhibitors induce apoptosis in some cells and inhibit it in others [88]. Specifically, in the case of the FLAP inhibitor, MK886, the concentration needed to induce apoptosis (5-10 μM) is approximately 100-times greater than the concentration required to inhibit FLAP/5-LO activity in neutrophils (approx. 3 nM) [77,80,89]. Therefore, at these higher concentrations, MK886 may be acting independently of FLAP. Datta and colleagues have shown that MK886 induces apoptosis in cells that do not express FLAP [89]. In addition the more specific and potent FLAP inhibitor, MK591 (an MK886 structural analog), is only 50% as effective as MK886 in inducing apoptosis in FL5.12 cells, an IL-3- dependent mouse prolymphoid progenitor cell line. In fact, MK886 may be inducing cell death by stimulating oxidative stress in the cancer cells. MK886 was shown to induce a 4-fold increase in oxidation after one hour, while N-acetylcysteine (NAC), an antioxidant, blocked MK886-induced apoptosis in the FL5.12 cells. Studies have shown that both MK886, and NDGA induce apoptosis of murine FL5.12 cells independently of 5-LO activity [90], while another study showed that NDGA and another 5-LO inhibitor, caffeic acid, do not induce apoptosis of rat monocytic cells [91,92]. The effects of these lipoxygenase inhibitors on apoptosis may be cell and/or species specific. Alternatively, FLAP, which binds fatty acids, and therefore acts similarly to other

FABPs, may affect fatty acid induced signal transduction pathways that regulate cell growth and differentiation [93]. Blocking FLAP and therefore these signal transduction pathways may induce apoptosis. In addition, FLAP may have other functions unrelated to leukotriene synthesis. For example, FLAP and 5-LO are not always expressed in the same cells [45,46,94,92], and transfection of the human colonic adenocarcinoma cell line, HT29, with FLAP induced an increase in COX activity and expression, but not 5-LO product synthesis [95]. Therefore, FLAP may act independently of 5-LO or FLAP inhibitors such as MK886 may be acting independently of FLAP to induce apoptosis. Although the mechanism for the effect of 5-LO inhibitors on cancer cell apoptosis has not been elucidated, several studies have shown the significance of 5-LO metabolites for promoting cancer cell growth. In addition, since several distinct inhibitors, targeting either 5-LO or FLAP, induce apoptosis of PC3 cells, this suggests that this effect is mediated by 5-LO.

1.3 5-Oxo-ETE

5-Oxo-ETE is synthesized from 5-HETE by the enzyme 5-hydroxyeicosanoid dehydrogenase (5-HEDH), and has greater biological activity than its precursor. Both 5-oxo-ETE and 5-HETE activate the same GPCR, but the receptor has about 100 times higher affinity for 5-oxo-ETE. Synthesized by numerous types of human cells, 5-oxo-ETE induces biological responses in a variety of inflammatory cells as well as some structural cells. 5-Oxo-ETE may be an important proinflammatory mediator, and ongoing studies are trying to elucidate its pathophysiological role. In addition to human cells, 5-oxo-ETE has also been found to be produced by bovine neutrophils [96], mouse macrophages [97] and rat brain [12].

1.3.1 5-HEDH

5-HEDH was originally identified by Powell and colleagues as a novel enzyme that synthesizes 5-oxo-ETE when they were conducting studies on the metabolism of LTB_4 in human neutrophils [98]. In neutrophils, LTB_4 is rapidly

metabolized by 20-hydroxylase (a cytochrome P-450 enzyme) to 20-hydroxy-LTB_4 and subsequently to ω-carboxy-LTB_4. The biologically inactive isomers of LTB_4, 6-trans-LTB_4 and 12-epi-6-trans-LTB_4, also undergo ω-oxidation, but they were also found to be converted to 6,7-dihydro metabolites via a 5-oxo intermediate, 5-oxo-6,7-dihydro-LTB_4 [99,100]. Since the 6-trans-LTB_4 isomers are synthesized non-enzymatically from LTA_4 and do not have biological activity, further studies were carried out by Powell and colleagues to determine whether other eicosanoids were more efficiently oxidized. They determined that 5(S)-HETE which is converted to 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE), is the preferred substrate for the enzyme, 5(S)-hydroxyeicosanoid dehydrogenase (5-HEDH) [98].

1.3.1.1 Properties of 5-HEDH

5-HEDH is present in the microsomal fraction (Figure 1.2) of human cells and requires $NADP^+$ as a cofactor. It is highly specific for eicosanoids containing a 5-hydroxyl group in the S configuration, followed by a 6-trans double bond with 5(S)-HETE being its preferred substrate [98]. The enzyme has not been purified or identified and therefore only the properties of the crude membrane-bound enzyme are known. Studies were carried out with cell lines to determine enzyme properties. The U937 and HL-60 monocytic cell lines, have similar 5-HEDH activity to isolated human monocytes and neutrophils [101]. Differentiation of U937 cells toward macrophages by PMA resulted in an over 3-fold increase in the V_{max} for 5-HEDH activity after 4 days, while the K_m remained unchanged. Differentiation of HL-60 cells to macrophage-like cells by 1,25-dihydroxy-vitamin D_3 (dh-VitD_3) resulted in a 2-fold increase in 5-HEDH activity after 3 days, whereas PMA induced only a slight increase.

5-HEDH is highly selective for $NADP^+$, which is 10,000 times more potent than NAD^+ in promoting 5-oxo-ETE synthesis [101]. The reaction occurs via a ping-pong reaction mechanism, where either the cofactor or the substrate binds to the enzyme, and then leaves, followed by binding of the other. It would seem likely that $NADP^+$ binds first, and is then released after oxidizing the

enzyme. Subsequently, 5-HETE could bind and would be oxidized by the oxidized enzyme. The K_m and V_{max} for the synthesis of 5-oxo-ETE from 5-HETE are respectively, 2-fold and 8-fold greater, than the reverse reaction (5-oxo-ETE → 5-HETE). pH affects the forward and reverse reactions differently, with the forward reaction 2-fold greater at physiological pH, 7.4. The reverse, reduction reaction is favoured at higher pH. The levels of NADPH in unstimulated U937 cells are 12-fold higher than $NADP^+$ and NADPH competitively inhibits 5-oxo-ETE formation by 5-HEDH in these cells [101]. Consequently, the ratio of $NADP^+$ to NADPH rather than their specific concentrations is important in regulating the rate of 5-oxo-ETE synthesis.

1.3.1.2 5-HEDH substrate specificity

As previously mentioned, 5-HEDH is highly specific for eicosanoids containing a 5-hydroxyl group in the S configuration, followed by a 6-trans double bond [98,102]. 5(R)-HETE, a closely related compound to 5(S)-HETE, and LTB_4, which has a 6-*cis* double bond, are not metabolized by this enzyme. In addition, regioisomers of 5-HETE, including 8-HETE, 9-HETE, 11-HETE, 12-HETE and 15-HETE are not oxidized. However, other eicosanoids that meet the positional and stereochemical requirements of 5-HEDH are also substrates, including 6-trans-LTB_4, 12-epi-6-trans-LTB_4, and 5(S),15(S)-diHETE [98]. A recent study by our group showed that the 18-carbon compound sebaleic acid (5,8-octadecadienoic acid) can be converted to 5-HODE (5(S)-hydroxy-6E,8Z-octadecadienoic acid) by human neutrophils and the latter substance can be oxidized to 5-oxo-ODE (5-oxo-6E,8Z-octadecadienoic acid) by human neutrophils and keratinocytes [103]. We assume that 5-HEDH is the enzyme responsible for the formation of 5-oxo-ODE, since the substrate 5-HODE has a 5(S)-hydroxyl group and 6-*trans* double bond and the enzyme activity is found in the microsomal fraction and requires $NADP^+$ as a cofactor [103].

1.3.2 Regulation of 5-oxo-ETE synthesis by 5-HEDH

5-HEDH is present in numerous types of human inflammatory cells including eosinophils [104], dendritic cells [105], neutrophils [98,106,107], platelets [108], and monocytes [109,110]. It is also found in structural cells, including aortic endothelial cells [111], airway epithelial cells, airway smooth muscle cells [112], and keratinocytes [103]. In all of these cell types, the synthesis of 5-oxo-ETE is limited by the availability of $NADP^+$.

1.3.2.1 Respiratory burst stimulates 5-Oxo-ETE synthesis

Phagocytic cells express NADPH oxidase-2 (NOX-2), which is inactive in resting cells. NOX comprises six subunits that assemble when activated and catalyzes the reduction of molecular oxygen to superoxide as a defence mechanism against bacteria [113]. The large amount of superoxide that is formed by this reaction, known as the respiratory burst, can be converted to H_2O_2 either spontaneously or enzymatically by superoxide dismutase (SOD) [114]. Phorbol myristate acetate (PMA) activates protein kinase C (PKC), which phosphorylates the $p47_{phox}$ subunit of NOX. Then, phosphorylated $p47_{phox}$ along with $p67_{phox}$ translocates from the cytosol to the membrane, where it interacts with the NOX complex [115].

PMA stimulates the synthesis of 5-oxo-ETE in neutrophils [116, 117], eosinophils [104], dendritic cells [105], monocytes [109], and unfractionated lymphocytes [109]. Although neutrophil microsomes contain high levels of 5-HEDH activity, intact unstimulated neutrophils do not synthesize appreciable amounts of 5-oxo-ETE, but rather convert 5(S)-HETE primarily to 5(S),20-diHETE by ω-oxidation. However, PMA stimulates the production of 5-oxo-ETE from 5-HETE by 6-fold, while inhibiting the formation of 5,20-diHETE [116]. PMA does not directly act on 5-HEDH, since it has no effect on 5-oxo-ETE synthesis by neutrophil microsomes [117]. Inhibitors of PKC and NOX, staurosporine, and diphenylene iodonium (DPI), respectively, both block PMA-stimulated 5-oxo-ETE production suggesting that its actions are mediated by the generation of $NADP^+$ by NOX-2 [116,117]. Consistent with this, the actions of PMA can be

mimicked by phenazine methosulfate (PMS), which induces the nonenzymatic conversion of NADPH to $NADP^+$ (Figure 1.3). PMA also stimulates 5-oxo-ETE production from neutrophils incubated with exogenous AA, A23187, and serum-treated zymosan. In unstimulated cells, the major products from exogenous AA are 15-HETE and 5-HETE, with minimal amounts of 5-oxo-ETE and LTB_4. PMA stimulates LTB_4 and 5-oxo-ETE synthesis by 4-fold, whereas the levels of 5-HETE and 15-HETE do not increase as much [116]. The relative amounts of the products formed from neutrophils incubated with AA and PMA, are 5-HETE > 5-oxo-ETE > LTB_4. Stimulation of neutrophils with A23187 alone leads to the formation of LTB_4, 5-HETE and 5-oxo-ETE from endogenous AA, and this is increased in the presence of PMA. Although more LTB_4 than 5-oxo-ETE is initially formed, 5-oxo-ETE is metabolized more slowly so that it predominates at longer time points.

Dendritic cells also express NADPH oxidase and generate superoxide when stimulated with PMA [118]. Dendritic cells, differentiated from peripheral blood monocytes by treatment with IL-4 and GM-CSF for 4 days, followed by treatment with lipopolysaccharide (LPS) for 24 h, were found to synthesize significant amounts of 5-oxo-ETE (15-fold more than neutrophils in the absence of any stimulus) from exogenous 5-HETE [105]. PMA and A23187 both stimulated the synthesis of 5-oxo-ETE from endogenous 5-HETE in dendritic cells, while other agonists, including C5a, PAF, fMLP, GM-CSF, RANTES, and IL-5 did not.

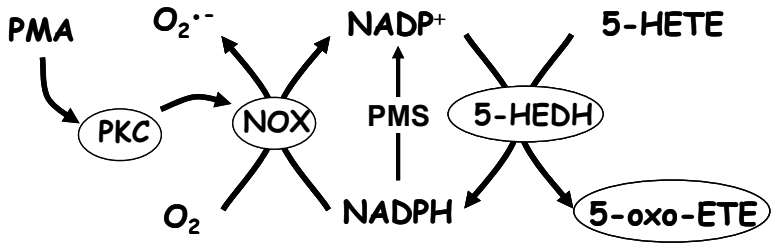


Figure 1.3: Respiratory burst promotes 5-oxo-ETE production. PMA activates NOX, via PKC, stimulating 5-oxo-ETE synthesis by elevating $NADP^+$ levels. PMS induces the same effect by directly oxidizing NADPH to $NADP^+$.

1.3.2.2 Oxidative stress stimulates 5-oxo-ETE synthesis

Cells maintain very high levels of both GSH and NADPH under normal conditions as a protective, antioxidant, mechanism. However, exposure to high levels of ROS induces rapid increases in GSSG and $NADP^+$ levels via the glutathione redox pathway (described in section 1.1.2). As previously mentioned (Section 1.3.1.1) NADPH inhibits the production of 5-oxo-ETE by 5-HEDH [101], and 5-oxo-ETE synthesis is low in resting cells but increases when the redox state of the cell is altered to increase the ratio of $NADP^+$ to NADPH. Promoting oxidative stress, by stimulating U937 cells with t-butyl hydroperoxide (tBuOOH), increases the ratio of $NADP^+$ to NADPH from 0.082 to 2.94 (a 36-fold increase) and induces a 6-fold increase in 5-oxo-ETE synthesis [101].

Similar to their effects on monocytes, both H_2O_2 and tBuOOH, significantly stimulate 5-oxo-ETE production by approximately 5-fold in human aortic endothelial cells (HAEC) [111] in conjunction with rapid increases in $NADP^+$ and GSSG levels. Diamide, which non-enzymatically converts GSH to GSSG, has a similar effect as H_2O_2, stimulating 5-oxo-ETE synthesis [111,112], further supporting the role of the glutathione redox cycle in H_2O_2-induced 5-oxo-ETE synthesis. Inhibitors of the glutathione redox cycle, BCNU, which inhibits glutathione reductase (GR), and NEM which alkylates sulfhydryl groups, block the effect of H_2O_2 [111,112].

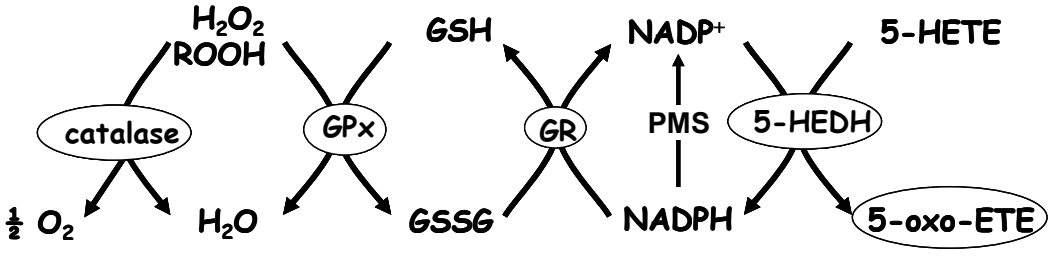


Figure 1.4: H_2O_2 stimulates 5-oxo-ETE productions via the glutathione redox pathway. H_2O_2 is neutralized by GPx while oxidizing GSH to GSSG, which is then reduced again by GR. NADPH is converted to $NADP^+$ by GR providing the cofactor for 5-oxo-ETE production by 5-HEDH.

1.3.2.3 The pentose phosphate pathway regulates 5-oxo-ETE synthesis

The initial step of the pentose phosphate pathway is oxidation of glucose 6-phosphate by glucose-6-phosphate dehydrogenase. This enzyme requires $NADP^+$ as a cofactor, and NADPH is generated during the reaction [119]. The dehydrogenase can be inhibited by dehydroepiandrosterone (DHEA), thereby blocking the conversion of $NADP^+$ to NADPH. Consequently, DHEA augments the stimulatory effect of H_2O_2 on $NADP^+$ and GSSG levels in HAEC cells thereby increasing 5-oxo-ETE production [111]. This provides further support that the intracellular levels of NADPH and $NADP^+$ regulate 5-HEDH activity.

1.3.3 Alternate pathways for 5-oxo-ETE production

5-Oxo-ETE may also be synthesized from substrates other than 5-HETE during nonenzymatic lipid peroxidation. Polyunsaturated fatty acids may be auto-oxidized under conditions of oxidative stress to hydroperoxides [120], which can subsequently be reduced to their corresponding alcohols (hydroxy) or dehydrated to ketones (oxo), especially in the presence of heme-containing compounds such as haemoglobin [121]. In this way, 5-HpETE, can be nonenzymatically converted directly to 5-oxo-ETE [122,12]. Murine macrophages synthesize 5-HETE and 5-oxo-ETE from endogenous AA when stimulated with calcium ionophore, A23187. However, 5-oxo-ETE is probably formed non-enzymatically from 5-HpETE in this case, since these cells do not convert 5-HETE to 5-oxo-ETE and therefore probably do not express 5-HEDH [97]. Further evidence for a different mechanism for 5-oxo-ETE synthesis in these cells was that the conversion of 5-HpETE to 5-oxo-ETE occurred in the cytosolic fraction rather than in the presence of microsomes. In addition, 5-oxo-ETE synthesis was independent of pyridine nucleotides, and the reaction was not sterospecific (both 5(R)- and 5(S)-HpETE were substrates). The proposed mechanism for the conversion of 5-HpETE to 5-oxo-ETE was via catalysis by an iron containing factor, such as haemoglobin, with the initial reduction to the 5-alkoxy radical followed by the removal of the C-5 hydrogen [122]. Recently, Farias and colleagues identified elevated levels (18-fold, 48-fold, and 10-fold, respectively) of unesterified AA, 5-

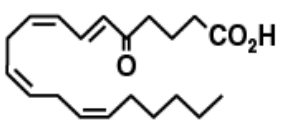
oxo-ETE and 5-HETE in rat brains after cerebral ischemia compared to controls [12]. The stimulation of 5-oxo-ETE production may be due to the formation of ROS which occurs during brain ischemia [123], but it is not clear in this case whether 5-oxo-ETE was formed nonenzymatically or via 5-HEDH.

1.3.4 Isomers of 5-oxo-ETE

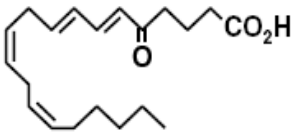
5-Oxo-7E,9E,11Z,14Z-eicosatetraenoic acid (also named 5-oxo-ETE in the literature, but in this thesis is referred to as 5-oxo-7,9,11,14-ETE), is formed by non-enzymatic hydrolysis of LTA_4, and is an isomer of 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid formed from 5-HETE by 5-HEDH [124,125]. The isomers differ in the location and configuration of 2 of their double-bonds (Figure 1.5). LTA_4, an allylic epoxide is converted enzymatically and non-enzymatically to several products depending on the cell type. 5-Oxo-7,9,11,14-ETE is produced by neutrophils incubated with calcium ionophore and exogenous arachidonic acid, but not with ionophore alone. Under physiological conditions, 6-trans-LTB_4 and 12-epi-6-trans-LTB_4 are the major nonenzymatic LTA_4 hydrolysis products (72%) whereas 5,6-diHETE and 5-oxo-7,9,11,14-ETE represent approximately 14% each. However, increasing the pH to 9.5 favours the formation of 5-oxo-7,9,11,14-ETE [125]. Blocking the 5-LO pathway with the FLAP inhibitor, MK-886 inhibits 5-oxo-7,9,11,14-ETE synthesis, indicating that it is formed by the hydrolysis of 5-LO-synthesized LTA_4. This isomer has some biological activity and inhibits 5-LO activity with an IC_{50} of 1.5 μM compared to 5-oxo-ETE (IC_{50} >10 μM) which cannot effectively inhibit 5-LO activity [124]. 5-Oxo-7,9,11,14-ETE also induces calcium mobilization in neutrophils by activating the LTB_4 receptor, BLT1, as this response can be blocked by LTB_4–induced desensitization and by the LTB_4 receptor antagonist, LY223982. However, it is much less potent than 5-oxo-ETE in eliciting this response. Further studies on this compound have not been reported.

Another isomer, 8-*trans*-5-oxo-ETE (5-oxo-6E,8E,11Z,14Z-eicosatetraenoic acid) (Figure 1.5), is a degradation product of 5-oxo-ETE [126]. Although 8-*trans*-5-oxo-ETE can activate the 5-oxo-ETE receptor, it is less potent

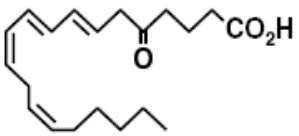
than 5-oxo-ETE in inducing calcium mobilization (6-fold) and cell migration (3-fold) in neutrophils.



5-oxo-6E,8Z,11Z,14Z-ETE (5-oxo-ETE)



5-oxo-6E,8E,11Z,14Z-ETE (8-trans-5-oxo-ETE)



5-oxo-7E,9E,11Z,14Z-ETE

Figure 1.5: Isomers of 5-oxo-ETE. 5-oxo-ETE is synthesized by 5-HEDH from 5-HETE and activates OXE-R. 8-trans-6E,8E,11Z,14Z-eicosatetraenoic acid is a degradation product of 5-oxo-ETE and weakly activates OXE-R. 5-oxo-7E,9E,11Z,14Z-eicosatetraenoic acid is formed by non-enzymatic hydrolysis of LTA_4 in neutrophils and weakly activates BLT1.

1.3.5 Metabolism of 5-oxo-ETE

Eicosanoids are metabolized via many enzymatic pathways and several have been discovered for 5-oxo-ETE in both human and murine cells. Some of these pathways are cell specific and involve cytochrome P450 enzymes, other lipoxygenases, as well as some other enzymes (Figure 1.6). Similar to other eicosanoids, conversion of 5-oxo-ETE to its metabolites via these pathways generally results in biological inactivation.

1.3.5.1 Reduction to 5-HETE

The oxidation of 5-HETE to 5-oxo-ETE is reversible and 5-oxo-ETE can be converted to 5(S)-HETE in the presence of NAPDH by neutrophil microsomes [98]. The reaction is highly stereospecific as no 5(R)-HETE is synthesized and is probably catalyzed by 5-HEDH. The enzymatic conversion of 5-HETE to 5-oxo-ETE at neutral pH is favored by an 8-fold higher V_{max}, although the K_m for the reduction reaction is about one-half that for the oxidation reaction [101]. Lowering the pH (maximal pH 6) favors the conversion of 5-oxo-ETE to 5-HETE

by monocytic cell microsomes, whereas raising the pH has the opposite effect [101]. Unstimulated platelets also convert 5-oxo-ETE to 5-HETE [108].

1.3.5.2 ω-Oxidation by cytochrome P450 enzymes

The CYP4A and CYP4F families of cytochrome P450 catalyze the hydroxylation of AA and its metabolites at the 20-carbon position (also called ω-hydroxylation) [127]. These metabolites usually have lower biological activity than their precursors, so these pathways can serve to limit the biological activities of lipid mediators. 5-Oxo-ETE is metabolized in human neutrophils by ω-oxidation to 5-oxo-20-hydroxyeicosatetraenoic acid (5-oxo-20-HETE) resulting in 99% loss of biological activity [116,126,128]. The cytochrome P450 enzyme, LTB_4 20-hydroxylase (CYP4F3), which is specifically expressed in neutrophils [129, 127], is thought to catalyze this reaction [126]. CYP4F3 also metabolizes 5-HETE to 5,20-diHETE, which is the main 5-HETE metabolite in unstimulated neutrophils [116,130], in contrast to PMA-stimulated neutrophils which convert 5-HETE principally to 5-oxo-ETE. However, 5-HETE and 5-oxo-ETE are not as good substrates as LTB_4 for CYP4F3 [116].

Murine macrophages metabolize 5-oxo-ETE to several 18-hydroxy and 19-hydroxy compounds through a series of oxidation and reduction reactions [97]. 20-Hydroxy metabolites of 5-oxo-ETE are not formed in these cells. Although 5-oxo-18-HETE and 5-oxo-19-HETE were not identified in the study, it is thought that 5-oxo-ETE initially undergoes direct ω-1 and ω-2 oxidation to form these metabolites. Subsequent reduction of the keto group by 5-ketoreductase produces 5,19-diHETE and 5,18-diHETE. Alternatively, the Δ^6-double bond of 5-oxo-ETE can be reduced by 5-oxo-eicosanoid Δ^6-reductase, followed by ω-1 and ω-2 oxidation to give 5-oxo-18-HETrE and 5-oxo-19-HETrE (Figure 1.6) [97]. 5-Oxo-18-HETrE was further metabolized to produce 5,18-diHETrE by a 5-ketoreductase. The specific enzymes involved in the metabolism of 5-oxo-ETE in murine macrophages are not precisely known. The murine 5-ketoreductase could not be identical to 5-HEDH because these murine cells do not express this enzyme [97]. These metabolites do not have any biological activity (intracellular calcium

release), and therefore the metabolism of 5-oxo-ETE by these pathways also results in its inactivation. These metabolic pathways are similar to those observed in rat neutrophils which convert LTB_4 to 18- and 19- but not 20-hydroxy metabolites [131].

1.3.5.3 Esterification into cellular lipids

5-HETE and other eicosanoids are incorporated mainly into triglycerides and to a lesser extent into phospholipids in neutrophils and other cells [132]. O'Flaherty and colleagues determined that 5-oxo-ETE must first be reduced to 5-HETE before it is esterified into triglycerides by intact neutrophils. Only 5-HETE was recovered in cell extracts treated with the enzyme triacylglycerol lipase, which releases fatty acids [128]. However, in isolated neutrophil membrane fractions, 5-oxo-ETE was directly esterified into triglycerides, since 5-oxo-ETE could subsequently be recovered from these membranes following treatment with triacylglycerol lipase. In addition, triacsin C, an inhibitor of acyl CoA synthetase, blocked the incorporation of 5-HETE and 5-oxo-ETE into triglycerides [128].

1.3.5.4 Reduction by an olefin reductase

Cytosolic eicosanoid Δ^6-reductase, present in neutrophils, catalyzes the reduction of the 6,7-double bond of 5-oxo-ETE, converting it to its 6,7-dihydro metabolite, 5-oxo-ETrE (5-oxo-8Z,11Z,14Z eicosatrienoic acid) [100]. Metabolism of 5-oxo-ETE by this enzyme results in loss of biological activity since 5-oxo-ETrE is approximately 1000-fold less potent at inducing calcium mobilization in neutrophils. The Δ^6-reductase requires calcium and calmodulin and uses NADPH as a cofactor. This enzyme is distinct from the cytosolic NADPH-dependent PG Δ^{13}-reductase, which reduces 15-oxo-metabolites of prostaglandins to 13,14-dihydro-15-oxo-prostaglandins and does not require calcium or calmodulin. In addition, the Δ^6 and Δ^{13}-reductase activities could be separated by ion exchange chromatography [100]. Other 5-oxo compounds, including 5-oxo-15-HETE and 5-oxo-6-*trans*-isomers of LTB_4 can also be reduced to their corresponding dihydro metabolites by this Δ^6-reductase.

1.3.5.5 Metabolism by 12-lipoxygenase

Human platelets have high levels of microsomal 5-HEDH activity, and convert 5-HETE to 5-oxo-ETE [108]. However, platelets do not express 5-LO, and therefore, cannot synthesize 5-HETE or 5-oxo-ETE from AA. Neighbouring inflammatory cells, such as neutrophils or eosinophils could provide 5-HETE to platelets. Platelets co-incubated with neutrophils that are stimulated with PMA and A23187 would be expected to synthesize more 5-oxo-ETE than neutrophils alone. However, the opposite is true, and studies have shown that this is due to the further metabolism of 5-oxo-ETE to 5-oxo-12-HETE by 12-LO [108], which is highly expressed in platelets and is activated following cell stimulation [133]. Thus, platelets stimulated with A23187 or thrombin convert 5-oxo-ETE to 5-oxo-12-HETE, whereas unstimulated platelets convert 5-oxo-ETE mainly to 5-HETE probably due to the action of 5-HEDH. 5-Oxo-12-HETE does not induce calcium mobilization in neutrophils, but instead blocks the response to 5-oxo-ETE and thus has antagonist-like properties [108].

1.3.5.6 Metabolism by 15-lipoxygenase

Eosinophils express high levels of 15-lipoxygenase, and convert 5-oxo-ETE to 5-oxo-15-HETE [134]. 5-Oxo-15-HETE may also be synthesized from 5(S),15(S)-diHETE by 5-HEDH in neutrophils [98]. Soybean 15-LO converts AA to 5-oxo-15-HETE along with the major product of 15-HETE and several other products [134].

1.3.5.7 Glutathionylation by LTC_4 synthase

Murine peritoneal macrophages and human platelets convert 5-oxo-ETE to a glutathione adduct, FOG_7 (5-oxo-7-glutathionyl-8,11,14-eicosatrienoic acid) [135,136]. Abundant in these cells, LTC_4 synthase catalyzes the 1,4-Michael addition of GSH to the 7-position of 5-oxo-ETE to form FOG_7. This reaction is inhibited by the FLAP antagonist MK-886, which also inhibits LTC_4 synthase. Like 5-oxo-ETE, FOG_7 induces actin polymerization and is a chemoattractant for human eosinophils and neutrophils. However, in contrast to 5-oxo-ETE, FOG_7

does not stimulate an increase in intracellular calcium levels, and seems to act via a distinct receptor [135]. Distinct 5-oxo-ETE glutathione conjugates can be formed by human placental and rat liver glutathione S-transferases. Although these conjugates have identical mass spectra and co-chromatograph with FOG_7, they do not induce actin polymerization in neutrophils and are presumably isomers of FOG_7 [136].

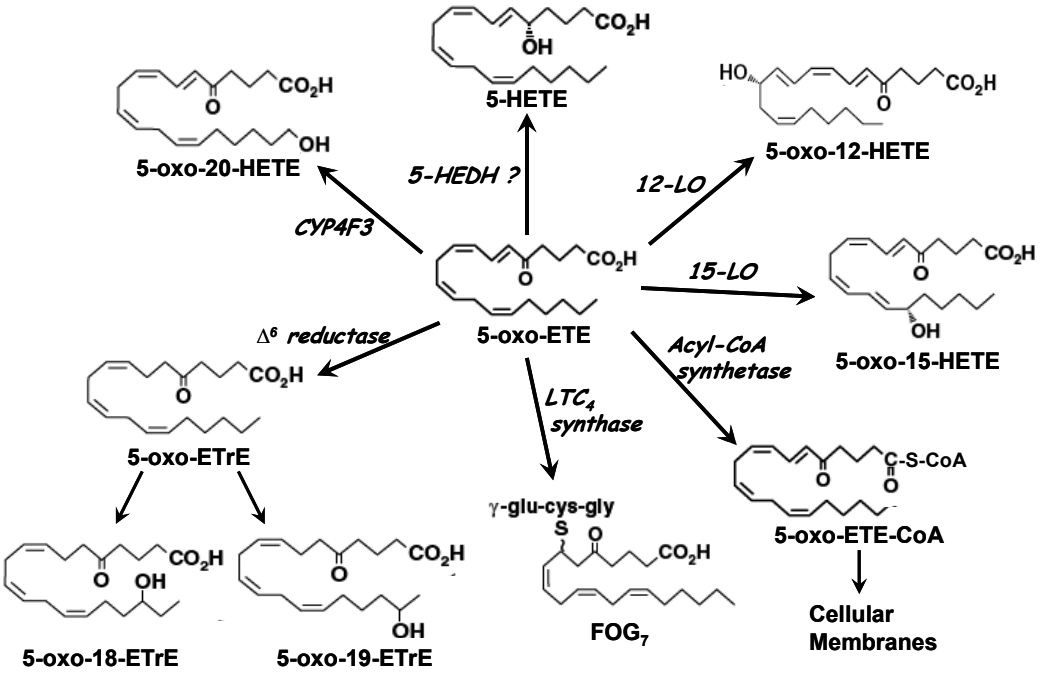


Figure 1.6: Metabolism of 5-oxo-ETE. 5-oxo-ETE can be metabolized by several enzymes, often leading to its biological inactivation.

1.4 Biological role of 5-oxo-ETE

Like most other biologically active eicosanoids and chemoattractants 5-oxo-ETE acts through a specific G-protein coupled receptor (GPCR) and stimulates downstream signaling and cellular responses in several different types of cells. 5-Oxo-ETE has much higher biological activity than its metabolic precursor, 5-HETE and is a potent chemoattractant with relative activity for eosinophils > neutrophils > basophils > monocytes.

1.4.1 The 5-oxo-ETE receptor

5-HEDH has high specificity for 5-HETE, and 5-oxo-ETE has much higher biological activity than its metabolic precursor. Previous studies have shown that 5-HETE induces biological responses including degranulation of neutrophils and calcium release from intracellular stores, but it had very weak activity (470-fold weaker than LTB_4) [137]. It was found to activate a receptor different from the receptors for PAF or LTB_4, since these responses were only subject to homologous desensitization, and were not affected by pre-treatment with PAF or LTB_4 [137]. However, the receptor was not known until studies identified the receptor for 5-oxo-ETE.

Initial studies on the biological responses to 5-oxo-ETE provided evidence that it acts via a selective GPCR. In neutrophils, eosinophils and monocytes, responses to 5-oxo-ETE including actin polymerization [138-140], calcium mobilization [141], chemotaxis [142] and degranulation [143] are subject to homologous desensitization by pre-treatment with 5-oxo-ETE, but not to heterologous desensitization by other chemoattractants such as LTB_4, PAF [138-143] or C5a [139]. In addition, the LTB_4 receptor antagonist, LY255283, only blocks LTB_4-induced responses and does not affect 5-oxo-ETE induced neutrophil degranulation [143,144], calcium mobilization [141] monocyte migration [142], or eosinophil lung infiltration in rats [145]. These studies revealed that 5-oxo-ETE was not activating the receptor for LTB_4, but was activating a distinct receptor. However, 5-oxo-ETE and 5-HETE metabolism and esterification into phospholipids made binding studies difficult. O'Flaherty used triacsin C to block esterification, and showed that 5-[^{3}H]oxo-ETE bound specifically and reversibly to neutrophil membranes [128]. It could only be displaced by 5-oxo-ETE, 5-HETE or 5,15-diHETE with potencies relative to their ability to induce calcium mobilization [128,146]. In agreement with previous inhibitor studies, LTB_4 was unable to displace bound 5-[^{3}H]oxo-ETE.

1.4.1.1 Identification of the 5-oxo-ETE receptor

The receptor for 5-oxo-ETE is a $G\alpha_{i/o}$-protein coupled receptor which inhibits adenylyl cyclase (blocks cAMP formation) and its activity can be blocked by pertussis toxin. The receptor was independently cloned around the same time by three groups performing *in silico* searches for putative orphan GPCRs for which the ligands are unknown. Hosoi and colleagues cloned the cDNA for the 5-oxo-ETE receptor (initially named TG1019) after performing database searches for consensus sequences for GPCRs with a peptide ligand [147]. Screening a library of natural bioactive compounds and related molecules using a TG1019-Gα-protein fusion system, Hosoi and colleagues determined that several unsaturated fatty acids and eicosanoids activated the binding of GTPγS to TG1019-$G\alpha_i$ [147]. Of the identified ligands, the most potent was 5-oxo-ETE (EC_{50}; 5.7 nM), 12-fold more potent than 5(S)-HpETE. Confirming the findings of O'Flaherty, 5-oxo-ETE did not activate the binding of GTPγS to TG1019-$G\alpha_s$ or TG1019-$G\alpha_q$. The order of the potency for activation of GTPγS binding to TG1019-$G\alpha_i$ was 5-oxo-ETE >> 5(S)-HpETE > AA = 5Z,8Z,11Z-eicosatrienoic acid (Mead acid) = 5(R,S)-HETE = 5(S)-HETrE [147]. Several other eicosanoids tested, including leukotrienes, prostaglandins, 12-HETE and 15-HETE did not activate this receptor.

At the same time, Jones and colleagues cloned the orphan GPCR, named R527 [148]. After screening approximately 2000 ligands, they identified 5-oxo-ETE as the most potent inducer of calcium mobilization in human embryonic kidney (HEK293) cells transfected with R527 [148]. 5(S)-HpETE was 100-fold less potent than 5-oxo-ETE, and 5(S)-HETE was a very weak agonist. R527 had 99.7% sequence identity with TG1019 with a single amino acid change at position 368 from a leucine to a valine. In addition, R527 was shorter by 39 amino acids truncated from the N-terminus of TG1019 (423 amino acids) [147,148]. These differences did not alter biological activity. A third group, Takeda and colleagues, also identified the 5-oxo-ETE receptor, (hGPCR48) while performing a search for GPCRs that did not contain introns [149-151].

The same groups performed tests to determine whether the cloned receptors were responsible for the 5-oxo-ETE-induced cellular responses. 5-oxo-ETE induced calcium mobilization and chemotaxis of Chinese hamster ovary (CHO) cells transfected with the 5-oxo-ETE receptor. Both responses were blocked by pertussis toxin [151] and by treatment with a phospholipase C inhibitor, U73122 [152]. The phosphoinositide-3 kinase (PI3K) inhibitor, LY294002, also blocked chemotaxis induced by 5-oxo-ETE [152]. Early studies using biochemical analysis confirmed the coupling of the 5-oxo-ETE receptor to a $G_{i/o}$-protein. 5-Oxo-ETE inhibited forskolin-stimulated cAMP production in CHO cells transfected with TG1019 [147], R527 [148] or hGPCR48 [151] confirming the coupling of the receptor to $G\alpha_i$. This effect could be reversed by pre-treatment with pertussis toxin in agreement with previous studies in eosinophils [139], neutrophils [140,153,154], monocytes [142] and basophils [155] carried out before the receptor was cloned. Although GPCRs that mediate calcium mobilization are often coupled to pertussis toxin-insensitive $G\alpha_q$ proteins, calcium mobilization has been reported to be mediated by $G\alpha_{i/o}$ for other chemoattractants, such as IL-8 and fMLP in mice [156,157].

The 5-oxo-ETE receptor gene maps to 2p21 [147,148] and the IUPHAR Nomenclature Committee for Leukotriene and Lipoxin Receptors designated the 5-oxo-ETE receptor, the OXE receptor [158]. The OXE receptor (OXE-R) is present in a wide variety of human tissues and is most highly expressed in peripheral leukocytes, lung, kidney, liver and spleen [147,148]. Eosinophils express approximately 200-fold more R527 than lung macrophages with relative expression on eosinophils > neutrophils > macrophages (200:6:1) [148].

1.4.1.2 Downstream signaling of the 5-oxo-ETE receptor

Although different cells may express the same receptors, the activated downstream signaling pathways may differ depending on the type and activation state of the cell. One of the downstream signaling pathways activated by binding of 5-oxo-ETE to the OXE-R appears to be phospholipase C (PLC) since the PLC inhibitor, U73122, concentration dependently blocked 5-oxo-ETE induced

calcium mobilization and chemotaxis in cells stably expressing OXE-R [152]. In contrast, a Rho kinase inhibitor (Y27632), a MEK inhibitor (PD98059), and a phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002) did not affect 5-oxo-ETE induced calcium mobilization. However, in eosinophils obtained from subjects with mild asthma, the MEK inhibitor blocked 5-oxo-ETE induced calcium mobilization [159]. Conversely, the PI3K inhibitor and to a lesser extent, the MEK inhibitor blocked 5-oxo-ETE induced chemotaxis. This indicates that chemotaxis does not depend solely on calcium mobilization [152]. Consistent with the inhibitory effects of PI3K inhibitors, 5-oxo-ETE also activates PI3K in neutrophils as measured by increased phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) levels [153,160], which seems to mediate adhesion. 5-Oxo-ETE also dose dependently induced the rapid phosphorylation (activation) of p42/p44 extracellular signal-regulated kinases (ERK-1/2) in neutrophils [144,154], eosinophils [144,159], PC3 cells [161] and CHO cells transfected with the OXE-R [152]. In addition, 5-oxo-ETE also induces the phosphorylation of Akt [152,161]. Activation of p42/p44 ERK in the CHO transfected cells as well as eosinophils was inhibited by the MEK inhibitor PD98059 [152,159] and only partially inhibited by the PI3K inhibitor LY294002 [152]. Akt activation was inhibited by the PI3K inhibitor only, whereas a Rho kinase inhibitor did not affect the activation of either Akt or p42/p44 ERK [152]. PKCδ and to a lesser extent, PKCζ, have been shown to mediate eosinophil migration and intracellular responses [159]. Inhibition of PKCδ with Rottlerin in eosinophils resulted in decreased 5-oxo-ETE-induced migration, MMP-9 secretion, and CD11b [159]. In contrast, inhibition of PKCζ decreased 5-oxo-ETE induced migration and slightly increased CD11b, but did not have an effect on the other responses. Therefore, the intracellular responses mediating migration via PKCζ is unclear. The role of PKCδ is also unclear since Rottlerin is not a specific PKCδ inhibitor [162]. The above studies indicate that 5-oxo-ETE can activate several downstream signaling pathways that differentially mediate its cellular responses (Figure 1.7).

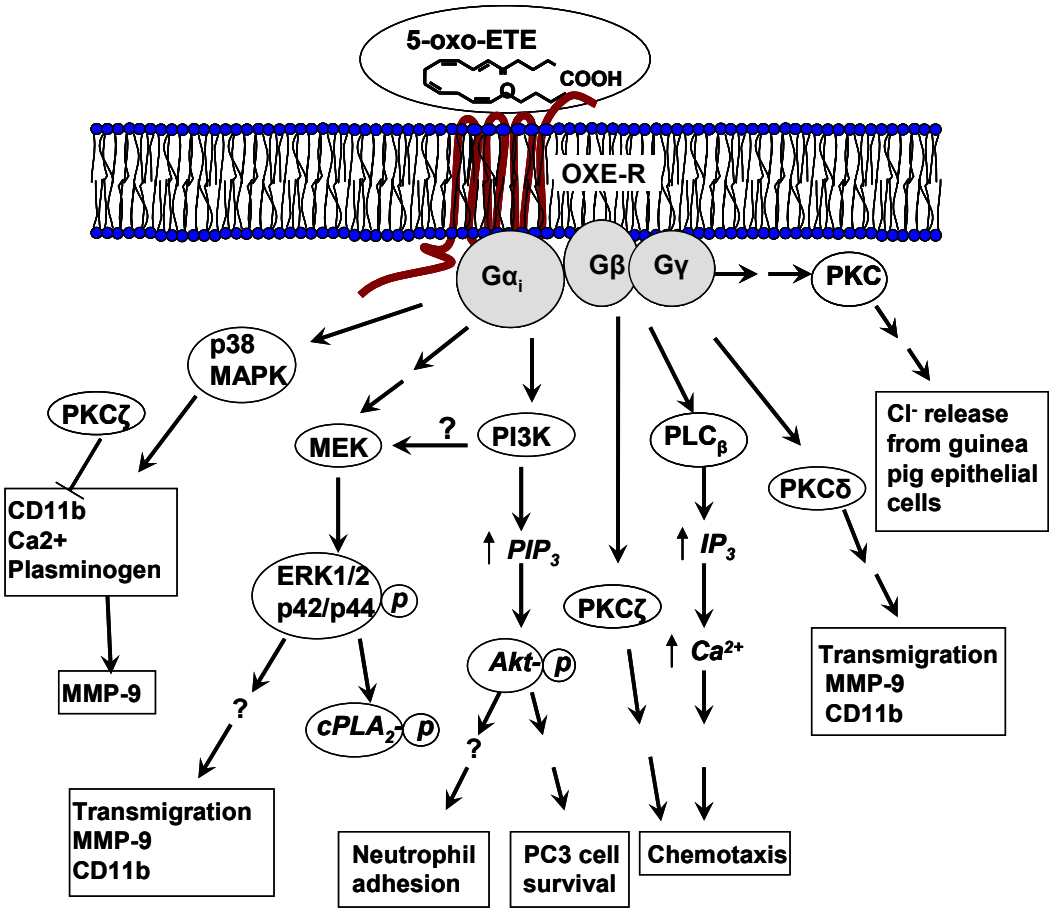


Figure 1.7: Intracellular signaling by OXE-R activated by 5-oxo-ETE. OXE-R is a GPCR, and studies in neutrophils, PC3 cells, and CHO cells transfected with OXE-R have elucidated some of the pathways activated by 5-oxo-ETE.

1.4.1.3 Structural requirements for OXE receptor activation

To gain a better understanding of the role that 5-oxo-ETE plays in inflammatory processes, it is important to know the specificity of the OXE-R. Fatty acids that are structurally similar to 5-oxo-ETE and produced by human cells or synthesized chemically may act either as agonists or antagonists. 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid (DHA), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid (EPA), dihomo-γ-linolenic acid, and 11Z,14Z,17Z-eicosatrienoic acid, were reported to have antagonist-like properties, as they were found to block 5-oxo-ETE induced cell activation [147]. Activation of OXE-R is

highly specific to 5-oxo-ETE as its metabolites and precursors have much lower potencies, as discussed in section 3.5. Furthermore, fatty acids with hydroxyl or oxo groups at positions other than the 5-position, such as 15-HETE, 5,15-diHETE and 15-oxo-ETE do not activate OXE-R [128,141,144,154]. Several studies have shown that the hydrophobic ω-end of 5-oxo-ETE is required for biological activity [126,163], since the ω-oxidation product, 5-oxo-20-HETE, is 100-fold less active [126]. On the other hand, the configuration of the Δ^8-double bond has relatively little effect on activity since 8-*trans*-5-oxo-ETE is only slightly less potent than 5-oxo-ETE (8-*cis*). Methylation of the carboxyl group reduces potency by 20-fold [126].

Members of our group recently investigated the relative abilities of different 5-oxo fatty acids to induce Ca^{2+} mobilization, CD11b expression, migration of neutrophils, and actin polymerization in eosinophils to gain a better understanding of the structural requirements for OXE-R activation, [163]. Dr. Joshua Rokach at the Florida Institute of Technology (F.I.T.) synthesized a series of 5-oxo fatty acids of different carbon chain lengths (12 to 20 carbons) containing 6-*trans* and 8-*cis* double bonds (5-oxo-dienoic acids) [163]. The 12- and 14- carbon compounds did not have any activity, whereas the 16-carbon dienoic acid was a weak agonist and the 18-carbon dienoic acid compound (5-oxo-ODE) was almost as potent as 5-oxo-ETE (20 carbons) [103,163]. 20-Carbon 5-oxo fatty acids with zero to five double bonds were also synthesized. The fully saturated fatty acid as well as the fatty acid with a single 6-trans double bond had very weak or no activity [163]. The trienoic acid 5-oxo-6E,8Z,11Z-eicosatrienoic acid, which was shown to be synthesized by neutrophils from Mead acid (5Z,8Z,11Z-eicosatrienoic acid) [163], which accumulates in essential fatty acid deficiency [164], and its 8-*trans* isomer were approximately equipotent with 5-oxo-ETE. These studies greatly enhanced our understanding of the structural requirements for activation of OXE-R.

The ligands for OXE-R are not limited to eicosanoids or lipids. Another group identified 2 peptides that are agonists of this receptor [165]. Using a cell-free assay to screen large libraries of peptides for their ability to bind to GPCRs,

Sasaki and colleagues screened a library of hexapeptides (6 amino acids) with random sequences for their ability to bind the OXE-R [165]. Two peptides, $^{-}$OOC-HMQLYF-NH_2 and $^{-}$OOC-HMWLYF-NH_2, which differ by only one amino acid, both stimulated binding of GTPγS to OXE-$G\alpha_i$. The two peptides also induced chemotaxis and Ca^{2+} mobilization in CHO cells transfected with the OXE-R, but 1000- and 100- fold higher levels of the peptides compared to 5-oxo-ETE were required to induce these responses. The responses were specific because they were not induced by a similar peptide, $^{-}$OOC-HMQLDF-NH_2 [165]. The authors suggested that there were potentially several hundred other hexapeptides with agonist activites since they did not perform a complete screen [165].

1.4.2 Activation of PPARs by 5-oxo-ETE

Peroxisome proliferator-activated receptors (PPARs) are ligand activated transcription factors. They form heterodimers with retinoid X receptor (RXR) and bind PPAR-responsive elements activating transcription of genes regulating lipid metabolism and energy homeostasis [166]. There are three types including PPARα, PPARβ/δ, and PPARγ and numerous endogenous and exogenous ligands have been identified, including eicosanoids and other fatty acids [167]. O'Flaherty and colleagues determined that at high concentrations (100 μM), 5-oxo-ETE directly activates PPARγ in breast cancer cells [168]. The structurally related compound 5-oxo-15-HETE was slightly more potent, but 5-HETE was inactive. In these cells, 5-oxo-ETE had no effect on PPARα or PPARβ/δ.

1.5 Biological effects of 5-oxo-ETE

Since the original discovery of 5-oxo-ETE, its biological effects have been examined. Most of the biological responses attributed to 5-HETE prior to the discovery of 5-oxo-ETE are probably due either to its conversion to 5-oxo-ETE, or to its weak activity through the OXE-R. 5-Oxo-ETE is considerably more potent than 5-HETE and activates eosinophils, neutrophils, and monocytes, and also induces responses in several other types of cells (Figure 1.8).

1.5.1 Tissue infiltration of granulocytes

Granulocytes are important in host defence against bacteria and parasites and are recruited to sites of infection by chemotactic stimuli. Elevated numbers of eosinophils and/or neutrophils are found in sites of inflammation. Chemoattractants stimulate blood leukocytes to roll and then adhere to the vascular endothelium, exit the circulation, and subsequently migrate through the basement membrane into the tissue at the site of infection. This process involves changes in cytoskeletal structure, including the polymerization of actin. Increases in cytosolic calcium levels also appear to be involved because calcium chelation in eosinophils and calcium depletion in neutrophils were shown to block cell migration [169]. Activated selectins, specifically L-selectin on the surface of granulocytes, are adhesion proteins that facilitate the rolling of leukocytes along the vascular endothelium. The surface expression of β_2-integrins on leukocytes is upregulated resulting in tight binding of leukocytes to vascular endothelial cells and transmigration through the basement membrane into the tissue [170]. During transmigration, activated leukocytes release proteinases, including matrix metalloproteases (MMPs), to digest the extracellular matrix [1] which facilitates their entry into the tissue.

1.5.2 Effects of 5-oxo-ETE on eosinophils

1.5.2.1 Eosinophil migration

5-Oxo-ETE exerts numerous effects on eosinophils and is more potent than many other lipid mediators and chemokines. 5-Oxo-ETE is a potent (EC_{50} = 20 nM) eosinophil chemoattractant and induces the *in vitro* migration of these cells in a Boyden chamber [104,134,144], inducing a greater maximal response than most other known eosinophil chemoattractants, including the peptide chemokines, eotaxin-1 and RANTES (Regulated on Activation Normal T Cell Expressed and Secreted), which act through CC chemokine receptors. The order of potency of known eosinophil chemotactic agents for inducing migration of eosinophils in a Boyden chamber are eotaxin > 5-oxo-ETE > C5a > PAF > fMLP > RANTES $\geq$ C3a > LTB_4 > LTD_4 [104,144]. Although 5-oxo-ETE is

approximately 10-fold less potent than eotaxin, the maximal response is approximately 50% higher [171]. In this study, RANTES was only slightly weaker than 5-oxo-ETE, but suboptimal levels of eotaxin and RANTES acted synergistically with 5-oxo-ETE, increasing its potency by 4-fold and slightly increasing the maximal response induced by 5-oxo-ETE [171]. Another study by Schratl and colleagues showed the effects of pre-incubating eosinophils with chemoattractants on their migration *in vitro* [172]. The authors found that 5-oxo-ETE primes eosinophils to migrate toward eotaxin, but in contrast, eotaxin does not alter cell responsiveness to 5-oxo-ETE [172]. The lipid mediator PGD_2 enhanced the responsiveness of eosinophils to both eotaxin and 5-oxo-ETE, but inhibited these responses in basophils. Alternatively, both 5-oxo-ETE and eotaxin inhibited PGD_2 induced eosinophil migration. The inhibitory effect of 5-oxo-ETE on PGD_2 stimulated eosinophil migration was found to be mediated by p38 MAPK [172]. Schratl and colleagues incubated the cells in the top well in the Boyden chamber with the priming chemoattractant [172] which may explain the differences in results from the study where the two chemoattractants were placed in the lower well [171].

1.5.2.2 Eosinophil transmigration

As a potent eosinophil chemoattractant, 5-oxo-ETE also potently induces *in vitro* transmigration of eosinophils, from both normal and asthmatic subjects through a Matrigel basement membrane [159,173,174]. IL-5, which promotes eosinophil survival, enhances this response by approximately 50% making 5-oxo-ETE 10-fold more potent than PAF. However, unlike 5-oxo-ETE, PAF does not induce the transmigration of eosinophils *in vitro* in the absence of IL-5 [173,175]. 5-Oxo-ETE induces a greater maximal response than eotaxin. However, eotaxin induced a greater response in eosinophils from asthmatics compared to healthy subjects, whereas there was no difference with 5-oxo-ETE [176], suggesting that OXE receptor expression is not altered in asthmatics. 5-Oxo-ETE also potentiated the IL-5/Matrigel stimulated surface expression of the activation marker CD69 [177], but had no effect on the expression of CD44.

Cell migration through Matrigel requires the release of proteinases such as MMPs (matrix metalloproteinases) [178] and 5-oxo-ETE induces the expression and activity of MMP-9 [159,174]. A monoclonal anti-MMP-9 antibody and the general MMP inhibitor, BB-103, both partially inhibited 5-oxo-ETE-induced eosinophil transmigration, but had no effect on the response to eotaxin [173,176]. In addition, the responses to 5-oxo-ETE and eotaxin were partially inhibited with a monoclonal antibody against CD87, the membrane receptor for urokinase plasminogen activator (uPA: a serine protease). These studies indicate that MMP-9 induced by the plasmin/plasminogen system [159] are required for the effect of 5-oxo-ETE on the migration of eosinophils through the basement membrane [173].

The effect of 5-oxo-ETE induced transmigration may partially be mediated by cysLTs and the CysLT1 receptor. 5-Oxo-ETE was shown to stimulate the release of LTC_4 from eosinophils [174] and the CysLT1 receptor inhibitor, Montelukast, and the FLAP inhibitor, MK-0591, were shown to block 5-oxo-ETE induced migration of eosinophils through Matrigel by approximately 30%. The effect of 5-oxo-ETE on the release of LTD_4 was not measured, but LTD_4 affects 5-oxo-ETE induced transmigration. Although LTD_4 induces minimal eosinophil migration in a Boyden Chamber [179, 180], and does not induce migration through Matrigel [174], it acts synergistically with suboptimal levels of 5-oxo-ETE (1 nM) to increase the migration of eosinophils through Matrigel [174]. LTD_4 also partially reversed the inhibitory effect of MK-0591 on 5-oxo-ETE induced eosinophil transmigration. 5-Oxo-ETE induced expression of uPAR and MMP-9 is blocked by the CysLT1R antagonist, Montelukast, but not by MK-0591 [174]. It is therefore not clear whether Montelukast is acting through a separate activity from its role as a CysLT1R antagonist to mediate the effects of 5-oxo-ETE.

1.5.2.3 *In vivo* effects

Intradermal injection of 5-oxo-ETE into asthmatic and nonasthmatic humans induces infiltration of eosinophils, neutrophils, and macrophages into the skin after 24 hours [181]. Compared to vehicle, the largest dose of 5-oxo-ETE induces approximately 8- and 43- fold increases in the number eosinophils at the site of injection in control subjects and asthmatic subjects, respectively. Asthmatics have more circulating eosinophils which may account for the differences observed [181], or the eosinophils from asthmatics may be more responsive to 5-oxo-ETE. The 5-oxo-ETE-induced infiltration of neutrophils was similar for both control and asthmatic subjects after 24 hours, but the response was lower than for eosinophils. Only the highest dose of 5-oxo-ETE induced the infiltration of macrophages, with no significant recruitment of lymphocytes. This corresponds with *in vitro* effects, as 5-oxo-ETE is a more potent chemoattractant for eosinophils than neutrophils or macrophages. The number of infiltrating eosinophils in response to 5-oxo-ETE was lower than the number of cells measured in other studies in response to eotaxin [182] and RANTES [183] which also induce eosinophil infiltration into skin. Similar to its effects *in vitro*, 5-oxo-ETE also induces some degranulation of eosinophils *in vivo* as determined by extracellular MBP staining [144,181].

Intratracheal instillation of 5-oxo-ETE to Brown Norway rats strongly induces eosinophil migration into lung tissues for up to 36 hours [145,184]. The maximal response to 5-oxo-ETE is almost 2-fold higher than to LTB_4, whereas LTD_4 and LTE_4 have no effect. 5-oxo-ETE also induces slightly more eosinophil lung infiltration than PGD_2 [184], and acts independently of the LTB_4 and PAF receptors. The 5-oxo-ETE induced infiltration was inhibited by in vivo administration of antibodies to the integrins CD11a and CD49d, but not CD11b [145].

1.5.2.4 Other biological responses

Calcium mobilization and actin polymerization are often associated with cell migration. Probably contributing to its chemotactic effect, 5-oxo-ETE (EC_{50} = 19 nM) induces a rapid and transient increase in cytosolic calcium levels with an order of potency of PAF > 5-oxo-ETE ≈ eotaxin ≈ LTB_4 [138,139,171]. 5-Oxo-ETE also induces a rapid increase in actin polymerization [138,139,171], with an EC_{50} of 6.3 nM. It is equipotent with PAF and somewhat less potent than LTB_4 and eotaxin, but has a maximal response 3-fold greater than PAF and LTB_4, and 2-fold higher than eotaxin [138,171].

5-Oxo-ETE also stimulates L-selectin shedding and increased surface expression of the β_2-integrin CD11b but at a slower rate than calcium and actin polymerization [138,171]. 5-Oxo-ETE (EC_{50} = 5.0 nM) is approximately 10-, 18-, and 14-fold more potent at stimulating L-selectin shedding than PAF, LTB_4 and 5-HETE, respectively [138] and induces a 5-fold greater maximal response compared to eotaxin [171]. In contrast, eotaxin, LTB_4, and PAF are respectively, 100-, 7-, and 2- fold more potent inducers of CD11b surface expression than 5-oxo-ETE (EC_{50} = 9.5 nM). However, the maximal response induced by 5-oxo-ETE is greater than for eotaxin and LTB_4 [138,171].

5-Oxo-ETE also induces eosinophil degranulation and the release of eosinophil peroxidase and arylsulfatase [144], whereas 5-oxo-15-HETE and 5-HETE are approximately 10-30-fold and 100-300-fold weaker, respectively, than 5-oxo-ETE [144]. This response is strongly enhanced following priming with GM-CSF [144]. In addition, 5-oxo-ETE acts synergistically with PAF, C5a, and LTB_4 to induce eosinophil degranulation [144]. Eosinophils isolated from asthmatics were shown to release LTC_4 in response to 5-oxo-ETE [174] as well as to produce superoxide and H_2O_2 [139].

The activity of 5-oxo-ETE is affected by other cells present in whole blood. Erythrocytes in whole blood have been shown to reduce the potency of eotaxin 10-30 fold [185]. This may be a regulatory mechanism to limit its biological activity when in circulation. Schratl and colleagues measured shape change, which occurs when leukocytes are stimulated with chemoattractants. The

authors determined that eotaxin, 5-oxo-ETE and PGD_2 are 8-, 10-, and 2-fold, respectively, more potent in isolated (plasma-free) eosinophils compared to leukocytes [172].

1.5.3 Neutrophils

5-Oxo-ETE induces similar responses in neutrophils as in eosinophils including migration in a Boyden chamber but with less potency (EC_{50} = 90 nM) [126,141]. It also potently (EC_{50} = 2 nM) induces the rapid and transient mobilization of calcium from intracellular stores in neutrophils but is less potent than LTB_4 [141,153,186]. In addition, 5-oxo-ETE (EC_{50} = 10 nM) induces rapid actin polymerization [140,153], shape change [172] and aggregation [186] in these cells. 5-Oxo-ETE also stimulates the expression of CD11b and to a lesser extent, CD11c [140] and another study found that it also induces the adhesion of neutrophils mediated by CD18 [160]. Similar to eosinophils, 5-oxo-ETE stimulates neutrophil degranulation [143,154,186] as measured by the release of lysozyme and β-glucuronidase. 5-Oxo-ETE has 100-fold less potency in inducing this response in neutrophils compared to eosinophils, in contrast to LTB_4, which is 5-fold more potent in stimulating neutrophil degranulation. 5-Oxo-ETE enhances neutrophil degranulation in response to PAF and DAG but not LTB_4. However, GM-CSF, TNFα and to a lesser extent, G-CSF increase the potency of 5-oxo-ETE in inducing neutrophil degranulation [143,144,154]. 5-Oxo-ETE also elicits superoxide (O_2^-) production in GM-CSF-treated neutrophils, but has little [153,154] or no effect [154] in its absence. Similar to LTB_4, PAF, and fMLP, 5-oxo-ETE increases the exocytosis of secretory vesicles as measured by increased surface alkaline phosphatase activity [154].

5-Oxo-ETE was also shown to stimulate the phosphorylation of $cPLA_2$, which is required for its activation, but is less potent than GM-CSF, LTB_4, and G-CSF [154,187]. Consistent with this, 5-oxo-ETE induces the release of very low levels of AA from neutrophils [154]. GM-CSF and fMLP act synergistically with 5-oxo-ETE to induce $cPLA_2$ phosphorylation and AA release [154,187]. This

response to 5-oxo-ETE may lead to further production of pro-inflammatory AA metabolites.

1.5.4 Monocytes

5-Oxo-ETE has more limited effects on monocytes than neutrophils or eosinophils. Although it does not induce calcium mobilization, it induces both actin polymerization and migration of monocytes *in vitro* [142]. The magnitude of 5-oxo-ETE-induced actin polymerization is comparable to the response to the CC chemokine, monocyte chemotactic protein-1 (MCP-1). The magnitude of 5-oxo-ETE induced chemotaxis of monocytes is similar to neutrophils, but monocytes are more sensitive. However, the efficacy of 5-oxo-ETE is only 40% that of fMLP and the effect of 5-oxo-ETE on monocyte migration is enhanced in the presence of suboptimal levels of MCP-1 and MCP-3, with which it acts synergistically [142]. 5-Oxo-ETE also synergistically enhanced the MCP-1-induced release of AA but did not affect MCP-1 stimulated calcium mobilization or CD18 expression.

5-Oxo-ETE also induces the release of GM-CSF from monocytes. While performing experiments with purified eosinophils, Stamatiou and colleagues in our lab, determined that 5-oxo-ETE promotes the survival of eosinophils [188]. However, further studies revealed that this effect was only observed in the presence of small numbers of contaminating monocytes. The conditioned media from 5-oxo-ETE-treated monocytes contained elevated levels of GM-CSF and promoted the survival of monocyte-depleted eosinophils, an effect that was blocked by inclusion of an antibody against GM-CSF [188]. GM-CSF exerts numerous effects on inflammatory cells, including enhanced survival and activation of eosinophils [189], and basophils [190], as well as increased expression of both 5-LO [191] and FLAP [192] in neutrophils. Therefore, 5-oxo-ETE may have more as yet undiscovered roles in the inflammatory process.

1.5.5 Basophils

Basophils accumulate in tissues during late-phase allergic reactions following allergen challenge and may play a role in the pathogenesis of allergic inflammation. Similar to eosinophils and neutrophils, basophils must transmigrate across the endothelium and basement membrane into tissues. Compared to PGD_2, 5-oxo-ETE weakly activates basophils and induces the expression of the adhesion molecule, CD11b (EC_{50}, 95 nM versus 11 nM), and the activation marker, CD203c (EC_{50}, 37 nM versus 10 nM) [193]. 5-Oxo-ETE is only a weak inducer of Ca^{2+} flux in basophils and consequently does not induce histamine release [155,193,194], which requires increased levels of intracellular Ca^{2+}. However, 5-oxo-ETE is an effective chemoattractant for these cells [155,194] inducing a magnitude of response similar to MCP-1 and eotaxin, the most potent basophil chemoattractant, although it is 3-fold less potent than eotaxin. Both 5-oxo-ETE-induced chemotaxis [155] and Ca^{2+} flux [194] are pertussis toxin sensitive, indicating that its effects are mediated through a GPCR, presumably the OXE receptor. In agreement with this, basophils express both mRNA [155,194] and protein [194] for this receptor.

Similar to eosinophils, 5-oxo-ETE induces the transmigration of IL-3-activated basophils through Matrigel [195], but it is 20-fold less potent than RANTES and basophils are less sensitive to 5-oxo-ETE than eosinophils. Although inhibition of the β_2-integrin CD18 and MMP-2 and/or MMP-9 blocked 5-oxo-ETE induced transmigration of IL-3-activated basophils, it was not established whether 5-oxo-ETE stimulates their expression.

1.5.6 Platelets

Platelets contain 5-HEDH and can oxidize 5-HETE to 5-oxo-ETE, which can be further converted to 5-oxo-12-HETE by platelet 12-LO [108]. 5-Oxo-ETE (IC_{50} = 1.5 μM) was found to completely inhibit AA-induced aggregation whereas 5-oxo-12-HETE inhibited it by approximately 50% [108]. The physiological significance of this response is unclear, because of the high concentration of 5-oxo-ETE required.

1.5.7 Airway smooth muscle cells

5-oxo-ETE produces a concentration-dependent relaxing effect on human bronchial explants (airway smooth muscle; ASM) pre-contracted with methacholine (IC_{50} = 0.39 μM), arachidonic acid (IC_{50} = 0.60 μM) or histamine [196]. The effect of 5-oxo-ETE is approximately 80% of the relaxation effect observed with norepinephrine. This indicates that in humans 5-oxo-ETE may be a bronchodilator, in contrast to its TXA_2-mediated bronchoconstrictor effect on guinea pig bronchial rings [197]. In addition, 5-oxo-ETE induces hyperpolarization of the ASM from the resting membrane potential (IC_{50} = 0.43 μM). The relaxation and hyperpolarization induced by 5-oxo-ETE on human ASM is blocked by pre-treatment with iberiotoxin, a large conducting Ca^{2+}-activated K^+ channel (BK_{Ca}) inhibitor. The relaxing effect of 5-oxo-ETE is not pertussis-toxin sensitive, indicating that it is not mediated by the OXE receptor, consistent with the absence of mRNA for the OXE receptor in human ASM cells [148,196]. Further studies with reconstituted BK_{Ca} channels, show that 5-oxo-ETE concentration-dependently activates them, increasing the length of time that the channels are open and conversely decreasing the duration that the channels were closed. These responses to 5-oxo-ETE indicate that it directly acts on the channels either by binding to the cell membrane or by interacting with the protein subunits of the BK_{ca} channels.

1.5.8 Epithelial cells

Epithelial cells in the mammalian gut, stimulated by the presence of bacteria, secrete anions, particularly Cl^- ions. This results in the release of water from the cells and a subsequent decrease in cell volume. Prostaglandins and cysLTs, which can be formed in the lamina propria in the mammalian gut can induce the release of Cl^- by adjacent epithelial cells [198-200]. 5-HETE can also stimulate Cl^- secretion from intestinal epithelial cells [201], and consequently 5-LO inhibitors block ion secretion from these cells [202]. Therefore, Macleod and colleagues tested the cellular responses elicited by 5-oxo-ETE, since it could potentially be present in the lamina propria [203]. Compared to other known

stimuli of intestinal epithelial cell ion flow, 5-oxo-ETE potently (EC_{50} = 20 pM) induced a rapid decrease in the volume of guinea pig jejunal crypt epithelial cells [203]. It was 250-, 50- and 10- fold more potent than 5-HETE, LTD_4, and bradykinin, respectively, and induced a larger maximal decrease in crypt cell volume than 5-HETE. The effects of 5-oxo-ETE and 5-HETE on crypt cell volume are inhibited by Cl^- and K^+ channel blockers as well as by two PKC inhibitors [203]. These results indicate that 5-oxo-ETE and 5-HETE, activate Cl^- and K^+ channels and that this effect is mediated by PKC.

1.5.9 Proliferation of cancer cells

As previously mentioned (Section 1.2.5), many types of cancer cells express elevated levels of 5-LO compared to normal cells [18], and inhibitors of the 5-LO pathway induce apoptosis of cancer cells [77-80]. Corresponding with elevated levels of 5-LO, the levels of 5-HETE have been found to be 2.2-fold higher in malignant tissue as compared to that of benign tissue from prostate cancer patients [18]. In addition, prostate cancer cells were reported to convert exogenous AA to 5-HETE, which was detected by radioimmunoassay [77] but the levels of 5-oxo-ETE were not measured.

Since AA stimulates the growth of prostate cancer cells, and 5-LO inhibitors induce apoptosis, numerous studies have looked at the effect of different 5-LO products on cell growth. Ghosh and Myers showed that 5-oxo-ETE, 5-HpETE and 5-HETE but not LTB_4 [80] or the cysLTs [77], reverse the effects of the 5-LO pathway inhibitors and promote prostate cancer cell (PC3 and LNCaP) proliferation. 5-Oxo-ETE also reverses apoptosis of prostate cancer cells induced by selenium, which may act by inhibiting 5-LO [87]. In addition to prostate cancer cells, 5-oxo-ETE induces the proliferation of breast (MDA-MB-231 and MCF7) and ovarian cancer cells (SKOV) [168]. The structurally related compounds, 5-oxo-15-HETE and 5-HETE have less activity than 5-oxo-ETE. 5-Oxo-ETE seems to act via its receptor, since all of these cell lines express the OXE receptor, and its proliferative effect is blocked by pertussis toxin [168]. In addition, treating PC3 cells with siRNA against OXE-R causes a dose-dependent

decrease in OXE-R expression and a corresponding decrease in cell viability [204]. The expression of the OXE receptor may be linked to proliferation since the more metastatic PC3 cells express 3.4-fold higher levels compared to LNCaP cells [204]. In contrast, normal prostate epithelial cells (PrEC) do not have detectable levels of OXE mRNA [204]. This provides further support that 5-oxo-ETE promotes prostate cancer cell growth by activating its receptor.

Ghosh and colleagues found that 5-HETE has approximately 50% less efficacy than 5-oxo-ETE and 5-HpETE for promoting the survival and proliferation of prostate cancer cells [77]. In contrast, in another study, O'Flaherty and colleagues reported that 5-HETE is more potent than 5-oxo-ETE in promoting PC3 cell growth and reversing the effects of 5-LO inhibitors [161]. The downstream signaling induced by 5-HETE was blocked by pertussis toxin, indicating that this effect was mediated by a GPCR. The authors suggested that 5-HETE may be acting via a GPCR distinct from the OXE receptor [161]. However, later studies by this group and studies by Ghosh and colleagues indicate that 5-oxo-ETE is more potent than 5-HETE [168]. The above discrepancy may be due to the experimental conditions such as the length of time the cells were incubated with the enzyme inhibitors, or the concentrations and confluency of the cells.

Members of the stress-activated protein kinase family are reportedly involved in apoptosis, and are stimulated by certain factors or by the removal of growth factors. The transcription factor c-Jun promotes and regulates several genes involved in cell proliferation and induces apoptosis when it is activated [205]. c-Jun N-terminal kinase (JNK) phosphorylates and activates c-Jun and inhibitors of the 5-LO pathway, MK886, MK591, and AA861 induce the rapid phosphorylation of JNK as well as c-Jun, with only a small increase in phosphorylation of p38-MAPK [206]. 5-HETE was shown to reverse or block the MK886-induced phosphorylation of JNK [206]. The effect of 5-oxo-ETE was not tested and whether it inhibits JNK phosphorylation has not been determined to date. However, since 5-HETE acts via OXE-R, 5-oxo-ETE will probably have the same effect. Both 5-HETE and 5-oxo-ETE were also shown to induce the

rapid phosphorylation of ERK1/2 and Akt in PC3 cells [161]. Akt is anti-apoptotic and promotes the survival of prostate cancer cells [207], and therefore may mediate the responses to 5-oxo-ETE in these cells.

Further studies are required to determine the role of 5-oxo-ETE in cancer cell growth, since other activities have been discovered. O'Flaherty and colleagues determined that much higher concentrations (100 μM) of 5-oxo-ETE and 5-oxo-15-HETE, but not 5-HETE, inhibit cell proliferation, inducing apoptosis [168]. These effects are not sensitive to pertussis toxin and are therefore presumably independent of the OXE receptor. Similar to 15-deoxy-PGJ_2 which also induces apoptosis of cancer cells, 5-oxo-ETE and 5-oxo-15-HETE both activate PPARγ directly at concentrations above 10 μM, and this is blocked by inhibitors of PPARγ [168]. Conversely, 5-HETE does not activate PPARγ [168,208,209] and does not induce apoptosis [168]. PPARγ is expressed by several types of tumor cells and its activation by specific ligands has been shown to inhibit cancer cell proliferation or induce apoptosis *in vitro* [210]. In addition, 5-LO pathway inhibitors, MK886 and NDGA, which inhibit cell growth, have also been shown to upregulate the expression of PPARγ and PPARα in breast cancer cells [78]. However the role for PPARs is still unclear since *in vivo* studies have shown that other ligands can either inhibit or promote cancer cell growth [167,210]. The stimulatory effects of 5-oxo-ETE on apoptosis may not be physiological because of the high concentrations required. Although a PPARγ inhibitor was shown to block activation of PPARγ by 5-oxo-ETE, it did not block the 5-oxo-ETE (or 15-deoxy-PGJ_2 or 5-oxo-15-oxo-ETE)-induced apoptosis [168], indicating that this effect is not due to PPARγ activation. The authors suggest that instead 5-oxo-ETE may be involved in a Michael addition reaction with SH residues of IκB kinase or the p50 subunit of NFκB, thus blocking their role in cell survival [168]. This has previously been proposed for 15-deoxy-PGJ_2 and other α,β-unsaturated ketones [211]. Therefore, the role of 5-oxo-ETE-induced PPARγ activation and the mechanism for its induction of apoptosis at high concentrations have yet to be elucidated.

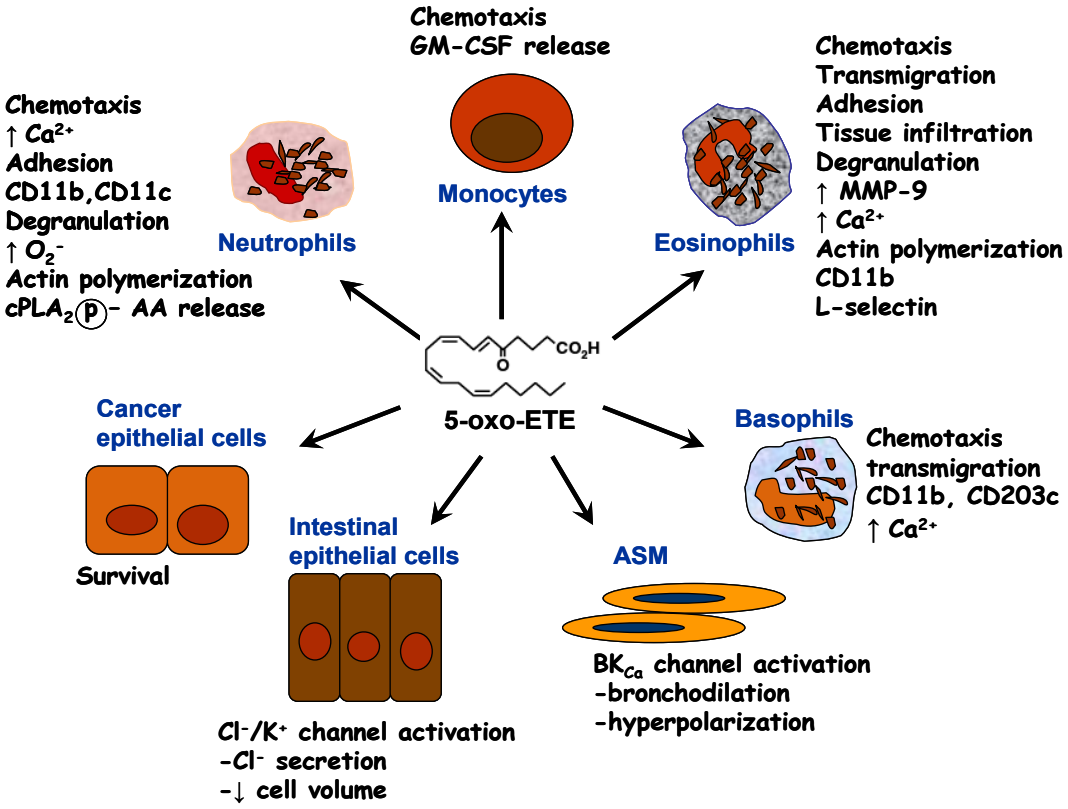


Figure 1.8: Biological effects of 5-oxo-ETE in human cells.

1.6 Summary

The biologically active 5-LO product, 5-oxo-ETE, induces effects on a variety of cell types. It is a potent chemoattractant for eosinophils, and other inflammatory cells, and induces intracellular calcium mobilization, eosinophil and neutrophil degranulation and GM-CSF release from monocytes. 5-Oxo-ETE also activates Cl^- and K^+ channels in epithelial cells, and promotes the proliferation of cancer cells in addition to a number of other effects. Synthesized from 5-HETE by the microsomal enzyme 5-HEDH, 5-oxo-ETE is more potent than its precursor and acts via the selective OXE receptor. The intracellular oxidative environment tightly regulates the synthesis of 5-oxo-ETE; however, many stimuli can promote its production. The actions of 5-oxo-ETE are limited by its metabolism via numerous enzymatic pathways. Studies into the production of 5-oxo-ETE are important to further understand its role in various pathological conditions.

Chapter 2: Aim of Study

2.1 5-oxo-ETE synthesis by human B lymphocytes

Many inflammatory cells have been shown to synthesize 5-oxo-ETE [5] and B lymphocytes express 5-LO and FLAP, and synthesize LTB_4 and 5-HETE [43,45-48,50]. These studies showed that under normal conditions, these cells had very low 5-LO activity and stimulation by oxidative stress was required to induce activity. In addition, previous studies by our lab have shown that oxidative stress increases the production of 5-oxo-ETE in monocytes, epithelial cells and endothelial cells [101,110-112]. We hypothesized that B cells express 5-HEDH and synthesize 5-oxo-ETE, and that its levels would be elevated under conditions of oxidative stress. Therefore, determined whether B cells have 5-HEDH activity and synthesize 5-oxo-ETE when incubated with AA and A23187 and whether this would be enhanced by oxidative stress. We investigated the synthesis of 5-oxo-ETE from 5-HETE or exogenous AA and A23187 in primary human tonsillar B cells, as well as several B cell lines. In addition the effect of oxidative stress induced with H_2O_2 was also evaluated. Since the ratio of $NADP^+$ to NADPH regulates 5-oxo-ETE synthesis [101], we examined the effect of oxidative stress on the levels of the pyridine nucleotides, $NADP^+$ and NADPH, as well as the levels of reduced and oxidized glutathione. The correlation between the effect of H_2O_2 on these cellular products and 5-oxo-ETE synthesis was determined.

2.2 Regulation of 5-oxo-ETE synthesis by prostate cancer cells

Many cancer cells express elevated levels of 5-LO and FLAP, and inhibitors of this pathway have been shown to induce cell death, while 5-oxo-ETE has been shown to promote their survival [77,80,87,168]. However, it is not known whether these cells express 5-HEDH and synthesize 5-oxo-ETE. In addition, inflammatory cells, and specifically eosinophils, have been found in the resident tissue of tumors [66,67]. We hypothesized that prostate cancer and other types of cancer cells express 5-HEDH and synthesize 5-oxo-ETE which could promote the infiltration of inflammatory cells into cancerous tissues. In addition,

cancer cells are often subject to oxidative conditions, which could influence the production of 5-oxo-ETE which we also determined. The effect of anti-cancer agents such as tamoxifen and DHA which alter the intracellular environment were also evaluated to determine whether they had an affect on 5-oxo-ETE synthesis. Although cancer cells express 5-LO and FLAP they synthesize very low levels of 5-LO products, and we performed co-incubations of neutrophils with prostate cancer cells to determine whether 5-HETE synthesized from inflammatory cells could be converted to 5-oxo-ETE by resident cancer cells.

2.3 Purification of 5-HEDH from human monocytes and neutrophils

Numerous studies have identified the presence of 5-HEDH in several types of cells, and determined the regulation of 5-oxo-ETE synthesis by this enzyme [101,110-112]. 5-HEDH, an integral membrane protein, has not been isolated or sequenced, which has limited studies on its expression in different pathologies. Identifying the sequence of 5-HEDH and its gene will further our understanding of the role of 5-oxo-ETE in different physiological conditions. We attempted to purify 5-HEDH from human U937 cells and neutrophils, using numerous chromatographic purification techniques with a goal to sequence the enzyme for future studies using specific antibodies or in situ hybridization to study its expression, localization and transcriptional regulation. In collaboration with Dr Joshua Rokach at F.I.T., we also attempted to purify 5-HEDH using affinity chromatography with synthetic 5-HETE analogs attached to solid phase particles [5]. We tracked the elution of 5-HEDH using an enzymatic activity assay and compared this to protein levels. We subsequently sequenced tryptic peptides in fractions containing partially purified 5-HEDH activity and searched the database for dehydrogenases.

Chapter 3: Materials and Methods

3.1 Materials

5-HETE [212] and 5-oxo-ETE [213] were prepared by total organic synthesis by Dr Joshua Rokach (Florida Institute of Technology, Melbourne, FL). 13S-hydroxy-9Z,11E-octadecadienoic acid (13-HODE) was produced by incubating linoleic acid (Nu-Chek Prep Inc., Elysian, MN) with soybean lipoxygenase (Sigma-Aldrich, St. Louis, MO)[214] Prostaglandin B_2 (PGB_2), and 8,11,14-eicosatrienoic acid (ETrA) were obtain from Cayman Chemical (Ann Arbor, MI). Arachidonic acid (AA) and 4,7,10,13,16,19-docosahexaenoic acid (DHA) were obtained from Nu-Chek Prep Inc.

Diamide, dimethyl sulfoxide (DMSO), o-phthalaldehyde (OPT), tamoxifen, 1,2-bis[2-chloroethyl]-1-nitrosourea (BCNU), N-ethylmaleimide (NEM), phorbol 12-myristate 13-acetate (PMA), phenazine methosulfate (PMS), NAD+, 3-[(3-cholamidopropyl)-dimethylammonio]1-propanesulfonate (CHAPS), and n-octyl-β-d-glucopyranoside (octylglucoside) were obtained from Sigma-Aldrich (St-Louis, MO). Phenylmethanesulfonyl fluoride (PMSF) was obtained from ICN Biomedicals Inc. (Aurora, OH). Glycerol, and HPLC grade acetonitrile, and methanol were purchased from Fisher Scientific (Nepean, ON). Triacsin C was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Complete mini EDTA-free protease inhibitor cocktail tablets and $NADP^+$, NADPH and NADH, were purchased from Roche Diagnostics (Laval, QC). Products used for cell culture, including cell growth media, antibiotics, fetal bovine serum (FBS) and trypsin were purchased from Invitrogen (Burlington, ON). Acrylamide/bisacrylamide solution, ammonium persulfate (APS), N,N,N",N'-tetramethylethylenediamine (TEMED), Coomassie Brilliant Blue R-250 and all the apparatus used for gel electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA, USA)

3.2 Preparation of cells and culture conditions

3.2.1 Culture of U937 cells and B cell lines

The B cell lines, Ramos, U266, and CESS, were obtained from ATCC (Manassas, VA) and cultured in RPMI 1640 medium containing sodium bicarbonate (1.5 g/L), sodium pyruvate (1 mM), and L-glutamine (2 mM), supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were cultured at a density between 2 x 10^5 and 10^6cells/ml in 5% CO_2 at 37 ^oC.

U937 cells obtained from ATCC were cultured in modified RPMI 1640 medium containing sodium bicarbonate (1.5 g/L), glucose (4.5 g/L), HEPES (25 mM), sodium pyruvate (1 mM), and L-glutamine (2 mM), supplemented with 10% (v/v) FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were maintained at a density between 10^5 and 1.5 x 10^6 cells/ml. The U937 cells (10^6 cells/ml) were terminally differentiated with PMA (18 nM) for 4 days. The PMA-differentiated cells became adherent, and were resuspended in cold 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 pH 7.4) by scraping with a rubber policeman.

3.2.2 Culture of epithelial cancer cell lines

The epithelial cancer cell lines, PC3, A427, and MCF7 were obtained from ATCC and cultured in RPMI 1640 medium (RPMI 1640 medium containing sodium bicarbonate (1.5 g/L), sodium pyruvate (1 mM), and L-glutamine (2 mM)), supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml). A549 cells were cultured in DMEM/F12 medium containing sodium bicarbonate (1.5 g/L), sodium pyruvate (1 mM), and L-glutamine (2 mM), supplemented with 10% FBS. The cells were grown in culture flasks until 80% confluence and then trypsinized (0.25% trypsin supplemented with EDTA •4Na) and plated at a density of 1.5 x 10^4 cells/cm^2. Cells were sub-cultured to a maximum of 20 passages.

3.2.3 Isolation of tonsillar B lymphocytes

Primary B lymphocytes were isolated from human tonsils discarded after surgery. The tonsils were thoroughly minced, resuspended in RPMI 1640 medium supplemented with 2% FCS (Hyclone Laboratories Inc, Logan, Utah), penicillin (50 U/ml), streptomycin (50 μg/ml), and amphotericin B (1/500 wt/vol) from Life Technologies (Burlington, ON) and layered onto a Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) gradient. Tonsil lymphocytes were separated by rosetting with neuraminidase-treated sheep red blood cells and subsequent Ficoll-Paque density centrifugation. Monocytes were removed from the E-rosette-negative fraction by adherence depletion. The remaining B cells were routinely >98% pure on flow cytometry by CD20 staining, with <1% CD141 and <1% CD31.

3.2.4 Isolation of neutrophils

Neutrophils were isolated from whole blood as previously described [98]. Whole blood from healthy donors was treated with Dextran T-500 (Sigma-Aldrich) followed by centrifugation for 30 min over Ficoll-Paque (Pharmacia Biotech) to remove mononuclear cells. Any remaining red blood cells present in the pellet containing neutrophils were removed by hypotonic lysis with distilled water. The isolated neutrophils were resuspended in PBS^-.

3.3. Measurement of eicosanoids in intact cells by RP-HPLC

3.3.1 Incubation conditions

To determine cellular 5-HEDH activity, B cells (2 x 10^6 cells/ml) suspended in PBS^+ (PBS^- supplemented with calcium (1.8 mM) and magnesium (1 mM)) were incubated for various times at 37 ^oC in a shaking water bath with 5-HETE (1 μM) in the presence or absence of H_2O_2. 5-HETE and H_2O_2 were added at the same time.

To measure the 5-LO metabolites of AA, B cells were suspended in PBS^+ at a concentration of 10^7 cells/ml. The cells were treated with vehicle, H_2O_2 (100 μM unless otherwise indicated) or diamide (250 μM) followed immediately by

the addition of AA (50 μM) and the calcium ionophore, A23187 (5 μM). Incubations were performed at 37 ^{o}C in a shaking bath for the times indicated. All reactions with non-adherent cells were stopped by addition of ice cold methanol (630 μl) followed by cooling at 0 ^{o}C. The samples were then diluted with distilled water to a final concentration of 30% methanol and stored at -80 ^{o}C before analysis by RP-HPLC. Either 13-HODE (100 ng) or PGB_2 (100 ng) were added as internal standards to the samples before analysis by HPLC.

Adherent epithelial cancer cells (PC3, A427, and MCF7) were plated in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 3 x 10^5 cells/well in 6-well plates (3.1 x10^4 cells/cm^2) and left overnight to adhere. The cells in the wells (duplicate) were counted the day of the experiment to monitor the exact number of cells present for the incubations and it was determined that the cells did not proliferate during this time. On the day of the experiment, the RPMI was removed from the wells and replaced with either RPMI supplemented with 0.5% FBS (1 ml/well) prior to incubations or PBS^+ (1 ml/well). To determine the effects of various agents on 5-oxo-ETE production, the cells were incubated at 37 ^{o}C with 5-HETE (4 μM) in the presence and absence of various substances, including H_2O_2 (100 μM unless otherwise specified), DHA (40 μM), tamoxifen (50 μM), MK886 (20 μM), doxorubicin (10 μM), or triacsin C (20 μM) for various times (5 min to 24 hrs). To stop the incubations, the medium in the well was transferred to a tube after adding either PGB_2 (100 ng) or 13-HODE (100 ng) as internal standards. The wells were then rinsed with ice-cold methanol (630 μl) which was then added to the medium in the tubes. The samples were diluted with water to a final concentration of 30% methanol and then stored at -80 ^{o}C before analysis by RP-HPLC. Cells were trypsinized in selected wells and counted using a hemocytometer.

The production of 5-LO metabolites by adherent PC3 cells co-incubated with neutrophils and neutrophils alone was measured. PC3 cells were plated in complete RPMI medium in 6-well plates at 6 x 10^5 cells/well (6.2 x 10^4 cells/cm^2), and left to adhere overnight. RPMI was removed from the wells on the day of the experiment, and replaced with PBS^+. PC3 cells were pre-incubated

for 10 min at 37ºC with vehicle or NEM (100 μM) which was then removed and then incubated for 10 min with vehicle or H_2O_2 (100 μM) in 1ml PBS^+. Freshly isolated neutrophils (3 x 10^6 cells) were then added to the PC3 cells with AA (40 μM) and A23187 (5 μM) in 1ml PBS^+ and incubated at 37 ºC for 30 min. As controls, neutrophils (3 x 10^6 cells) were incubated in the 6-well plates with the same conditions as the coincubations. The reactions were stopped following the same method used for adherent cells. PGB_2 (100 ng) was added as an internal standard.

3.3.2 RP-HPLC analysis of eicosanoids

Eicosanoids were analyzed by precolumn extraction combined with reverse-phase HPLC [215] using a modified Waters 2695 Alliance system (Waters Corp., Mississauga, ON) with a photodiode array detector (Waters model 2996). 5-HETE metabolites were separated on a 150 x 3.9 mm Nova-Pak C18 (octadecylsilyl; ODS) column (Waters Corp; 4 μm particle size) maintained at 35 ºC, with a flow rate of 1 ml/min using a linear gradient between solvent A (water containing 0.02% (v/v) acetic acid) and solvent B (acetonitrile containing 0.02% acetic acid) (Table 1) as follows: 0 min: 65% B; 1.5 min: 65% B; 7 min, 82% B. All solvents were of HPLC grade. 5-HETE, and 13-HODE were quantitated at 235 nm, and 5-oxo-ETE was quantitated at 280 nm. The products were quantitated using the ratio of the areas of the UV absorbance peaks at their λ_{max} versus the area of the peak for the internal standard, 13-HODE, and correcting for differences in their extinction coefficients (Table 2)

Table 3.1: RP-HPLC solvents

Solvent	Composition	Analysis
A	Water containing 0.2% (v/v) acetic acid	**Eicosanoids**
B	Acetonitrile containing 0.2% (v/v) acetic acid	
C	Methanol containing 0.2% (v/v) acetic acid	
D	100 mM Citric acid containing 4 mM TBAH; pH 2.2	**$NADP^+$ and NAD^+**
E	Acetonitrile	
F	200 mM NH_4OAc containing 10 mM TBAH; pH 6.0	**NADPH and NADH**
G	Methanol	
H	Water containing 0.05% (v/v) TFA	**GSH and GSSG**
I	Acetonitrile containing 0.05% (v/v) TFA	

Table 3.2: Extinction coefficients for eicosanoids analyzed by RP-HPLC

Eicosanoid	λmax (nm)	Extinction Coefficient
13-HODE	235	23,000
5-HETE	235	27,000
5-oxo-ETE	280	22,100
PGB_2	280	28,680
LTB_4	280	39,500
12-HHTrE	235	33,400

Because AA is converted to a wider array of eicosanoids than 5-HETE, a column temperature of 30 ^oC was used as well as a longer gradient between solvents A, B, and C (methanol containing 0.02% acetic acid) as follows: 0 min: 65% A, 24% B, 11% C; 4 min: 65% A, 24% B, 11% C; 39 min: 11% A, 38% B,

51% C. The stationary phase was the Novapak C18 column described above. 5-HETE, 13-HODE, 12-HHTrE were analyzed at 235 nm, whereas 5-oxo-ETE, PGB_2 and LTB_4 were analyzed at 280 nm. The products were quantitated as previously described, using PGB_2 as the internal standard and the extinction coefficients shown in Table 2.

3.4 Measurement of pyridine nucleotide levels in intact cells by RP-HPLC

Intracellular $NADP^+$ and NAD^+ levels were measured in the lymphoblastoid CESS B cell line, tonsillar B cells, and PC3 cells. Intracellular NADPH and NADH levels were measured in CESS and tonsillar B cells. Cells were incubated separately to measure the oxidized and reduced forms.

$NADP^+$ and NAD^+ were converted to fluorescent naphthyridine derivatives [216] and measured by RP-HPLC. CESS and tonsillar B cells (8×10^6 cells/ml), resuspended in 250 µl PBS^+, were incubated with various concentrations of H_2O_2 at 37 ^oC for specified times. PC3 cells in PBS^+ in 6-well plates (3.1×10^4 cells/cm^2) were stimulated with H_2O_2. An equivalent volume of ice-cold methanol/water (1:1) containing 50 mM acetophenone and 3 N KOH was added to the cells to stop the reactions. The internal standard, deamino-NAD^+ (100 ng), was immediately added and the samples left on ice in the dark for 20 minutes. The samples were then incubated on ice for another 5 minutes with formic acid (2.3 N) followed by extraction with ethyl acetate (2 x 1.5 ml). The final aqueous phase was then treated with phenazine methosulfate (100 µM) for 10 min at 37 ^oC. Samples were stored at -80 ^oC before analysis by RP-HPLC. The fluorescent NAD^+ and $NADP^+$ derivatives were separated on a 150 x 4.6 mm Ultracarb 5 µm ODS column (Phenomenex, Torrance, CA) with a flow rate of 1.25 ml/min using a gradient between solvent D (citric acid (100 mM) containing 4 mM tetrabutyl ammonium hydroxide (TBAH) at pH 2.2) and solvent E (acetonitrile) as follows: 0 min: 1% E; 1 min: 1% E; 12 min: 25% E at 35 ^oC. The fluorescent derivatives were analyzed with a Waters model 2475 fluorescence detector (λ_{Ex} 371 nm, λ_{Em} 438 nm). The concentrations of $NADP^+$ and NAD^+ were determined from a standard curve obtained from mixtures of different

amounts of NAD^+ and $NADP^+$ with a constant amount (100 ng) of the internal standard deamino-NAD^+.

The levels of NADPH and NADH were measured following incubation of CESS and tonsillar B cells suspended in 100 µl PBS^+ (7.5×10^6 cells/ml) with various concentrations of H_2O_2 at 37 °C for specified times. Reactions were stopped by adding 0.5 volumes of ice-cold 0.2 M Na_3PO_4 and immediately cooling to 0 °C. Samples were stored at -80 °C before analysis by RP-HPLC. NADPH and NADH were separated on a 150 x 3.0 mm Gemini C18 column (Phenomenex; 3 µm particle size) with a flow rate of 0.5 ml/min using a gradient composed of solvent F (200 mM ammonium acetate containing 10 mM TBAH pH 6.0) and solvent G (methanol) as follows: 0 min, 8% G; 15 min, 17% G at 35 °C. NADPH and NADH were measured with a fluorescent detector (λ_{Ex} 325 nm, λ_{Em} 450 nm) and quantitated using an external standard curve.

3.5 Measurement of GSH and GSSG levels in intact cells by RP-HPLC

The intracellular levels of the oxidized and reduced forms of glutathione were measured in CESS and tonsillar B cells by RP-HPLC using a modified post-column derivatization method [217]. B cells were diluted in PBS^+ to a concentration of 2×10^6 cells/ml, and PC3 cells were incubated in 1 ml of PBS^+ in 6-well plates (3.1×10^4 cells/cm^2). Incubations were terminated by addition of an equal volume of ice-cold 200 mM phosphoric acid containing 12 mM CHAPS and cooling to 0 °C to stop the incubations. Samples were stored at -80 °C before analysis by RP-HPLC.

GSH and GSSG, separated on a 150 x 4.6 mm Ultracarb 5 µm ODS column (Phenomenex), were subjected to automated postcolumn derivatization by mixing the column eluate with o-phthalaldehyde (OPT; 372 µM) in tribasic sodium phosphate, pH 12, in a water bath at 70 °C as previously described [111,112]. Under these conditions, GSH and GSSG are converted to a fluorescent isoindole adduct, which was measured with a fluorescence detector (λ_{Ex} 336 nm, λ_{Em} 420 nm). Using a flow rate of 1 ml/min, the mobile phase was a linear gradient between Solvent H (water containing 0.05% trifluoroacetic acid (TFA))

and Solvent I (acetonitrile containing 0.05% TFA) as follows: 0 min: 0% solvent I; 10 min: 15% I. The amounts of GSH and GSSG were determined from an external standard curve using the pure compounds.

3.6 Real-time PCR analysis of OXE receptor mRNA expression

mRNA expression of OXE-R in B cells was determined by real-time PCR. Total cellular RNA was isolated from purified tonsillar B cells with TRIZOL (Invitrogen) and dissolved in nuclease-free water and subsequently reverse transcribed. The RNA was treated with DNAse to remove any contaminating genomic DNA since the OXE-R gene does not contain any introns [147]. cDNA strands were generated in 20 µl reaction mixture containing 1 µg total RNA as a template, 0.5 µg oligo(dT)12-18, 0.25 mM dNTP, and 200 U of M-MLV reverse transcriptase (Invitrogen) in the presence of a ribonuclease inhibitor for 50 min at 42 ^{o}C.

Quantitation of mRNA coding for OXE-R was performed by real-time polymerase chain reaction (PCR) using the Roche LightCycler PCR system (Roche Diagnostics, Laval, QC). The specific OXE-R primers used were 5'-CCTCTGCTCCCTCTGCCTTTAC – 3' and 3' – GGTAGCGGTTGAGTGCGATG – 5' (Alpha DNA, Montreal, QC) based on published sequences [147,148]. Real-time PCR reactions were performed in 200 µl containing 2 µl of B cell cDNA, 4 µM of each primer, and 10 µl of Quantitect SYBR Green PCR buffer (Qiagen Inc.) containing Taq DNA polymerase, dNTP, 5 mM $MgCl_2$, and SYBR Green I. The activation of the HotStarTaq DNA polymerase was performed at 95 ^{o}C for 15 min. The thermal cycling program consisted of denaturation for 15 sec at 95 ^{o}C, annealing for 20 sec at 60 ^{o}C, and extension for 20 sec at 72 ^{o}C and was repeated for 55 cycles. Results were analyzed with the LightCycler software version 3.5.3, and the melting curve was used to check for specificity of the amplification products. Quantitation of the PCR product was performed using a standard curve obtained with serial dilutions of the amplicon. β_2-microglobulin was measured as the housekeeping gene and quantitated as a control to correct for variations in cDNA content among samples. Values for OXE-R were normalized to β_2-microglobulin in each sample.

3.7 Partial purification of 5-HEDH

The experimental conditions for each purification step were tested and optimized in an attempt to purify 5-HEDH. All of the purification steps were carried out at 4^{o}C unless otherwise specified. The isolated microsomes and purified protein fractions were stored at -80 ^{o}C for further analyses.

3.7.1 Measurement of 5-HEDH activity

5-HEDH enzymatic activity was measured in microsomes, solubilized microsomes, and fractions from purification procedures. Aliquots from elution samples were incubated with 5-HETE (1 μM) and $NADP^{+}$ (100 μM) in 20 mM sodium phosphate, pH 7.4 (**Buffer A**), containing 15 mM octylglucoside at 37 ^{o}C for between 10 and 60 min, depending on the purification step. The reactions were stopped at 0 ^{o}C by adding ice cold methanol (630 μl) and placing the tube immediately on ice. The samples were subsequently diluted to 30% methanol with water and stored at -80 ^{o}C until analysis by precolumn extraction coupled to RP-HPLC as described in section 3.3.1, using13-HODE (100 ng) as an internal standard.

3.7.2 Measurement of 5-ketoreductase activity

Purified protein fractions were incubated with 5-oxo-ETE (4 μM) and NADPH (100 μM) for 60 minutes at 37 ^{o}C to test for 5-ketoreductase activity (5-oxo-ETE → 5-HETE) in contrast to dehydrogenase activity (5-HETE → 5-oxo-ETE). The reactions were stopped and 5-HETE measured by RP-HPLC as previously described in section 3.3.1 for dehydrogenase activity incubations.

3.7.3 Measurement of protein

Protein concentrations for all purification steps were measured using either the Bio-Rad DC (detergent compatible) protein assay kit (Bio-Rad Laboratories), which is based on the Lowry assay [218], or the Bio-Rad Bradford protein assay [219]. Protein levels were determined using an external standard curve of bovine serum albumin (BSA).

3.7.4 Concentration of protein and detergent exchange

Amicon (Millipore) molecular weight cut-off (MWCO) centrifugal filters that retained 5-HEDH activity were used to concentrate the protein as well as to remove any salts or exchange detergents or buffers after different purification steps. 5-HEDH activity was measured in both the filtrate and the retentate. 5-HEDH activity in the solubilized microsomes was retained and thereby concentrated by filtration using 50 kDa MWCO Amicon centrifugal filters (4 ml tube; 3 cm^2 membrane area) centrifuged at 3,350 x g. 5-HEDH fractions obtained from further steps of purification (e.g. DEAE Sepharose, blue Sepharose) were combined and concentrated using 30 kDa MWCO centrifugal filters (15 ml tube; 7.6 cm^2 membrane area). To remove any salt used for elution, the concentrated protein was always washed with several volumes of detergent and centrifuged again.

3.7.5 Preparation of microsomal fractions by differential centrifugation

Neutrophils or PMA-differentiated U937 cells were suspended in 22 ml PBS^- supplemented with 1 mM PMSF and subsequently disrupted by sonication at 40 cycles/second on ice pulsing 5 x 8 sec with intervals of 1 min to allow for cooling [98]. The sonicates were centrifuged at 1,500 x g at 4 ^oC for 10 min to remove intact cells and nuclei. The supernatants were then centrifuged at 10,000 x g for 10 min to remove granules, and then at 150,000 x g for 120 min at 4 ^oC using a Beckman (Mississauga, ON) ultracentrifuge (rotor Type 50.2Ti). The final pellet containing the microsomal fraction was resuspended by homogenization (using a glass homogenizer) in Buffer A.

3.7.6 Solubilization of microsomes

Microsomes were solubilized with either 30 mM octylglucoside or 6 mM CHAPS in Buffer A at 4 ^oC for 90 to 180 min and diluted to 5 ml. To remove unsolubilized microsomes, the solubilized microsomes were subsequently centrifuged at 200,000 x g for 60 min at 4 ^oC using a Beckman ultracentrifuge

(rotor Type 90Ti). The supernatant containing the solubilized microsomal protein was concentrated using the MWCO filters.

3.7.7 Size exclusion chromatography

Solubilized microsomal proteins (2 mg) were separated on the basis of size using a 7.8 x 300 mm BioSep 5 µm SEC3000 (Phenomenex) gel filtration column at room temperature or on ice. Using the Waters 2695 Alliance system, different mobile phases containing various phosphate buffer concentrations and detergents were tested using isocratic conditions to obtain optimal 5-HEDH elution and purification. Protein standards (10 µg), including bovine serum albumin (BSA; 68 kDa, ovalbumin; 44 kDa, and cytochrome c; 12.5 kDa), were injected separately to determine their retention times. The protein levels were monitored at 280 and 215 nm and fractions (1 ml) were collected using the automated Waters Fraction Collector II to test for 5-HEDH and 5-ketoreductase activity.

3.7.8 Diethylaminoethyl Sepharose

Diethylaminoethyl (DEAE) Sepharose (Sigma) was used to partially purify 5-HEDH using a batch method. DEAE Sepharose (3 mg) was weighed into a 15 ml Falcon tube and was washed and equilibrated according to the manufacturer's instructions. DEAE Sepharose was centrifuged at 2000 rpm (300 x g) for 2.5 min at 4 ^{o}C after each wash to remove and exchange buffers. Several binding and elution conditions were tested to optimize the purification of 5-HEDH. Prior to loading the protein, the stationary phase was equilibrated with Buffer A containing 6 mM CHAPS. Solubilized microsomal protein (~1.5 mg/ml DEAE) was then incubated with DEAE Sepharose for 5 min with continuous rotation at 4-7 ^{o}C to allow the proteins to bind. The tube was then centrifuged and the supernatant collected. The DEAE Sepharose was then washed 5 times with 11 ml of **Buffer B** (Buffer A with 20% glycerol) containing 6 mM CHAPS. 5-HEDH was eluted with 3 x 11 ml of 20 mM NaCl in Buffer B containing 30 mM octylglucoside. Any remaining bound protein was eluted with 2 x 11 ml of Buffer B containing 1 M NaCl and 30 mM octylglucoside.

3.7.9 Carboxymethyl Sepharose

Carboxymethyl (CM) Sepharose CL-6B (GE Healthcare Bio-Sciences Corp.), a weak cation exchanger, was equilibrated according to the manufacturer's instructions. Solubilized microsomes (450 µg) were incubated with CM Sepharose (500 µl) and the batch method was used to optimize 5-HEDH binding and elution conditions varying the buffers (Tris pH 8.2 and phosphate buffer pH 7.4), detergents (6 mM CHAPS and 30 mM octyglucoside) and NaCl (0.05 to 1 M) for elution .

3.7.10 Red Sepharose

Several 5-HEDH binding and elution conditions were tested using the red dye bound to Sepharose (Red Sepharose CL-6B; GE Healthcare Bio-Sciences Corp., Baie d'Urfé, QC). The batch method was used to optimize elution conditions. The freeze dried red Sepharose was hydrated in distilled water for 30 min, and then repeatedly washed with distilled water to remove any preservatives according to manufacturer's instructions. The binding and elution conditions to obtain optimal purification of 5-HEDH were tested, varying the ratio of protein to Sepharose, the time for binding (5 and 60 min), the detergents (CHAPS and octyglucoside), and the concentration of NaCl or $NADP^+$ for elution.

3.7.11 Blue Sepharose

Blue Sepharose CL-4B (GE Healthcare Bio-Sciences Corp.) was rehydrated, washed and equilibrated according to the manufacturer's instructions. All purification steps were carried out at 4 ^{o}C, and the blue Sepharose was centrifuged at 400 x g for 4 min to remove and exchange buffers. Blue Sepharose was saturated with distilled water, and then equilibrated with Buffer A containing 30 mM octylglucoside. Solubilized microsomes were incubated with blue Sepharose for 5 min while rotating. The tube was then centrifuged and the supernatant collected. The blue Sepharose was then washed sequentially with 1 x 11 ml Buffer B containing 30 mM octylglucoside, 2 x 11 ml Buffer B containing 0.8 M NaCl and 3 mM CHAPS, and 2 x 11 ml Buffer B containing 6 mM

CHAPS. 5-HEDH was eluted with 3 x 11 ml of 1.3 M NaCl and 6 mM CHAPS in Buffer B. Remaining bound protein was eluted with 2 x 11 ml 2 M NaCl and 6 mM CHAPS in Buffer B.

Fractions containing the highest 5-HEDH activity were combined and concentrated with 30 kDa MWCO Amicon centrifugal filters. The retentate was stored at -80 ^{o}C for further purification.

3.7.12 Hydroxylapatite

CHT hydroxylapatite (Bio-Rad Laboratories) was equilibrated according to the manufacturer's instructions. Solubilized microsomes (~1 mg) were incubated with hydroxylapatite (~250 mg) in a low concentration phosphate buffer (10 mM). The binding and elution conditions for 5-HEDH were optimized by varying the detergent (6 mM CHAPS and 30 mM octylglucoside) and buffer concentrations (10 to 400 mM phosphate buffer, pH 7.4).

3.7.13 Affinity chromatography

Several 5-HETE analogs, modified at the ω-end were synthesized by Dr. Joshua Rokach at the Florida Institute of Technology. A spacer was added, and the analogs were either attached to 1) biotin, subsequently bound to streptavidin, or 2) Affigel-10 (Bio-Rad Laboratories), a cross-linked agarose gel bead support derivatized with N-hydroxy-succinimide ester on a 10-atom long spacer arm. Lineweaver-Burk experiments were performed to determine whether the 5-HETE analogs were substrates for 5-HEDH. Serial dilutions of 5-HETE or the analogs (0.1 to 6 μM) were incubated with microsomes (50 μg) with 100 μM $NADP^{+}$ at 37 ^{o}C for 5 min and the amounts of 5-oxo-ETE or the 5-oxo product of the analog produced were measured by RP-HPLC.

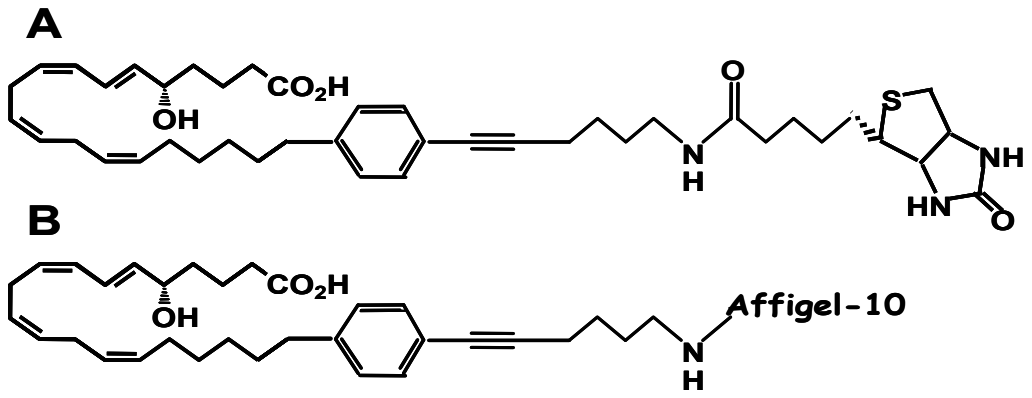


Figure 3.1: 5-HETE analogs used for affinity chromatography. A) The biotin-5-HETE analog and **B**) Affigel-5-HETE analog were synthesized by Dr. Joshua Rokach at the Florida Institute of Technology.

Experiments were performed on ice, with Affigel-5-HETE or biotin-5-HETE (100 µl – 300 µl) washed repeatedly with methanol, to remove isopropanol in which they were stored. They were then equilibrated with 5-HEDH binding buffer, before being incubated with DEAE purified fractions containing 5-HEDH, or solubilized microsomes (80 – 150 µg). Due to the hydrophobicity of the analogs, methanol (10 – 20 % (v/v)) or glycerol (10 % (v/v)), were added to all of the buffers. Numerous conditions for 5-HEDH binding were tested including pH (6.8, 7.4), detergents (15 mM or 30 mM octylglucoside or 3 mM or 6 mM CHAPS), temperature (6 – 8 ^{o}C or room temperature) and time (5 min to 60 min). In addition, similar conditions were tested to elute 5-HEDH, including adding NaCl (1 – 2 M), NADPH or $NADP^{+}$ (10 µM) or 5-HETE (1 – 10 µM).

3.7.14 Native gel electrophoresis/Blue native gel electrophoresis

As an additional attempt to purify 5-HEDH, solubilized microsomes or fractions containing partially purified 5-HEDH were loaded onto non-denaturing (native) gels to permit detection of 5-HEDH activity following electrophoresis. Electrophoresis was performed using the Mini-PROTEAN 3 (Bio-Rad) cell apparatus. The effects of adding octylglucoside(15 mM or 30 mM) or CHAPS (3 mM or 6 mM), and/or glycerol to the gels as well as several loading buffers

(detergent concentrations), and acrylamide gradients were evaluated. The protein samples were diluted (2:1) in sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 30% (v/v) glycerol, 0.01% (w/v) bromophenol blue with or without detergent (3-6 mM CHAPS or 10-40 mM octylglucoside). Protein samples were loaded onto 4% (w/v) stacking gels (1 or 1.5 mm thickness) and separated with resolving gels (4-16% (w/v) gradient or 7% (w/v) acrylamide) using 0.2 M Tris-HCl (pH 8.8) prepared with acrylamide-bisacrylamide (37.5:1) and polymerized with 0.1% (v/v) TEMED and 0.05% (w/v) APS. The samples were then electrophoresed at 4 ^{o}C for 2-3 hrs at 100 V in running buffer (25 mM Tris and 190 mM glycine, pH 8.3).

Alternatively, blue native gel electrophoresis (BN-PAGE), based on the technique developed by Schagger [220,221] to separate mitochondrial membrane complexes, was tested for separating active 5-HEDH from other microsomal proteins. In an attempt to optimize the separation of proteins, several gel concentrations and acrylamide gradients (4-18%) were also evaluated. Concentrated solubilized microsomes or fractions purified with DEAE or blue Sepharose were mixed with sample buffer (37.5 mM ε-aminocaproic acid, 0.25% Coomasie Blue G-250). A 4% acrylamide stacking gel and a gradient of 4-18% acrylamide resolving gel containing 500 mM ε-aminocaproic acid, 50 mM Bis-Tris (pH 7.0) were polymerized with 0.1% (v/v) TEMED and 0.1% (w/v) APS. The samples were electrophoresed using blue cathode buffer (50 mM Tricine, 15 mM Bis-Tris, 0.02% Coomassie Blue G-250, pH 7.0) and anode buffer (50 mM Bis-Tris, pH 7.0) at 4 ^{o}C at 100 V. Once the blue sample running front was one-third to one-half of the total running distance, the blue cathode buffer was exchanged for a colourless cathode buffer (50 mM Tricine, 15 mM Bis-Tris, pH 7.0), and the electrophoresis was continued. The gel is destained during the rest of the run and the protein bands are easily detected.

The gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories) or using the ProteoSilver^{TM} Silver Stain Kit (Sigma). For staining with Coomassie Blue, the gel was washed twice in distilled water, then stained in 0.025% (w/v) Coomassie Blue dissolved in 40% (v/v) methanol and 7% (v/v)

acetic acid shaking for 30 min to overnight at room temperature. The gel was then destained in 40% methanol containing 7% acetic acid for 30 min and then destained until the background was clear in a 5% methanol solution containing 7% acetic acid. A picture of the gels was captured using a FluorChem 8000 imaging system and AlphaEase FC software, version 3.1.2 (Alpha Innotech Corporation, San Leandro, CA).

3.7.15 In-gel 5-HEDH activity assay

Samples separated by native electrophoresis were loaded onto two lanes and one lane was stained with Coomassie blue or silver stain and the other lane was used to determine the distribution of 5-HEDH activity. Ten equal sized gel bands were excised from the second lane and placed into tubes containing 500 µl of 15 mM octylglucoside and incubated with 2 µM 5-HETE and 100 µM $NADP^+$ for 60 min.

A formazan precipitation assay, used to detect $NADP^+$-dependent enzymatic activity [222], was evaluated for its use in detecting 5-HEDH activity in the intact gels (Figure 3.2). Intact gel lanes containing solubilized microsomes containing 5-HEDH activity were cut from the gel and washed with 20 mM phosphate buffer (pH 7.4) buffer for 10 min at room temperature. Fresh phosphate buffer was added and the reaction started by the addition of 100–500 µM $NADP^+$, 40-200 µg/ml PMS, 10-100 µM 5-HETE, and either 0.4 mg/ml iodonitrotetrazolium (INT) or 0.4 mg/ml nitroblue tetrazolium (NBT). The gel was incubated for 20 min to 2 hrs with shaking in the dark at room temperature until a precipitate was observed. Due to limited availability of 5-HETE, the method was optimized and tested using cytosolic protein isolated from PMA-differentiated U937 cells and testing for glucose-6-phosphate dehydrogenase activity following addition of the substrate glucose-6-phosphate instead of 5-HETE. Duplicate lanes were stained with Coomassie Blue.

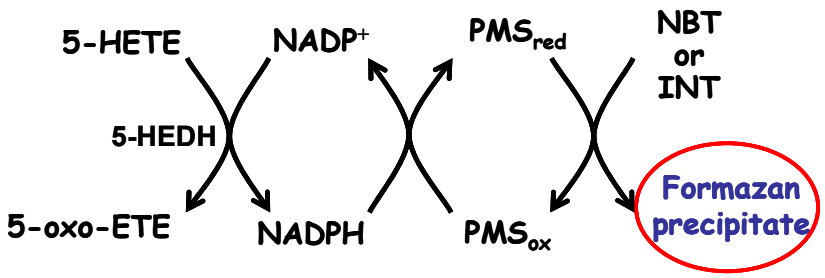


Figure 3.2: Formazan precipitation assay for 5-HEDH activity. 5-HEDH converts 5-HETE to 5-oxo-ETE in the presence of $NADP^+$. PMS converts the NADPH produced back to $NADP^+$. Reduced PMS then interacts with tetrazolium salt (NBT or INT) which is converted to a blue insoluble formazan precipitate.

3.7.16 SDS-Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was used to separate membrane proteins in the purified fractions by their size. For samples to be analyzed by LC-MS/MS, prior to loading the purified 5-HEDH protein fractions on the gel, the fractions were concentrated further using a 10 kDa MWCO filter in addition to exchanging the detergent for 10 µM surfactin. The protein samples (30 µg) were diluted (2:1) in Laemmli sample buffer [223] containing 62.5 mM Tris-HCl (pH 6.8), 25% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, and 5% (v/v) β-mercaptoethanol and heated to 37 ^oC for 30 min. Denatured samples were then loaded onto a 6% (w/v) acrylamide stacking gel (125 mM Tris-HCl pH 6.8) with a 10% acrylamide resolving gel (375 mM Tris-HCl pH 8.8) containing 0.1% SDS prepared with acrylamide-bisacrylamide (37.5:1) and electrophoresed at room temperature for 2 h at 120 V in running buffer (25 mM Tris, 190 mM glycine and 0.1% SDS, pH 8.3). The medium size PageRuler^{TM} unstained protein ladder (Fermentas, Burlington, ON) was loaded onto the gel and used as size standards. The gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories) or using the ProteoSilver^{TM} Silver Stain Kit (Sigma).

2D electrophoresis, performing denaturing SDS-PAGE after native or BN-PAGE was also evaluated. The proteins in the native gel lane were denatured by

soaking the gel in 0.5 M Tris HCl pH 6.8 containing 1% (v/v) SDS and 1% (v/v) β-mercaptoethanol for 30 min at room temp. The native gel lane or individual bands (1 mm thickness) were then placed on the SDS stacking gel and electrophoresed using the same conditions as the SDS-PAGE described above and subsequently stained.

3.8 Identification of potential candidate proteins for 5-HEDH

3.8.1 In-gel digestion of proteins from the SDS-PAGE gel

Ten protein bands of approximate equal size (1-1.5 mm) were excised from the SDS-PAGE gel (stained with Coomassie Brilliant Blue R-250) between 25 kDa and 70 kDa according to the molecular weight markers. The modified Mann protocol was used for the in-gel digestion of proteins prior to mass spectrometric analysis [224]. This includes reducing the protein disulfide bonds and alkylating the cysteines prior to their trypsinization.

Briefly, the excised bands were washed successively with water, 50% acetonitrile, 100% acetonitrile, and 100 mM ammonium bicarbonate (Solution 2), after which they were dried in a speed-vac. The protein disulfide bonds were then reduced with dithiothreitol (DTT; 10 mM) in Solution 2 at 56 ^{o}C for 45 min, and the free sulfhydryl groups were subsequently alkylated with 55 mM iodoacetamide in Solution 2 at room temperature in the dark. The samples were washed with Solution 2 and then incubated with a 50% acetonitrile in Solution 2 and dried in a speed-vac. Subsequently the samples were digested with 13 ng/µl trypsin in 10mM ammonium bicarbonate containing 10% (v/v) acetonitrile) on ice for 45 min after which they were incubated with the same solution without trypsin overnight at 37 ^{o}C. The samples were acidified with 2% TFA for 2 min, and the supernatant was removed and saved. The peptides in the gel slices were recovered by successive extractions by sonication (30 min) with 0.1 % TFA in water, 0.1% TFA/30% acetonitrile and 0.1% TFA/60% acetonitrile. The supernatants were combined with the 2% TFA supernatant, and dried in a speed-vac and reconstituted in the initial mobile phase.

3.8.2 LC-MS/MS

To sequence and identify the proteins in the purified fractions, the digested peptides were separated by nanoflow chromatography and analyzed by MS/MS (performed by Dr Bernard Gibbs at the Sheldon Biotechnology Centre, McGill University, Montreal, QC) [225]. The trypsin digested peptides were injected and desalted using an Isocratic Agilent 1100 series pump (Agilent Technologies Canada Inc.; Mississauga, ON) on a 5 x 0.3 mm Zorbax 300SB-C18 column (Agilent) with a flow rate of 15 mL/min for 10 min. Subsequently, the digested peptides were separated using an Agilent 1100 series nanopump on a 10 x 0.075 mm Biobasic C18 PicoFrit column (New Objective, Woburn, MA, USA) at a flow rate of 200 nl/min. Peptides were eluted using a gradient with solvent A (0.1% FA) and solvent B (95% acetonitrile: 0.1% FA) and analyzed with a QTRAP 4000 MS/MS system (Applied Biosystems/MDS Sciex Canada, Streetsville, ON).

Enhanced MS scans were acquired between 350–1500 m/z with a source voltage of 2075 and scan speed of 4000 amu/s and active dynamic fill time. Information-dependent MS/MS analysis was performed on the three most intense multiply charged ions; a dynamic exclusion of 120 sec was used to limit resampling of previously selected ions to two events. MS/MS scans were acquired between 70–1700 m/z at a scan speed of 4000 amu/s. Fixed fill time was set at 25 ms with Q0 trapping and rolling collision energy of $\pm$ 3 eV. Three scans were averaged and peak lists were generated with MASCOT (Matrix Science Inc., Boston, MA, USA) script version 16 from Analyst 1.4.1 software (Applied Biosystems/MDS Sciex Canada). Spectral processing included peak smoothing and centroiding without deisotoping.

3.8.3 Identification and analysis of peptide sequences

Database searches (Swiss-Prot) were performed with MASCOT 1.9 using carbamidomethyl (CAM) as a fixed modification and methionine oxidation as a variable modification. Proteins were identified from peptide sequences available for all species.

3.9 Data analysis

Results are presented as means ± S.E. The Hill equation was used to calculate EC_{50} values. Paired t-tests were used for the comparison of vehicle to H_2O_2-treated B cells incubated with 5-HETE as well as for the PC3 cells cocultured with neutrophils. A $p < 0.05$ was considered significant and "n" refers to the number of independent experiments performed. A different donor was used for each tonsillar B cell experiment. Purification of 5-HEDH was assessed by determining specific activity (pmol product/min/μg protein).

Chapter 4: Results

4.1 Synthesis of 5-oxo-ETE in B cells

4.1.1 B lymphocytes synthesize 5-oxo-ETE

To determine whether B cells possess 5-HEDH activity, CESS B cells were incubated with 5-HETE (1 μM) for 10 min in PBS^+ and the products were analyzed by RP-HPLC (Figure 4.1A). 5-HETE (t_R - 5.1 min, detected at 235 nm) was converted to a single product (t_R – 5.7 min) with a maximum absorbance at 280 nm. The UV spectrum of this product was identical to that of 5-oxo-ETE (max – 280 nm) and co-eluted with pure 5-oxo-ETE (data not shown). Human tonsillar B cells as well as the B cell lines, Ramos and U266, also synthesized 5-oxo-ETE when incubated with 5-HETE (1 μM). CESS cells had the highest capacity, synthesizing 96 ± 26 pmol 5-oxo-ETE/10^6 cells, while U266 cells synthesized only 7.1 ± 0.3 pmol 5-oxo-ETE/10^6 cells (Figure 4.1C). Isolated tonsillar B cells and Ramos cells synthesized similar levels of 5-oxo-ETE (28 ± 5 pmol/10^6 cells and 23 ± 5 pmol/10^6 cells, respectively (Figure 4.1C)).

4.1.2 Oxidative stress enhances 5-oxo-ETE synthesis from 5-HETE in B cells

Hydrogen peroxide (H_2O_2) was added to the cells at the same time as 5-HETE to determine whether oxidative stress stimulates 5-oxo-ETE production in these cells. H_2O_2 (100 μM) appreciably stimulated the production of 5-oxo-ETE from 5-HETE after 10 min in CESS cells (Figure 4.1B) as well as all of the B cells investigated (Figure 4.1C).

All further experiments determining the regulation of 5-oxo-ETE synthesis in B cells were only done with CESS cells and tonsillar B cells to compare an immortalized cell line with primary cells.

Although tonsillar B cells have a much lower capacity to synthesize 5-oxo-ETE compared to CESS cells, H_2O_2 was a more potent stimulator of 5-oxo-ETE production in tonsillar B cells with an EC_{50} of 1.0 ± 0.1 μM whereas the EC_{50} was 10.3 ± 2.3 μM in CESS cells (Figure 4.2). The maximal response to H_2O_2 was observed at 10 μM in tonsillar B cells at approximately 100 μM to 315

μM in CESS cells. Cells were pre-incubated (10 min) with 250 μM BCNU, a glutathione reductase (GR) inhibitor, to determine whether the H_2O_2-stimulated increase in 5-oxo-ETE production was due to activation of the glutathione redox cycle. BCNU blocked the increase in 5-oxo-ETE synthesis in both CESS cells and tonsillar B cells stimulated with H_2O_2 concentrations up to 315 μM (Figure 4.2).

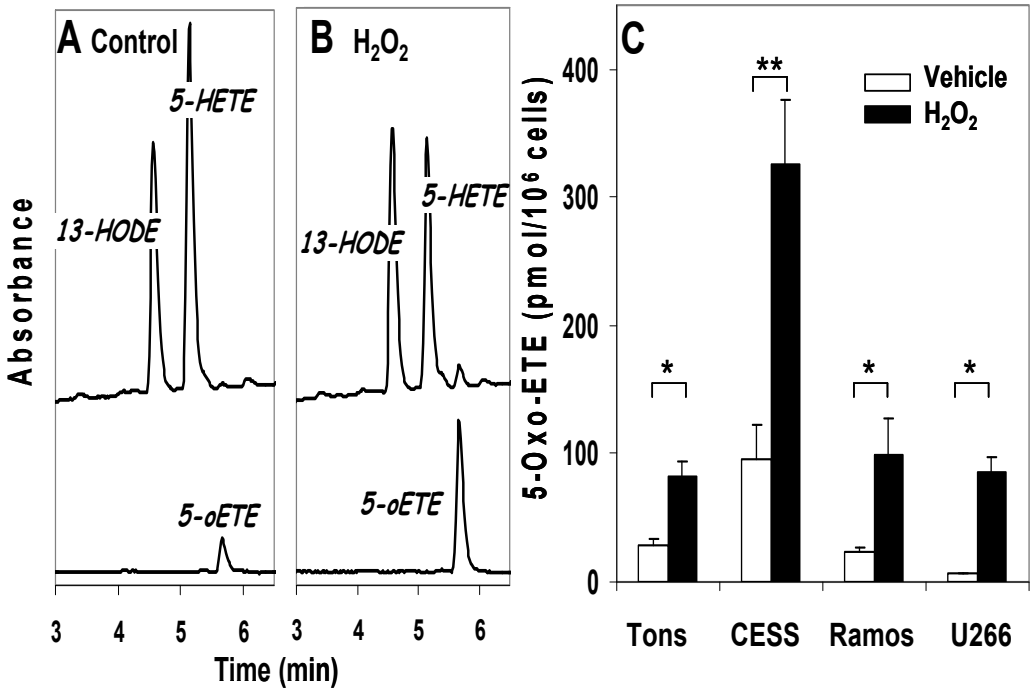


Figure 4.1: 5-oxo-ETE synthesis from 5-HETE in B cells is stimulated by H_2O_2. Representative RP-HPLC chromatograms of 5-oxo-ETE synthesized by CESS cells (1 x 10^6 cells) incubated for 10 min in PBS^+ with 5-HETE alone (**A**) or with 100 μM H_2O_2 (**B**). The internal standard, 13-HODE (100 ng), was added to the samples before analysis by HPLC to enable quantitation of 5-HETE and 5-oxo-ETE. **C**) H_2O_2 stimulates 5-oxo-ETE synthesis in Ramos cells, U266 cells, CESS cells and primary tonsillar B cells compared to controls. B cells (2 x 10^6 cells/ml) were incubated with 1 μM 5-HETE with vehicle (open bars; □) or with H_2O_2 (100 μM, filled bars; ■) for 10 min in PBS^-. (n = 4 for all cells except n = 3 for Ramos cells) Values presented are mean ± S.E. **, $p < 0.005$; *, $p < 0.05$

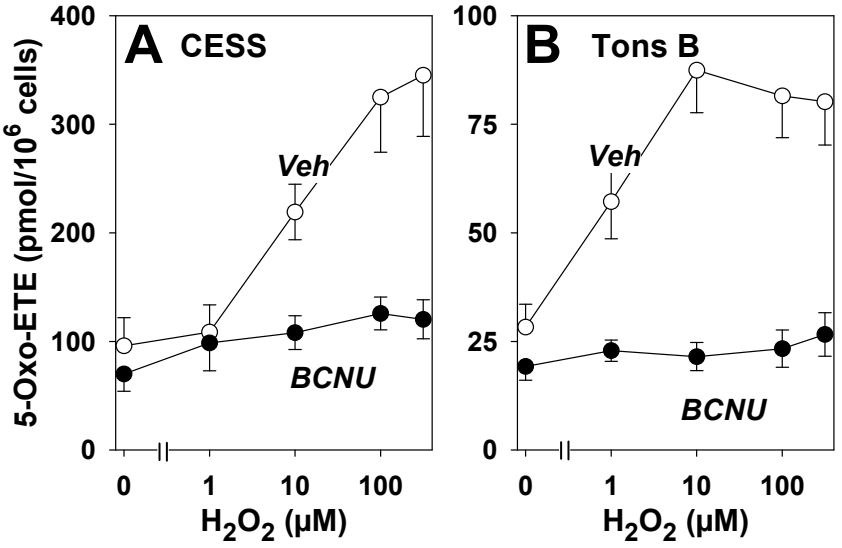


Figure 4.2: H_2O_2 concentration-dependently stimulates 5-oxo-ETE production in B cells and this effect is blocked by BCNU. A) CESS cells and **B**) isolated tonsillar B cells were preincubated (10 min) with vehicle (○) or BCNU (250 μM; ●) before adding 5-HETE (1 μM) and increasing concentrations of H_2O_2 (0, 1, 10, 100, or 315 μM) for 10 min in PBS^+. The cells were incubated at a concentration of 2 x 10^6 cells/ml. Values presented are mean ± S.E. (CESS; n = 4, tonsillar B cells; n = 5)

4.1.3 Metabolism of arachidonic acid by B cells stimulated with H_2O_2

B cells stimulated with exogenous AA and A23187 were previously shown to synthesize 5-HETE and LTB_4 [43,45]. Therefore, we wanted to determine whether AA and A23187 stimulated cells would synthesize 5-oxo-ETE as well. Tonsillar B cells and CESS cells (5 x 10^6 cells) were incubated with 50 μM AA and 5 μM calcium ionophore (A23187) with and without 100 μM H_2O_2 for up to 30 min. As shown in Figure 4.3A, CESS cells incubated with exogenous AA and A23187 synthesized low levels of 5-oxo-ETE, in addition to LTB_4, 5-HETE and 12-HHT. Stimulating the cells with H_2O_2 for 20 minutes induced a dramatic increase in 5-oxo-ETE production (Figure 4.3B). The levels of AA metabolites synthesized by CESS cells reached a maximum at around 5 minutes (Figure 4.4A), and the levels of 5-oxo-ETE (3.2 ± 0.8 pmol/10^6 cells) were similar

to the levels of LTB_4 (4.1 ± 1.4 pmol/10^6 cells) (Figure 4.4A). The levels of 12-HHT, a COX metabolite of AA, were higher than all of the 5-LO metabolites synthesized.

H_2O_2 selectively stimulated the production of 5-oxo-ETE (7-fold), and induced lower increases in 5-HETE (2-fold) and LTB_4 (2-fold) production from AA in CESS cells (Figure 4.4B). The levels of 5-oxo-ETE reached a maximum at 5 min (22 ± 2 pmol 5-oxo-ETE/10^6 cells), and then slowly declined over 25 min. The levels of 5-HETE were still higher than 5-oxo-ETE and reached a maximum at 2.5 min (31 ± 6 pmol 5-HETE/10^6 cells) (Figure 4.4B). The levels of LTB_4 increased from 4.1 ± 1.4 pmol/10^6 cells in vehicle-treated cells to 9.2 ± 1.2 pmol/10^6 cells in H_2O_2-treated cells in 5 min (Figure 4.4B). Conversely, H_2O_2 had no effect on 12-HHT levels.

To determine whether direct stimulation of the glutathione redox cycle could mimic the effect of H_2O_2 on 5-oxo-ETE production, CESS cells were treated with diamide (250 μM), which non-enzymatically converts reduced glutathione (GSH) to the disulfide (GSSG). This results in elevated $NADP^+$ levels as GSSG is converted back to GSH by glutathione reductase, an NADPH-dependent enzyme. Diamide stimulated an even greater increase (10-fold) in 5-oxo-ETE levels than H_2O_2, peaking at 5 min (31 ± 4 pmol 5-oxo-ETE/10^6 cells) which then slowly decreased over the next 25 min (Figure 4. 4C). The levels of 5-HETE and LTB_4 in cells stimulated with diamide were comparable with the levels in cells stimulated with H_2O_2. Diamide stimulated a slight increase in 12-HHT levels (Figure 4.4C).

Tonsillar B cells have lower 5-LO activity than CESS cells and therefore, when stimulated with AA and A23187 synthesized lower levels of 5-oxo-ETE compared to CESS cells (Figure 4.4D). The difference was even greater with tonsillar B cells stimulated with H_2O_2 or diamide compared to CESS cells. In addition, the synthesis of 5-oxo-ETE was much slower than in CESS cells. The levels of 12-HHT (1.8 ± 0.5 pmol/10^6 cells) were similar to 5-oxo-ETE (2.0 ± 0.3 pmol/10^6 cells) and less than 5-HETE in vehicle-treated tonsillar B cells. This is in contrast to the high levels of 12-HHT synthesized in CESS cells. However,

similar to CESS cells, H_2O_2 significantly stimulated 5-oxo-ETE synthesis (4-fold) in tonsillar B cells to 8.3 ± 1.4 pmol 5-oxo-ETE/10^6 cells plateauing after 10 min (Figure 4.4E). H_2O_2 stimulated 5-HETE synthesis to a lesser extent (6.8 ± 1.6 pmol/10^6 cells), whereas, LTB_4 synthesis was increased 4-fold to 3.5 ± 0.5 pmol LTB_4/10^6 cells compared to vehicle after 10 min. Similar to CESS cells, 12-HHT levels were not affected by H_2O_2 stimulation of tonsillar B cells.

Similar to CESS cells, diamide stimulated increased 5-oxo-ETE production, however, the levels increased continually over 30 min to 16 ± 3 pmol 5-oxo-ETE/10^6 cells (Figure 4.4F). Diamide also stimulated an initial increase in 5-HETE production to a greater extent than H_2O_2 to 14 ± 3 pmol 5-HETE/10^6 cells after 5 minutes, after which the levels decreased. Diamide had a similar effect to H_2O_2 on LTB_4 levels. 12-HHT levels were not affected by diamide.

The maximal response to H_2O_2 for all of the 5-LO products measured was observed at 100 μM in both CESS cells and tonsillar B cells (Figure 4.5A and B). H_2O_2 was a similarly potent stimulator of 5-oxo-ETE production in tonsillar B cells (EC_{50} of 16 ± 5 μM) and CESS cells (EC_{50} = 22 ± 2 μM) (Figure 4.5A and B).

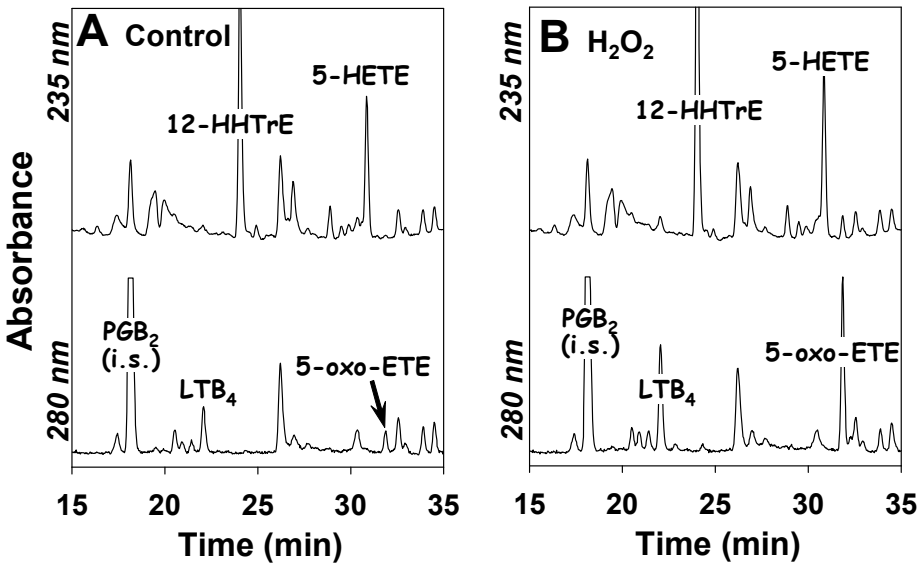


Figure 4.3: Effects of H_2O_2 on 5-oxo-ETE synthesis in B cells incubated with exogenous AA and A23187. Representative RP-HPLC chromatograms of CESS cells (5 x 10^6 cells) incubated with AA (50 μM) and A23187 (5 μM) in PBS^+ with vehicle (**A**) or 100 μM H_2O_2 (**B**) for 20 min. The internal standard, PGB_2 (100 ng) was added prior to analysis by RP-HPLC. The levels of LTB_4, 12-HHTrE, 5-HETE and 5-oxo-ETE were quantitated by RP-HPLC. Internal standard (i.s.) is PGB_2.

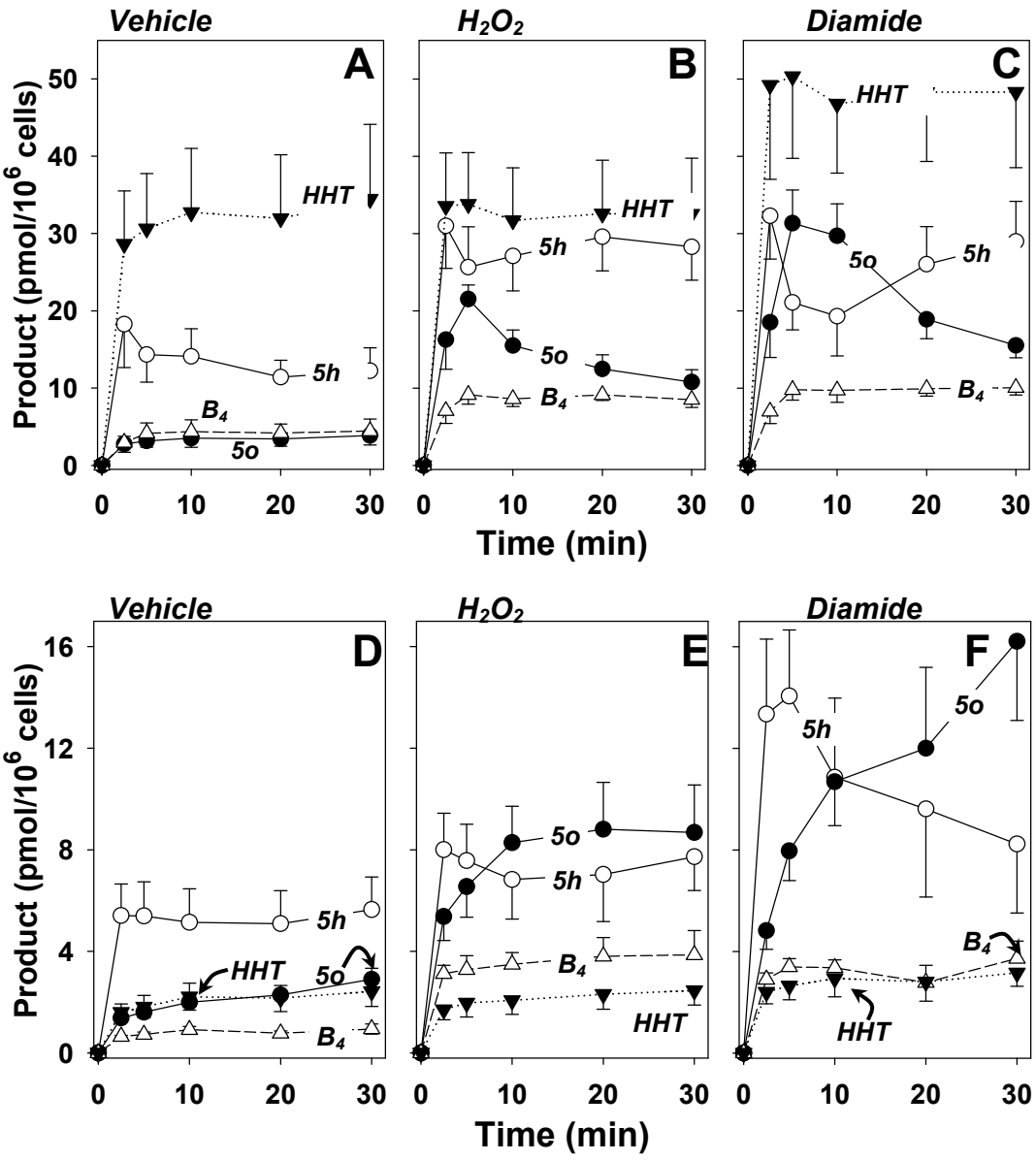


Figure 4.4: Time course for the effect of H_2O_2 and diamide on the formation of eicosanoids by CESS cells and tonsillar B cells incubated with AA and A23187. CESS cells (**A-C**) or tonsillar B cells (**D-F**) (10 x 10^6 cells/ml) incubated with AA (50 μM) and A23187 (5 μM) were treated with vehicle (**A,D**), 100 μM H_2O_2 (**B,E**) or 250 μM diamide (**C,F**) for 2.5, 5, 10, 20 and 30 min in PBS^+. 5-oxo-ETE (5o; ●), 5-HETE (5h; ○), LTB_4 (B_4; Δ), 12-HHT (HHT; ▼) were quantitated by RP-HPLC. Values are mean ± S.E. (CESS; n = 6, tonsillar B cells; n = 5)

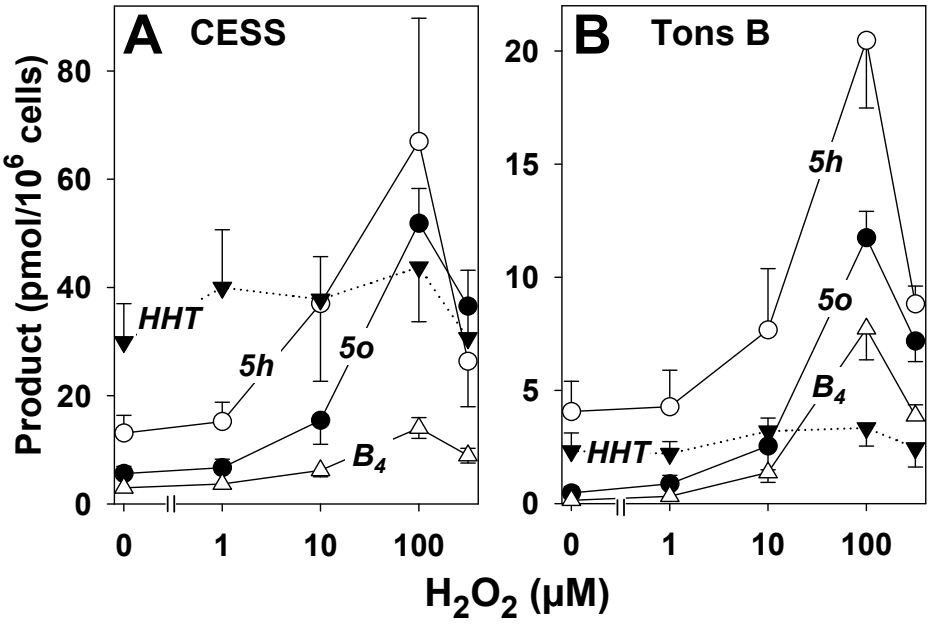


Figure 4.5: H_2O_2 concentration dependently stimulates 5-oxo-ETE synthesis from AA and A23187 in B cells. A) CESS cells and **B**) isolated tonsillar B cells (2 x 10^6 cells/ml) were incubated with AA (50 μM) and A23187 (5 μM) and stimulated with H_2O_2 (0, 1, 10, 100, or 315 μM) for 5 min in PBS^+. 5-oxo-ETE (5o; ●), 5-HETE (5h; ○), LTB_4 (B_4; Δ), 12-HHT (HHT; ▼) were quantitated by RP-HPLC. Values are mean ± S.E. (CESS; n = 4, tonsillar B cells; n = 3)

4.1.4 Effects of H_2O_2 on GSH and GSSG levels in B cells

The glutathione redox cycle appears to be involved in the H_2O_2-induced synthesis of 5-oxo-ETE in B cells, since BCNU, the glutathione reductase inhibitor, blocked the stimulation of 5-oxo-ETE production (Figure 4.2). Additionally, diamide, which non-enzymatically converts GSH to GSSG, had an even greater stimulatory effect than H_2O_2 on 5-oxo-ETE production. Therefore, we measured the intracellular levels of GSH and GSSG by RP-HPLC in B cells (2 x 10^6 cells/ml) stimulated with H_2O_2 for up to 20 min to determine whether they were affected by H_2O_2.

The basal levels of GSH were much lower in tonsillar B cells (0.29 ± 0.04 nmol/10^6 cells) compared to CESS cells (4.6 ± 1.1 nmol/10^6 cells). However, H_2O_2 induced a rapid increase in GSSG levels in both types of cells. The increase

in GSSG levels reached a maximum between 30 and 90 sec with a concurrent decrease in GSH for both cell types (Figure 4.6A and B). The levels of both GSH and GSSG subsequently returned slowly towards baseline. This process was more rapid in CESS cells, since the level of GSSG was only 63% the maximum in tonsillar B cells compared to 29% in CESS cells after 20 min.

In CESS cells, the concentration-response curve for H_2O_2 was determined after 90 sec since this was when GSSG levels were maximal. The concentration response curves were similar to those for H_2O_2-induced 5-oxo-ETE production, and the maximal conversion of GSH to GSSG was observed with approximately 100 μM H_2O_2 (Figure 4.6C and D). The tonsillar B cell GSSG levels were less sensitive to H_2O_2 than 5-oxo-ETE production from exogenous AA. The EC_{50} was 12 ± 2 μM for tonsillar B cells (compared to an EC_{50} of 1.0 ± 0.1 μM for 5-oxo-ETE synthesis) and 15 ± 2 μM for CESS cells.

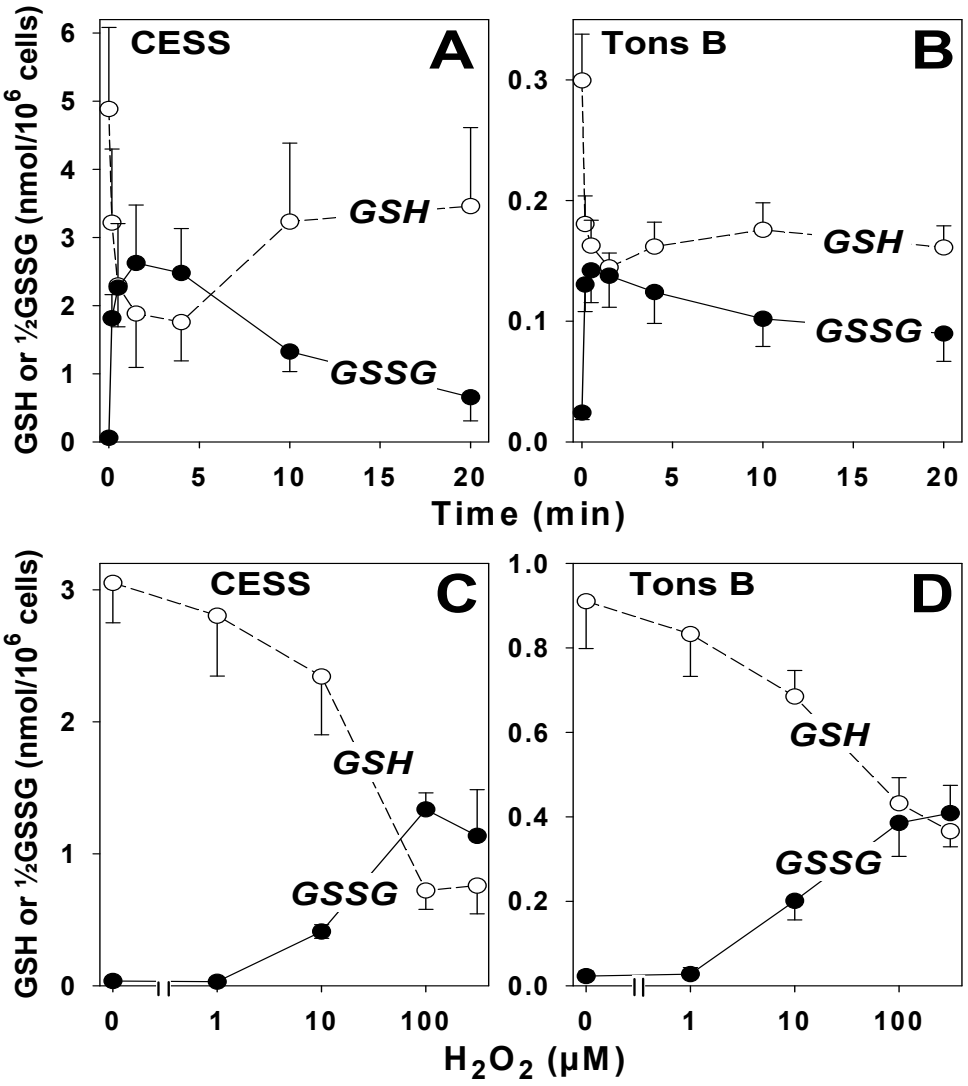


Figure 4.6: H_2O_2 concentration dependently induced rapid increases in GSSG levels in CESS cells and tonsillar B cells. The effect of H_2O_2 on GSSG and GSH levels over time was measured by RP-HPLC in CESS cells (**A)** and tonsillar B cells (**B**). The cells (2 x 10^6 cells/ml) were incubated with 100 μM H_2O_2 for up to 20 min. (CESS; n = 5, tonsillar B cells; n = 4) The concentration-response for H_2O_2 (0, 1, 10, 100, and 315 μM) treatment on GSSG and GSH levels after 90 sec was determined in CESS cells (**C**) and tonsillar B cells (**D**). GSSG (●) and GSH (○) were quantitated by RP-HPLC followed by post-column derivatization. (n = 3 for concentration-response curves). Values presented are mean ± S.E.

4.1.5 Effects of H_2O_2 on $NADP^+$ and NADPH levels

$NADP^+$, the cofactor for 5-oxo-ETE production by 5-HEDH, is produced during the glutathione redox cycle as GSSG is converted back to GSH by glutathione reductase (GR) which uses NADPH as a cofactor. To determine whether changes in these cofactors could explain the effects of H_2O_2 on 5-oxo-ETE synthesis we measured their levels by HPLC. $NADP^+$ levels increased rapidly after the cells were stimulated with H_2O_2, and reached 87% and 81% of their maximum by 10 sec in CESS cells and tonsillar B cells, respectively (Figure 4.7A and B). After 30 sec, the $NADP^+$ levels started to decrease in both types of cells, with a slower decrease observed in tonsillar B cells (Figure 4.7B). Similar to the resting levels of GSH, the basal levels of NADPH were approximately 10-fold higher in CESS cells (183 ± 31 pmol/10^6 cells) than in tonsillar B cells (19 ± 4 nmol/10^6 cells). This was also true for the resting levels of $NADP^+$ which was 19 ± 6 nmol/10^6 cells in CESS cells and only 3.4 ± 0.7 nmol/10^6 cells in tonsillar B cells. The concentration-response curve for H_2O_2 was measured after 90 sec (Figure 4.7C and D), and H_2O_2 more potently stimulated the conversion of NADPH to $NADP^+$ in tonsillar B cells (EC_{50}; 1.9 ± 0.2 μM) compared to CESS cells (EC_{50}; 9.7 ± 3.2 μM).

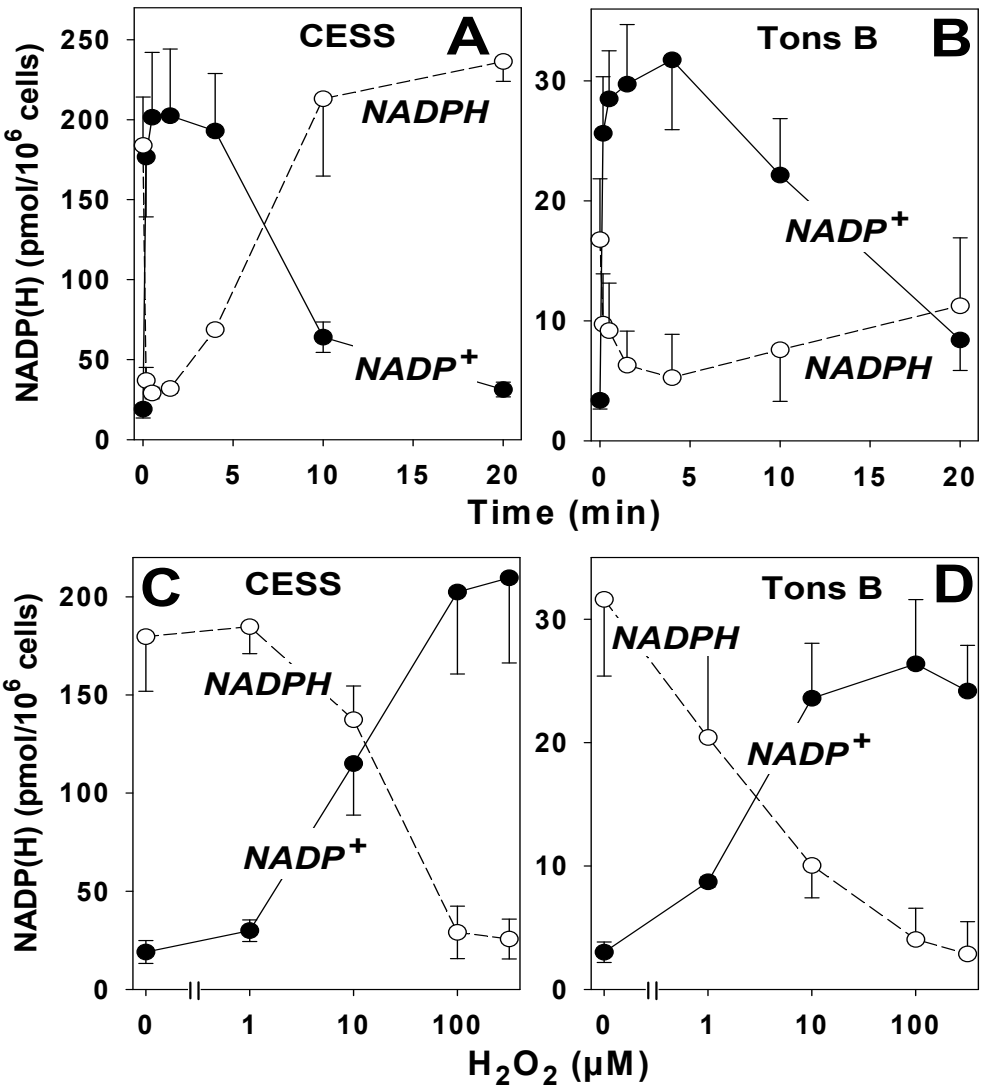


Figure 4.7: H_2O_2 concentration dependently induced rapid increases in $NADP^+$ levels in CESS cells and tonsillar B cells. The effect of H_2O_2 on $NADP^+$ and NADPH levels over time was measured by RP-HPLC in CESS cells (**A**) and tonsillar B cells (**B**). The cells (8 x 10^6 cells/ml) were incubated with 100 μM H_2O_2 for up to 20 minutes. (CESS; n = 5 (NADPH), n = 4 ($NADP^+$), tonsillar B cells; n = 3 (NADPH), n = 3 ($NADP^+$)) The concentration-response for H_2O_2 (0, 1, 10, 100, and 315 μM) treatment (90 sec) on $NADP^+$ and NADPH levels were determined in CESS cells (**C**) and tonsillar B cells (**D**). $NADP^+$ (●) and NADPH (○) were quantitated by RP-HPLC. Values are mean ± S.E. (n = 3 for concentration-response curves)

4.1.6 OXE receptor mRNA expression in B cells

To determine whether B cells express the receptor for 5-oxo-ETE, OXE-R, real time-PCR was carried out with unstimulated tonsillar B cells and cells activated with ant-CD40 and IL-4. The mRNA for OXE-R was present in all samples, and seemed to be elevated in anti-CD40/IL-4 activated B cells compared to unstimulated cells (Figure 4.8). However, there was greater variability of expression levels in the activated cells.

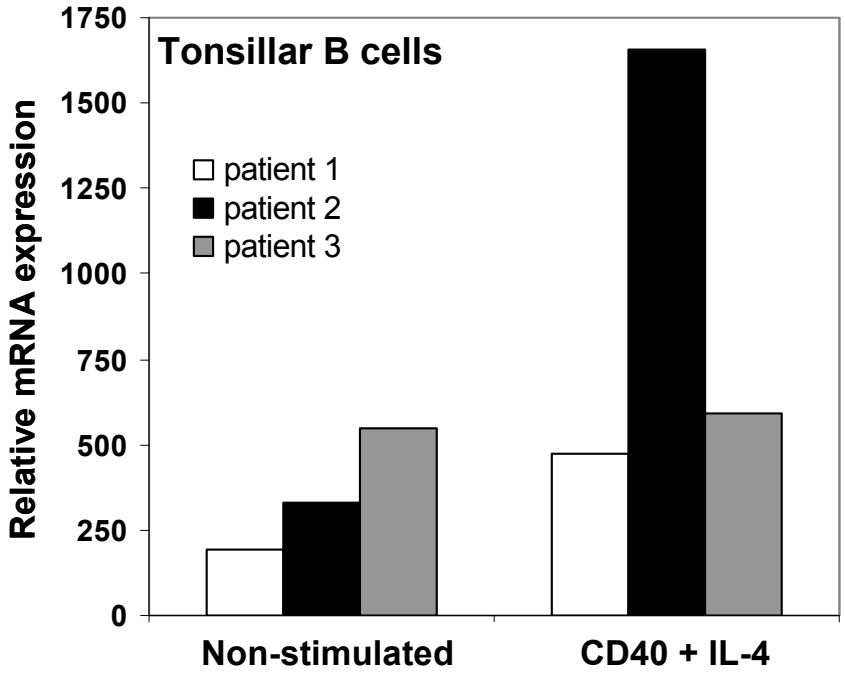


Figure 4.8: Normal and anti-CD40/IL-4 activated human tonsillar B cells express OXE-R mRNA. Real-time PCR of cDNA from tonsillar B cells from three patients was performed using primers specific for OXE-R as described in Materials and Methods (Chapter 3, pg 57). Results are expressed as the ratio of tonsillar B cell OXE-R mRNA over the expression of the housekeeping gene, β_2-microglobulin.

4.2 Synthesis of 5-oxo-ETE and its regulation in cancer epithelial cells

4.2.1 Prostate cancer cells (PC3) synthesize 5-oxo-ETE

5-Oxo-ETE has been shown by several groups to promote prostate cancer cell survival [77,80,168]. To determine whether these cells express 5-HEDH and were able to synthesize 5-oxo-ETE from 5-HETE, prostate cancer (PC3) epithelial cells (3×10^5 cells/well) were incubated with 5-HETE (2 μM) in PBS^+ over 60 min. PC3 cells synthesized low levels of 5-oxo-ETE (10 min; 0.08 ± 0.03 nmol/10^6 cells) which slowly increased after 60 min (0.26 ± 0.03 nmol/10^6 cells) (Figure 4.9A).

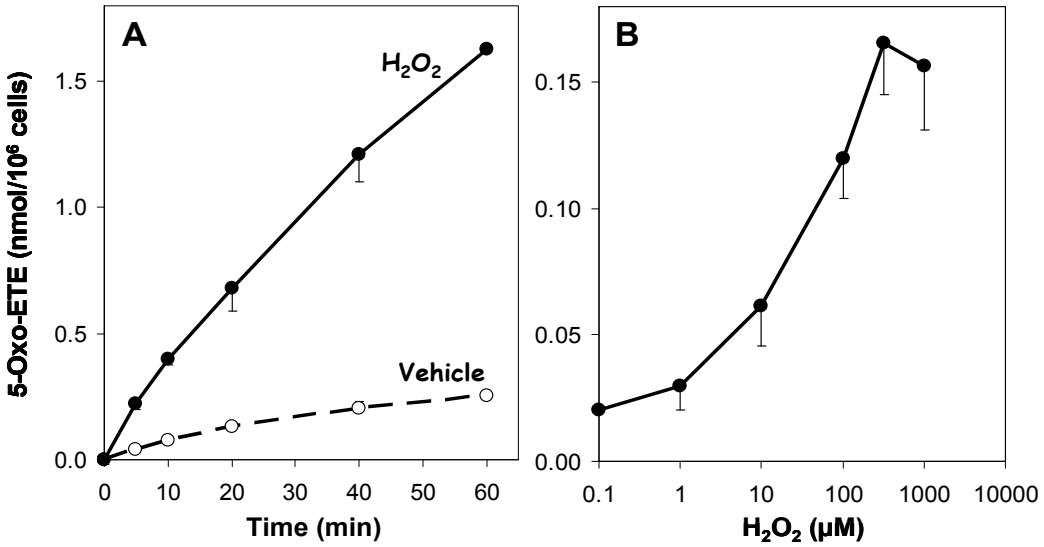


Figure 4.9: H_2O_2 induces an increase in 5-oxo-ETE production from 5-HETE in PC3 cells. A) PC3 cells (3×10^5 cells/well) were incubated with 5-HETE (2 μM) and stimulated with vehicle (○) or H_2O_2 (100 μM; ●) for 5 to 60 min in PBS^+. (n = 5) **B**) The concentration response (0, 1, 10, 100, 315 and 1000 μM) for H_2O_2 treatment (10 min) on 5-oxo-ETE production was determined. (n = 3) 5-Oxo-ETE was analyzed by RP-HPLC. Values presented are mean ± S.E.

4.2.2 Oxidative stress enhances the metabolism of 5-HETE by PC3 cells

The effect of oxidative stress on the synthesis of 5-oxo-ETE was determined by stimulating the cells with H_2O_2 (100 μM) for up to 60 min. H_2O_2 induced a steady increase in 5-oxo-ETE synthesis (1.6 ± 0.4 nmol/10^6 cells) compared to vehicle-treated cells (0.26 ± 0.03 nmol/10^6 cells) after 60 min (Figure 4.9A). The effect of H_2O_2 on 5-oxo-ETE synthesis was concentration dependent inducing maximal effects at 315 μM, with an EC_{50} of 38 ± 3 μM after measured at 10 min (Figure 4.9B).

4.2.3 Effects of H_2O_2 on GSH, GSSG and $NADP^+$ levels in PC3 cells

The intracellular GSH and GSSG levels in PC3 cells (3 x 10^5 cells/well) stimulated with H_2O_2 (100 μM) were measured by RP-HPLC. The levels of GSH (40 ± 2 nmol/10^6 cells) decreased rapidly in H_2O_2-treated cells reaching a minimal level by 7 minutes (5.4 ± 0.6 nmol/10^6 cells) (Figure 4.10A). A concurrent rapid increase in GSSG levels from 0.12 ± 0.01 nmol/10^6 cells to 27 ± 2 nmol/10^6 cells by 7 min was observed.

The basal levels of $NADP^+$ in PC3 cells, measured by RP-HPLC, were 22 ± 4 pmol/10^6 cells. H_2O_2 stimulated an initial rapid increase in $NADP^+$ to 231 ± 16 pmol/10^6 cells within 1 min, after which the levels continued to increase up until 20 min (610 ± 66 pmol/10^6 cells) (Figure 4.10B). Alternatively, the basal levels of NAD^+ were high (2039 ± 420 pmol/10^6 cells) and after H_2O_2 stimulation the levels remained stable for up to 3 min and then decreased to 1012 ± 186 pmol/10^6 cells by 20 min.

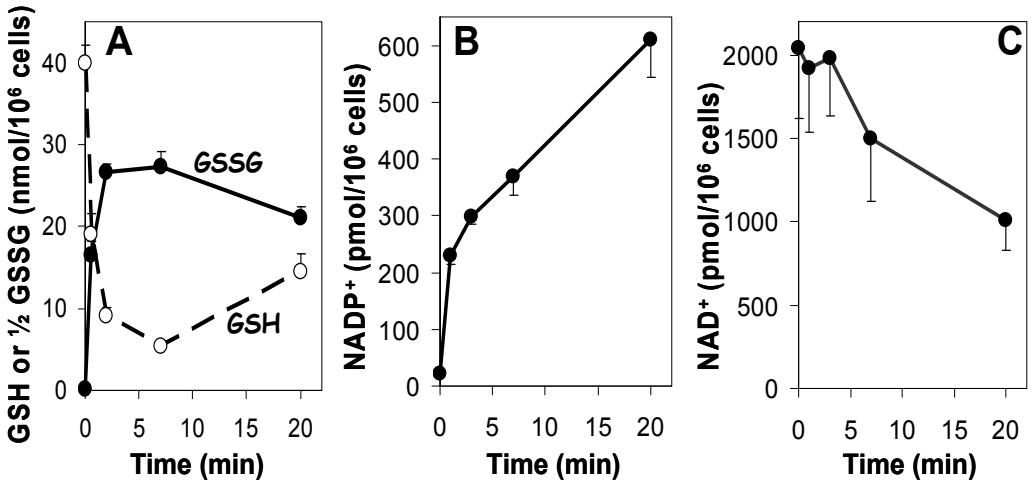


Figure 4.10: Effect of H_2O_2 on GSH, $NADP^+$, and NAD^+ levels in PC3 cells. The time course of the effect of H_2O_2 on (**A**) GSSG (○) and GSH (●) levels and (**B**) $NADP^+$ (●) and C) NAD^+ (●) levels were measured by RP-HPLC in PC3 cells. The cells (3 x 10^5 cells/well) were incubated with 100 μM H_2O_2 for up to 20 min. GSH, GSSG and $NADP^+$ were measured by RP-HPLC. (GSH, GSSG; n = 3, $NADP^+$, NAD^+; n = 6) Values presented are mean ± S.E.

4.2.4 5-LO products synthesized by neutrophils coincubated with PC3

PC3 cells express 5-LO and FLAP [226], however, we were unable to detect 5-LO products when PC3 cells were incubated with exogenous AA and A23187. Since inflammatory cells are associated with many types of tumors, we wanted to determine whether PC3 cells could synthesize 5-oxo-ETE from 5-HETE produced by adjacent leukocytes. PC3 cells (6 x 10^5 cells/well) were co-incubated with neutrophils (3 x 10^6 cells) in PBS^+ with AA (50 μM) and A23187 (5 μM) for 30 min. PC3 cells were preincubated with or without H_2O_2 (100 μM) before the reaction was started with addition of the neutrophils and AA and A23187. These results were compared to neutrophils incubated alone in 6-well plates. 5-Oxo-ETE levels were slightly higher when neutrophils were coincubated with PC3 cells (110 ± 31 pmol/10^6 cells) compared to neutrophils alone (83 ± 15 pmol/10^6 cells) (Figure 4.11B and E). H_2O_2 had no effect on the

levels of 5-oxo-ETE synthesized by neutrophils alone, but stimulated a significant increase when neutrophils were co-incubated with PC3 cells (171 ± 26 pmol 5-oxo-ETE/10^6 cells). The levels of 5-HETE (398 ± 117 pmol/10^6 cells) and LTB_4 (88 ± 19 pmol/10^6 cells) were lower in the coincubations compared to neutrophils alone (475 ± 93 pmol 5-HETE/10^6 cells and 125 ± 38 pmol LTB_4/10^6 cells) (Figure 4.11A, C, D, F). In the coincubations, the levels of 5-HETE slightly decreased in response to H_2O_2, and its levels slightly increased with neutrophils alone, whereas LTB_4 levels were not affected.

Since the neutrophils adhered to the plastic 6-well plates, they may have become activated and therefore were not the ideal control. To verify whether 5-oxo-ETE was being synthesized by PC3 cells in addition to the neutrophils, the levels of glutathione were depleted by preincubating the PC3 cells with NEM (100 μM) prior to stimulation with H_2O_2. NEM has previously been shown to block H_2O_2-induced 5-oxo-ETE synthesis by other cells [110-112]. NEM was removed before the PC3 cells were incubated with H_2O_2 and neutrophils to ensure that it only affected the synthesis of 5-oxo-ETE by the PC3 cells. NEM induced a significant decrease in 5-oxo-ETE levels in the coincubations stimulated with both vehicle (50 ± 13 pmol/10^6 cells) and H_2O_2 (43 ± 11 pmol/10^6 cells) (Figure 4.11B). In neutrophils alone, the levels of 5-oxo-ETE in NEM- treated cells, with or without H_2O_2, did not change compared to vehicle-treated cells (Figure 4.11E). Alternatively, NEM had no effect on the levels of 5-HETE or LTB_4 in the coincubations. However, in neutrophils alone, NEM induced a decrease in 5-HETE and a 2-fold increase in LTB_4 for both vehicle and H_2O_2 stimulated cells.

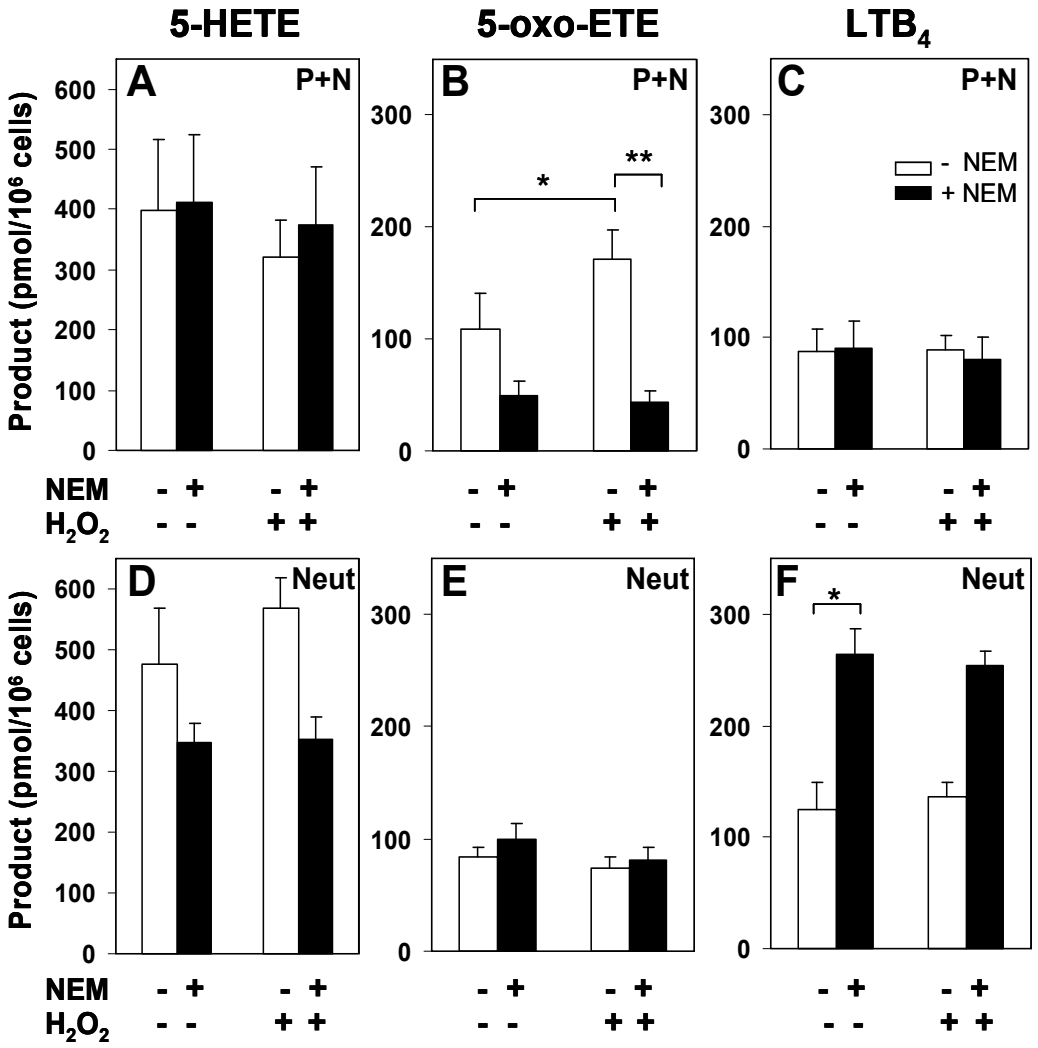


Figure 4.11: Higher levels of 5-oxo-ETE are synthesized when neutrophils were coincubated with PC3 cells compared to neutrophils alone incubated with AA and A23187. PC3 cells (6 x 10^5 cells/well) coincubated with neutrophils (3 x 10^6 cells/ml) (**A-C;** P+N) or neutrophils alone (3 x 10^6 cells/ml) (**D-F;** Neutr) were incubated with AA (50 μM) and A23187 (5 μM) for 30 min in PBS^+. For the coincubations, PC3 cells were preincubated with vehicle (□) or 100 μM NEM (■) followed by vehicle or 100 μM H_2O_2 before the neutrophils were added and the reactions started. Neutrophils with preincubated with NEM followed by H_2O_2 prior to incubation with AA and A23187. The levels of 5-oxo-ETE (**A,D**), 5-HETE (**B,E**) and LTB_4 (**C,F**) were quantitated by RP-HPLC. (n = 5) Values presented are mean ± S.E.

4.2.5 Anti-cancer agents stimulate 5-oxo-ETE synthesis by PC3 cells

Recent studies in our lab have determined that neutrophils undergoing apoptosis have a greater capacity to synthesize 5-oxo-ETE. Therefore, we determined whether docosahexaenoic acid (DHA), a ω-3 PUFA, and MK886, a 5-LO/FLAP inhibitor, which have been shown to induce PC3 cell death [77,227,228], would stimulate 5-oxo-ETE synthesis. PC3 cells were incubated with 5-HETE (4 μM) in the presence of vehicle, DHA (40 μM) or MK886 (20 μM) for up to 24 hrs in RPMI medium containing 0.5% FBS. DHA and to a lesser extent MK886 stimulated 5-oxo-ETE synthesis by 2 hrs (1.0 ± 0.3 nmol/10^6 cells and 0.84 ± 0.19 nmol/10^6 cells, respectively) compared to vehicle-treated cells which reached maximal levels by 2 hrs (0.24 ± 0.03 nmol/10^6 cells) (Figure 4.12A). In vehicle-treated cells, 5-oxo-ETE remained constant up to 6 hrs, but was not detected after 24 hrs. 5-Oxo-ETE levels reached maximal levels by 6 hrs (2.5 ± 0.6 nmol/10^6 cells) in DHA-treated cells, while after 24 hrs, the levels decreased slightly (1.9 ± 0.7 nmol/10^6 cells). The levels of 5-oxo-ETE in MK886-treated cells increased up to 6 hrs (1.4 ± 0.3 nmol/10^6 cells), and remained constant up to 24 hrs (Figure 4.12A). The DHA-treated PC3 cells were completely non-adherent by 6 hrs and 24 hrs, and could not be counted with a haemocytometer as they seemed too fragile, whereas the vehicle-treated cells remained adherent and viable. The majority of the MK886-treated cells were non-adherent after 6 and 24 hrs.

5-Oxo-ETE, 5-HETE, and DHA can be incorporated into cellular membranes [128], by acyl CoA synthetase, and DHA may potentially block esterification of the former compounds resulting in their prolonged availability. We treated the cells with triacsin C, an acyl CoA synthetase inhibitor [229]. We also compared the total 5-HETE plus 5-oxo-ETE levels for the different treatments. Triacsin C (20 μM) did not induce cell death, but stimulated 5-oxo-ETE synthesis only slightly more than vehicle (0.43 ± 0.04 nmol/10^6 cells) at 6 hrs and considerably less than DHA and MK886 (Figure 4.12A). The total 5-HETE plus 5-oxo-ETE levels decreased considerably more rapidly in vehicle-treated cells compared to the other conditions, reaching 25 % of the initial levels

in 6 hrs and < 1% by 24 hrs (Figure 4.12B). The decrease was slower in DHA-, MK886-, and triacsin C-treated cells, with > 75 % still present after 6 hrs for all treatments. DHA and MK886 stimulated cells had > 50 % of initial levels present after 24 hrs, whereas triacsin C-treated cells had 39% (Figure 4.12B). Although triacsin C did not stimulate 5-oxo-ETE synthesis or cell death, the levels of 5-HETE and 5-oxo-ETE were maintained at a similar level as in the presence of DHA or MK886.

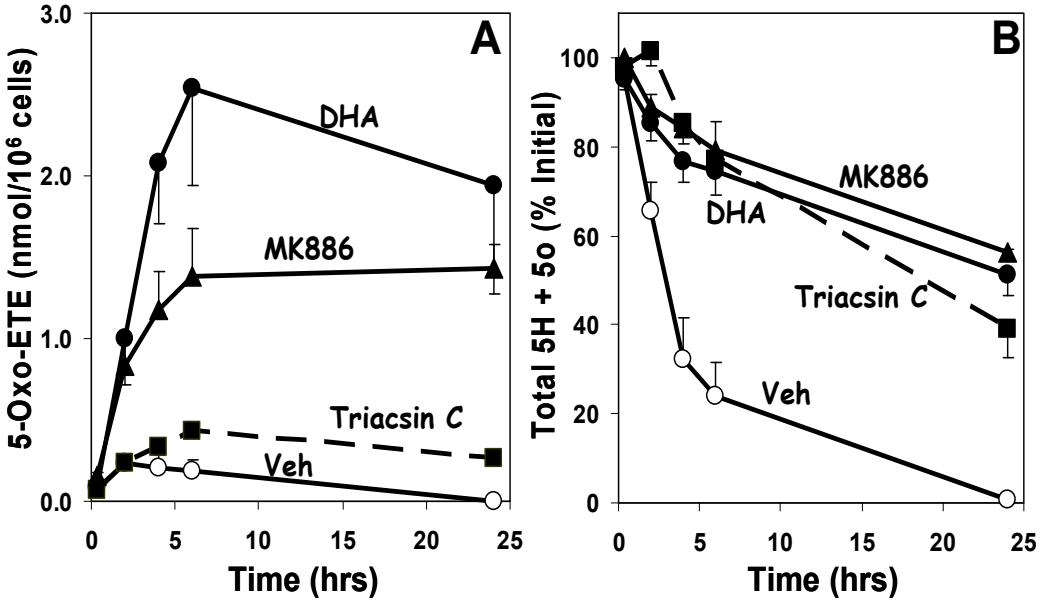


Figure 4.12: Time course for Effects of DHA on 5-oxo-ETE synthesis in PC3 cells incubated with 5-HETE. A) 5-oxo-ETE and **B)** total 5-HETE plus 5-oxo-ETE levels (% Initial) were measured in PC3 cells (3 x 10^5 cells/well) incubated with 4 μM 5-HETE in RPMI 1640 medium supplemented with 0.5% FBS and treated with vehicle (○), 40 μM DHA (●), 20 μM MK886 (▲), or 20 μM Triacsin C (■) for 2, 4, 6 and 24 hrs. 5-HETE and 5-oxo-ETE were analyzed by RP-HPLC. Values presented are mean ± S.E. (Vehicle, DHA, MK886; n = 4, Triacsin C; n = 3)

To determine whether this increased 5-oxo-ETE production could be induced by other cytotoxic compounds, we incubated PC3 cells with tamoxifen (50 μM), a selective estrogen receptor modulator, and doxorubicin (10 μM), an anthracycline antibiotic, with 5-HETE (4 μM) for up to 24 hrs. Similar to DHA, tamoxifen induced PC3 cell death under the incubation conditions within 24 hrs. Additionally, tamoxifen stimulated 5-oxo-ETE synthesis, but to a lesser extent than DHA (1.5 ± 0.3 nmol/10^6 cells compared to 2.3 ± 0.8 nmol/10^6 cells at 6 hrs) (Figure 4.13A). 5-Oxo-ETE levels decreased more rapidly in tamoxifen-treated cells, reaching 5-fold lower levels compared to DHA-treated cells after 24hrs. However, this did not correlate with the total levels of 5-HETE plus 5-oxo-ETE, which were higher at all time points in tamoxifen-treated cells compared to DHA (Figure 4.13B). Doxorubicin did not induce cell death, as the cells were still adherent and viable, as determined by trypan blue exclusion after 24 hrs. Doxorubicin had no effect on 5-oxo-ETE synthesis or the total levels of eicosanoids (Figure 4.13A and B) which were similar to vehicle-treated cells.

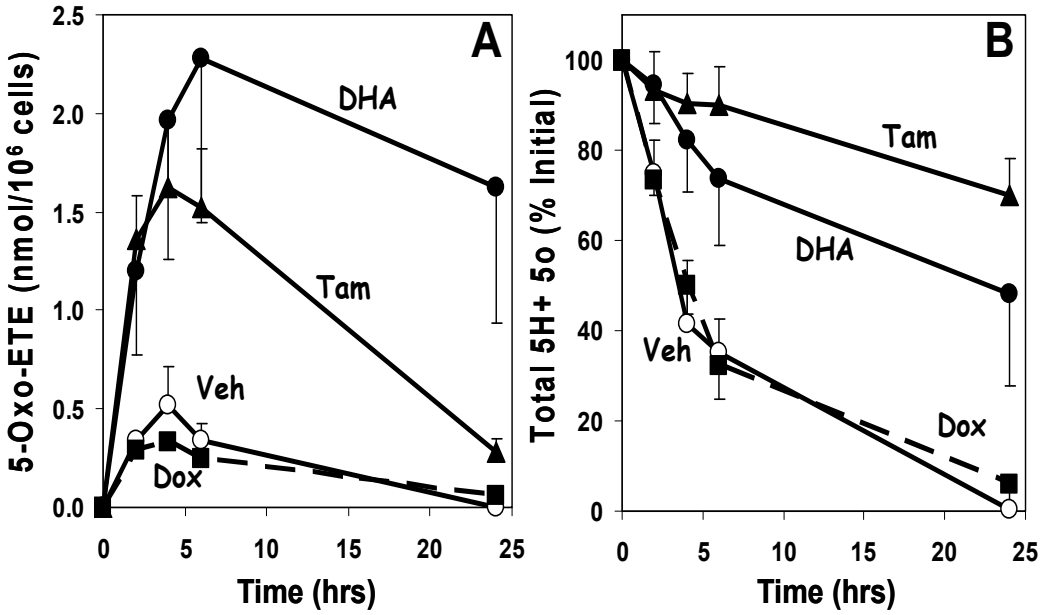


Figure 4.13: Effects of DHA, tamoxifen, and doxorubicin on 5-oxo-ETE synthesis in PC3 cells incubated with 5-HETE. A) 5-oxo-ETE and **B**) the total 5-HETE plus 5-oxo-ETE levels (% Initial) were measured in PC3 cells (3 x 10^5 cells/well) incubated with 5-HETE in RPMI 1640 medium supplemented with 0.5% FBS and treated with vehicle (Veh; ○),40 μM DHA (●), 50 μM tamoxifen (Tam;▲), or 10 μM doxorubicin (Dox; ■) for 2, 4, 6 and 24 hrs. 5-HETE and 5-oxo-ETE were analyzed by RP-HPLC. Values presented are mean ± S.E. (Veh, Tam; n=4, DHA, Dox; n = 3)

4.2.6 Effects of DHA, tamoxifen and MK-886 on 5-oxo-ETE synthesis

To evaluate whether the effects of DHA and the other agents were specific to PC3 cells, we evaluated 5-oxo-ETE synthesis in other types of epithelial cancer cells. We incubated breast cancer cells (MCF7) and lung cancer cells (A427) with 4 μM 5-HETE with and without DHA (40 μM), tamoxifen (50 μM) or MK886 (10 μM) for 24 hrs. MCF7 and A427 both synthesized low levels of 5-oxo-ETE (0.49 ± 0.04 nmol/10^6 cells and 0.59 ± 0.03 nmol/10^6 cells, respectively, at 6 hrs) compared to PC3 cells (Figure 4.14A and C). Similar to its effects on PC3 cells, DHA stimulated the greatest increase in 5-oxo-ETE synthesis by both

cell types, but induced a greater response in A427 cells (3.7 ± 0.5 nmol/10^6 cells at 6 hrs) compared to MCF7 cells (2.1 ± 0.4 nmol/10^6 cells at 6 hrs). After 24 hrs, the 5-oxo-ETE remained elevated. At 6hrs, tamoxifen stimulated a greater maximal response (1.7 ± 0.6 nmol 5-oxo-ETE/10^6 cells) than MK886 (0.98 ± 0.43 nmol 5-oxo-ETE/10^6 cells) in MCF7 cells, whereas MK886 induced higher 5-oxo-ETE synthesis (2.6 nmol/10^6 cells) than tamoxifen (1.70 ± 0.02 nmol/10^6 cells) in A427 cells (Figure 4.14A and C). After 24 hrs, the levels of 5-oxo-ETE remained elevated in the MK886-treated cells, whereas, the levels decreased in the tamoxifen-treated cells.

Similar to PC3 cells, the total levels of 5-HETE plus 5-oxo-ETE only decreased to 2% the initial levels in vehicle-treated cells (Figure 4.14B and D). The greater 5-oxo-ETE synthesis by A427 cells corresponded to the elevated total eicosanoid levels after 24 hrs which remained at > 70% the initial levels. In MCF7 cells, the total eicosanoids levels decreased more rapidly than in A427 cells, reaching > 40% by 24 hrs when treated with tamoxifen and MK886 and > 70% when treated with DHA.

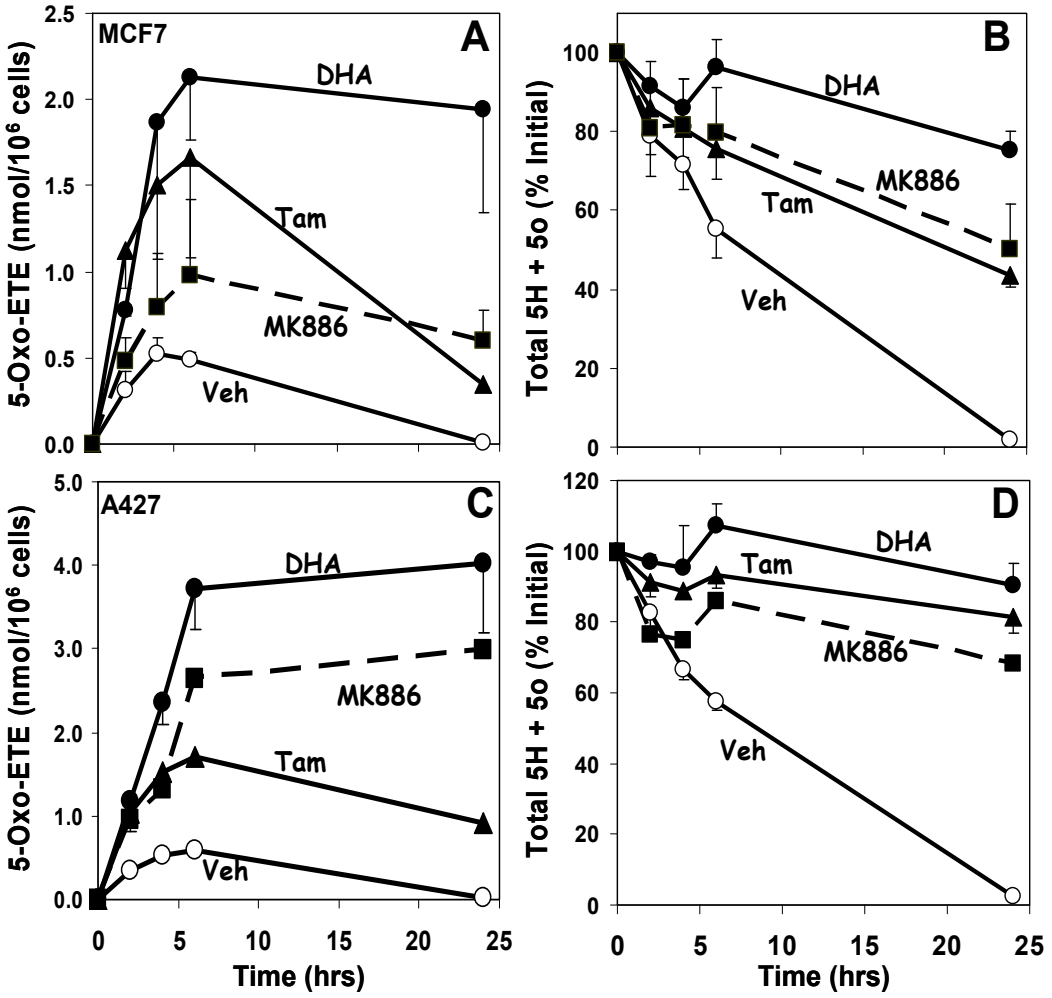


Figure 4.14: Effects of DHA, tamoxifen, and MK886 on 5-oxo-ETE synthesis in MCF7 and A427 cells incubated with 5-HETE. **A,C**) 5-oxo-ETE and **B,D**) the total 5-HETE plus 5-oxo-ETE levels (% Initial) were measured in MCF7 cells (**A,B**) or A427 cells (**C,D**) (3 x 10^5 cells/well) incubated with 4 µM 5-HETE in RPMI 1640 medium supplemented with 0.5% FBS and treated with vehicle (Veh; ○), 40 µM DHA (●), 50 µM tamoxifen (Tam; ▲), or 10 µM MK886 (■) for 2, 4, 6 and 24 hrs. 5-HETE and 5-oxo-ETE were analyzed by RP-HPLC. Values presented are mean ± S.E. (MCF7: Veh, DHA, MK886; n = 3, Tam; n = 2) (A427: Veh, DHA, Tam; n = 3, MK886; n = 1)

4.3 Partial purification of 5-hydroxyeicosanoid dehydrogenase (5-HEDH)

In order to characterize 5-HEDH and its regulation and expression, identification of the sequence will be necessary which can best be accomplished by mass spectrometry. To facilitate this, we have attempted to at least partially purify 5-HEDH prior to analysis. Several chromatographic and separation techniques were tested and evaluated to determine their efficiency and ultimate value in purifying 5-HEDH sufficiently for analysis by mass spectrometry. The best techniques were then combined to obtain a partially purified preparation of the enzyme. As no antibody against 5-HEDH is available, we were only able to detect it on the basis of its enzyme activity which precluded the use of denaturing conditions. All purification methods were carried out in the presence of the complete protease inhibitor cocktail at 4 – 8 ^{o}C unless otherwise specified.

4.3.1 Sources of 5-HEDH

The monocytic cell line, U937, which was terminally differentiated with PMA (18 nM) for 4 days, was used as a source of 5-HEDH since it was previously found to have 4-fold more 5-HEDH activity than undifferentiated U937 cells or human neutrophils [101]. To compare enzymes identified by mass spectrometry, neutrophils isolated from whole blood obtained from healthy donors was used as another source of 5-HEDH.

5-HEDH is a membrane bound protein, and the majority of 5-HEDH activity is present in the microsomal fractions of cells [98]. The cells were lysed by sonication and then the microsomal fraction was isolated by differential centrifugation, resulting in removal of proteins from other subcellular fractions.

4.3.2 Solubilization of microsomal proteins

Since 5-HEDH is a hydrophobic membrane bound enzyme, solubilization of the microsomal proteins with detergents is required to remove lipids before it could be purified. Since non-ionic detergents interfere less with purification techniques than ionic detergent, we tested digitonin, and octylglucoside, and the zwitterionic detergent, CHAPS for their ability to solubilize microsomal proteins.

They were also tested to ensure that they did not inhibit 5-HEDH activity or interfere with the enzyme assay (ie. absorb UV at 280 nm) which is required to determine where the enzyme elutes. CHAPS (6 mM), a zwitterionic detergent, was able to solubilize the majority of microsomal proteins while maintaining 5-HEDH activity (Table 4.1) however, there was considerable variability between preparations. 30 mM octylglucoside, a non-ionic detergent and digitonin (0.5% (w/v)) were also able to efficiently solubilize the microsomal proteins while maintaining 5-HEDH activity. Addition of deoxycholate resulted in loss of 5-HEDH activity and the non-ionic detergents, Triton X-100 and NP-40 both absorb at 280 nm and therefore interfered with the activity assay. Further solubilizations were carried out using either 6 mM CHAPS or 30 mM octylglucoside.

Table 4.1: Solubilization of 5-HEDH by non-ionic and zwitterionic detergents.

Detergent	Concentration	% Recovery (5-HEDH activity)
CHAPS	2.5 mM	54
	5 mM	68
	6 mM	35 - 93
	10 mM	16
Digitonin	0.5% (w/v)	93
Octylglucoside	30 mM	35 - 87
Deoxycholate	1% (w/v)	Loss of activity

4.3.3 Concentration 5-HEDH activity by centrifugation

Since it was necessary to concentrate the protein between purification steps as well as to exchange detergents or remove salt, Amicon centrifugal filters (Fisher) were used. Low molecular weight solutes (eg. salts) pass through the membrane, while solutes larger than the membrane pore size are retained. Since the size of 5-HEDH is unknown, we evaluated 30, 50 and 100 kDa molecular weight cutoff (MWCO) filters with solubilized microsomes for their ability to

retain 5-HEDH activity. Nearly all of the unpurified 5-HEDH activity was retained with all three filters (Figure 4.15A). However, when 5-HEDH was first partially purified by treatment with blue Sepharose, only 40% of the activity was retained by the 100 kDa MWCO filter (Figure 4.15B), whereas 100% was retained by the 50 kDa MWCO filter. However, when DEAE purified fraction containing 5-HEDH activity were filtered with 50 kDA MWCO filters, 7% of the activity passed through (Figure 4.15C). The filters may not retain proteins of sizes slightly higher than cutoff, therefore the results indicate that the size of 5-HEDH is > 30 kDa and < 100 kDa and probably slightly larger than 50 kDa.

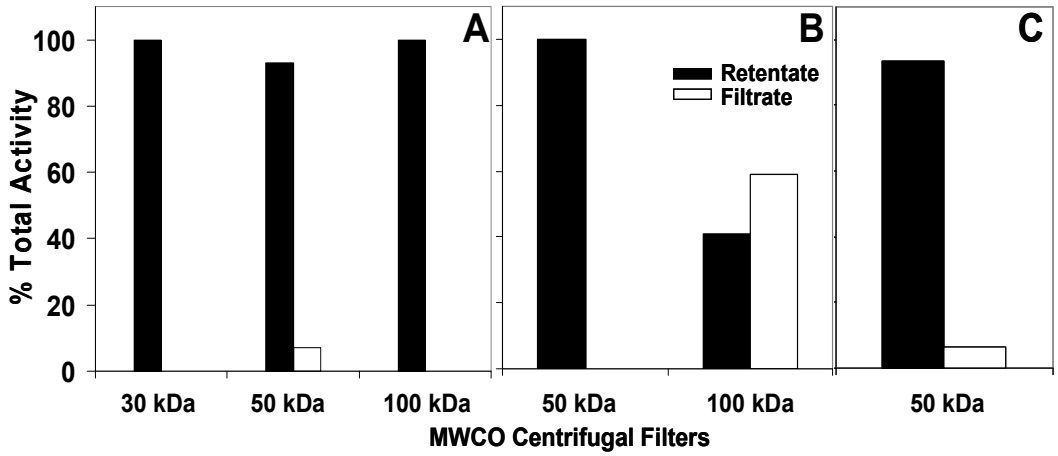


Figure 4.15: MWCO centrifugal filters can be used to concentrate fractions containing 5-HEDH activity A) Solubilized microsomes (25 μg) were filtered through 30, 50, and 100 kDa MWCO filters and centrifuged for 10 minutes at 3350 x g. **B**) Blue Sepharose fractions containing 5-HEDH activity were filtered through 50 and 100 kDa MWCO filters. **C**) DEAE purified fractions containing 5-HEDH activity were filtered through a 50 kDa MWCO filter. Retentate (■); Filtrate (□) (n = 2)

4.3.4 Size exclusion chromatography

Size exclusion chromatography (SEC), which separates proteins based on size, was tested for its effectiveness in separating 5-HEDH from other proteins. Large proteins elute first, taking the shortest path through the column and smaller proteins are retained on the column longer. A BioSep SEC3000 (Phenomenex) HPLC column was used to separate solubilized microsomal protein (2 mg) isolated from U937 cells and 1 minute fractions were collected to measure 5-HEDH activity. Several detergent and buffer concentrations were tested to optimize the separation of 5-HEDH from other proteins. However, under several test conditions, 5-HEDH activity consistently eluted over several fractions (minutes), and did not seem to be sufficiently separated from the majority of proteins. Figure 4.16 illustrates a representative separation using the SEC column. The mobile phase was 50 mM phosphate buffer pH 7.0 containing 6 mM CHAPS at a flow rate of 1.0 ml/min, with the column placed on ice to preserve 5-HEDH activity. Protein standards (10 μg), including bovine serum albumin (BSA; 68 kDa), ovalbumin (44 kDa), and cytochrome c (12.5 kDa) were injected separately to determine their retention times (8.0 min, 8.3 min, and 9.3 min, respectively) (Figure 4.16A). These could then be used to determine the apparent molecular weight of 5-HEDH, according to its retention time. 5-HEDH activity eluted between 8 to 12 min (Figure 4.16B), which corresponded to the elution of the majority of the loaded protein. According to this method, the size of 5-HEDH was probably lower than that of ovalbumin (44 kDa), however, the presence of detergent may have affected the elution of 5-HEDH and the activity eluted over 4 min, making the results unreliable. Another experiment using DEAE purified 5-HEDH did not yield conclusive results since 90% of the enzymatic activity was lost, and was near the detection limits of our method. Further experiments were not carried out, since this method did not result in any purification of 5-HEDH.

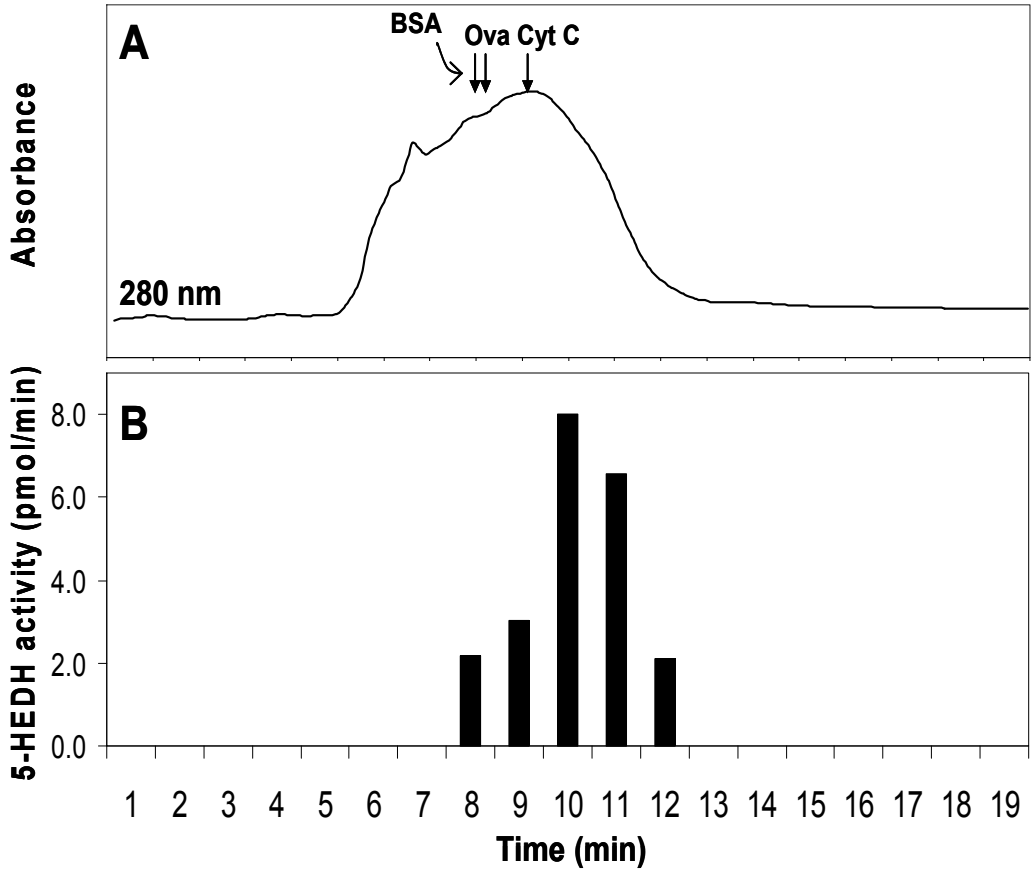


Figure 4.16: Size exclusion chromatography of U937 cell solubilized microsomal protein containing 5-HEDH activity. **A**) Chromatograph of solubilized microsomal protein (2 mg) loaded onto a 7.8 x 300 mm BioSep SEC3000 5 μm (Phenomenex) column. The proteins were separated using isocratic conditions of 50 mM phosphate buffer pH 7.4 containing 6 mM CHAPS at a flow rate of 1.0 ml/min. Protein levels were monitored using a diode array detector at 280 nm. The arrows indicate the elution of standard proteins, BSA (8.0 min), ovalbumin (Ova; 8.3 min), and cytochrome C (Cyt C; 9.3 min). **B**) Fractions (1-19; 1 min) were collected and assayed for 5-HEDH activity by incubating with 5-HETE (1 μM) and $NADP^+$ (100 μM) for 1 hr at 37 ^oC.

4.3.5 Carboxymethyl (CM) Sepharose chromatography

The weak cation exchanger, carboxymethyl (CM) Sepharose was tested for its capacity to bind and purify 5-HEDH using a batch method. The pI of the protein determines at which pH they will bind the cation exchanger. At pHs below a protein's pI, it will be positively charged and should interact with the negatively charged carboxymethyl Sepharose. The pI of 5-HEDH is unknown, therefore, different buffers, buffer concentrations, pHs, and detergents were tested. Under the different conditions tested, 5-HEDH never bound to CM Sepharose. Figure 4.17 includes two separate experiments (batch method). In the experiments presented, solubilized microsomes (450 µg) were incubated with CM Sepharose (750 µl) in Buffer A containing 30 mM octylglucoside (Figure 4.17A) or 50 mM phosphate buffer (pH 7.4) containing 3 mM CHAPS (Figure 4.17B) at 6 – 8 ^{o}C for 5 min. To elute 5-HEDH, increasing amounts of NaCl (0.05, 0.5, and 1 M) in the respective buffers was added to the protein/CM Sepharose solution. However, all of the activity eluted in the first fraction indicating that 5-HEDH was not retained along with the majority of the protein. As CM Sepharose did not bind 5-HEDH and therefore, did not increase result in increased purity, further testing was not carried out.

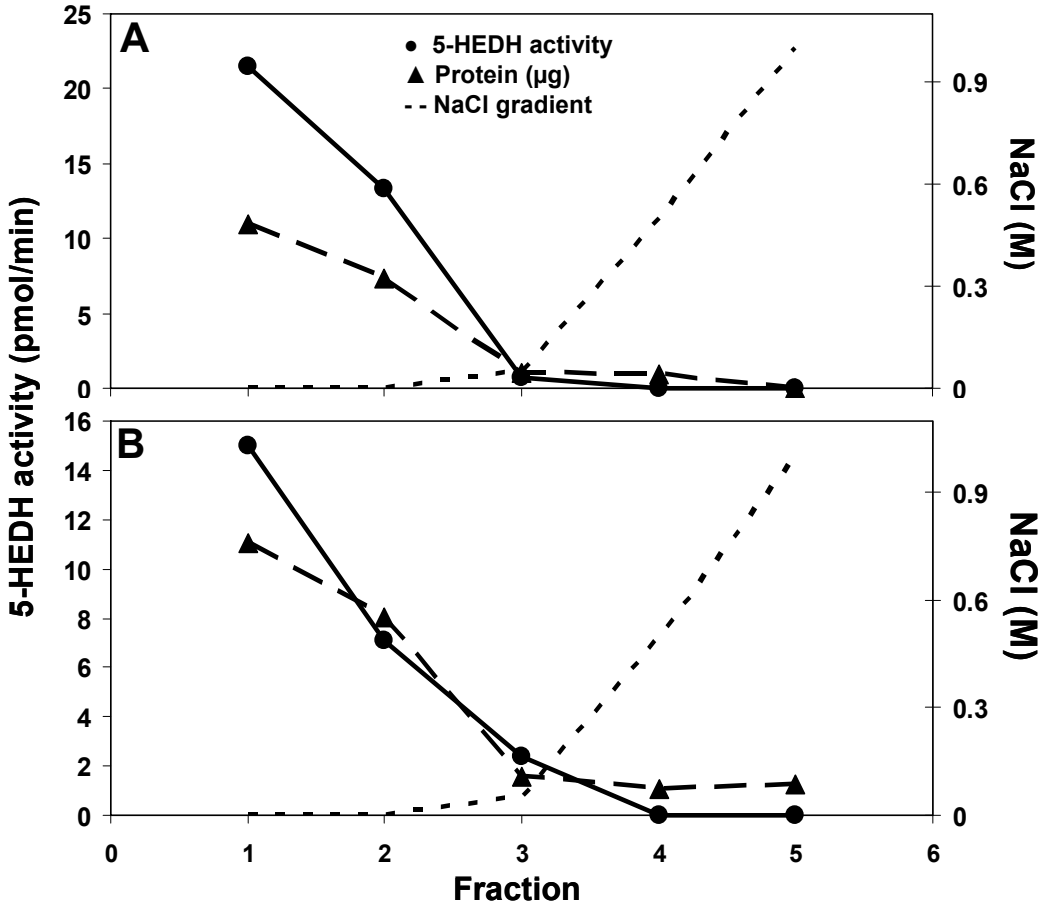


Figure 4.17: Purification of 5-HEDH by CM Sepharose. Solubilized microsomes (450 µg) in 30 mM octylglucoside (**A**) or 3 mM CHAPS (**B**) in 50 mM phosphate buffer, pH 7.4 was incubated with CM Sepharose (750 µl) for 5 min at 6-8 ^{o}C. The washes contained the same detergent that was used for binding for each experiment. The CM Sepharose with bound 5-HEDH was then washed with buffer containing detergent (Fractions 1,2), and subsequently with 0.05 M, 0.5 M and 1 M NaCl (fractions 3,4 and 5, respectively) (dotted line). 5-HEDH activity (●) and protein levels (▲) were measured in collected fractions as described in Materials and Methods (Chapter 3, pg 58).

4.3.6 Diethylaminoethyl (DEAE) Sepharose chromatography

The anion exchanger, diethylaminoethyl (DEAE) Sepharose was used to successfully partially purify 5-HEDH (Figure 4.18). A batch (other than a column) technique was used for simplicity. A variety of buffers containing detergents and conditions were initially tested to determine the optimal conditions for binding of 5-HEDH to the stationary phase and its subsequent elution. Buffer A containing detergent and 20 % glycerol was used for all binding and elution steps. It was determined that the binding of 5-HEDH to the DEAE Sepharose improved when incubated with 6 mM CHAPS compared to 30 mM octylglucoside. Therefore, microsomes solubilized (4.7 mg) with either octylglucoside or CHAPS were incubated with DEAE Sepharose (2.5 ml) at 6 - 8 ^{o}C for 5 min in the presence of 6 mM CHAPS. The DEAE Sepharose was washed with sufficient buffer containing 6 mM CHAPS to remove all unbound proteins. The majority of 5-HEDH activity eluted with 20 mM NaCl in 30 mM octylglucoside, with relatively low amounts of protein (Figure 4.18). The majority of the protein was either not initially bound to the DEAE Sepharose, or could only be eluted with a higher concentration of NaCl (1 M). A small amount of 5-HEDH also eluted with this high salt concentration (Figure 4.18). This protocol resulted in ~75 % recovery of total 5-HEDH activity loaded onto the DEAE Sepharose and ~7-fold increase in specific activity. DEAE Sepharose was used for further purification of 5-HEDH.

4.3.7 Red Sepharose

The dyes in red and blue Sepharose are similar in structure to NAD^+ and $NADP^+$, respectively. They therefore retain enzymes by binding to their cofactor binding sites. Several experiments were carried out to determine whether these chromatographic media could be used in the purification of 5-HEDH. Figure 4.19 illustrates a representative experiment showing the binding and elution of 5-HEDH activity from Red Sepharose relative to the amount of protein using a batch method. Solubilized microsomes (2.4 mg) were incubated with Red Sepharose (400 mg) in 30 mM octylglucoside for 10 min at 6 - 8 ^{o}C. The majority of protein was

not retained whereas the majority of 5-HEDH activity was retained (Figure 4.19) and could be eluted from red Sepharose with 1 M NaCl in 5 mM CHAPS. However, a significant amount of protein was unfortunately present in these fractions as well, therefore not resulting in significant purification of 5-HEDH. Other conditions were also examined in which the elution buffers were modified by varying the detergents, NaCl concentration and by addition of glycerol. However, they did not improve the separation of 5-HEDH from other proteins and the results were not reproducible, therefore it was concluded that Red Sepharose was not an efficient method to purify 5-HEDH.

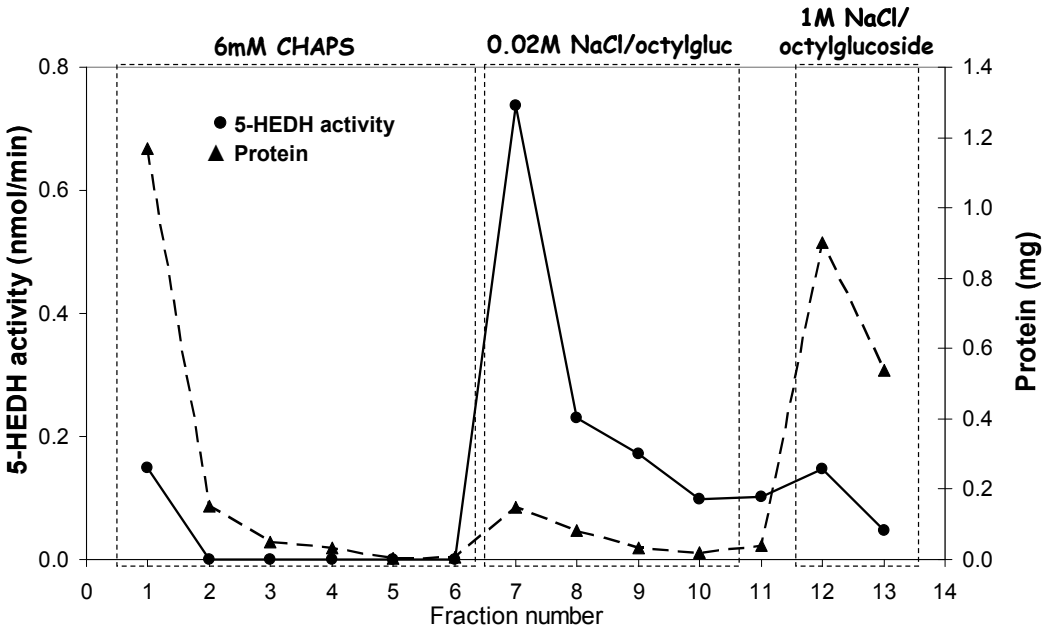


Figure 4.18: DEAE Sephorose separated 5-HEDH activity. Solubilized microsomes (4.7 mg) diluted in 6 mM CHAPS were incubated with DEAE Sepharose (2.5 ml) for 5 min at 4^{o}C. Unbound protein was washed off with several washes of 20 mM phosphate buffer containing 6 mM CHAPS/20% glycerol (fractions 1-6). 5-HEDH activity was eluted with 0.02 M NaCl in 30 mM octylglucoside/20% glycerol (fractions 7-10). Fraction 11 contained 0.04 M NaCl, and the remainder of bound protein was eluted with 1 M NaCl. 5-HEDH activity (●) and protein levels (▲) were measured as described in Materials and Methods (Chapter 3, pg 58).

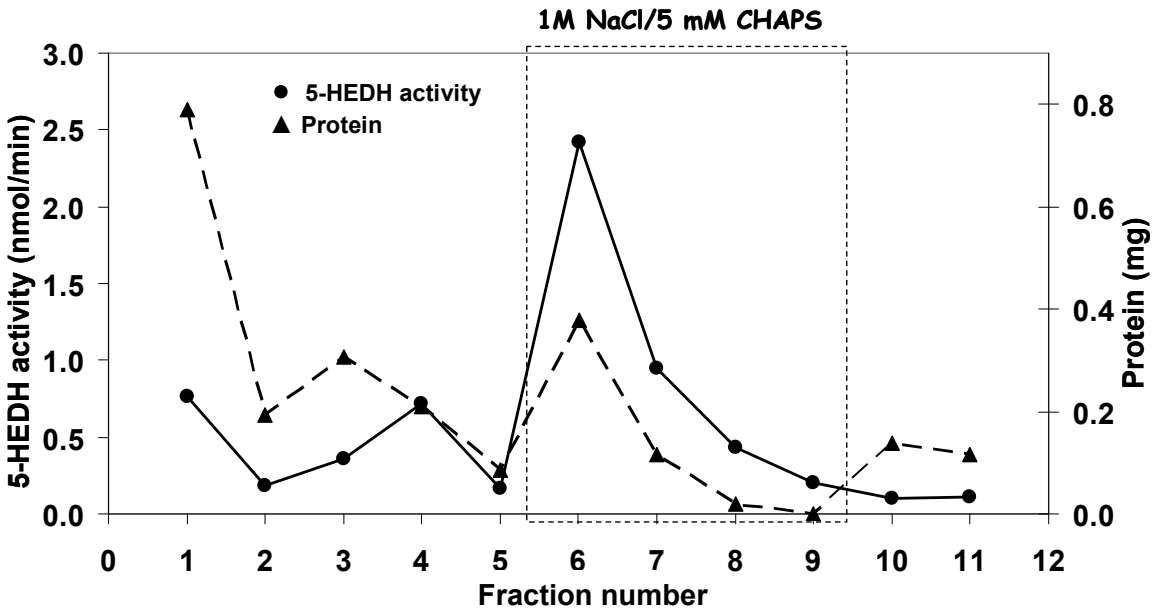


Figure 4.19: Purification of 5-HEDH by Red Sepharose. Using a batch method, 5-HEDH bound to red Sepharose when solubilized microsomes (2.4 mg) were incubated in 30 mM octylglucoside for 10 min at 6 - 8 ^{o}C. Unbound proteins were washed off with the binding buffer containing 30 mM octylglucoside (fractions 1-2). Further proteins were eluted with 0.1 M NaCl in 5 mM CHAPS (fractions 3-5). The majority of 5-HEDH activity eluted with 1 M NaCl in 5 mM CHAPS. The remaining protein was removed with 2 M NaCl/5 mM CHAPS. 5-HEDH activity (●) and protein levels (▲) were measured in collected fractions as described in Materials and Methods (Chapter 3, pg 58).

4.3.8 Blue Sepharose

Blue Sepharose was tested since it is similar in structure to $NADP^+$ and should therefore retain 5-HEDH via its cofactor binding site. Optimization of the binding and elution conditions (detergents, NaCl concentrations) using solubilized microsomes resulted in effective partial purification of 5-HEDH. DEAE Sepharose partially purified 5-HEDH bound to Blue Sepharose when incubated in 30 mM octylglucoside, while the majority of the activity could be eluted with 1.3 M NaCl in 6 mM CHAPS containing 20% glycerol (Figure 4.20). This resulted in

a ~3–fold increase in specific activity from DEAE Sepharose. The majority of the protein was either un-retained or eluted prior to 5-HEDH with lower concentrations of NaCl and in 30 mM octylglucoside. Elution of 5-HEDH with $NADP^+$ (10 μM) instead of NaCl was also tested but did not result in appreciable purification of the enzyme and was also not reproducible. The recovery of total 5-HEDH activity incubated with Blue Sepharose was generally at least 70%. Combining purification of 5-HEDH using DEAE Sepharose followed by Blue Sepharose resulted in ~21-fold increase in specific activity.

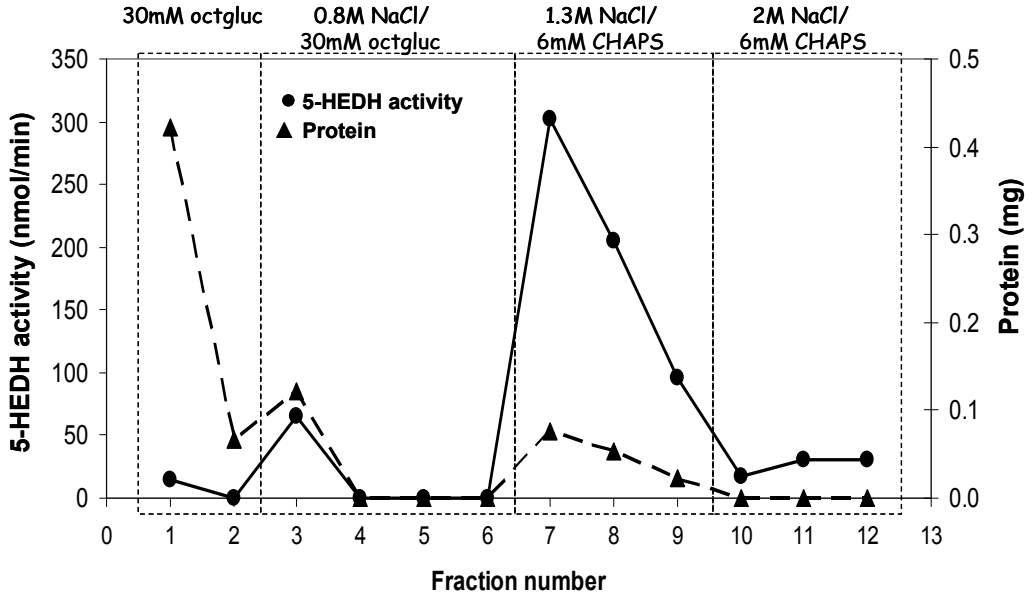


Figure 4.20: Purification of 5-HEDH by Blue Sepharose. Using a batch method, 5-HEDH bound to blue Sepharose (400 mg) when DEAE purified 5-HEDH (2.7 mg) were incubated in 30 mM octylglucoside for 5 min at 6 – 8 ^oC. Unbound proteins were washed off with the binding buffer containing 30 mM octylglucoside (fractions 1-3). Further unwanted proteins were eluted with 0.8 M NaCl in 30 mM octylglucoside/20% glycerol (fractions 4-5). 5-HEDH activity eluted with 1.3 M NaCl in 6 mM CHAPS/20% glycerol, while the remaining protein was removed with 2 M NaCl/6 mM CHAPS/20% glycerol. 5-HEDH activity (●) and protein levels (▲) were measured in collected fractions as described in Materials and Methods (Chapter 3, pg 58).

4.3.9 Hydroxylapatite

Hydroxylapatite (HA) functions as a "mixed-mode" ion exchanger where positively charged calcium ions and negatively charged phosphate ions interact nonspecifically with the negatively charged carboxyl groups and the positively charged amino groups, respectively, on proteins. Proteins are bound to hydroxylapatite at a low buffer concentration and eluted by increasing the buffer concentration. Using the batch method, several binding and elution conditions, including varying detergents and buffer concentrations were tested to optimize 5-HEDH purification using this technique. Figure 4.21 includes two separate experiments in which solubilized microsomes (4.9 mg) were incubated with HA (250 mg) in 10 mM phosphate buffer pH 7.4 containing 6 mM CHAPS, and subsequently washed with 125 mM, 200 mM and 400 mM buffer containing 6 mM CHAPS. A significant amount of 5-HEDH was not retained by HA, and 200 mM and 400 mM phosphate buffer induced its elution along with a large proportion of the protein. 5-HEDH binding and elution varied greatly between experiments using similar conditions (Figure 4.21) as well as repeated experiments. Therefore, it was not possible to predict the enzyme binding and elution with certainty and this technique did not prove to be useful for the purification of 5-HEDH.

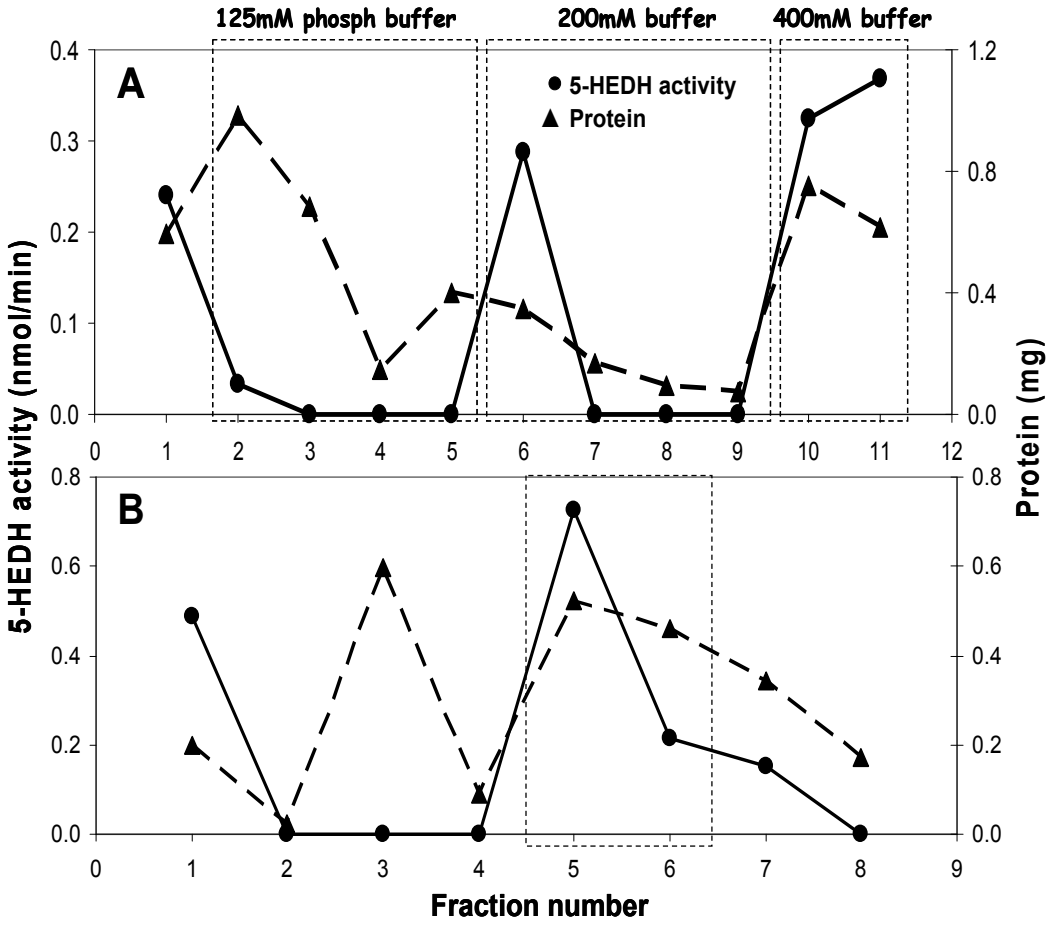
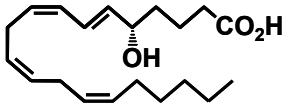
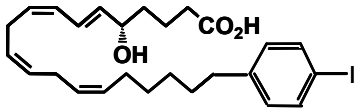
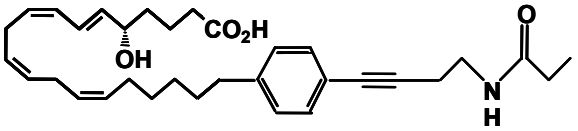
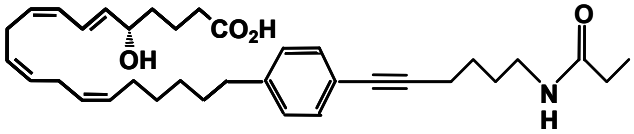
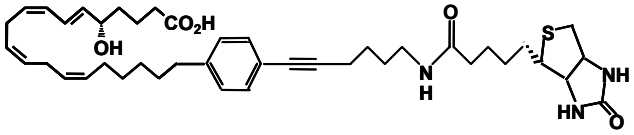
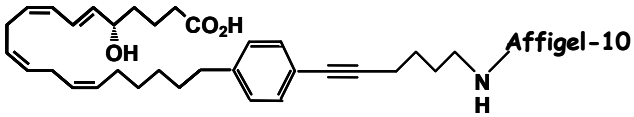


Figure 4.21: 5-HEDH purification by hydroxylapatite. **A**) Solubilized microsomes (4.9 mg) were incubated with hydroxylapatite (250 mg) in 10 mM potassium phosphate buffer containing 6 mM CHAPS for 10 min at 6 – 8 ^{o}C. Increasing concentrations of phosphate (125 mM (Fractions 2-5), 200 mM (Fractions 6-9), and 400 mM (Fractions 10-11), pH 7.5) were added sequentially to elute 5-HEDH. **B**) Another example in which solubilized microsomes were incubated with hydroxylapaptite under similar conditions (125 mM (Fractions 2-4), 200 mM (Fractions 5-6), and 400 mM (Fractions 7-8), pH 7.5), but resulted in a different 5-HEDH elution profile. 5-HEDH activity (●) and protein levels (▲) were measured in collected fractions as described in Materials and Methods (Chapter 3, pg 58).

4.3.10 Affinity chromatography

Affinity chromatography, using a 5-HETE analog should purify 5-HEDH with more specificity than the other chromatographic techniques. Several 5-HETE analogs modified at the ω-end, were synthesized by Dr. Joshua Rokach's group (Dr Pranav Patel) at the Florida Institute of Technology [230]. Chemical modification of the carboxylic group would be more straightforward, however 5-HETE methyl ester is a very weak substrate for 5-HEDH, indicating that the carboxylic group is required for binding to the enzyme [5,102]. Therefore, 5-HETE could only be coupled at the ω-end to ensure the analogs could bind the enzyme. 5-HETE analogs either contained biotin or were coupled to Affigel-10, a solid support (Figure 3.1, pg 63) via carbon chains of different length. To be effective, the 5-HETE analogs needed to bind to 5-HEDH, either as a substrate or as an inhibitor, and several analogs with different groups added to the linker chain were analyzed to determine whether they bound the enzyme. To determine whether the analogs were substrates, we used Lineweaver-Burk plots to determine the Michaelis-Menton constant (K_m) and the maximum reaction velocity (V_{max}) (Table 4.1). Increasing concentrations of the analogs and 5-HETE were incubated with microsomal protein (50 μg) with $NADP^+$ (100 μM) for 5 min. Compound 2, (Table 4.2), which was subsequently modified and coupled to Affigel-10 or biotin, was coincubated with 5-HETE (1 μM) and $NADP^+$. Compound 2 was determined to be a competitive inhibitor of 5-HEDH, and dose-dependently blocked the formation of 5-oxo-ETE (Figure 4.22A), and at 0.3 μM, blocked 5-HETE conversion to 5-oxo-ETE by 60%. Alternatively, compound 2 was not a good substrate since its V_{max} was much lower than that of 5-HETE (Figure 4.22B and C), similar to the majority of the analogs (Table 4.2). The oxidation of Affigel-5-HETE by 5-HEDH could not be measured since Affigel is an agarose and therefore, not soluble.

Table 4.2: Lineweaver-Burk analysis of 5-HEDH with 5-HETE analogs. Microsomes (50 µg protein) were incubated with increasing amounts of 5-HETE or the synthesized 5-HETE analogs plus 100 µM $NADP^+$ for 5 min. The K_m and V_{max} values were determined by linear regression.

5-HETE analog	Analog Structures	K_m (µM)	V_{max} (pmol/(min x µg protein)
5-HETE		0.8	1.8
1		0.5	2.1
2		0.2	0.4
3		0.6	0.6
Biotin 5-HETE		0.2	0.5
Affigel 5-HETE		--	--

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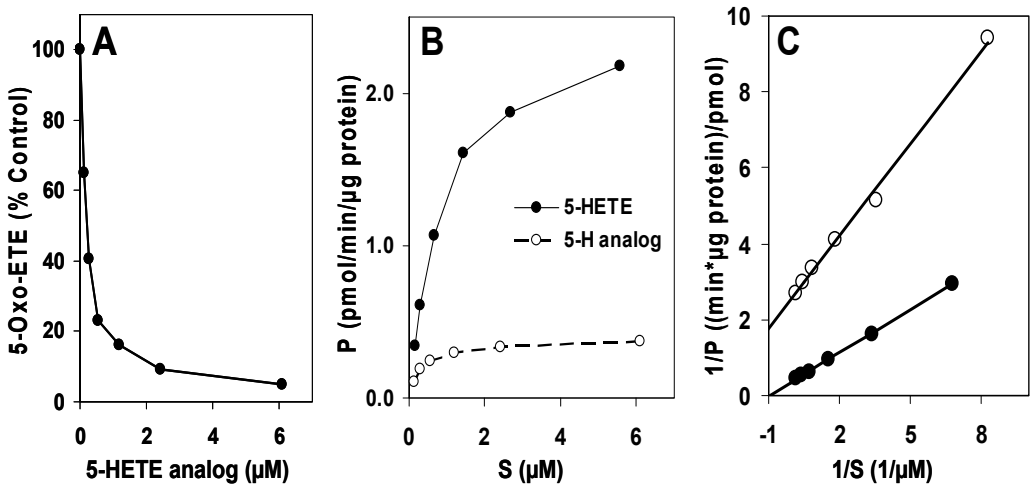


Figure 4.22: The 5-HETE analog, compound 2, is not a substrate of 5-HEDH and acts as an inhibitor. **A**) Inhibition of oxidation of 5-HETE by 5-HEDH by compound 2. Microsomes (50 μg protein) were incubated for 5 min with 5-HETE (1 μM) and $NADP^+$ (100 μM) in the presence of increasing amounts of the 5-HETE analog. The amounts of 5-oxo-ETE produced were measured by RP-HPLC. **B**) Product (P) versus substrate (S) concentration. The effects of substrate concentration on the oxidation of 5-HETE (●) and compound 2 (○) were determined by incubating increasing concentrations of these compounds with microsomal fractions as described above. **C**) Lineweaver-Burk plot of 1/P versus 1/S for oxidation of 5-HETE (●) and compound 2 (○). (n = 1)

The amount of 5-HETE analog coupled to Affigel differed between batches ranging from 38% to 60%, and we found that lower amounts of coupling resulted in better purification of 5-HEDH. However, the results between experiments were not consistent. Numerous 5-HEDH binding and elution conditions (buffer, pH, temperature, time, and detergent) for Affigel-5-HETE or streptavidin-biotin-5-HETE were evaluated for either solubilized microsomes, or DEAE Sepharose purified 5-HEDH. Figure 4.23 presents several experiments using different batches of Affigel-5-HETE with 38%, 45% and 60% bound 5-HETE. 5-HEDH has greater dehydrogenase activity at higher pH [101], therefore, Tris buffer (50mM, pH 8.8) was tested to determine if the enzyme

interacted better with the Affigel-5-HETE at this pH. In addition, phosphate buffer (20 mM pH 7,4) containing 10% (v/v) glycerol and detergent (CHAPS (3 mM) or octylglucoside (15 mM)) were tested for binding of 5-HEDH. The majority of the 5-HEDH activity appeared not to bind to the Affigel and was present in the first fraction (Figure 4.23). After binding, the Affigel-5-HETE was washed with the same binding buffer to ensure unbound proteins were removed (second bar in Figure 4.23). To elute 5-HEDH, the Affigel-5-HETE was washed with 1 M NaCl in both 15 mM and 30 mM octyglucoside on ice and at 37 ^{o}C, respectively, followed by 10 μM 5-HETE for 30 min at 37 ^{o}C. Several previous experiments had indicated that 5-HEDH was bound very tightly to the Affigel-5-HETE when the incubations were carried out at 6 – 8 ^{o}C. Therefore, we tested incubating the Affigel-5-HETE at 37 ^{o}C (with NaCl or 5-HETE, last 2 bars in Figure 4.23) to facilitate the release of 5-HEDH. The lower % bound Affigel-5-HETE (45% and 38%, Figure 4.23A and B respectively), incubated with higher pH buffer, bound slightly more 5-HEDH than 60% bound Affigel-5-HETE (Figure 4.23C) or using a buffer with lower pH (Figure 4.23D). However, the recovery of 5-HEDH activity was very low (< 10%) which was not affected by the temperature of incubations. Several problems occurred with this purification method. For example, the majority of the activity did not bind to the Affigel-5-HETE for many experiments, or the majority of the activity was lost during the purification procedure. In addition, the results could not be replicated when the experiments were repeated. However, due to the potential benefits of this procedure in purifying 5-HEDH, further testing will be required for developing a purification protocol.

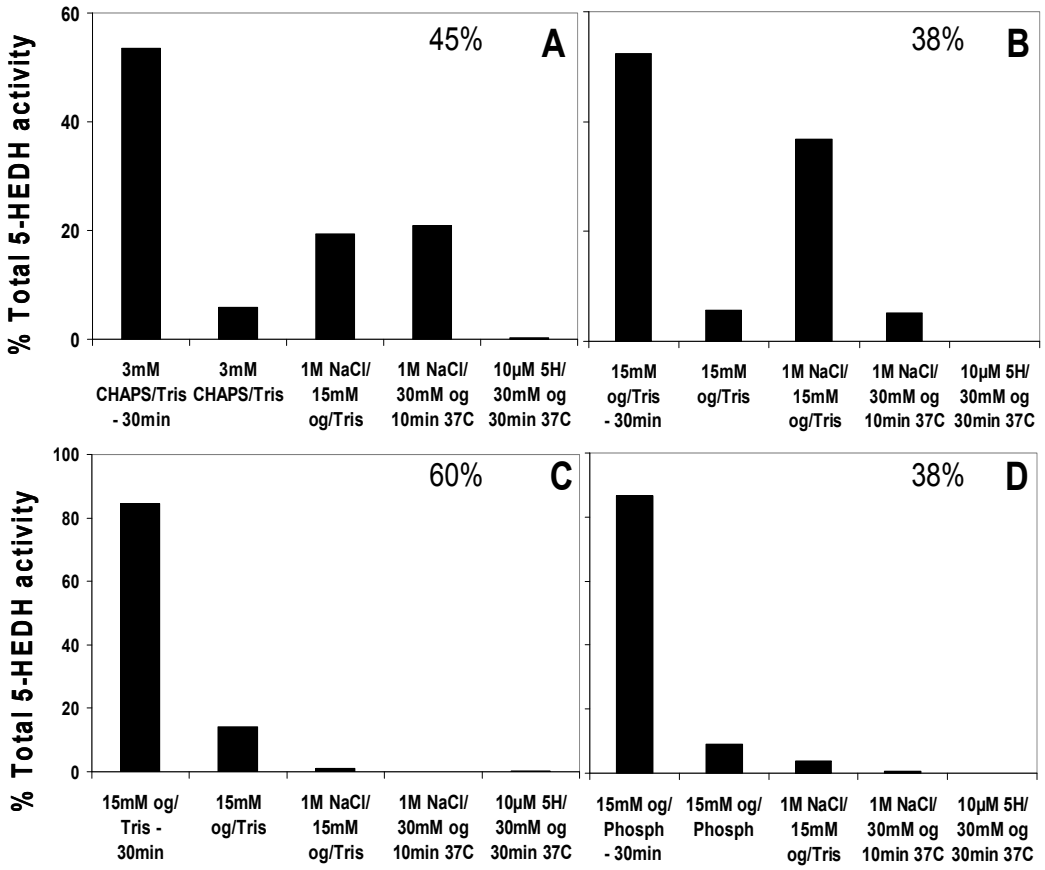


Figure 4.23: Affinity chromatography of 5-HEDH. Using a batch method, 4 different batches **A**) 45%, **B**) 38%, **C**) 60%, and **D**) 38% of Affigel-5-HETE (~150 µl) were incubated with DEAE purified 5-HEDH fractions (80 µg) for 20 min at 6 – 8 ^{o}C. Binding buffers included 50 mM Tris buffer (pH 8.8) (**A-C**) and 20 mM phosphate buffer (pH 7.4) (**D**) contained detergent (CHAPS or octyglucoside). To elute 5-HEDH, the Affigel-5-HETE was washed with 1 M NaCl in both 15 mM and 30 mM octyglucoside on ice and at 37 ^{o}C, respectively, followed by 10 µM 5-HETE for 30 min at 37 ^{o}C. 5-HEDH activity was measured in as described in Materials and Methods (Chapter 3, pg 58).

4.3.11 Native/blue native gel electrophoresis

Native gel electrophoresis, used to separate non-denatured proteins, was also tested for potentially purifying 5-HEDH. Identifying 5-HEDH migration by measuring its activity directly in native gels could potentially provide more

information about its size. Additionally, once its location in the native gel was determined, it could then be purified by denaturing electrophoresis (SDS-PAGE) for subsequent analysis by mass spectrometry to determine its sequence.

Numerous conditions for native gel electrophoresis were tested with a goal to maintain 5-HEDH activity with optimal separation from other proteins. For example various concentrations of different detergents (CHAPS and octylglucoside) and/or glycerol were added to the gel mixtures before polymerization. In addition, different gel loading buffers and acrylamide gradients were also tested. A 4% stacking gel which included the same detergent as the resolving gel being tested was always used. Another technique, blue native electrophoresis (BN-PAGE) which uses Coomassie Blue in the resolving buffer, and was designed to separate membrane proteins, was also tested.

For each gel, samples (solubilized microsomes, or fractions obtained following partial purification of 5-HEDH on DEAE or Blue Sepharose) were loaded onto two lanes each. After the electrophoresis, one lane for each sample was stained with Coomassie Brilliant Blue R-250 (Bio-Rad) and the other lane was used to measure 5-HEDH activity. Before loading onto the gel, the samples were diluted in 20 mM phosphate buffer containing detergent (CHAPS or octylglucoside) depending on the conditions being tested. Two proteins, glutathione reductase (100 kDa) and BSA (66.4 kDa), were used as molecular weight markers. The gels were run at 6 – 8 ^{o}C at 100 V in running buffer until migration was complete.

To measure 5-HEDH activity in the native gels, ten equally sized bands excised from the gels were placed into separate incubation tubes. The bands were then incubated with 5-HETE (2 μM) and $NADP^{+}$ (100 μM) for 60 min. Fortunately, 5-HEDH remained active and its migration in the gels was easily determined. As a membrane protein, we thought that the solubility and therefore, migration and activity of 5-HEDH might be enhanced by adding a non-denaturing, non-ionic detergent to the electrophoresis gel. Adding detergents improved the detection of 5-HEDH activity measured directly in the gel compared to when no detergents were added. Figure 4.24A and C includes 2 sample gels,

that were polymerized with 6 mM CHAPS and 2 different acrylamide gradients (4 – 12% and 4-16%, respectively). 5-HEDH activity was highest in gels supplemented with 6 mM CHAPS compared to those supplemented with octylglucoside (15 mM or 30 mM) or a lower concentration of CHAPS (3 mM). The amount of detergent in the loading buffer did not affect migration (Figure 4.23A), but had an effect on 5-HEDH activity, where samples loaded in 20 mM CHAPS had greater activity than 10 mM CHAPS or 40 mM octylglucoside (Figure 4.24B). Addition of glycerol, induced a slight shift in migration and also increased the 5-HEDH activity measured (Figure 4.24C and D). Unfortunately, 5-HEDH activity was not concentrated in a small band, but was spread through several bands (Figure 4.24B and D). Numerous conditions were tested to concentrate the activity into one band, however, a similar 5-HEDH activity profile was always obtained. Therefore, this was not helpful for purifying 5-HEDH or identifying its MW.

The results with BN-PAGE were similar to those with native electrophoresis, where 5-HEDH activity was spread through several bands. Figure 4.25 presents one example of BN-PAGE of solubilized microsomes and 5-HEDH that had been partially purified using DEAE and blue Sepharose. The gel stained with Coomassie blue showed that the protein was smeared throughout the lane and the measured 5-HEDH activity was similarly spread across a large portion of the lanes (Figure 4.25B). We were unable to optimize the conditions so that 5-HEDH was concentrated into one band (or section) of the gels.

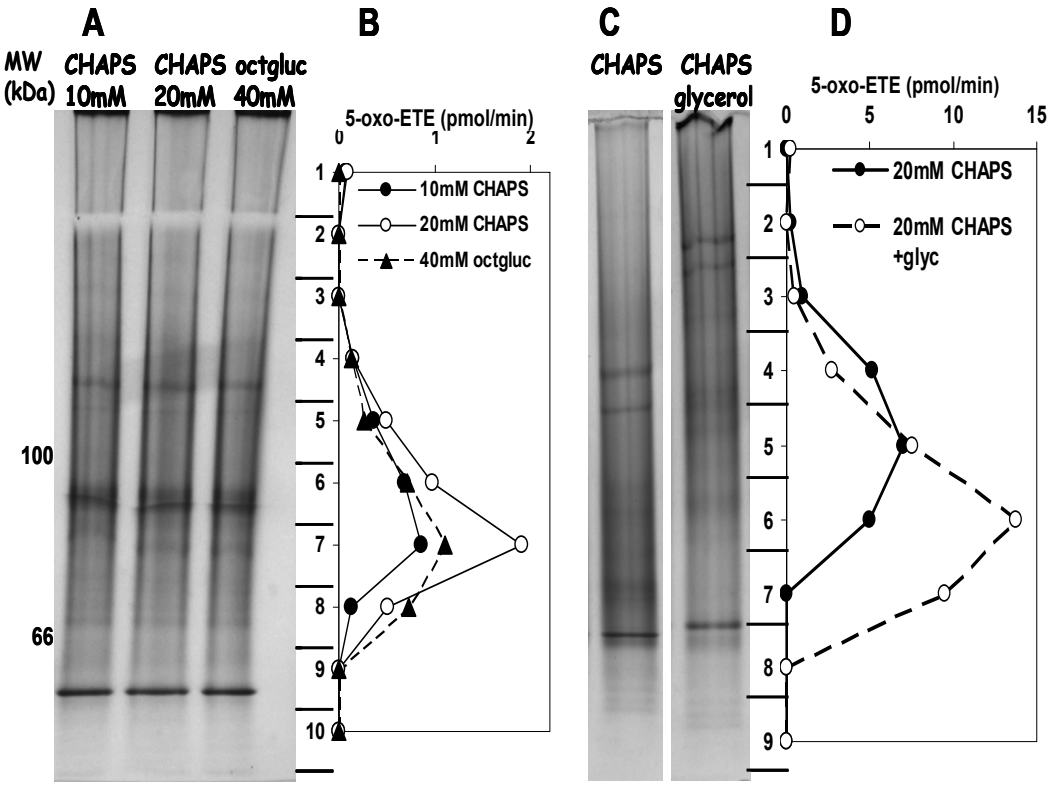


Figure 4.24: Distribution of 5-HEDH activity in solubilized microsomes loaded onto a native acrylamide gel. **A,B**) Solubilized microsomes (20 µg) from U937 cells were loaded onto several lanes of a native acrylamide gel containing 6 mM CHAPS (4% stacking; 4-12% gradient resolving gel). The detergent was varied in the loading buffer (10 mM (●) or 20 mM CHAPS (○), or 40 mM octylglucoside (▲)). The gel was run for 2 hrs at 100 volts (6-8 ^{o}C). **B**) 5-HEDH activity was measured in bands excised from duplicate lanes. **C,D**) Solubilized microsomes were loaded onto two native gels in 20 mM CHAPS loading buffer. The 4-16% acrylamide gradient gels contained 6 mM CHAPS (●) and one gel also contained 10% glycerol (○). **D**) 5-HEDH activity measured in the bands excised from duplicate lanes showed differences in distribution of 5-HEDH activity. To measure 5-HEDH activity in the excised bands, they were placed into incubation tubes, with 15 mM octylglucoside (500 µl). The bands were incubated with 5-HETE (2 µM) and $NADP^{+}$ (100 µM) for 1 h at 37 ^{o}C. (n=1)

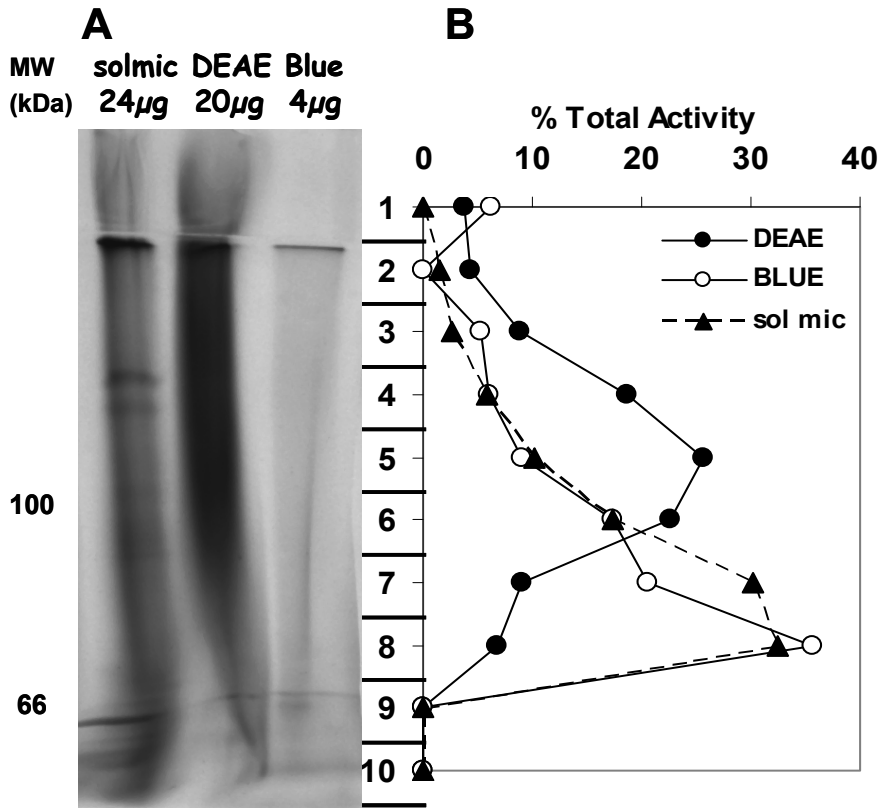
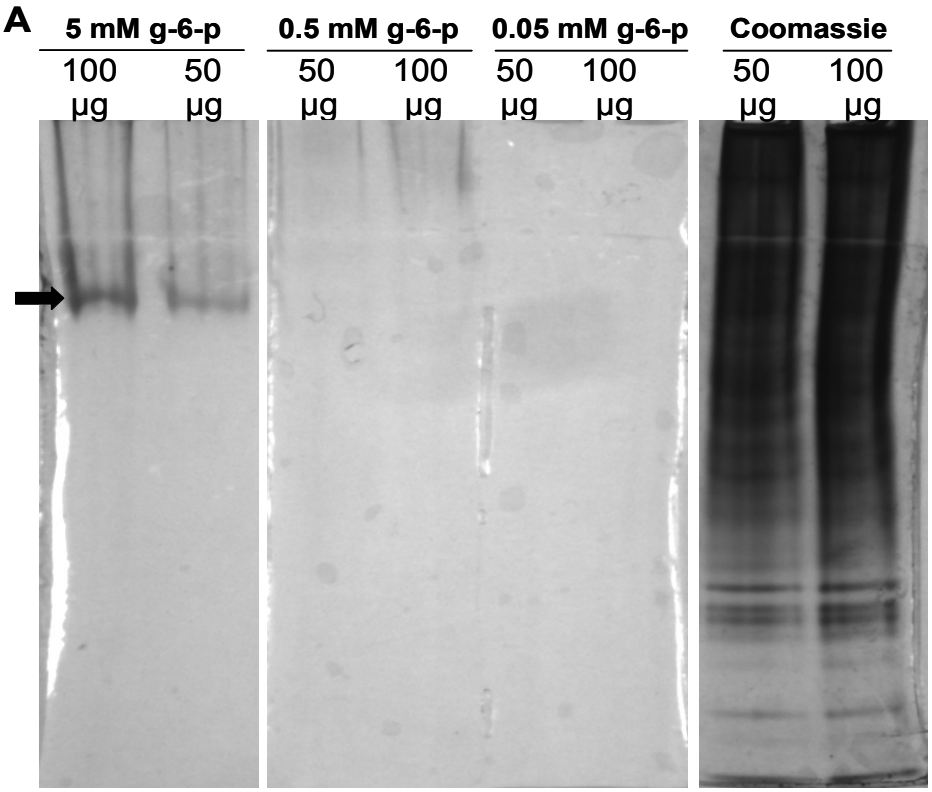


Figure 4.25: Migration of proteins and 5-HEDH activity by BN-PAGE. A) Solubilized microsomes (solmic; 24 µg) from U937 cells, DEAE purified protein (DEAE; 20 µg), and DEAE-Blue Sepharose purified protein (Blue; 4 µg) was loaded onto several lanes of a blue-native acrylamide gel containing (4% stacking; 4-16% gradient resolving gel). The gel was run for 2 hrs at 100 volts (4 ^{o}C) and the blue cathode buffer was replaced when the running front was at half of the total running distance as described in Materials and Methods (Section 3.7.14). **B**) 5-HEDH activity was measured in bands excised from duplicate lanes. The bands, placed in tubes, were incubated in 15 mM octylglucoside (500 µl) with 5-HETE (2 µM) and $NADP^{+}$ (100 µM) for 1 hr at 37 ^{o}C. (n = 1)

4.3.12 In-gel formazan precipitation enzymatic assay

As an alternative to excising native gel lane bands and then incubating them separately to determine 5-HEDH activity, an in-gel formazan precipitation assay was evaluated. This would provide faster results, and potentially result in more accurate determination of 5-HEDH location within the gel. The relative amounts of substrate (5 – 15 mM), tetrazolium salt (0.2 - 0.8 mg/ml), $NADP^+$ (100 – 500 μM) and PMS (40 – 200 μg/ml) needed to be optimized, to determine the minimum amounts required for a given amount of protein. Control lanes were incubated either without substrate, or lanes without protein were included with the incubations. Since quite large amounts of substrates are requires (μM - mM), the feasibility of the assay was tested using cytosolic glucose-6-phosphate dehydrogenase (G-6-PD) (Figure 4.26A) due to limited amounts of 5-HETE. Cytosolic protein obtained by differential centrifugation of PMA-differentiated U937 cell sonicates (50, 100 μg) was loaded onto a native gel, and electrophoresed. Gel lanes were incubated separately in the presence of INT (0.4 mg/ml), $NADP^+$ (500 μM), PMS (0.2 mg/ml), and substrate, glucose-6-phosphate (g-6-p; 0.05, 0.5 or 5 mM) for up to 2 h. A band of formazan precipitate was only observed for incubations with the highest amount of g-6-p (5 mM). Even the lowest amount of substrate (0.05 mM), would utilize too much 5-HETE for a 5-HEDH assay.

Even though large amounts of 5-HETE would be required, the assay was tested for 5-HEDH in solubilized microsomes (90, 180 μg) separated by native gel electrophoresis (8% acrylamide) (Figure 4.26B). 5-HEDH has high affinity for 5-HETE and might not require as high levels of substrate as G-6-PD. 5-HETE (15 μM) was incubated with the gel lane, in the presence of INT (0.4 mg/ml), $NADP^+$ (500 M), PMS (0.2 mg/ml), however, the assay did not reveal the location of the enzyme subjected to native electrophoresis. This may be due to an insufficient amount of 5-HETE or more likely to the spread of the enzyme over a large portion of the gel lanes resulting in insufficient formazan precipitate formed in a concentrated area to be visualized.


A
5 mM g-6-p
0.5 mM g-6-p
0.05 mM g-6-p
Coomassie
100 µg
50 µg
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50 µg
100 µg

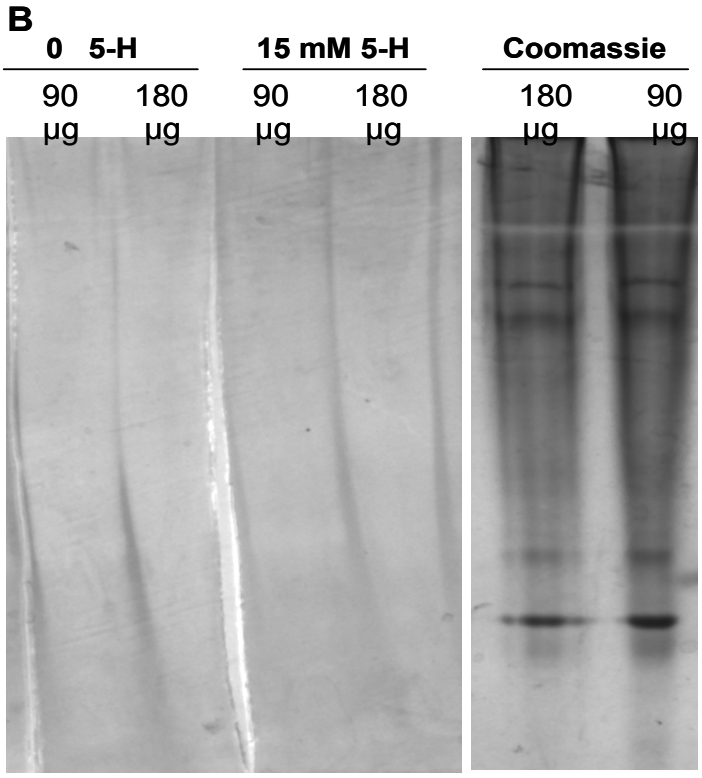
B
0 5-H
15 mM 5-H
Coomassie
90 µg
180 µg
90 µg
180 µg
180 µg
90 µg

Figure 4.26: In-gel 5-HEDH activity formazan precipitation assay of G-6-PD and 5-HEDH. A) Cytosolic protein or **B**) solubilized microsomes were submitted to native gel electrophoresis (8% resolving gel). **A**) Lanes were either stained with Coomassie Blue or subjected to formazan precipitation. Gel lanes were incubated with glucose-6-phosphate (0.05, 0.5 or 5 mM), $NADP^+$ (500 μM), PMS (0.2 mg/ml), and INT (0.4 mg/ml) in PBS^- for 2 h at room temp. in the dark. The arrow indicates staining for g-6-pd. **B**) Gel lanes were either stained with Coomassie Blue or incubated with $NADP^+$ (500 μM), PMS (0.2 mg/ml), INT (0.4 mg/ml) and with or without 5-HETE (15 mM) in 15 mM octylglucoside in phosphate buffer (2.5 ml) for 2 hrs.

4.3.13 2D-Electrophoresis of partially purified 5-HEDH

Combining two separate electrophoresis gels results in greater separation of proteins and 2D electrophoresis (native followed by denaturing) was performed on DEAE purified 5-HEDH protein fractions. Figure 4.27 presents an example of a 2D-gel of native electrophoresis separated proteins subsequently separated in an SDS gel. Bands (9) from a triplicate blue-native gel lane (Figure 4.25A; DEAE lane) were excised and loaded onto a denaturing SDS Tricine gel (10% resolving), and electrophoresed. Each band from the native gel contained numerous bands that could be separated by denaturing electrophoresis, and, differences are observed in the bands visualized. For example, bands 2 to 4 contain many protein bands greater than 100 kDa, whereas bands 5 and 6 contain fewer bands at this size. Denaturing gels separate proteins solely based on size, since proteins do not retain their tertiary structure. Although native gels do not separate proteins based exclusively on size, larger proteins do not migrate as far as smaller proteins. The limitations of this method are due to the spread of 5-HEDH activity in the native gel electrophoresis step.

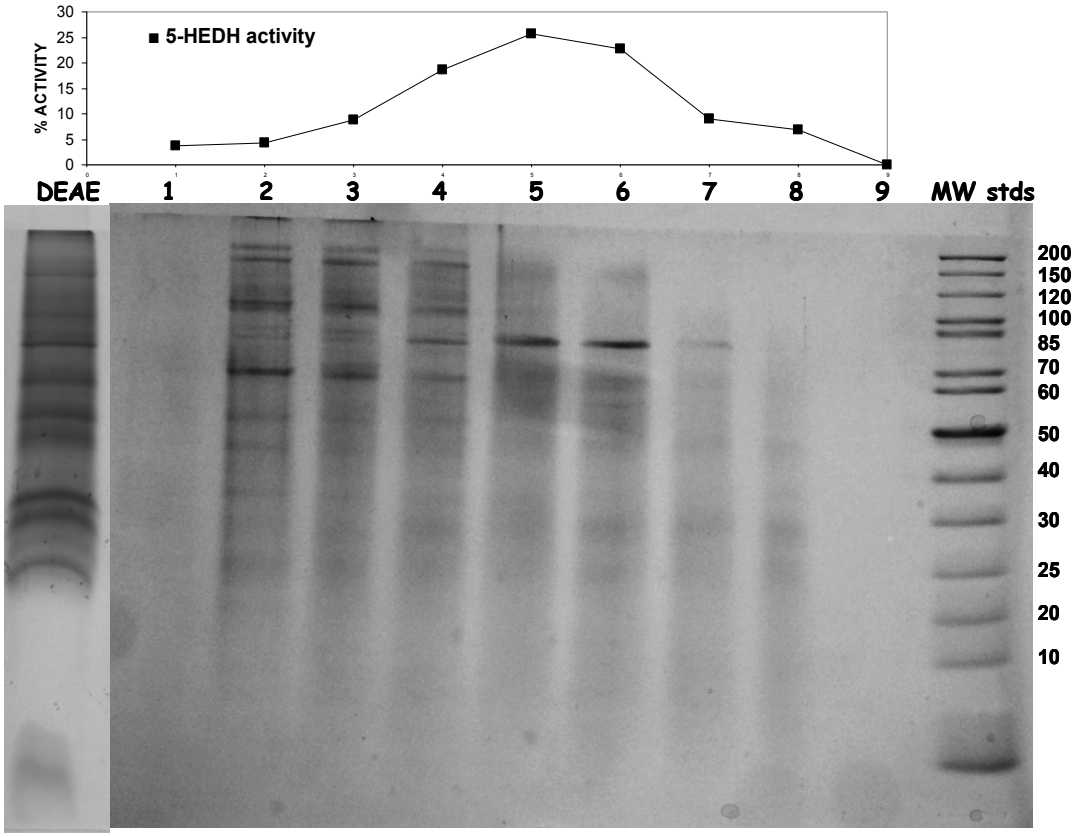

Figure 4.27: 2D-PAGE – BN-PAGE followed by SDS-PAGE of DEAE purified 5-HEDH. Bands excised from a blue native gel (4-16% acrylamide) that was loaded with DEAE purified 5-HEDH (Figure 4.25A) were equilibrated in denaturing 0.5 M Tris-HCl pH 6.8 buffer containing SDS (1% (v/v)) and β-mercaptoethanol (1% (v/v)) for 30 min at room temp. The lanes were then placed on the top of the stacking gel (6%), and electrophoresed at 120 V for ~2 h. The gel was stained with Coomassie Blue. The graph presents the 5-HEDH activity (■) in the individual bands from the blue native gel.

4.3.14 SDS-PAGE separation of 5-HEDH purified fractions

5-HEDH fractions purified by DEAE, followed by Blue Sepharose were concentrated using a 10 kDa MWCO filter and the detergent was exchanged for 10% surfactin which is more compatible with mass spectrometric sequencing. The proteins (32 µg) were separated on a 10% (w.v) acrylamide Tris-glycine gel (Figure 4.28) with a 6% (w/v) loading gel. Partially purified 5-HEDH from two

sources (solubilized microsomes from PMA-differentiated U937 cells or neutrophils isolated from whole blood) was analyzed separately. Since the actual molecular weight of 5-HEDH is unknown, ten equally sized bands were excised from the gels between 30 kDa and 70 kDa, based on the retention of 5-HEDH activity with the MWCO filters. The tryptic peptides from the excised bands were then analysed by mass spectrometry.

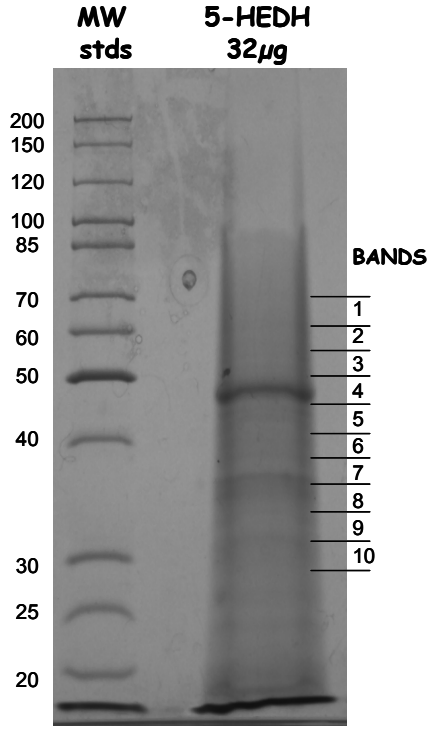

Figure 4.28: SDS-PAGE separation of 5-HEDH protein partially purified sequentially by DEAE and blue Sepharose. Protein fractions (32 µg) containing partially purified (DEAE – Blue Sepharose) 5-HEDH from U937 cells were loaded onto a 10% Tris-glycine denaturing SDS gel (4% (v/v) loading gel). Molecular weight standards were loaded onto another well and the proteins were allowed to migrate at 110 volts for 2 hrs, until the loading dye reached the bottom of the gel. The gel was stained with Coomassie blue. Bands 1-10 were cut from the 5-HEDH protein lane for subsequent analysis by mass spectrometry.

4.3.15 LC/MS/MS analysis of proteins in purified fractions

In an attempt to identify 5-HEDH, tryptic peptides from the 10 bands excised from the SDS gels were sequenced by LC-MS/MS. The sequenced digested peptides were then identified by MASCOT, a search engine that matches mass spectrometry data to available primary sequence databases (Swiss-Prot) to identify proteins. Numerous known proteins as well as proteins with assumed functions based on sequences were identified, including several dehydrogenases, for each cell type. Some examples are listed in Table 4.3. Of the dehydrogenases identified, dehydrogenase/reductase (SDR family) member 7 (DHRS7), a member of the short-chain dehydrogenase superfamily of proteins was identified in both U937 cells and neutrophils. Although the alternative name for this enzyme is retinol short-chain dehydrogenase/reductase (retSDR4), the substrate for this enzyme is unknown. Different peptides were sequenced for this protein, and Table 4.4 shows the peptide sequences obtained from 5-HEDH purified from U937 cells and neutrophils. Further evaluations will be needed to confirm these findings.

Table 4.3: Dehydrogenase proteins identified by SDS-PAGE and LC-MS/MS analysis from U937 cells and primary human neutrophils.

Cells	Theor. MW (Da)	SwissProt accession no.	Identified Protein (alternative names)	Substrate	Sequence coverage
U937, neutrophils	38673	Q9Y394	Retinol short-chain dehydrogenase/ reductase (retSDR4); dehydrogenase/ reductase (SDR family) member 7 (DHRS7)	Unknown specificity	4 % U* 11 % N*
U937	35791	Q8TC12	Androgen-regulated short-chain dehydrogenase/ reductase 1; prostate short-chain dehydrogenase/ reductase 1; retinol dehydrogenase 11 (RDH11); retinol reductase 1	Retinal (reductase activity)	11 %
U937	42159	Q15738	NAD(P) dependent steroid dehydrogenase-like (NSDHL); Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating	3-β-hydroxyl-4-β-methyl-5-α-cholest-7-ene-4-α-carboxylate	40 %
U937	83000 (subunit)	P40939	Trifunctional enzyme subunit alpha, mitochondrial; long-chain enoyl-CoA hydratase;	(S)-3-hydroxy-acyl-CoA	5 %
U937	47151	Q8NBX0	Putative saccharopine dehydrogenase (SCC_DH)	N(6)-(L-1,3-dicarboxypropyl -L-lysine	12 %
neutrophils	27329	Q9BUP3	Oxidoreductase HTATIP2; HIV-1 TAT-interactive protein 2; TIP30	Oxidoreductase required for tumor suppression	13 %

* U, U937 cells; N, neutrophils

Table 4.4: Peptides sequenced by LC-MS/MS that correspond to DHRS7

Cells	Sequenced Peptides
U937 cells	IDILVNNGGMSQR - 13 aa
Neutrophils	LGVSLVLSAR - 10 aa AVLQEFGR - 8 aa CVLPHMIER - 9 aa LMLISMANDLK - 11 aa

4.3.16 Other purification methods evaluated for the purification of 5-HEDH

Several other purification techniques were tested which were unsuccessful in purifying 5-HEDH included hydrophobic interaction chromatography, reverse phase HPLC, $NADP^+$-Sepharose and 2',5'-ADP-Sepharose, and trichloroacetic acid (TCA) precipitation. However, appropriate binding or elution conditions could not be established and/or significant enzymatic activity was lost. In an attempt to determine whether 5-HEDH was localized in the nuclear membrane, isolation of nuclear membrane fractions was attempted, and tested for 5-HEDH activity. However, several enzymatic assays would be required to ensure the purity of the nuclear membrane fraction. This would include assays to test whether enzymes normally present in other organelles or the plasma membrane were present. However, since 5-HEDH is a microsomal enzyme, it is probably localized to the endoplasmic reticulum, therefore, isolation of nuclear membrane fractions was not pursued further.

4.3.17 Elution of 5-HEDH dehydrogenase activity and ketoreductase activity

Many dehydrogenases are able to catalyze both oxidation and reduction reactions depending on the relative concentration of the substrate and product. Microsomal fractions from neutrophils and U937 cells catalyze the reduction of the 5-HEDH product 5-oxo-ETE to 5-HETE in the presence of NADPH [98], but it was not known whether this was due to 5-HEDH acting in the reverse (ketoreductase) direction (Figure 4.29C). We therefore determined whether 5-HETE dehydrogenase and 5-oxo-ETE ketoreductase activities co-eluted during different purification procedures.

Purified protein fractions were incubated with either 5-HETE (1 μM) plus $NADP^+$ (100 μM), or 5-oxo-ETE (2 μM) plus NADPH (100 μM) at pH 7.4 in 15 mM octylglucoside in Buffer A. When solubilized microsomes were fractionated using DEAE, the dehydrogenase and ketoreductase activities co-eluted in the same proportions in all of the fractions with the majority of both activities eluting in the 20 mM NaCl fractions (Figure 4.29A). Similarly, when the activity was

measured in fractions from a size exclusion column, both activities displayed similar elution profiles (Figure 4.29B).

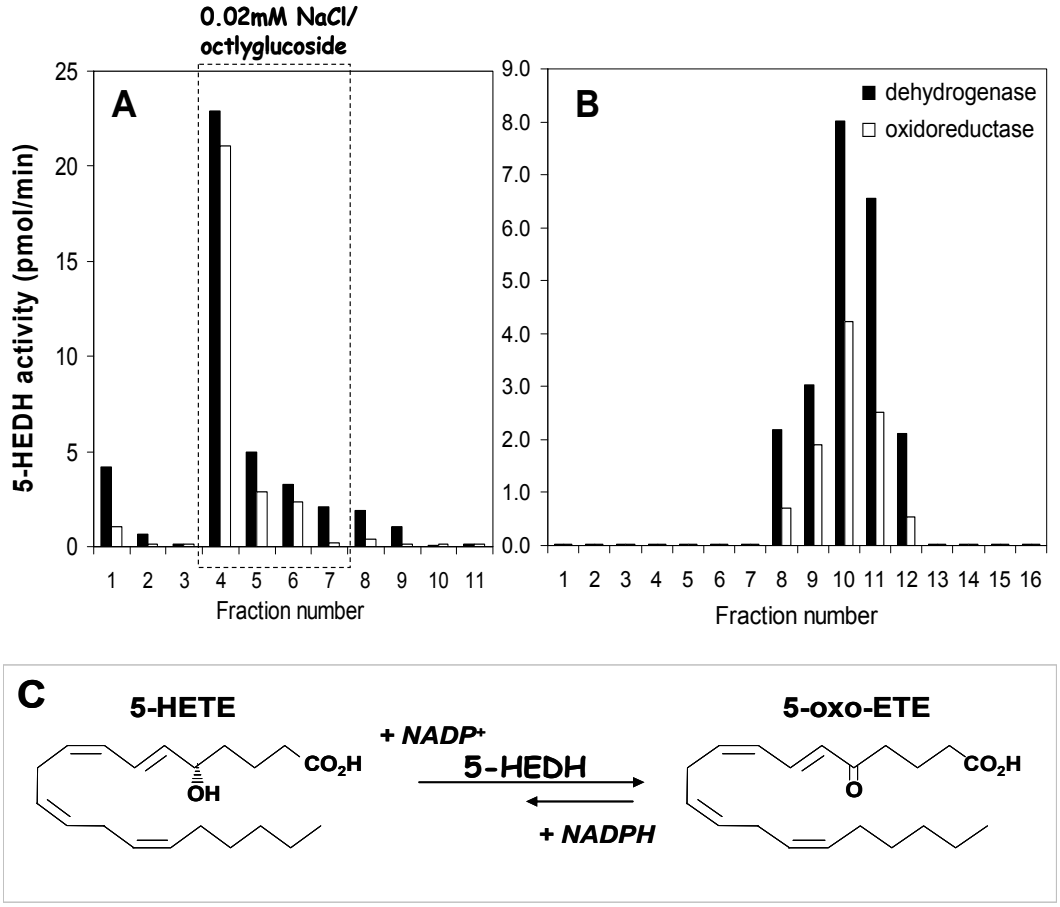

Figure 4.29: Elution of dehydrogenase and oxidoreductase activity by DEAE Sepharose and size exclusion chromatography. 5-HEDH dehydrogenase (■) and ketoreductase (□) activity in elution fractions from (**A**) DEAE Sepharose purification and (**B**) SEC separation of solubilized microsomes isolated from U937 cells. Aliquots were incubated with either 5-HETE (1 μM) plus $NADP^+$ (100 μM) or 5-oxo-ETE (2 μM) plus NADPH (100 μM) for 60 min at 37 ^oC in 15 mM octylglucoside in Buffer A. (**C**) Dehydrogenase and ketoreductase reactions catalyzed by 5-HEDH at physiological pH.

Chapter 5: Discussion

Previous studies have shown that 5-oxo-ETE is a potent leukocyte chemoattractant [126,134,142,174], promotes the survival of cancer cells in vitro [76,168], and induces many intracellular responses [5]. In addition, leukocytes, smooth muscle, epithelial, and endothelial cells possess 5-HEDH activity and convert 5-HETE to 5-oxo-ETE [5,110-112]. As a 5-LO metabolite of AA, the synthesis of 5-oxo-ETE is tightly regulated at several steps. A greater understanding about the regulation of its production would be beneficial in understanding its potential roles in various conditions or diseases. Studies to date have looked at the biochemical and cellular regulation of 5-oxo-ETE synthesis by 5-HEDH. The effects of the levels of the cofactor ($NADP^+$) as well as the structural requirements for metabolism by 5-HEDH have been studied in leukocytes [101,110] and structural cells [111,112]. Objectives of these studies were to determine whether 5-oxo-ETE can be synthesized by B lymphocytes and cancer cells, and if so, how this is regulated. Another objective was to partially purify 5-HEDH with the ultimate goal of obtaining its sequence.

5.1. B lymphocytes synthesize 5-oxo-ETE

Previous studies have shown that B cells express 5-LO and FLAP and synthesize 5-HETE and LTB_4 [43,45-47,50,231]. However, synthesis of 5-LO products by B cells required stimulation by oxidative stress as well as addition of exogenous AA and calcium ionophore, A23187. The requirement for exogenous AA may be due to the fact that only immature B cells and not mature B cells contain $cPLA_2$ [51]. Tonsillar B cells comprise both mature and naïve B cells, and therefore, this may account for their inability to metabolize endogenous AA to appreciable amounts of 5-LO products even though some of them express 5-LO and FLAP.

The aim of this study was to determine whether B cells synthesize 5-oxo-ETE and whether its synthesis is regulated by oxidative stress. We determined that primary isolated tonsillar B cells as well as three human B cell lines (CESS,

U266, and Ramos) express 5-HEDH and synthesize 5-oxo-ETE when incubated with its direct substrate 5-HETE. CESS cells had the greatest capacity to synthesize 5-oxo-ETE, whereas the other types of B cells synthesized lower levels.

To determine whether B cells synthesize 5-oxo-ETE from AA, only CESS and tonsillar B cells were tested. The CESS cell line is a proliferating EBV-immortalized IgG^+ lymphoblastoid B cell line that expresses surface $CD19^+$, $CD20^-$, $CD44^+$, $CD38^+$, $CD77^-$, and the cells are therefore similar to memory B cells [232,233]. In contrast, tonsillar B cells comprise a heterogeneous pool of B cells of different activation states and maturity, including naïve, pro-germinal center, germinal center and memory B cells [234]. We determined that CESS and tonsillar B cells synthesized 5-oxo-ETE when incubated with exogenous AA and calcium ionophore, A23187. However, there were differences in the relative levels of 5-HETE, 5-oxo-ETE, and LTB_4 produced between CESS and tonsillar B cells. Overall, the total amounts of 5-LO products synthesized by CESS cells were substantially greater than those produced by tonsillar B cells. 5-HETE was the major 5-LO product formed by both cell types, in agreement with previous reports [45,46], but CESS cells in the absence of oxidative stress synthesized similar amounts of 5-oxo-ETE and LTB_4 whereas tonsillar B cells synthesized about twice as much 5-oxo-ETE compared to LTB_4.

5.1.1 Oxidative stress induces 5-oxo-ETE synthesis in B cells

Several factors can induce ROS production or oxidative stress in B cells. PMA, protein A, cross-linking of B cell antigen, or anti-Ig mAb activate NADPH oxidase and stimulate oxidative burst in EBV transformed B cells as well as normal tonsillar B cells [56,235,236]. In addition, cyclosporin A which is given to transplant patients [237], the antibiotic antimycin A [55], and EBV infection [238] stimulate oxidative stress in B cells. Alternatively, ROS can be supplied to B cells by other cells such as granulocytes [55]. Production of 5-LO metabolites (5-HETE and LTB_4) in B cells was previously shown to require stimulation by ROS [45,48,52-54]. Since oxidative stress could affect 5-oxo-ETE synthesis at

the levels of both 5-LO and 5-HEDH, we wanted to determine its effects on the formation of this substance by B cells. As with monocytic cells [101], oxidative stress, induced by H_2O_2, stimulated the synthesis of 5-oxo-ETE from 5-HETE in tonsillar B cells and three B cell lines. The H_2O_2-stimulation of 5-oxo-ETE in these cells was concentration-dependent and tonsillar B cells were 10-fold more sensitive to H_2O_2 with an EC_{50} of 1.0 μM compared to 10.3 μM for CESS cells. The glutathione redox pathway has been shown to be involved in the stimulatory effect of oxidative stress on 5-oxo-ETE synthesis in other cells [110]. We also demonstrated the involvement of the GSH redox cycle in this study as the glutathione reductase inhibitor BCNU blocked the stimulatory effect of H_2O_2 on the oxidation of 5-HETE to 5-oxo-ETE in these cells.

Similar to previous studies [110,101], H_2O_2 also stimulated the production of 5-LO products by B cells incubated with AA and A23187. In CESS cells, 5-oxo-ETE reached maximal levels within 5 min and then declined, whereas in tonsillar B cells, the levels were maximal by 10 min and remained at this level for at least 30 min. Both types of cells synthesized greater amounts of 5-oxo-ETE compared to LTB_4, but the levels of 5-oxo-ETE were higher than 5-HETE only in tonsillar B cells. The levels of GSSG and $NADP^+$ corresponded well with the abilities of both cells to synthesize 5-oxo-ETE (Figure 5.2).

5.1.2 Oxidative stress activates the glutathione redox pathway in B cells

We measured the levels of GSH, GSSG, NADPH and $NADP^+$ by RP-HPLC in CESS and tonsillar B cells after stimulation with H_2O_2 to determine whether the glutathione redox cycle was involved in the H_2O_2-induced stimulation of 5-oxo-ETE production (Figure 5.1). The basal levels of GSH and NADPH were approximately 16-fold and 10-fold lower, respectively, in tonsillar B cells compared to CESS cells, indicating that CESS cells may have better anti-oxidant capacity. Our results are consistent with previous studies that have shown that malignant B cells have increased levels of GSH [239]. The basal levels of $NADP^+$ were also lower (6-fold) in tonsillar B cells compared to CESS cells. H_2O_2 induced a rapid decrease in GSH with a concurrent rapid increase in GSSG

levels, indicating interconversion by GPx. This was accompanied by a similarly rapid decrease in NADPH and a concurrent increase in $NADP^+$, presumably due to conversion of GSSG back to GSH by GR, which requires NADPH as a cofactor. Both GSSG and $NADP^+$ reached maximal levels within 90 sec in both cell types but they returned toward basal levels more rapidly in CESS cells than in tonsillar B cells. While the levels of GSSG decreased to 25% of the maximum in CESS cells over 20 min, the levels only decreased to 63% in tonsillar B cells with GSH levels remaining low (Figure 5.2). This indicates lower GR activity in tonsillar B cells versus CESS cells. In addition, the GPx activity was much higher in CESS cells inducing a 63-fold increase in GSSG from basal levels versus a 6-fold increase in tonsillar B cells.

The ratio of $NADP^+$ to NADPH is important for 5-HEDH activity [101] and the maximal ratio was 6-7 for both types of cells. However, the maximum ratio was reached by 30 sec in CESS cells, followed by a sharp decline, and by 4 min in tonsillar B cells (Figure 5.2) also consistent with lower GR activity in the latter. After 10 min, the ratio in tonsillar B cells was still above 3 whereas it was only 0.3 in CESS cells. This may be an indication of lower pentose phosphate pathway activity and therefore reduced conversion of $NADP^+$ to NADPH in tonsillar B cells even though the incubations were carried out in glucose-free PBS^+. This sustained higher ratio of $NADP^+$ to NADPH may account for the prolonged synthesis of 5-oxo-ETE in tonsillar B cells. The more rapid conversion of $NADP^+$ back to NADPH in CESS cells may account for the decrease in 5-oxo-ETE levels (Figure 5.2).

In both CESS cells and tonsillar B cells, H_2O_2 induced a concentration-dependent increase in LTB_4, 5-HETE and 5-oxo-ETE synthesis in cells incubated with AA and A23187, with maximal stimulation with 100 μM. The interconversion of GSSG/GSH and NADPH/$NADP^+$ also varied with the concentration of H_2O_2 with maximal stimulation with either 100 μM or 315 μM. H_2O_2 was more potent in inducing stimulation of $NADP^+$, and 5-oxo-ETE synthesis in tonsillar B cells compared to CESS cells, whereas the potency was similar for GSSG production. This may be due to differences in basal cofactor

levels and enzyme activities. In both types of cells, the synthesis of all three 5-LO products was decreased at the highest H_2O_2 concentration employed, presumably due to inhibition of 5-LO. In contrast, 5-HEDH was not inhibited by this concentration of H_2O_2 when the substrate was exogenous 5-HETE.

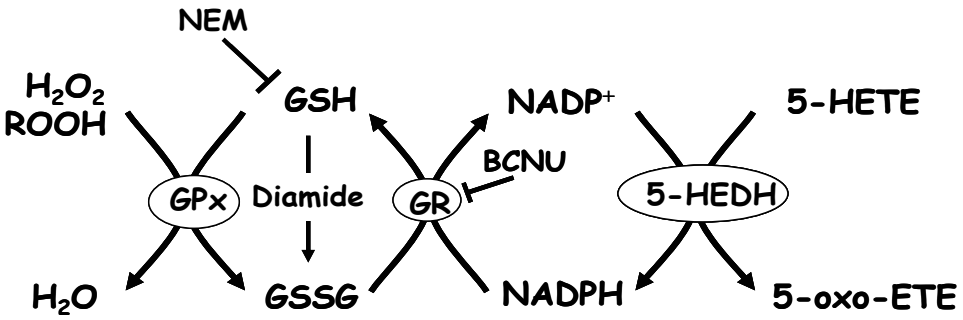

Figure 5.1: The proposed mechanism for the stimulation of 5-oxo-ETE by H_2O_2 by the glutathione redox cycle. H_2O_2 is reduced to water by GPx (glutathione peroxidase), which oxidizes GSH to GSSG, which is then reduced back to GSH by GR (glutathione reductase) which uses NADPH as a cofactor. The increased levels of $NADP^+$ are available for the 5-HEDH (5-hydroxyeicosanoid dehydrogenase) catalyzed conversion of 5-HETE to 5-oxo-ETE. NEM (N-ethylmaleimide) depletes GSH levels and BCNU inhibits GR. H_2O_2 can also be degraded by catalase.

In agreement with previous studies with B cells [45,48], diamide, which converts GSH to GSSG, stimulated the synthesis of 5-LO products in both CESS and tonsillar B cells, diamide increased 5-oxo-ETE levels more than other 5-LO products. As with H_2O_2, 5-oxo-ETE reached a maximum and then declined in CESS cells, but continued to increase up to at least 30 min in tonsillar B cells. This provides further evidence that the lower GPx activity in tonsillar B cells was limiting the production of 5-oxo-ETE since diamide bypasses this enzyme.

This study provides evidence that stimuli that induce oxidative stress in B cells may promote the production of 5-oxo-ETE. However, AA or 5-HETE may

need to be provided by adjacent cells since endogenous release of AA may be limited. Differences in the activities of enzymes involved in maintaining intracellular levels of NADPH and GSH as well as the activity of 5-HEDH itself determine the amounts of 5-oxo-ETE synthesized by B cells and presumably also other cells.

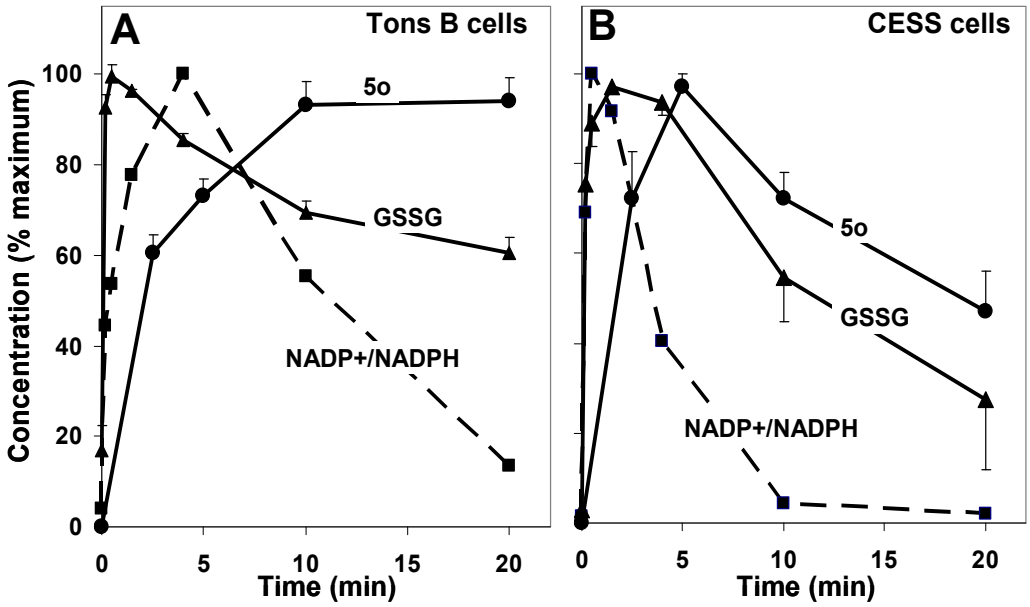

Figure 5.2: The ratio of $NADP^+$ to NADPH, and GSSG correspond to the levels of 5-oxo-ETE produced in oxidative stress stimulated B cells. A) Tonsillar B cells and B) CESS cells were stimulated with 100 µM H_2O_2. To measure 5-oxo-ETE, the cells were also incubated with AA (40 µM) and A23187 (5 µM). GSSG (▲, n = 4), $NADP^+$, NADPH (ratio $NADP^+$/NADPH; ■, n = 4), and 5-oxo-ETE (5o; •, n = 5) were measured by RP-HPLC as described in Materials and Methods (Chapter 3, pg 51-57). The % maximal responses are presented with S.E. Same day separate incubations were performed for $NADP^+$ and NADPH, therefore SE was not calculated for the ratio.

5.1.3 Effects of 5-LO products on B cells

Why do B cells have the capacity to synthesize 5-LO products, including 5-oxo-ETE? B cells express the BLT1 receptor [240], and LTB_4 induces many responses in these cells, including surface expression of the activation marker CD23, enhanced IgE production [241], proliferation, and differentiation of IL-4 stimulated B cells [242,243]. B cells also express the $CysLT_1$ receptor and LTD_4 induces calcium mobilization and immunoglobulin production [244]. We conducted some preliminary experiments to determine whether B cells also respond to 5-oxo-ETE. We found that tonsillar B cells express low levels of OXE receptor mRNA as measured by real-time PCR, and its expression was slightly increased in anti-CD40, IL-4 activated cells. However, we did not observe any biological responses using the cellular assays tested. 5-oxo-ETE did not induce actin polymerization, measured by flow cytometry in tonsillar B cells or Ramos cells. Neither did 5-oxo-ETE have an effect on calcium mobilization, measured by fluorescence, in CESS, Ramos or tonsillar B cells compared to the positive control, PAF, which induced responses in all of the cell types. Chemotaxis assays, performed in a Boyden chamber, were not conclusive. 5-Oxo-ETE may induce other biological responses in B cells which have not been tested or B cells of different maturity or activation states from those tested may respond to 5-oxo-ETE.

5.1.4 B cells synthesize 12-HHT

Studies have previously shown that B cells express COX-1 and COX-2 and synthesize PGE_2 and to a lesser extent $PGF_{2\alpha}$ [245]. In other cell types, the COX product PGH_2 is converted by TXA_2 synthase to 12-HHT, TXA_2 and malondialdehyde (MDA) [246]. Studies on the synthesis of TXB_2 by human B cells have been limited. Peripheral B cells have been shown to synthesize very minimal levels of TXB_2 (similar to background) using RIA while it was undetectable in a human B cell line, GM-130 [247]. Murine B cells have also been shown to synthesize TXB_2 [248]. The present study showed that B cells synthesize 12-HHT in addition to 5-LO products, when incubated with AA and

A23187. CESS cells synthesized more 12-HHT than any of the 5-LO products, whereas the amount of 12-HHT synthesized by tonsillar B cells was similar to that of 5-oxo-ETE (Figure 4.4 A and D). COX-2 expression has been shown to be upregulated in activated (CD40) B cells [245] and therefore, the synthesis of 12-HHT may be upregulated in activated cells, but we did not examine this possibility. H_2O_2 did not affect the levels of 12-HHT in either CESS or tonsillar B cells, but diamide induces a 50% increase in CESS cells. Since 12-HHT, TXA_2 and MDA are synthesized in approximately equal amounts, the level of 12-HHT has long been considered a marker for the synthesis of TXA_2, and was initially assumed to have little biological activity. However, a recent study showed that 12-HHT activates ERK in PC3 cells and human lung fibroblasts and also activates the epidermal growth factor receptor (EGFR) via activation of an MMP in PC3 cells [249]. That study did not identify the receptor directly activated by 12-HHT, which did not have an effect in as many cell types as the TXA_2 receptor agonist, I-BOP. Recently Okuno and colleagues showed that 12-HHT has higher affinity than LTB_4 for the BLT_2 receptor [4]. In this study, 12-HHT was shown to induce chemotaxis, calcium mobilization and decreased cAMP formation in cells transfected with the BLT_2 receptor [4]. Since B cells express this receptor [250], it is possible that 12-HHT may have an autocrine effect on these cells, but this was beyond the scope of this study and was not tested.

5.2 Cancer cells convert 5-HETE to 5-oxo-ETE

Prostate cancer cells have previously been shown to express higher levels of 5-LO and FLAP than normal epithelial cells [18]. The PC3 cell line is androgen-insensitive and highly metastatic, derived from metastatic lesions of bone [251]. Since inhibitors of the 5-LO pathway induce apoptosis of PC3 cells [77,80], and 5-oxo-ETE promotes their survival [80,87,168], it was important to determine whether these cells have the ability to synthesize 5-oxo-ETE and if so, how this is regulated. We observed that PC3 cells do indeed synthesize 5-oxo-ETE from 5-HETE and express 5-HEDH activity. Cancer cells have high metabolic activity, and in many cases produce large amounts of H_2O_2 with or

without stimulation [252]. Therefore, cancer cells may have a high capacity to synthesize 5-oxo-ETE since oxidative stress and H_2O_2 have been shown to enhance its synthesis by a variety of other cells, including B cells (Section 5.1), monocytes [101], bronchial epithelial cells and bronchial smooth muscle cells [112]. As observed with other cell types, H_2O_2 strongly stimulated 5-oxo-ETE synthesis from 5-HETE by PC3 cells with its levels increasing up to at least 60 min. This was accompanied by rapid increases in the levels of GSSG and $NADP^+$. The basal levels of GSH (~40 nmol/10^6 cells) were much higher than in B cells and were similar to those previously observed in keratinocytes [103] and normal human bronchial epithelial cells [112]. GSH levels in PC3 cells have previously been reported to be about twice those in the slower growing androgen-sensitive prostate cancer cell line, LNCaP [253]. This elevated antioxidant capacity of PC3 cells may allow for greater survival, proliferation or metastatic activity. Unlike B cells, the levels of GSSG diminished very slowly over time following the addition of H_2O_2, and the levels of $NADP^+$ continued to increase up to at least 20 min. This is similar to the effects of H_2O_2 on 5-oxo-ETE synthesis and GSSG and $NADP^+$ levels in normal human bronchial epithelial cells (NHBE) [112]. The prolonged increase in 5-oxo-ETE up to at least 60 min may be due to the increased availability of $NADP^+$, the cofactor for 5-HEDH. This may be a result of a reduced rate of degradation of H_2O_2, since PC3 cells have lower catalase than less metastatic cells [254] or a reduced rate of reduction of $NADP^+$ by the pentose phosphate pathway. $NADP^+$ increased in a biphasic manner after stimulation with H_2O_2. There was initially a very rapid increase up to about 1 min, followed by a slower sustained increase up to at least 20 min. Although the levels of NADPH were not measured, it would seem likely that the initial increase in $NADP^+$ was due to the rapid glutathione reductase-catalyzed oxidation of NADPH, following reduction of H_2O_2 by glutathione peroxidase (Figure 5.1). The slower sustained increase in $NADP^+$ may be due to activation of NAD kinase, a ubiquitously expressed enzyme that is required for the synthesis of NADP(H) and is activated as a result of depletion of NADPH [255]. Evidence for this is the

continued decrease in NAD^+ levels starting about 3 min after H_2O_2 stimulation and continuing until at least 20 min.

5.2.1 Transcellular biosynthesis of 5-oxo-ETE

We were unable to detect any 5-LO products by UV detection separated by RP-HPLC when we incubated PC3 cells with AA and A23187 in the presence or absence of H_2O_2. Prostate cancer cells have been reported to express 5-LO and FLAP [18], and synthesize 5-HETE [18,80]. However, these studies used radioimmunoassay to measure 5-HETE levels, which may not be accurate. However, the 5-HETE identified in prostate cancer tissue may be synthesized by resident inflammatory cells. In our studies, the levels of 5-LO products may be below the detection limit of our methods (UV detection), and it is possible that they could be detected using mass spectrometry. Alternatively, the intracellular redox environment could block 5-LO activity in these cells although this can be drastically affected by H_2O_2.

Inflammatory cells including macrophages, neutrophils and eosinophils [66,67] have been shown to be present within tumor environments. Activation of these cells could lead to the release of 5-HETE which could then be converted to 5-oxo-ETE by the same cells or the cancer cells. However, coincubation of neutrophils with PC3 cells led to only slightly higher levels of 5-oxo-ETE compared to neutrophils alone, cultured on plastic rather than in the presence of PC3 cells. Neutrophils adhere to plastic, and it is possible that they could be activated under these conditions. Therefore, neutrophils incubated alone in plastic wells may not be an appropriate control to determine how much 5-oxo-ETE is synthesized via transcellular biosynthesis by PC3 cells cultured in the presence of neutrophils.

To distinguish between neutrophil-derived and PC3 cell-derived 5-oxo-ETE, we attempted to selectively stimulate or inhibit 5-oxo-ETE formation by PC3 cells. Our initial experiments demonstrated that H_2O_2 stimulates the conversion of 5-HETE to 5-oxo-ETE by PC3 cells, in contrast to neutrophils, which show little increase in 5-oxo-ETE synthesis in the presence of H_2O_2 [110].

When we preincubated PC3 cells with H_2O_2 prior to the addition of AA, A23187 and neutrophils, we found that there was a significant increase in 5-oxo-ETE synthesis. In contrast, H_2O_2 did not affect the formation of 5-oxo-ETE from AA by neutrophils alone. Therefore, the increase in 5-oxo-ETE synthesis in PC3 cell/neutrophil coincubations in the presence of H_2O_2 is presumably due to conversion of neutrophil-derived 5-HETE to 5-oxo-ETE. To provide further evidence for the contribution of PC3 cells to 5-oxo-ETE synthesis in these coincubation experiments we selectively blocked the glutathione redox cycle in PC3 cells by depleting GSH with the alkylating agent NEM, which has previously been shown to block 5-oxo-ETE synthesis by H_2O_2-stimulated NHBE cells, BSMC [112], and human aortic endothelial cells [111]. The NEM was then removed so that it could not affect the metabolism of AA by neutrophils. Addition of neutrophils and AA/A23187 to NEM-treated PC3 cells resulted in 55% inhibition of 5-oxo-ETE synthesis in the absence of H_2O_2 and 75% inhibition in the presence of H_2O_2. We assume that the amount of 5-oxo-ETE that could potentially be inhibited by NEM under these conditions was all due to conversion by PC3 cells of neutrophil-derived 5-HETE to 5-oxo-ETE. The majority of non-NEM inhibitable 5-oxo-ETE synthesized in the coincubations was probably derived from neutrophils, but a small portion could also have been derived from PC3 cells, as NEM did not completely block 5-oxo-ETE synthesis in other cells types [110,112].

Treatment of neutrophils alone, with NEM did not affect either the total levels of 5-LO products, or the levels of 5-oxo-ETE. However, NEM induced a decrease in the levels of 5-HETE and a significant increase in the levels of LTB_4. This effect on LTB_4 synthesis has previously been reported in rat macrophages incubated with exogenous AA [256]. The increased LTB_4 levels and reduced 5-HETE levels may be due to the depletion of GSH, which is required for the peroxidase-catalyzed reduction of 5-HpETE to 5-HETE. This would prolong the lifetime of 5-HpETE, permitting a greater proportion to be converted to LTA_4 by 5-LO and consequently increased LTB_4 synthesis.

5.2.2 5-oxo-ETE synthesis increases with cell death

Recently members of our lab have observed that neutrophils undergoing cell death have a much higher ability to synthesize 5-oxo-ETE (Graham *et al*., submitted). We therefore examined the effect of agents that induce cancer cell death on 5-oxo-ETE synthesis. We incubated PC3 cells with 5-HETE and DHA [227], tamoxifen [257], doxorubicin [258] and MK886 [77], which have been shown to induce cancer cell apoptosis by differing mechanisms. PC3 cells synthesized low levels of 5-oxo-ETE up to 6 hrs. DHA, MK886 and tamoxifen all stimulated the synthesis of 5-oxo-ETE from 5-HETE to different extents up to 6 h compared to vehicle-treated cells, whereas doxorubicin which did not induce cell death under the condition employed had no effect. There are several potential mechanisms that could explain the stimulatory effects of cytotoxic agents on 5-oxo-ETE synthesis. DHA could competitively inhibit esterification of eicosanoids into cellular lipids by acyl CoA synthetase since it is also a substrate for this enzyme [259]. In addition, apoptosis of the cells in response to any of the cytotoxic agents could inactivate acyl CoA synthetase, also inhibiting esterification of 5-HETE and 5-oxo-ETE. To estimate the degree of uptake of 5-HETE and 5-oxo-ETE by esterification into lipids, we calculated the total amounts of these two products remaining after incubation of 5-HETE with cells for different times. The total levels 5-HETE and 5-oxo-ETE decreased rapidly in vehicle- and doxorubicin-treated cells, reaching < 50% of initial levels after 6 h with < 10% present after 24 h presumably due to esterification. In contrast, the total levels of 5-HETE and 5-oxo-ETE in DHA, tamoxifen, or MK886-treated cells decreased at a much slower rate with > 50% still present after 24 h. However, the reduced rate of uptake of 5-oxo-ETE and 5-HETE does not seem to be sufficient to explain the large increase in 5-oxo-ETE synthesis, as the levels of total unesterified 5-HETE plus 5-oxo-ETE did not correlate with the levels of 5-oxo-ETE. For example, tamoxifen stimulated 5-oxo-ETE synthesis (6-fold more than vehicle) much less than DHA (17-fold over vehicle), but was more effective than DHA in inhibiting the loss of 5-oxo-ETE + 5-HETE.

To further investigate the relationship between the degree of uptake of 5-oxo-ETE/5-HETE and 5-oxo-ETE synthesis, we examined the effects of triacsin C, an acyl CoA synthetase inhibitor, on these processes. Although triacsin C has been shown to induce apoptosis of different types of cancer cells including hepatic [260] and colon cancer cells [261,262], the levels we used (20 μM) did not induce apoptosis within 24 h, as the cells were still viable as determined by trypan blue exclusion. Although, triacsin C blocked the decrease in free 5-HETE + 5-oxo-ETE in PC3 cells cultured for 24 h to an extent similar to DHA and MK886-treated cells, it had little effect on 5-oxo-ETE synthesis. Thus the effect of DHA and other cytotoxic agents cannot be explained solely by their inhibitory effects on uptake of 5-oxo-ETE by esterification.

The effect of the above agents was not limited to PC3 cells, because they also stimulated 5-oxo-ETE synthesis in the lung cancer cell line, A427 and the breast cancer cell line, MCF7. As with PC3 cells, DHA induced the greatest response in both A427 and MCF7 cells with tamoxifen being about half as effective. Lower levels of MK886 (10 μM) induced similar responses in these cells compared to the levels required for PC3 cells (20 μM).

We attempted to measure the levels of $NADP^+$ in tumor cells to determine whether the effects of the cytotoxic agents on 5-oxo-ETE synthesis could have been mediated by elevation of the levels of this cofactor. However, the levels of $NADP^+$ were lower than our level of detection, since the incubations were carried out in 1 ml of media whereas normally we remove the media for adherent cells (Section 3.4). Since the cells are nonadherent and not viable when treated with the cytotoxic agents, we could not remove the media by centrifugation, as the cells were not intact, and any $NADP^+$ would be in the media. The alternative was to plate the cells at a higher confluency to have higher concentrations of $NADP^+$, however higher concentrations of the cytotoxic agents were required to induce the same effects and the results may not have been comparable. Preliminary studies incubating DHA in combination with catalase to eliminate H_2O_2, did not lower DHA-induced 5-oxo-ETE synthesis indicating that extracellular H_2O_2 was not involved.

The compounds tested all induce cell death via different pathways. Tamoxifen induces PC3 cells death independent of estrogen, via inhibition of Ca^{2+}-dependent PKC [228]. Doxorubicin intercalates DNA, but induces cell death at longer time points [258]. MK886 is a 5-LO pathway inhibitor [57], and DHA can induce lipid peroxidation, inhibits eicosanoid formation and could affect the properties of cell membranes [227,263]

Cormier and colleagues looked at tumor associated eosinophils and noted that eosinophils primarily localized to the internal necrotic region of the tumors (human melanoma injected into mice) [67]. These necrotic regions released an unidentified factor that was strongly chemotactic for eosinophils (greater than eotaxin-1 or -2) and induced eosinophil degranulation. The factor was also released from these cells and other types of cancer cells *in vitro* only when they were stressed or dying. The authors suggested that the factor released from the necrotic regions of tumors played a role in the recruitment of eosinophils may be 5-oxo-ETE or another eosinophil chemotactic lipid mediator [67]. The present study demonstrates that cancer cells subjected to oxidative stress or undergoing apoptosis have a greater capacity to synthesize 5-oxo-ETE. Activated inflammatory cells present in the tumor environment may supply 5-HETE to the dying tumor cells which may synthesize appreciable amounts of 5-oxo-ETE. The 5-oxo-ETE may then promote further infiltration of inflammatory cells or promote the survival of adjacent proliferative tumor cells.

5.3 Partial purification of 5-HEDH

Cloning 5-HEDH, the enzyme required for 5-oxo-ETE synthesis, and determining its protein and gene sequences will be vital for the further elucidation of its transcriptional and biochemical regulation. In addition, this will aid in a greater understanding of the role that 5-oxo-ETE plays in normal and pathological conditions.

5.3.1 The biochemical approach versus the molecular biological approach

There are two main approaches to sequence proteins. The biochemical approach uses various purification and chromatographic techniques to isolate the protein of interest from a particular tissue or cell type. These techniques take advantages of different biochemical and structural properties of proteins to separate them. For example, proteins can be separated according to size, or by their cofactor or substrate binding sites. Alternatively, the molecular biological approach involves identifying known sequences of proteins with similar functions or enzymatic activity from the same or different species and using degenerate primers based on sequences from conserved regions of these known proteins. Degenerate primers are required since several codons can code for a single amino acid and there are differences in codons used between species. Then intermediate steps are required to acquire full length clones. The full length clones would then be amplified in cells and tested for the desired activity. However, this can be challenging since enzymes with similar functions may have very low sequence homology, although functional domains (such as cofactor binding sites) may maintain some homology.

5.3.2 Partial purification of 5-HEDH

We decided to attempt to partially purify 5-HEDH and then sequence it by mass spectrometry, as we thought this might be feasible since we have a fairly simple enzymatic activity assay to monitor its elution. However, since 5-HEDH is an integral membrane protein, purification of 5-HEDH proved very challenging. Many membrane proteins have not been purified due to the challenges posed by their hydrophobicity.

We used two different cellular sources of 5-HEDH, PMA-differentiated U937 cells which express considerable levels of 5-HEDH activity and isolated neutrophils, to identify dehydrogenases identified by mass spectrometry that were present in both types of cells. As an integral membrane protein, 5-HEDH was solubilized with non-ionic detergents to disrupt interactions between the hydrophobic proteins since they tend to aggregate in solution. Several detergents

were determined to solubilize 5-HEDH efficiently including a zwitterionic detergent, CHAPS (6 mM), and the non-ionic detergents, octylglucoside (30 mM) and digitonin (0.5%). In addition, hydrophobic proteins may aggregate if they are not solubilized completely, which may interfere with interactions with the chromatographic stationary phases used to purify them.

Several purification and chromatographic techniques were tested for their efficacy for purification of 5-HEDH from other microsomal proteins. These included size exclusion, ion exchange, hydrophobic interaction, immobilized dyes, and affinity chromatography. For each purification step, there were numerous conditions that needed to be optimized including but not limited to buffer (concentration and pH), detergent (concentration), addition of a stabilizing reagent (glycerol), 5-HEDH binding conditions (temperature and duration), and elution conditions (buffer, detergent and salt concentration).

DEAE Sepharose was found to effectively purify 5-HEDH partially from other proteins. DEAE Sepharose, a weak anion exchanger, separates proteins based on their isoelectric point. At pHs above the proteins pI, the protein will be negatively charged while DEAE Sepharose is positively charged separating proteins based on their charges at different pHs. At pHs above a proteins pI, the protein should be positively charged. Since we do not know the pI of 5-HEDH, we tried several pHs, buffers and detergents. Phosphate buffer, pH 7.4 containing CHAPS was used to bind 5-HEDH to DEAE Sepharose. This indicates that the pI of 5-HEDH should be less than 7.4. However, using the same buffer but containing octylglucoside resulted in less efficient binding of 5-HEDH. Therefore, even though the detergents were non-ionic (octylglucoside) or zwitterionic (CHAPS) and should not have altered the charge on the protein, they were affecting the interactions of 5-HEDH with DEAE Sepharose. In addition, to elute 5-HEDH, low concentrations of salt in octylglucoside were used to selectively elute 5-HEDH. Alternatively, CM Sepharose, a cationic exchanger, which binds positively charged proteins at pHs below their pI, did not bind 5-HEDH under any of the conditions we tried.

Blue Sepharose also effectively purified 5-HEDH partially from other microsomal proteins. Structurally, blue and red dyes resemble the structure of the cofactors NAD^+ and $NADP^+$. Therefore, they bind to the NAD^+ or $NADP^+$ binding domains of enzymes, as well as binding lipoproteins, albumin and interferon and have been used extensively in the purification of a multitude of dehydrogenases and other enzymes that bind NAD^+ or $NADP^+$. Similar to DEAE Sepharose, the detergent used for binding altered the results. The results obtained with Red Sepharose were not reproducible between experiments, even when altering the detergents. Blue Sepharose yielded better results. In the presence of octylglucoside, 5-HEDH bound to Blue Sepharose effectively, however in the presence of CHAPS, it did not bind as effectively, and the results were not reproducible. Therefore, to elute 5-HEDH, CHAPS containing salt was used. Blue Sepharose was used as the second step in the purification of 5-HEDH. 5-HEDH partially purified fractions from DEAE Sepharose were purified further with Blue Sepharose resulting in a ~21-fold increase in specific activity.

5.3.2.1 Affinity chromatography

Affinity chromatography has the best advantage for the greatest purification of proteins since it takes advantage of a unique property of the protein, the substrate. Only the enzyme of interest which has high affinity for the substrate attached to the solid support should be retained, while all other proteins should not interact and elute immediately.

The lab of Dr Joe Rokach (F.I.T), synthesized several 5-HETE analogs, with different spacer arms, modified at the ω-end since the carboxylic acid group seems to be required for binding to 5-HEDH [5]. The final affinity ligands synthesized included a biotinylated 5-HETE analog and an Affigel-10-5-HETE analog. The biotinylated 5-HETE was bound to streptavidin (attached to beads), which non-covalently binds biotin with high affinity. Biotin-5H was not a good substrate of 5-HEDH as determined by Lineweaver-Burk analysis. Evaluating numerous binding conditions, including temperature (37 ^oC), longer incubation times, and different detergents and buffers, 5-HEDH did not bind and was always

eluted in the unretained fraction. Similar problems occurred with the ω-derived 5-HETE analog bound to Affigel-10. This method would eliminate any potential problems with the binding of biotin-5-HETE to the streptavidin bead. Again the binding and elution conditions were optimized, however, we observed a significant decrease in the recovery of 5-HEDH activity. Some conditions, higher pH, would promote binding of 5-HEDH to the Affigel-10-5-HETE, however, the results could often not be repeated. All of these experiments were carried out using the batch method, and therefore the manipulations may affect the results. A column method may result in improved binding of 5-HEDH, since the movement of protein through the column may promote interactions with the solid support, whereas with the batch method, the Affigel-5-HETE was rotating during the incubations which may disrupt the interactions with the enzyme.

5.3.2.2 Native gel electrophoretic separation of 5-HEDH

Denaturing gel electrophoresis separates proteins based on size, since proteins are denatured, and ionized so that shape, configuration and charge do not affect mobilization through the gel. When purifying enzymes, ideal conditions keep the enzyme intact and active to allow monitoring of its location. Therefore, native electrophoresis which separates proteins in their native (active) state by size would be a great addition to the purification of 5-HEDH. However, although we were able to measure 5-HEDH activity, it was unsuccessful in concentrating 5-HEDH. The migration of 5-HEDH activity was found to be broadly spread throughout the gel lanes. This may be an indication that the solubilized enzyme either aggregated with itself and/or other proteins which affected its mobility in the gel. Several different, loading buffers, gel concentrations, gel gradients, and different detergents incorporated into the gel were tested resulting in differences in measured enzyme activity and slight variations in 5-HEDH migration. However, the broad distribution of enzyme activity could not be corrected.

Blue-native gel electrophoresis was originally used to separate membrane-bound mitochondrial complexes [220]. Since 5-HEDH is an integral membrane bound enzyme, it was thought that this technique might be useful for separating it

from other proteins while maintaining its activity for identification. However, similar problems occured as with the native gels, where the activity was broadly distributed, which could not be resolved.

In-gel activity assays are used to identify the presence of intact enzymes, for example matrix metalloproteinases and several studies have also shown the use of in-gel formazan precipitation assays for dehydrogenases [222,264]. An in-gel activity assay would significantly facilitate the purification and identification of 5-HEDH and the feasibility of using this assay was evaluated. However, as determined by tests performed with cytosolic glucose-6-phosphate dehydrogenase, large amounts of substrate (> 50 μM) were required. In addition, due to the limitations of the native gels, the in-gel 5-HEDH assay was unsuccessful since the activity was spread throughout the gel lanes. A blue precipitate was obtained, indicating that the reaction worked, however, it was probably too diffuse in the gel to be detected. 5-HEDH has high affinity for 5-HETE, and if the enzyme could be concentrated into a narrow band in the native gels, the formazan precipitation assay may work.

5.3.2.3 Other purification techniques

Several other techniques commonly used to purify proteins were tested, including reverse-phase HPLC (C4) and trichloroacetic acid precipitation. However, even in the presence of protease inhibitors, the activity of 5-HEDH was lost or not recovered. Microsomes comprise the endoplasmic reticulum, and contain considerably higher 5-HEDH activity than the other subcellular fractions although, granules also have some activity [112]. Depending on the severity of the cell disruption, the microsomal fraction may also contain the nuclear envelope and the membranes of other organelles. Therefore, 5-HEDH may be present in other cellular membranes, and may be present on the nuclear envelope where 5-HpETE, LTA_4, and LTC_4 are synthesized. Attempts were made to isolate nuclear membrane fractions from U937 cells, however, assays were required to ensure that there were no contaminating membranes from other organelles. Preliminary results were inconclusive, however, there was some 5-HEDH activity present in

the nuclear membrane fraction. Determining the further subcellular localization of 5-HEDH would allow further understanding of the regulation of 5-oxo-ETE synthesis. Isolation of nuclear fractions, or the use of sucrose gradients may provide information into the subcellular localization of 5-HEDH. The combination of these methods with the DEAE - Blue Sepharose separation, would eliminate more proteins that interfere with the analysis by mass spectrometry

5.3.3 Size range of 5-HEDH

Two techniques were used to determine the size range (30 < MW < 100 kDa) for 5-HEDH, including size exclusion chromatography and using several filters that retained proteins of different molecular weights (MWCO filters). The size exclusion column did not prove useful since 5-HEDH activity was not concentrated. Filters with 30, 50, and 100 KDa cutoffs were used to concentrate 5-HEDH. 5-HEDH activity in solubilised microsomes was retained by all three filter sizes, probably due to the enzyme in larger detergent micelles with other proteins. However, when it was partially purified by DEAE alone, > 50% of the activity passed through the 100 KDa MWCO filter, indicating that the enzyme was less than 100 kDa. When further concentrated with Blue Sepharose, minimal levels of 5-HEDH activity passed through the 50 kDa MWCO filter, and no activity passed through the 30 KDa MWCO filter. As the enzyme was purified, it had fewer proteins to interact with, and the micelles were probably smaller, and more representative of its true size.

5.3.4 Co-elution of 5-HEDH dehydrogenase and ketoreductase activity

Previous studies have shown that the ketoreductase activity (conversion of 5-oxo-ETE to 5-HETE in the presence of NADPH) is present in neutrophil microsomes [98], and that NADPH inhibits the dehydrogenase activity. This indicated that 5-HEDH probably catalyzes both reactions, with the forward dehydrogenase activity favored at physiological pH [101]. This study provides further evidence that 5-HEDH also catalyzed the conversion of 5-oxo-ETE to 5-HETE since both activities (dehydrogenase and ketoreductase) co-eluted in the

same relative amounts when purified by both size exclusion chromatography and DEAE Sepharose. This is consistent with many other dehydrogenases that catalyze both the forward and backward reaction, with one direction favored.

5.3.5 Mass spectrometry results for partially purified 5-HEDH

LC-MS/MS analysis was performed on 10 bands cut from SDS-PAGE gels of 5-HEDH purified from solubilized microsomes by DEAE Sepharose followed by Blue Sepharose. Since these methods were not very specific, and blue Sepharose binds to the NAD^+ or $NADP^+$ binding domains of enzymes, several dehydrogenases were identified. To narrow down the potential candidates for 5-HEDH, the analysis was performed on partially purified 5-HEDH obtained from both PMA-differentiated U937 cells and neutrophils. It should be possible to eliminate any proteins that are highly expressed by one of these cells but not the other. For example in fractions purified from U937 cells, the most abundant protein identified was NAD(P)-dependent steroid dehydrogenase-like (NSDHL) protein, but this protein was not present in neutrophil fractions. Several enzymes have the potential to catalyze more than one reactions, for example, LTA_4 hydrolase catalyzes the hydrolysis of LTA_4 to LTB_4, but also has an aminopeptidase activity that is not related to the first reaction [265]. Therefore, 5-HEDH may be a dehydrogenase with a previously identified substrate, which may have greater affinity for 5-HETE, or it may be an orphan dehydrogenase whose sequence is known, but not its enzymatic activity. Therefore, enzymes identified by mass spectrometry cannot be ruled out completely if they have a known substrate.

The enzyme dehydrogenase/reductase (SDR family) member 7 (DHRS7) (or retinol short-chain dehydrogenase/reductase (retSDR4), was identified in partially purified 5-HEDH fractions from both U937 cells and neutrophils. It is a member of the short-chain dehydrogenase/reductase (SDR) family, which is one of the largest known protein families, with over 3000 members in all species [266]. Members of the SDR family are involved in the metabolism of steroids, prostaglandins, retinoids and fatty acids (e.g. 15-hydroxyprostaglandin

dehydrogenase). These enzymes have about 15 – 30% sequence homology amongst one another and to date 71 members have been identified in humans [267] which may use NAD^+ and/or $NADP^+$ as cofactors. The active sites and cofactor binding sites have consensus sequences (Table 5.1). The mechanism of the reactions catalyzed by these enzymes, involves binding of the cofactor first which remains enzyme bound while the substrate binds the active site [268]. In contrast, previous studies for 5-HEDH, suggested that it catalyzes the conversion of 5-HETE to 5-oxo-ETE by a ping-pong mechanism, based on biochemical studies [101].

Table 5.1: SDR sequence motifs found in DHRS7 (Swiss-Prot; Q9Y394)
The amino acid numbers presented are specific for DHRS7.

Sequence Motifs	Function
$T_{56}G_{57}XXXG_{61}XG_{63}$	Cofactor binding site Maintenance of central β-sheet
$N_{137}N_{138}G_{139}G_{140}$	Stabilization of central β-sheet
N_{162}	Active site
S_{190}-Y_{203}-K_{207}	Active site

1 MNWELLLWLL VLCALLLLLV QLLRFLRADG DLTLLWAEWQ GRRPEWELTD
51 MVVWVTGASS GIGEELAYQL SK**LGVSLVLS** **AR**RVHELERV KRRCLENGNL
101 KEKDILVLPL DLTDTGSHEA ATK**AVLQEFG** **R**IDILVNNGG MSQRSLCMDT
151 SLDVYRKLIE LNYLGTVSLT K**CVLPHMIER** KQGKIVTVNS ILGIISVPLS
201 IGYCASKHAL RGFFNGLRTE LATYPGIIVS NICPGPVQSN IVENSLAGEV
251 TKTIGNNGDQ SHKMTTSRCV R**LMLISMAND** **LK**EVWISEQP FLLVTYLWQY
301 MPTWAWWITN KMGKKRIENF KSGVDADSSY FKIFKTKHD

BOLD – neutrophils Underline – U937 cells

Figure 5.3: Complete sequence of DHRS7 with sequenced peptides. Peptides for DHRS7 were sequenced by mass spectrometric analysis of 5-HEDH purified from neutrophils (**bold**), and U937 cells (Underline).

DHRS7 has 339 amino acids, a molecular weight of 38673 Da and based on its sequence probably uses $NADP^+$ instead of NAD^+ as a cofactor [267]. These correspond with the properties of 5-HEDH, however, the subcellular location of DHRS7 has not been established. The gene is located on chromosome 14 (14q23.1). DHRS7 was originally discovered by searching databases of ESTs (Expressed Sequence Tags) from retina and the pineal gland [269]. mRNA for this enzyme was expressed in all tissues tested including skeletal muscle, heart, kidney, pancreas, brain, lung, liver and pancreas [269].

In the present study, four peptides derived from DHRS7 were sequenced from tryptic digests of neutrophil fractions, whereas, only one peptide was identified in fractions from U937 cells. The confidence that these sequences were correct and specific to this enzyme were high, based on correlation of the mass of the sequenced peptides as well as the fragment ions. However, it would still be desirable to repeat the 5-HEDH purification and peptide sequencing from U937 cells to confirm the presence of this enzyme and to determine whether there are any other viable candidates. Incorporation of an additional chromatographic step would also be beneficial, as this could eliminate more proteins, in particular NSDHL, which was present in large amounts in the 5-HEDH fractions from U937 cells (Table 4.3). Another alteration that may be made would be to cover more area of the SDS gel when trypsinizing the gel bands. Although we have a good idea of the size range for 5-HEDH, (30 kDa < MW ≈ 50KDa), we only included bands up to 70 kDa. Trypsinizing bands taken up to 100 kDa will increase the confidence that we are not missing any potential candidate proteins.

After confirming the presence of DHRS7 in U937 cells, studies would be required to determine whether it is 5-HEDH and catalyzes the conversion of 5-HETE to 5-oxo-ETE. Several approaches may be pursued, including cloning the full length protein sequence, and measuring activity in the whole cells and isolated microsomes. CHO cells, in which studies on OXE-R were carried out, and which may not express 5-HEDH [152] could be used for these studies. In addition, siRNA studies downregulating the expression of DHRS7 could be

performed on cells that express 5-HEDH (e.g. U937 cells), with subsequent determination of enzymatic activity.

5.4 Conclusions and implications of these findings

The physiological role of 5-oxo-ETE has not been completely elucidated to date, however, its biological action as a potent survival and chemotactic factor for leukocytes, and in particular eosinophils, indicates that it primarily acts as an inflammatory mediator. The initial inflammatory response functions to protect organisms against infection and invading parasites by initiating the recruitment of granulocytes which release granules containing cytotoxic enzymes. However, tissue damage occurs with prolonged inflammation. Consequently, 5-oxo-ETE may be involved in the pathogenesis of inflammatory diseases associated with eosinophil accumulation such as asthma, in addition to its role in promoting the survival of tumor cells. Relatively little information is available on the levels of 5-oxo-ETE in biological fluids partly because it is metabolized via numerous pathways, making its detection difficult. However, several methods have been developed that will make its detection possible [270,271]. A recent study reported elevated levels of 5-oxo-ETE in the lungs from subjects with severe pulmonary hypertension, which was associated with increased oxidative stress and increased numbers of inflammatory cells [272].

The present study has shown that B cells express 5-HEDH and that 5-oxo-ETE is a major product of AA metabolism, particularly after stimulation with H_2O_2. This effect of H_2O_2 correlates with the ratio of $NADP^+$ to NADPH which has been shown to be important for 5-oxo-ETE production from 5-HETE [110]. Oxidative stress is often associated with inflammation which could stimulate 5-oxo-ETE synthesis by B cells at sites of inflammation. In addition, stimulation of the B cell receptor leads to H_2O_2 production, presumably through activation of NADPH oxidase [273] which may also promote 5-oxo-ETE synthesis. Further studies with B cells may reveal a role for 5-oxo-ETE synthesis by these cells. These cells were shown here to express OXE-R, and 5-oxo-ETE may exert autocrine responses. LTB_4 induces many responses in B cells [240], and it is

feasible that 5-oxo-ETE may play another role in asthma, by inducing responses in IgE producing B cells which have been linked to the severity of asthma [274].

The present study has also shown that several tumor cell lines, PC3 (prostate), MCF7 (breast), and A427 (lung) express 5-HEDH and synthesize 5-oxo-ETE. Similar to its effect in B cells, H_2O_2 stimulates 5-oxo-ETE synthesis by these cells but for prolonged periods. Several agents cytotoxic to these cancer cells, docosahaenoic acid, tamoxifen, and MK886 were shown to stimulate 5-oxo-ETE production over prolonged periods much longer than have been observed with other stimuli. The effects of these agents on 5-oxo-ETE synthesis corresponded with the induction of cell death, and also with decreased metabolism of both 5-HETE and 5-oxo-ETE. This effect on 5-oxo-ETE synthesis may play a role in the recruitment of inflammatory cells to the tumor site, since tumor microenvironments comprise numerous types of inflammatory cells in addition to proinflammatory mediators [66,67]. In addition, increased synthesis of 5-oxo-ETE in the necrotic region of tumors may promote the survival of nearby tumor cells. Since 5-LO pathway inhibitors have been shown to induce cancer cell death [80,87,206], and cancer cells synthesize 5-oxo-ETE, 5-HEDH inhibitors and/or OXE-R antagonists may be useful treatments for cancer. In addition, the exact mechanism for the stimulation of 5-oxo-ETE synthesis by these cytotoxic agents should be studied further.

The metabolism of AA is also highly regulated by the activation status of $cPLA_2$ and 5-LO. In this study, the 5-LO activity in B cells and PC3 cells, which both express 5-LO and FLAP, is very tightly regulated, and these cells synthesize low levels of 5-LO metabolites from endogenous AA. In this study we did not detect 5-LO products formed by PC3 cells, however a recent study measured 5-HETE synthesized from these cells [275]. However, we observed transcellular biosynthesis of 5-oxo-ETE between neutrophils and PC3 cells. This indicates that cells which express 5-HEDH but do not express 5-LO or do not have high 5-LO activity, such as endothelial cells or epithelial cells may synthesize 5-oxo-ETE from 5-HETE produced by neighbouring cells. In this way, considerable levels of 5-oxo-ETE may be synthesized by resident tissue cells from 5-HETE produced by

activated inflammatory cells resulting in further recruitment of inflammatory cells leading to tissue damage as is observed in asthma [2]. In addition, AA has also been shown to be transferred between neutrophils and platelets, and therefore, AA or 5-HETE may be transferred to B cells by neighbouring leukocytes resulting in 5-oxo-ETE synthesis by both types of cells. 5-HEDH is more widely expressed than 5-LO, and therefore, understanding the cellular tissue infiltrates in pathological conditions may aid in determining the relative production of individual eicosanoids.

To date, studies on the regulation of 5-oxo-ETE have been performed using biochemical studies [101,110-112]. Recently, inhibitors of 5-HEDH have been synthesized by our lab [102] and these will be invaluable in identifying the role that 5-oxo-ETE plays in normal and pathological conditions such as asthma and cancer. However, to further understand the role of 5-oxo-ETE in these diseases, identifying the sequence of 5-HEDH will be important to study its expression and regulation. To that end, we have developed a protocol to partially purify 5-HEDH, and have performed peptide sequence analysis by mass spectrometry. One potential candidate, enzyme dehydrogenase/reductase (SDR family) member 7 (DHRS7) has been identified from 5-HEDH partially purified from two cell sources. The molecular weight of this protein (39 kDa) is within the range that we determined for 5-HEDH. We expected to identify numerous candidate proteins by this method, and further experiments will be needed to confirm the presence of this enzyme in U937 cells, for which only one peptide was identified. Eventually when more candidate proteins are identified, and/or the presence of DHRS7 is confirmed in U937 cells, the cDNA for these proteins will be cloned from cells that preferably do not express 5-HEDH, for example murine macrophages [136]. Comparisons of activity levels will reveal which candidate protein has 5-HEDH activity, allowing confirmation of its identity.

Purifying and sequencing 5-HEDH will provide new approaches to determine its expression as well as the localization of 5-oxo-ETE synthesis. For example, antibodies may be raised to 5-HEDH to enable immunohistochemical analyses or alternatively, in situ hybridization may be performed. The effect of downregulating or blocking the enzyme, using antibodies or siRNA, may aid in

elucidating the physiological role of 5-oxo-ETE. Although much is known about 5-oxo-ETE synthesis, identifying 5-HEDH will considerably broaden our understanding of the physiological role of 5-oxo-ETE.

Contributions to Original Research

1. Primary B lymphocytes and B cell lines express 5-HEDH and synthesize 5-oxo-ETE from 5-HETE and exogenous AA with A23187. 5-oxo-ETE synthesis is stimulated in these cells by oxidative stress which elevates the intracellular $NADP^+$ through the glutathione redox pathway. B cells also synthesize the BLT2 receptor ligand, 12-HHT

2. Tonsillar B lymphocytes express low levels of OXE-R mRNA, which are slightly elevated in anti-CD40/IL-4 activated cells.

3. Various cancer cell lines, including PC3 (prostate), A427 (lung) and MCF7 (breast) express 5-HEDH. The synthesis of 5-oxo-ETE is stimulated by oxidative stress through the glutathione redox-mediated elevation of intracellular $NADP^+$.

4. PC3 cells can utilize neutrophil-derived 5-HETE to synthesize 5-oxo-ETE by transcellular biosynthesis.

5. Cytotoxic agents for tumor cells, including docosahexaenoic acid, tamoxifen, and MK886, stimulate 5-oxo-ETE synthesis in PC3 (prostate), A427 (lung) and MCF7 (breast) cancer cells. Triacsin C and doxorubicin which did not induce cell death had minimal effects on 5-oxo-ETE synthesis.

6. DEAE Sepharose followed by Blue Sepharose chromatography results in partial purification of solubilized 5-HEDH.

7. The size of 5-HEDH was determined to be between 30 kDa and 100 kDa and is probably closer to 50 kDa and a potential candidate for 5-HEDH

with a MW of 39 kDa was identified, dehydrogenase/reductase (SDR family) member 7 (DHRS7).

8. 5-HEDH appears to catalyze both the dehydrogenase (5-HETE $\rightarrow$5-oxo-ETE) and ketoreductase (5-oxo-ETE $\rightarrow$ 5-HETE) activities since both activities co-elute during a variety of purification procedures.

List of Publications

Manuscripts in preparation

- **Grant, G.E.**, Gravel, S., Guay, J., Rokach, J., Mazer, B., Powell, W.S. Oxidative stress induces the production of 5-oxo-ETE from arachidonic acid in B cells.

- **Grant, G.E.**, Gravel, S., Rubino, S., Rokach, J., Powell, W.S. Metabolism of 5-HETE by tumor cells: Stimulation of 5-oxo-ETE formation by oxidative stress, docosahexaenoic acid and antiproliferative agents.

Publications

The work described in the following papers was not included in this thesis.

- Patel, P., Cossette, C., Anumolu, J.R., Erlemann, K-R., **Grant, G.E.**, Rokach, J., Powell, W.S. (2009) Substrate selectivity of 5-hydroxyeicosanoid dehydrogenase and its inhibition by 5-hydroxy-Δ^6-long chain fatty acids. J. Pharmacol. Exp. Ther. 339(1):335-41

  - Performed cell culture and isolated the microsomal protein.

- Patel, P., Lee, G-J., Kim, S., **Grant, G.E.**, Powell, W.S., Rokach, J. (2008) Enantio and Stereospecific Syntheses of 15(R)-Me-PGD_2, a Potent and Selective DP2-Receptor Agonist. *J. Org. Chem.* 73(18):7213-18

  - Developed the RP-HPLC method and analyzed the synthesized 15(R)-Me-PGD_2.

- Erlemann, K.R., Cossette, C., **Grant G.E.**, Lee, G.J., Patel, P., Rokach, J., Powell, W.S. (2007) Regulation of 5-hydroxyeicosanoid dehydrogenase activity in monocytic cells. *Biochem. J.* 403(1):157-165

  - Performed cell culture and isolated the microsomal protein.

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Appendix – Research Compliance Certificates

Centre universitaire de santé McGill
McGill University Health Centre
Les meilleurs soins pour la vie
The Best Care for Life

May 16, 2008

William S. Powell, PhD
Room 308
Meakins-Christie Laboratories

Dear Dr. Powell,

Thank-you for submitting the 5-year report to the Biomedical-C Research Ethics Board on the progress of the protocol entitled "Biosynthesis and role of eicosanoids in inflammation."

The study documents have been reviewed. There are no impediments to extending the project's approval for another year.

TITLE:	"Biosynthesis and role of eicosanoids in inflammation."
P.I.:	William S. Powell, PhD
Initially Approved:	April 2, 1998
Reapproved:	April 29, 1999 / April 29, 2000 / April 29, 2001 April 29, 2002 / April 29, 2003 / May 6, 2004 May 10, 2005 / May 10, 2006 / May 10, 2007
Most Recently Approved:	May 16, 2008
Expiry Date:	May 16, 2009

Sincerely,

Pierre Ernst, MD
Chair, Biomedical-C
Research Ethics Board of the MUHC.

McGill University

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

Projects involving potentially biohazardous materials should not be commenced without approval from Environmental Health & Safety. Submit applications before 1) starting new projects, 2) renewing existing projects, or 3) changing the nature of the biohazardous materials within existing projects.

1. PRINCIPAL INVESTIGATOR: William S. Powell PHONE:)94071
DEPARTMENT: Medicine FAX: 7483
ADDRESS: Meakins-Christie Laboratories, 3626 St. Urbain St. E-MAIL: William.Powell@McGill.ca

PROJECT TITLE: Biosynthesis and role of eicosanoids in inflammation

2. EMERGENCY: Person(s) designated to handle emergencies

Name: Gail Grant Phone No: work: 094088 home: 630-0515
Name: Sylvie Gravel Phone No: work: 094088 home: 354-5185

3. FUNDING SOURCE OR AGENCY (specify): CIHR

Grant No.: MOP-6254 Beginning date: 1 Oct, 2003 End date: 30 Sept, 2008

4. Indicate if this is

☐ Renewal: procedures previously approved without alterations.

Approval End Date: _____

☐ New funding source: project previously reviewed and approved under an application to another agency.

Agency: _____ Approval End Date: _____

☒ New project: project not previously reviewed.

☐ Approved project: change in biohazardous materials or procedures.

☐ Work/project involving biohazardous materials in teaching/diagnostics.

CERTIFICATION STATEMENT: Environmental Health & Safety approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in Health Canada's "Laboratory Biosafety Guidelines" and in the "McGill Laboratory Biosafety Manual".

Containment Level (select one): ☐ 1 ☒ 2 ☐ 2 with additional precautions ☐ 3

Principal Investigator or course director: _____ (SIGNATURE) date: 4 (day) 2 (month) 05 (year)

Approved by Environmental Health & Safety: _____ (SIGNATURE) date: 07 (day) 02 (month) 05 (year)

Expiry: 30 (day) 09 (month) 08 (year)

*as defined in the "McGill Laboratory Biosafety Manual"

5. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Job Title/Classification	Trained in the safe use of biological safety cabinets within the last 3 years? If yes, indicate training da
Gail Grant	Medicine	PhD student	No
Sylvie Gravel	Medicine	Technician	No

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues, toxins of biological origin) & designated biosafety risk group

We are requesting approval to use two B lymphocyte cell lines:

1) CESS cells (ATCC Number: TIB-190) were isolated from the peripheral blood of an adult Caucasian male patient with myelomonocytic leukemia after incubation with Epstein-Barr Virus. These cells have been designated as Biosahety level 2
2) SKW 6.4 cells (ATCC Number: TIB-215) were obtained by transforming human B lymphocytes with Epstein-Barr virus (EBV

ii) the procedures involving biohazards

The cell lines will be grown and maintained in culture. The cells will be stimulated with various agents such as hydrogen peroxide and arachidonic acid. The cells will then be lysed with alcohol and the amounts of lipid metabolites in the extract will be measured. Oxidative stress will be evaluated by measuring the levels of oxidized glutathione and $NADP^+$.

iii) the protocol for decontaminating spills

Any spills of cells or solutions that come in contact with cells will be cleaned with 10% bleach and 70% ethanol.

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) that could increase the hazards?

No

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

NA

9. What precautions will be taken to reduce production of infectious droplets and aerosols?

All work with these cells will be performed under sterile conditions in a type A/B3 class II biological safety cabinet. After each use the work area will; be cleaned with 70% ethanol and exposed to UV light.

10. Will the biohazardous materials in this project expose members of the research team to any risks that might require special training, vaccination or other protective measures? If yes, please explain.

No

11. Will this project produce combined hazardous waste – i.e. radioactive biohazardous waste, biohazardous animal carcasses contaminated with toxic chemicals, etc.? If yes, please explain how disposal will be handled.

No

12. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
Meakins-Christie Labs	SS17	NuAire	NU425 600	79734	Sept 17, 2003

Public Health Agency of Canada
Centre for Emergency Preparedness and Response

Agence de santé publique du Canada
Centre de mesures et d'interventions d'urgence

Permit no.-Permis no.

P- 10719

Permit to import human pathogen(s)

Permis d'importation d'agent(s) anthropopathogène(s)

Under the authority of the Human Pathogens Importation Regulations.

Sous le régime du Règlement sur l'importation des agents anthropopathogènes.

Importer-Name, address and postal code - **Importateur**-Nom, adresse, et code postal

Meakins-Christie Laboratories - McGill University
3626 St-Urbain Street
Montreal, QC
H2X 2P2
Attn.: William S. Powell

Fascimilie-Télécopieur: (514) 398-7483

Telephone no.- No. de téléphone: (514) 398-3864 ext.: 0940

Supplier-Name and address - **Fournisseur**-Nom et adresse

American Type Culture Collection
10801 University Blvd.
Manassas, VA 20108 USA

Name(s) of Port(s) of Entry- To Clear Customs at Port(s) of entry
Nom(s) de(s) point(s) d'entrée -Dédouanement au(x) point(s) d'entrée de

Montréal

Description of Pathogen(s)-For the importation of- **Description de(s) agents anthropopathogène(s)**-Pour l'importation de

Following human cell lines which have been transformed with Epstein-Barr virus: CESS (#TIB-190) and SKW 6.4 (#TIB-215).

On the following terms and conditions as marked:-Celles des conditions suivantes qui sont indiquées:

1.	Work involving any of the imported material shall be limited to *in vitro* laboratory studies.	■ Les travaux auquels la matière importée est destinée doivent se limiter à des études de laboratoire *in vitro*.
2.	Domestic animals, including poultry, cattle, sheep, swine and horses, shall not be directly or indirectly exposed to infection by any of the imported material.	■ Les animaux domestiques, y compris les volailles, bovins, ovins, porcins et chevaux, ne doivent pas être exposés, directement ou indirectement, à l'infection par la matière importée.
3.	All animals exposed to infection by any of the imported material shall be so exposed and held only in isolated insect-and rodent-proof facilities.	☐ Les animaux exposés à l'infection par la matière importée doivent y être exposés et être gardés uniquement dans des installations isolées à l'abri des insectes et des rongeurs.
4.	All equipment, animal pens, cages, bedding, waste and other articles under the importer's control, that come in direct or indirect contact with any of the imported material, shall be sterilized by autoclaving or incinerated.	■ L'équipement, les enclos pour animaux, les cages, les litières, les déchets et tout autre article sous la responsabilité de l'importeur qui viennent en contact direct ou indirect avec la matière importée doivent être stérilisés par autoclavage ou incinérés.
5.	Packaging materials, containers and all unused portions of the imported material shall be sterilized by autoclaving or incinerated.	☐ Le matériel d'emballage, les récipients et toute partie inutilisée de la matière importée doivent être stérilisés par autoclavage ou incinérés.
6.	No work on the imported material shall be done, except work conducted or directed by the importer in the facilities described in the application for this permit. NO HUMAN PATHOGEN BELONGING TO RISK GROUP 3 OR 4 MAY BE REMOVED TO ANOTHER LOCATION, OR TRANSFERRED INTO THE POSSESSION OF A PERSON OTHER THAN THE IMPORTER, WITHOUT THE PERMISSION OF THE DIRECTOR.	■ La matière importée ne peut servir qu'aux travaux effectués ou dirigés par l'importateur dans les installations décrites dans la demande de permis. AUCUN AGENT ANTHROPOPATHOGÈNE DU GROUPE DE RISQUE 3 OU 4 NE PEUT, SANS LA PERMISSION DU DIRECTEUR, ÊTRE TRANSPORTÉ VERS UN AUTRE LIEU OU ÊTRE MIS EN LA POSSESSION D'UNE AUTRE PERSONNE QUE L'IMPORTATEUR.
7.	On completion of the importer's work involving the imported human pathogen, the pathogen and all its derivatives shall be destroyed.	☐ Au terme des travaux de l'importateur auxquels a servi l'agent anthropopathogène importé, celui-ci et tous ses dérivés doivent être détruits.

8. **This permit is valid only for:**
Le présent permis n'est valide que pour:

☐ a) a single entry into Canada or une seule entrée au Canada ou

■ b) importations at intervals of / les importations effectuées à intervalles de — during the period beginning on / au cours de la période commençant le **February 15, 2005** — and ending on / et se terminant le **February 28, 2006**

Authorization-Signature of Director
Autorisation-Signature du Directeur

M. Best

Date February 15, 2005

Note: Transporting and otherwise dealing with imported material are subject to federal, provincial and municipal laws (if any), to the extent that, those laws apply in respect of that material

Remarque: Les opérations relatives à la matière importée, y compris le transport, sont assujetties aux lois fédérales, provinciales et aux règlements municipaux applicables.

Canada