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IDENTIFICATION OF ARACHIDONIC ACID METABOLITES FORMED THROUGH THE 15-LIPOXYGENASE-1 PATHWAY

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Stockholm 2013

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ISBN 978-91-7549-026-7

ABSTRACT

The polyunsaturated fatty acid arachidonic acid is the precursor of many biologically active lipid mediators. This thesis is focused on the arachidonic acid metabolites formed through the 15-lipoxygenase-1 (15-LO-1) pathway.

The formation and the biological effects of mediators formed through cyclooxygenases and 5-lipoxygenase pathways are well characterized. The 15-LO-1 is less studied but several lines of evidence suggest a role for 15-LO-1 in asthma and other inflammatory diseases. In this thesis, eosinophils, mast cells, airway epithelial cells and the Hodgkin L1236 cell line were found to express 15-LO-1. In mast cells and airway epithelial cells, IL-4 stimulation increased the expression of 15-LO-1. Stimulation of eosinophils with pro-inflammatory agents and osmotic activation of mast cells by mannitol resulted in activation of the 15-LO-1 pathway. Bacterial infection as well as mechanical injury of the epithelial cells in the respiratory tract are well-known triggers of asthma attacks. These stimuli also increased the formation of 15-HETE in primary airway epithelial cells. Increased amounts of 15-LO-1 were found in bronchial biopsies from asthmatic patients compared to healthy volunteers. 15-LO-1 was also positively stained in Hodgkin lymphoma biopsies localized in the Hodgkin-Reed Sternberg cells, indicating a therapeutic or diagnostic relevance for 15-LO-1 in Hodgkin lymphoma.

Analysis with LC-MS/MS identified the novel metabolite 14,15-LTC_4. This metabolite was given the name eoxin C_4 (EXC_4) since eosinophils were a rich source of this metabolite. In addition, mast cells, airway epithelial cells, nasal polyps and L1236 cells could also produce EXC_4. This metabolite was converted to EXD_4 and EXE_4 in eosinophils and L1236 cells. The cysteinyl-eoxins were shown to be 100 times more potent than histamine and almost as potent as cysteinyl-leukotrienes to increase the transendothelial permeability. Increased permeability and vascular leakage is a hallmark of inflammation, which is an important feature of asthma. Both LTC_4 synthase and certain soluble glutathione S-transferases were found to catalyze the conjugation of glutathione with EXA_4 leading to the formation of EXC_4. The animal ortholog of 15-LO-1, the 12/15-LO, was also found to generate EXC_4, indicating that studies on animals can be predictive of the function of 15-LO-1 in human.

Besides the cysteinyl-eoxins, the Hodgkin L1236 cell line also converted arachidonic acid into the 14,15- hepoxilin (Hx) A_3 11(S) and 14,15-HxB_3 13(R) as well as 14,15-HxA_3-C and 14,15-HxA_3-D. The 14,15-HxA_3 11(S) and 14,15-HxB_3 13(R) were also identified in eosinophils, dendritic cells and nasal polyps. The Hodgkin lymphoma tumor only consists of a minority of malignant cells and the main part is inflammatory cells, such as eosinophils and mast cells. A potential role of 15-LO-1 could be to facilitate the inflammatory features of this disease.

In essence, this thesis demonstrates that the 15-LO-1 pathway can convert arachidonic acid to many different metabolites with potential pro-inflammatory effects.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CID	Collision induced dissociation
DAD	Diode array detector
DiHETE	Dihydroxy-eicosatetraenoic acid
ESI	Electrospray ionization
EX	Eoxin
GC	Gas chromatography
GST	Glutathione S-transferase
HETE	Hydroxy-eicosatetraenoic acid
HL	Hodgkin lymphoma
HPETE	Hydroperoxy-eicosatetraenoic acid
HPLC	High performance liquid chromatography
H-RS	Hodgkin Reed-Sternberg
Hx	Hepoxilin
IgE	Immunoglobulin E
IL	Interleukin
KETE	Keto-eicosatetraenoic acid
LC	Liquid chromatography
LO	Lipoxygenase
LT	Leukotriene
LTC_4S	Leukotriene C_4 synthase
LX	Lipoxin
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
m/z	Mass-to-charge
NADP	Nicotinamide adenine dinucleotide phosphate
NHL	Non-Hodgkin lymphoma
NSAID	Non-steroidal anti-inflammatory drug
PG	Prostaglandin
PMNL	Polymorphonuclear leukocyte
RAM	Radioactivity monitoring
RT-PCR	Real time-poly chain reaction
SRM	Single reaction monitoring
STAT	Signal transducer and activator of transcription
Trx	Trioxilin
TX	Thromboxane
UV	Ultraviolet

LIST OF CHEMICAL ABBREVIATIONS

5(S)-HETE	5(S)-hydroxy-6,8,11,14(E,Z,Z,Z)-eicosatetraenoic acid
12(S)-HETE	12(S)-hydroxy-5,8,10,14(Z,Z,E,Z)-eicosatetraenoic acid
15(S)-HPETE	15(S)-hydroperoxy-5,8,11,13(Z,Z,Z,E)-eicosatetraenoic acid
15(S)-HETE	15(S)-hydroxy-5,8,11,13(Z,Z,Z,E)-eicosatetraenoic acid
15-KETE	15-keto-5,8,11,13(Z,Z,Z,E)-eicosatetraenoic acid
EXA_4	14(S),15(S)-epoxy-5,8,10,12(Z,Z,E,E) eicosatetraenoic acid
EXC_4	14(R)-glutathionyl-15(S)-hydroxy-5,8,10,12(Z,Z,E,E)-eicosatetraenoic acid
EXD_4	14(R)-cysteinyl-glycyl-15(S)-hydroxy-5,8,10,12(Z,Z,E,E)-eicosatetraenoic acid
EXE_4	14(R)-cysteinyl-15(S)-hydroxy-5,8,10,12(Z,Z,E,E)-eicosatetraenoic acid
LTA_4	5(S),6(S)-epoxy-7,9,11,14(E,E,Z,Z)-eicosatetraenoic acid
LTB_4	5(S)12(R)-dihydroxy-6,8,10,14(Z,E,E,Z)-eicosatetraenoic acid
LTC_4	5(S)-hydroxy-6(R)-glutathionyl-7,9,11,14(E,E,Z,Z)-eicosatetraenoic acid
LTD_4	5(S)-hydroxy-6(R)-cysteinyl-glycyl-7,9,11,14(E,E,Z,Z)-eicosatetraenoic acid
LTE_4	5(S)-hydroxy-6(R)-cysteinyl-7,9,11,14(E,E,Z,Z)-eicosatetraenoic acid
14,15-HxA_3	11(R/S)-hydroxy-14,15-epoxy-5,8,12(Z,Z,E)-eicosatrienoic acid
14,15-HxA_3-C	11(S),15(S)-dihydroxy-14(R)-glutathione-5,8,12(Z,Z,E)-eicosatrienoic acid
14,15-HxA_3-D	11(S),15(S)-dihydroxy-14(R)-cysteinyl-glycyl-5,8,12(Z,Z,E)-eicosatrienoic acid
14,15-HxB_3	13(R/S)-hydroxy-14,15-epoxy-5,8,12(Z,Z,E)-eicosatrienoic acid
HxA_3	8(R/S)-hydroxy-11(S),12(S)-epoxy-5,9,14(Z,E,Z)-eicosatrienoic acid
HxA_3-C	11(R)-glutathionyl-8(R/S),12(S)-dihydroxy-5,9,14(Z,E,Z)-eicosatrienoic acid
HxA_3-D	11(R)-cysteinyl-glycyl-8(R/S),12(S)-dihydroxy-5,9,14(Z,E,Z)-eicosatrienoic acid
HxB_3	10(R/S)-hydroxy-11(S),12(S)-epoxy-5,8,14(Z,Z,Z)-eicosatrienoic acid
$TrxA_3$	8(R/S),11(R),12(S)-5,9,14(Z,E,Z)-trihydroxy-eicosatrienoic acid
$TrxB_3$	10(R/S),11(R),12(R)-5,8,14(Z,Z,Z)-trihydroxy-eicosatrienoic acid

1 INTRODUCTION

The aim of the work presented in this thesis was to increase the knowledge of the expression and biological function of the enzyme 15-lipoxygenase type 1 (15-LO-1).

1.1 FATTY ACIDS

Membrane and compartments of the cells consist of a phospholipid bilayer with a hydrophilic exterior and a hydrophobic interior. The construction of the phospholipid is a backbone of typically a glycerol with a phosphorylated alcohol hence being hydrophilic and two hydrophobic fatty acids. Fatty acids consist of a carboxyl group with an even numbered aliphatic chain which are either saturated or unsaturated. Although the fatty acid composition of the phospholipid is different in various organs and tissues, one common fatty acid in mammalian cells is arachidonic acid (20:4, ω6) which consists of 20 carbon atoms and four cis double bonds, positioned at carbon 5, 8, 11 and 14 (5,8,11,14(*Z*,*Z*,*Z*,*Z*)-eicosatetraenoic acid). Arachidonic acid is dietary obtained or synthesized from the essential fatty acid linoleic acid (18:2, ω6). Arachidonic acid is almost completely incorporated in the membrane and only a limited amount is free in the cytosol. The release of fatty acids from phospholipids is catalyzed by phospholipase A_2 (PLA_2), an enzyme present as different isoforms in various cell types [1]. Arachidonic acid is a substrate for cyclooxygenases, lipoxygenases and cytochrome P450 enzymes and the metabolites derived from this fatty acid are collectively called eicosanoids since arachidonic acid contains 20 carbon atoms.

1.2 CYCLOOXYGENASES

Two functional cyclooxygenase (COX) isoenzymes, COX-1 and COX-2, are known. In addition, a splice variant of COX-1 is identified as COX-3 [2]. COX-1 is constitutively expressed in most mammalian cells, whilst COX-2 is an inducible enzyme in macrophages and other cells activated at sites of inflammation. COX converts arachidonic acid to the hydroperoxide-endoperoxide prostaglandin (PG) G_2 which is subsequently reduced to PGH_2, which in turn is the precursor to PGE_2, PGD_2, PGI_2 and $PGF_{2\alpha}$ (Figure 1). These prostaglandins are formed via specific terminal prostaglandin synthases [3]. PGH_2 is also the precursor to thromboxane (TX) A_2 and this conversion is catalyzed by TXA_2 synthase [4].

1.3 LIPOXYGENASES

Lipoxygenases (LO) are a family of non-heme iron containing enzymes which stereospecifically introduce molecular oxygen into polyunsaturated fatty acids, such as arachidonic acid. In humans, at least six functional genes have been identified and the enzymes are named according at which carbon atom oxygen is introduced in arachidonic acid. Hence the human enzymes are 5-LO, 12 (S)-LO, 12(R)-LO, 15-LO type 1 (15-LO-1), 15-LO-2 and epidermis-type 3-LO (e-LOX-3) [5].

The 5-LO is most thoroughly investigated, and this enzyme catalyzes the formation of 5(S)-hydroperoxy-eicosatetraenoic acid (5(S)-HPETE) from arachidonic acid. This formation is a free radical reaction initiated by hydrogen abstraction at carbon 7 of arachidonic acid, followed by a migration of the radical to carbon 5 and hence forming a Δ6-trans double bond. Molecular oxygen is then introduced at carbon 5 forming the

5(S)-HPETE. This metabolite can be either reduced to the 5(S)-hydroxy-eicosatetraenoic acid (5(S)-HETE) or the enzyme can catalyze the formation of the epoxide leukotriene (LT) A_4. In this latter reaction, a subsequent abstraction of hydrogen at carbon 10 of the 5(S)-HPETE occurs followed by a radical migration to carbon 6, yielding three conjugated double bonds. The radical finally merges with the hydroperoxy group and a dehydration forms the unstable epoxide of LTA_4 [6]. LTA_4 can be either hydrolyzed enzymatically to LTB_4 or conjugated with glutathione to form the LTC_4. This conjugation is catalyzed by the membrane associated glutathione transferase LTC_4 synthase (LTC_4S). LTC_4 can be further metabolized by γ-glutamyl transpeptidase and dipeptidase to LTD_4 and LTE_4, respectively [7] (Figure 1). LTC_4, LTD_4 and LTE_4 are collectively referred to as the cysteinyl-leukotrienes.

1.4 PROSTAGLANDINS

Prostaglandins are locally acting mediators with strong varied physiological effects, for example contraction and relaxation of smooth muscle tissue. PGE_2 is the most abundantly produced prostaglandin and this metabolite is important in regulating inflammatory processes, fever and inflammatory induced pain. The inflammation associated formation of PGE_2 is mainly catalyzed by the membrane PGE synthase-1 (mPGES-1) [8]. PGI_2 and TXA_2 play an important role in hemostasis [9]. Non-steroidal anti-inflammatory drugs (NSAIDs), such as acetyl salicylic acid and diclofenac, inhibit prostaglandin synthesis and these drugs are commonly used to treat fever and pain. Celebrex® is a selective COX-2 inhibitor, and thereby causes less gastro-intestinal side effects, and is used for the treatment of patients which cannot tolerate non-specific NSAID. Treatment with selective COX-2 inhibitors, however, leads to more cardiovascular side effects than therapy with non-specific NSAID [10].

1.5 LEUKOTRIENES

The cysteinyl–LTs are potent bronchoconstrictors and airway constriction is a general feature of asthma. Other actions caused by the cysteinyl–LTs are mucus secretion in the airways, vasoconstriction and vascular leakage causing edema [11]. A role in airway remodeling (airway collagen deposition and smooth muscle thickening) is also described for the cysteinyl–LTs [12]. Singulair® and Accolate® are cysteinyl-LT receptor 1 antagonists, and these drugs are used to treat asthma. In US, a 5-LO inhibitor named Zyflo® is also used for asthma treatment.

1.6 HEPOXILINS

In animals, 12-LO converts arachidonic acid into the unstable 12(S)-HPETE, followed by an internal isomerization of the hydroperoxy to monohydroxy epoxide, leading to the formation of metabolites called hepoxilins. The name hepoxilin (Hx) is a combination of the structure (hydroxy epoxide) with the first established biological activity (insulin release). Two hepoxilins have been identified, HxA_3 and HxB_3, with the hydroxyl positioned at carbon 8 and 10, respectively. These metabolites are quite unstable and are further metabolized, either by conjugation with glutathione forming the HxA_3-C or hydrolysis of the epoxide forming metabolites containing three hydroxyl groups named trioxilin (Trx) A_3 and B_3. HxA_3-C is metabolized in analogy with the cysteinyl-LTs and forms the HxA_3-D [13].

The activity of 12(R)-LO in human skin is quite low in normal human epidermis but the synthesis of 12(R)-HETE and HxB_3 are strongly elevated in the inflammatory and proliferative skin disease psoriasis. eLOX3 lacks oxygenase activity but can metabolize 12(R)-HPETE and form a specific hepoxilin, which itself, or as a precursor, have a role to maintain a normal skin barrier. Mutations of the genes coding for 12(R)-LO or eLOX3 have been linked to certain types of ichtyosis, characterized of flaky skin [14].

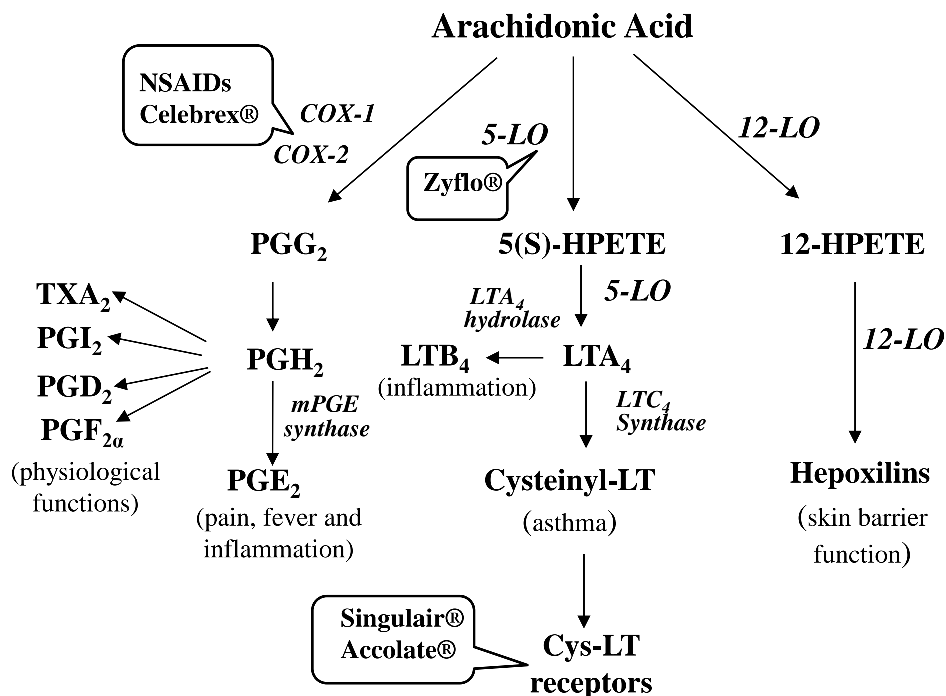

Figure 1. *Overview of the arachidonic acid metabolism. Three metabolizing pathways are displayed, consisting of different enzymes yielding biologically important eicosanoid mediators. Drugs on the market to inhibit the formation or action of these metabolites are indicated in boxes.*

2 15-LIPOXYGENASE-1

Two types of 15-LO is expressed in human cells. The amino acid sequence similarity between 15-LO-1 and 15-LO-2 is only 40% and the biological functions are quite different [15]. 15-LO-1 is predominantly expressed in airway epithelial cells, reticulocytes, eosinophils and dendritic cells [16] (Table 1) whereas 15-LO-2 is expressed in hair roots, prostate, lung and cornea. 15-LO-1 can oxygenate arachidonic acid at carbon 15 but also at carbon 12, leading to the formation of 15(S)-HETE and 12(S)-HETE at a ratio of 9:1. In contrast, 15-LO-2 appears to catalyze the formation of 15(S)-HETE only. There are also differences in substrate specificity between the two 15-LO enzymes [17]. This thesis deals with the expression and chemical properties of 15-LO-1.

Cell type	15-LO-1 expression
Normal cells:	
Airway epithelial cells	+++
Eosinophils	+++
Cornea epithelial cells	++
Mast cells	+(+)
Alveolar macrophages	+(+)
Dendritic cells	++
Reticlocytes	++
Synovial cells	+
Neutrophils	+
Endothelial cells	+*
Fibroblasts	+
Semianl fluid	+
Malignant cells:	
Hodgkin Reed-Sternberg cells (L1236)	+++
Colon carcinoma cells	+
Prostate cancer	+

*[18]

Table 1. *Relative* e*xpression of 15-LO-1 in human cells [19].*

2.1 STRUCTURE

Human 15-LO-1 is a 74.8 kDa protein and contains non-heme iron. The crystal structure of the rabbit reticulocyte 15-LO-1 has been solved. The 15-LO-1 is comprised of two domains: a small N-terminal, β-barrel domain, and a lager C-terminal domain which is predominantly α-helix. The sequence, size and structure of the N-terminal domain have similarities with domains in other mammalian lipases. The C-terminal domain is the catalytic domain coordinating the catalytic non-heme iron. The fatty acid is believed to enter with the methyl group first into the binding pocket [20]. The size and the shape of the substrate binding pocket are essential for the position of oxygenation, favoring the 15-LO over the 12-LO activities [21].

2.2 REGULATION

The gene for 15-LO-1 is named ALOX15 and is located at 17p13.3 in the genome. Expression of 15-LO-1 is highly regulated at the transcriptional, translational, post-translational and epigenetic level [15]. Interleukin (IL)-4 and IL-13 induce the

expression of 15-LO-1 via the signal transducer and activator of transcription (STAT)-6/ Janus kinase signaling pathway [22]. The STAT-6 activation is crucial for the transcriptional activation of 15-LO-1 [23]. STAT-1 and -3 are also indicated to regulate transcription of 15-LO-1 [24]. Furthermore, the histone 3 lysine 4 methylation status is of importance for the expression of the 15-LO-1 gene [25]. Translational regulation is described during the maturation of erythrocytes. 15-LO-1 can undergo suicide inactivation as a post-transcriptional regulation; due to a 15-LO-1 produced arachidonic acid metabolite intermediate that binds covalently to the binding pocket [26].

2.3 ACTIVITY

In resting cells, 15-LO-1 is present in the cytosol but after Ca^{2+} stimulation the enzyme can translocate to intracellular membranes, although the affinity to Ca^{2+} is rather low, with a Kd of 0.2-0.5 mM. The association with the membrane seems to consist of unspecific hydrophobic bonds. The bonds are strengthened by calcium building salt bridges between positive amino acids and the negative phospholipid head [27].

2.3.1 Substrates

Compared to 5-LO, 15-LO-1 is a promiscuous enzyme regarding substrate specificity. In addition to the arachidonic acid also linoleic acid, linolenic acid and other polyunsaturated fatty acids independent of chain length are substrates. Besides the free fatty acids, esterified fatty acids in the membrane can also be oxygenated by 15-LO-1. However, the reaction rates with various substrates are quite different. For instance, the free linoleic acid is five times more effectively oxygenated compared to esterified linoleic acid [15].

2.3.2 Catalytic reaction

The non-heme iron is the catalyst in the 15-LO-1 pocket and the redox status of iron changes from Fe^{2+} to Fe^{3+} by increased peroxide tonus. The 15-LO-1 substrates all have a common feature with a bis-allylic methylene structure, and from the methyl group a hydrogen is abstracted leaving a radical (Figure 2). The radical can then delocalize to a more energetically favorable position, most likely two carbons away, yielding a conjugated double bond. In arachidonic acid, a free radical is initially formed at carbon 13, and is then delocalized primarily to carbon 15. Molecular oxygen is then inserted at that position leading to the formation of 15(S)-hydroperoxy-eicosatetrenoic acid (15(S)-HPETE). The return of the hydrogen also gets back the activated iron (Fe^{3+}). Subsequently, a homolytic cleavage of the peroxy forms an alkoxy radical which in turn initiates an array of secondary products, such as epoxyhydroxyls and keto-dienes [28]. The 15-LO-1 can also abstract a hydrogen from carbon 10 of 15(S)-HPETE and this will result in three conjugated double bonds and an epoxide positioned at carbon 14,15, a metabolite called 14,15-LTA_4. In addition, another oxygenation can occur to form dihydroxy metabolites like the 8(R/S),15(S)-dihydroxy eicosatetraenoic acids (8,15-DiHETEs) and 14(R/S),15(S)-dihydroxy eicosatetraenoic acids (14,15-DiHETEs). The 8,15-DiHETEs can also be degradation products of the 14,15-LTA_4 [29] (Figure 3). A sum of four 8,15-DiHETEs enantiomers can be formed and the quartet pattern is for example described in human airway epithelial cell incubated with arachidonic acid [30].

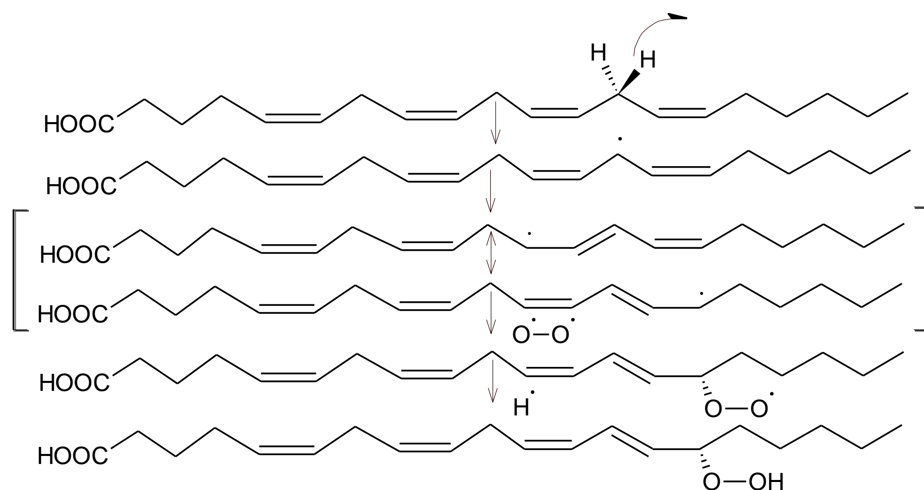

Figure 2. *15-LO-1 catalyzes the conversion of arachidonic acid to 15(S)-HPETE. The hydrogen at carbon 13 is abstracted, yielding a radical which is stabilized by the conjugated double bond intermediate. The radical present at carbon 15 pairs with molecular oxygen forming a peroxy radical and subsequent addition of hydrogen forms the hydroperoxy.*

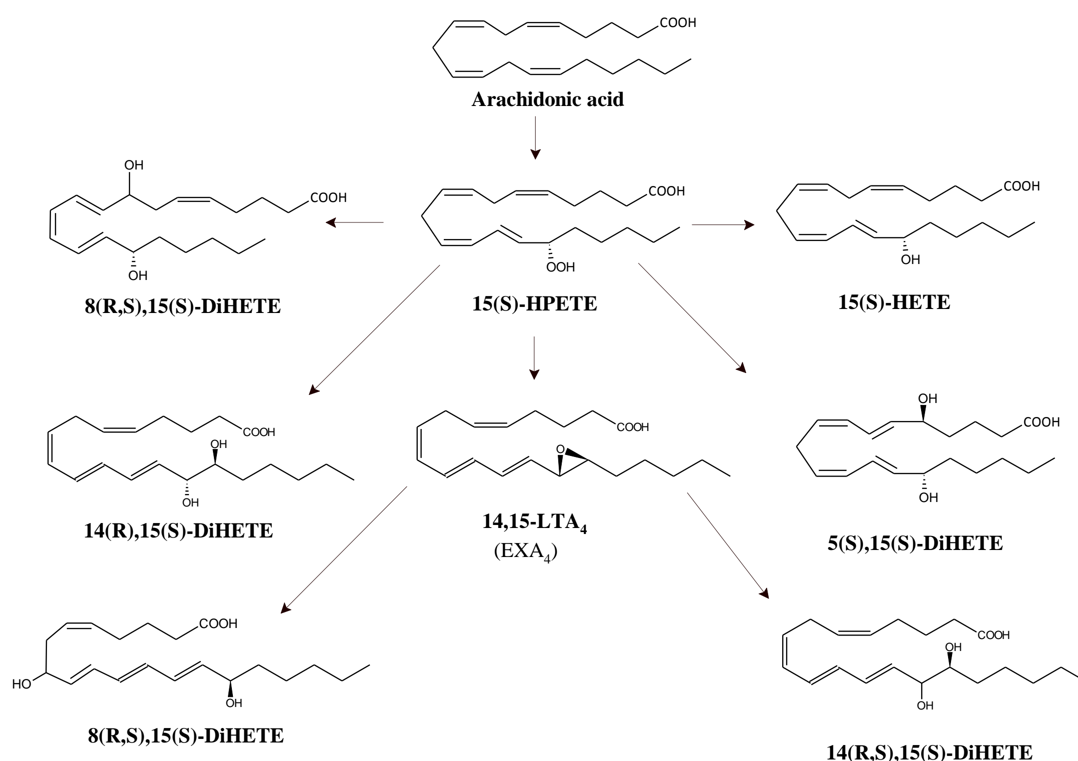

Figure 3. *An overview of arachidonic acid metabolites formed through the15-LO-1 pathway (known before the papers included in this thesis were published). The 8(R,S),15(S)-DiHETEs are in sum four metabolites, a quartet, which elute separately from the LC column. Thereafter, the other DiHETEs elutes and finally the monohydroxy 15(S)-HETE. The DiHETEs contain three conjugated double bonds and have a UV absorbance maximum around 270 nm and the 15(S)-HETE, which contains two conjugated double bonds, at 235 nm. The DiHETEs and 15(S)-HETE have m/z 334 and 319, respectively, in negative ion mode MS.*

2.4 SPECIES HOMOLOGEOUS

The animal ortoholog to human 15-LO-1, apart from rabbit reticulocyte 15-LO-1, is the leukocyte 12-LO with similar enzymatic expression, distribution and regulation, but possess mainly 12-LO activity [15]. The leukocyte 12-LO is expressed in cow, pig, rat and mouse. Since the leukocyte 12-LO primarily converts arachidonic acid to 12(S)-HETE and secondarily to 15(S)-HETE the enzymes are often referred to as a 12/15-LO. Similar to human 15-LO-1, linoleic acid and esterified fatty acids in membranes are also substrate for the animal 12/15-LO [31].

2.5 BIOLOGICAL ROLE- ASSOCIATED DISEASES

15-LO-1 has been implicated to possess several biological roles and to be involved in both physiological and pathophysiological processes (Table 2) due to catalyzing the conversion of free arachidonic to various metabolites. In addition, the capability of 15-LO-1 to oxygenate fatty acids in biomembranes and lipoproteins should also be considered when discussing the biological importance of this enzyme.

2.5.1 Cell differentiation

15-LO-1 was first identified in rabbit reticulocytes and a physiological role for 15-LO-1 was suggested. The reticulocyte is precursor to the erythrocyte, the red blood cell, and the maturation process includes a degradation of the mitochondria which coincides with the expression of 15-LO-1. The 15-LO-1 oxygenation of the mitochondrial membranes and the enzyme´s role in forming pores in the membrane are suggested to increase permeability which contributes to the mitochondrial degradation during erythropoiesis [32]. The 15-LO-1 also specifically degrades organelles in the eye lens by integrating with biomembranes allowing the release of proteases [33].

Disease	Reference
Asthma	19, 37, 39
Chronic obstructive pulmonary disease (COPD)	52
Atherosclerosis	53
Pulmonary arterial hypertension	54
Obesitas	55
Diabetes	56
Stroke	57
Alzheimer´s disease	48
Osteoporosis	58
Hodgkin lymphoma	19
Colorectal cancer	59
Prostate cancer	60

Table 2. *A summary of diseases associated with 15-LO-1 expression.*

2.5.2 Inflammation and asthma

Arachidonic acid metabolites formed via the 5-LO and COX-2 pathways are involved in inflammatory conditions in man. Also 15-LO-1 has been suggested to be primarily involved in inflammation, specifically in airway inflammation. Increased expression of 15-LO-1, both the gene and the enzyme, has been detected in the bronchial epithelial cells in asthmatics compared to healthy subjects [34,35]. Formation of 15(S)-HETE was higher in chopped human bronchi, incubated with arachidonic acid, from

asthmatics compared with non-asthmatics. Airway epithelial cells are the main source of 15(S)-HETE production in the respiratory tract is [36]. In bronchoalveolar lavage (BAL) fluid, 15(S)-HETE was detected and the levels were higher in asthmatics compared to healthy subjects. Increased level of 15(S)-HETE was associated with tissue eosinophil numbers, increased sub-basement thickness and increased 15-LO-1 expression in bronchial epithelium [37]. Overexpression of 15-LO-1 in the airway epithelial cell line A549 leads to increased release of chemokines and to increased recruitment of cells involved in the inflammatory response [38]. 15(S)-HETE also stimulates mucus secretion in airway epithelial cells [39]. Intranasal challenge with osmotic activation by mannitol increased 15(S)-HETE content of the nasal lavage fluid and the levels correlated with nasal symptoms [40]. In a mice knock-out study of the 15-LO-1 ortholog, the 12/15-LO, the allergen induced airway inflammation and remodeling was attenuated [41]. Although all these observations suggest a role for 15-LO-1 in airway inflammation, the mechanism of action is not clarified and receptors that specifically bind 15-LO derived metabolites have not yet been identified. However, one 15-LO-1 derived arachidonic acid product, 5-oxo-15(S)-HETE, has been reported as a potent chemotactic agent for eosinophils and neutrophils [42].

2.5.3 Cancer

Various cancer forms, such as colorectal, prostate and breast cancer, have been associated with 15-LO-1 expression [43]. Nuclear hormone receptors that regulate gene expressions upon fatty acid binding are often suggested to be involved when the correlation between cancer and 15-LO-1 is discussed.

2.5.4 Atherosclerosis

A pro-atherogenic role is suggested for the ortholog 12/15-LO in mice [44] but the relevance in humans is not clear. 12/15-LO is overexpressed in atherosclerotic lesions in mice and is modifying the high density lipoprotein 3 in human endothelial cells and hence is suggested to be involved in atherosclerosis [45]. The ability of 15-LO-1 to oxygenate lipoproteins and the 15-LO-1 expression in macrophages are the reason that the correlation between 15-LO-1 and atherosclerosis has been studied. However, atherosclerotic lesions in human appear to correlate more with the expression of 15-LO-2, than with15-LO-1 [46, 47].

2.5.5 Alzheimer’s disease

Increased expression of 15-LO-1 was identified in brain affected with Alzheimer’s disease compared to non-effected brain [48]. Furthermore, in cerebrospinal fluid from patients with Alzheimer´s disease 15(S)-HETE was detected in increased levels compared to samples from healthy subjects [49]. However, the biological role of 15-LO-1 in the disease is not clear.

2.5.6 Anti-inflammatory effect and lipoxins

Although there are many studies indicating a pro-inflammatory action of 15-LO-1 there are also several indications of an anti-inflammatory action for this enzyme. For example, in human asthmatic bronchitis the increased level of 15(S)-HETE inhibits LTB_4 formation [50].

Since 15(S)-HPETE and 5(S)-HPETE contain two doubly allylic methylene groups they may serve as lipoxygenase substrates for a secondary oxygenation. These double oxygenated products are called lipoxin (LX) A_4 and LXB_4 and are triple hydroxyls. Lipoxins are formed by the action of two different lipoxygenases present in different cells, so called trans-cellular metabolism. Lipoxins reduce inflammation and promote resolution [51].

3 CELLS

Different cell types were investigated with the purpose of increasing the knowledge of 15-LO-1. A short description of these cells is presented below.

3.1 EOSINOPHILS

Eosinophils are involved in the immune response against parasites and play a central role in certain inflammatory responses. Normally1-3% of the circulating leukocytes are eosinophils but many patients with severe asthma or aspirin-intolerant asthma have increased number of eosinophils in the respiratory tract. Eosinophils can release cytokines, chemokines and lipid mediators upon activation.

3.2 MAST CELL

Mast cells are key effectors in allergic reactions. The characteristic of the mast cell is the rich cytoplasmic granule which contains histamine, tryptase, proteases, proteoglycans and peptidase. Mast cells are of hemapoetic origin and circulate in peripheral blood and finally mature when entering a tissue exposed to the external environment. The life span of the mast cell is long compared to other inflammatory cells and can survive in tissue for months. Environmental allergens trigger immunoglobulin E (IgE) overproduction which bind and activate mast cells. Upon activation a mast cell degranulates and releases the stored pro-inflammatory mediators. Furthermore it can synthesize PGD_2, cysteinyl- leukotrienes and various HETEs. Cytokines and chemokines are both stored in the granule and can be *de novo* synthesized [61].

3.3 AIRWAY EPITHELIAL CELLS

The lining of the respiratory tract consists of airway epithelial cells and they act as protective barrier. The airway epithelial cells are composed of basal cells, ciliated cells and mucus cells. The mucus cells, also called the goblet cells, produce and secrete mucus, which can trap potential pathogens and foreign particles in the airway. The ciliated cells effectively move the mucus up the respiratory tract. Airway epithelial cells can also produce and release biologically active compounds including lipid mediators, growth factors and a variety of cytokines/chemokines important in the pathogenesis of airway disorders [62].

3.4 LYMPHOCYTES

Lymphomas are cancers originating from lymphoid cells, mainly B lymphocytes, and these malignant cells are classified as either Hodgkin lymphoma (HL) or non-Hodgkin lymphomas. Hodgkin lymphoma is a well-defined disease, whereas non-Hodgkin lymphomas are a less defined heterogenic group of lymphomas. L1236 is a Hodgkin lymphoma derived cell line [63]. A morphologically characteristic cell in a Hodgkin lymphoma tumor is called Hodgkin Reed-Sternberg (H-RS) cell, a multinucleated giant cell. The Hodgkin lymphoma tumor consists only of 1-2% of H-RS cells and the rest is infiltrating inflammatory cells like T-cells, B-cells, neutrophils, eosinophils and mast cells [64].

4 THE MASS SPECTROMETER

The mass spectrometer (MS) measures the mass-to-charge ratio of ions in the gas phase. MS has long been a valuable tool for the structural determination of the eicosanoids.
A typical mass spectrometer consists of three components which are all controlled by a computerized system (Figure 4). The first component, the ion source, is responsible for forming ions and/or to transfer ions into the gas phase and subsequently introduce the ions into the mass analyzer. The mass analyzer separates ions according to their mass-to-charge (*m/z*) ratio. Finally the ion beam current is measured by a detector. The design of the different components gives the characteristics to each kind of mass spectrometer. The mass spectrometer components work under reduced pressure or vacuum.

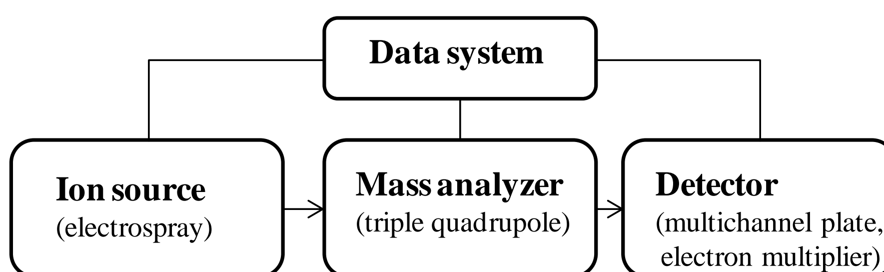

Figure 4. *The components of the mass spectrometer.*

4.1 ION SOURCE

Sample introduction from atmospheric pressure (760 torr) into vacuum (10^{-6} torr) needs to be in such a way that the vacuum system is minimally disturbed. At the beginning of mass spectrometry history, the analytes were transformed into the gas phase by heat, which demands derivatization of thermally labile molecules. The ions were formed by electron impact (EI) which is a rather harsh method where the analyte interacts with a beam of electrons. A gas chromatograph (GC) allows separation of the sample components on a column prior to their MS analyses [65]. This combination, GC-MS, is still widely used for thermally stable and non-polar compounds. A softer ionization technique called chemical ionization (CI) was later developed, where EI was applied on a reagent gas, which subsequently reacts with the analytes [66]. The ionization technique called fast atom bombardment (FAB) achieves ionization by accelerated atoms, typically argon or xenon, bombarding the analyte dissolved in a non-volatile matrix. FAB ionization combined with protective environment of the matrix enables polar and thermally labile molecules to survive the ionization process [67].

The combination of liquid chromatography (LC), where sample components are separated on a column in the liquid phase, and MS, was revolutionized when the electrospray ionization (ESI) technique was developed in late 1980s [68, 69]. In ESI the ions are already present in the liquid phase and when the solvent is vaporized, ions in gas phase can enter the mass analyzer. The solvent used is chosen depending on the solubility of the analyte and its capacity to evaporate. The liquid flow enters the ion

source through a capillary to which a voltage is applied. The voltage produces an electrical gradient in the fluid which separates the charges at the liquid surface, forming a fine spray. At the tip of the spray a cone develops, and highly charged droplets leave the spray when electrostatic repulsion exceeds the surface tension of the liquid. Droplets are then attracted to the mass analyzer entrance due to the oppositely charged voltages at the entrance, and the pressure gradient. During this transition the droplets gradually reduce in size until the ions enter the gas phase. There are two theories regarding the exact mechanism for the formation of ions from charged droplets [70].

4.2 MASS ANALYZERS

There are several types of mass analyzers, but the most commonly used are quadrupoles, ion traps and time-of-flight (TOF) analyzers. In the present work, MS instruments with triple quadrupole analyzers were used. A triple quadrupole MS consists of three sequential quadrupoles. A quadrupole consists of four parallel rods to which a direct current (DC) voltage and a superimposed radio-frequency (RF) potential are applied. The specific RF to DC ratio determines which *m/z* ions can pass the electrical field. Hence, the voltage settings allow only the ions of interest to reach the detector [71]. Triple quadrupole instruments have good linearity of signal response and are therefore suitable for quantitative studies but the disadvantage is the low spectral resolution.

4.3 MS ANALYSIS

In the triple quadrupole mass spectrometer several types of scans/experiments can be performed by operating each quadrupole differently (Figure 5). The first and third quadrupole can be operated in a scanning or static mode which allows a mass range or only one mass, respectively, to pass. The second quadrupole can either transmit the ions or perform collision induced dissociation (CID) by adding inert gas. The selected ions collide with the gas which induces fragmentation. All this makes it possible to perform MS scan, MS/MS scan, neutral loss scan, parent ion scan or single reaction monitoring (SRM) [72]. The first four scans are suitable for structure elucidation analysis and the fifth for quantitative analysis.

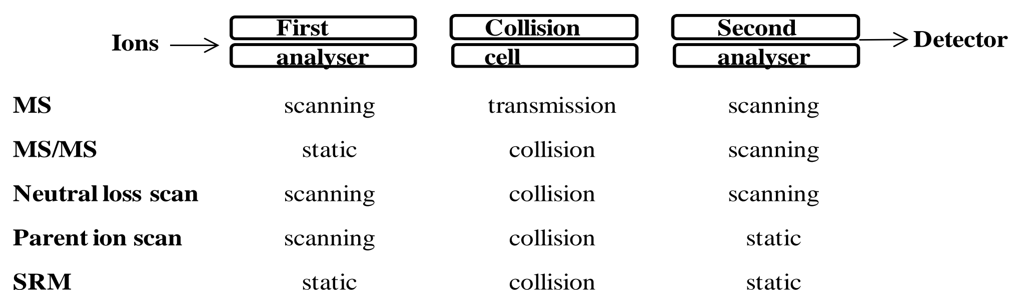

Figure 5. *An overview of the different scan types, and the settings of each quadrupole, which could be executed on a triple quadrupole instrument.*

4.4 OTHER DETECTORS

The LC-MS set up allows other detectors to be used simultaneously. In series with the LC-MS, an ultraviolet (UV) or a diode array detector (DAD) can be connected. This

equipment detects the UV absorbance capability of the compound and can give a characteristic spectrum which can be useful in the structure elucidation of the compound. A radioactivity monitoring (RAM) detector could be used in parallel with the MS instrument. The destiny of a radioactive labeled substrate is easier to follow than an unlabeled substrate.

4.5 SAMPLE HANDLING

The samples intended for LC-MS analysis often need some preparation prior to the analysis. High salt content should be diluted, sample with debris should be centrifuged and also extraction techniques are often used to extract the analytes of interest and get a more concentrated and cleaner sample. Samples are usually stored at -70°C prior analysis to minimize sample degradation.

4.6 EICOSANOID ANALYSIS

Mass spectrometry has been a central tool in the structural characterization of all of the eicosanoids starting in the 1960s with the structural determination of the prostaglandins [73]. Initially analysis was performed by techniques, such as GC/MS and EI and later FAB but more recently with ESI. Electrospray ionization allows analysis of both volatile and nonvolatile eicosanoids. Most eicosanoids give rise to both the protonated $[M+H]^+$ and deprotonated $[M-H]^-$ molecular ion and hence can be analyzed in both negative and positive ion mode. However, the intensity for oxygenated eicosanoids is usually higher in negative ion mode, and for glutathione conjugated eicosanoids the highest intensity is achieved in the positive ion mode.

Quantification of eicosanoids has mainly been performed by LC-MS/MS due to the accurate quantification within a reasonable run time. Isobaric eicosanoids need to be separated by LC prior to the mass spectrometer and/or give rise to different fragmentation products in order to be analyzed accurately. Quantification is usually performed by a triple quadrupole mass spectrometer, using the selectively/ sensitivity of the SRM/MRM scan. However, eicosanoid quantification in biological fluids can be challenging due to their low levels, down to the pico-molar range [74].

5 OBJECTIVES

The overall objective of this thesis was to characterize various arachidonic acid metabolites formed via the 15-LO-1 pathway in different cell types. Furthermore, the focus was also on the expression of the enzyme in various cell types and the biological functions of the arachidonic acid metabolites formed through the 15-LO-1 pathway.

The specific objective for each respective paper was:

- To investigate if 15-LO-1 is expressed in mast cells and if so which arachidonic acid metabolites are formed (paper I).

- To investigate which arachidonic acid 15-LO-1 derived metabolites are formed in eosinophils and how the 15-LO-1 pathway in eosinophils can be activated. Furthermore, the aim of the study was also to elucidate the pro-inflammatory properties of these metabolites in an inflammation *in vitro* model (paper II).

- To investigate if eoxins are formed by the Hodgkin lymphoma derived cell line L1236 and examine if 15-LO-1 is expressed in biopsies derived from Hodgkin lymphoma patients (paper III).

- To investigate the formation and identity of other metabolites than eoxins formed through the 15-LO-1 pathway in the Hodgkin lymphoma derived cell line L1236 (paper IV).

- To investigate if the animal ortholog to 15-LO-1, the 12/15-LO expressed in eosinophils derived from mini pigs, can form eoxins (paper V).

- To investigate the effect of certain stimulus on 15-LO-1 activity in human airway epithelial cells. Furthermore, to investigate if eoxins are formed in airway epithelial cells and examine which glutathione-S-transferase (s) is responsible for the conjugation of glutathione with EXA_4 (paper VI).

6 METHODOLOGY

The general procedure to investigate the activity of 15-LO-1 in a cell type was to incubate the cells with unlabeled/labeled arachidonic acid and identify the metabolites with LC-MS/MS in combination with UV/DAD and RAM detection. The eosinophils/ the polymorphonuclear leucocytes (PMNL) fraction were isolated from whole blood and in order to produce mast cells, mononuclear cells were isolated from cord blood and differentiated *in vitro*. The cells were treated with various stimuli prior to the incubation and/ or certain inhibitors. The metabolites were confirmed by authentic standards, and some metabolites, the 14,15-HXs, were synthesized. PCR or microarray and western blot analyses were performed to evaluate the expression of the 15-LO-1 gene and the enzyme, respectively. Immunohistochemical studies were performed to explore the expression *in vivo* of 15-LO-1 and other enzymes in cells or tissues. A model to evaluate the metabolite´s pro-inflammatory potential was tested. The model measures the change in permeability of an endothelial monolayer caused by the metabolites. For experimental details, please read the material and methods section in each of the listed papers.

7 RESULTS

7.1 PAPER I

Cord blood derived mast cells were prepared by two protocols, with or without serum, and similar results were obtained independently of protocol. Mast cells stimulated with IL-4 for 120 hours express 15-LO-1, but not 15-LO-2. This was shown at both mRNA and protein level. A time course of 15-LO-1 expression after stimulation with IL-4 for 24-120 hour showed positive protein detection at 72 hour and further expression at 120 hour. Mast cells incubated with labeled arachidonic acid was analyzed with high performance liquid chromatography (HPLC) connected with UV and RAM detection. Only IL-4 stimulated mast cells produced detectable amounts of arachidonic acid metabolites.

The main metabolite detected was 15-keto-eicosatetraenoic acid (15-KETE). The identity was confirmed by MS/MS analysis in negative ion mode by nanospray infusing the collected peak and compared with authentic standard. The MS/MS spectrum was clearly different from the 5-KETE standard spectrum. Besides the 15-KETE, also 15-HETE was formed in a ratio of about 1:9 compared to 15-KETE. In contrast, homogenized mast cells formed mainly 15-HETE. The formation of 15-KETE and 15-HETE were not inhibited with indomethacin demonstrating that these metabolites were not formed via the COX pathway. To evaluate the mechanism of 15-KETE formation, mast cells were incubated with either 15(S)-HPETE or 15(S)-HETE, and the production of 15-KETE seems to be possible from both metabolites.

Mast cells were incubated with mannitol in order to mimic the mechanism of increased osmolartity that occurs in airways following exercise. The 15(S)-HETE was measured by a 15(S)-HETE enzyme immunoassay and the 15(S)-HETE level was significantly increased after mannitol stimulation. Immunohistochemical staining of mast cells and 15-LO-1 was performed with antibodies against tryptase and 15-LO-1, respectively. (The antibody against 15-LO-1 do not detect 5-LO, 12-LO or 15-LO-2.) Cord blood derived mast cells, skin from a patient with atopic dermatitis and human bronchial tissue were sequential double stained. The study demonstrated that 15-LO-1 was expressed in mast cells in all investigated tissues.

7.2 PAPER II

Eosinophils are known to be a rich source for 15-LO-1 and these cells were isolated and incubated with arachidonic acid. The LC-UV chromatogram revealed peaks which corresponded to the quartet of the 8,15-DiHETEs and also to the 14,15-DiHETEs as well as a small peak of LTC_4. The complete quartet indicated formation of 14,15-LTA_4 since two of the 8,15-DiHETEs are degradation products of 14,15-LTA_4. In addition to these metabolites, a more polar metabolite was detected. The UV spectrum revealed it to contain three conjugated double bonds with a UV absorbance maximum at 282 nm. The formation of the metabolite was not inhibited by 5-LO or COX inhibitors. The metabolite was also formed when eosinophils were incubated with 14,15-LTA_4. To further characterize the structure of this metabolite, it was analyzed with MS/MS in

positive ion mode. The *m/z* 626.2 and several of the fragments of the metabolite were similar as for LTC_4.

The data presented above suggested that the metabolite could be 14,15-LTC_4 (14(R)-glutathionyl-15(S)-hydroxy-5,8,10,12(Z,Z,E,E)-eicosatetraenoic acid). This was confirmed with comparison to synthetic standard. The MS/MS analysis displayed that the cleavage adjacent to the glutathionyl position in LTC_4 and 14,15-LTC_4, gave rise to fragments, *m/z* 189 and 205, respectively, that distinguish between these metabolites. The 14,15-LTC_4 could also be further metabolized by the eosinophils and to14,15-LTD_4 (14(R)-cysteinyl-glycyl-15(S)-hydroxy-5,8,10,12(Z,Z,E,E)-eicosatetraenoic acid) and 14,15-LTE_4 (14(R)-cysteinyl-15(S)-hydroxy-5,8,10,12(Z,Z,E,E)-eicosatetraenoic acid). To avoid confusion with the leukotrienes and since the 14,15-LTs were identified in eosinophils, we suggested the name eoxins (EX). Thus, these novel metabolites were named EXA_4, EXC_4, EXD_4 and EXE_4. The formation of EXC_4 and EXD_4 were also detected in mast cells with enzyme immunoassay and MS/MS. Surgically removed nasal polyps spontaneously released EXC_4. The conjugation reaction between EXA_4 and glutathione occurred in the membrane fraction in eosinophils.

Eosinophils challenged with arachidonic acid produced predominately EXC_4. In contrast, cells stimulated with ionophore A23187 only produced LTC_4. Allergen stimulated mast cell produce LTC_4 and PGD_2 and these mediators as well as IL-5 stimulated the formation of EXC_4 from the endogenous arachidonic acid pool in eosinophils.

An *in vitro* permeability assay based on the assessment of changes in the transendothelial electric resistance across human endothelial monolayer can be used as a marker of changes in vascular permeability [75]. Increase in vascular permeability leading to vascular leakage is a hallmark of inflammation. In order to evaluate the potential pro-inflammatory role of eoxins, these mediators were tested in this *in vitro* model. Interestingly, all eoxins were capable of inducing permeability. Although the eoxins were less potent than cysteinyl-LTs, the eoxins were about 100 times more potent than histamine. EXD_4 was the most potent eoxin. The time response curve for the eoxins resembles the pattern observed for directly acting agonists such as histamine and LTC_4.

7.3 PAPER III

The Hodgkin lymphoma derived cell line L1236 incubated with arachidonic acid produced 15-HETE and 12-HETE in a ratio of about 9:1, which is in agreement with a 15-LO-1 catalyzed formation of these metabolites. The identity and quantification of these mediators were determined with LC-UV analysis. Other Hodgkin lymphoma derived cell lines examined such as L428, KMH2 and L570 did not produce these metabolites. RT-PCR and western blot revealed expression of 15-LO-1, but not 15-LO-2, in L1236 cells. Western blot analysis of subcellular fractions of L1236 cells revealed that in the presence of Ca^{2+}/ Mg^{2+}, with or without calcium ionophore, the majority of 15-LO-1 was found in the membrane fraction although a substantial amount was also present in the cytosolic fraction. The capacity to convert arachidonic acid to 15-HETE and 12-HETE, however, did not correlate with the western blot results. Incubation of

the cytosolic fraction with arachidonic acid led to higher levels of 15-HETE than similar incubations with the membrane fraction. These results indicate that 15-LO-1 in the cytosol has higher enzyme activity than membrane bound 15-LO-1.

Incubation of L1236 cells with arachidonic led to the formation of 8,15-DiHETEs, 14,15-DiHETE and 5,15-DiHETE, a precursor to the chemotactic 5-oxo-15(S)-HETE. The formation of EXC_4 and EXD_4 was confirmed by MS/MS. In addition, a small peak eluted prior EXC_4 and EXD_4, respectively, but with identical MS/MS spectra to synthetic EXC_4 and EXD_4. The UV absorbance maximum, however, was slightly lower, indicating a change in double bond configuration. These metabolites were postulated to be 8-trans-EXC_4 and 8-trans-EXD_4. In order to establish if EXE_4 also was formed, the cells were incubated with EXA_4. Indeed EXE_4 was formed and this was established with MS/MS. The maximum formation of EXC_4 and EXD_4 was achieved after incubation with arachidonic acid for 5 and 30 min, respectively. EXC_4 and EXD_4 formation were detected after incubation with 1µM arachidonic acid and a plateau was reached at 40 µM.

The L1236 cell line contains the characteristic giant Hodgkin Reed-Sternberg (H-RS) cells. To evaluate if H-RS cells *in vivo* also express 15-LO-1, biopsies from HL lymph nodes were immunohistochemically stained. H-RS cells were positively stained for 15-LO-1 in 17 of 20 tumors but also in tissue macrophages and eosinophils. The strongest staining in H-RS cells was from three cases of nodular sclerosis subtype II HL and in one case of mixed cellularity HL. Biopsies from non-Hodgkin lymphoma tumors did not contain any H-RS cells and no 15-LO-1 staining was observed.

7.4 PAPER IV

In order to fully characterize the metabolic profile of products generated through the 15-LO-1 pathway in L1236, these cells were incubated with ^{14}C-labeled arachidonic acid. The 8,15-DiHETEs and the eoxins have already been identified to be produced by the L1236 cell line. In addition, in the radioactivity chromatogram two other major metabolites were detected. The most polar metabolite was produced in similar amounts as EXC_4. These metabolites did not have an absorbance maximum above 200 nm, and were therefore only vaguely detected in the UV chromatogram. The lack of UV absorbance demonstrated that these metabolites not contain any conjugated double bonds. Maximal amount of the more polar metabolite was obtained after two minutes of incubation and the less polar metabolite after 10 minutes of incubation. Furthermore, these metabolites were also formed after L1236 cells were incubated with 15(S)-HPETE but not with EXA_4.

Analysis of the metabolites by MS revealed a molecular weight of the polar and less polar metabolite of plus 18 Da compared to EXC_4 and EXD_4, respectively. This indicated an additional hydroxyl group in comparison to the cysteinyl eoxins. The MS/MS spectrum of the polar metabolite revealed a fragment ion at *m/z* 308, which represent the glutathione cleaved adjacent to the sulfur with the charge retention on the glutathione part [76]. The MS/MS spectrum of the less polar metabolite contained a fragment ion at m/z 179, which represent the cysteinyl-glycine complex. These

fragment ions revealed that the polar metabolite is transformed into the less polar metabolite by cleaving of the glutamate, as for the conversion of EXC_4 to EXD_4.

The additional fragment ions in the spectra of these metabolites were mostly derived from the peptide part and not from the fatty acid part of the molecule; hence the position of the hydroxyl group was not clear. The 13(R/S)-hydroxy-14,15-epoxy-eicosatrienoic acid is also known as 14,15-hepoxilin B_3 (14,15-HxB_3) and this metabolite is produced through the 15-LO-1 pathway in human airway epithelial cells [77]. Therefore, the two 13(R) and 13(S) enantiomers of the 14,15-HxB_3 conjugated with glutathione (14,15-HxB_3–C) was synthesized. The LC retention time of the 13(R) enantiomer was identical to the most polar metabolite. However, the MS/MS spectrum was clearly different from the polar metabolite spectrum.

The 11(R/S)-hydroxy-14,15-epoxy-eicosatrienoic acid also known as the 14,15-Hepoxilin A_3 (14,15-HxA_3) is produced through the 15-LO-1 pathway in garlic root [78]. The 14,15-HxA_3 is more unstable than 14,15-$HxAB_3$ since it is acid sensitive and degrades to a trihydroxy acids. The two 11(R) and 11(S) enatiomers of the 14,15-HxA_3 conjugated with glutathione (14,15-HxA_3–C) were synthesized. The 14,15-HxA_3–C with the hydroxyl group at carbon 11 in S configuration had identical retention time and MS/MS spectrum as the polar metabolite. The polar metabolite was therefore suggested to be 11(S),15(S)-dihydroxy-14(R)-glutathione-5,8,12(Z,Z,E) eicosatrienoic acid (14,15-HxA_3–C 11(S)). Since the less polar metabolite appeared to be formed after removal of glutamate from the polar metabolite, the synthesized 14,15-HxA_3–C 11(S) was incubated with ɣ-glutamyl transpeptidase or L1236 cells. The product formed co-eluted and had similar MS/MS spectrum as the less polar metabolite. The identity of the less polar metabolite was therefore 11(S),15(S)-dihydroxy-14(R)-cysteinyl-glycyl-5,8,12(Z,Z,E) eicosatrienoic acid (14,15-HxA_3–D 11(S)).

Subcellular fractionation of L1236 cells demonstrated that the supernatant and not the membrane fraction converted 14,15-HxA_3 into 14,15-HxA_3-C. Therefore the capacity to catalyze the conversion of several soluble recombinant glutathione S-transferases (GSTs) was tested. The highest capacity had GST M1-1b, P1-1 (Ile), M2-2 and P1-1 (Val). In contrast, LTC_4S had relatively low capacity to catalyze this reaction. The enzyme GST M1-1 is highly expressed in L1236 cells (unpublished data).

Neither of the other 15-LO-1 expressing cells or tissues, that is eosinophils, dendritic cells and nasal polyps, produced the cysteinyl containing 14,15-Hx. However, these cells, as the L1236 cells, converted arachidonic acid to 14,15-HxA_3 11(S) and 14,15-HxB_3 13(R). Also recombinant 15-LO-1 enzyme incubated with arachidonic acid converted arachidonic acid stereoselectively to 14,15-HxA_3 11(S) and 14,15-HxB_3 13(R), indicating that human 15-LO-1 itself has an intrinsic hepoxilin synthase activity.

7.5 PAPER V

The animal ortholog to 15-LO-1, the 12/15-LO, has mainly 12-LO activity and hence catalyzes the formation of partly different metabolites than the human enzyme although the animal enzyme also possesses 15-LO activity to some extent. The aim of this study

was to evaluate whether the minor 15-LO activity in the animal enzyme could catalyze the formation of eoxins. Three mini pigs were infected with the parasite *Ascaris suum* to increase the eosinophil numbers since eosinophils contain abundant amounts of 12/15-LO [79]. On four consecutive days (1-4) after infection, blood was drawn and the polymorphonuclear leukocyte (PMNL) fraction, which contain eosinophils, were isolated. The highest levels of eosinophils were detected at day 1 after infection.

The PMNL fractions, pre-incubated with 5-LO and COX inhibitors, were incubated with ^{14}C-labeled arachidonic acid and analyzed with LC-UV/RAM and by LC-MS/MS. The 8,15-DiHETE quartet and 14,15-DiHETE were identified. The EXC_4 metabolite was detected in the radioactivity and UV chromatograms and had identical MS/MS spectrum as authentic EXC_4 standard. Thus, PMNL isolated from porcine had the capacity to produce EXC_4. The amount of EXC_4 was quantified by LC-MS/MS multi reaction monitoring (MRM) by the utilizing *m/z* 626 to *m/z* 301 and 205 transitions. The formation of 12-HETE was highest day 1 and then decreased while the levels of 15-HETE were constant during the studied period. Taken together, although porcine PMNL produced about five times more 12-HETE than 15-HETE, the cells could generate significant amounts of EXC_4.

7.6 PAPER VI

The human airway epithelial cells highly express 15-LO-1 and several reports describe an increased expression and activity of the enzyme in the respiratory tract of asthma patients. The commercially available MucilAir cells are a model of the human lung epithelium. It consists of basal cells, ciliated cells and mucus cells, and are fully differentiated and cultivated on micro-porous filters in an air-liquid interface [80]. Human epithelial airway MucilAir cells were used in this study and were firstly analyzed by western blot for the presence of 15-LO-1. The 15-LO-1 enzyme was highly expressed and the expression was further enhanced after stimulation with IL-4. The mRNA expression was investigated by whole genome microarray analysis and a high hybridization signal for 15-LO-1 indicated a high gene expression.

Epithelial cells were incubated with arachidonic acid and the 15-HETE and EXC_4 metabolites were identified by LC-MS/MS. 15-HETE was detected in un-stimulated cells, however the levels of 15-HETE were significantly elevated after IL-4 stimulation. The activity of 15-LO-1 was investigated after treating the cells with the bacteria *Pseudomonas aeruginosa*, condensed smoke extract or by scratching the cell surface with a pipette to mimic epithelial injury. Challenge of the cells with *P. aeruginosa* and scratching stimulated the formation of 15-HETE by a factor of 10, but condensed smoke extract did not increase the formation of 15-HETE.

The conversion of EXA_4 to EXC_4 occurred mainly in the cytosol. Therefore, it was of interest to examine which soluble GST that is expressed in airway epithelial cells. Microarray analysis of soluble GSTs demonstrated that GST P1-1 had the highest expression. Recombinant soluble GSTs (M1b-1b, M2-2, M3-3, M4-4, P1-1(Val), P1-1(Ile), T1-1, A1-1, A2-2, A3-3, A4-4) were incubated with EXA_4 and glutathione, and subsequently the EXC_4 formation was quantified. Both isoforms of GST P1-1 effectively converted EXA_4 to EXC_4. Immunohistochemistry staining for 15-LO-1 and

GST P1-1 was performed on human airway epithelial cells from bronchial biopsies derived from healthy volunteers and asthmatic patients. Sequential cuts indicate co-localization of 15-LO-1 and GST P1-1 in epithelial cells lining the bronchi, showing enzymes required for EXC_4 formation is expressed in the human airway epithelial cells. The airway epithelial had higher extent of positively 15-LO-1 stained cells in biopsies from asthmatic patients compared to healthy volunteers. The intensity after GST P1-1 staining was equally high in both groups.

8 DISCUSSION

The expression and activity of 15-LO-1 was investigated in eosinophils, mast cells and airway epithelial cells. These cells have been reported to have a role in the pathophysiology of asthma. The Hodgkin lymphoma L1236 cell line, which has a high constitutive expression of 15-LO-1, was also investigated. The identified arachidonic acid metabolites formed through the 15-LO-1 pathway are presented in Figure 6.

8.1 EOSINOPHILS (PAPER II, IV & V)

Eosinophils which contain high amounts of 15-LO-1 [81] formed EXC_4, EXD_4 and EXE_4, in addition to the earlier described mono- and dihydroxy metabolites derived from arachidonic acid. The formation of the eoxins from arachidonic acid have not been demonstrated earlier in human cells, however, in rat basophils the conjugation of synthetic 14,15-LTA_4 to 14,15-LTC_4 have been shown [82]. The positively identification was based on formation from EXA_4, UV triene spectrum and finally identical retention time and MS/MS spectrum compared to synthetic standards.

The EXC_4 MS/MS spectrum contained fragments derived from the glutathione part, such as *m/z* 497, 319, 308 and 301. The many functional groups of the glutathione part make it easier to fragment than the carbon chain of the fatty acid, which partly consists of conjugated double bonds. These fragments were also present in the MS/MS spectrum of LTC_4 and have been identified with deuterium and MS^3 experiments with an ion trap mass spectrometer. The *m/z* 319, 308, 301 are all derived from cleaving adjacent to the glutathione sulfur, whilst the *m/z* 497 is cleaving of a amide bond resulting a loss of the glutamic residue in the glutathione adduct [83].

Although many similarities of the EXC_4 and LTC_4 MS/MS spectra, two high-intensity fragment ions differed between the spectra. The EXC_4 and LTC_4 MS/MS spectra contained *m/z* 205 and 189, respectively. The LTC_4 *m/z* 189 represents the lipid part after a cleavage of C6-C7 carbon bond of the lipid backbone adjacent to the position of the glutathione. Primarily the *m/z* 319 is formed by cleavage of the carbon-sulfur bond and forming a protonated epoxide, which subsequently is cleaved forming a resonance stabilized alkyl cation *m/z* 189 [83]. The EXC_4 fragment ion *m/z* 205 was suggested to be a result of a cleavage of the C13-C14 bond in the lipid backbone and is possibly formed in a similar way. The *m/z* 205 and 189 was also present in the MS/MS spectra of the D and E metabolites. Hence, the *m/z* 205 and 189 fragment ions could be used to distinguish between the cys-EX and cys–LT. The fragments could be used in qualitative analysis but also in quantitative analysis to ensure specificity.

The EXA_4 formation in eosinophils was also verified by the presence of the full quartet of 8,15-DiHETEs. EXA_4 is formed from 15-HPETE and could possibly be catalyzed by the 12-LO activity of the 15-LO-1. The catalytic process would then be induced by the removal of hydrogen at carbon 10 [84]. The EXA_4 conjugation with glutathione to form EXC_4 occurred in the membrane fraction and LTC_4S can catalyze the conversion in Sf9cells (unpublished results). This indicates LTC_4S as responsible for the conjugation in eosinophils. The EXC_4 was also detected in nasal polyps, probably due to high eosinophil infiltration in the nasal polyps.

Increase in vascular permeability leading to vascular leakage is a hallmark of inflammation. All eoxins were capable of increasing permeability in a model system based on the resistance across a human endothelial monolayer, thus indicating a capacity to induce vascular leakage *in vivo*. The eoxins were less potent than the cysteinyl-LTs, although the eoxins were about 100 times more potent than histamine. The permeability increasing effect of the cys-LTs and histamine is achieved by triggering intracellular calcium mobilization and cytoskeleton rearrangement leading to paracellular gaps [85]. The eoxins could possible increase vascular permeability in a similar way.

The eosinophils also produced 14,15-HxA_3 11(S) and 14,15-HxB_3 13(R) in a stereoselective manner and the 15-LO-1 was indicated to have intrinsic hepoxiln synthase capability.

Porcine eosinophils are a rich source of the 12/15-LO [79], which is the animal ortholog to 15-LO-1. The human 15-LO-1 possess mainly 15-LO activity whilst the 12/15-LO, mainly possess 12-LO activity. Porcine PMNL fractions, enriched with eosinophils, were incubated with arachidonic acid. The cells produced the 8,15-DiHETE quartet and 14,15-DiHETE, which is in agreement with earlier reports [86]. Also the EXC_4 were identified and the only probable enzyme in the PMNL fraction to facilitate the conversion of arachidonic acid into EXA_4 is the 12/15–LO. Murine eosinophils which contain 12/15-LO also convert arachidonic acid to EXA_4 [87]. The formation of EXA_4 was possible facilitated by a combination of 15- and 12-LO activity. The 15-LO activity forms the 15-HPETE and from 15-HPETE the 12-LO activity removes a hydrogen at carbon 10, followed by the homolytical cleaved hydroperoxy forming an alkoxy radical which is cyclized and forms the epoxide of EXA_4 [84,88]. Purified 12-LO from porcine leukocytes have earlier been shown to have EXA_4 synthase activity with 15-HPETE as a substrate [79].

The enzyme in porcine PMNL fraction responsible for the glutathione conjugation of EXA_4 forming EXC_4 was not established. Expression of LTC_4S is not described for porcine leucocytes however the conjugation can also be catalyzed by soluble GSTs. The PMNL fraction produces 15-HETE:12-HETE at a ratio of 1:5, compared to the human 15-LO-1 which produces the hydroxy acids at a ratio of 9:1 [15]. The 12-HETE and 15-HETE formation were not connected, the 15-HETE formation was rather constant while high levels of 12-HETE were detected on day 1. This could be due to platelet contamination with high 12-LO activity of the PMNL fraction. Studies of animal 12/15-LO can probably to some extent be used to increase our knowledge of the human 15-LO-1 function.

8.2 MAST CELLS (PAPER I & II)

Cord blood derived mast cells expressed 15-LO-1, but not 15-LO-2, only after IL-4 stimulation, indicating the importance of this interleukin for 15-LO-1 expression in mast cells. The stimulatory effect of IL-4 on the 15-LO-1 has also been shown in monocytes, epithelial and dendritic cells and alveolar macrophages [89-91]. The 15-LO-1 containing mast cells transformed arachidonic acid into 15-KETE and 15-HETE. 15-KETE and 15-HETE eluted closely but still separated on the reverse phase C18

column used and they had different UV absorbance maximum at 280 and 236 nm, respectively. Both retention time and UV absorbance maximum was identical as for synthesized standards. The peaks were collected and infused by nanospray ionization and analyzed in negative ion mode. The MS scan of 15-KETE contained *m/z* 317 which is in agreement of the molecular weight of 15-KETE (318 g/mol). The MS/MS scan gave rise to several fragments e.g. *m/z* 113, 139, 219 and 273, as was also the case for the synthetic standard. The *m/z* 113 and 219 fragments were not detected in the MS/MS spectrum of 5-KETE, clearly separating 15-KETE and 5-KETE. The *m/z* 113 and 219 fragment was suggested to be a result of a cleavage of the C6-C7 and C14-C15 bond, respectively, with the charge retention on the carboxyl acid part. Experiments performed with $^{18}O_2$ or D_8 labeled 15-HPETE confirm the *m/z* 219 being formed after a rearrangement of the double bonds forming a conjugated triene ion. However, the labeled experiments indicate that the *m/z* 113 is a cleavage of the C13-C14 with the charge retention on the omega end [92].

The main metabolite in intact cells was 15-KETE but 15-HETE was the main metabolite produced in in homogenized cells. This indicates the requirement of an intact cell structure for the 15-KETE formation. A $NADP^+$ dependent dehydrogenase converts the 5-LO corresponding metabolite 5(S)-HETE to 5-KETE in neutrophils and monocytes [93]. 5-KETE is a potent chemoattractant for neutrophils, however, no recruitment effect on neutrophils is found for 15-KETE [94]. Recombinant thromboxane synthase and prostacyclin synthase can form a mixture of 15-KETE and 15-HETE from 15-HPETE, a mechanism suggested to occur via hemolytic and heterolytic cleavage of 15-HPETE [95]. In rabbit lung prostaglandin dehydrogenase has been suggested to convert 15-HETE to 15-KETE [96], however no expression of this enzyme has been reported in mast cells. If a contribution of another enzyme besides 15-LO-1 is required for the formation of 15-KETE was not established. In addition to 15-KETE formation mast cells also formed EXC_4 through the 15-LO-1 pathway.

In nasal lavage fluid from patients with allergic rhinitis mannitol inhalation increase the 15-HETE production compared to placebo inhalation. The 15-HETE increase is related to a decreased nasal peak inspiratory flow in patients with active rhinitis [37]. The mannitol treatment of mast cells significantly increased the 15-HETE release. The release was measured with enzyme immunoassay, although the cross reactivity of the antibody to 15-KETE is not known and 15-KETE could therefore contribute to the released value. The value of 15-HETE however is in the range of other reported values; less than prostaglandin D_2 but more than cysteinyl-LTs [97]. The expression of active 15-LO-1 mast cells *in vitro* was clearly indicated and to investigate if mast cells express 15-LO-1 *in vivo* immunohistochemistry was performed. Mast cells in human bronchial tissue and in skin from a patient with atopic dermatitis also expressed 15-LO-1.

8.3 AIRWAY EPITHELIAL CELLS (PAPER VI)

Human airway epithelial cells contain abundant amounts of 15-LO-1. The airway epithelium is directly involved in the pathology of asthma, as an essential controller of inflammatory, immune and regenerative responses to allergens, viruses and environmental pollutants [98]. Therefore it is of great interest to investigate the airway epithelial cells. The cultivated human airway epithelial MucilAir cells expressed 15-

LO-1 and the expression was further induced by IL-4. The 15-LO-1 was active and transformed arachidonic acid into 15-HETE and EXC_4. In order to resemble physiological conditions in asthmatic patients, the cells were treated with various stimuli that trigger an asthma attack and the effect on 15-LO-1 activity was evaluated. *P. aeruginosa* is a medically significant Gram-negative bacterial pathogen distinguished for its antibiotic resistance. Incubation of epithelial cells with *P. aeruginosa* stimulated markedly the formation of 15-HETE. Characteristic for asthmatics are the disrupted and injured bronchial epithelial. To mimic epithelial injury the airway epithelial cells were scratched with a pipette, which led to increased formation of 15-HETE. Cigarette smoke is one component that could cause injured epithelium, however the cells treated with condensed smoke extract did not produce more 15-HETE.

The glutathione conjugation of EXA_4, leading to the formation of EXC_4, was in human airway epithelial cells catalyzed by a soluble GST. Microarray analysis and incubation experiments with 12 soluble GST both indicated the two isoforms of GST P1-1 (Val and Ile) were responsible for this conjugation. The high expression of GST P1-1 mRNA and enzyme in human bronchi is in alignment with earlier reports [99]. Immunohistochemistry on bronchial biopsies from asthmatic and healthy subjects indicated co-localization of 15-LO-1 and GST P1-1. The expression of 15-LO-1 was higher in biopsies from asthmatic patients than in healthy volunteers, whereas the expression of GST P1-1 was high in both groups. The co-localization in the epithelial cells lining the bronchi suggests a role for GST P1-1 in the formation of the EXC_4 in the human airway epithelial cells. In conclusion, enzymes required for the EXC_4 formation is present in human airway epithelial cells and the 15-LO-1 pathway is activated after challenge with stimuli that cause an asthma attack.

8.4 HODGKIN LYMPHOMA CELLS (PAPER III & IV)

The HL derived L1236 cell line had high 15-LO-1 activity and catalyzed the conversion of arachidonic acid into EXA_4 which was further converted to the cysteinyl-EXs. The sequential cleavage of EXC_4 forming EXD_4 and EXE_4 was more rapid than in human eosinophils. This is probably due to a higher content of the catalyzing enzymes, ɣ-glutamyl transpeptidase and dipeptidase, respectively, in the L1236 cells. The EXC_4, EXD_4 and EXE_4 were identified by LC-MS/MS and in addition the 8-trans–EXC_4 and 8-trans–EXD_4 were detected. The double bond isomerization probably occurred non-enzymatically in the L1236 cells.

15-LO-1 was present in the cytosolic fraction in resting L1236 cells cultivated in a calcium free medium. When calcium is present, with or without ionophore, the main 15-LO-1 protein was detected in the membrane fraction. However, when the different subcellular fractions were incubated with arachidonic acid, the majority of 15-HETE and 12-HETE was formed in the cytosolic fraction. This was unexpected since membrane bound 15-LO-1 has earlier been found to possess higher activity than cytosolic 15-LO-1 protein [100]. A possible explanation could be that exogenous added arachidonic acid is easier accessible for the cytosolic 15-LO-1 enzyme. Another possibility is that the membrane 15-LO-1 enzyme might be inactivated by 15-HPETE since the metabolite is not as rapidly reduced in the membrane fraction as in the

cytosolic fraction. The translocation of 15-LO-1 to the membrane upon calcium challenge is in accordance with reported findings in eosinophils [100].

Immunohistochemistry showed that 15-LO-1 was expressed in 85% of the HL lymph node biopsies analyzed, but not in any of the NHL biopsies. The 15-LO-1 could therefore serve as a diagnostic tool to distinguish between HL and NHL.

L1236 were the only of the investigated HL cell lines that expressed 15-LO-1. However the L1236 cell line is the only cell line which is clonally related to the original tumor [101]. Several of the HL cell lines, such as L1236, have autocrine stimulation of IL-3, and therefore have constitutively expression of the transcription factor STAT-6. The STAT-6 is required for transcription of 15-LO-1 [24]. Although other HL cell lines expressed STAT-6 but not 15-LO-1 might be due to epigenetic control of the 15-LO-1 promotor.

The formation of arachidonic acid metabolites derived through the 15-LO-1 pathway in H-RS cells might contribute to the inflammatory features of HL. Eoxins increase vascular permeability and 5-oxo-15-HETE, putatively formed, is chemotactic for eosinophils, which are infiltrated in many HL tumors. 15-HETE has been reported to be both pro-inflammatory and anti-inflammatory in various studies [15,16]. One can also speculate that 15-LO-1 could be involved in formation of the H-RS giant cells by modulating the intercellular membrane. The 15-LO-1 is described to be involved in the degradation of mitochondrial membranes in the erythropoiesis [15] and membranes during the differentiation of keratinocytes and the eye lens [33].

L1236 cells was incubated with ^{14}C-labeled arachidonic acid and two hitherto unknown 15-LO-1 derived metabolites were identified as 14,15-HxA_3–C 11(S) and 14,15-HxA_3–D 11(S). The MS/MS spectra of both metabolites demonstrated several water losses, suggesting a loss of the additional hydroxyl group at carbon 11, hence forming a more stable configuration. The initial water loss enables the subsequent formation of the *m/z* 205 fragment ion. The mechanism of formation is probably the same as for the eoxins, by cleavage of the C13-C14 of the lipid backbone which charge retention on the lipid moiety. The 14,15-HxA_3–C 11(S) is formed by the L1236 cells at the same magnitude as the EXC_4. However, the other tested 15-LO-1 expressing cells did not produce 14,15-HxA_3–C 11(S) and 14,15-HxA_3–D 11(S) and the relevance of these metabolites need to be further assessed. The corresponding 12-LO formed HxA_3-C is reported to induce vascular contraction of guinea pig isolated trachea and increase vascular permeability in rat skin [102,103].

The 14,15-hepoxilins are described as hydroperoxide isomerase products in garlic root [78] and in rabbit aorta the 14,15-HxA_3 is formed by a CYP2J2 functioning as a 15-HPETE isomerase [104]. The 14,15-hepoxilins are also described as non-enzymatic degradation products from 15-HPETE [105]. However, the formation of 14,15-HxA_3 11(S) and 14,15-HxB_3 13(R) in L1236 cells, eosinophils, dendritic cells and nasal polyps was stereoselective, which indicated an enzymatic catalyzed formation. This is consistent with the results from human epithelial cells where the 14,15-HxB_3 with the hydroxyl group in R configuration is most prominent [77]. The stereospecific 14,15-hepxilins were also formed when arachidonic acid was incubated solely with

recombinant 15-LO-1, indicating an intrinsic 14,15-Hx activity, without the presence of an isomerase. Purified lipoxygenase from rabbit reticulocytes have also an intrinsic capacity to convert arachidonic acid into 15-HPETE and 14,15-HxB_3 [29]. The 12-LO Fe^{3+} is described to catalyze the formation of the arachidonic acid peroxide radical which rearranges to HxB_3 [106]. The 14,15-Hxs was proposed to be catalyzed by the 15-LO-1 Fe^{2+} where the 15-HPETE hydroperoxide is homolytical cleaved and an oxygen radical is formed and spontaneous cyclization forms an epoxyallylic radical having electron density principally at carbon 11 and 13. Oxygen rebound at these carbons results in the formation of 14,15-HxA_3 and 14,15-HxB_3, respectively.

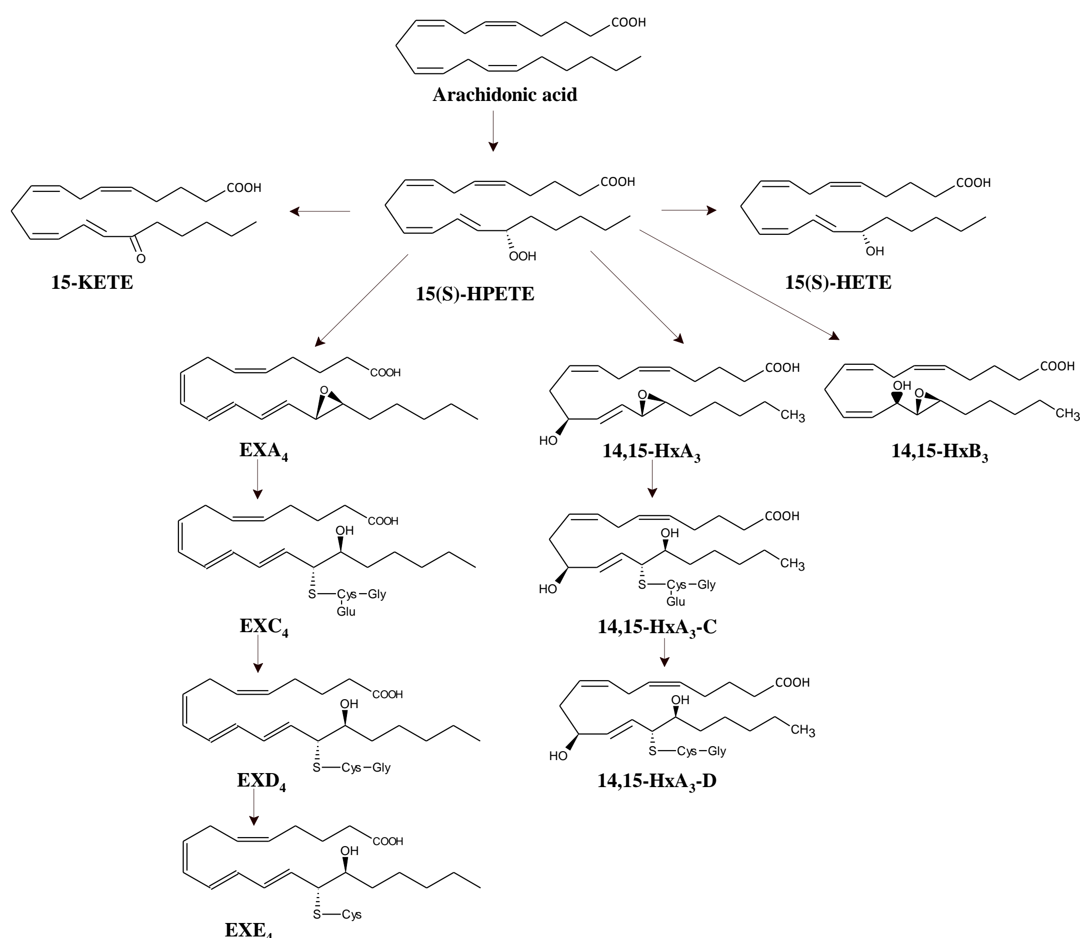

Figure 6. *An overview of arachidonic acid metabolites formed through the 15-LO-1 pathway described in this thesis. The 15-KETE was identified in cord blood derived mast cells. The cysteinyl-eoxins were identified to be formed by eosinophils, mast cells, airway epithelial cells, nasal polyps and the Hodgkin L1236 cell line.* EXC_4 *was also identified as a product formed by the animal ortholog to 15-LO-1, the 12/15-LO. The formation of the cysteinyl-14,15-hepoxilins were only established in the L1236 cell line, however the 14,15-*HxA_3 *11(S) and the 14,15-*HxB_3 *13(R) were stereoselectively formed by L1236 cells as well as by eosinophils, dendritic cells and nasal polyps.*

9 ACKNOWLEDGEMENTS

I would like to express my greatest gratitude to my main supervisor Hans-Erik Claesson, without you I would never had completed my PhD. It has been a long journey in many constellations and with a lot of fun on the way.

I would also like to thank my co-supervisor Bill Griffiths for your support. I hope to see you soon at ASMS.

I would like to thank Suzanne Iverson and Anna Sandholm for encouraging me to finalize my PhD.

Great thanks to all co-authors of the papers included in my thesis; Stina Feltenmark, Hélène Leksell, Linda Backman, Malin Johannesson, Erik Andersson, Ylva Tryselius, Mats Hamberg, Gunnar Nilsson, Ilkka Harvima, Magdalena Gulliksson, Barbro Dahlén, Maria Kumlin, Anna James, Narinder Gautam, Charlotte Edenius, Lennart Lindbom, Magnus Björkholm, Frida Schain, Jan Sjöberg, Anna Porwit, Bengt Mannervik and Christian Giske. I would also like to thank Meta Andersson for your support.

I would like thank all colleagues and friends that I have interacted with during the years.

Most of all, thanks to Fredrik, Siri and Eira for supporting me while I was "writing a book".

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