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STUDIES ON THE ACTIVATION OF CYTOSOLIC PHOSPHOLIPASE A_2 AND 15-LIPOXYGENASE-1 IN HEMATOPOIETIC CELLS

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ABSTRACT

Arachidonic acid is a polyunsaturated fatty acid that has four double bonds and belongs to the omega-6 family of fatty acids. Although it can be formed in humans from one essential fatty acid (linoleic acid), most of the arachidonic acid in the body comes with the intake of food. In mammalian cells, arachidonic acid is found esterified in cellular membranes. Phospholipases release arachidonic acid upon cellular stimulation. There are several different enzyme families of mammalian lipases, but only cytosolic phospholipase A_2 (cPLA$_2$-α) has been shown to preferentially release arachidonic acid. Depending on the cell and stimuli, arachidonic acid can be further metabolized into biologically active fatty acids, including prostaglandins, thromboxan A_2, leukotrienes and eoxins.

The first part of this thesis investigates the arachidonic acid release induced by cPLA$_2$-α in human platelets after stimulation with polychlorinated biphenyls (PCBs). The release of arachidonic acid by PCBs was shown to be cPLA$_2$-α dependent, since it was completely blocked by the cPLA$_2$-α inhibitors AACOCF3 or pyrrolidine-1. Two anti-estrogens, nafoxidin and tamoxifen - but not 17β-estradiol - inhibited PCB-induced arachidonic acid release. Platelets incubated with PCBs did not aggregate, even though a robust release of arachidonic acid was observed.

A unique feature of 15-LO-1 is that it translocates to internal cellular membranes and oxygenates free fatty acids, as well as fatty acids esterified into lipids. 15-LO-1 is expressed in lung epithelial cells, eosinophils, reticulocytes, mast cells and interleukin-4 (IL-4) stimulated monocytes and dendritic cells. In the second part, the 15-lipoxygenase type 1 (15-LO-1) was investigated in different hematopoietic cells. The enzyme translocated to the plasma membrane in IL-4 stimulated human dendritic cells upon calcium stimulation.

In addition, 15-LO-1 bound to certain phospholipids, particularly phosphatidylinositols in a lipid-overlay assay. A vesicle assay model was set up, and kinetic assays were performed. The V_{max} was shown to be unchanged, but the apparent K_m of 15-LO-1 towards arachidonic acid was significantly lower in the presence of PI(4.5)P_2 or PI(3.4)P_2 in the vesicles.

The expression of 15-LO-1 was investigated in biopsies from Hodgkin lymphoma (HL) tumors. 15-LO-1 was found in Hodgkin Reed-Sternberg cells, which constitutes only 1-2% of all tumor cells and is believed to orchestrate the infiltration of eosinophils, mast cells, neutrophils and T-cells into the HL tumor. The HL cell line L1236 also expressed 15-LO-1 constitutively and produced the inflammatory eoxins. In addition, the IL-13 induction of 15-LO-1 was investigated in the non-Hodgkin-lymphoma cell line Karpas-1106P, which is originating from a primary mediastinal B-cell lymphoma (PMBCL). Upon IL-13 stimulation, this cell line acquired several pro-inflammatory features that made the PMBCL cells more like the HL cell line L1236, demonstrating the biological similarities between the two diseases.

In summary, the results presented in this thesis demonstrate that 15-LO-1 and cPLA$_2$-α have important functions in the arachidonic acid cascade. The discovery of 15-LO-1 in the HL disease and the human cell lines supports the pro-inflammatory role of the enzyme and establishes a cellular model system that can help to further elucidate the biological role of human 15-LO-1.

LIST OF PAPERS

I. Pontus K.A. Forsell, Anders O. Olsson, **Erik Andersson**, Laxman Nallan, Michael H. Gelb. Polychlorinated biphenyls induce arachidonic acid release in human platelets in a tamoxifen sensitive manner via activation of group IVA cytosolic phospholipase A_2-α. *Biochemical pharmacology 71 (2005) 144–155*

II. **Erik Andersson**, Frida Schain, Märta Svedling, Hans-Erik Claesson, Pontus K.A. Forsell. Interaction of human 15-lipoxygenase-1 with phosphatidylinositol bisphosphates results in increased enzyme activity. *Biochimica et Biophysica Acta 1761 (2006) 1498-1505*

III. Hans-Erik Claesson, William J. Griffiths, Åsa Brunnström, Frida Schain, **Erik Andersson**, Stina Feltenmark, Hélène A. Johnson, Anna Porwit, Jan Sjöberg and Magnus Björkholm. Hodgkin Reed–Sternberg cells express 15-lipoxygenase-1 and are putative producers of eoxins in vivo. *FEBS Journal 275 (2008) 4222–4234*

IV. **Erik Andersson**, Frida Schain, Jan Sjöberg, Magnus Björkholm, Hans-Erik Claesson. A mediastinal B-cell lymphoma cell line shares several phenotypic features with Hodgkin lymphoma after treatment with interleukin-13: similar morphology, metabolism of arachidonic acid and release of cytokines *(manuscript)*

CONTENTS

LIST OF ABBREVIATIONS

12-HETE	12-hydroxy-(5,8,10,14)-eicosatetraenoic acid
12-HHT	12-hydroxy-(5,8,10)-heptadecatrienoic acid
13-HODE	13-hydroxyoctadeca-9,11-dienoic acid
13-HPODE	13-hydroperoxyoctadeca-9,11-dienoic acid
15-HETE	15-hydroxy-(5,8,11,13)-eicosatetraenoic acid
15-LO-1	Human 15-lipoxygenase type 1
AD	Alzheimer's disease
ALX-R	Lipoxin A_4 receptor
C1P	Ceramide-1-phosphate
Ca^{2+}	Calcium ions
COX	Cyclooxygenase
$cPLA_2$-α	Cytosolic phospholipase A_2 alpha
CysLT1	Cysteinyl-leukotriene receptor type 1
DLBCL	Diffuse large B-cell lymphoma
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
ER	Endoplasmic reticulum
EX	Eoxin
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HL	Hodgkin lymphoma
H-RS	Hodgkin Reed-Sternberg
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-13Rα1	Interleukin-13 receptor alpha 1
IL-4Rα	Interleukin-4 receptor alpha
IP-10	Small inducible cytokine B10
JAK	Janus-kinase
LDL	Low density lipoprotein
LO	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
M-CSF	Macrophage colony-stimulating factor
MDC	Macrophage derived chemokine
MEC	Mucosae-associated epithelial chemokine
Mg^{2+}	Magnesium ions
MIP	Macrophage inflammatory protein
NDGA	Nordihydroguaiaretic acid
NHL	Non-Hodgkin lymphoma
NSAID	Non-steroidal anti-inflammatory drug
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PG	Prostaglandin
PI	Phosphatidylinositol

PLA_2	Phospholipase A_2
PMBCL	Primary mediastinal B-cell lymphoma
PPAR	Peroxisome proliferator-activated receptors
PtdCho or PC	Phosphatidylcholine
PtdEtn or PE	Phosphatidylethanolamine
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
RP-HPLC	Reversed phase – high pressure liquid chromatography
Sn-1/Sn-2	Stereospecific numbering-1/-2
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TARC	Thymus activation-regulated cytokine
TGF	Tumor growth factor
TNF	Tumor necrosis factor
TXA_2	Thromboxane A_2
WHO	World health organization

1 INTRODUCTION

This thesis summarizes four studies about the activation of two enzymes, cytosolic phospholipase A2-α (cPLA$_2$-α) and 15-lipoxygensase type 1 (15-LO-1), in human hematopoietic cells. Although the enzymes catalyze different reactions and therefore have different biological functions, they share several features. Both are involved in the arachidonic acid cascade, which the scientific community has studied for several decades.

1.1 ARACHIDONIC ACID

Arachidonic acid, a twenty carbon long fatty acid with four double bonds (20:4), belongs to the group of omega-6 fatty acids (Figure 1). The concentration of free arachidonic acid in cells is limited; it is almost completely esterified into the sn-2 position of phospholipids. The amount of arachidonic acid varies among different cells and membrane compartments of the cell. Arachidonic acid is the major fatty acid in position sn-2 of phosphatidylserine (73%) and phosphatidylinositols (76%) when analyzing total cellular amounts of phospholipids in human platelets[1]. However, phosphatidylinositols represent only 6% of total phospholipids.

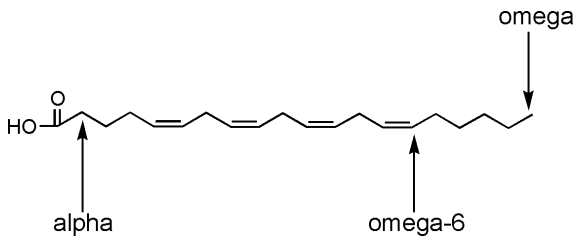

Figure 1. Arachidonic acid is an omega-6 polyunsaturated fatty acid.

1.2 PHOSPHOLIPASE A$_2$

The phospholipase A$_2$ (PLA$_2$) is a family of enzymes that are defined by the catalytic hydrolysis of the sn-2 ester bond of phospholipid substrates[2,3]. The products of the PLA$_2$ reaction are one free fatty acid and one lysophospholipid, both of which can act as messenger molecules (Figure 2).

Three main types of PLA$_2$s can be distinguished when classifying the family of PLA$_2$ enzymes according to their biological properties: the secretory PLA$_2$ (sPLA$_2$), the intracellular calcium-dependent PLA$_2$ (cPLA$_2$) and the intracellular calcium-independent PLA$_2$ (iPLA$_2$)[3].

Although all PLA$_2$s can release arachidonic acid, cPLA$_2$-α has been shown to be of particular importance in the formation of arachidonic acid metabolites. The cPLA$_2$-α preferentially releases arachidonic acid. Mouse knock-out studies confirmed its role in the biosynthesis of prostaglandins and leukotrienes, as well as in inflammatory diseases[4]. The cPLA$_2$-α gene is highly conserved in different species, and the human and mouse homologues share 95% amino acid identity[5]. The cPLA$_2$-α is expressed ubiquitously in human tissues[6].

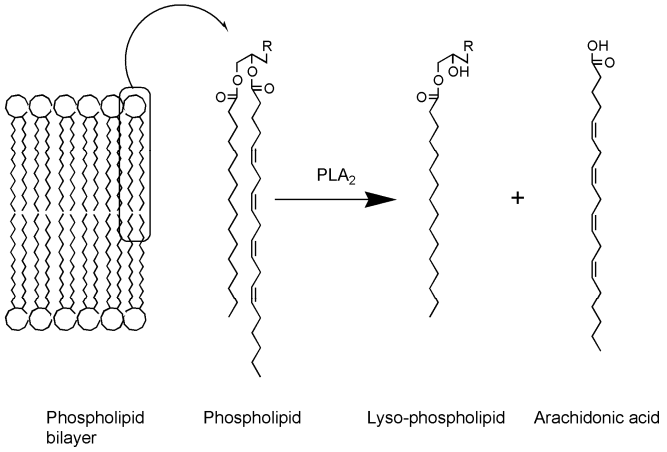

Figure 2. In the phospholipid bilayer, PLA_2 cleaves the ester bond of the fatty acid in sn-2 position generating a lyso-phospholipid and free arachidonic acid. The R is commonly a choline, ethanolamine, serine or inositol.

In mammalian cells, approximately 20 genes have been identified that encode PLA_2 enzymes, and new genes are being added to the list[6]. Thus, other PLA_2s besides cPLA$_2$-α might be important in inflammatory diseases. For example, the V sPLA$_2$ group has been reported to be involved in releasing arachidonic acid in human eosinophils, which produces leukotrienes[7].

1.3 LIPOXYGENASES

Lipoxygenases (LO) are enzymes that catalyze the incorporation of molecular oxygen into arachidonic acid, as well as into other polyunsaturated fatty acids[8]. Six functional genes that code for LOs have been found in humans. In accordance with tradition, the enzymes have been named according to the carbon in arachidonic acid to which the enzyme incorporates the oxygen: 5-LO, platelet type 12-LO, 12(R)-LO, 15-LO-1 and 15-LO-2[8]. The sixth LO is an epidermis-type 3-LO that acts in sequence with the 12(R)-LO to generate hydroxyepoxyeicosatrienoic acids[9].

15-LO-1 is expressed in epithelial cells of the upper airways, eosinophils, reticulocytes, dendritic cells, mast cells and macrophages [10,11]. 15-LO-2, which has low homology to 15-LO-1, is expressed in hair roots, prostate, lung and cornea. The enzymatic activity also differs between the enzymes. 15-LO-1 oxygenates free arachidonic acid to 15(S)-HETE and 12(S)-HETE in the ratio of 9:1 while 15-LO-2 produces 15(S)-HETE only. In addition, 15-LO-1 but not 15-LO-2 is able to use esterified arachidonic acid as substrate[12].

1.4 LEUKOTRIENES, PROSTAGLANDINS AND EOXINS

Leukotrienes, prostaglandins and eoxins are potent lipid mediators derived from arachidonic acid (Figure 3). Non-steroidal anti-inflammatory drugs, such as acetyl salicylic acid and diclofenac, inhibit prostaglandin synthesis and are used to treat fever and pain. The cysteinyl-leukotriene receptor antagonists, such as Singulair®, are used to treat asthma. The eoxins were discovered recently, and no drugs are currently available that inhibit their formation.

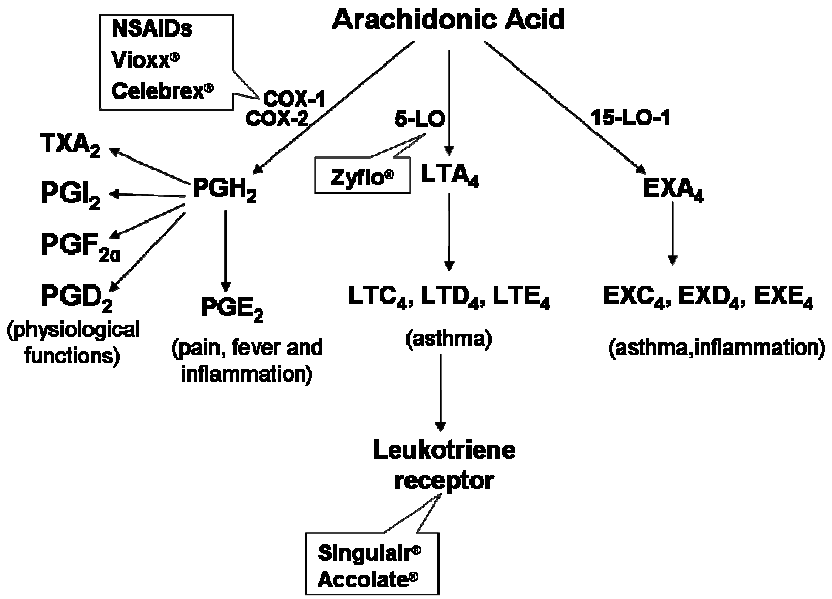

Figure 3. Arachidonic acid can be metabolized by different enzymes, leading to signaling molecules in different pathways. Drugs which block the formation or action of arachidonic acid metabolites are available to treat pain, fever and asthma (as indicated by the boxes).

In the pathway leading to leukotriene synthesis, 5-LO oxygenates arachidonic acid to 5-HPETE, which is then transformed to the epoxide LTA_4. Thus, 5-LO has both an oxygenating activity and an epoxide synthase activity. The LTA_4 can be further metabolized by either LTA_4-hydrolase, which generates LTB_4, or to LTC_4-synthase, which generates cysteinyl-leukotrienes[13].

The prostaglandin synthesis is initiated by the conversion of arachidonic acid into PGH_2, which is catalyzed by either COX-1 or COX-2[14]. Terminal prostaglandin synthases are responsible for the further metabolism of PGH_2 into PGE_2, PGD_2, PGI_2 and PGF_2-α. Another enzyme, the thromboxane A_2 synthase (TXA_2 synthase), also uses the PGH_2 as substrate and generates TXA_2 and 12-HHT (plus malondialdehyde) in an equimolar ratio in platelets[15].

2 CYTOSOLIC PHOSPHOLIPASE A_2 ALPHA

2.1 BIOLOGICAL FUNCTIONS

The $cPLA_2$-α has been extensively studied *in vitro*. Although the enzyme was believed to be important in inflammation, the biological functions of $cPLA_2$-α were revealed when the gene was knocked out in mice[4]. The knock-out female mice became pregnant less frequently, and the pups had an increased mortality rate. The mice had lesions in the small intestine, and they had problems concentrating urine when water-deprived. Otherwise the knock-out mice seemed to develop normally. Decreased capacity to produce prostaglandins and leukotrienes was found in cells, such as mast cells and peritoneal macrophages, which were isolated from the knock-out mice, compared to wild-type mice. Upon stimulation the effect on prostaglandin and leukotriene synthesis was immediate in cells from $cPLA_2$-α knock-outs, compared to wild type mice. However, from $cPLA_2$-α knock-out mice, it was possible to quantify prostaglandins and leukotrienes after stimulation of the cells for 12 hours. This could be interpreted as that $cPLA_2$-α is involved in the acute release of arachidonic acid metabolites[4]. The knock-out mice were less responsive in different disease models, such as anaphylaxis, acute lung injury, brain ischemia and polyposis[4].

Inherited mutations of the $cPLA_2$-α gene was discovered in one human subject[16]. This resulted in platelet dysfunction and impaired synthesis of TXB_2 and 12-HETE. The $cPLA_2$-α deficiency was also associated with decreased eicosanoid synthesis in leukocytes and small intestinal ulceration. Thus, it is probable that $cPLA_2$-α has a similar biological role in mice and humans.

2.2 REGULATION OF EXPRESSION

The gene coding for $cPLA_2$-α is found on chromosome 1, location 1q25. The $cPLA_2$-α is constitutively expressed in most cells and tissues but has also been reported to be induced upon inflammation[17]. Several stimuli, including IL-1α, IL-1ß, LPS, EGF and M-CSF are involved in the expression of $cPLA_2$-α[17]. In addition, the expression of $cPLA_2$-α can be attenuated by glucocorticoids, which also block eicosanoid production[6].

2.3 CRYSTAL STRUCTURE AND ENZYMATIC REACTION

The $cPLA_2$-α crystal structure has been determined[18]. The enzyme is an 85-kDa protein and has two structural domains, one N-terminal and one C-terminal. The domains are connected by a linker peptide that promotes rotational freedom of the domains relative each other.

The N-terminal domain (138 amino acids) is a C2-domain, found in several membrane active enzymes. The C2-domain of $cPLA_2$-α binds two calcium ions[18]. The C-terminal domain (611 amino acids) contains the catalytic site. The catalytic domain of $cPLA_2$-α differs from the α/β hydrolase fold found in multiple lipases. Only one central β-sheet in the vicinity of the catalytic site is in common with the α/β hydrolase fold. The active site funnel is lined with hydrophobic residues and penetrates one-third of the catalytic domain. The bottom of the cleft contains the residues, serine-228 and aspartate-549, that are essential for catalysis. A substrate mediated lid-opening exposed the active site to water which revealed conformational changes of the catalytic domain[19]. It was concluded that large regions of the catalytic domain do not penetrate the membrane. Furthermore, a single phospholipid diffused into the active site upon lid-opening, whereby free arachidonic acid was liberated.

The catalytic mechanism of $cPLA_2$-α is independent of calcium and appears to function as a serine hydrolase[18]. In the proposed mechanism, arginine-200 stabilizes the phosphate group and the aspartate-549 activates the serine-228, which attacks the sn-2 ester bond. When incubating $cPLA_2$-α with vesicles that contain certain phospholipids, as well as natural membranes, the enzyme released arachidonic acid over 20 times more efficiently than shorter polyunsaturated fatty acids[17]. The enzyme's preference for arachidonic acid is believed to stem from the formation of the active site funnel[6].

2.4 MEMBRANE INTERACTION

The $cPLA_2$-α preferentially cleaves phospholipids in a lipid membrane rather than as dissolved phospholipid monomers[20]. Upon calcium stimulation, $cPLA_2$-α translocates primarily to the nuclear membranes, ER and Golgi, but the enzyme has been shown to translocate to the plasma membrane as well[21-23]. A recent study showed that only the C2-domain bound two calcium ions, which induced conformational changes of this domain[24].

Both the C2-domain and the catalytic domain of $cPLA_2$-α interact with phospholipids. It has been suggested that the C2-domain is involved in membrane targeting and that the catalytic domain prolongs the enzyme's residence at the membrane[22]. When $cPLA_2$-α was truncated, the C2-domain was shown to preferentially bind to phosphatidylcholine vesicles[25]. The C2-domain did not discriminate between saturated and non-saturated fatty acids in position sn-1 or sn-2, despite the fact that $cPLA_2$-α preferentially releases arachidonic acid[25].

The catalytic domain has a $PI(4.5)P_2$ binding site that increases the enzymatic activity independent of calcium[26,27]. The C2-domain of $cPLA_2$-α was also shown to bind to another lipid, the ceramide-1-phosphate (C1P) in a calcium-dependent manner[28]. Surprisingly, no synergistic effect in enzyme activity was observed when the two lipids were combined in a vesicle based assay. Instead, the high affinity of $cPLA_2$-α to C1P increased the residence time on the membrane and competed with binding to $PI(4.5)P_2$, which raised the catalytic efficiency of the enzyme by increasing membrane penetration[29].

2.5 POST-TRANSLATIONAL REGULATION

The catalytic domain of $cPLA_2$-α has three functionally important phosphorylation sites: serine-505, serine-727 and serine-515[30-32]. Phosphorylation of serine-505 *in vitro* increases the hydrolyzation of sn-2-arachidonyl-phosphatidylcholine and the enzyme's affinity for phosphatidylcholine vesicles[33,34]. However, another group reported that the *in vitro* phosphorylation of serine-515, but not serine-505, increased the enzymatic activity[31]. Mitogen-activated protein kinases have been shown to be responsible for the phosphorylation of $cPLA_2$-α.

2.6 $CPLA_2$-ALPHA IN DISEASE

cPLA2-α has been shown to be involved in the pathogenesis of a variety of diseases, such as allergic reactions, acute lung injury, pulmonary fibrosis, brain injury and arthritis, as well as cancers[6]. Inflammation is a common component in all these disorders. The enzyme has been proposed to be a drug target, and inhibitors have reduced inflammation in animal models[35]. However, since $cPLA_2$-α is expressed constitutively in many cells and the enzyme is active in the first steps of arachidonic acid signaling cascades, there is a risk that a $cPLA_2$-α inhibitor would cause severe adverse effects in humans.

3 15 LIPOXYGENASE TYPE 1

3.1 15-LO-1 AND INFLAMMATION

The human 15-LO-1 is expressed constitutively in a few cell types, such as reticulocytes, eosinophils and airway epithelial cells[36]. However, its expression can be induced in monocytes, dendritic cells and mast cells after stimulation with IL-4 or IL-13[37-39]. Thus, 15-LO-1 is expressed in tissues and cells involved in inflammation. But since the enzyme can produce both inflammatory and anti-inflammatory mediators, a debate is ongoing about whether the biological role of 15-LO-1 in humans is pro-inflammatory or anti-inflammatory.

The 15-LO-1 pathway can be viewed as another branch in the arachidonic acid cascade, together with the pathways of the enzymes COX-1, COX-2 and 5-LO, which produce pro-inflammatory mediators. The 15-LO-1 product 15(S)-HETE has been shown to be elevated in tissues during inflammation, but no receptor has been found and the physiological relevance of 15(S)-HETE is not clear[10,36]. It has also been reported that 15-LO-1 in eosinophils can produce 5-oxo-15-hydroxy-ETA, a mediator that is chemotactic for eosinophils, mast cells and neutrophils[40]. The eoxins were recently discovered in eosinophils and the HL cell line L1236 following arachidonic acid stimulation[39,41]. The eoxins could also be formed endogenously after LTC_4, PGD_2 or IL-5 stimulation of the eosinophils. Eoxins induced increased permeability of endothelial cells *in vitro*, indicating that they are pro-inflammatory mediators. Furthermore, eoxins have been detected in porcine eosinophils, which indicates that these metabolites can be formed in species other than humans[11].

Lipoxins were first reported to be inflammatory mediators but are now believed to be involved in the resolution of inflammation. Lipoxins are formed from arachidonic acid by oxygenation in two enzymatic steps: 5-LO followed by 15-LO or 12-LO[42]. However, other enzymes, such as acetylated COX-2, have also been shown to be involved in the production of lipoxins. A lipoxin receptor named ALX-R, also binds pro-inflammatory peptides, including N-formyl peptide, LL-37, amyloid ß(Aß) and the human prion protein[43]. Thus, it remains to be established whether this receptor is the true lipoxin receptor or a peptide signaling receptor.

3.2 STRUCTURE

The crystal structure of the rabbit reticulocyte 15-LO has been determined and it is the only crystallized mammalian LO structure that has been published (there is a crystal structure of the human 12-(S)-LO at www.pdb.org but the article has not yet been published). The rabbit reticulocyte enzyme was shown to contain two domains - an N-terminal C2-domain and a C-terminal catalytic domain - separated by a flexible link[44].

The N-terminal C2-domain involves the first 110 amino acids. The C2-domain is a structure found in other enzymes that bind to lipid bilayers, such as lipases [45]. The relative amino acid homology in the C2-domain of 15-LO-1 compared to that of lipases was 23%, which is the common degree of homology found between lipases [44].

The C-terminal catalytic domain has been found to share structural homology only with plant LOs[44]. The catalytic non-heme iron is coordinated by four histidines and the C-terminal isoleucine into an octahedral geometry. The active site is boot shaped and lined with hydrophobic amino acids. Three bulky amino acids at the bottom of the active site have been shown to prevent arachidonic acid from sliding further into the active site[46]. The size of the substrate binding pocket was also shown to determine the positional introduction of oxygen into arachidonic acid, with 15-LO favored over 12-LO activity.

When the original crystallographic data of the rabbit reticulocyte 15-LO was reinterpreted, two forms of the enzyme were revealed[47]. One form of the enzyme had bound an inhibitor into the active site, which induced a conformational change such that the opening of the enzymatic cleft was blocked. In the second form without inhibitor, the entrance to the active site was open. The conclusion was that 15-LO-1 undergoes a conformational change upon ligand binding into the active site, which also induces movement of a surface helix. Thus, the structure of 15-LO-1 allows conformational flexibility, which was confirmed in another study that also concluded that the enzyme was stabilized by the interaction with lipid bilayers[48]. Besides the structural flexibility of the catalytic domain, it has also been shown that the link between the two domains allows them to move relative to each other, which could be important when the enzyme binds to intracellular membranes[49].

3.3 ENZYMATIC ACTIVITY

The 15-LO-1 oxygenates free polyunsaturated fatty acids, as well as fatty acids esterified into lipids (Figure 4). The mechanisms of the catalytic cycle of 15-LO-1 are generally believed to be the same as for all mammalian LOs, since the amino acids around the non-heme iron in the catalytic cleft are conserved[50,51]. However, due to other properties of the active site, such as size, hydrophobicity and probably conformational changes, the mammalian LOs have different substrate specificities and product profiles.

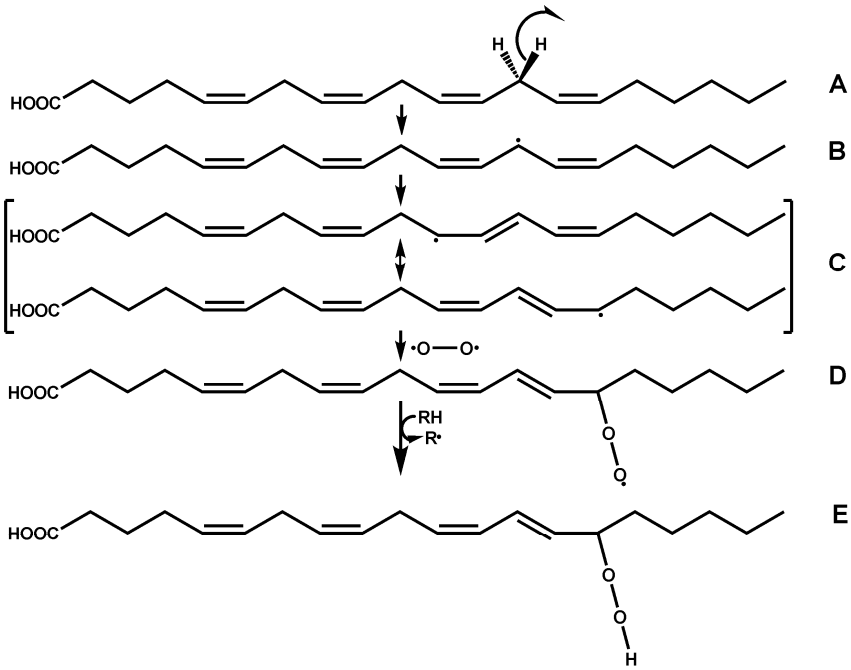

Figure 4. Oxygenation of arachidonic acid by 15-LO-1leads to the formation of 15(S)-HPETE. (A) The reaction is initiated by the hydrogen abstraction at carbon thirteen by 15-LO-1. (B) A radical is created, which is stabilized by the conjugated bonds in (C). (D) A peroxy radical is created when molecular oxygen reacts with the unpared electron at carbon fifteen. (E) The 15(S)-HPETE is formed by abstraction of one hydrogen atom.

3.3.1 The catalytic cycle of lipoxygenases

If the non-heme iron in the catalytic center of LOs is in the ferrous state (+II), it must be activated into the ferric state (+III)[51]. This LO activation can be accomplished by hydroperoxides in the cell, including LO products. Common to all LO substrates is the bis-allylic methylene structure, a methyl group between two double bonds, from which the activated iron(+III) abstracts one hydrogen (Figure 4). The iron is then reduced to iron(+II). At the same time a pentadienyl radical is created. The hydrogen abstraction is the rate-limiting step in the catalytic cycle. The radical is then rearranged two carbons away from the abstracted hydrogen, either towards the carbonyl group (-2) or towards the methyl terminus (+2) of the fatty acid. The next step is oxygen insertion at either carbon (-2) or (+2). When the fatty acid hydroperoxide is formed by taking a hydrogen, the iron is activated as it is oxidized back to its ferric state, iron(+III). Another feature of the LO catalytic reaction is that the hydrogen is removed from a methyl group that is on the opposite face of where oxygen is inserted[52]. After studying the rabbit reticulocyte crystal with molecular dynamics simulations, it was proposed that the oxygen enters the active site via a channel in the enzyme[53]. However, the oxygen channel hypothesis may need to be reconsidered after reinterpretation of the crystal due to the conformational changes[47].

3.3.2 Fatty acid substrates of 15-LO-1

15-LO-1 oxygenates the substrate arachidonic acid (20:4, omega-6), as well as other omega-6 and omega-3 fatty acids, such as linoleic acid (18:2, omega-6) and linolenic acid (18:3, omega-3). The fatty acid is believed to slide with the methyl end first into the catalytic site of 15-LO-1[44].

15-LO-1 catalyzes the conversion of arachidonic acid to 15(S)-HPETE by hydrogen abstraction at position carbon-13. A unique property of 15-LO-1 is that it can also remove one hydrogen at position carbon-10 in arachidonic acid, which produces 12(S)-HPETE. These two hydroperoxy fatty acids are then reduced to 15(S)-HETE and 12(S)-HETE, which are the major metabolites from arachidonic acid. 15(S)-HETE and 12(S)-HETE are formed in the ratio of 9:1. This ratio is governed by the bulky amino acids at the bottom of the cleft, preventing the fatty acid from sliding deeper into the enzyme.

Analogously to 5-LO, 15-LO-1 can also use 15(S)-HPETE as a substrate in the formation of the epoxide of EXA_4, probably by a hydrogen removal at position carbon-10[54] (Figure 5). It was shown recently that EXA_4 can be conjugated with glutathione by LTC_4-synthase into EXC_4, which after cleavage by γ-glutamyltransferase forms EXD_4, which in turn can be processed by a dipeptidase into EXE_4[39]. The intermediate EXA_4 was also shown to be involved in suicidal activation of 15-LO-1[55]. In addition, 15(S)-HPETE is the substrate in the 15-LO-1 catalyzed formation of 8(R,S),15(S)-DiHETEs, 5(S),15(S)-DiHETE as well as14(R,S),15(S)-DiHETE. The 8(R,S),15(S)-DiHETE and the 14(R,S),15(S)-DiHETE are also formed non-enzymatically from EXA_4.

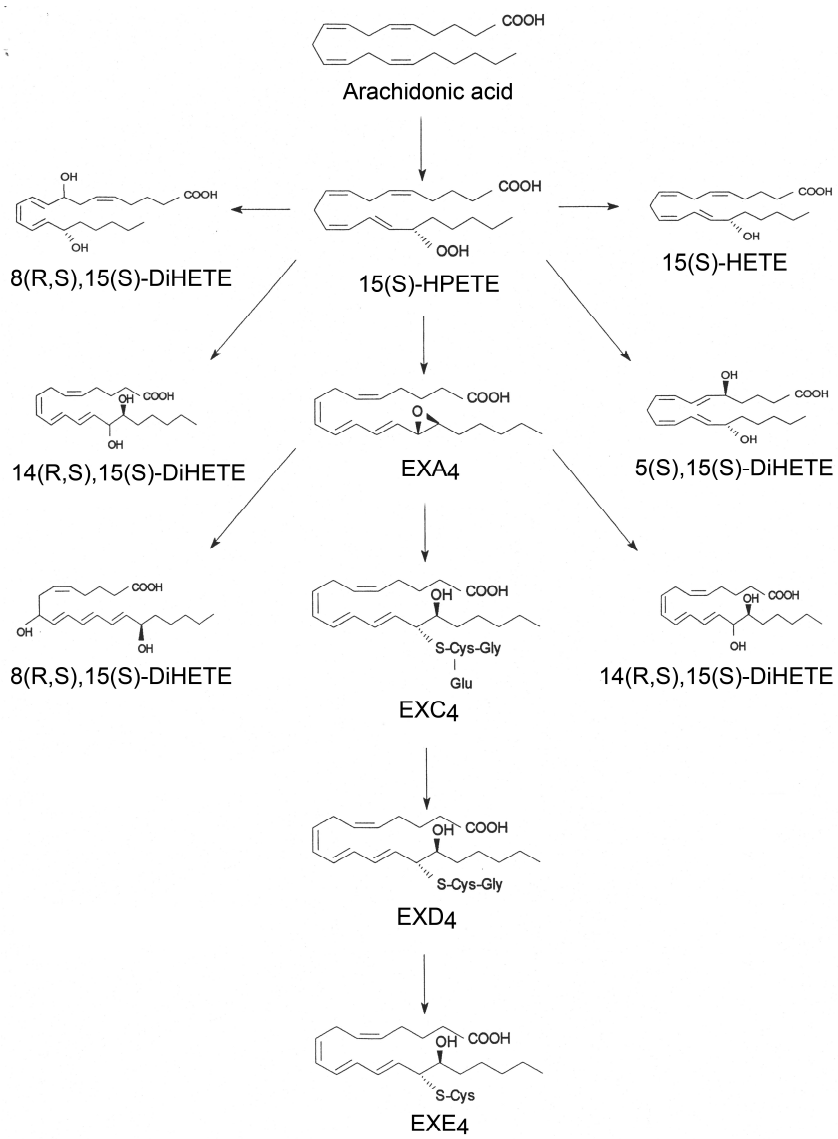

Figure 5. 15-LO-1 catalyses the conversion of arachidonic acid to 15(S)-HPETE, which can be reduced to 15(S)-HETE or be double-oxygenated to 5,15-, 8,15- or 14,15-DiHETEs. 15(S)-HPETE can be further metabolized into EXA_4 which after conjugation with glutathione forms EXC_4, which in turn can be converted further to EXD_4 and EXE_4.

3.3.3 Lipid substrates

It has been shown *in vitro* that 15-LO-1 can oxygenate different cellular membranes, including rat liver mitochondrial membranes, beef heart submitochondrial particles, rat liver endoplasmic membranes and erythrocyte plasma membranes[56]. It has also been found that the enzyme interacts with lipid particles such as LDL as measured by an increase in the oxidation of LDL in the presence of 15-LO-1[57].

The membrane binding of 15-LO-1 has been described to be a concerted action of hydrophobic amino acids on the surface of both the C2-domain and the catalytic domain[45]. This conclusion was based on the finding that the enzyme could still bind to membranes and was active after a truncation of the C2-domain.

Purified 15-LO-1 oxygenates fatty acids esterified into position sn-2 of phospholipids, lyso-phospholipids and lipoproteins[56,58,59]. The main product after incubation of mitochondrial particles with the rabbit reticulocyte 15-LO was 15(S)-HETE in phosphatidylcholine[56]. 15-LO-1 oxygenates arachidonic acid as a free fatty acid as well as when esterified into a phospholipid (Figure 6). Therefore, 15(S)-HETE can be formed by the liberation of arachidonic acid by PLA_2 followed by 15-LO-1 activity. Alternatively, 15-LO-1 can oxygenate the esterified arachidonic acid into esterified 15(S)-HETE, which is then liberated by a PLA_2.

The lipid oxygenating activity of 15-LO-1 has been shown in monocytes and bronchial epithelial cells stimulated with IL-4 and IL-13, respctively[60,61]. In both cell types, after ionophore stimulation, the main 15-LO-1 product was 15(S)-HETE, which was esterified into phosphatidylethanolamine. However, the oxygenation rates of esterified linoleic acid in phospholipids and low-density lipoproteins are 20% and 1-2%, respectively, compared to the oxygenation rate of free linoleic acid[36]. The biological significance of stereospecific oxygenation of various lipids is a unique property of 15-LO-1, which biological function remains to be determined.

3.4 CALCIUM

In unstimulated cells, 15-LO-1 is mainly found in the cytoplasm. Upon calcium stimulation the enzyme translocates to the plasma membrane[62]. The enzyme's affinity for calcium has been estimated to be relatively low, with a Kd of 0.2-0.5 mM[63]. It has been proposed that the calcium ions form salt bridges between the negatively charged head groups of phospholipids and the negatively charged amino acids at the enzyme-membrane interface[63]. It has been suggested that translocation of the enzyme is reversible although some 15-LO-1 is bound to the membrane surface before calcium stimulation[62]. The rabbit reticulocyte 15-LO has been shown to increase both the membrane oxygenating activity as well as the fatty acid activity after calcium stimulated translocation to submitochondrial particles[62].

3.5 REGULATION OF EXPRESSION

Because of its limited presence in human cells, the regulation of 15-LO-1 expression has been extensively studied on the transcriptional and translational levels. The gene of 15-LO-1 is named ALOX15 and is found in location 17p13.3 in the genome. The total length of the gene is 10.7 kb. It is processed into an mRNA with a length of 2.7 kb which is translated into the 74.8 kDa enzyme consisting of 662 amino acids.

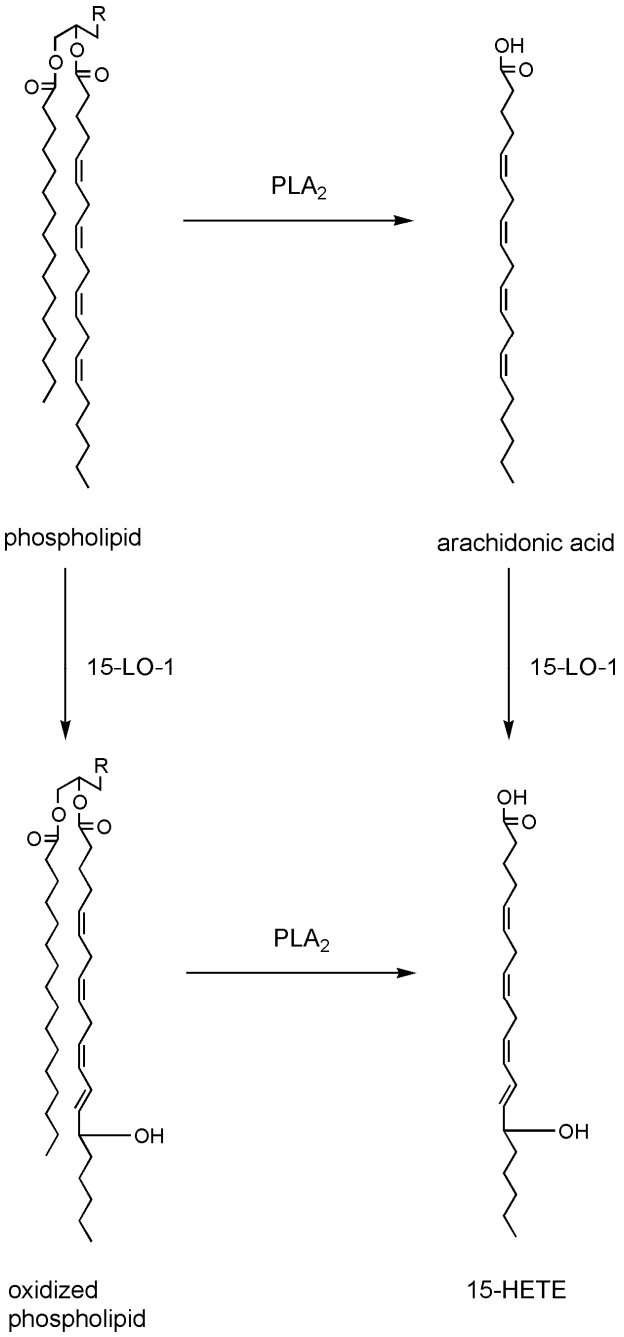

Figure 6. 15-LO-1 oxygenates polyunsaturated fatty acids either bound into a phospholipid or as a free fatty acid. The R is commonly a choline, ethanolamine, serine or inositol.

3.5.1 IL-4, IL-13 and STAT6 activation

The signal transducer and activator of transcription 6 (STAT6) is a monomeric protein found in most cell types[64]. Even though STAT6 signaling can be activated by several different ligands the classical signaling pathway is associated with the cytokines IL-4 and IL-13.

The receptor for IL-4 consists of two subunits, the IL-4Rα and the γ-chain, that dimerize upon IL-4 binding[65]. The IL-13 receptor also dimerizes upon ligand binding and consists of the IL-13Rα1 subunit and the same IL-4Rα subunit as in the IL-4 receptor[66]. Since the two cytokine receptors share the subunit that binds IL-4, this cytokine can signal by both receptors. Both the dimerized IL-4 receptor and IL-13 receptor signal by the same mechanism as described below.

Upon IL-4 or IL-13 binding, the receptor dimerizes and activates a family of tyrosine kinases called Janus-kinases (JAK)[65]. The JAKs, which are believed to be permanently associated with the cytoplasmic tails of the receptors, phosphorylate certain tyrosine residues on the tails, which then become docking sites of STAT6 monomers. When bound to the phosphorylated receptor tails, the STAT6 monomers become phosphorylated, which enables them to form dimers. The STAT6 dimers have thereby been activated. After translocation to the nucleus, the dimers regulate the transcription of certain genes, such as the 15-LO-1 and the CysLT1 receptor[67,68].

A constitutive STAT6 activation is not enough for expression of 15-LO-1 protein, given that the enzyme is not expressed in the PMBCL cell line Karpas-1106P[69,70]. In line with this observation, an epithelial cell line stimulated with IL-4 or transfected with STAT6 vector did not express 15-LO-1. However, cells that were transfected with the STAT6 vector and stimulated with IL-4 expressed the enzyme[67]. Another example is the monocyte cell lines in which 15-LO-1 expression was not induced by IL-4 stimulation even though they expressed IL-4 receptors[71]. In human orbital fibroblasts, IL-4 induced expression of 15-LO-1 mRNA, however, IL-4 was also shown to stabilize the 15-LO-1 mRNA[72].

Thus, other signaling events following IL-4 or IL-13 stimulation are necessary for expression of 15-LO-1. For example, the STAT6 is one of seven different STAT proteins, and it has been shown that STAT1 and STAT3 can be activated and induce 15-LO-1 expression in monocytes stimulated by IL-13[73]. Further studies are necessary to clarify the role of different STAT proteins in the 15-LO-1 gene expression.

3.5.2 Epigenetics

The field of epigenetics investigates how the modification of DNA or histones by acetylation, methylation, phosphorylation, ubiquitination and ADP ribosylation regulates gene expression[74]. Since IL-4 or IL-13 stimulation alone does not induce 15-LO-1 expression, the influence of the epigenetic mechanisms acetylation and methylation has been investigated[75,76]. How the other epigenetic mechanisms influence 15-LO-1 expression remains to be studied.

DNA is wrapped around histone proteins that form small subunits of the chromosomes. The amino-terminal part of the histone is exposed for post-translational modifications. By acetylating lysine residues on the amino-terminal tail of histones, the DNA is unwrapped and the genes are exposed for transcription factors. The 15-LO-1 gene has been shown to be regulated by acetylation of histones[75]. After IL-4 stimulation of the lung carcinoma cell line A549 and STAT6 activation, the histones and STAT6 itself were acetylated, which induced 15-LO-1 expression. The mRNA of 15-LO-1 was detected after 11 hours. Since activation of STAT6 by phosphorylation occurs within

minutes, the delay in gene expression was concluded to be due to the acetylation mechanism.

Gene expression can also be regulated by methylation of DNA and histones. The influence of DNA methylation and the expression of 15-LO-1 were investigated in five different cell lines. The A549 cells, which was the only one cell line without a methylated 15-LO-1 promoter, expressed mRNA after IL-4 stimulation[76]. In colon cancer cells, transcription of 15-LO-1 was shown to be regulated by histone methylation[77].

Thus, the selective expression of 15-LO-1 mRNA in certain cells can be explained by the epigenetic mechanisms histone acetylation, histone methylation, promoter methylation together with external stimuli by IL-4 or IL-13 and STAT6 activation.

3.5.3 Translational regulation

The 15-LO-1 mRNA and protein have been shown to be expressed during erythroid cell maturation in rabbits and humans[78,79]. During the maturation of pro-erythrocytes into mature erythrocytes, the cells undergo a regulated transformation in the bone marrow where hemoglobin synthesis is initiated, followed by ceased cell division and nucleus extrusion[80]. After enucleation, the cells are released into the blood as immature reticulocytes. During reticulocytes' maturation into final erythrocytes, mRNA translation is shut off and the mitochondria are broken down. The 15-LO-1 mRNA is expressed as long as the nucleus is present. The translation of mRNA into the 15-LO-1 enzyme coincides with mitochondria degradation in the reticulocytes.

It has been shown that 15-LO-1 mRNA is expressed but silenced by two proteins - the heterogeneous nuclear ribonucleoprotein K (hnRNP K) and hnRNP E1/E2 - during erythrocyte maturation. These two proteins bind to the 3'untranslated region of 15-LO-1 mRNA which not only prevents translation of the protein, but saves the mRNA from degradation.

The strictly controlled expression of 15-LO-1 in erythropoiesis has not been described in other cells that express the enzyme. There are no reports that STAT6 is involved in hnRNPs expression. Until further studies have been conducted, the translational regulation of 15-LO-1 must be regarded as unique to the maturation of erythrocytes.

3.6 POST-TRANSLATIONAL REGULATION

In the catalytic cycle of 15-LO-1, the iron in the catalytic site is activated by a hydroperoxy fatty acid that changes the oxidation state of the iron from the inactive ferrous (+II) state to the active ferric (+III) state[81]. During enzyme incubations this activation leads to a lag phase, which may be viewed as a post-translational regulation.

15-LO-1 undergoes suicide inactivation. This was proposed to be due to the oxidation of methionine-590[82]. Indeed, the enzyme was inactivated when methionine-590 was oxidized by 13(S)-HPODE. However, when this amino acid was replaced by leucine, the enzyme could still be self-inactivated. Another suicidal activation mechanism of 15-LO-1 was shown to depend on the arachidonic acid product 15(S)-HPETE, which covalently modified the enzyme in the active site[55].

An allosteric site has been proposed to influence the substrate specificity of 15-LO-1[83]. However, the location of the allosteric site remains to be determined.

There are no other known post-translational modifications of the human 15-LO-1 such as phosphorylations or glycosylations.

3.7 SPECIES DIFFERENCES BETWEEN 15-LO-1 HOMOLOGUES

In a phylogenetic tree, human 15-LO-1 is grouped together with the rabbit reticulocyte 15-LO and the 12/15-leukocyte LOs found in pig rabbit, cow, rat and mouse[84].

Two rabbit enzymes are found in this group, the reticulocyte type that has 99% of the amino acids in common with the leukocyte type[85]. The rabbit LO found in leukocytes converts arachidonic acid, mainly to 12(S)-HETE, as the orthologues enzymes found in rat and mice[86]. These 12-LOs have similarities in enzymatic properties, expression and regulation to human 15-LO-1, but these enzymes convert arachidonic acid, as the name suggests, primarily to 12(S)-HETE and secondarily to 15(S)-HETE. The enzymes are also active on linoleic acid, as well as on esterified substrates in cellular membranes. Thus, the presence of two very similar rabbit enzymes is probably due to gene duplication.

To summarize, the rabbit homologues to human 15-LO-1 are two very similar 12/15-LOs. One is expressed in reticulocytes (15-LO) and the other in leukocytes (12-LO). In mice and rats, there is one leukocyte 12-LO, which is the orthologue to human 15-LO-1, but this enzyme has mainly 12-LO activity. Thus, these enzymes are often named 12/15-LO in animals.

3.8 THE ROLE OF 15-LO-1 IN DISEASES

15-LO-1, or its orthologues in animals, is expressed in certain cells or under certain circumstances. Below is a brief description of different diseases in which 15-LO-1 is believed to have a biological role.

In humans, the epithelial cells in the upper airways express 15-LO-1 constitutively[87,88]. In addition, several studies indicate an increased expression of 15-LO-1 and production of 15(S)-HETE in bronchial asthma[89,90]. In mice, the 15-LO-1 orthologue 12/15-LO is expressed in the airway epithelial cells and is induced by IL-13 and activation of STAT6[91]. Also, knock-out studies in mice suggest that the enzyme has a pro-inflammatory role in the airways[92,93]. Thus, in epithelial cells 15-LO-1 expression correlates with 12/15-LO expression in mice. In addition, the enzymes play a role in airway inflammation.

15-LO-1 expression was shown to be increased compared to controls in human brain affected by Alzheimer's disease (AD)[94]. In addition, the 15-LO-1 derived products 15(S)-HETE and 12(S)-HETE were elevated in the cerebrospinal fluid of patients with AD, compared with controls[95]. This suggests that 15-LO-1 plays a role in AD. However, it has been proposed that the anti-inflammatory substance neuroprotectin D1, which can be formed via 15-LO-1, is found in neuronal cells cultivated *in vitro* in an AD model[96]. Therefore it remains to be proven whether 15-LO-1 is promoting or protecting development of AD.

In mice, the 12/15-LO, the orthologue to human 15-LO-1, was shown to regulate bone mass[97]. Both knock-out studies of the gene, as well as pharmacological inhibition of the enzyme, increased bone mass in mice. In humans, three investigations have concluded that the gene coding for the platelet type 12-LO was associated to osteoporosis, but not 15-LO-1[98-101]. However, two other publications have concluded that 15-LO-1 was associated with human osteoporosis[102,103]. Thus, it is an open question if osteoporosis in humans is dependent on 15-LO-1 or platelet type 12-LO.

Since 15-LO-1 can oxygenate LDL and can be up regulated in macrophages, the enzyme has been studied in different animal models for its involvement in atherosclerosis[104]. There seems to be a role for 12/15-LO in the plaque formation of mice, but whether 15-LO-1 is involved in human atherosclerosis remains controversial[101]. Association studies were conducted of 15-LO-1 SNPs and myocardial

infarction and coronary artery disease[105,106]. The studies did not find a correlation between 15-LO-1 and an increased risk of atherosclerosis.

The expression of 15-LO-1 has been associated with various cancers, such as colorectal cancer, prostate carcinoma and breast cancer[107,108]. Peroxisome-proliferator-activated receptors (PPARs), which are nuclear hormone receptors that regulate gene expressions upon fatty acid binding, are often involved when 15-LO-1 has been associated with cancers[109]. However, the PPARs can bind several different oxygenated fatty acids and the effects depend on cell type. Thus, it is difficult to conclude whether 15-LO-1 influences cancer development via PPARs.

4 BLOOD CELLS

Hematopoiesis is the formation of all blood cells that originate from hematopoietic stem cells in the bone marrow[110] (Figure 7). The blood cells are divided into two lineages: 1) the lymphoid cells (T-cells, B-cells and NK-cells) which are important in adaptive immunity, 2) the myeloid cells (basophils, neutrophils, eosinophils, macrophages and dendritic cells), which are involved in both adaptive and innate immunity, as well as blood clotting (platelets) and oxygen transport (erythrocytes). The blood cells that are important for this work are described in more detail below.

4.1 PLATELETS

Platelets, or thrombocytes, are the smallest human blood cells. These cells are involved not only in the formation of blood clots but also in inflammation and host defence[111]. In the adult human bone marrow, the hormone thrombopoietin stimulates megakaryocytes to produce 10^{11} platelets daily. The aggregation of platelets is stimulated by several different ligands, including thrombin, TXA_2, collagen and fibrinogen. The resting platelet's shape is discoid, but the morphology changes upon activation, granules secrete their content and fibrinogen binds to its receptor. Platelet activation is then amplified and fibrin formation by thrombin generates blood clots.

Upon calcium stimulation of the platelet, arachidonic acid is released from the membranes by $cPLA_2$-α and subsequently metabolized by COX-1 to PGH_2. The PGH_2 is a substrate to TXA_2-synthase, which produces TXA_2 and 12-HHT. Platelet aggregation is induced by binding of TXA_2 to the platelet's TXA_2-receptor. Inhibition of COX-1 in platelets, for instance with aspirin, leads to prolonged bleeding time[111].

4.2 DENDRITIC CELLS

Dendritic cells are rare and are found mainly in lymphoid tissues, but they are also present elsewhere in the human body. The function of the dendritic cell is to capture pathogens, digest them and present the protein fragments on the cell surface to T-cells and B-cells in the lymph nodes[112]. Immature dendritic cells mature in the tissue upon antigen processing, followed by migration to the lymph nodes. The myeloid dendritic cells originate from the bone marrow stem cells that mature into monocytes, which differentiate into macrophages or dendritic cells. Immature dendritic cells can be prepared from peripheral blood monocytes *in vitro* by treating them with IL-4 and GM-CSF. The cells differentiate into mature dendritic cells after further exposure to TNF-α, IL-1β, IL-6 and PGE_2.

4.3 EOSINOPHILS

Eosinophils are involved in the innate immune response against parasites[113]. However, eosinophils also play a central role in diverse inflammatory responses, such as asthma, as well as modulators of innate and adaptive immunity. Eosinophils are produced in the bone marrow, and their differentiation is induced by the transcription factors GATA-1, PU.1 and C/EBP. The development of eosinophils is also regulated by the cytokines IL-3, IL-5 and GM-CSF. Only 1-3% of the circulating leukocytes are eosinophils, which

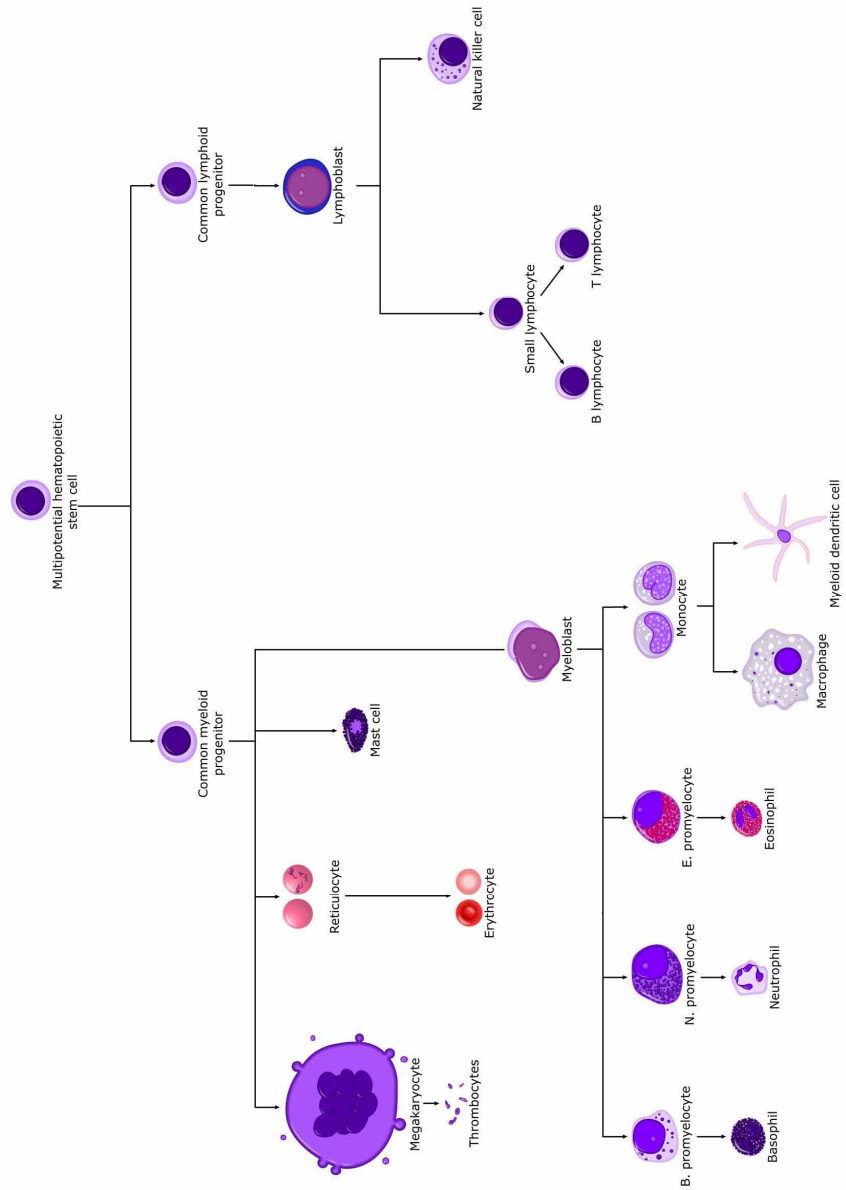

Figure 7. An overview of the hematopoiesis with the myeloid and lymphocyte lineages.

reside predominantly in the gastrointestinal tract. During a parasite infection, numerous cytokines and chemokines are released to direct eosinophils from the bone marrow to the sites of inflammation. However, only IL-5 and eotaxin selectively regulate the eosinophils trafficking, and they do so in synergy with each other. Upon activation, the eosinophils secrete an array of proinflammatory cytokines, cytotoxic granules, chemokines and lipid mediators. Eosinophils can also act as antigen-presenting cells and initiate immune responses, as well as activate mast cells. In addition, eosinophils play an important role in the pathogenesis of asthma and airway-hyper responsiveness. Inhibition of IL-5 using a monoclonal anti IL-5 antibody significantly reduced the number of severe exacerbations in patients with refractory eosinophilic asthma[114].

4.4 B-CELLS

The B-cell is a part of the adaptive immune system. Its function is to make antibodies against antigens, perform antigen presentation with the B-cell receptor and differentiate into a memory B-cell or plasma cell, after antigen interaction [115]. T-cells and B-cells originate from a common hematopoietic stem cell. In adults, T-cell differentiation and maturation takes place in the thymus while B-cell lymphopoiesis takes place in the bone marrow. During B-cell differentiation, the cell undergoes rearrangements of its immunoglobulin genes, and the B-cell is classified as immature when IgM is expressed on the surface. The maturation then proceeds in the spleen or lymph nodes, and the B-cell is classified as mature when it expresses both IgM and IgD proteins. During the maturation process of the B-cell, a positive selection takes place to ensure that the rearranged genes result in the production of functionally adequate antibodies. If not, the B-cell undergoes apoptosis. A similar negative selection takes place to prevent the B-cell receptor recognizing self-antigens. Class switch recombination is a further maturation step of the B-cell where it is stimulated by a T-helper cell to go from IgM and IgD expression into IgG, IgA and IgE production. This isotype switching is irreversible.

4.5 LYMPHOMAS

Lymphomas are cancers that originate from lymphocytes (B-cells, T-cells and NK-cells), which often form tumors in the lymph nodes[116]. Lymphomas have traditionally been classified as Hodgkin lymphoma (HL), which is well characterized, and non-Hodgkin-lymphomas (NHL), which constitute a heterogeneous group of several different subtypes. A correct subclassification of lymphomas is mandatory for adequate treatment decisions and prediction of outcome. The WHO classification of lymphomas, which was updated recently, defines the distinct lymphoma entities by morphology, immunophenotype, genetic features, clinical presentation and clinical course[116].

4.5.1 Hodgkin lymphoma and the Hodgkin Reed-Sternberg cell

In 1832, the pathologist Thomas Hodgkin published the first article about a disease of the lymphoid system[116]. Independent of each other, Carl Sternberg (1898) and Dorothy Reed (1902) described the characteristic multinucleated giant cell that came to be called the Reed-Sternberg cell. The annual incidence of HL in the Western world is 2-3 cases per 100,000 people[117]. The disease has a bimodal age pattern, with a first peak around 25 years of age and an increasing incidence after 55-60 years of age. Common clinical features are enlarged lymph nodes, splenomegaly, fever, weight loss and night sweats. Eighty to ninety percent of patients survive their disease following the currently available chemotherapy/radiotherapy.

Identification of the Hodgkin Reed-Sternberg (H-RS) cell is still used in the diagnosis of HL[117]. In the HL tumor, the H-RS cells account for only 1-2% percent of all cells (Figure 8). The HL tumor is characterized by an inflammatory infiltrate of many different types of cells of the immune system, including T-cells, B-cells, plasma cells, neutrophils, eosinophils and mast cells. The release of an array of inflammatory cytokines from the H-RS cells attracts the immune cells into the tumor and probably gives rise to several of the characteristic symptoms. Among the cytokines released from H-RS cells are IL-5, IL-6, IL-8, IL-10, IL-13, MEC, TNF-α, IFN-γ, TGF-ß, GM-CSF, galectin, MDC, TARC, IP-10, RANTES, MIP1-α and MIP3-α[117-119]. In particular, autocrine IL-13 signaling and constitutive STAT6 activation are rather unique features of H-RS cells.

The H-RS cell is probably derived from a germinal center B-cell with disadvantageous Ig light chain mutations that escaped apoptosis. In contrast to other B-cell lymphomas, the H-RS cell has undergone extensive gene reprogramming, lost most B-cell typical genes and acquired expression of genes that are typical of other cells in the immune system. Multiple signaling pathways, including NF-κB, Jak-STATs, PI3K-Akt, Erk, AP1 notch 1 and receptor tyrosine kinases, result in a deregulated activity in H-RS cells. In the Western world, the Epstein Barr virus is found in the H-RS cells of about 40% of HL tumors.

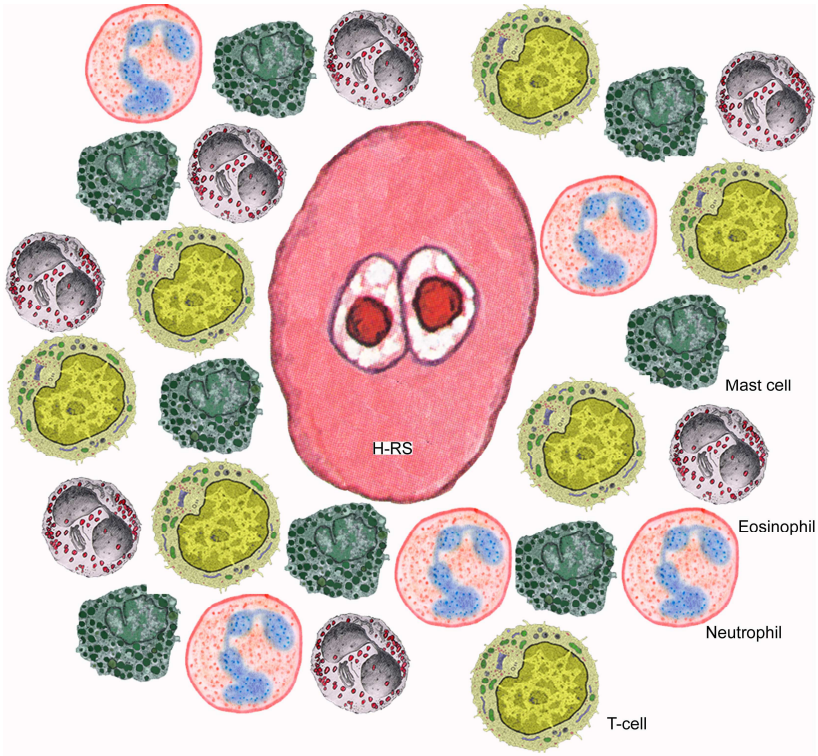

Figure 8. The HL tumor consists of few H-RS cells surrounded by an infiltrate of inflammatory cells.

4.5.2 Diffuse large B-cell lymphoma and primary mediastinal B-cell lymphoma

Diffuse large B-cell lymphoma (DLBCL) is the most common type of lymphoid cancer, accounting for 25-30% of all NHLs[120]. Primary mediastinal B-cell lymphoma (PMBCL) is an uncommon DLBCL subtype characteristically found in young females. Gene expression profiling suggests that this disease resembles HL more than other types of DLBCL[121,122]. PMBCL showed high expression of genes in the IL-13/IL-4 signaling pathways and STAT6 dependent genes, such as CD23, NF-IL13, FIG1 and the IL-4-induced gene 1, was expressed. A constitutive STAT6 activity in PMBCL cells was also confirmed *in vitro*[123].

5 MATERIALS AND METHODS

For additional experimental details, see the material and method sections in respective paper.

5.1 PAPER I

Polychlorinated biphenyls induce arachidonic acid release in human platelets in a tamoxifen sensitive manner via activation of group IVA cytosolic phospholipase A_2-α. *Biochemical pharmacology 71 (2005) 144–155*

Human blood was collected into EDTA-containing vaccutainer tubes and the platelets were obtained after centrifugation at 200 x g for 15 minutes. The platelets were washed in 1 mM EGTA in PBS (without calcium/magnesium) and centrifuged at 1000 x g for 15 minutes. The pellet was resuspended to 2-4 x 10^8 platelets/ml in the buffer used in respective assay.

In order to measure arachidonic acid release in platelets, the cells were labeled with ^{14}C-arachidonic acid. The platelets were incubated in PBS with 50 µM acetyl salicylic acid, 100 µM NDGA and ^{14}C-arachidonic acid for 60 minutes at 37ºC. After washing three times in the same buffer, the platelets were resuspended in the buffer used in respective assay.

Incubations of platelets were performed at 37ºC with DMSO control and inhibitors as described. PBS or calcium ionophore were added followed by incubation at 37ºC for 10 minutes and termination by adding methanol.

In broken cell assays, platelets were centrifuged and resuspended in 1 mM EGTA in PBS (without calcium/magnesium) and sonicated 2 x 5 seconds. After preincubation with substances for 20 minutes at 37ºC, 20 µM arachidonic acid was added followed by incubation for 10 minutes at 37ºC and the incubation was terminated with methanol.

Platelet aggregation was measured in an aggregometer, at 37ºC, by resuspending the cells in PBS with calcium and magnesium. After the addition of ionophore or CB-52 the light transmission was measured relative a blank.

Measurement of intracellular calcium was performed by loading the cells with 10 µM FURA2-AM for 45 minutes at 20ºC. After washing twice and the addition of stimuli, excitation was measured at 335 nm and 363 nm while emission was set at 510 nm.

Subcellular fractionation was performed after incubating platelets with CB-52 or vehicle at 37ºC for 10 minutes. The platelets were centrifuged at 1000 x g for 10 minutes and resuspended in 20 mM TRIS-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT and 10% glycerol added with 1 mM phenylmethanesulfonyl fluoride. After homogenization 2 x 5 seconds the homogenate was centrifuged at 100,000 x g for six minutes. The supernatant was saved and the pellet was washed once and resuspended in the buffer above.

The calcium dependent/independent PLA_2 activity after subcellular fractionation was analyzed by the addition of 2 µM of the substrate mixture 1:1 of PtdEtn:PtdCho both with 1-palmitoyl-2-(1-^{14}C)-arachidonyl. In the calcium dependent PLA_2 assay the buffer was 80 mM glycine, pH 9.0, 5 mM Ca^{2+}, 0.5 mM DTT, 1 mg/ml albumin and 10% glycerol. The calcium independent assay was performed with the buffer used in the subcellular fractionation supplemented with 1 mg albumin/ml. The PLA_2 incubations were performed at 37ºC for 60 minutes and terminated with two volumes of methanol containing 0.5% acetic acid and 40 µM stearic acid.

Prior to the RP-HPLC analysis of arachidonic acid, 12-HETE and 12-HHT the samples were centrifuged, applied to and eluted from solid-phase C18 extraction cartridges.

5.2 PAPER II

Interaction of human 15-lipoxygenase-1 with phosphatidylinositol bisphosphates results in increased enzyme activity. ***Biochimica et Biophysica Acta 1761 (2006) 1498-1505***

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque Plus density gradient centrifugation. The PBMCs were plated in complete medium and incubated for 60 minutes at 37ºC in 5% CO_2. The adherent cells were washed twice and cultured in complete medium supplemented with IL-4 and GM-CSF for 24 hrs. The cells were cultured for another period of 42 hrs in complete medium containing TNF-α, IL-1β, IL-6 and PGE_2. The dendritic cells were immunophenotyped by flow cytometry and were >80% CD40+ VD83+, >70% DC-sign+ and >95% CD14-.

The localization of 15-LO-1 in calcium stimulated dendritic cells was investigated by incubating the cells in PBS with calcium at 37ºC for 5 minutes with and without ionophore A23187. The cells were cytocentrifuged onto SuperFrostPlus glass slides and fixed with paraformaldehyde for 10 minutes. Detection of 15-LO-1 was performed with rabbit antiserum or preimmunserum plus a FITC-conjugated antirabbit antibody before examination in a confocal microscope.

Subcellular fractionation of dendritic cells was performed to investigate the relative quantity of 15-LO-1 translocation. The cells were incubated in PBS with calcium at 37ºC for 5 minutes with ionophore A23187, ionophore A23187 plus EGTA or a control with buffer only. The cells were homogenized by sonication and centrifuged for 10 minutes at 1500 x g and the supernatant was ultracentrifuged at 145,000 x g for 60 minutes at 4ºC. The supernatants were saved and the pellets resuspended to their initial volumes. The detection of 15-LO-1 in supernatants and pellets was performed by western-blot and polyclonal rabbit antiserum.

To investigate if 15-LO-1 binds to certain phospholipids, recombinant enzyme was incubated with PIP-Strips, Sphingo-Strips and PIP-Arrays. The strips and the array were blocked in PBS-T plus BSA, washed three times and then incubated over night at 4ºC with 15-LO-1 350 ng/ml PBS-T. After washing, the strips and arrays were detected for 15-LO-1 with polyclonal rabbit antiserum.

A vesicle assay was set up to elucidate if lipids, that 15-LO-1 bound to in the PIP-strip assay, influence the enzyme activity. The vesicles were made of 1-palmitoyl-2-oleoylphosphatidylcholine + one additional phospholipids, shown to bind 15-LO-1, and the substrate arachidonic acid. The lipids were dissolved in chloroform and the solvent was evaporated under nitrogen gas before the addition of buffer, 20 mM TRIS-HCL, pH 7.5, 0.2 M sucrose, 1 mM Ca^{2+}. The lipids were resuspended by freezing in ethanol and dry ice, thawing and gentle vortexing. The lipid suspension was pushed through an extruder with 400 nm pores.

Validation of the vesicle assay was performed by adding ^{14}C-arachidonic acid or ^{14}-C-phosphatidylcholine in chloroform. The vesicles were made as described above but diluted in buffer without sucrose and ultracentrifuged at 145,000 x g for 60 minutes at 20ºC. The supernatant and pellet were separated and the ^{14}C content was measured on a Minibeta scintillation counter. About 80% of the radioactivity was found in the pellets of both lipid compositions.

Vesicle activity assays were performed by incubating vesicles with 15-LO-1 in buffer containing 1 mM Ca^{2+} or the addition of an excess of EGTA. After 10 minutes

incubation at room temperature the reaction was terminated by adding methanol. The mixture was directly injected onto a RP-HPLC and detected at 235 nm. Kinetic assays were performed as described above but the incubation time was six minutes.

5.3 PAPER III

Hodgkin Reed–Sternberg cells express 15-lipoxygenase-1 and are putative producers of eoxins in vivo. ***FEBS Journal 275 (2008) 4222–4234***

The HL cell lines L1236, HDLM2, KMH2 and L428 were cultivated in RPMI 1640 medium, supplemented with 2 mM L-glutamine, 10 percent FBS, penicillin (100 U/ml) and streptomycin (100µg/ml), in a humified atmosphere with 5 percent CO_2 at 37°C**.**

Diagnostic HL-involved lymph node biopsies were collected at the Karolinska University Hospital. Paraffin sections of the biopsies, 10 HL nodular sclerosis subtype, 10 HL with mixed cellularity and ten NHLs were para-formaldehyde fixed before immunodetection of 15-LO-1 and alkaline phosphatase staining. The biopsies were also classified according to the number of infiltrating eosinophils.

The mRNA was extracted from the cells and RT-PCR was performed with specific primers to investigate if L1236 cells expressed 15-LO-1 and 15-LO-2. The expression of 15-LO-1 in L1236 cells was also visualized by 15-LO-1 antibody staining and immunocytochemistry of cytocentrifuged and para-formaldehyde fixed cells.

Calcium dependent translocation of 15-LO-1 has been studied in eosinophils and IL-4 stimulated monocytes. After washing L1236 cells with PBS, with or without calcium and calcium plus ionophore, subcellular fractionation was performed by sonication of the cells followed by centrifugation at 1500 x g and then 100,000 x g. Aliquotes from the membrane and supernatant fractions were analyzed by western-blot and detection by 15-LO-1 specific antibody and ECL. The same aliquots were also incubated with arachidonic acid for quantifying 15-LO-1 activity.

Cellular activity assays were performed with arachidonic acid to investigate the metabolites formed via the 15-LO-1 pathway in L1236 cells. The cells were washed twice and diluted in PBS before pre-warming the samples at 37°C for two minutes and addition of arachidonic acid. Methanol was used to stop the incubations and the samples were purified by solid phase C18 extraction. Monohydroxy fatty acids were analyzed on RP-HPCL-PDA with the mobile phase methanol:water:TFA (69:31:0.07) while eoxins and dihydroxy fatty acids were analyzed with the mobile phase acetonitrile:methanol:water:acetic acid (28:18:54:1, pH 5.6).

Mass spectrometry was used to structurally identify the different 15-LO-1 metabolites formed after cellular activation assays. Positive mode was used and mass spectra were monitored for the parent ions EXC_4:626, EXD_4:497 and EXE_4:440 (m/z).

5.4 PAPER IV

A mediastinal B-cell lymphoma cell line shares several phenotypic features with Hodgkin lymphoma after treatment with interleukin-13: similar morphology, metabolism of arachidonic acid and release of cytokines ***(manuscript)***

The HL cell line L1236 and the NHL cell line Karpas-1106P were cultivated in RPMI 1640 medium, supplemented with 2 mM L-glutamine, 10 percent FBS, penicillin (100 U/ml) and streptomycin (100µg/ml), in a humified atmosphere with 5 percent CO_2 at 37°C**.** The stimulation of Karpas-1106P was performed with 10 ng/ml of recombinant human IL-4 or IL-13.

Karpas-1106P cells were stimulated with IL-4 or IL-13 and compared with L1236 cells to monitor any morphology changes. The cells were washed twice in PBS with Ca^{2+}/Mg^{2+} containing 10 % FBS before 0.1 x 10^6 cells were cytospinned onto Super Frost Plus glasses and stained by May-Grünewald/Giemsa.

The cell lines were incubated with arachidonic acid to detect and quantify 15-LO-1 metabolites by RP-HPLC-PDA. The samples were preincubated for two minutes in a water bath at 37°C with shaking and subsequently arachidonic acid was added and the incubation proceeded for 10 min. The reactions were stopped with ice-cold methanol. To detect the eoxins, the samples were purified by solid phase C18 extraction. In the analysis of soluble and esterified 15-LO-1 metabolites, ^{14}C –arachidonic acid was used and the cells were fractionated by ultracentrifugation followed by saponification of the lipids

Western-blot analysis of 15-LO-1 was performed to confirm the results from the activity assays, and the findings were compared with the expression of 15-LO-1 protein. Total protein was extracted from the cell lines and 10 µg per sample was loaded on the gel. Detection of the 15-LO-1 enzyme was performed with a primary 15-LO-1 antibody and ECL detection.

Investigation of IL-4 and IL-13 receptor expression in the cell lines was performed by flow cytometry. The cells were washed, incubated with antibodies against the receptors and isotype controls before fixation in para-formaldehyde and FACS analysis.

A calcium flux receptor assay was performed to investigate if the IL-4 and IL-13 stimulated Karpas-1106P cells express functional CysLT receptors in a similar manner as L1236 cells after stimulation with different eicosanoids. The cells were loaded with the fluorophore Calcium 3 for one hour at 37°C before the eicosanoids were added to the cells. In the inhibition assay, zafirlukast and montelukast were incubated with the cells during the last 30 minutes along with the fluorophore.

Quantification of cytokines involved in HL was performed in L1236 cells as well as interleukin stimulated and unstimulated Karpas-1106P cells. The cells were cultivated 16 hours in RPMI 1640. The cytokines were measured in the supernatant with Bioplex kits.

6 SUMMARY

6.1 PAPER I

Polychlorinated biphenyls induce arachidonic acid release in human platelets in a tamoxifen sensitive manner via activation of group IVA cytosolic phospholipase A_2-α. *Biochemical pharmacology 71 (2005) 144–155*

In this study the release of arachidonic acid and formation of 12-HETE and 12-HHT (TXA_2) in human platelets after challenge with polychlorinated biphenyls (PCB) was investigated.

Human platelets were stimulated with four different PCBs, four different PCB mixtures and ionophore A23187 was used as control. Two of the PCBs, CB-52 and CB-47, induced arachidonic acid release to a higher extent than calcium ionophore. Also the PCB mixtures, three out of four, did induce 12-HETE and 12-HHT formation in the platelets.

The PCB that induced the highest arachidonic acid release, CB-52, was incubated with homogenized platelets prior to the addition of arachidonic acid. Compared with a DMSO control, the 12-HHT level was 30% lower and the 12-HETE level was 31% higher. Thus, CB-52 inhibited the 12-HHT formation and arachidonic acid was shunted to the 12-LO pathway. The time curve for the 12-HETE formation was shown to have a lag-phase and reached a plateau after 20 minutes. The 12-HHT formation showed no lag-phase and reached the plateau after 10 minutes. The dose-response curve of CB-52 gave a half maximal response of 35 µM for both 12-HHT and 12-HETE.

To identify which PLA_2 that released arachidonic acid upon CB-52 stimulation, the following inhibitors were used: pyrrolidone-1 (selective $cPLA_2$-α inhibitor), BEL ($iPLA_2$ inhibitor) and AACOCF3 (inhibits both $cPLA_2$-α and $iPLA_2$). Pyrrolidone-1 inhibited 12-HETE formation with an IC50 of 38 nM and ^{14}C-arachidonic acid release with an IC50 of 5 nM. BEL inhibited 12-HETE by 52% at 30 µM. AACOCF3 inhibited 12-HETE formation with an IC50 of 6 µM.

Prior to catalyze the release of arachidonic acid from phospholipids, the $cPLA_2$-α translocates to internal membranes. In agreement with this, the majority of $cPLA_2$-α was found in the membrane fraction, after CB-52 stimulation, while in unstimulated platelets the enzyme was detected in the cytosolic fraction. In parallel, ^{14}C-arachidonic acid release was measured and it correlated with the translocation of $cPLA_2$-α. The western-blots after the subcellular fractionations showed two parallel bands for $cPLA_2$-α. This different migration of the enzyme is due to phosphorylations of the enzyme.

Platelets were treated with EGTA, EGTA + BAPTA-AM or 1 mM calcium in order to investigate if CB-52-induced $cPLA_2$-activity was calcium dependent. EGTA + BAPTA-AM induced the highest formation of both 12-HHT and 12-HETE. The PCB did not increase the calcium levels when platelets were loaded with FURA2-AM and subsequently treated with either CB-52 or calcium ionophore.

Since PCBs can mimic hormones, platelets were pre-incubated with 17β-estradiol, tamoxifen and nafoxidin before the CB-52 incubation. Both anti-estrogens, but not the 17β-estradiol, inhibited the formation of 12-HETE. In an *in vitro* assay none of the anti-estrogens inhibit the $cPLA_2$-α activity.

The amount of 12-HHT is an indirect measure of TXA_2 formation, since TXA_2 synthase catalyze the conversion of PGH_2 to TXA_2 and 12-HHT in a ratio of 1:1. Since TXA_2 induce aggregation, platelets were assayed in an aggregometer and stimulated with CB-52 or ionophore. Only the ionophore induced aggregation of the platelets. When the platelets were pre-incubated with CB-52 followed by the addition of calcium

ionophore platelet aggregation was detected, even if it was reduced compared with only ionophore stimulation.

These results show that $cPLA_2$-α activity in platelets can be induced by mechanisms independent of calcium.

6.2 PAPER II

Interaction of human 15-lipoxygenase-1 with phosphatidylinositol bisphosphates results in increased enzyme activity. ***Biochimica et Biophysica Acta 1761 (2006) 1498-1505***

The sub-cellular translocation of 15-LO-1 in eosinophils and IL-4 stimulated monocytes has earlier been reported. This study shows that 15-LO-1 translocates to the plasma membrane also when monocytes have been differentiated to dendritic cells.

Most of the 15-LO-1 was detected in the membrane fraction when dendritic cells were stimulated with calcium ionophore. In the presence of EGTA, however, the enzyme was predominately found in the cytosolic fraction. In unstimulated cells approximately the same amount of 15-LO-1 was found in the membrane and cytosolic fractions.

A lipid dot-blot assay was performed to investigate if 15-LO-1 binds to certain phospholipids. Recombinant 15-LO-1 was incubated with dot-blots, nitrocellulose membranes with dots of phospholipids, and detected with 15-LO-1 antiserum. The 15-LO-1 was shown to bind several phospholipids, especially phosphatidylinositols.

A vesicle assay was set up to investigate whether those lipids that bound 15-LO-1 in the dot-blot assay also influenced the enzymatic activity. Vesicles were made of phosphatidylcholine and free arachidonic acid or linoleic acid. One phospholipid from the dot-blot assay was also added to each vesicle sample. The vesicles were incubated with 15-LO-1 in the absence or presence of calcium.

The enzymatic activity increased in the presence of calcium when either $PI(4.5)P_2$ or $PI(3.4)P_2$ were added to the vesicles. In the absence of calcium, the enzymatic activity was independent of the lipid composition of the vesicles. The outcome of the assay was the same for arachidonic acid or linoleic acid.

A kinetic assay was performed with arachidonic acid and vesicles containing $PI(4.5)P_2$ or $PI(3.4)P_2$ compared to phosphatidylcholine only. Similar V_{max} were obtained with the addition of either phosphatidylinositol but lower apparent Km was obtained in the presence of phosphatidylcholine only.

These results indicate that the 15-LO-1 activity is regulated by the lipid composition of lipid bilayers.

6.3 PAPER III

Hodgkin Reed–Sternberg cells express 15-lipoxygenase-1 and are putative producers of eoxins in vivo. ***FEBS Journal 275 (2008) 4222–4234***

The HL cell lines L1236, L428, KMH2 and L570 were incubated with arachidonic acid to elucidate the eicosanoid production. Only L1236 was shown to produce the monohydroxy fatty acids 15(S)-HETE and 12(S)-HETE in the ratio 9:1, which is characteristic of 15-LO-1 activity.

RT-PCR analysis confirmed that L1236 indeed expressed mRNA from 15-LO-1 but not from 15-LO-2. Immunohistochemistry also confirmed that the L1236 cells expressed 15-LO-1, which was distributed in the cytosol in unstimulated cells.

Western-blot detection and activity assays were performed on the cytosolic and membrane fractions after calcium stimulation and subcellular fractionation of L1236

cell. As expected, 15-LO-1 translocated to the membranes upon calcium stimulation. However, the amount of 15-LO-1 protein in the pellet and supernatant fractions did not correlate with the enzymatic activity. Instead, the enzymatic activity was always higher in the supernatant fractions.

Incubations of L1236 with arachidonic acid that were analyzed by RP-HPLC and mass spectrometry provided evidence that the cells produced eoxins as well as various dihydroxy fatty acids. The amount of EXC_4 peaked around five minutes and then declined during the formation of EXD_4, which reached a maximum concentration at 30 minutes.

Biopsies from HL and NHL tumors were investigated for the expression of 15-LO-1 by immunohistochemistry. 15-LO-1 was detected in the H-RS cells in 85% of the HL biopsies and in all eosinophils, which were found in every biopsie. However, none of the NHL samples expressed 15-LO-1.

These results show that 15-LO-1 is expressed in H-RS cells and indicate that the enzyme play a role in the inflammation in HL.

6.4 PAPER IV

A mediastinal B-cell lymphoma cell line shares several phenotypic features with Hodgkin lymphoma after treatment with interleukin-13: similar morphology, metabolism of arachidonic acid and release of cytokines *(manuscript)*

The PMBCL cell line Karpas-1106P has been shown to express proteins that normally are induced by IL-4 stimulation due to constitutively expressed STAT6. Therefore the Karpas-1106P and the HL cell line L1236 were investigated with regard to the expression of IL-4 and IL-13 receptors. By flow cytometry both cell lines were shown to express both types of receptors.

The L1236 cells have the characteristic morphology of H-RS cells. When unstimulated and IL-4/IL-13 stimulated Karpas-1106P cells were stained with May-Grünewald /Giemsa the stimulated cells showed a similar morphology to that of L1236 cells including giant cell forms with multiple nucleoli.

Karpas-1106P cells were cultivated for 11 days to investigate if IL-4 or IL-13 stimulation of the cells induced 15-LO-1. Samples were taken every day from the cultures and activity assays with arachidonic acid as well as western-blot detection of 15-LO-1 were performed. The interleukin stimulated Karpas-1106P cells expressed active 15-LO-1 and both the amount of enzyme and the activity increased until a plateau was reached after eight days. The stimulated Karpas-1106P cells also produced eoxins.

The formation of 15-HETE did not increase with increasing cell densities of Karpas-1106P cells or L1236 cells. Instead, the soluble amount of 15-HETE increased with up to 5 million cells/ml and then declined. When performing a similar assay but with ^{14}C-arachidonic acid followed by subcellular fractionation and saponification of the lipids, it was revealed that the amount of the different 15-LO-1 products varied with cell density.

Different eicosanoids were added to L1236, unstimulated and IL-4/IL-13 stimulated Karpas-1106P cells to measure receptor activities by calcium flux measurements. Only LTC_4 and LTD_4 were shown to induce calcium flux in L1236 and interleukin stimulated Karpas-1106P cells but not in unstimulated cells. The IL-4 stimulated Karpas-1106P cells were pre-incubated with zafirlukast and montelukast that dose dependently inhibited LTD_4 stimulated calcium flux, showing that the signal was conducted through the CysLT1 receptor.

In order to elucidate if interleukin-treated Karpas-1106P cells and L1236 release similar cytokines, the culture media from these cells was analysed by the bioplex detection kit. These results indicate that the cytokines TNF-α, Rantes, IL-6, IP-10 and IFN-γ were released by interleukin stimulated Karpas-1106P cells as well as L1236 cells. In contrast, these cytokines were not released by unstimulated Karpas-1106P cells.

These results further support a close relationship between HL and PMBCL.

7 GENERAL DISCUSSION

Arachidonic acid is not generally a free fatty acid in cells but is rather found esterified into phospholipids in intracellular membranes. In the first study of this thesis, data about the release of arachidonic acid by cPLA$_2$-α from platelets membranes upon PCB stimulation is presented. The second study presents novel findings about the membrane interaction of 15-LO-1 and shows that the composition of the phospholipid membrane influences the kinetics of the enzyme. The third and forth studies present data about the constitutive expression of 15-LO-1 in HL and the inducible expression in a NHL cell line, which acquired features characteristic of the HL-cell line L1236.

It is well established that calcium promotes translocation of cPLA$_2$-α to intracellular membranes. In addition, the cPLA$_2$-α membrane interaction also depends on the lipids C1P and PI(4.5)P$_2$ in a calcium-dependent and calcium-independent manner, respectively. In the first study, the PCB induced translocation and activation of cPLA$_2$-α was shown to be independent of calcium. Since phosphatidylinositol lipids are present in platelets, one explanation of the membrane translocation and enzymatic activity could be the binding of cPLA$_2$-α to PI(4.5)P$_2$.

The enzymatic activity of cPLA$_2$-α has been shown to increase by phosphorylations. However, the mechanism of phosphorylated enzyme activation is unknown. The PCB CB-52 induced phosphorylation of cPLA$_2$-α. Since phosphorylation introduces negative charges on the enzyme, this should rather prevent the enzyme from interacting with membranes in the absence of calcium. The cPLA$_2$-α has been shown to undergo conformational changes by interacting with the lipid substrates. Therefore, it is possible that phosphorylation can also induce conformational changes of cPLA$_2$-α. This could hypothetically improve the interaction of hydrophobic amino acids on the enzyme's surface with cell membranes and thereby increase the amount of enzyme in the pellet fraction.

The drugs tamoxifen and nafoxidine are amphiphilic cations with a tertiary amine that have been used in the treatment of breast cancer. Both drugs are modulators of estrogen receptors. Tamoxifen has been shown to bind to negatively charged phosphatidylinositols, which inhibited phospholipase C activity[124]. By a similar mechanism, this could explain the inhibitory effect of tamoxifen, and perhaps of nafoxidine as well, on CB-52 induced cPLA$_2$-α activity. If tamoxifen binds to PI(4.5)P$_2$ in the platelets, it would prevent cPLA$_2$-α from binding to PI(4.5)P$_2$ in a calcium-independent manner. Another possible explanation for the inhibitory effect of tamoxifen may be that it interferes with the phosphorylation of cPLA$_2$-α, which could inhibit activation of the enzyme.

Activation of cPLA$_2$-α by PCBs in platelets is an artificial assay setup. Nevertheless, this model system might be a useful tool to investigate the calcium-independent translocation and membrane interaction of cPLA$_2$-α.

The 15-LO-1 translocation to internal membranes upon calcium stimulation has also been well established, as in the case of cPLA$_2$-α. 15-LO-1 translocation has been studied in eosinophils and IL-4 stimulated monocytes where the enzyme bound predominantly to the plasma membrane. This study also found that 15-LO-1 translocated to the plasma membrane upon calcium ionophore stimulation in IL-4 stimulated dendritic cells and L1236 cells. The enzyme was distributed fairly equally between the membrane and cytosolic fractions in unstimulated cells, while most 15-LO-1 was in the cytosolic fraction in the absence of calcium.

Surprisingly, 15-LO-1 enzymatic activity in the cytosolic fraction was always higher than in the membrane fraction even though most 15-LO-1 protein was bound in the membrane. This was an unexpected finding, given that membrane-bound 15-

LO-1 has been found to possess higher activity than the cytosolic enzyme on both phospholipids and fatty acid substrates[56]. It is believed that 15-LO-1 binds to membranes with the opening to the catalytic site toward the phospholipid bilayer. Thus, exogenously added arachidonic acid might not have the same access to the active site of the membrane-associated 15-LO-1 as in the case of the soluble, cytosolic enzyme. Alternatively, the membrane-associated 15-LO-1 undergoes suicidal inactivation during oxygenation of membrane lipids. In addition, 15-LO-1 might be inactivated by 15-HPETE, which is likely to be rapidly inactivated in the cytosol, whereas 15-HPETE generated by membrane-bound 15-LO may persist and inactivate the membrane-bound enzyme. The human 5-LO has also been reported to be inactivated by ionophore-stimulated translocation, followed by subcellular fraction in neutrophils[125]. Thus, it appears that LO translocation to membranes can regulate enzymatic activity.

Some 15-LO-1 was always found in the membrane fraction, suggesting that the enzyme can bind with high affinity to the membranes. Thus, alternative mechanisms also contribute to the membrane association of 15-LO-1. Therefore, a lipid dot-blot assay was performed with recombinant 15-LO-1. The enzyme was shown to bind certain phospholipids, particularly phosphatidylinositols. When two of the lipids, PI(4.5)P_2 and PI(3.4)P_2, that bind 15-LO-1 were part of a vesicle membrane, the enzymatic activity of arachidonic acid increased in the presence of calcium. This suggests that calcium mediates the translocation of 15-LO-1 to the plasma membrane, where it can also interact with PI(4.5)P_2 and PI(3.4)P_2 to increase its activity at certain membrane compartments. Other phosphatidylinositols, such as PI(3.5)P_2, did not increase enzymatic activity, which suggests specificity in the binding of 15-LO-1 to PI(4.5)P_2 and PI(3.4)P_2 (Figure 9). The rabbit reticulocyte 15-LO has been crystallized, but no structural homology to other characterized PI(4.5)P_2 binding sites was found in the first published crystal structure. However, now that the crystal data has been re-interpreted, the new structures might reveal novel lipid binding sites.

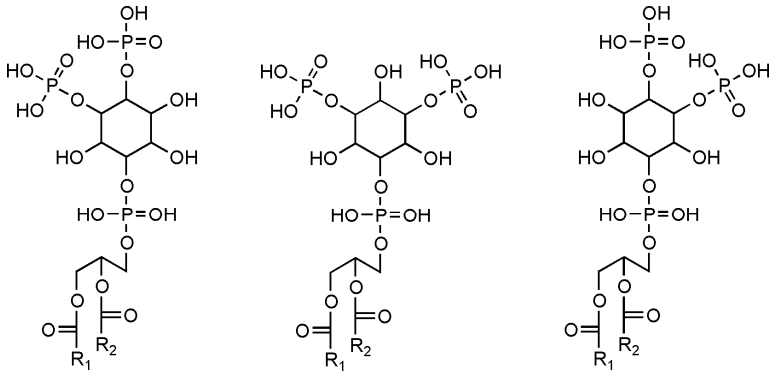

Figure 9. PI(4.5)P_2 and PI(3.4)P_2 - but not PI(3.5)P_2 - increased the enzymatic activity of 15-LO-1. The R_1 is usually a saturated fatty acid, while R_2 is a polyunsaturated fatty acid, such as arachidonic acid.

When comparing the results of the lipid interactions of 15-LO-1 with cPLA$_2$-α, the enzymes turn out to have a lot in common. For example, cPLA$_2$-α has also been found to interact with certain phospholipids in a lipid dot-blot assay, while vesicle assays and a PI(4.5)P$_2$ binding site have been characterized[126]. Both enzymes have a lid that covers the active site, and conformational changes occur by calcium ions, lipid bilayer interactions and substrate binding. The cPLA$_2$-α has been more thoroughly studied than 15-LO-1. Thus, LO researchers can learn and draw inspiration from the phospholipase A$_2$ research field. In addition, future studies will elucidate whether these enzymes also interact with each other in arachidonic acid metabolism.

15-LO-1 expression in H-RS cells was studied in the third paper. The enzyme was found in H-RS cells in 85% of the HL biopsies examined. 15-LO-1 was not expressed in nine different subentities of NHL. In addition, the only HL cell line that has been proven to be clonally related to the original tumor tissue, the L1236 cell line, abundantly expressed 15-LO-1 and produced eoxins. The expression of 15-LO-1 and the putative formation of eoxins by H-RS cells in vivo are likely to contribute to the inflammatory features of HL. These findings may have important diagnostic and therapeutic implications in HL. Furthermore, the discovery of the high 15-LO-1 activity in L1236 cells demonstrates that this cell line comprises a useful model system to study the chemical and biological roles of 15-LO-1.

The two lymphomas HL and PMBCL have some clinical similarities and have been proven to be closely related in genomic association studies. In particular, STAT6 signaling has been documented to be a feature in both lymphomas. However, the histopathologic inflammatory features of HL are absent in PMBCL. In the fourth study, stimulation of the PMBCL cell line Karpas-1106P by IL-4 or IL-13 changed the cellular morphology, induced 15-LO-1 and eoxin production, up-regulated the CysLT1 receptor and stimulated the secretion of inflammatory cytokines. Thus, the PMBCL cell line acquired several inflammatory features and became more like the HL cell line L1236 during cultivation with IL-13. This further supports the relationship between the two diseases HL and PMBCL[121,122]. In addition, these results can yield clues about the appearance of mediastinal gray zone lymphomas, which have features that are transitional between HL and PMBCL[127].

In murine cancer models, IL-4 treatment has been shown to improve the survival rate of treated animals[128,129]. In humans, IL-4 has been investigated in phase 1 clinical trials for the treatment of various cancers[130,131]. Since the overall survival of HL patients is better than that of patients with PMBCL, it may be of interest to investigate whether IL-4 or IL-13 treatment of PMBCL would improve the clinical outcome.

Although only one human being with inactive cPLA$_2$-α has been identified, the results of that study suggest that the enzyme plays the same biological role in humans and mice[4,16]. For example, the human subject had multiple small intestinal ulcers which were also found in the cPLA$_2$-α knock-out mice. However, the biological function of 15-LO-1 in humans is not clear, and several attempts have been made to elucidate its role by means of animal knock-out models. It is still unknown whether there would be any biological similarities between a human with an inactive 15-LO-1 enzyme compared to any of the 12/15-LO knock-out animals. One reason is that the receptors of 15-LO-1 metabolites have not been identified, rendering it unclear whether these metabolites have the same effects in different species. Another reason is that the biological function of 15-LO-1 oxygenated phospholipids has not yet been fully determined.

Therefore, 15-LO-1 researchers will need to perform more studies in man and use clinically relevant *in vitro* models to clarify the enzyme's role in humans. Human airway epithelium is one such model, given that the airway epithelial cells are

accessible, the enzyme is abundantly expressed in these cells and several papers indicate that 15-LO-1 plays a role in asthma.

The results presented in this thesis also validate the cell line L1236, with a constitutive 15-LO-1 expression, and the IL-13 stimulated Karpas-1106P cell line as a novel *in vitro* model. Both cell lines are suitable for elucidating the biochemical features of the enzyme and, which is of great importance, this *in vitro* model is related to the inflammatory disease HL.

The most direct way of identifying the biological function of 15-LO-1 in humans would be the administration of a specific 15-LO-1 inhibitor. Awaiting this to happen, the use of the *in vitro* models presented here can hopefully be of great value to 15-LO-1 researchers.

8 CONCLUSIONS

Certain PCBs induce arachidonic acid release in platelets, and the induction phosphorylated $cPLA_2$-α, as well as activated the enzyme in a calcium-independent way. The anti-estrogens nafoxidin and tamoxifen inhibited the $cPLA_2$-α induction by PCBs. The presented model could be useful for studying the calcium-independent activation of $cPLA_2$-α in platelets.

15-LO-1 translocates to the plasma membrane after calcium stimulation of IL-4 stimulated dendritic cells and L1236 cells. However, the enzymatic activity with exogenous substrate did not correlate with the western-blot results after subcellular fractionation of L1236 cells. 15-LO-1 binds to certain phospholipids in a lipid dot-blot assay, while $PI(3.4)P_2$ and $PI(4.5)P_2$ increase 15-LO-1 enzymatic activity in a vesicle assay. The vesicle assay is a novel tool for studying 15-LO-1 activity *in vitro*.

15-LO-1 is expressed in HL tumor biopsies and the HL cell line L1236. These cells also produced eoxins. IL-4 or IL-13 stimulation of the PMBCL cell line Karpas-1106P induced 15-LO-1 expression and eoxin production. In addition, interleukin stimulation of PMBCL cells changed the morphology, as well as inducing expression of CysLT1 receptors and secretion of pro-inflammatory cytokines, which – taken together - made the cells more like the HL cell line L1236.

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