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# STUDIES ON THE ACTIVATION OF CYTOSOLIC PHOSPHOLIPASE $A_2$ AND 15-LIPOXYGENASE-1

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# ABSTRACT

Arachidonic acid is a polyunsaturated fatty acid, which has four double bonds and belongs to the omega-6 family of fatty acids. Even if it can be formed in humans from one essential fatty acid, the 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 mammalian lipases but the $cPLA_2$-α has been shown to preferentially release arachidonic acid. Depending on which cell and what stimuli, arachidonic acid can be further metabolized into for example prostaglandins, thromboxan $A_2$ or leukotrienes.

The first publication 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. Interestingly, platelets incubated with PCBs did not aggregate despite the fact that a robust release of arachidonic acid was observed.

The second publication investigates the 15-LO-1, which catalyzes the metabolism of several polyunsaturated fatty acids of both the omega-6 and omega-3 families. A unique feature of 15-LO-1 is that it translocates to internal cellular membranes and oxygenates fatty acids also when they are esterified into lipids. The 15-LO-1 is expressed in lung epithelial cells, eosinophils, reticulocytes and IL-4 stimulated monocytes and dendritic cells. In the second publication, 15-LO-1 was found to translocate to the plasma membrane in IL-4 stimulated human dendritic cells upon calcium stimulation. The 15-LO-1 was shown to bind the membrane even when calcium was chelated.

In a lipid-overlay assay, 15-LO-1 bound to certain phospholipids and especially phosphatidylinositols. The vesicle assay model was set up to investigate if phosphatidylinositols also influenced the enzyme activity of 15-LO-1. In the presence of calcium, the addition of phosphatidylinositol-4.5-bisphosphate ($PI(4.5)P_2$) or $PI(3.4)P_2$ to the vesicles increased the 15-LO-1 activity on the substrate arachidonic acid. Kinetic assays were performed and the Vmax was shown to be unchanged but the apparent Km 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.

Taken together, this thesis demonstrates that 15-LO-1 and cPLA2-α share several regulatory properties even though they catalyze different reactions in the arachidonic acid metabolism.

# LIST OF PUBLICATIONS

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 2006, Article in press*

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 12/15-LO | 12/15-lipoxygenase |
| 12-LO | 12-lipoxygenase |
| 15-LO-1 | 15-lipoxygenase type 1 |
| 15-LO-2 | 15-lipoxygenase type 2 |
| 5-LO | 5-lipoxygenase |
| 12-HETE | 12-hydroxy-(5,8,10,14)-eicosatetraenoic acid |
| 12-HHT | 12-hydroxy-(5,8,10)-heptadecatrienoic acid |
| 13-HPODE | 13-hydroperoxyoctadeca-9,11-dienoic acid |
| 13-HODE | 13-hydroxyoctadeca-9,11-dienoic acid |
| 15-HETE | 15-hydroxy-(5,8,11,13)-eicosatetraenoic acid |
| $Ca^{2+}$ | Calcium ions |
| COX-1 | Cyclooxygenase type 1 |
| COX-2 | Cyclooxygenase type 2 |
| $cPLA_2$-α | Cytosolic phospholipase $A_2$ |
| DMSO | Dimethylsulfoxide |
| DTT | Dithiothreitol |
| ER | Endoplasmatic reticulum |
| EGTA | Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| IL-1β | Interleukin-1β |
| IL-4 | Interleukin-4 |
| IL-6 | Interleukin-6 |
| $iPLA_2$ | Calcium-independent phospholipase $A_2$ |
| $LTA_4$ | Leukotriene $A_4$ |
| $LTB_4$ | Leukotriene $B_4$ |
| $LTC_4$ | Leukotriene $C_4$ |
| LDL | Low density lipoprotein |
| $Mg^{2+}$ | Magnesium ions |
| NDGA | Nordihydroguaiaretic acid |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate buffered saline |
| PtdCho | Phosphatidylcholine |
| PtdEtn | Phosphatidylethanolamine |
| $PI(3.4)P_2$ | Phosphatidylinositol-3,4-bisphosphate |
| $PI(3.5)P_2$ | Phosphatidylinositol-3,5-bisphosphate |
| $PI(4.5)P_2$ | Phosphatidylinositol-4,5-bisphosphate |
| $PLA_2$ | Phospholipase $A_2$ |
| PCB | Polychlorinated bisphenyl |
| $PGI_2$ | Prostacyclin |
| $PGD_2$ | Prostglandin $D_2$ |
| PGE2 | Prostglandin $E_2$ |
| $PGF_2$-α | Prostglandin-$F_2$ α |
| RP-HPLC | Reversed phase – high pressure liquid chromatography |

| | |
|---|---|
| Sn-1/Sn-2 | Stereospecific numbering-1/-2 |
| $TXA_2$ | Thromboxane $A_2$ |
| TNF-α | Tumor necrosis factor-α |

# 1 INTRODUCTION

## 1.1 ARACHIDONIC ACID

Arachidonic acid is a twenty carbon long fatty acid with four double bonds (20:4) that belongs to the group of omega-6 fatty acids, see figure 1. The concentration of free arachidonic acid in cells is very low, instead, it is almost completely esterified into the sn-2 position of phospholipids. The amount of arachidonic acid varies between different cells and different 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.

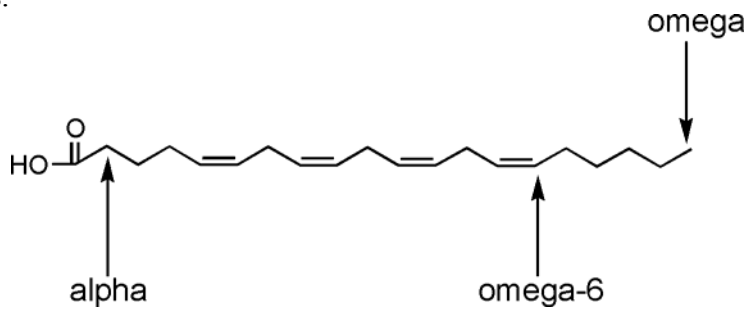


Figure 1. Arachidonic acid is an omega-6 polyunsaturated fatty acid.

## 1.2 PHOSPHOLIPASE $A_2$

The phospholipase $A_2$s ($PLA_2$) are a family of enzymes, 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, which both could act as messenger molecules, see figure 2.

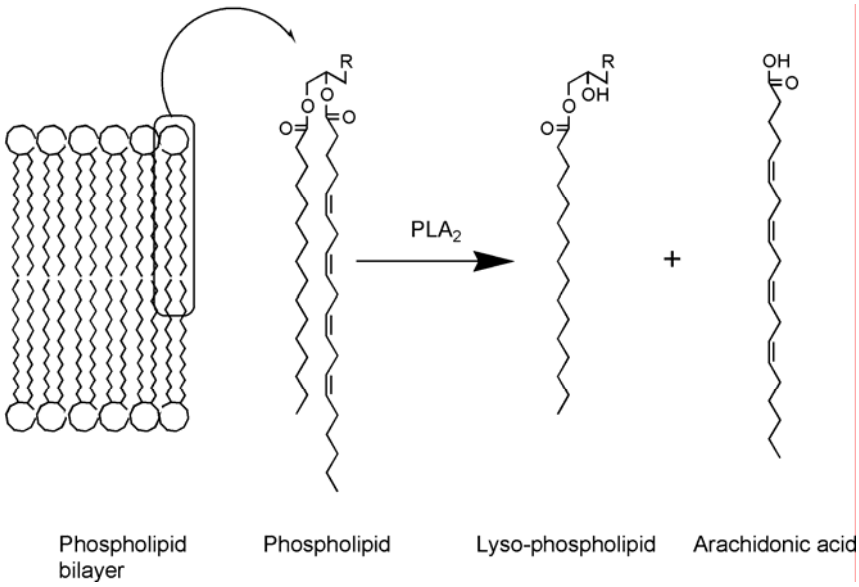


Figure 2. In the phospholipid bilayer, $PLA_2$ cleaves the ester bond of the fatty acid in sn-2 position generating a lyso-phospholipid and free arachidonic acid. The R is commonly a choline, ethanolamine, serine or inositol.

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 $PLA_2$, 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 important in the formation of arachidonic acid metabolites. The $cPLA_2$-α preferentially releases arachidonic acid and mouse knock-out studies has 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].

## 1.3 LIPOXYGENASES

Lipoxygenases are enzymes that catalyze the incorporation of molecular oxygen into arachidonic acid, but also into other polyunsaturated fatty acids [7]. Six functional genes coding for lipoxygenases have been found in humans. They have been named according to which carbon in arachidonic acid the enzyme incorporates the oxygen, namely: 5-lipoxygenase (5-LO), the platelet type 12-lipoxygenase (12-LO), 12(R)-lipoxygenase, 15-lipoxygenase type 1 (15-LO-1), 15-lipoxygenase type 2 (15-LO-2) and epidermis-type 3-lipoxygenase [7].

The 15-LO-1 is expressed in epithelial cells of the upper airways, in eosinophils, reticulocytes, dendritic cells and macrophages [8]. The 15-LO-2, which has low homology to 15-LO-1, is expressed in hair roots, prostate, lung and cornea. 15-LO-2 also differs from the 15-LO-1 by oxygenating free arachidonic acid to 15-HETE only and by not being active on esterified arachidonic acid [9].

## 1.4 LEUKOTRIENES AND PROSTAGLANDINS

Leukotrienes and prostaglandins are potent lipid mediators derived from arachidonic acid, see 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 anti-leukotriene drugs, for example montelukast, are used to treat asthma.

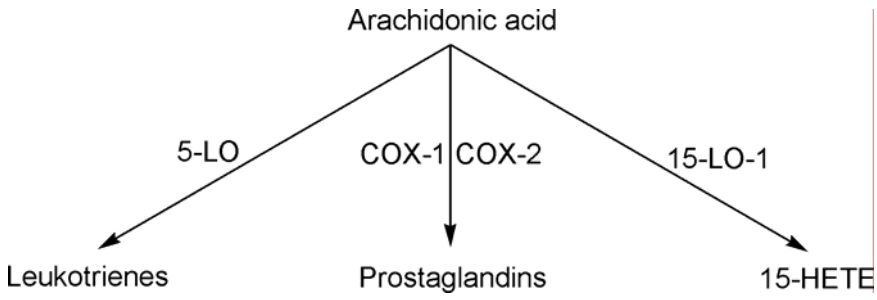


Figure 3. The arachidonic acid can be metabolized by different enzymes leading to signaling molecules in different pathways.

In the pathway leading to leukotriene synthesis, the 5-LO oxygenates arachidonic acid to 5-HPETE and then to $LTA_4$, which can be further metabolized by either the $LTA_4$-hydrolase which generates $LTB_4$, or the $LTC_4$-synthase which generates cysteinyl-leukotrienes [10].

The prostaglandin synthesis begins with the conversion of arachidonic acid into $PGH_2$ catalyzed by either COX-1 or COX-2 [11]. 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 thromboxan-$A_2$ ($TXA_2$) and 12-HHT + malondialdehyde in an equimolar ratio in platelets [12].

# 2 $CPLA_2$- A

## 2.1 STRUCTURE

The $cPLA_2$-α is an 85-kDa protein which crystal structure has been determined [13]. The enzyme has two structural domains, one N-terminal and one C-terminal, connected by a linker peptide that promotes some 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 [13].

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 to the α/β hydrolase fold. The active site funnel is lined with hydrophobic residues and penetrates one-third into the catalytic domain. The bottom of the cleft contains the residues, which are essential for catalysis, serine-228 and aspartate-549.

## 2.2 ENZYMATIC REACTION

The catalytic mechanism of $cPLA_2$-α is independent of calcium and appears to function as a serine hydrolase [13]. In the proposed mechanism the phosphate group is stabilized by arginine-200 and the aspartate-549 activates the serine-228, which attacks the sn-2 ester bond. The $cPLA_2$-α has been shown to release arachidonic acid in sn-2 position of cellular phospholipids almost 20-fold more effective than other fatty acids [5].

## 2.3 MEMBRANE INTERACTION

### 2.3.1 Calcium

The $cPLA_2$-α preferentially cleaves phospholipids in a lipid membrane rather than as dissolved phospholipid monomers [14]. The translocation of $cPLA_2$-α to phospholipid membranes upon calcium stimulation is dependent on the C2-domain of the enzyme. Upon calcium stimulation, $cPLA_2$-α translocates primarily to the nuclear membranes, ER and Golgi but the enzyme has been shown to also translocate to the plasma membrane [15-17]

### 2.3.2 Lipid interactions

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 residence at the membrane [16].

When the enzyme was truncated, the C2-domain was shown to preferentially bind phosphatidylcholine vesicles [18]. The C2-domain did not discriminate between saturated or non-saturated fatty acids in position sn-1 or sn-2 despite the fact that $cPLA_2$-α preferentially releases arachidonic acid [18].

The catalytic domain has a phosphatidylinositol binding site that increases the enzymatic activity independently of calcium [19, 20].

## 2.4 POST-TRANSLATIONAL REGULATIONS

The catalytic domain of $cPLA_2$-α has three functionally important phosphorylation sites: serine-505, serine-727 and serine-515 [21-23]. Phosphorylation of serine-505 *in vitro* increases the hydrolysation of sn-2-arachidonylphosphatidylcholine by 30% and the enzymes affinity for phosphatidylcholine vesicles [24, 25]. In contrast, another group reported that the *in vitro* phosphorylation of serine-515 but not serine-505 increased the enzymatic activity [22].

## 2.5 BIOLOGICAL FUNCTIONS

The biological functions of $cPLA_2$-α has been studied in knock-out mice [4]. The knock-out female mice became less frequently pregnant and there was an increased mortality rate of the pups. The mice had lesions in the small intestine and when water deprived, the mice had problems with concentrating urine. Otherwise the knock-out mice seemed to develop normally.

Decreased amounts of prostaglandins and leukotrienes were found in cells, such as mast cells and peritoneal macrophages, isolated from the knock-out mice in comparison to wild-type mice. The effect on prostaglandin and leukotriene synthesis was immediate in cells from $cPLA_2$-α knock-outs. However, over time the amount of prostaglandins and leukotrienes released from cells from $cPLA_2$-α knock-out mice differed less than cells from control animals. 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, for example anaphylaxis, acute lung injury, brain ischemia and polyposis [4].

# 3 15-LO-1

## 3.1 STRUCTURE

The 15-LO-1 is a 75 kDa protein consisting of 661 amino acids. The crystal structure of rabbit reticulocyte 15-LO has been determined. The enzyme was shown to contain two domains separated by a flexible link, an N-terminal C2-domain and a C-terminal catalytic domain [26]. The rabbit reticulocyte enzyme is the only mammalian lipoxygenase, which has been crystallized.

The N-terminal C2-domain involves the first 110 amino acids. The homology of C2-domains found in lipases is low and the C2-domain of 15-LO-1 has the same percent of amino acid homology, 23%, as lipases [26].

The C-terminal catalytic domain is unique and has only structural homology to plant lipoxygenases [26]. 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 in the bottom of the active site have been shown to prevent arachidonic acid to slide further into the active site [27].

## 3.2 ENZYMATIC REACTIONS

The 15-LO-1 can oxygenate free polyunsaturated fatty acids as well as fatty acids esterified into lipids, see figure 4.

### 3.2.1 Fatty acid substrates

The 15-LO-1 oxygenates the substrate arachidonic acid but also other omega-6 and omega-3 fatty acids. The fatty acid is believed to slide with the methyl end first into the catalytic site of 15-LO-1 [26].

The 15-LO-1 catalyses the conversion of arachidonic acid to 15-HETE and 12-HETE in the ratio 9:1. This ratio is governed by the bulky amino acids in the bottom of the cleft, which prevent the fatty acid to slide deeper into the enzyme. This also explains why the 15-LO-1 also oxygenates shorter fatty acids such as linoleic acid (18:2, omega-6) or linolenic acid (18:3, omega-3).

### 3.2.2 Lipid substrates

In addition to using free polyunsaturated fatty acids as substrates, 15-LO-1 also oxygenates fatty acids esterified into position sn-2 of phospholipids or lipoproteins [28, 29]. However, the oxygenation rate of esterified fatty acids is slower. 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.

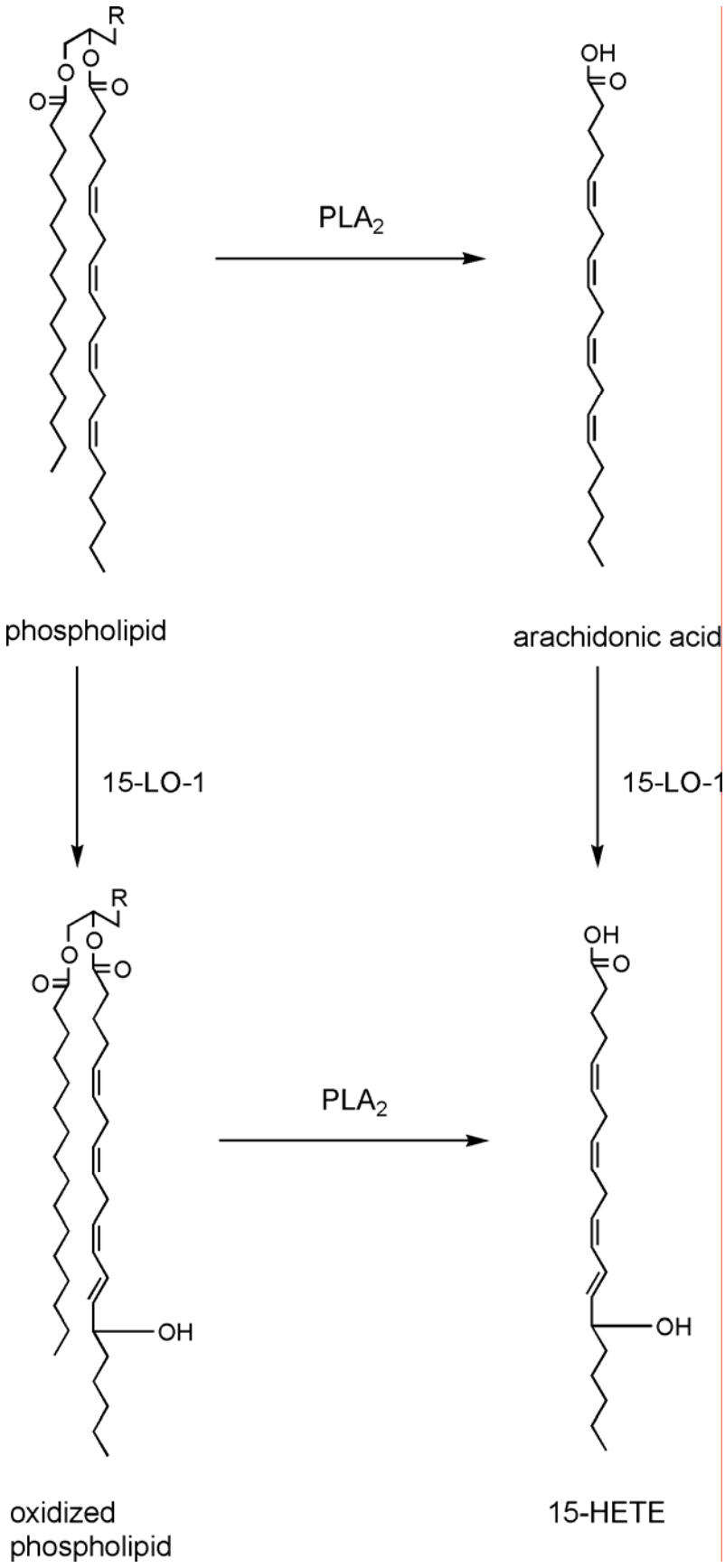


Figure 4. The 15-LO-1 oxygenates polyunsaturated fatty acids either bound into a phospholipid or as a free fatty acid. In both cases it is carbon in position fifteen in the fatty acid that is oxygenated. The R is commonly a choline, ethanolamine, serine or inositol.

## 3.3 MEMBRANE INTERACTION

### 3.3.1 Calcium

In unstimulated cells, 15-LO-1 is mainly found in the cytoplasm but upon calcium stimulation the enzyme translocates to the plasma membrane [30]. The enzyme's affinity for calcium has been estimated to be low, with a Kd of 0.2-0.5 mM [31]. 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 [31]. Although some 15-LO-1 is bound to the membrane surface before calcium stimulation it has been suggested that translocation of the enzyme is reversible [30].

### 3.3.2 Lipid interactions

It has been shown *in vitro* that 15-LO-1 can oxygenate different cellular membranes, for example rat liver mitochondrial membranes, beef heart sub mitochondrial particles, rat liver endoplasmic membranes and erythrocyte plasma membranes [28] . It has also been found that the enzyme is capable of interacting with lipid particles such as LDL as measured by an increase in the oxidation of LDL in the presence of 15-LO-1 [32].

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 [33]. This conclusion was based on the finding that after a truncation of the C2-domain, the enzyme still could bind to membranes and was enzymatically active.

## 3.4 POST-TRANSLATIONAL REGULATIONS

In the catalytic cycle of 15-LO-1, the iron in the catalytic site is activated by a hydroperoxy fatty acid which changes the oxidation state of the iron from the inactive ferrous (+II) state to the active ferric (+III) state [34]. During enzyme incubations this activation is seen as a lag phase, which could be viewed as a post-translational regulation.

The 15-LO-1 undergoes suicide inactivation and this was proposed to be due to the oxidation of methionine-590 [35]. Indeed, when methionine-590 was oxidized by 13-HPODE the enzyme was inactivated. However, when this amino acid was mutated to a leucine the enzyme could still be self-inactivated.

There are no other known post-translational modifications of the human 15-LO-1.

## 3.5 SPECIES DIFFERENCES

The rabbit 15-LO-1 reticulocyte lipoxygenase, which has been crystallized, has high amino acid homology to the human 15-LO-1. Rabbits also express another lipoxygenase, which has 99% of the amino acids in common with the reticulocyte type, which is expressed in peripheral monocytes [36]. This second rabbit lipoxygenase, which has been classified as a leukocyte-type, converts arachidonic acid mainly to 12-HETE. This latter enzyme is also the ortholog to human 15-LO-1 in rat and mice [37]. This 12-LO has similar enzymatic properties, expression and regulation as the human

15-LO-1 but it converts arachidonic acid, as the name suggests, primarily to 12-HETE and less 15-HETE, and is also active on linoleic acid as well as membranes

To summarize, the rabbit orthologs to human 15-LO-1 are two very similar 12/15-LOs of which one is expressed in reticulocytes (15-LO) and the second in leukocytes (12-LO). In mice and rats there is one leukocyte 12-lipoxygenase with high homology to the human 15-LO-1, but this enzyme is a 12-LO. There is no evidence of a reticulocyte 15-LO in rats or mice. Thus, the enzyme is often named 12/15-LO in animals.

## 3.6 BIOLOGICAL FUNCTIONS

The biological function of 15-LO-1 is not clear and several attempts have been made to elucidate its role in animal knock-out models. Below is a short description of four different areas where 15-LO-1 is believed to play a role. A more detailed discussions is found in a review by H. Kuhn et al. [8].

The 15-LO-1 is another branch in addition to COX and 5-LO, in the arachidonic acid metabolism. The 15-LO-1 products 13-HODE and 15-HETE have been shown to be elevated in tissues during inflammation but the physiological relevance of these metabolites is not clear.

The epithelial cells in the upper airways express 15-LO-1 and several studies indicate an increased expression of this enzyme in bronchial asthma. The metabolite 15-HETE has been shown to induce bronchial contraction *in vitro* but inhaled 15-HETE did not have any effect.

The expression of 15-LO-1 has been associated with different cancers, such as colorectal cancer, prostate carcinoma and breast cancer. The role of 15-LO-1 in cancer is not clear especially not in colorectal cancer.

Since 15-LO-1 can oxygenate LDL, the enzyme has been studied in different animal models for the involvement in atherosclerosis. There seems to be a role for 15-LO-1 in the plaque formation in mice but whether the enzyme is involved in human atherosclerosis remains controversial. Immunostaining of human arteries showed very low amounts of 15-LO-1 and the expression did not correlate with atherosclerotic plaque formation [38]. However, significant amounts of 5-LO was detected suggesting a role of leukotrienes in the disease.

# 4 MATERIAL AND METHODS

## 4.1 PUBLICATION 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.

To measure arachidonic acid release in platelets, they 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 ones 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 with 0.5% acetic acid and 40 µM stearic acid.

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

## 4.2 PUBLICATION II

**Interaction of human 15-lipoxygenase-1 with phosphatidylinositol bisphosphates results in increased enzyme activity.** ***Biochimica et Biophysica Acta 2006, Article in press***

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 added 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 they 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 SUMMARY

## 5.1 PUBLICATION 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 $TXA_2$ in human platelets after challenge with PCB was investigated.

Human platelets were stimulated with four different PCBs, four different PCB mixtures and ionophore A23187 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 $TXA_2$ 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 levels were 30% lower and the 12-HETE levels 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 the $^{14}$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.

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. Interestingly, the EGTA + BAPTA-AM samples 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, could inhibit the formation of 12-HETE. In an *in vitro* assay none of the anti-estrogens inhibit the $cPLA_2$-α activity.

The level of 12-HHT is an indirect measure of $TXA_2$ formation. Since $TXA_2$ aggregates platelets, they were assayed in an aggregometer and stimulated with CB-52 or ionophore. Only the ionophore induced aggregation of the platelets. When the same assay was performed but the platelets were preincubated with CB-52 followed by the addition of calcium ionophore platelet aggregation was detected, even if it was less than with ionophore only.

## 5.2 PUBLICATION II

**Interaction of human 15-lipoxygenase-1 with phosphatidylinositol bisphosphates results in increased enzyme activity.** ***Biochimica et Biophysica Acta 2006, Article in press***

The subcellular translocation of 15-LO-1 in eosinophils and IL-4 stimulated monocytes has earlier been reported [30]. 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 Vmax were obtained with the addition of either phosphatidylinositol but lower apparent Km was obtained in the presence of phosphatidylcholine only.

# 6 GENERAL DISCUSSION

Arachidonic acid is generally not a free fatty acid in cells but rather found esterified into phospholipids in intracellular membranes. One part of this thesis presents new data about the release of arachidonic acid by cPLA$_2$-α from platelets membranes upon PCB stimulation. The other part presents novel findings about the membrane interaction of the 15-LO-1 and that the composition of the phospholipid membrane influences the kinetics of the enzyme.

It is well established that calcium promotes translocation of cPLA$_2$-α to intracellular membranes. Even though several publications have presented data about cPLA$_2$-α phosphorylations and that it might increase the enzymatic activity no one has been able to explain the mechanism. In the case of calcium, the idea that the positive calcium ions promote membrane binding by interacting with the negatively charged head groups of the membranes phospholipid bilayer and the cPLA$_2$-α amino acids. However, phosphorylation of the enzyme introduces negatively charges and without calcium this should rather prevent the enzyme from interacting with the membranes. One interpretation could be that the hydrophobic interaction, of the surface amino acids of cPLA$_2$-α, with the lipid bilayer is even more important than expected. The activation of cPLA$_2$-α by PCBs in platelets might be a useful tool to investigate the translocation from a new point of view.

One possible explanation to the inhibitory effect of tamoxifen on CB-52 induced cPLA$_2$-α activity could be that the drug binds to phosphatidylinositols [39]. Tamoxifen is an amphiphilic cation that binds to the negatively charged phosphatidylinositols and thereby preventing phosphatidylinositol kinases from product inhibition and explains phospholipase C inhibition. Since cPLA$_2$-α has a phosphatidylinositol binding site this could be the mechanism of the tamoxifen inhibition of CB-52 induced 12-HETE formation.

Another interesting finding from the cPLA$_2$-α study is that the platelets do not aggregate despite the release of arachidonic acid and the production of TXA$_2$. This was shown in an *in vitro* assay and it is still an open question if this occurs *in vivo*.

The translocation to internal membranes of 15-LO-1 due to calcium stimulation is also well established, as in the case of cPLA$_2$-α. The 15-LO-1 translocation has been studied in eosinophils and IL-4 stimulated monocytes where the enzyme was bound to the plasma membrane. In this thesis the 15-LO-1 was also found to translocate to the plasma membrane upon calcium ionophore stimulation in IL-4 stimulated dendritic cells. The enzyme was distributed equally between the membrane and cytosolic fractions in unstimulated cells while most 15-LO-1 was in the cytosolic fraction when EGTA was added. Interestingly, some enzyme was always found in the membrane fraction suggesting that the translocation is not entirely reversible. Thus, other mechanisms are also contributing to the membrane association of 15-LO-1.

That is why a lipid dot-blot assay was performed with recombinant 15-LO-1. The enzyme was shown to bind certain phospholipids and especially phosphatidylinositols.

When two of the lipids, PI(4.5)$P_2$ and PI(3.4)$P_2$, shown to bind 15-LO-1 was 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 also can 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 the enzymatic activity which suggests specificity in the binding of 15-LO-1 to PI(4.5)$P_2$ and PI(3.4)$P_2$, see figure 5. The rabbit reticulocyte 15-LO ha been crystallized but no structural homology to other characterized PI(4.5)$P_2$ binding sites was found.

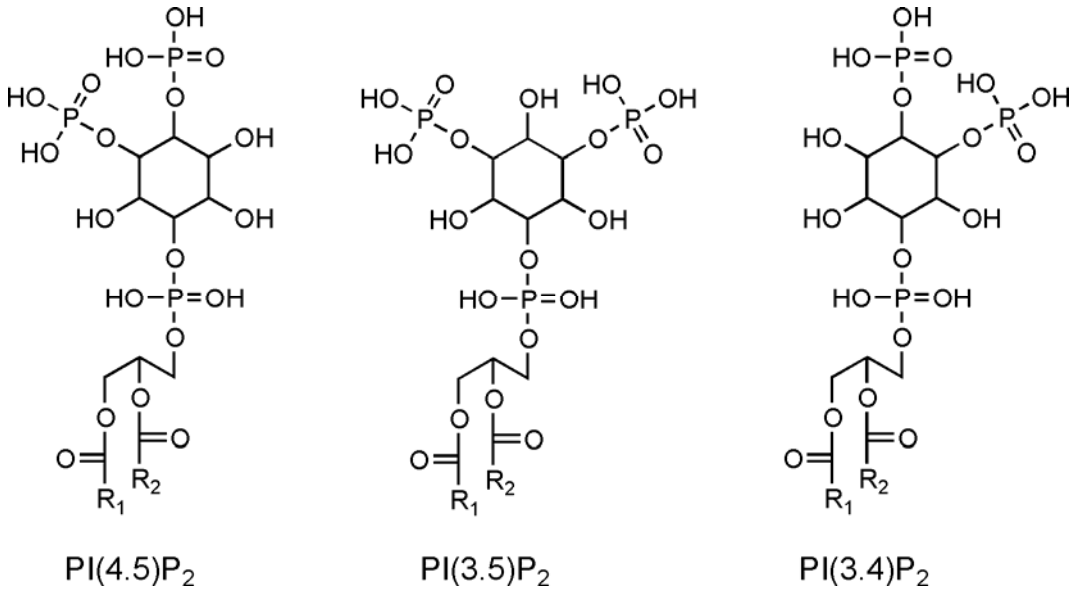


Figure 5. PI(4.5)$P_2$ and PI(3.4)$P_2$ but not PI(3.5)$P_2$ increased the enzymatic activity of 15-LO-1. The $R_1$ is usually a saturated fatty acid while $R_2$ is a polyunsaturated fatty acid, such as arachidonic acid.

When comparing the results from the lipid interactions of 15-LO-1 with cPLA$_2$-α, the enzymes have a lot in common. For example, also cPLA$_2$-α has been found to interact with certain phospholipids in a lipid dot-blot assay and a PI(4.5)$P_2$ binding site has been characterized [20]. If one consider that a pool of arachidonic acid is found in the position sn-2 of PI(4.5)$P_2$ in the cells, then it is probably not a coincidence that both enzymes increase their activities when binding to that phospholipid.

the 15-LO-1 metabolite 15-HETE was specifically incorporated into phosphatidylinositols in pulmonary epithelial cells stimulated with IL-4 [40]. This could be one way to eliminate 15-HETE or to build up a pool of 15-HETE in the membrane, which could alter the membrane permeability or be released upon other cellular stimuli.

Taken together, the 15-LO-1 and cPLA$_2$-α have been shown to share several regulatory properties even though they catalyze different reactions. Future studies will elucidate if these enzymes interact with each other in the arachidonic acid metabolism.

# 7 CONCLUSIONS

Certain PCBs induce arachidonic acid release in platelets and the induction activates $cPLA_2$-α in calcium independent way. The anti-estrogens nafoxidin and tamoxifen inhibited the $cPLA_2$-α induction by PCBs. Interestingly, the platelets released $TXA_2$ without aggregating. This could be a useful model to study the calcium independent activation of $cPLA_2$-α in platelets.

The 15-LO-1 translocates to the plasma membrane after calcium stimulation of IL-4 stimulated dendritic cells. 15-LO-1 binds to certain phospholipids and $PI(3.4)P_2$ and $PI(4.5)P_2$ increase the 15-LO-1 enzymatic activity in a vesicle assay. The vesicle assay is a novel tool for studying lipoxygenase activity *in vitro*.

This thesis highlights that the membrane interactions of $cPLA_2$-α and 15-LO-1 are important in the regulation of the arachidonic acid cascade. A better understanding of the mechanisms behind the enzyme's membrane interactions will hopefully lead to better treatment of inflammatory diseases.

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