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Regulation of the activity of 5-lipoxygenase, a key enzyme in leukotriene biosynthesis

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ABSTRACT

5-Lipoxygenase (5LO) catalyzes two steps in the biosynthesis of leukotrienes (LTs), lipid mediators of inflammation derived from arachidonic acid. LTs function in normal host defense, and have pathophysiological roles in chronic inflammatory diseases as asthma and atherosclerosis. Also, possible effects of 5LO products in relation to tumorigenesis have been described. Thus, insight regarding the biochemistry of 5LO is relevant for better understanding of normal physiology, and for development of therapy.

1. Introduction

Inflammation is necessary for life and mostly beneficial, but sometimes inflammatory reactions can become excessive, and lead to harmful outcome. In several endemic diseases chronic inflammation is part of the pathophysiology. Examples are the synovia in rheumatoid arthritis, the atherosclerotic vessel wall, inflammation of infarcted myocardium, and the hyper-reactive chronically inflamed lung tissue of the asthma patient. The signs of inflammation include accumulation of leukocytes, and leakage of plasma from small vessels in inflamed tissue. Leukotrienes (LTs) constitute one family of inflammatory mediators, formed from arachidonic acid (AA) [1]. LTB_4 is chemotactic for several types of leukocytes (e.g. neutrophils, dendritic cells, T-cells), and LTC_4 leads to increased vasopermeability of postcapillary venules. In addition, more recent work has implicated LTs in functions of antigen presenting cells. Regarding inflammatory diseases, LTs are established mediators in asthma and allergic rhinitis, and antiLTs (mainly Cys-LT1 receptor antagonists) are used in asthma treatment [2,3]. Also in atherosclerosis LTs are important in the pathophysiology, and 5-lipoxygenase products are considered regarding development and progression of cancer.

5-Lipoxygenase (5LO) is one of six human lipoxygenases. In LT biosynthesis 5LO catalyzes oxygenation of AA to 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE), and further dehydration to the allylic epoxide LTA_4. LTA_4 is further converted by LTA_4 hydrolase to the dihydroxy acid LTB_4, and by LTC_4 synthases to the glutathione conjugate LTC_4 (Fig. 1). 5LO is expressed primarily in various leukocytes: polymorphonuclear leukocytes (neutrophils and eosinophils), monocytes/macrophages, dendritic cells, mast cells, B-lymphocytes, and in foam cells of human atherosclerotic tissue.

2. 5-Lipoxygenase: a catalytic domain binding iron and a C2-like β-sandwich

Mammalian 5LOs are monomeric enzymes with 672 or 673 amino acids. A model structure of human 5LO, based on the crystal structure (1LOX) of the ferrous form of rabbit reticulocyte 15LO [4], consists of a N-terminal β-sandwich (residues 1–114) and a C-terminal catalytic domain (residues 121–673) (Fig. 2). The catalytic domain, composed of several α-helices, binds the prosthetic iron. By EPR, the iron of purified recombinant 5LO is ferrous, treatment with 5-HPETE or other lipid hydroperoxides gave ferric 5LO. EPR also indicated a flexible iron ligand arrangement, and selenide inhibited 5LO by binding to iron thus abolishing the EPR signal ($g = 6.2$) typical for active 5LO. In the lipoxygenase reaction mechanism, the iron acts as electron acceptor and donor, during hydrogen abstraction and peroxide formation. In many mononuclear non-heme iron(II) enzymes a 2-His-1-carboxylate facial triad anchor the iron. Mutagenesis indicated that two conserved His (H372, H550) and the C-terminal Ile-673 constitute such a triad in 5LO. In addition, H367 and N554 might function as replaceable ligands to iron. A C-terminal loop (hydrogen bond Asn-669 to His-399) may stabilize the C-terminal iron ligand.

The 5LO β-sandwich was also modelled on the basis of the C2-like domain of *C. perfringens* α-toxin (1QMD, a phospholipase C) [5]. Sequence and topology similarities between Polycystin-1, Lipoxygenase, and α-Toxin led to the definition of the "PLAT"

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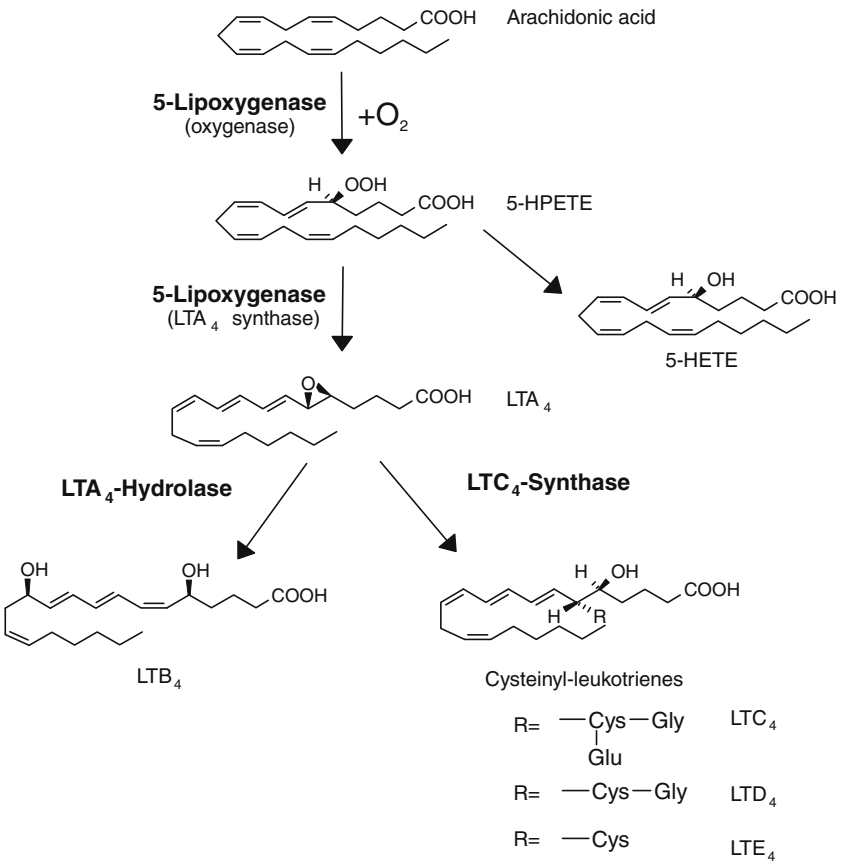

Fig. 1. Conversion of AA to LTs and 5-HETE.

domain as a subset within the C2 family [6]. Residues in the ligand binding loops of the β-sandwich bind Ca^{2+}, cellular membranes, and coactosin-like protein (CLP). Also the RNaseIII Dicer (involved in miRNA biosynthesis) binds to the C2-like β-sandwich of 5LO, and this association modified the miRNA precursor processing activity of Dicer [7]. ATP binds to 5LO and increases enzyme activity, apparently by stabilizing the structure, see [8] for additional references.

3. Activation of 5LO *in vitro* by Ca^{2+} in the presence of scaffold factors

Ca^{2+} was found to activate 5LO purified from human leukocytes [9]. The EC_{50} for Ca^{2+} activation of purified 5LO is quite low (1–2 μM), full activation is reached at 4–10 μM. 5LO binds Ca^{2+} in a reversible manner, for the intact enzyme a K_d close to 6 μM was determined by equilibrium dialysis and the stoichiometry averaged two Ca^{2+} per 5LO. Similar results (two Ca^{2+} per 5LO, K_{Ca} 7–9 μM) were obtained for the His-tagged C2-like domain (residues 1–115). Mutagenesis indicated that residues in ligand binding loop 2 of the 5LO C2-like domain are important for Ca^{2+} binding, and for Ca^{2+} activation of enzyme activity [10], but also other Ca^{2+} binding sites have been suggested, see Discussion in [11]. Mg^{2+}, at concentrations which are present in cells (mM), can activate 5LO *in vitro*. 5LO has some basal activity in the absence of Ca^{2+} and Mg^{2+}, the divalent cation is not part of the catalytic mechanism.

3.1. Membranes containing phosphatidylcholine

Many C2 domains are known to mediate Ca^{2+}-induced membrane association. The enzyme activity of 5LO from human leukocytes depended on microsomal membranes [9], and synthetic PC vesicles could replace the cellular membrane fraction as a stimulatory factor. Binding of 5LO to synthetic PC liposomes was induced by Ca^{2+}, and Ca^{2+} (as well as Mg^{2+}) increased the hydrophobicity of 5LO in a phase partition assay. The isolated 5LO C2-like β-sandwich had a high affinity for zwitterionic PC vesicles and it was suggested that the PC selectivity directs 5LO to the nuclear envelope [5]. Accordingly, the β-sandwich was required for translocation of GFP–5LO constructs to the nuclear membrane in HEK 293 cells [12]. In similarity to rabbit 15LO [13], residues also in the 5LO catalytic domain may contribute to membrane binding of the intact enzyme.

Ca^{2+}-induced binding to PC stabilized the structures of both 5LO protein and the membrane, and it was found that 5LO can bind also to cationic phospholipids [14]. This binding was stronger and occurred in the absence of Ca^{2+}, but Ca^{2+} was required for 5LO activity. It was suggested that 5LO can bind to membranes in "productive or nonproductive modes", i.e. membrane binding *per se* might not confer 5LO activity. Increased membrane fluidity favored 5LO association, and it was argued that this may be the factor directing 5LO to the AA enriched nuclear envelope. Interestingly, addition of cholesterol to a membrane preparation *in vitro* reduced 5LO activity by half [15], and cholesterol sulfate could also inhibit 5LO in intact cells.

3.2. Coactosin-like protein

Human CLP (142 amino acid residues) is similar to *Dictyostelium discoideum* coactosin, a member of the ADF/Cofilin group of actin binding proteins. Binding of CLP to 5LO was found by the yeast two-hybrid system. *In vitro* binding stoichiometry was 1:1, and

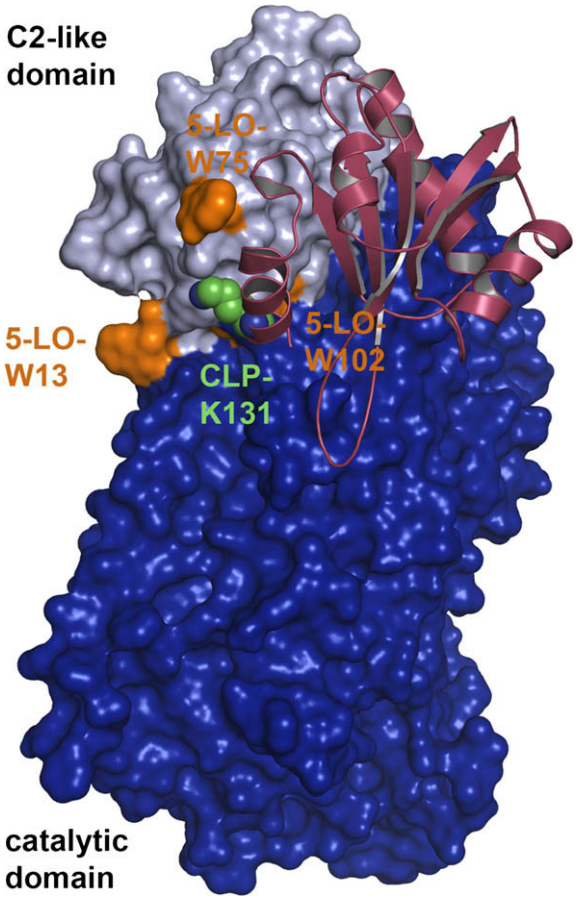

Fig. 2. Model structure of the CLP–5LO complex. Generated from a model structure of 5LO and a structure of CLP (PDB: 1WNJ), as described in [11]. Gray, 5LO C2-like β-sandwich. Blue, 5LO catalytic domain. Trp residues 13, 75, and 102 are in orange. The CLP backbone is in purple, with Lys-131 indicated by green spheres.

coimmunoprecipitation indicated binding also in intact cells [16]. Human CLP also binds F-actin, but a 5LO–CLP-actin trimer has not been demonstrated. Two Lys residues involved in binding to F-actin and 5LO, respectively, are close in the CLP structure, indicating overlapping binding sites. CLP can upregulate and modulate the 5LO pathway *in vitro* [11,17]. Thus, in the absence of PC, CLP supported Ca^{2+}-induced 5LO activity leading to 5-HETE, but not to LTA_4. When CLP was present together with PC, there was a 3- to 5-fold increase of the amount of LTA_4 formed, compared to PC only (Fig. 3). CLP can bind to 5LO in the absence of Ca^{2+}. This association stabilizes 5LO and prevents non-turnover inactivation of the enzyme *in vitro*, over time and at elevated temperature. However, by itself CLP could not induce 5LO activity, rather CLP (as PC) functions as a scaffold for Ca^{2+}-induced 5LO activity.

As an indication for association of CLP and 5LO in cells, the subcellular localizations of the two proteins were compared. After stimulation of PMNL with Ca^{2+} ionophore, both CLP and 5LO were recovered in a nuclear fraction, while in resting cells, CLP and 5LO were cytosolic [17]. Similar results were obtained for Mono Mac 6 cells, when primed with phorbol ester [11]. Thus, it seems that migration of CLP is connected with the previously established migration of 5LO. In the cell, 5LO may be in complex with CLP, and when activated by Ca^{2+} (or Mg^{2+}) this complex is capable of producing 5-HPETE. Formation of LTA_4 is determined by the well established translocation of 5LO to the nuclear membrane, CLP might comigrate with 5LO in this translocation. The 5LO inhibitor hyperforin (from St. John's wort) interrupted binding of 5LO to CLP *in vitro*, and impaired nuclear translocation in human neutrophils [18].

3.3. Trp residues in the 5LO C2-like β-sandwich bind phosphatidyl choline and/or CLP

Binding of PC to the 5LO β-sandwich involves three Trp residues (W13, W75, W102) [5]. Mutagenesis of these residues (to Ala) reduced the affinity of the isolated 5LO C2-like domain to PC, substitution of Trp-102 gave the most prominent effect with 20-fold reduced affinity [5]. However, when all these residues were mutated to Ala in intact 5LO, Ca^{2+}-induced enzyme activity in presence of 25 μg/ml PC was the same as for wt-5LO. Only at relatively low concentrations of PC (2.5 μg/ml) and AA (10 μM) the activity of the triple mutant was reduced to about 25% of wild type 5LO [17]. For each of the three single Trp-mutants, the activity at low PC and AA was about 50% of wild type 5LO [11]. Thus, all three Trp residues seem to contribute similarly, for PC to support 5LO enzyme activity. Apparently, Trp-102 is more important for PC-binding of the isolated 5LO β-sandwich, than for PC to support enzyme activity of intact 5LO. This would be compatible with Trp-102 being hidden in the cleft between the two domains in intact 5LO (compare below), while in the isolated 5LO C2-like domain Trp-102 is exposed.

On the other hand, for CLP to support 5LO activity, Trp-102 in 5LO was essential, and mutagenesis of this residue obliterated binding of CLP to 5LO [11]. Trp-102 is part of a stretch of conserved surface residues (FPCYRW) [6] partially hidden in the cleft between the two domains, as previously demonstrated for the corresponding residue (Trp-100) in 12/15LO [19]. Thus, binding of CLP to 5LO may have an allosteric effect on the association of the two domains in 5LO, these may open up for CLP to directly access Trp-102. Alternatively, as suggested by a model of the CLP–5LO complex (Fig. 2) 5LO-W102 may not bind CLP-K131 directly, but via an interaction network involving 5LO-R165. This model also suggested a direct π-cation interaction of CLP-K131 with 5LO-F14, as well as six hydrogen bonds between 5LO and CLP, three of which involve residues in the 5LO catalytic domain, while the other three involve residues in the β-sandwich. In either case, CLP may influence the relative positions of the two domains in 5LO. Also the association of 5LO to PC membranes is visualized to span the entire protein, with effects on structure [14,15].

3.4. Efficient LTA_4 formation of 5LO requires both phosphatidyl choline and CLP

For the most efficient Ca^{2+}-induced LTA_4 production *in vitro*, it was required that both PC and CLP were present [11,17]. This suggests a complex, comprising 5LO, Ca^{2+}, PC, and CLP. As discussed [17] one could visualize a complex where 5LO binds CLP, and membrane is bound by residues in both 5LO and CLP. A precedent for such complex is the association of pancreatic lipase, colipase, and membranes [20]. Inactive lipase binds to the small colipase (95 amino acids) via its C-terminal β-sandwich, while contact with a lipid–water interphase generates an active form where colipase also contacts residues in the lipase catalytic domain, and "opening of a lid" permits access to the active site. In the active complex, residues both in lipase and in colipase contribute to micelle binding. Interestingly, the C-terminal β-sandwich of pancreatic lipase is virtually identical to the N-terminal β-sandwich of rabbit 15LO, and thus very similar to the 5LO C2-like domain. CLP can bind to 5LO in absence of Ca^{2+}, in the modelling of the CLP–5LO complex (Fig. 2) Ca^{2+} was not included. In the active four-partner complex (containing also Ca^{2+} and PC) the mode of binding between 5LO and CLP may become modified.

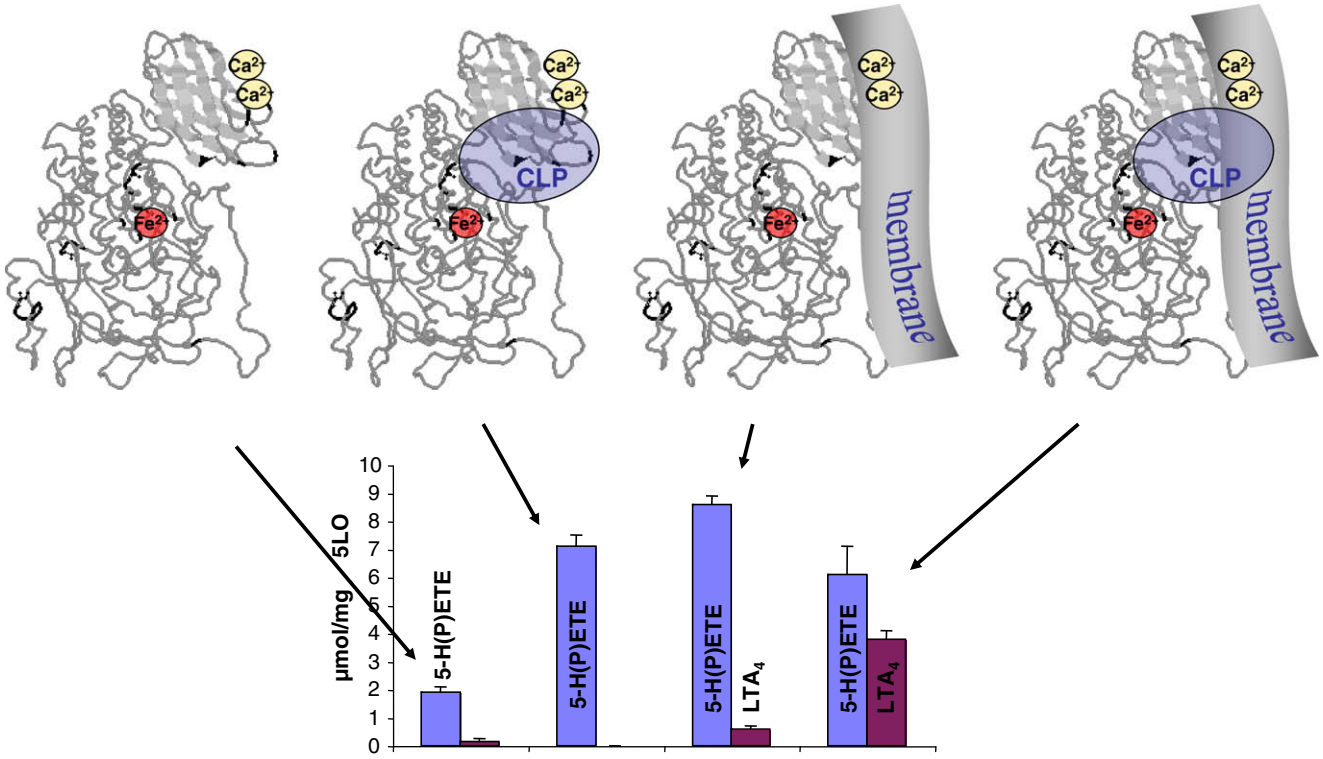

Fig. 3. Ca^{2+}-induced formation of 5-H(P)ETE and LTA_4, by 5LO in presence of scaffold factors CLP (stoichiometry 1:1) and/or phosphatidylcholine (25 μg/ml). From Ref. [11]. Similar data in [17].

4. Regulation of 5LO activity in the cell

Stimuli which induce LT formation (e.g. fMLP, PAF, opsonized zymosan, ionophores) activate both 5LO and cytosolic phospholipase A_2 (cPLA$_2$). In fact, both enzymes share structural (C2 domain) and regulatory properties [8,21,22]. Thus, 5LO and cPLA$_2$ are regulated in two ways, by divalent cations and by phosphorylations, and both activation modes influence the subcellular localization of the enzymes. In activated leukocytes 5LO and cPLA$_2$ associate with the nuclear membrane, where they become close to 5LO activating protein (FLAP), and 5LO gets access to endogenous substrate.

4.1. 5LO, a mobile enzyme

At the nuclear membrane conversion of endogenous AA to LTA_4 can be particularly prominent, and upon cell stimulation, both 5LO and cPLA$_2$ migrate to this locale, where cPLA$_2$ liberates AA from phospholipids. Membrane-bound FLAP binds AA and may facilitate transfer of AA to 5LO. In cells lacking FLAP or when FLAP is inhibited, transformation of endogenous AA by 5LO is blocked [23]. Free AA supplied from exogenous sources, e.g. by transcellular mechanisms from neighbouring cells, can be converted also by cytosolic 5LO. It was suggested that 5LO may be in different cellular loci when exogenous or endogenous AA is metabolized.

In resting cells, 5LO resides either in the cytosol (e.g. in neutrophils, eosinophils, peritoneal macrophages) or in a nuclear soluble compartment associated with chromatin (e.g. in alveolar macrophages, Langerhans cells, rat basophilic leukemia cells). Nuclear import sequences (NIS), rich in basic amino acids, are present both in the N-terminal domain of 5LO, and close to the C-terminus [24]. Priming of resting cells by glycogen or cytokines, or by cell adhesion to surfaces, causes nuclear import of 5LO, in many cell types this confers an increased capacity for subsequent LT biosynthesis [25]. An exception is eosinophils, here nuclear localization suppressed 5LO activity. It was suggested that the multiple NIS in 5LO may allow for a modulated nuclear import [24], in this manner cells may regulate the capacity for subsequent LT production. Also nuclear export sequences are present in 5LO, and inhibition by leptomycin b indicate exportin-1 mediated export [26,27]. For intact cells, phosphorylations of 5LO modulate both nuclear import and export, contributing to regulation of 5LO activity.

4.2. Phosphorylations of 5LO

5LO can be phosphorylated at three residues: Ser-271, by MAPKAP kinases; Ser-663 by ERK2; and Ser-523 by PKA catalytic subunit.

There are several isoforms of p38 Mitogen-activated protein kinase (p38 MAP kinase), which are activated by cell stress or inflammatory cytokines. Activated p38 MAKP in turn phosphorylates MAPKAP kinases 2 and 3 (MK2/3). By in-gel kinase assays 5LO was found to be a substrate for MK2/3, and these 5LO kinases were activated upon stimulation of MM6 cells, PMNL, and B-lymphocytes. Mutation of Ser-271 to Ala in 5LO abolished MK2 catalyzed phosphorylation *in vitro*. Also, phosphorylation by kinases prepared from stimulated PMNL and MM6 cells was clearly reduced, indicating that this is a major site for cellular phosphorylation of 5LO. Compared to the established MK2 substrate heat shock protein 27, 5LO was only weakly phosphorylated *in vitro* by MK2. However, addition of unsaturated fatty acids (AA or oleic acid) strongly upregulated phosphorylation of 5LO *in vitro*. Ser-271 is located within in nuclear export sequence [26,27].

Cell stress can induce LT biosynthesis in leukocytes. Sodium arsenite (chemical stress) was the most efficient MK2/3 stimulus in a B-lymphocyte cell line and in human PMNL. Also other stress stimuli (osmotic stress, heat shock) activated p38 MAPK and stimulated 5LO activity in human PMNL. Sodium arsenite and osmotic

stress were effective also after chelation of Ca^{2+} [28]. Apparently, MK2/3 catalyzed phosphorylation of 5LO is a pathway for stimulation of 5LO in stress-stimulated leukocytes, which is different in character from Ca^{2+} activation of 5LO in ionophore treated cells, see [21,22] for reviews.

Another MAP kinase (ERK2) phosphorylated 5LO *in vitro*, at Ser-663. Also this phosphorylation was stimulated by unsaturated fatty acids [29]. Phosphorylation at this site is probably related to PMA-primed 5LO activity in Mono Mac 6 cells. Without PMA-priming there was actually no translocation of 5LO to the nucleus when MM6 cells were stimulated with ionophore [30]. For PMNL, both ERK2 and p38 MAP kinase activities were important for 5LO activity when the cells were stimulated with AA only (no ionophore). It appears reasonable that cellular activation of 5LO is the result of both phosphorylations and elevated Ca^{2+}, as described for cytosolic phospholipase A_2. Intracellular Ca^{2+} concentrations connected with active 5LO are considerably lower than found for purified 5LO *in vitro* [31].

Phosphorylation at Ser-523 by PKA directly suppresses 5LO catalysis *in vitro* as well as in the cell, and prevents 5LO nuclear localization by interfering with a NIS close to the kinase motif [32]. This provides a molecular basis for the 5LO suppressive effects of exogenous adenosine and increased cAMP, which activate PKA [33]. Interestingly, polyunsaturated fatty acids such as AA, which promote phosphorylation at Ser-271 and Ser-663, prevented cAMP-mediated inhibition of 5LO translocation and product formation in neutrophils, apparently by interaction at a region close to the catalytic site [34].

4.3. 5-Lipoxygenase activating protein (FLAP)

The compound MK-886 was found to inhibit 5LO in intact human leukocytes, but not in broken cells. Using a photoaffinity analog to MK-886, and an MK-886 affinity gel, the 18 kDa protein (FLAP) could be purified from neutrophils, for review see [23]. In osteosarcoma cells transfected with cDNA for 5LO and/or FLAP, both proteins were required for A23187 induced LT production from endogenous AA. FLAP bound to a photoaffinity analog of AA, and *cis*-unsaturated fatty acids as AA competed with inhibitors (BAY X1005, MK-886) for binding to FLAP. Thus, FLAP is thought to function as an AA transfer protein, and FLAP is crucial for conversion of endogenous substrate by 5LO. FLAP also stimulated the utilization of exogenous AA, and greatly (190-fold) stimulated utilization of another exogenous substrate (12(*S*)-HETE). FLAP promotes conversion of 5-HPETE to LTA_4, this is a feature shared with CLP, otherwise there are no obvious similarities between FLAP and CLP. Interestingly, CLP can bind the 5LO product 5(*S*)-HETE, but was not reported to bind AA [35].

In RBL-2H3 cell extracts, FLAP mono-, di-, and trimers were found, and also mixed complexes of FLAP and LTC_4 synthase were described [36]. In neutrophils FLAP was present as mono- and dimers, the dimer stage correlated with LT biosynthesis [37]. In the crystal structure, inhibitor-bound FLAP is a homotrimer, each monomer has four transmembrane helices, connected by two cytosolic and one lumenal loop [38]. The inhibitors bound to membrane-embedded pockets in FLAP, suggesting how they might prevent binding of AA. Association between FLAP and 5LO could be demonstrated in mouse synovial PMN and in IgE-stimulated RBL-2H3 cells, using a membrane-permeant cross-linker [39]. Also, sophisticated antibody-based Fluorescence Lifetime Imaging Microscopy suggested different FLAP complexes on inner and outer nuclear membranes. It was concluded that also FLAP may function as a 5LO scaffold protein.

Most of the FLAP is associated with the nuclear membrane, but also with endoplasmatic reticulum. Lipid rafts are important in membrane trafficking and signalling events. Interestingly, in RBL-2H3 cells FLAP co-localized with the lipid-raft marker flotillin-1, and methyl-β-cyclodextrin which depletes cells of cholesterol and disrupts the membrane rafts, reduced LT biosynthesis [40]. Recently, also 5LO was associated with lipid rafts, in mantle cell lymphoma [41].

4.4. Gender specific 5LO activity in human neutrophils

LT formation in stimulated whole blood or neutrophils from males was found to be substantially lower as compared to females [42]. This was accompanied by changed 5LO trafficking. For female neutrophils, the previously determined (and well established) pattern of 5LO subcellular redistribution was confirmed, meaning that 5LO resides in the cytosol of resting neutrophils, and translocates to the nuclear membrane upon stimulation. However, in male neutrophils, a substantial part of 5LO was located at the perinuclear region already in resting cells, 5LO only marginally redistributed upon stimulation, and less 5LO products were formed. Also the subcellular distribution of CLP was gender-dependent, as CLP colocalized with 5LO in nuclear/non-nuclear fractions from male and female neutrophils. The different 5LO location was related to gender-specific differential activation of extracellular signal-regulated kinases (ERKs), in turn directly related to male/female testosterone levels [42]. This regulation of ERKs and LT formation by androgens may provide a molecular basis for gender differences in the inflammatory response, and in inflammatory diseases as asthma.

5. Concluding remarks

5LO requires scaffold factors, and in addition to PC, also membrane-bound FLAP and soluble CLP have such functions. Available data indicate that CLP binds to and chaperones 5LO in the cytosol of i.e. neutrophils. When 5LO is active in the cytosol [30] and possibly at other sites as lipid bodies [43], CLP may function as the scaffold for activity. When cells are stimulated, CLP redistributes to a nuclear fraction, similar to 5LO. However, it is unclear whether CLP actually binds to 5LO at the nuclear membrane, or if CLP "delivers" 5LO to FLAP. CLP also binds to F-actin, and the nucleus has an actin-network [44] which may bind CLP. When 5LO is active at the nuclear membrane, FLAP may be the sole scaffold, or somehow function together with CLP, how these proteins function together in detail to support 5LO remains to be understood. Extended knowledge about mechanisms for 5LO regulation, including the gender difference in LT biosynthesis, may lead to new possibilities regarding development and use of compounds interfering with the 5LO pathway.

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