5-Lipoxygenase: mechanisms of regulation[1]

Olof Rådmark[2] and Bengt Samuelsson

Department Medical Biochemistry and Biophysics, Division of Physiological Chemistry II, Karolinska Institutet, S-17177 Stockholm, Sweden

Abstract **5-Lipoxygenase (5-LO) catalyzes two steps in biosynthesis of leukotrienes (LTs), a group of lipid mediators of inflammation derived from arachidonic acid (AA). LT antagonists are used in treatment of asthma; more recently a potential role also in atherosclerosis has raised considerable interest. Furthermore, possible effects of 5-LO metabolites in relation to tumorigenesis have emerged. Thus, an understanding of the biochemistry of this lipoxygenase has potential implications for treatment of various diseases.—** Rådmark, O., and B. Samuelsson. **5-Lipoxygenase: mechanisms of regulation. *J. Lipid Res.* 2009.** 50: **S40–S45.**

Supplementary key words arachidonic acid • leukotriene • inflammation

Leukotrienes (LTs) are inflammatory mediators causing, for example, phagocyte chemotaxis and increased vascular permeability. In leukotriene biosynthesis 5-lipoxygenase (5-LO) catalyzes oxygenation of arachidonic acid (AA) to 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE), and further dehydration to the allylic epoxide leukotriene A_4 (1). As one of six human lipoxygenases, 5-LO is expressed primarily in various leukocytes: polymorphonuclear leukocytes (neutrophils and eosinophils), monocytes/macrophages, mast cells, B-lymphocytes, dendritic cells, and foam cells of human atherosclerotic tissue. LTA_4 is further converted by LTA_4 hydrolase to the dihydroxyacid LTB_4, and by LTC_4 synthases to the glutathione conjugate LTC_4. The other cysteinyl-LTs are formed by hydrolytic removal of γ-Glu and Gly from LTC_4 (yielding LTD_4 and LTE_4). In proinflammatory contexts, LTs typically stimulate cellular responses, which are quick in onset and of short duration (as smooth muscle contraction, phagocyte chemotaxis, increased vascular permeability). These are mediated via G-protein coupled receptors, BLT1/2 for LTB_4 and CysLT1/2 and GPR17 for the cys-LTs. Antileukotrienes (CysLT1 receptor antagonists) are used in asthma treatment (2, 3). For more complete references, see (4–6).

REGULATION OF 5-LO EXPRESSION

The large 5-LO gene (71.9 kbp, **Fig. 1**) is located on chromosome 10, and contains 14 exons (7). The promoter region contains eight GC-boxes but lacks TATA and CAT boxes, resembling promoters of typical house-keeping genes. Nevertheless, 5-LO expression is mainly restricted to leukocytes. Methylation-specific DNA sequencing showed that the 5-LO core promoter is completely methylated in the cell lines U-937 and HL-60TB (do not express 5-LO protein), while it is unmethylated in HL-60 cells (which express 5-LO protein upon differentiation) (8). In vitro methylation of the promoter strongly inhibited activity in reporter genes assays, while treatment of U-937 and HL-60TB cells with the demethylating agent 5-aza-2'deoxycytidine restored 5-LO expression. Thus, DNA methylation determines if a cell type can express 5-LO or not. In addition to the normal expression in various leukocytes, 5-LO is also found in many epithelial tumor cells (5, 9). Possibly, DNA methylation is responsible for suppression of 5-LO expression in most cell types, while aberrant methylation may lead to up-regulated 5-LO expression in tumor cells.

GC-boxes in the 5-LO gene

Eight GC-boxes are found in the proximal part of the human 5-LO gene promoter (Fig. 1). Five of these are arranged in tandem and are recognized by transcription factors Sp1 and Egr-1, as shown by gel-shift assays. Expression of endogenous 5-LO (in Mono Mac 6 cells) was reduced by mithramycin, a drug that blocks GC-boxes. Also, a novel Sp1 binding site was found, beside an initiator-like sequence, which includes the major transcription initiation site (TIS) (10). In early studies, the proximal GC-rich part of the promoter was crucial for the expression of reporter genes. In a more recent promoter analysis, the five GC-boxes in tandem were excised while keeping upstream promoter sequence. This resulted in only a minor (47%) decrease of promoter activity; instead the solitary GC-box closer to the TIS seemed more important (11). Epigenetic mechanisms

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[1] This paper is dedicated to Professor E. J. Corey in connection with his 80th birthday.

[2] To whom correspondence should be addressed.
e-mail: olof.radmark@ki.se

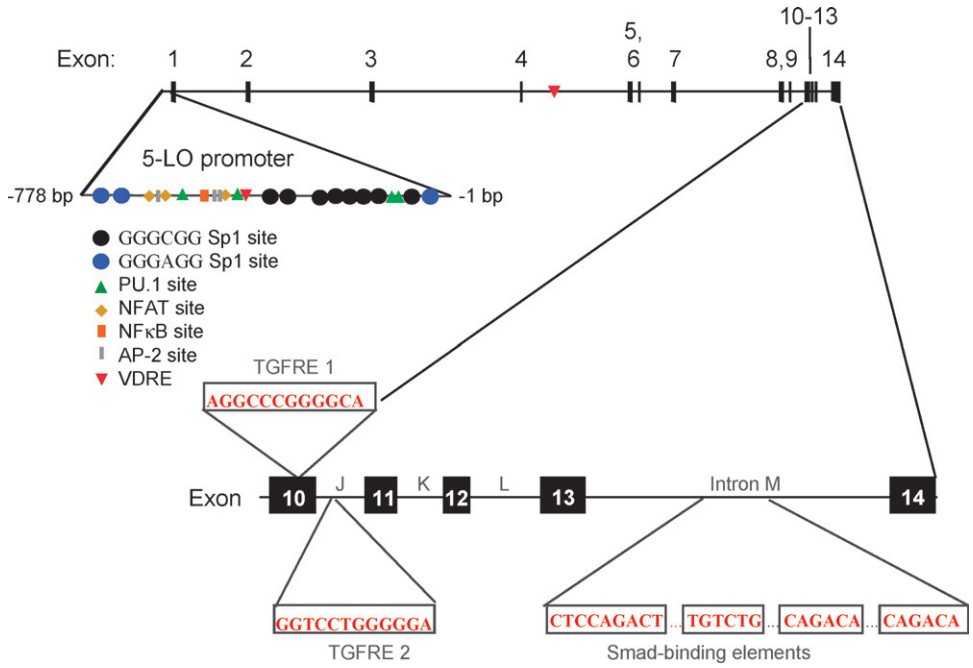

Fig. 1. The human 5-Lipoxygenase (5-LO) gene (71.9 kilobases) is localized on chromosome 10, and it is divided into 14 exons.

regulate the access of transcription factors; ChIP analysis showed that the histone deacetylase inhibitor trichostatin A increased binding of Sp1/Sp3, as well as of RNA polymerase II to the core part of the 5-LO gene promoter, in Mono Mac 6 cells (11).

Naturally occurring mutations in the human 5-LO promoter consist of deletions or additions of Sp1-binding sites, to the normally five tandem GC boxes (12). A pharmacogenetic association was found between the mutant genotypes and responses to a 5-LO inhibitor (ABT-761) in an asthma clinical trial (13). This finding implies that the mutations should lead to reduced 5-LO expression. This may be in accordance with the finding that eosinophils from asthmatics carrying a mutated non5/non5 genotype expressed less 5-LO mRNA and produced less LTC_4 (14), and the increased susceptibility to tuberculosis for carriers of ALOX5 variants (15). On the other hand, in relation to atherosclerosis, mutations of the GC-boxes in tandem were connected with increased intima-media thickness and increased plasma level of C-reactive protein (16). This would seem compatible with an increased production of proinflammatory LTs, due to up-regulated 5-LO expression. The effects of these mutations on 5-LO reporter gene expression have varied between cell lines. Together, the effect of mutations in this part of the GC-rich human 5-LO gene promoter is not easily understood. In the mouse 5-LO gene there are no GC-boxes in tandem, the core promoter contains only one Sp1/3 binding site (17).

Strong up-regulation of 5-LO expression by TGFβ and $1,25(OH)_2D_3$

TGFβ and $1,25(OH)_2D_3$ give a strong (100-fold) up-regulation of 5-LO protein in differentiating Mono Mac 6 cells. However, no induction of 5-LO promoter activity by TGFβ and $1,25(OH)_2D_3$ could be detected for reporter gene constructs containing various parts of the promoter, apparently the −6079 to +53 promoter region mediates basal 5-LO transcription. This is in accordance with earlier data suggesting that the effect of $1,25(OH)_2D_3$ and TGFβ was due to posttranscriptional events, such as transcript elongation and maturation.

However, $1,25(OH)_2D_3$ and TGFβ did up-regulate the response for reporter gene plasmids containing the complete 5-LO coding sequence plus the introns J-M (18). Peculiarly, these effects of $1,25(OH)_2D_3$ and TGFβ were independent of 5-LO promoter sequence. Using this assay system, two functional response elements for the TGFβ effectors Smad3 and 4 were identified in the distal part of the 5-LO gene (exons 10–14, Fig. 1) (18). Recently, also a downstream vitamin D responsive element (VDRE) was found (in intron 4, at +42,000), which was characterized as one of the strongest of the human genome (reporter gene response in MCF-7 cells) (19). In the genomic context, the function of these downstream Smad and VDREs is unclear, but it can be speculated that there is a role for 5-LO transcript elongation and maturation. VDREs are present also in the promoter (−779 to −229); binding of the vitamin D receptor was demonstrated by EMSA, DNA footprinting, and chromatin immunoprecipitation assays (19, 20). By DNA looping the downstream elements may contact the promoter and regulate transcription (19).

THE 5-LO PROTEIN, FACTORS DETERMINING ENZYME ACTIVITY IN VITRO

Mammalian 5-LOs are monomeric enzymes containing 672 or 673 amino acids. A model structure, based on the

crystal structure (1LOX) of the ferrous form of rabbit reticulocyte 15LO (40% sequence identity with 5-LO) (21), consists of a N-terminal β-sandwich (residues 1–114) and a C-terminal catalytic domain (residues 121–673) binding the prosthetic iron (**Fig. 2**). As for many mononuclear nonheme iron(II) enzymes a 2-His-1-carboxylate facial triad (22) anchor the iron also in 5-LO. For 5-LO, mutagenesis studies thus indicate that two conserved His (H372, H550) and the C-terminal Ile-673 constitute the triad (Fig. 2). In addition, H367 and N554 might function as replaceable ligands to iron (23). The function of the C terminus as one of the iron ligands is stabilized by a hydrogen bond (Asn-669 to His-399), forming a C-terminal loop. Electron paramagnetic resonance (EPR) studies have showed that the iron of purified recombinant 5-LO is ferrous; treatment with 5-HPETE and also other lipid hydroperoxides gave ferric 5-LO. Similar to soybean LO-1, human 5-LO has an axial EPR signal (g = 6.2).

Ca^{2+}, PC, and CLP

The N-terminal β-sandwich of 5LO resembles a C2 domain with typical ligand binding loops (24). Residues in these loops bind Ca^{2+}, cellular membranes, and coactosin-like protein (CLP). Calcium (Ca^{2+}) can activate 5-LO by inducing binding to phosphatidyl choline membranes and/or CLP. ATP binds to 5-LO and increases enzyme activity, but hydrolysis of ATP is not required. Instead, it appears that ATP stabilizes the enzyme. ATP affinity is used for purification of 5-LO, although the binding site has not been determined.

Ca^{2+} activates 5-LO purified from human leukocytes (25). The EC50 for Ca^{2+} activation of purified 5-LO is low (1–2 μM); full activation is reached at 4–10 μM. 5-LO 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 of maximum binding averaged around two Ca^{2+} per 5-LO. Similar results (two Ca^{2+} per 5-LO, K_{Ca} 7–9 μM) were obtained for the His-tagged C2-like domain (residues 1–115). Mutagenesis indicated that residues in the ligand binding loop 2 (Asn-43, Asp-44, Glu-46, Fig. 2) of the 5-LO C2-like domain are important for Ca^{2+} binding, and for Ca^{2+} activation of enzyme activity (24). Also Mg^{2+}, at millimolar concentrations present in cells, can activate 5-LO in vitro. 5-LO has some basal activity in the absence of Ca^{2+} and Mg^{2+}, the divalent cation is not part of the catalytic mechanism.

Many C2 domains mediate Ca^{2+}-induced membrane association. The enzyme activity of 5-LO from human leukocytes depended on microsomal membranes (25), and synthetic phosphatidyl choline (PC) vesicles could replace the cellular membrane fraction as a stimulatory factor. Binding of 5-LO to synthetic PC liposomes was induced by Ca^{2+}, and Ca^{2+} (as well as Mg^{2+}) increased the hydrophobicity of 5-LO in a phase partition assay. The isolated 5-LO C2-like β-sandwich had a high affinity for zwitterionic PC vesicles and three Trp residues in the ligand binding loops (W13, W75, W102, Fig. 2) were important for PC binding (26). It was suggested that the PC selectivity directs 5-LO to the nuclear envelope. Accordingly, rhe β-sandwich was required for translocation of GFP-5-LO constructs to the nuclear membrane in HEK 293 cells (27).

Ca^{2+}-induced binding to PC stabilized the structures of 5-LO protein and the membrane, and it was found that 5-LO can bind also to cationic phospholipids (28). This binding was stronger and occurred in the absence of

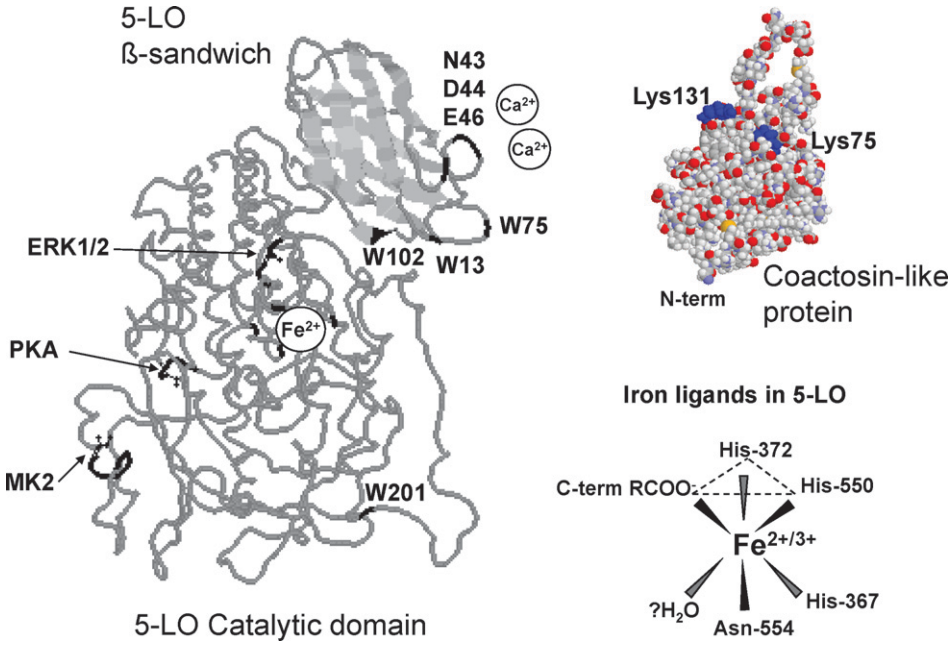

Fig. 2. Model structure of 5-LO (generated with RasMol) based on the crystal structure for the ferrous form of rabbit reticulocyte 15-LO (PDB: 1LOX) (21). ERK1/2, extracellular signal-regulated kinase; MK2, mitogen-activated protein kinase activated protein (MAPKAP) kinase 2; PKA, protein kinase A. Inserts show the ligands to iron in 5-LO and the structure of coactosin-like protein (CLP) (PDB: 1WNJ) (51).

Ca^{2+}, but Ca^{2+} was required for 5-LO activity. It was suggested that 5-LO can bind to membranes in "productive or nonproductive modes" (i.e., membrane binding per se might not confer 5-LO activity). Increased membrane fluidity favored 5-LO association, and it was argued that this should be the factor directing 5-LO to the AA enriched nuclear envelope. Interestingly, addition of cholesterol to a membrane preparation in vitro reduced 5-LO activity by half, and cholesterol sulfate could also inhibit 5-LO in intact cells.

Human CLP (142 amino acids) is similar to *Dictyostelium discoideum* coactosin, a member of the ADF/Cofilin group of actin binding proteins. Binding of CLP to 5-LO was found by the yeast two-hybrid system (29). In vitro binding stoichiometry was 1:1, and coimmunoprecipitation indicated binding also in intact cells (30). Human CLP also binds F-actin, and mutagenesis showed the involvement of Lys-75 and Lys-131 in binding to F-actin and 5-LO, respectively. These Lys residues are close in the CLP structure indicating overlapping binding sites (Fig. 2). CLP can up-regulate and modulate the 5-LO pathway in vitro (31). When present together with PC, CLP gave a 3-fold increase of the amount of LTA_4. These effects required protein interaction via Trp residues in ligand binding loops of the 5-LO β-sandwich; binding and stimulatory effects of CLP were abolished for the 5-LO-W13A,W75A,W102A triple mutant. CLP can bind to 5-LO in the absence of Ca^{2+} (30), but Ca^{2+} was required for 5-LO activity. After stimulation of polymorphonuclear leukocytes (PMNL) with Ca^{2+} ionophore, CLP and 5-LO were recovered in a nuclear fraction, while in resting cells, CLP and 5-LO were cytosolic (31). Cellular 5-LO may always 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 5-LO to the nuclear membrane; CLP might comigrate with 5-LO in this translocation. A recent finding is that CLP can bind the 5-LO product 5(S)-HETE (32).

REGULATION OF 5-LO ACTIVITY IN THE CELL

Considering the biological actions of LTs, it is reasonable that cellular 5-LO activity is tightly controlled. The amount of free AA available as substrate for 5-LO as well as its accessibility for 5-LO are determinants for LT biosynthesis. Regulation of cellular LT production involve intracellular migration of 5-LO as well as of cytosolic phospholipase A_2 ($cPLA_2$); in activated leukocytes both these enzymes associate with the nuclear membrane.

5-LO, a mobile enzyme

At the nuclear membrane conversion of endogenous AA to LTA_4 can be particularly prominent (33), and upon cell stimulation, 5-LO and $cPLA_2$ migrate to this locale, where $cPLA_2$ liberates AA from phospholipids. Membrane-bound 5-LO-activating protein (FLAP) may facilitate transfer of AA to 5-LO, in cells lacking FLAP or when FLAP is inhibited, transformation of endogenous AA by 5-LO is blocked (33). In the recent crystal structure, FLAP is a homotrimer (34). In cell extracts, various FLAP multimers were found, and, interestingly, mixed complexes of FLAP and LTC_4 synthase have been detected (35). Free AA supplied from exogenous sources (e.g., from plasma or by transcellular mechanisms from neighboring cells) can be converted also by cytosolic 5-LO. In fact, 5-LO might be in different cellular loci when exogenous or endogenous AA is metabolized.

In resting cells, 5-LO 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, rich in basic amino acids, are present in the N-terminal domain of 5-LO and close to the C terminus (36, 37). Priming of resting cells by glycogen or cytokines, or by cell adhesion to surfaces, causes nuclear import of 5-LO; in many cell types this confers an increased capacity for subsequent LT biosynthesis. An exception is eosinophils, in this cell type nuclear localization suppressed 5-LO activity. It was suggested that the multiple nuclear import sequences in 5-LO may allow for a modulated nuclear import (36); in this manner cells may regulate the capacity for subsequent LT production. Nuclear export sequences have also been identified in 5-LO (38). For intact cells, phosphorylations of 5-LO modulate nuclear import and export, and contribute to regulation of 5-LO activity.

Phosphorylations of 5-LO

5LO can be phosphorylated in vitro on three residues: Ser-271, by mitogen-activated protein kinase activated protein (MAPKAP) kinase 2 (39); Ser-663 by ERK2 (40); and Ser-523 by PKA catalytic subunit (41).

p38 Mitogen-activated protein kinase (p38 MAP kinase) exists in several isoforms, which are activated by cell stress or inflammatory cytokines. Activated p38 MAKP in turn phoshorylates 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 alanine 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 with 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) up-regulated phosphorylation of 5LO by active MK2 in vitro.

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+} (42). Apparently, MK2/3 catalyzed phoshorylation 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 Ref. 4 for review.)

Another MAP kinase (ERK2) was found to phosphorylate 5LO in vitro on Ser-663. Also this phosphorylation was stimulated by unsaturated fatty acids (40). Phosphorylation of 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. For PMNL, it was appeared that ERK2 and p38 MAP kinase activities were important for AA-induced 5LO product formation (no ionophore).

Phosphorylation at Ser-523 by PKA directly suppresses 5-LO catalysis in vitro as well as in the cell (41, 43) and prevents 5-LO nuclear localization by inhibiting the nuclear import function of a nuclear import sequences close to the kinase motif (43). This appears to be the molecular basis for the 5-LO suppressive effects of exogenous adenosine and increased cAMP, which activate PKA (44). Interestingly, polyunsaturated fatty acids such as AA, which promote phosphorylation at Ser-271 and Ser-663, prevented cAMP-mediated inhibition of 5-LO translocation and product synthesis in activated neutrophils, apparently by interaction at a region close to the catalytic site (45).

Gender-specific 5-LO activity in human neutrophils

A recent finding of considerable interest is that LT formation in stimulated whole blood or neutrophils from males was substantially lower as compared with females (46). This was accompanied by changed 5-LO trafficking. For female neutrophils, the previously determined (and well-established) pattern of 5-LO subcellular redistribution was confirmed, meaning that 5-LO resides in the cytosol of resting neutrophils and translocates to the nuclear membrane upon stimulation. However, in male neutrophils, a substantial part of 5-LO was located at the perinuclear region already in resting cells, 5-LO only marginally redistributed upon stimulation, and less 5-LO products were formed. Previous findings suggest that 5-LO already associated with the nuclear envelope at the time of cell stimulation may be less active and/or display a changed substrate specificity (47, 48). Thus, the perinuclear 5-LO localization induced by 5α-DHT may be a regulatory mechanism in males, which attenuates LT formation at sites of inflammation.

This different 5-LO location was related to gender-specific differential activation of extracellular signal-regulated kinases (ERKs), in turn directly related to male/female testosterone/5α-dihydrotestosterone levels (46). Apparently, regulation of ERKs and leukotriene formation by androgens provides a molecular basis for gender differences in the inflammatory response and in inflammatory disease as asthma. This may explain the better efficiency for the cysLT-1 antagonist montelukast as asthma treatment in girls reaching puberty compared with boys at same age (49). Also, in a European multicentre study, females dominated among severe asthma patients (50).

PERSPECTIVES

Although 5-LO has been studied intensely since the enzyme activity was first described in 1976, several issues remain unresolved. The mechanism behind the prominent increase of 5-LO expression during differentiation of Mono Mac 6 cells with TGFβ and $1,25(OH)_2D_3$, involving distal parts of the gene is unclear. Can the mutations regarding the number of GC-boxes in tandem in the 5-LO gene promoter link to increased or decreased protein expression, or to other mechanisms connecting to asthma drug response and atherosclerosis? Intranuclear localization of 5-LO confers a higher activity in most cell types. How this occurs is unclear as well as if there is some other role for 5-LO inside the nucleus. The structure of 5-LO has not been determined; the model structure is based on rabbit 15-lipoxygenase. How does ERK attenuate 5-LO activity in male neutrophils? Although inhibition of LT biosynthesis may be beneficial in inflammatory diseases, only one 5-LO inhibitor (Zileuton) has reached the market. New findings regarding 5-LO enzyme activation, and the gender difference in LT biosynthesis, may lead to new possibilities regarding development and use of 5-LO inhibitors.

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