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Mammalian lipoxygenases and their biological relevance

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Abstract

Lipoxygenases (LOXs) form a heterogeneous class of lipid peroxidizing enzymes, which have been implicated in cell proliferation and differentiation but also in the pathogenesis of various diseases with major public health relevance. As other fatty acid dioxygenases LOX oxidize polyunsaturated fatty acids to their corresponding hydroperoxy derivatives, which are further transformed to bioactive lipid mediators (eicosanoids and related substances). On the other hand, lipoxygenases are key players in regulation of the cellular redox homeostasis, which is an important element in gene expression regulation. Although the first mammalian lipoxygenases were discovered 40 years ago and although the enzymes have been well characterized with respect to their structural and functional properties the biological roles of the different lipoxygenase isoforms are not completely understood. This review is aimed at summarizing the current knowledge on the physiological roles of different mammalian LOX-isoforms and their patho-physiological function in inflammatory, metabolic, hyperproliferative, neurodegenerative and infectious disorders.

Keywords

eicosanoids; inflammation; atherosclerosis; cancer; brain; stroke; infection

1. Introduction

Lipoxygenases (LOXs) are non-heme iron-containing dioxygenases [1, 2] that catalyze dioxygenation of polyunsaturated fatty acids containing at least two isolated cis-double bonds (Fig. 1). In mammalian cells linoleic acid (C18:Δ2, n-6) and arachidonic acid (C20:Δ4, n-6) are the most abundant polyenoic fatty acids that serve as substrates for the different mammalian LOX-isoforms. In general, mammalian LOXs prefer free fatty acids as substrate over polyenoic fatty acid containing ester lipids but the cellular concentration of free fatty acids is rather low. Thus, an active LOX pathway requires liberation of substrate fatty acids from the ester lipids localized in the cellular membranes. After hydrolytic cleavage of the membrane ester lipids catalyzed by cytosolic phospholipase A2 [3] the

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liberated fatty acids [mainly arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] are alternatively oxygenated by cyclooxygenases (COX) to G-prostaglandins (PGG2 in case of AA, PGG3 in case of EPA, PGG4 in case of DHA) or by LOX isoforms to various hydroperoxy derivatives of the substrate fatty acids [2]. The primary products of the LOX pathway are subsequently converted to a large array of bioactive lipid mediators, which include leukotrienes [4], lipoxins [5], hepoxilins [6], eoxins [7], resolvins [8], protectins [9] and others. However, the classical concept of the arachidonic acid cascade may not be the only way, by which LOXs exhibit their bioactivity. There are at least two alternative scenarios (Fig. 2): i) Some LOX isoforms are capable of oxygenating polyenoic fatty acids if they are constituents of phospholipids [10] or cholesterol esters [11]. The introduction of a hydrophilic peroxide group into the hydrophobic tail of a fatty acid changes the physico-chemical properties of the ester lipids. Clustering of oxidized lipids within the lipid bilayer of a biomembrane leads to the formation of “hydrophilic pores”. By this mechanism the barrier function of the membrane is impaired which may lead to cellular dysfunction. ii) The cellular redox state is of major cell physiological relevance. It impacts the gene expression pattern of a given cell population [12] on transcriptional and post-transcriptional levels and thus determines the cellular phenotype. In each cell the redox homeostasis is maintained by the balanced equilibrium of pro- and anti-oxidative processes and LOXs constitute some of the key pro-oxidative players in the redox homeostasis. LOX-catalyzed formation of hydroperoxy lipids impacts the activity of redox-dependent transcription and/or translation factors [13], which in turn leads to up- and/or down-regulation of the expression of redox sensitive genes.

The molecular details of how the different LOX-isoforms exhibit their bioactivity have been explored for many years and a large number of reports employing various loss-of-function (siRNA-mediated expression knockdown, knockout mice) as well as gain-of-function (cellular transfection studies, transgenic animals) strategies have provided a deeper insight into the biological importance of LOXs in health and disease. Nonetheless, our knowledge of the biological role of various LOX-isoforms, in particular for *ALOX15*, *ALOX15B*, and *ALOX12* is still somewhat limited. This review is aimed at summarizing and critically evaluating the experimental data characterizing the physiological and patho-physiological roles of various LOX-isoforms in mammals. Of course, LOXs have been the topic of previous reviews and a PubMed search with the key words “lipoxygenase and review” yielded some 1700 hits. However, most of these reviews cover selected areas of LOX research such as LOX enzymology [1], *ALOX5* pathway and leukotriene signaling [2] or LOX in bone disease [14]. To the best of our knowledge there is no recent review paper summarizing the current knowledge of the biological role of mammalian LOX isoforms in health and disease.

During the past decades LOX research has developed rapidly and a PubMed search with the keyword “lipoxygenase” gave some 15,600 hits. Since 2003 about 500 articles have been published annually and because of space limitations it was not possible to reference here even 10% of these reports. Thus, although we tried to make a balanced selection we might have overlooked important articles and we apologize to those distinguished colleagues whose work we have not had sufficient space to reference.

2. Lipoxygenase distribution, classification and properties

LOX occur in two (bacteria, eukarya) of the three domains of terrestrial life [1, 15] but their occurrence in archaea remains unclear (Fig. 3). The genomic sequences of selected archeae (*Methanococcus voltae*, *Halorubrum kocurii*) also contain LOX-like sequences but in the absence of any functional data it remains unclear if these sequences encode for a functional LOX-isoform. When we performed multiple amino acid alignments of these putative LOX sequences with the primary structure of well-characterized pro- and eukaryotic LOXs we observed only low (<25%) degrees of amino acid conservation. Moreover, we did not find conservation of the iron liganding residues suggesting that the sequences of interest may not encode for functional LOXs. The occurrence of LOX in single cell organisms, in plants and lower metazoa [15–17] has been reviewed before but distribution of LOX isoforms in multicellular vertebrates has not been summarized systematically.

2.1. Classification of mammalian lipoxygenases and LOX genes

The human genome involves six functional LOX genes (ALOX15, ALOX15B, ALOX12, ALOX12B, ALOXE3, ALOX5), which encode for six different LOX-isoforms [18]. Except for the ALOX5 gene, which was mapped to chromosome 10, all other LOX genes are localized in a joint gene cluster on chromosome 17. The corresponding mouse genes [18] were detected in syntenic regions on chromosome 6 (alox5) and 11 (other LOX-isoforms). Originally, the human LOX isoforms were classified with respect to their specificity of arachidonic acid oxygenation but this nomenclature turned out to be misleading and caused confusion among scientists not working in the LOX field [1]. These days the gene nomenclature is frequently employed to define the LOX isoenzyme and Table 1 summarizes human and murine LOX-isoforms and assigns names of the genes to the different isoenzymes. For this review we will use the names of the genes also when we talk about the corresponding enzymes. To differentiate between genes and proteins we employ italic letters when referring to enzymes but use normal letters when referring to the genes.

The ALOX15 gene encodes for the 12/15-LOX, which is expressed at high levels in eosinophils [19], broncho-alveolar epithelial cells [20] and interleukin 4 treated monocytes [21]. The ALOX15B gene encodes for 15-LOX2, which is highly expressed in epithelial cells [22, 23]. The ALOX12 gene encodes for the platelet-type 12-LOX (pl12-LOX), which is expressed at high levels in blood platelets [24] but also occurs in the skin [25]. The ALOX12B gene [26, 27], which encodes for a 12R-lipoxygenating enzyme and the ALOXE3 gene [28, 29] encode for two distinct epidermis-type LOX isoforms, which are co-expressed in the skin. These enzymes have been implicated in epidermal differentiation [30] and appear to be important for the development of the epidermal water barrier [31, 32]. The ALOX5 gene encodes for a 5-lipoxygenating enzyme, which plays a major role in leukotriene biosynthesis [2, 33].

In mice the situation is somewhat different (Table 1). Mouse *Alox12*, *Alox12b*, *Aloxe3* and *Alox5* share high degrees of amino acid conservations with their human orthologs and exhibit similar enzymatic properties. However, this is not the case for mouse *Alox15* and mouse *Alox15b*. In fact, mouse *Alox15* is a 12-lipoxygenating enzyme converting arachidonic acid mainly to 12S-HpETE [34]. In contrast, the human ortholog exhibits a 15-

lipoxygenating activity [35]. Because of its reaction specificity and its high-level expression in murine leukocytes mouse *Alox15* has previously been named leukocyte-type 12-LOX but this nomenclature should not be used any more. In general, LOXs, which have previously been named leukocyte-type 12-LOXs [mice [34], rats [36], pigs [37] cattle [38], macaca [39] and other mammals), should be classified as 12-lipoxygenating *ALOX15* isoforms. Analysis of the completely sequenced genomes of these and additional mammalian species did not provide any evidence for the simultaneous existence of separate ALOX15 and leukocyte-type 12-LOX genes in a single mammalian species. Even in rabbits, where 15- and 12-lipoxygenating *ALOX15* variants are expressed [40], only a single copy ALOX15 gene exists. For the time being it remains unclear how a single ALOX15 gene is able to encode in a tissue specific manner [40] for two functionally distinct enzyme species, but post-translational mRNA modification [41] might be involved. It should explicitly be stressed here that in humans there is a single copy ALOX15 gene but there is no additional gene encoding for a leukocyte type 12-LOX. On the other hand, mice, rats, pigs, cattle, macaca and others express 12-lipoxygenating *ALOX15* isoforms. The molecular basis for the variable reaction specificity of *ALOX15* orthologs from different species has been explored in detail [39, 42] and multiple mutagenesis studies have indicated that single amino acid exchanges at critical positions convert the 15-lipoxygenating human *ALOX15* into a 12-lipoxygenating isoform [1, 43]. Inversely, the 12-lipoxygenating mouse *Alox15* (formerly called mouse leukocyte-type 12-LOX) can easily be converted into a 15-lipoxygenating enzyme by L353F exchange [44].

Human *ALOX15B* converts arachidonic acid almost completely to 15S-HpETE [22]. In contrast, the mouse ortholog, which shares a high degree of overall amino acid conservation with the human enzyme, exhibits an arachidonic acid 8S-lipoxygenating activity [23]. Site directed mutagenesis of Tyr603 and His604 of human *ALOX15B* to the corresponding residues present at these positions in murine *Alox15b* (Tyr603Asp+His604Val) leads to a complete shift in the positional specificity of arachidonic acid oxygenation from 15S-HpETE to 8S-HpETE formation [45]. The inverse mutagenesis strategy starting with human *ALOX15B* leads to partial alterations in the reaction specificity [45]. When we compared (data not shown) the *ALOX15B* amino acid sequences of different mammals (man, chimpanzee, gorilla, orangutan, macaca, baboon, cattle, pigs, rat) we found that all of them share the human motif (Asp-Val or Asp-Ile). Only mice have a Tyr-His combination at these positions. Thus, among mammals mice are somewhat unique and although not tested for other mammals arachidonic acid 15-lipoxygenation may be predicted for other (chimpanzee, gorilla, orangutan, macaca, baboon, cattle, pigs, rat) mammalian *ALOX15B* orthologs. It would be of mechanistic interest to experimentally test this prediction and explore in more detail the biological background of this unusual reaction specificity of mouse *Alox15b*.

2.2. Enzymatic properties of mammalian lipoxygenases

Mammalian LOXs are single polypeptide chain proteins that fold into a two-domain structure (Fig. 4). The small (about 15 kDa) N-terminal domain consists of several parallel and anti-parallel β-sheets and has been implicated in activity regulation and membrane binding. The C-terminal catalytic domain consists of several helices and contains the catalytic nonheme iron localized in the putative substrate-binding pocket. For mammalian

LOXs complete crystal structures are currently available for rabbit *ALOX15* [46, 47], which serves as a suitable model for the human ortholog; for a stabilized version of the human *ALOX5* [48]; for the catalytic domain of porcine *ALOX15* [49]; and for human *ALOX15B* [50]. In addition, X-ray data have been published for a phosphorylation-mimicking mutant (Ser663Asp) of the stabilized version of human *ALOX5* [51]. However, these data need to be interpreted with care since the functional consequences of the phosphorylation mimicking mutations (Ser663Asp exchange converts the reaction specificity of the stabilized human *ALOX5* from 5- to 15-lipoxygenation) could not be confirmed for native *ALOX5* orthologs of man, mice and zebrafish [52].

In aqueous solutions the structure of proteins is less rigid than in crystals. To compare the degree of motional flexibility of rabbit *ALOX15* and soybean-LOX1, small angle X-ray scattering (SAXS), dynamic fluorescence, and fluorescence resonance energy transfer measurements were carried out. The results suggest that rabbit *ALOX15* is more susceptible to temperature-induced structural alterations and exhibits a higher degree of global conformational flexibility [53]. There are several processes contributing to global structural flexibility of rabbit *ALOX15*: i) Interdomain movement: Comparative SAXS measurements on aqueous solutions of recombinant rabbit *ALOX15* and its catalytically active N-terminal truncation mutant (no N-terminal β-barrel domain) suggested the possibility of interdomain movement [54]. Such interdomain movement was not confirmed for the soybean enzyme [55]. Although SAXS data in general can be interpreted in different ways [56] more recent molecular investigations into the dimerization behavior of rabbit *ALOX15* [57] and molecular dynamics simulations [58] confirmed the possibility of interdomain movement for this enzyme. For the solution structure of human *ALOX12* a similar interdomain movement has been suggested [59]. ii) Alternative conformers: Reevaluation [47] of the X-ray data set obtained for the crystallized rabbit *ALOX15* [46] suggested that the enzyme undergoes conformational changes when binding an inhibitor at the active site. Helices surrounding the catalytic center appear to relocate upon ligand binding. However, it remains unclear whether these structural rearrangements are peculiar to the active site probe (inhibitor) used for crystallization or whether binding of substrate fatty acids at the active site also induces similar structural alterations. iii) Allosteric properties: Human *ALOX5* [60], *ALOX15* and *ALOX15B* [61, 62] appear to exhibit allosteric properties. Although the binding sites for allosteric effectors have not been identified for *ALOX15* and *ALOX15B* kinetic data suggest the existence of different enzyme conformers. Interestingly, the allosteric regulators of human *ALOX15* do not affect the catalytic activity of the less flexible soybean *LOX1* [63]. iv) Enzyme dimerization: For a long time LOXs have been suggested to function as monomeric enzymes. However, more recent data on recombinant human *ALOX15* [64], human *ALOX12* [59] and human *ALOX5* [65] suggests the existence of LOX dimers in aqueous solutions. Human *ALOX15* may undergo ligand-induced dimerization and molecular dynamics simulations suggested that LOX dimers are surprisingly stable in the presence of substrate fatty acids. Introduction of negatively charged residues (Trp181Glu, His585Glu, LeuL183Glu, Leu192Glu) at the protein surface disturbs monomer interactions compromising the catalytic activity of the mutants [64]. In addition, the rabbit *ALOX15* forms oligomers upon membrane binding [66].

The catalytic cycle of the LOX reaction involves four consecutive elementary reactions [1, 2]: i) hydrogen abstraction from a bisallylic methylene forming a carbon centered fatty acid radical, ii) rearrangement of the fatty acid radical, iii) introduction of molecular dioxygen forming a oxygen-centered peroxy radical, iv) reduction of the hydroperoxy radical to the corresponding anion. Hydrogen abstraction appears to be the rate-limiting step of the catalytic cycle and this elementary reaction involves hydrogen tunneling [67, 68]. Thus, LOXs may be considered quantum chemical enzymes and some aspects of the reaction mechanisms cannot adequately be described employing traditional thermodynamics.

3. Biological function of mammalian LOX isoforms

Following the classical concept of the arachidonic acid cascade LOXs exert their bioactivity via the formation of lipid mediators that regulate the functional phenotype of a given cell population (Fig. 2). However, at least two alternative concepts have been introduced to explain LOX functionality: i) Several LOX isoforms are capable of oxidizing complex ester lipids and even lipid-protein assemblies (biomembranes, lipoproteins) modifying their structural and functional parameters. This concept is at least in part applicable for the roles of different LOX isoforms in erythropoiesis, epidermal differentiation and atherogenesis (see 3.1.1., 3.1.2., 3.3.2.2.). ii) LOXs are lipid peroxidizing enzymes and by forming lipid peroxides they modify the cellular redox state. Since the cellular redox equilibrium is an important regulator of cell proliferation and gene expression (3.1.3.) intracellular LOX-activity may impact cell functionality. Of course, LOXs are not the only enzymes modifiying the cellular redox state since a large number of pro- and anti-oxidative enzymes exist in mammalian cells. However, the catalytic activity of LOX clearly contributes to cellular redox homeostasis.

3.1. Lipoxygenases in cell development and proliferation

3.1.1. *ALOX15* in erythropoiesis—Normal erythrocytes and their immediate precursors (reticulocytes) do not contain sizable amounts of *ALOX15*. However, when erythropoiesis is challenged in rabbits [69] by either repeated bleeding or forced hemolysis (phenylhydrazine injection) reticulocytes express large amounts of *ALOX15*. In fact, rabbit reticulocytes are the richest natural source of *ALOX15* and model calculations suggested that up to 4 mg of *ALOX15* protein is present in 1 ml of packed reticulocytes [70]. Interestingly, the enzyme is almost undetectable in young stress reticulocytes but during in vitro maturation of these cells expression of the enzyme parallels the maturational decline of cellular respiration [71]. These anti-parallel biological dynamics (increase in *ALOX15* expression vs. decrease in cellular respiration) and the observation that isolated *ALOX15* in vitro induces structural decomposition of rat liver mitochondria [72] implicated *ALOX15* in maturational breakdown of mitochondria during late erythopoiesis. Consistent with this hypothesis, oxidation products formed by *ALOX15* were found in reticulocyte membranes [73]. In vitro studies with the isolated rabbit *ALOX15* showed that the enzyme does not just bind to mitochondrial and other organelle membranes and oxidize the membrane lipids [74], but also directly permeabilizes them, forming pores in the membrane [66]. Freshly isolated reticulocytes matured in vitro degrade their mitochondria more slowly in the presence of a LOX inhibitor [75–77]. However, functional inactivation of the Alox15 gene in mice did not lead to major

functional defects in erythropoiesis [78], and we (Kühn, unpublished results) did not find significant differences in the standard hematological parameters (erythrocyte count, Hb, HK, MCHC, MCV) of Alox15-deficient mice when compared with Alox15-sufficient controls. In addition to intracellular degradation initiated by *ALOX15* [70], there are two competing hypotheses for how mitochondrial degradation occurs in erythroid cells [79]: i) engulfment and digestion within autophagic vacuoles [80]; and ii) exocytosis of mitochondria within exosomes [81]. There is experimental support for all three scenarios, but none of the proposed mechanisms appears to provide a complete answer [82]. It may simply be that inhibition of one pathway can be compensated for by one or both of the others. Indeed, *ALOX15* inhibition leads to an increase in autophagic vacuoles in cultured cells, and in the livers of Alox15 knockout mice in vivo [83]. Furthermore, exosome formation by in vitro matured reticulocytes is impaired by addition of a LOX inhibitor [83], suggesting there is considerable crosstalk between these pathways. Further studies of erythropoiesis under both stressed and non-stressed conditions are needed to investigate the relative contribution of each of the three mitochondrial degradation pathways.

3.1.2. Lipoxygenases in epidermal differentiation and skin development—

ALOX12B (12R-LOX) and *ALOXE3* (eLOX-3) have been implicated in late epidermal differentiation, particularly in maintenance of the *Stratum corneum* [84, 85]. Mammalian skin is composed of three principal layers (epidermis, dermis, subcutis) and the *Stratum corneum* constitutes the outermost layer of the epidermis. Its major function is to protect the organism from infection, irritants, and from loss of water. The *Stratum corneum* consists of specialized cells (corneocytes), which are according to the brick and mortar model [86] imbedded in a compact extracellular matrix consisting of cross-linked proteins and special extracellular lipids. Like red blood cells, corneocytes are anucleated cells, which do not contain intracellular organelles [87]. They originate from interfollicular epidermal stem cells localized in the *Stratum basale* of the epidermis and mature via keratinocytes into corneocytes. During this maturation process the cells migrate perpendicularly through the epidermis and their journey towards the surface of the skin takes approximately 14 days. In more basal layers of the *Stratum corneum* corneocytes are bridged together through specialized junctions (corneodesmosomes) but these junctions disintegrate as the cells mature resulting in desquamation. Corneocytes are characterized by the cornified envelope, which is formed beneath the plasma membrane [88, 89]. It consists of a 10 nm thick layer of highly crosslinked insoluble proteins and a 5 nm thick layer of ceramide lipids that are covalently bound to the proteins. Ceramides also occur in the extracellular space and here they organize the extracellular lipids into orderly lamellae. Together, the cornified envelope and extracellular lipid lamellae, are essential for effective physical and water barrier function in the skin [90].

In normal mouse skin five different LOX-isoforms (*Alox15b, Alox12, Alox12b, Aloxe3, Aloxe12*) are expressed. Targeted inactivation of the Alox12 [91], Alox12b [84] and Aloxe3 [85] genes led to an impaired water barrier function of the skin. Alox12 knockout mice are viable and reproduce normally [92] and thus, defective function of this enzyme is likely to be compensated by the other LOX-isoforms. In contrast, Alox12b and Aloxe3 knockout mice die shortly after birth because of rapid dehydration [84, 85].

In humans autosomal recessive congenital ichthyosis (ARCI) is a group of skin diseases, which is characterized by intense scaling [93]. Naturally occurring mutations in the ALOX12B and ALOXE3 genes have frequently been detected in ichthyosis patients [94, 95]. In a large group of 250 ARCI patients [96] 11 previously unidentified mutations have been described in the two LOX genes in 21 ARCI patients from 19 unrelated families. These data indicated that mutations in the two genes are the second most common cause for ARCI in this patient cohort. More detailed analysis of the sequence data revealed a high allelic heterogeneity for the ALOX12B gene, and two mutational hotspots in the ALOXE3 gene have been identified. Functional characterization of these mutations indicated a loss of catalytic activity suggesting a causal relation between the loss-of-function mutations and pathogenesis [96]. Unfortunately, the frequency of functionally deficient ALOX12B and ALOXE3 mutants in the average population (not ARCI patients) has not been determined. Of course, ALOX12B and ALOXE3 deficiencies are not the only reason for ARCI and mutations in other genes such as transglutaminase-1 do also contribute [97].

The question why defective ALOX12B and ALOXE3 expression in man and mice induces impaired formation of the water barrier of the skin is still a matter of discussion. The current understanding of the molecular mechanisms [30, 31, 98] is that under normal conditions *ALOX12B* catalyzes oxygenation of skin specific ceramides to their corresponding hydroperoxídes. These hydroperoxy lipids are subsequently converted by the hydroperoxide isomerase activity of *ALOXE3* [99] to hepoxilin-like secondary lipid peroxidation products [6]. This oxidative modification triggers preferential removal of the oxidized linoleate moieties from the ceramides resulting in the formation free ω-hydroxyceramides. These reactive lipids may subsequently be linked covalently to proteins contributing to the formation of the cornified envelope [88, 89]. Although there are still some mechanistic caveats (e.g. which lipid hydrolyzing enzymes prefer hepoxilin-containing ceramides over the non-oxidized counterparts) this pathogenetic scenario is supported by several lines of experimental observations [98]: i) Murine epidermis contains oxidized linoleate containing ceramides, which are almost absent in the skin of Alox12b knockout mice. ii) The oxidized linoleate residues in the ceramide lipids are chiral suggesting their enzymatic origin. iii) Covalently cross-linked ceramides in the epidermis of Alox12b knockout mice are severely reduced in the epidermis of these animals. iv) Aloxe3 knockout mice show a less severe phenotype when compared with Alox12b-deficient animals, which was associated with a reduction in covalently bound ceramides.

3.1.3. Lipoxygenases in cell proliferation and carcinogenesis—The role of LOX isoforms in cell proliferation and carcinogenesis appears to be very complex and the observed effects are sometimes controversial. *ALOX5* metabolites, such as 5-HETE, its oxidation product 5-oxo-ETE and peptido leukotrienes, stimulate cell proliferation and thus, may act as pro-carcinogenics [100]. 12-HETE, the major arachidonic acid oxygenation product of *ALOX12* and *ALOX12B*, which is also formed in smaller amounts by *ALOX15* orthologs, also exhibits pro-carcinogenic activities stimulating cell adhesion, metastasis and neoangiogenesis [101]. These data are consistent with the observation that overexpression of *ALOX15* in human prostate cancer cells increases tumorgenesis [102]. In contrast, the major *ALOX15* and *ALOX15B* metabolite of linoleic acid oxygenation (13-HODE) induces

apoptosis in human colorectal cancer and thus, exhibits anti-carcinogenic properties [103]. Expression of *ALOX15B* is reduced in human prostate, esophageal and skin carcinoma [104–106] and the enzyme was suggested as tumor suppressor protein [107]. It should, however, be stressed that there is no uniform expression regulation of different LOX isoforms in all types of malignancies. In contrast, expression regulation strongly depends on the kind of tumor and perhaps on its developmental stage. For instance, the enzymatic activity of *ALOX15* is down-regulated in colorectal carcinoma [108] but up-regulation was observed in prostate cancer [109].

If one reviews the relevant LOX literature four (*ALOX15, ALOX15B, ALOX12, ALOX5*) of the six human LOX isoforms have been implicated in regulation of cell proliferation and carcinogenesis. There are a number of reports exploring the roles of these LOX isoforms in different types of cancer [110–115], but the majority of the reports implicate LOXs in colorectal [116] and prostate carcinoma [117]. Thus, for this review we will focus on these two types of cancer. Carcinogenesis is a complex process that involves increased cell proliferation, reduced apoptosis, tumor associated neoangiogenesis, up-regulation of cellular adhesion and invasiveness (metastasis) as well as down-regulation or circumvention of immunological defense reactions. In all of these processes LOX isoenzymes have been implicated [118] but there is no unifying concept for the biological roles of the different LOX isoforms.

3.1.3.1. Lipoxygenases in colorectal cancer: Comparison of the steady state concentrations of LOX metabolites in normal, polyp and colorectal cancer mucosa did not identify significant differences in 12-HETE, 15-HETE and leukotriene B4 levels [119]. However, the tissue concentrations of 13S-HODE declined across this progressive sequence. In a separate study a strong correlation between *ALOX5* expression and increased polyp size as well as higher tumor grade suggested a role for this enzyme in early stages of colon cancer [120]. These data are consistent with the overexpression of *ALOX5* in colon polyps and carcinoma tissue described in another study [121]. Summarizing these results it was concluded that ALOX5 expression is an early event in the mechanistic sequence leading to colon cancer, with increased expression in adenoma, while *ALOX12* expression appears to be a later event, possibly mediating invasion and metastasis. The products of the ALOX5 pathway (leukotrienes) have been suggested to induce their biological effects as endocrine or paracrine mediators via binding at cell surface receptors of surrounding cells. For instance, leukotriene B4 (LTB4) regulates colon cancer growth via binding at the BLT1 receptor. A BLT1 receptor antagonist, and siRNA-induced expression silencing of this protein suppressed LTB4-induced cell proliferation [122]. On the other hand, autocrine signaling mechanisms, such as activation of endogenous transcription factors, have also been described for various LOX products [123, 124].

The role of *ALOX15* in colorectal carcinoma has been a matter of discussion for several years and still the picture is not clear. Transfection of HCT116 colon carcinoma cells with ALOX15 induced activation of the ERK protein kinase led to increased rates of cell proliferation. These data suggest a pro-carcinogenic activity of the enzyme [125]. Treatment of these cells with NDGA, a non-specific LOX inhibitor with antioxidant properties, appeared to block ERK activation, which is consistent with the pro-carcinogenic activity of

ALOX15 [125]. The underlying mechanisms have not been explored in detail but it might well be that NDGA-induced cell cycle arrest is related to an off-target effect of this compound. NDGA is a potent antioxidant and interferes with the redox state of the cells. Since cell cycle regulation is impacted by the redox equilibrium [126] the observed anti-proliferative effect might not directly be related to *ALOX15* inhibition. In similar cellular models of colorectal carcinoma (HCT116, HT29) *ALOX15* exhibited anti-carcinogenic properties, which was related to inhibition of the anti-apoptotic effect of the inflammatory transcription factor nuclear factor kappa B [127]. Here again, the molecular basis for the observed anti-carcinogenic affect is not completely understood but overexpression of ALOX15 inhibited the degradation of the inhibitor of kappa B, impaired nuclear translocation of p65 and p50, decreased DNA binding in the nucleus and reduced the transcriptional activity of NF-κB [128]. Unresolved chronic inflammation is a key process in tumor progression and thus, pro-resolving lipid mediators (eicosanoids and related metabolites of other polyenoic fatty acids) such as lipoxins [129], resolvins [130] and maresins [131] need to be discussed as regulators of carcinogenesis [132]. Resolving eicosanoids are generally believed to exhibit anti-tumor activities [133]. Chronic inflammation of colonic mucosa creates a pro-carcinogenic milieu and patients suffering from ulcerative colitis exhibit defective lipoxin biosynthesis [134]. Thus, the lack of pro-resolving mediators may drive carcinogenic transformation of normal epithelial cells during chronic inflammation. On the other hand, under certain conditions these mediators may also act in a pro-carcinogenic manner. For instance, depletion of regulatory T cells induced by cyclophosphamide treatment of patients with large established tumors caused significant tumor progression and this effect was suggested to be mediated by an increase in lipoxin A4 levels [135].

3.1.3.2.Lipoxygenases in prostate cancer: Four LOX isoforms (*ALOX5*, *ALOX15*, *ALOX15B*, *ALOX12*) have been implicated in the pathogenesis of prostate cancer and pro- as well as anti-carcinogenic effects have been reported. As pro-inflammatory enzyme *ALOX5* was suggested to be pro-carcinogenic. The enzyme is overexpressed in prostate adenocarcinoma [136] but the molecular mechanisms for the pro-carcinogenic effects are not completely understood. Inhibition of the enzyme triggers apoptosis in different types of prostate cancer [137–139] and pharmacological interference with other constituents of leukotriene signaling induced similar effects [140]. For instance, the cysteinyl leukotriene receptor 1 (cysLTR1) is overexpressed in prostate cancer and cysLTR1 antagonists inhibit prostate cancer cell growth by up-regulating apoptotic cell death [141]. Moreover, downregulation of the OXE receptor for 5-oxo-ETE reduced prostate cancer cell survival [142].

ALOX15 is expressed at variable levels in different prostate carcinoma cell lines [143] and formation of 13-HODE suggested functional activity of the enzyme [144]. Forced overexpression of *ALOX15* in cultured human prostate cancer cells augmented the rate of cell proliferation and subcutaneous transplantation of ALOX15-transfected PC3 cells into athymic nude mice increased the frequency of tumor formation and tumor size [102]. Similarly, conditional expression of human *ALOX15* in mouse prostate induces prostatic intraepithelial neoplasia [145]. Taken together, these data suggest a pro-carcinogenic

character of this enzyme. On the other hand, *ALOX15*-mediated metabolism of docosahexaenoic acid is required for apoptosis in prostate cancer cells [146] and *ALOX15* metabolites of docosahexaenoic acid inhibit prostate cancer cell proliferation and cell survival [147]. Hence, in the presence of DHA the enzyme might exhibit anti-carcinogenic properties.

ALOX15B is expressed in normal human adult prostate and its expression is impaired in prostate intraepithelial neoplasia and in prostate cancer [148]. In normal prostate cells the enzyme has been identified as negative cell cycle regulator and consequently, a function of ALOX15B as tumor suppressor has been suggested in prostate carcinoma [149]. Although the molecular basis for the tumor suppressive activity is not completely understood some mechanistic scenarios have been suggested. 15S-HETE inhibits proliferation in PC3 prostate carcinoma cells and this effect involves activation of peroxisome proliferator-activated receptor gamma by the ALOX15 product 15S-HETE [150]. Since 15-HETE can also be formed by ALOX15 these two enzymes may contribute to this protective effect. Expression of ALOX15B is cell-autonomously up-regulated in cultured prostate cells, and induction of enzyme expression was associated with cell senescence [151]. Moreover, transgenic expression of human ALOX15B in mouse prostate leads to hyperplasia and cell senescence [152]. It should be stressed at this point that the product specificity of mouse and human *ALOX15B* are different. For the human enzyme 15-HETE is the exclusive arachidonic acid oxygenation product [22], whereas mouse alox15b makes 8-HETE [23]. Thus, induction of cell senescence by ALOX15B overexpression may not be related to the formation of arachidonic acid oxygenation products. Expression of *ALOX15B* is tightly regulated in prostate cancer and several mechanisms have been described. The reaction product of human *ALOX15B* catalyzed arachidonic acid oxygenation (15-HETE) activates enzyme expression via activation of peroxisome proliferator activated receptor gamma (PPARγ) [153]. However, it remains unclear if similar observations can be made in murine experimental setups. Because of the different reaction specificities of human and mouse *ALOX15B* 8-HETE should be the preferred activator of murine PPARγ. Unfortunately, to the best of our knowledge such experimental data are currently not available. In addition, the tumor suppressive effects of *ALOX15B* may be related to the down-regulation of vascular endothelial growth factor in prostate carcinoma and to induction of tumor dormancy by the enzyme [154]. In other words, loss of *ALOX15B* functionality was suggested to represent a key step for prostate cancer cells to exit from dormancy and embark on malignant progression [154]. In prostate cancer expression of *ALOX15B* is strongly down-regulated but the underlying molecular mechanisms are not completely understood. Recently, it has been reported that glucocorticoid signaling may be involved in *ALOX15B* expression regulation and these data might be relevant for prostate cancer [155].

ALOX12 exhibits pro-carcinogenic activities in the prostate. It stimulates tumor growth and neoangiogenesis [101], increases the metastatic potential [156] and promotes tumor cell survival [157]. Although the mechanistic reasons for the tumor-promoting effects have not been studied in detail this activity might involve NFκB signaling [158, 159].

Most naturally occurring polyenoic fatty acids serve as LOX substrates and the biological activity of the products formed from a certain fatty acid may differ from that formed by the

same LOX isoform from a different fatty acid. For instance, the major *ALOX15* product of arachidonic acid metabolism (15-HETE) might exhibit different biological effects than the major *ALOX15* product of docosahexaenoic acid (17-HDHE). Thus, the alimentary supply of polyenoic fatty acids might modify the overall character of LOX isoforms in prostate cancer [160–162].

3.2. Lipoxygenases in inflammation

According to the classical concept of the arachidonic acid cascade LOXs are key enzymes in the biosynthesis of linear eicosanoids and related mediators originating from other polyenoic fatty acids (leukotrienes, lipoxins, resolvins, maresins, hepoxilins, eoxins etc.) and these compounds [6–11] been implicated as promoting and/or protecting against pathogenic inflammation. However, the patho-physiological role of LOX may not be restricted to the formation of signaling lipids. LOXs are lipid peroxidizing enzymes and their catalytic activity may impact the cellular redox state. Since the redox state is an important regulator of the cellular gene expression pattern the catalytic activity of the enzyme may alter the functional phenotype of mammalian cells [163]. In fact, transfection-induced overexpression of ALOX15 in U937 cells alters the gene expression pattern (GSE8173) but the physiological consequences of these expression alterations have not been characterized in detail.

3.2.1. Pro-inflammatory properties of lipoxygenases—Inflammation is a protective response of the organism aimed at fighting inflammation inducers. This fight requires a balanced activity of various cellular and humoral constituents of the adaptive and innate immune system, and inflammatory mediators have coordinating functions. There is a large array of lipid and non-lipid mediators regulating acute inflammation as well as its inflammatory resolution. Leukotrienes (LT) are classical pro-inflammatory mediators originating from the *ALOX5* pathway [2, 164]. They are biosynthesized in different types of leukocytes and other immune competent cells from free arachidonic acid. The key enzyme in the biosynthetic cascade is *ALOX5* [2, 165], which catalyzes the first two steps of leukotriene biosynthesis (oxygenation of arachidonic acid to 5S-HpETE and conversion of 5S-HpETE to LTA4). The leukotriene biosynthetic cascade has extensively been reviewed before [33, 166] and most enzymes and regulators involved in this pathway have been well characterized with respect to their structural and functional properties [2]. There are two principal classes of LTs (peptido LTs and peptide-free LTs) and additional non-LT *ALOX5* products such as 5-HETE, and 5-oxo-ETE [167]. The various LTs (Fig. 5) exhibit different bioactivities and preferentially act on different cells types:

i. Peptido-LTs: The cysteinyl LTs (LTC4, LTD4, LTE4) are constituents of the slow-reacting substance of anaphylaxis and play an important role for the pathogenesis of allergic diseases, such as bronchial asthma [168], rhinitis [169] and allergic eye disease [170]. On the molar basis cysLTs are at least 1000-times more effective as bronchoconstrictor than histamine [171], and nanomolar concentrations of cys-LTs cause plasma leakage and cell adherence in postcapillary venules leading to bronchial edema [172]. In addition, cysteinyl LTs induce mucus secretion in vitro and in vivo [173, 174] and may also alter mucus viscosity [174]. Taken together,

these effects implicated leukotrienes in the pathogenesis of pulmonary dysfunction, and after leukotriene synthesis inhibitors [175] and leukotriene receptor antagonists [176, 177] became available as drugs, anti-leukotriene therapy has been employed in the clinic to supplement glucocorticoid-base therapeutic schemes [178]. Nevertheless, as monotherapy, inhaled corticosteroids display superior efficacy to anti-leukotrienes in patients with persistent asthma and this superiority is particularly evident in patients with moderate airway obstruction [179]. Asthma patients who continue to experience symptoms despite being on regular inhaled corticosteroids represent a management challenge, and long-acting beta(2)-agonists or anti-leukotrienes are two treatment options that could be considered as add-on strategies. Direct comparison of these two therapeutic approaches suggest that long-acting beta(2)-agonist treatment might be superior to anti-leukotriene therapy in reducing oral steroid treated exacerbations [180]. However, the differences in lung function and quality of life scores were rather moderate but there was evidence of increased risk of serious adverse events under long-acting beta(2)-agonist treatment. In general, the beneficial effects of anti-leukotriene therapy have recently been challenged. On one hand, this therapeutic concept brings remarkable ease of anti-inflammatory treatment, administration and symptom improvement with minimal side effects to the management of adult asthma [181]. On the other hand, it was suggested to limit anti-leukotriene therapy to asthmatics, who are refractory to inhaled corticosteroids or cannot use inhalant devices. Considering the low incidence of these circumstances oral anti-leukotrienes should be more carefully considered for treating asthma in the clinical environment but several clinically relevant issues (effects of anti-leukotriene medication on peripheral airways and on airway remodeling, alternative administration concepts) remain to be clarified before anti-leukotriene therapy could serve as a more effective strategy in the treatment of bronchial asthma in adults [177]. In children the beneficial effects of anti-leukotriene therapy for bronchial asthma are even more difficult to evaluate because the number and the size of clinical trials carried out so far is rather limited [182]. The currently available data of randomized studies suggest that there is no firm evidence supporting the suggestion that adding leukotriene receptor antagonists (montelukast) to inhaled corticosteroid therapy is safe and effective to reduce the occurrence of moderate or severe asthma attacks in children taking low-dose inhaled corticosteroids [183]. After being on the market for more than 10 years, the limited number of available studies testing anti-leukotrienes in children, the absence of data on preschoolers, and the inconsistency of available trials reporting of efficacy and safety of clinical outcomes is disappointing and limit general conclusions [183]. However, considering the pro-inflammatory effects that leukotrienes have in experimental setups, it is rather surprising that the outcome of anti-leukotriene treatment are not better and that in some clinical trials only a minority of patients could be classified as full responders. This discrepancy might be explained by additional LT receptors that are not affected by the current drugs. In addition, there may be different phenotypes of bronchial asthma and some of them might involve LT to a lesser degree [182, 183].

ii. Peptide-free LTs: Leukotriene B4, the major bioactive peptide-free leukotriene, is a strong endogenous stimulator of the innate immune response [184]. It is released from polymorphonuclear leukocytes, monocytes and macrophages and induces cell aggregation and increases vascular permeability [185]. It stimulates chemotaxis and adherence of neutrophils to the vascular wall [186, 187]. It also binds to two major types of cell surface receptors (BLT1, BLT2) and induces G-protein dependent intracellular signaling cascades leading to activation of inflammatory cells [188, 189]. Although LTB4 is one of the most powerful pro-inflammatory mediators, neither LTB4 synthesis inhibitors (inhibitors of *ALOX5* or LTA4 hydrolase) nor BLT1/BLT2 receptor antagonist turned out to be effective anti-inflammatory drugs.

3.2.2. Anti-inflammatory properties of lipoxygenases—Termination of acute inflammation was previously considered a passive process, which became possible because of the decay of pro-inflammatory signals. However, during the past decade there has been a change in this paradigm. Today we consider inflammatory resolution an active process, which proceeds according to a biological program, aimed at reestablishing normal tissue homeostasis [190]. Inflammatory resolution is initiated by alterations of the cellular composition in the inflamed tissue (neutrophils and pro-inflammatory M1 macrophages are replaced by anti-inflammatory M2 macrophages that clean up the battle field) and by a switch in inflammatory signaling. For instance, formation of pro-inflammatory lipid mediators (prostaglandins, leukotrienes) is down-regulated whereas biosynthesis of anti-inflammatory (proresolution) mediators is switched on. Endogenous pro-resolving lipid mediators include a number of LOX products such as lipoxins [7, 191], resolvins [192], protectins [11], maresins [193], and others. The specific interaction of the pro-resolution mediators with G protein-coupled receptors (GPCR 32, ALX, BLT1) on the surface of immune cells induces a number of pro-resolution processes. For instance, leukocyte migration is reduced [194], vascular permeability returns to normal [195], pro-inflammatory neutrophils undergo apoptosis [196] and M2 macrophages phagocytose apoptotic neutrophils, bacterial remnants and necrotic debris [197]. While adequate inflammatory resolution prevents tissue injury leading to *restitutio ad integrum*, inadequate resolution results in chronic inflammation.

The proresolving eicosanoids and docosanoids are multiple oxygenation products of three major polyenoic fatty acids (arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid) that are biosynthesized by a concerted activity of various LOX isoforms with different positional specificity (*ALOX12*, *ALOX15*, *ALOX15B*, *ALOX5*). Since aspirin-treated COX2 exhibits a 15R-LOX activity [198] it also participates in the biosynthesis of these mediators [199] and the anti-inflammatory effect of aspirin may partly be related to this mechanism. In vivo, lipoxins are formed from arachidonic acid via transcellular biosynthetic mechanisms involving 5-, 12- and 15-lipoxygenating LOX isoforms [200]. However, LxB4 can also formed by double oxygenation of 15S-HETE methyl ester by purified rabbit *ALOX15* alone [201]. Resolvins and protectins are biosynthesized from the omega-3 fatty acids mainly from eicosapentaenoic and docosahexaenoic acid. These fatty acids occur in high concentrations in marine organisms (fish oil) and their anti-inflammatory properties [202, 203] are well known. Lipoxins, they are biosynthesized via multiple oxygenations of arachidonic acid by

aspirin treated COX-2 and/or several LOX-isoforms (*ALOX12*, *ALOX15*, *ALOX15B*). Protectins, previously called neuroprotectins since they were discovered in the brain [204], have been implicated in resolution of neuroinflammation. However, they also occur in peripheral tissues. Maresins are macrophage-derived mediators of inflammatory resolution [205], which are mainly formed from docosahexaenoic acid. Their name is an acronym for macrophage mediator in resolving inflammation, and as resolvins they exhibit potent anti-inflammatory properties. For instance, they prevent infiltration of pro-inflammatory neutrophils into inflamed tissues and stimulate phagocytosis of apoptotic neutrophils and cell debris by M2 macrophages [205]. In two different experimental models of arthritis systemic functional silencing of the ALOX15 gene induced uncontrolled inflammation and tissue damage. These data are consistent with an anti-inflammatory and tissue-protective role of the enzyme [206]. Although peritoneal macrophages of these animals produced significantly reduced levels of lipoxin A4 it remains unclear whether the formation of these pro-resolving mediators is the major reason for the anti-inflammatory effect. Alternatively, it was suggested that *ALOX15* may play an important role in development of osteoclasts but here again the molecular mechanisms are not well understood [207].

The anti-inflammatory properties of certain LOX isoforms (*ALOX12*, *ALOX15*, *ALOX15B*) should not be limited to their involvement in the biosynthesis of pro-resolving eicosanoids since alternative concepts may also be applicable. The primary products of linoleic acid and arachidonic oxygenation by *ALOX15* and *ALOX15B* (13S-HpODE, 15S-HpETE) exhibit anti-inflammatory activities in various inflammation models [208]. Moreover, LOX metabolites may activate PPAR signaling [209, 210] stimulating anti-inflammation via this pathway [211]. Oxidized phospholipids, which may be formed by *ALOX15* catalyzed oxygenation of membrane lipids [74], are capable of preventing the binding of agonists to toll-like receptors and thus, prevent activation of the innate immune response [212]. Unilateral somatic gene transfer of ALOX15 in an experimental model of glomerulonephritis suppresses inflammation and preserved kidney function in the transfected kidney [213]. Although the mechanism of this effect has not been explored in detail the data are consistent with an anti-inflammatory effect of *ALOX15*.

3.3. Lipoxygenases in the cardio-vascular system

3.3.1. Lipoxygenases in blood pressure regulation and hypertension—15-lipoxygenating LOX-isoforms (*ALOX15*, *ALOX15B*) have been implicated in regulation of vascular tone and thus, may play a role in blood pressure regulation and hypertension [214–216]. More than 20 years ago it was reported that arachidonic acid induces endothelium-dependent relaxation of rabbit aorta [217]. Since this effect was not seen in the presence of the unspecific LOX inhibitor nordihydroguaiaretic acid LOX metabolites have been suggested as molecular inducers of vasorelaxation. Similar effects have been reported for bovine coronary arteries [218] and later on these metabolites have been identified as 11,14,15- and 11,12,15-trihydroxyeicosatrienoic acids [219]. Although the source of these metabolites has not been identified at this stage of research [219] later expression silencing studies [220] and adenovirus mediated somatic gene transfer [221, 222] suggested the involvement of *ALOX15*. Interestingly, chronic hypoxia and hypercholesterolemia enhanced *ALOX15* mediated vasorelaxation in rabbit arteries [223, 224]. More direct evidence for the

in vivo relevance of *ALOX15* in blood pressure regulation was recently provided by experiments with ALOX15-deficient mice [225]. Although systolic blood pressures did not differ between these mice and wild-type controls Alox15$^{-/-}$-mice exhibited higher resistance towards L-NAME- and high-salt-induced hypertension than corresponding controls. The *ALOX15* inhibitor nordihydroguaiaretic acid attenuated this resistance suggesting the involvement of lipid peroxidation. The molecular basis for this effect has not been explored and it remains unclear whether or not it is related to the vasomotor properties of *ALOX15* products. Interestingly, injection of wild-type peritoneal macrophages, which are a major source of *ALOX15* in mice, into ALOX15-deficient animals abolished their resistance toward L-NAME-induced hypertension. Inversely, wildtype mice acquired resistance to L-NAME-induced hypertension after depletion of macrophages by clodronate injection [225].

3.3.2. Lipoxygenases in atherogenesis—Three different LOX-isoforms (*ALOX5*, *ALOX15*, *ALOX15B*) have been implicated in the pathogenesis of atherosclerosis [226–228]. *ALOX5* and *ALOX15B* are expressed at high levels in advanced atherosclerotic lesions whereas ALOX15 mRNA was only present in small amounts [229]. However, low levels of lesional expression of *ALOX15* in advanced human plaques do not necessarily exclude involvement of the enzyme in atherogenesis. If *ALOX15* is involved in maturation and differentiation of macrophages it might contribute to atherogenesis without being expressed in the lesion: i) If the enzyme is involved in early stage of hematopoietic differentiation (monocyte/macrophage maturation), which proceeds in the bone marrow, functionally different macrophages are likely to be generated and thus, foam cell formation may be impacted. ii) If the enzyme is expressed in cells not present in the lesions it might contribute to systemic (not local) LDL oxidation, which is considered a risk factor for atherogenesis. iii) If *ALOX15* is only involved in early stages of lesion formation [230, 231] it may be absent in advanced lesions but still might contribute to early stages of lesion development. In all these cases expression silencing and pharmacological intervention with *ALOX15* pathway may impact lesion formation without lesional expression of the enzyme.

3.3.2.1. ALOX5, leukotriene signaling and vascular inflammation: Already 25 years ago the formation of LTB4 in human atherosclerotic lesions was demonstrated [232]. Moreover, in human atherosclerotic coronary arteries key enzymes of leukotriene biosynthesis (*ALOX5*, *ALOX5AP*, LTA4H) have been detected and the arteries exhibited a contractile response when challenged with LTC4 and LTD4 [233]. More recently, high levels of expression of all enzymes of the leukotriene biosynthetic cascade were found in human atherosclerotic plaques and the expression levels of *ALOX5* and LTA4H correlated with symptoms of lesional instability [234].

In different murine atherosclerosis models variable and inconclusive data were obtained with respect to the patho-physiological relevance of leukotriene signaling. For instance, when fed a lipid rich diet ALOX5-deficient mice are not protected from lipid deposition in the vessel wall but show an increased tendency for the development of aortic aneurysms [235]. These data link the leukotriene pathway to inflammatory disturbance of vessel wall remodeling [236] rather than to lipid homeostasis However, in another mouse model of aneurysm formation (angiotensin II treatment) genetic and pharmacological interference

with leukotriene biosynthesis did not show significant effects [237]. Deletion of the BLT1 receptor reduced lesion formation during early stages of plaque development, but was without any effect at more advanced disease stages [238]. On the other hand, BLT1 deficient mice were protected from aortic aneurysm formation in the angiotensin II model [239] and a selective BLT1 antagonist protected against the early phase aneurysm development [240]. For the time being it remains unclear what might be the reasons for the inconsistent effects observed in humans and mouse atherosclerosis models. It is well known that hyperlipidemic mouse atherosclerosis models do not adequately mirror all aspects of human atherosclerosis. Thus, more relevant animal atherosclerosis models (non-human primates) and more detailed clinical trials are required to assess the therapeutic potential of anti-leukotrienes therapy in treatment and prevention of human atherosclerosis, aortic aneurysms and myocardial infarction.

3.3.2.2. ALOX15 and lipoprotein modification: In the early 1990s the LDL oxidation hypothesis was introduced [241, 242] and its refined version was more recently critically reviewed [243]. This hypothesis suggested that oxidized LDL exhibits strong pro-atherogenic activities because it is rapidly taken up by macrophages via scavenger receptor mediated pathways. Since these pathways are not feedback-controlled excessive intercellular lipid deposition may occur and macrophages develop into lipid-laden foam cells. These cells then accumulate in the subendothelial space of the arteries to form fatty streaks which are considered early atherosclerotic lesions [244]. Since *ALOX15* is capable of modifying LDL [245] and other lipoproteins [246] by oxidizing their ester lipids the enzyme has been implicated in atherogenesis. In atherosclerotic lesions of rabbits [231] and humans [230, 247] esterified specific LOX-products (mainly 13S-HODE) have been detected but the biosynthetic origins of these products have not been explored in detail. In particular, the question whether they are formed by *ALOX15*, *ALOX15B*, or alternative biosynthetic pathways has not been answered conclusively. Several studies employing ALOX15 deficient mice supported a pro-atheorgenic role of *ALOX15* [248–253]. On the other hand, overexpression of ALOX15 in two rabbit and one mouse atherosclerosis models suggested anti-atherogenic effects [254–256]. In one of these studies it was suggested that *ALOX15* activity in the local milieu afforded atheroprotection via the formation of proresolving lipid mediators [256] and this was later on suggested as more general paradigm [257]. Taken together, as discussed for the *ALOX5* pathway the role of *ALOX15* in atherosclerosis is controversial [258, 259] and this may be related to mechanistic differences of the various animal atherosclerosis models.

3.3.2.3. ALOX15B in atherogenesis: As indicated above gene expression studies in advanced human atherosclerotic lesions suggested high-level expression of ALOX15B [229, 260] and these data suggested a role of this LOX isoform in atherogenesis. Macrophage expression of *ALOX15B* has been reported under hypoxic conditions [261] and hypoxia inducible factor (HIF) has been implicated [260]. Catalytic activity of this enzyme was related to chemokine release [262] and the enzyme has been suggested as tissue marker for atherosclerotic carotid artery [263]. More recently, polymorphisms in the ALOX15B gene have been associated with coronary artery disease [228] but the underlying molecular mechanisms remain unclear. Functional characterization of the mutant enzymes did not

reveal major defects and thus, *ALOX15B* products might not be involved [228]. Nevertheless, lesional expression of *ALOX15B*, which accepts arachidonic acid, linoleic acid and other polyenoic fatty acid as substrate [264, 265], may contribute to the formation of specific LOX products detected in the lesion lipids [230, 231, 247].

3.3.3. Lipoxygenase in platelet function and atherothrombosis—Two LOX isoforms (*ALOX12, ALOX15B*) have been suggested to impact platelet function and atherothrombosis. The first LOX-isoforms detected in animals was the *ALOX12*, which is present in large amounts in human platelets [266]. Unfortunately, the precise role of this enzyme for blood platelet physiology is still a matter of discussion since pro- and antithrombotic activities have been reported. Blood platelets of ALOX12-deficient mice exhibited an increased sensitivity for ADP-induced aggregation suggesting the *ALOX12* pathway as down-regulator for platelet aggregation (anti-thrombotic effect) [92]. In contrast, more recent studies suggested that *ALOX12*-derived 12-HETE [267] and possibly other oxylipins [268] may play an important role as prothrombotic mediators in atherothrombosis. Although the molecular basis for the anti-thrombotic effect of the *ALOX12* pathway has not been studied in detail at this time more recent data suggest the involvement of protein kinase C. To determine the functional interaction between protein kinase C and *ALOX12* during platelet activation pharmacological interventions studies were carried out using specific modulators of the two pathways [269]. Separate inhibition of *ALOX12* and PKC resulted in impaired secretion of dense granule and in attenuation of both aggregation and αIIbβ(3) activation. However, activation of PKC downstream of *ALOX12* inhibition rescued agonist-induced aggregation and integrin activation. Inhibition of *ALOX12* had no effect on PKC-mediated aggregation indicating that *ALOX12* is localized upstream of PKC in the signaling cascade. Taken together these studies support an essential role for PKC downstream of *ALOX12* activation in human platelets and suggest *ALOX12* as a possible target for antiplatelet therapy [269]. In a similar study pharmacological interference with *ALOX12* activity resulted in attenuation of platelet aggregation, selective inhibition of dense granule secretion, and inhibition of platelet adhesion [270]. ALOX12-deficient mice showed attenuated integrin activity. These data confirm the role of *ALOX12* in regulating platelet function and thrombosis and provide the basis for the development of innovative strategies for the therapy of thrombosis [270, 271]. Moreover, dihydroxylated metabolites derived from alpha-linoleic acid inhibit platelet function [272] and a geometric isomer of protectin D also prevented platelet aggregation at submicromolar concentrations when induced by either collagen, arachidonic acid or thromboxane [273].

Although *ALOX15B* is not expressed in human platelets this enzyme has recently been implicated in the regulation of platelet functionality [274]. Impedance aggregometry indicated that the major oxygenation products of arachidonic acid conversion by *ALOX15B* (15-HETE, 15-HpETE) stimulated platelet aggregation. Moreover, platelet aggregation was augmented by the addition of cell lysates of ischemic human macrophages, which express large amounts of *ALOX15B*, whereas platelet aggregation was reduced when lysates of ALOX15B siRNA treated macrophages were used. These data suggest that *ALOX15B* expression in human plaques may be involved in thrombus formation [274].

3.4. Lipoxygenases in the central nervous system

3.4.1. Physiological roles of lipoxygenases in the CNS

3.4.1.1. Lipoxygenase expression in the CNS: *ALOX15* is the major LOX isoform in both rat [275] and canine brain [276]. Various cell types in the brain express *ALOX15* [276], but the expression levels under normal conditions are rather low. Depending on disease state and type of oxidative stress, it can also be up-regulated in different cell types (see below). In contrast to *ALOX15, ALOX12* does not appear to be expressed in significant amounts in rat brain [275, 277]. The mouse ortholog of human ALOX12B was reported to be expressed in adult brain cortex, however its possible function there remains elusive [278]. In zebrafish, an atypical 12-lipoxygenating enzyme species was found to be essential for normal brain development, but it is at present unclear which mammalian LOX-isoform this enzyme corresponds to [279].

3.4.1.2. 12-HETE and 12-HpETE as second messengers of semaphorin signaling: interactions with the actin cytoskeleton and growth cone turning/collapse: Both 12-HETE and 12-HpETE have been implicated separately as signaling mediators in axon guidance, indicating a direct function of 12-lipoxygenating LOX isoforms in brain development [280–283]. These eicosanoids function as classic second messengers [284], relaying and amplifying the signal produced by external stimuli including the axon guidance molecule semaphorin 3A (Sema3A) [280]. We recently showed that both 12-HETE and 12-HpETE can function as potent messengers in the Sema3A pathway, with 12-HpETE being the more efficient [285]. 5-HETE was not able to replace 12-HETE and 12-HpETE in this assay. 12(R)-HETE and 15-HETE have not been tested. At present, the further components downstream of 12-HETE/12-HpETE are not clear; both direct binding of 12-HETE to the actin cytoskeleton [286], and the involvement of the monooxygenase MICAL (Molecule Interacting with CasL) as mediator [287] have been documented. A separate line of evidence suggests the involvement of a protein kinase C (PKCε and MARCKS [288].

In addition to these effects on neuronal architecture, *ALOX15* influences synaptic signaling by its effects on long-term depression [289–291] and long-term potentiation [292, 293], which are core elements of interneuronal communication. The latter effect was mediated by 12-HpETE acting on L-type calcium channels [290]. Nonetheless, ALOX15 knockout mice do not show any overt behavioral defects, suggesting that either these effects can be bypassed, or the knockouts have found a way to compensate, for example by up-regulating one of the other 12-lipoxygenating isoforms. In line with this latter possibility, the brains of ALOX15$^{(-/-)}$ mice still generate 12-HETE, albeit at much reduced levels [291]. Residual 12-HETE appears to be the (S)-isomer, according to our findings using a stereospecific enzyme immunoassay (Pekcec and van Leyen, unpublished results).

3.4.2. Patho-physiological roles of lipoxygenases in the CNS (ischemia, neurodegeneration)—Neurons are especially vulnerable to oxidative stress, and oxidative stress-related pathology is a hallmark of several CNS diseases, including stroke, Parkinson's, and Alzheimer's Disease. LOXs are both activated by and contribute to oxidative stress, and are thus likely to be major players in these pathologies. *ALOX15* has been linked to apoptotic cell death in cultured primary neurons [22, 294–296] as well as

several brain-derived cell lines, including the human neuroblastoma cell line SH-SY5Y [297, 298] and the mouse hippocampal cell line HT22 [22, 299, 300]. The mechanism is apoptotic, but likely mediated by mitochondrial damage and apoptosis-inducing factor (AIF), rather than by caspase activation [299, 301, 302]. Similar damaging effects can be elicited *in vivo* by direct injection of arachidonic acid into the brain, which causes edema [303, 304]. Injecting glutathione disulfide, which is the oxidized version of glutathione, likewise induces brain damage via 12/15-LOX [305].

3.4.2.1. Oxidative stress and ALOX15 in the developing brain: periventricular leukomalacia: Periventricular leukomalacia (PVL) is a white matter injury in infants that is the dominant pathological factor in determining long-term cognitive and motor deficits in premature infants. It is characterized by necrotic lesions, and a diffuse type of injury involving microglia. In a recent study, we showed that *ALOX15* expression is increased in the brains of PVL infants [306]. Several cell types were affected, including microglia (Fig. 6) and oligodendrocyte precursor cells. Importantly, some of these cells were TUNEL-positive, indicating these were injured cells and suggesting that *ALOX15* contributed to disease pathology. This hypothesis is supported by cell culture studies, where oligodendrocyte precursors are vulnerable to an *ALOX15*-dependent form of cell death when cultured in the absence of cysteine [307, 308]. Similarly, LOX inhibitors protect oligodendrocytes against hyperoxia [309]. The mechanism is likely similar to that in neurons, with AIF translocation to the nucleus as apoptotic effector [310]. It will be interesting to see if LOX inhibitors are protective in animal models of PVL.

3.4.2.2. Genetic associations with stroke: In 2004 polymorphisms in the gene encoding *ALOX5* activating protein (ALOX5AP) were linked to an increased risk for ischemic stroke [311]. The protein encoded by ALOX5AP is required for ALOX5 activity [312]. In the aftermath, numerous replication studies were carried out in several different ethnic populations, with variable outcomes. For example, the findings originally made in the Icelandic cohort were replicated in Scottish and Spanish [313], but not in a Swedish [314] population. One U.S.-based study did not confirm this connection [315], while another found an association for Americans of European, but not of African descent [316]. Similarly, studies of Chinese populations in some cases confirmed the original results, in others did not; but a recent meta-analysis concluded that the link could be confirmed in the Chinese population [317]. A genetic study of English and German patients reported significant associations for several genes of the leukotriene pathway [318]. It should be emphasized that increased risk of stroke does not imply an increased severity of strokes as well. Those types of study, which could uncover target genes relevant for treatment of stroke, are very difficult to carry out, and none have so far been reported.

3.4.2.3. ALOX15 in stroke: The strongest evidence for any LOX isoform causing injury to the CNS exists in stroke. Early studies in the 1970s showed an increase in free fatty acids including arachidonic acid in a rat model of ischemia [319], an indication of phospholipase activity that suggested proteins of the arachidonic acid cascade might contribute to ischemic injury. In 1984, Moskowitz and colleagues reported increased levels of leukotrienes, as well as the *Alox15* metabolite 12-HETE, in the brains of gerbils subjected to forebrain ischemia

[320]. A large body of evidence has since then accumulated to demonstrate the involvement of *ALOX15* in causing brain injury following stroke [321]. Several events converge to favor the activation of *ALOX15*. Arachidonic acid liberated from phospholipids by cytosolic phospholipase A2 (cPLA2) provides additional substrate and, together with increased levels of reactive oxygen species (ROS) activates *ALOX15* [322]. Glutathione levels drop, removing one of the antioxidant pathways that serve to keep *ALOX15* activity in balance [323]. Conversely, intracellular calcium rises, favoring membrane binding of the activated enzyme [324]. In addition, the protein levels of *ALOX15* increase specifically in the penumbra region surrounding the core infarct, the brain region which is vulnerable to delayed cell death [325]. The factors leading to transcriptional up-regulation in the ischemic brain have not been determined yet, but may include members of the STAT family of transcriptional activators, which regulate ALOX15 expression in several other cell types [326–328].

Increased *ALOX15* in the ischemic cortex is accompanied by increased pro-apoptotic AIF, in both human stroke patients [329], as well as mouse models of stroke [301]. *ALOX15* also co-localizes with MDA2, an antibody that recognizes malonedialdehyde-modified lysisne residues, indicative of phospholipid oxidation [329]. Taken together, these findings document that *ALOX15* is part of a major cell death pathway that is activated in the ischemic brain. Consistent with these observations, ALOX15 gene knockout protects mice against stroke [325, 330], and also reduces leakage of the blood-brain barrier and edema formation [331]. Importantly, these protective effects could be replicated by pre-treatment with LOX inhibitors. While those early inhibitors also had strong antioxidant activity, we have since introduced newer inhibitors with low antioxidant activity [308, 332], and found those to be protective even when given four hours after onset of the experimental stroke [329]. These compounds may be clinically useful in treating stroke.

3.4.2.4. ALOX5 in stroke: Early reports of LOX activity in animal models of stroke emphasized *ALOX5* products over those of *ALOX15*, although the latter were also detected [320]. The increased leukotrienes were later shown to be blood-derived, rather than being formed in the brain parenchyma [333]. Several *ALOX5* inhibitors were reported to be neuroprotective, but these were typically strong antioxidants, which lack major isoform-specificity [334]. A later study showed that in two different ischemia models, ALOX5 knockout mice had an equal amount of injury compared to wild-type mice [335]. Since then, the focus has shifted more to *ALOX15* and its effects on stroke severity. Nonetheless, it is not unlikely that *ALOX5* and its products are involved in specific subsets of stroke pathology, especially those featuring an increased inflammatory component.

3.4.2.5. Neuroprotection through lipoxygenase metabolites in stroke: In contrast to the damaging effects of LOX activity, a separate line of investigations is exploring the restorative potential of LOX-derived mediators including lipoxins and protectins. For example, neuroprotective effects of rosiglitazone were related to *ALOX5*-dependent formation of lipoxin A4 [336]. In line with these findings, an agonist of the lipoxin A4 receptor provided neurovascular protection in a rat model of ischemic stroke [337]. This was

accompanied by a reduction of the matrix metalloproteinase MMP-9, which was also diminished in another study by administration of lipoxin A4 methyl ester [338].

An extensive body of work has accumulated to demonstrate the potential of protectins, specifically neuroprotectin D1 (NPD1), to reduce injury in animal models of stroke [339]. The underlying principle is the conversion of docosahexaenoic acid to NPD1 by a succession of LOX-mediated oxidation reactions, and infusion of DHA has been shown to be protective in experimental stroke [340]. Intriguingly, a closely related isomer termed AT-NPD1 can be generated in situ in the brain by administration of aspirin with docosahexaenoic acid, and this AT-NPD1 also has equivalent neuroprotective properties [341].

3.4.2.6. Lipoxygenase involvement in Alzheimer's and other neurodegenerative diseases: In Alzheimer's, both *ALOX5* and *ALOX15* have been implicated, but their precise roles are far from clear. *ALOX15* expression is increased in the brains of Alzheimer's patients [342], along with increased levels of 12- and 15-HETE in the cerebrospinal fluid of patients with Alzheimer's or mild cognitive impairment pathology [343]. Consistent with a damaging function of *ALOX15* in Alzheimer's, degenerative defects in the transgenic Alzheimer's mouse model tg2576 were reduced when ALOX15 was absent [344]. Conversely, in another study *ALOX15* expression was reduced in the hippocampus of Alzheimer's patients [345] and this effect was paralleled by reduced neuroprotectin D1 levels. In contrast, *ALOX5* was increased in hippocampus and cortex of Alzheimer's patients [346]. Rao et al. found elevated levels of *ALOX12* and *ALOX15*, but no increase in *ALOX5* when comparing Alzheimer's brains to those with no pathology [347]. There are several possible reasons for these discrepancies, which may be related to the complexity of disease progression. Other factors may include small sample size, differences in brain regions and different techniques used for analysis. Further studies are needed to get a clearer picture of differential LOX expression and its consequences in Alzheimer's brains.

In cell culture models, an amyloid beta-derived peptide was found to cause cell death in primary neurons. This effect was blocked by inhibition of *ALOX15* and *ALOX12* with baicalein, and similar protective effects were observed when an antisense oligonucleotide targeting *ALOX15* expression was employed [348, 349]. Again somewhat at odds with these findings, another line of experiments suggested that miRNA125b, a micro-RNA that binds at the 3'-UTR of ALOX15 mRNA and down-regulates its translation, was increased in Alzheimer's patients. In primary neuronal-glial cells treated with IL1β and amyloid β1–42, an increase of miRNA125b led to down-regulation of *ALOX15* [350]. An antagomir to miRNA125b that restored *ALOX15* protected these cells. In both of these cases, a direct causative link to Alzheimer's pathology remains to be established.

In other CNS Diseases, much remains to be studied about possible LOX involvement in the respective pathology. Somewhat surprisingly, in a mouse model of multiple sclerosis, experimental allergic encephalomyelitis, knockout mice deficient in either ALOX5 or ALOX15 actually do worse [351]. After spinal cord injury, *ALOX15* was increased 25-fold in rats, compared to a 1.7-fold up-regulation detected for COX-2 [352]. But whether or not this increase contributes to the injury is presently unknown.

3.5. Lipoxygenases in metabolic disorders

3.5.1. Diabetes—A number of LOX metabolites and several LOX isoforms have been implicated in the pathogenesis of diabetes. Both *ALOX5* generated leukotriene B4, and 12-HETE generated by either *ALOX15* or *ALOX12* was elevated in diabetic patients with severe cardiac ischemia [353]. Especially for *ALOX15* a substantial body of evidence has accumulated to link the enzyme to the pathogenesis of diabetes [354–362]. Since LOXs are pro-oxidative enzymes producing hydroperoxy lipids, LOX-induced oxidative stress and subsequent mitochondrial dysfunction might account for much of the increased pathology detected in diabetic cardiomyopathy and other vascular diseases [363]. Increased levels of 12S-HETE were linked to coronary artery disease in type 2 diabetic patients [364]. More generally, *ALOX15* has been shown to be up-regulated in both cell culture and animal models of diabetes [365].

Insulin secretion of cultured human islet cells was reduced by nanomolar concentrations of 12(S)-HETE and 12HpETE. These data suggest that 12(S)-HETE reduces insulin secretion in human islets but it remains unclear, which LOX-isoforms contribute to the in vivo production of 12S-HETE [354]. Hepoxilin A3, generated from 12-HpETE by hepoxilin A3 synthase, can induce insulin secretion in pancreatic beta cells and islets [366]. Furthermore, HXA3 protects the rat insulinoma cell line RINm5F against oxidative stress-induced cell death, although the mechanism needs further study [367].

3.5.1.1. LOX in Type 1 diabetes: There is only limited information currently available on the potential role of LOX isoforms in type-1 diabetes. Female nonobese diabetic (NOD) mice are a suitable model for this disease [368]. Remarkably, the NOD-ALOX15^{null} strain, in which ALOX15 is absent, is almost completely protected [369]. These results suggest *ALOX15* contributes to the pathology, and may be related to effects of *ALOX15* on islet cell and/or macrophage functionality [370]. Similarly, Alox15$^{(-/-)}$ mice are resistant to induction of Type 1 diabetes by streptozotocin [357]. In addition, increased levels of ALOX5 metabolites have been detected in diabetic rats [371], but the biological relevance of this observation remains unclear. To the best of our knowledge there is no detailed study currently available characterizing the role of any LOX-isoform in type-1 diabetes in humans.

3.5.1.2. LOX in Type 2 diabetes: Nadler and colleagues investigated the role of *ALOX15* in adipocytes *in vivo* and in cultured cells [372]. The enzyme is induced in white epididymal adipocytes in mice fed a high-fat diet. On a cellular level, a similar up-regulation can be seen when 3T3-L1 adipocytes, which are treated with palmitate. The *ALOX15* products 12-HETE and 12-HpETE diminish the response of 3T3-L1 adipocytes to insulin, consistent with the resistence to insulin characteristic of type 2 diabetes [372].

3.5.2. Lipoxygenase isoforms in adipocyte maturation, obesity and metabolic syndrome—Adipocytes, the major cell type of adipose tissue, differentiate from mesenchymal stem cells via fibroblasts. A complex regulatory network controls adipogenesis, and PPARγ is a decisive factor in this process. 3T3-L1 cells are frequently employed as cellular model of adipocyte differentiation [373] and the LOX inhibitors nordihydroguaiaretic acid and baicalein inhibit adipocyte maturation of 3T3-L1 cells in vitro

[374]. The inhibitory effect of baicalein was prevented by administration of the PPARγ agonist rosiglitazone implicating LOX-isoforms and PPARγ in adipocyte maturation in this in vitro model [374]. Gene expression profiles indicated the presence of Alox15, Alox12 and Aloxe3 in white and brown adipose tissue, but among them only Aloxe3 is expressed at high levels in 3T3-L1 cells. Forced expression of this LOX isoform or addition of *ALOXE3* products (hepoxilins) stimulated adipogenesis and RNAi-mediated expression knockdown prevented adipocyte differentiation [374]. Although these data need to be confirmed for in vivo development of adipocytes the results suggested that *ALOXE3* might constitute an important player in adipogenesis and that specific *ALOXE3* inhibitors might be useful to interfere with this process.

In addition of *ALOXE3* other LOX-isoforms, such as *ALOX15, ALOX12 and ALOX5* have been implicated in adipogenesis [375]. 15-HETE, the major arachidonic acid oxygenation product of *ALOX15* and *ALOX15B*, induced angiogenesis in adipose tissue and thus, has been implicated in growth of adipose tissue [376]. In a limited (1215 subjects) Chinese genetic correlation study a polymorphism in the ALOX12 gene (rs2073438) was significantly associated with total and percentage fat mass ($p=0.007$ and $p=0.012$, respectively) suggesting that *ALOX12* might contribute to the variation of obesity phenotypes in young Chinese men [377]. Unfortunately, the underlying molecular basis has not been explored and it remains to be clarified whether there is a causal relation between *ALOX12* expression and body fat mass. Since adipositas is currently considered a chronic inflammation of the adipose tissue [378] the *ALOX5* pathway has also been implicated in the pathogenesis of this disorder. Blockade of leukotriene signaling by treatment with LOX inhibitors and leukotriene B4 antagonists as well as RNAi-induced expression silencing of leukotriene B4 receptors in human and mouse preadipocytes isolated from native adipose tissues showed acceleration of differentiation into mature adipocytes [379]. From these data the authors concluded that leukotriene B4 signaling may negatively regulate preadipocyte differentiation via induction of transforming growth factor expression [379].

Nonalcoholic fatty liver disease (NAFLD) is a major hepatic consequence of the metabolic syndrome. ALOX15 mRNA is up-regulated in $ApoE^{(-/-)}$ mice, which are frequently employed as model for NAFLD [380]. These days adipositas is considered a chronic low grade inflammatory disorder of the adipose tissue and the inflammatory activities of *ALOX12* and *ALOX15* in the adipose tissue have recently been reviewed [375]. Consistent with the inflammation hypothesis increased levels of *ALOX5* products have been interpreted as signs of low-grade inflammation in adipose tissue, which could contribute to pre-diabetic pathology [381]. Adipose tissue and adipocytes from obese Zucker rats, a model for metabolic syndrome, featured both increased *ALOX15* and *ALOX5* levels, as well as their eicosanoid products [382].

An important question is how the *ALOX15* expression is up-regulated in these low-grade inflammatory models. It is known that in several cell types including macrophages, IL-4 and IL-13 regulate *ALOX15* levels [326, 383]. The transcription factor PPARγ might be involved in expression regulation of ALOX15. Since this transcription factor is activated by the *ALOX15* metabolites 12- and 15-HETE there may exist a damaging feed forward mechanism [384].

Catalytic activity of *ALOX15* is tightly regulated on transcriptional, translational, and post-translational levels and anti-oxidative enzymes such as glutathione peroxidases GPX-1 and -4 have been implicated [385]. In the absence of GPX4 the activity of *ALOX15* is increased and may lead to cell death in a cellular model system [323]. However, it remains to be shown whether this is also the case in vivo. In any case, these findings along with loss of the glutathione substrate may be an important factor in the oxidative tissue damage found in metabolic syndrome.

4. Lipoxygenase isoforms in infectious diseases

When entering the body pathogenes induce an inflammatory host response and LOX products have been implicated as signaling molecules in this protective reaction complex [2, 33, 166]. In principle, the severity of the inflammatory response depends on the balance of pro- and anti-inflammatory mediators and both, hosts and pathogens may contribute to this equilibrium of signaling molecules. For the time being little is known about the role of different LOX-isoforms in infectious diseases and only scattered experimental data are currently available for viral, bacterial and parasite infections as well as for mycosis.

4.1. Lipoxygenase isoforms in viral infections

The human immune system responds to viral infections with an activation of inflammatory cells, and leukotrienes as classical inflammatory mediators [2, 33] have been implicated in this defense reaction. Although no functional LOX sequences have currently been described in major pathogenic viruses eicosanoid production of hosts cells might impact the infection process. The Epstein-Barr Virus (EBV) up-regulates the formation of pro-inflammatory leukotrienes in human peripheral mononuclear cells [386]. Moreover, EBV infection triggers malignant transformation of lymphocytes to Burkitt's lymphoma cells, which are characterized by an increased resistance to apoptosis [387]. Such apoptosis-resistant cells overexpressed *ALOX5* and *ALOX15* and Inhibitor studies as well as cell incubation in the presence of 5-HETE suggest that *ALOX5* and to a lesser extent other LOX-isoforms might be involved in EBV-mediated lymphoma progression [387].

A further example for the involvement of the *ALOX5* pathway in the pathogenesis of virus infection is the human cytomegalovirus (HCMV). In vitro, HCMV infection of human vascular smooth muscle cells strongly increased the expression of *ALOX5* mRNA (170-fold) and protein [388]. In vivo, HCMV-infected vascular smooth muscle cells express the *ALOX5* protein and these data suggest that leukotriene signaling might be upregulated during HCMV infections. However, it remains unclear whether *ALOX5* expression in vivo is directly induced by the virus or whether *ALOX5* expression is just a consequence of the accompanying inflammatory response.

When human neutrophils are infected in vitro with the dengue virus (DENV-2), *ALOX5* expression is upregulated and the cells biosynthesize significantly more leukotriene B4 [389]. In the presence of MK886 (*ALOX5* activating protein antagonist) the increase in leukotriene B4 biosynthesis is reduced indicating the relevance of the *ALOX5* pathway in dengue virus infection. Consistent with this conclusion is the observation that in vivo plasma

levels of leukotriene B4 were significantly elevated during the febrile stages of dengue infections [389].

The respiratory syncytial virus (RSV) induces bronchiolitis [390] and the resolution of this respiratory disorder is mediated by alternatively activated M2-macrophages. RSV infection of ALOX5 and ALOX15 deficient macrophages and mice failed to elicit differentiation of M2 macrophages. Interestingly, treatment of Alox5-deficient macrophages with lipoxin A4 and resolvin E1, but not with leukotriene B4 or leukotriene D4, restored the expression of M2-macrophage markers. From these data the authors concluded that patients with RSV infections might benefit from treatment with proresolving eicosanoids.

4.2. Lipoxygenase isoforms in bacterial infections

LOXs occur in selected bacteria [15] but other bacterial pathogens do not contain functional LOX sequences. For those bacteria carrying functional LOX genes [391] the corresponding enzymes may contribute to systemic eicosanoid formation during host-pathogen interaction. Puerperal sepsis is the leading cause of maternal mortality worldwide and *Streptococcus pyogenes* is the major etiologic agent of severe postpartum sepsis [392]. Mice lacking the *Alox5* pathway were significantly more vulnerable to puerperal sepsis when compared with corresponding wild-type controls [393]. Although the mechanistic basis of this deleterious effect of *Alox5* expression silencing has not been explored the failure of Alox5-deficient mice to synthesize pro-inflammatory leukotrienes may contribute to the increased vulnerability.

Acute pulmonary infection by *Streptococcus pneumoniae* is characterized by high bacterial numbers in the lung and a robust alveolar influx of polymorphonuclear leukocytes. *S. pneumoniae* infection induced expression of 12-lipoxygenating LOX isoforms in cultured pulmonary epithelium and in the lungs of infected mice [394]. Pharmacological (inhibitors) and genetic (Alox15 knockout mice) interference with the *Alox15* pathway reduced lung inflammation in vivo [394] and mechansitic studies suggest that pneumococcal pulmonary inflammation is paralleled by disruption of the lung epithelium via *Alox15* dependent hepoxilin A3 production. In the absence of *Alox15* lower amounts of hepoxilins were detected, which was suggested to contributed to reduced pathology [394]. In pneumococcus-induced otitis media *Alox5* appears to be of major patho-physiological relevance [395]. In an in vivo rat model of this disease Alox5 expression was strongly upregulated and this effect was paralleled by an increase in middle ear fluid. Here again, the mechansitic details have not been explored but the data relate alox5 expression to the pathogenesis of pneumococcus induced otitis media.

Anti-inflammatory lipoxins, which are biosynthesized in vivo by a concerted activity of various LOX-isoforms (*Alox5, Alox12, Alox15*), are key mediators in the resistance of host cells to *M. tuberculosis* infection [396]. High levels of lipoxin A4 were detected in the blood of infected wild-type mice but significantly lower levels were quantified in Alox5 deficient animals. Bacterial burdens in *Alox5* deficient lungs were significantly lower than in the organs of wildtype controls and the increased the resistance of $Alox5^{-/-}$ mice was counteracted by administration of a stable lipoxin A4 analog. From these data the authors concluded that the anti-inflammatory lipoxins negatively regulate the protective Th1

response against mycobacterial infection and suggested that inhibition of lipoxin biosynthesis could serve as a strategy for enhancing host resistance towards *M. tuberculosis* [396].

4.3. Lipoxygenase isoforms in parasite infections and mycosis

Toxoplasmosis is a parasitic disease caused by the protozoan *Toxoplasma gondii* and up to one third of the world's human population is estimated to carry a *Toxoplasma* infection [397]. The human ALOX12 gene has susceptibility alleles for human congenital toxoplasmosis and RNAi-mediated expression knockdown of this gene attenuated progression of *T. gondii* infection [398]. These data implicate *ALOX12* in host defense against *T. gondii* but the underlying molecular mechanisms remain unclear.

Schistosoma are parasitic flatworms. They induce Schistosomiasis, which is considered by the WHO the second most socioeconomically devastating parasitic disease (after malaria) with hundreds of millions infected people worldwide [399]. Periovular granuloma formation during *S. mansoni* infection is a complex immunologic response and LOX inhibitors reduced granuloma formation [400]. At the acute stage of infection, when granuloma formation is usually maximal, Alox5 deficient mice developed smaller granulomas around liver-deposited schistosome eggs compared with wild type and Alox15 deficient mice. These data suggest that *Alox5* but not *Alox15* may play a role in the host responses to schistosomiasis.

Paracoccidioidomycosis is a systemic mycosis caused by the thermodimorphic fungus *Paracoccidioides brasiliensis*. When Alox5 deficient mice and corresponding wild-type controls were intravenously inoculated with *P. brasiliensis* they exhibited an increased survival rate [401]. The disease resistance was associated with augmented nitric oxide production, reduced number of CD4(+)-CD25(+) regulatory T cells and higher levels of gamma interferon as well as interleukin-12 levels in the lungs. These results suggest that expression of *Alox5* increased the susceptibility of mice for *P. brasiliensis* suggesting that this pathway might constitute a potential target for therapeutic intervention.

Candida albicans is an opportunistic fungal pathogen that resides commensally on epithelial surfaces, but can cause severe inflammation in immunocompromized patients [402]. *C. albicans* is capable of biosynthesizing anti-inflammatory resolvins (RvE1) but in contrast to human cells there is not transcellular biosynthesis [403]. Although the biosynthetic mechanisms have not been clarified RvE1 in vitro enhanced phagocytosis of *C. albicans* by human neutrophils and augmented intracellular ROS generation and killing. Moreover, in a mouse model of systemic candidiasis RvE1 stimulated clearance of the fungus from circulating blood suggesting a possible role for RvE1 and its biosynthesizing machinery in *C. albicans* infections.

5. Lipoxygenase inhibitors as potential drugs

Since LOX isoforms have been implicated in the pathogenesis of major human diseases LOX inhibitors are of medical interest. According to their inhibitor mechanisms LOX inhibitors can be classified into 5 principal groups (Fig. 7): i) Redox inhibitors interfering with the valency change of the nonheme iron during the catalytic cycle. ii) Iron chelators

complexing the iron ion at the active site. iii) Active site probes competing with substrate fatty acids in the substrate binding pocket, iv) Suicide substrates leading to irreversible inactivation of the enzyme; v) Allosteric inhibitors that bind to a site other than the substrate binding pocket. Although a number of pharmaceutical companies including Abbott, Merck, Wellcome and ICI initiated LOX inhibitor programs the only LOX inhibitor that has made it to the clinics is the ALOX5 inhibitor zileuton [175]. This drug has been approved as anti-asthmatic but owing to its unfavorable pharmacokinetics and its side effects it has not reached wide spread acceptance. Originally, zileuton was available in two formulations under the brand names ZYFLO and ZYFLO CR. The immediate-release formulation ZYFLO is given at a dosage of 600 mg four times per day. The extended-release formulation (ZYFLO CR) is taken twice daily. Although there have been a number of promising LOX-inhibiting compounds other than zileuton their therapeutic potential was disappointing. Moreover, evaluation of the recent patent activities revealed only compounds with moderate inhibitory potency [404]. The most promising advances in drug development have been made for FLAP (*ALOX5* activating protein) antagonists and it might well be that such compounds will enter the market as anti-asthmatics [404]. There are a number of recent reviews summarizing the current knowledge in the field of LOX inhibitors [2, 404–406] and there is no need for re-reviewing them. However, we would like to briefly discuss three problems that might have contributed to the fact that LOX inhibitors have performed rather weakly in the clinics despite their promising experimental potential.

5.1. Isoform-specific LOX inhibitors

As indicated in Table 1 six functional LOX isoforms exist in humans. In the murine genome there are seven functional LOX genes. Although except for the *ALOXE3* the different human LOX isoforms catalyzed the same principal reaction (oxygenation of polyenoic fatty acids) they have been implicated in different physiological and patho-physiological processes. Consequently, inhibitors that impact the catalytic activity of several LOX isozymes are likely to have unwanted side effects. For instance, an *ALOX5* inhibitor, aimed at developing an anti-asthmatic drug [404], should not significantly impact the activity of *ALOX12B*. Otherwise problems with skin development may occur [84, 85]. This functional multiplicity of LOXs requires the development of isoform-specific inhibitors. Most experts working in this field are well aware of this problem and test the isoform-specificity of their compounds in different assay systems. However, the assay systems employed in the past are not strictly comparable and thus, the data might be misleading: i) The purified rabbit *ALOX15* has frequently been employed as model enzyme for the corresponding human ortholog but it still remains unclear whether the two enzymes have similar inhibitor sensitivities. The two *ALOX15* orthologs share a high degree of amino acid conservation (>80%) but there are amino acid differences that might impact inhibitor sensitivity. ii) In the past, impure enzyme preparations (cell lysates of different cells) were employed as enzyme sources for activity assays. For instance, lysates of rat basophilic leukemia cells were used as *ALOX5* source and platelet lysates were employed as source for human *ALOX12* [407]. Unfortunately, these assay systems are not strictly comparable since foreign proteins may bind the inhibitors, which impacts IC50 values. To circumvent these problems inhibitor studies should always be carried out with purified recombinant human enzyme preparations [408, 409]. Unfortunately, some human LOX-isoforms (*ALOXE3, ALOX12B*) are not well expressed as

recombinant proteins making this strategy difficult to follow. Alternatively, all LOX isoforms should be expressed in a single eukaryotic overexpression system (such as COS or HEK cells) and the cellular lysate may be employed as enzyme source. Recently, a systematic study was carried out, in which all rat 12-lipoxygenating LOX isoforms were overexpressed in HEK cells and an array of commercially available LOX inhibitors was tested using the cell lysates [410]. The data obtained indicate that the commercial LOX-inhibitors (NDGA, CDC, AA861, baicalein, PD146176) only exhibited a low degree of isoform specificity although some of them have previously suggested as isoform-specific LOX inhibitors. Most surprisingly, PD146176, which has been employed in experimental strategies as *ALOX15* specific inhibitor [323, 407, 411], did not at all inhibit rat *ALOX15* [410]. For the time being, it remains unclear why this compound effectively inhibits rabbit and human *ALOX15* [407] and not the rat ortholog [410] but it may be related to the fact that rat *ALOX15* is a 12-lipoxygenating enzyme [34] whereas rabbit and human orthologs are 15-lipoxygenating [35]. However, to avoid misinterpretations of experimental data it is most important to make sure that human *ALOX15*-inhibitors, which are scheduled to be employed as mechanistic probes in murine systems, have been tested for LOX inhibition in these experimental setups (rat, mouse or other species). It might well be that an inhibitor that effectively inhibits human *ALOX15* does not at all inhibit the orthologous enzymes of other species. Thus, effects of such compounds in mouse or rat experimental systems are probably due to off-target effects of the compounds although they might be interpreted as consequence of *ALOX15* inhibition.

5.2. Species-specific differences of LOX orthologs

To explore the patho-physiological roles of different LOX-isoforms murine disease models are required. Unfortunately, some murine LOX isoforms have different properties than their human ortholog and these differences may impact inhibitor sensitivity. For instance, murine *Alox15* isoforms (mouse, rat) are 12-lipoxygenating enzymes [34] whereas the human ortholog is 15-lipoxygenating [35, 412]. The molecular basis for this functional difference has previously been explored [44] and appears to be related to the volume of the substrate-binding pocket [1]. If this hypothesis is correct it may explain the differences in inhibitor sensitivity of rat [410] and human *ALOX15* orthologs [407]. There is a second species-specific difference between mouse and human LOX orthologs. Human *ALOX15B* converts arachidonic acid almost exclusively to 15-HpETE whereas the mouse ortholog is 8-lipoxygenating [45]. Here again, mutagenesis studies indicated that two critical amino acids make the functional difference between 15S- and 8S-lipoxygenation. Although there are currently no detailed experimental data comparing the inhibitor sensitivities of mouse and human *ALOX15B* it might be speculated that the differences in reaction specificity may also impact this enzyme property.

5.3. Off-target effects of LOX-inhibitors

Some LOX inhibitors, such as NDGA or propylgallate contain catechol structures and thus, exhibit anti-oxidative properties. Thus, in addition to being LOX inhibitors they may directly impact the redox homeostasis in biological systems. Since the cellular redox homeostasis is important for regulating the gene expression pattern on genetic [413] and epigenetic [12, 414] levels, it is difficult to discriminate which of the two functions (LOX

inhibition vs. redox activity) is the major reason for a biological effect. Consequently, results obtained with these types of LOX inhibitors need to be interpreted with care and to avoid misinterpretation inhibitor studies should always be confirmed by alternative loss-of-function strategies such as siRNA induced expression silencing and/or the use of genetic knock-out models.

6. Concluding remarks and perspectives

Because of the proposed biological roles of LOXs and their implication in the pathogenesis of public health-relevant human diseases these enzymes have received significant attention over the past decades. Although most human LOX isoforms can be expressed as recombinant proteins and have been well characterized with respect to their structural and functional properties there are a number of caveats that need to be addressed in the future. If one considers rabbit *ALOX15* as suitable structural model for the human ortholog, crystal data are now available for four (*ALOX15, ALOX12, ALOX5, ALOX15B*) of the six human LOX isoforms. Although there are subtle differences between the different isoforms the general fold of the enzymes is similar and on the basis of the current structural data reliable models may be constructed for the remaining LOX isoforms (*ALOX12B, ALOXE3*). When compared with other LOX isoforms the oxygenase activity of *ALOXE3* is limited and thus, the enzyme has been suggested to function as fatty acid peroxide isomerase. Although some studies have been performed to explore the molecular reasons for the lacking oxygenase activity of *ALOXE3* [415, 416] the structural basis remains unclear. Crystal data for *ALOXE3* might shed some light on this mechanistic detail. Furthermore, although dimerization of various LOX isoforms has recently suggested [64, 65] the mechanistic and biological consequences of this effect have rarely been studied. It might well be that enzyme dimerization may contribute to the allosteric properties that have been described for various LOX isoforms [62, 265]. Another problem in molecular LOX research is lacking direct structural information on LOX-substrate complexes. The currently available structural data on enzyme-ligand complexes [46–51] do not conclusively answer the questions on the structural basis for the reaction specificity of the different LOX-isoforms.

Although knockout mice are currently available for 5 LOX isoforms (Alox5, Alox15, Alox12, Alox12b, Aloxe3) the biological roles of *ALOX15* and *ALOX12* are not well understood. ALOX12 and ALOX15 knockout mice are viable and although irregular epididymal maturation has been reported for ALOX15-deficient sperms the animals breed well and corresponding mouse colonies can be established easily. On the other hand, challenging experiments suggest the involvement of the two enzymes in physiological processes. It might well be that the mild phenotypes of Alox15, Alox12 and Alox5 knockout mice may in part be related to the fact that no conditional knockouts are currently available for these two enzymes. One problem with non-conditional knockout mice is that their creation is somewhat selective (only embryonic stem cells that survived the genetic manipulation were selected for blastocyst injection) and that compensatory mechanisms during early embryogenesis cannot be ruled out. To overcome these problems inducible knockout systems should be established but such experiments are time consuming and quite expensive.

One of the most serious problems in LOX research in the past decade has been the lack of sensitive (in vivo IC50 in the lower nanomolar range) isoform-specific inhibitors that could be employed in animal disease models or in experimental clinical studies in humans. There are a large number of LOX inhibitor structures in the chemical databases but for most of them isoform-specificity has not been explored in strictly comparable assay systems. Only recently this problem was partly overcome [408, 409], but the new compounds have not yet reached widespread acceptance. In addition, the different functional characteristics of LOX orthologs in different mammalian species (12-lipoxygenating *Alox15* in mice vs. 15-lipoxygenating *ALOX15* in humans) make the situation even more complex. A broad experimental screening program for isoform-specific LOX inhibitors employing high throughput systems, which are based on purified recombinant human LOX isoforms, would help to approach these problems. Of course, such experiments and the following refinement strategies are time consuming and expensive and for the time being the pharmaceutical industry appears to be not interested in such strategies because of the controversial reports on the bioactivity of various LOX isoforms. However, the recent experimental data on the involvement of certain LOX isoforms in neurological disorders (especially *ALOX5* and *ALOX15*) and the potential use of *ALOX15* inhibitors for anti-stroke therapy [332] might change this situation.

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List of abbreviations

LOX	lipoxygenase
COX	cyclooxygenase
LT	leukotrienes
HETE	hydroxy eicosatetraenoic acid
PPAR	peroxysome proliferator activated receptor
MICAL	Molecule Interacting with CasL
ARCI	autosomal recessive congenital ichthyosis
GPCR	G-protein coupled receptors
NOD	nonobese diabetes
RSV	respiratory syncytial virus
NDGA	nordihydroguaiaretic acid
ALX	lipoxin receptor
OXE	oxo-eicosanoid receptor

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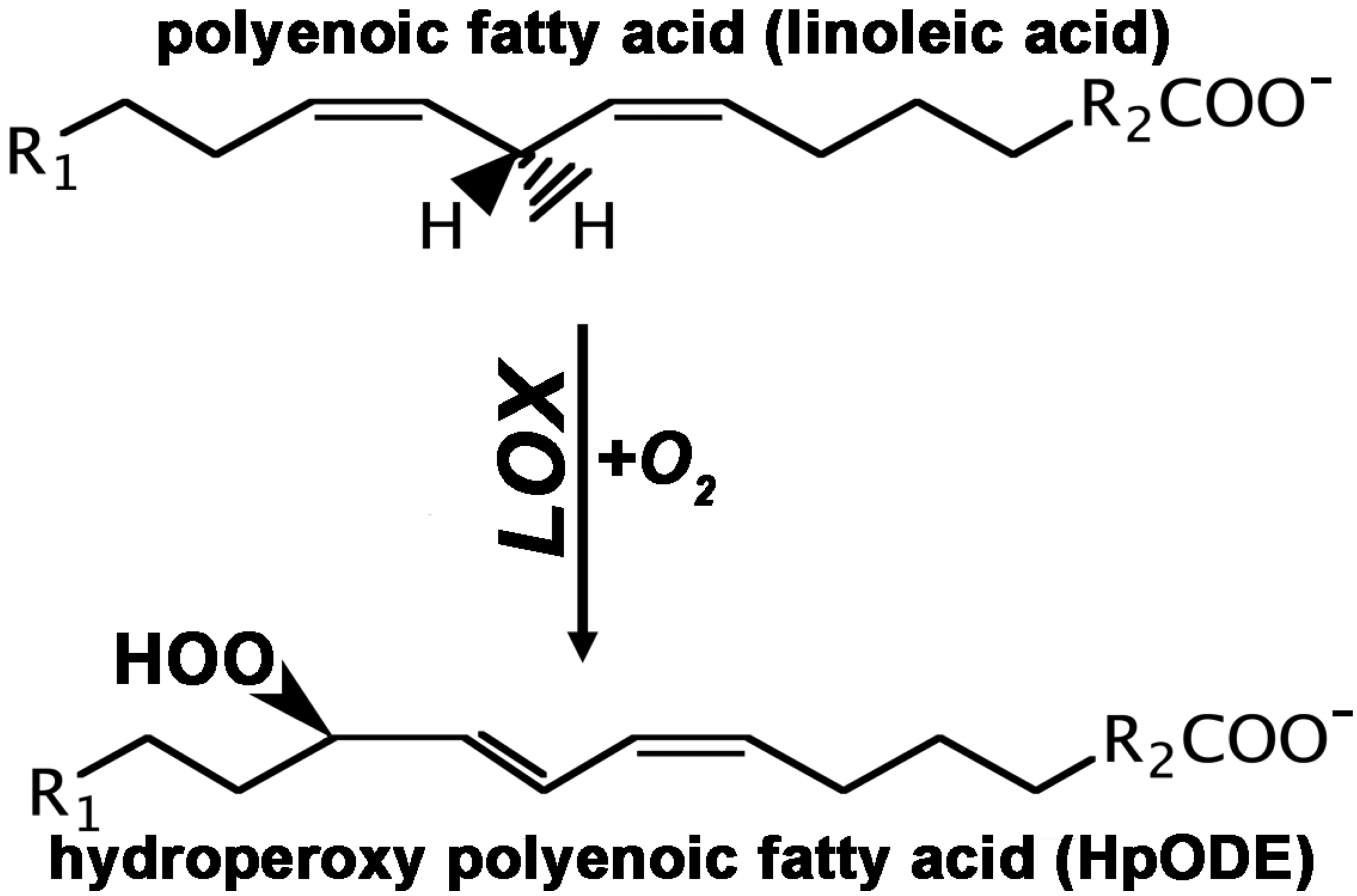

Fig. 1. Simplified scheme of the lipoxygenase reaction

LOXs convert polyenoic fatty acids containing at least one 1,4-pentadiene system to their corresponding hydroperoxy derivatives. Atmospheric oxygen serves as second substrate.

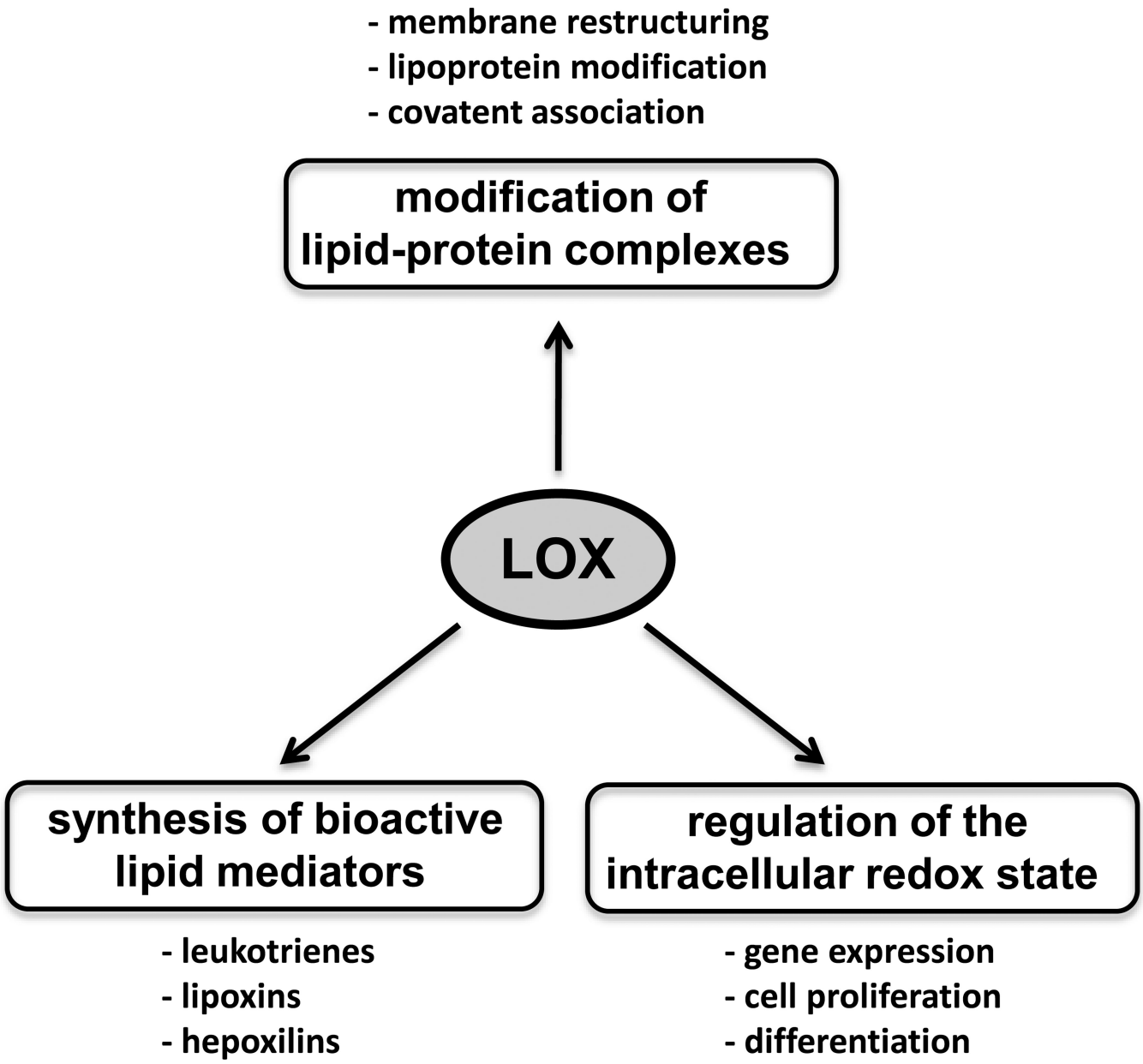

Fig. 2. Biological function of lipoxygenase

Lipoxygenases may exhibit their biological functionality via three different mechanistic scenarios. i) Formation of bioactive lipid mediators, ii) Structural modification of complex lipid-protein assemblies. iii) Modification of the cellular redox homeostasis, which alters the gene expression pattern.

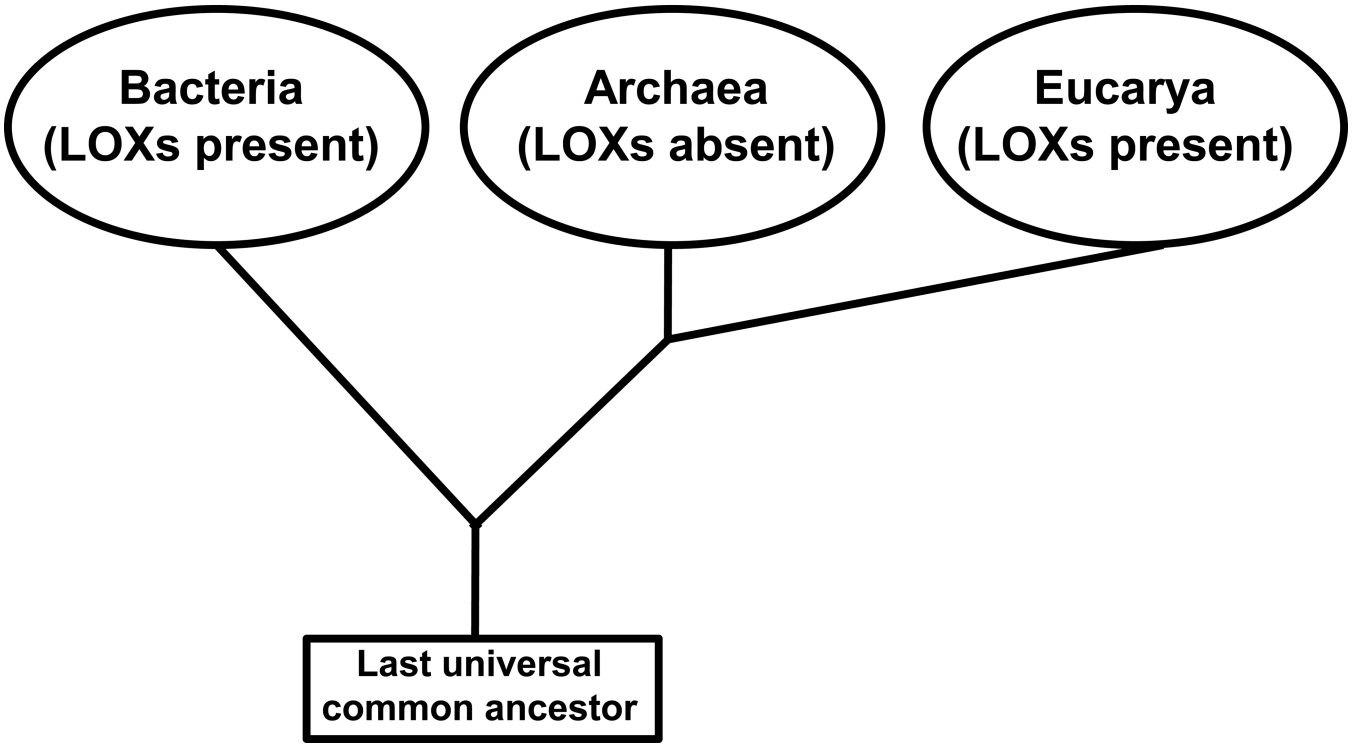

Fig. 3. Distribution of lipoxygenases in the kingdoms of terrestrial life

Lipoxygenase genes have been detected in two (bacteria, eukarya) of the three kingdoms of terrestrial life. Although LOX-like sequences have also been described in archaea, no functional LOX enzyme has been reported to occur.

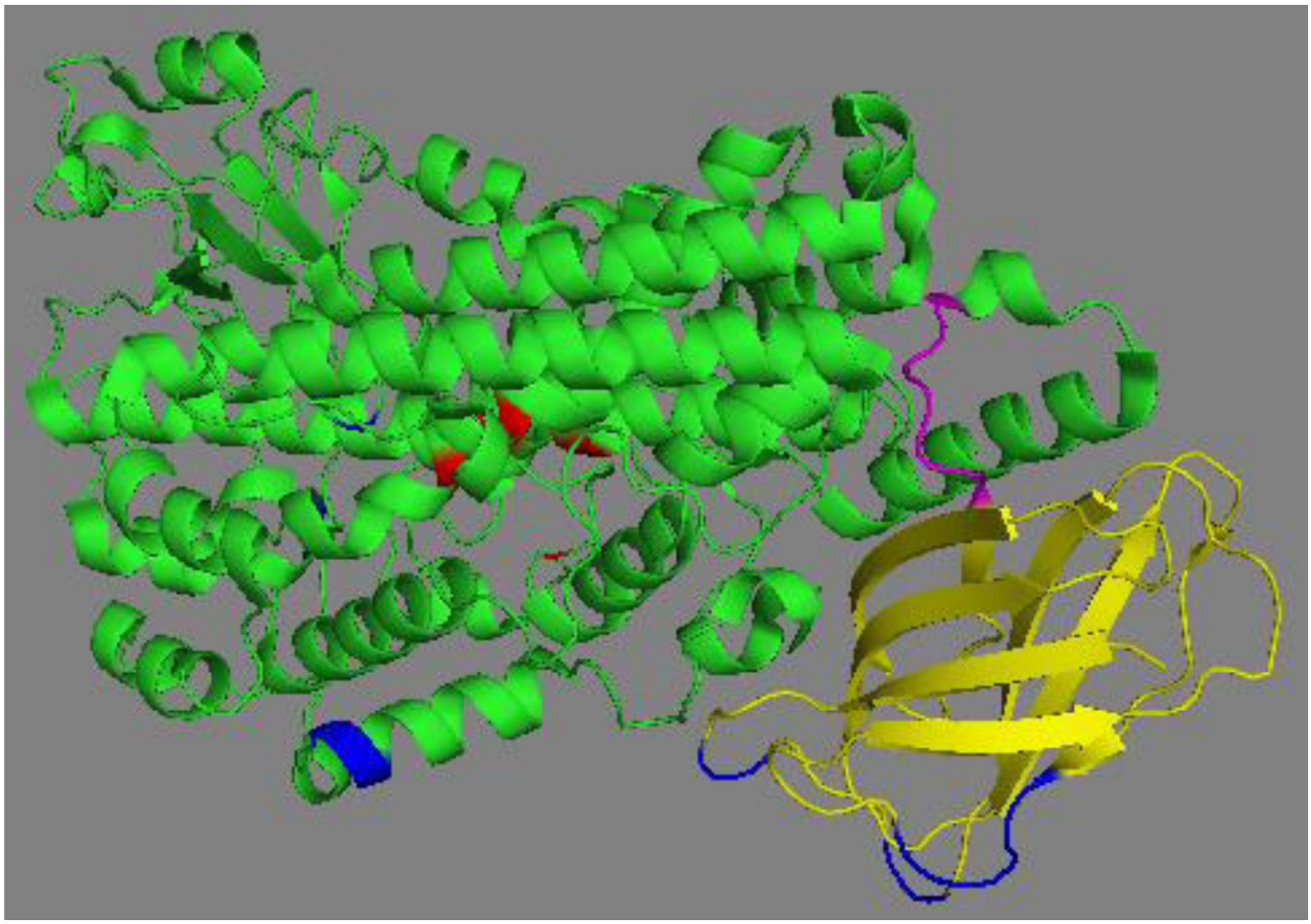

Fig. 4. Crystal structure of the stabilized version of human *ALOX5*

The N-terminal β-barrel domain is shown in yellow, the flexible inter-domain linker (D113-L118) in magenta, the C-terminal catalytic domain in green and the iron liganding residues in red. The residues mutated in wild-type *ALOX5* to get the stabilized version of the enzyme suitable for crystallization are indicated in blue. The image was constructed from the X-ray diffraction data using the PyMol software package.

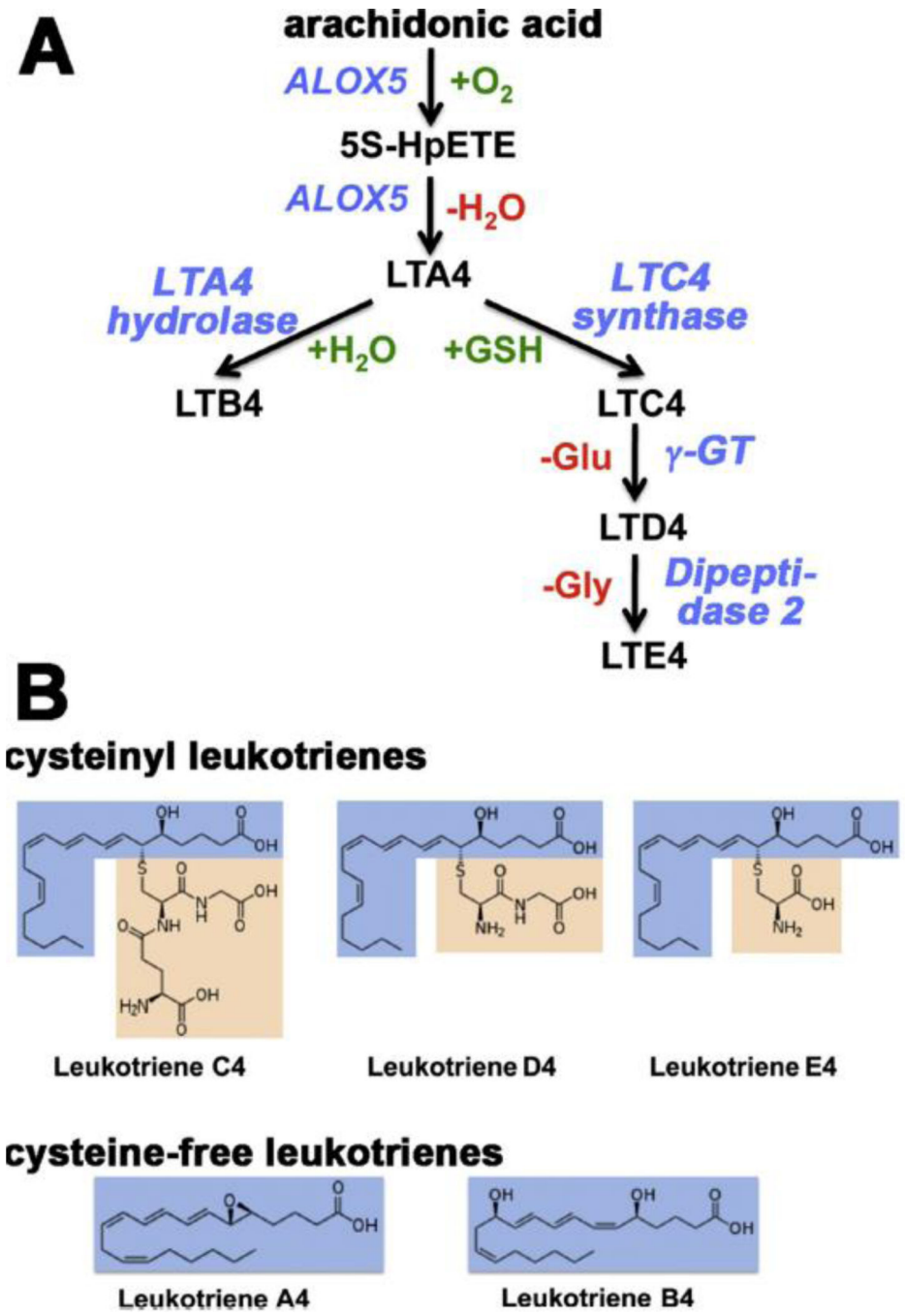

Fig. 5. Classification and structure of leukotrienes

A) Leukotriene biosynthesis, B) Structure of leukotrienes.

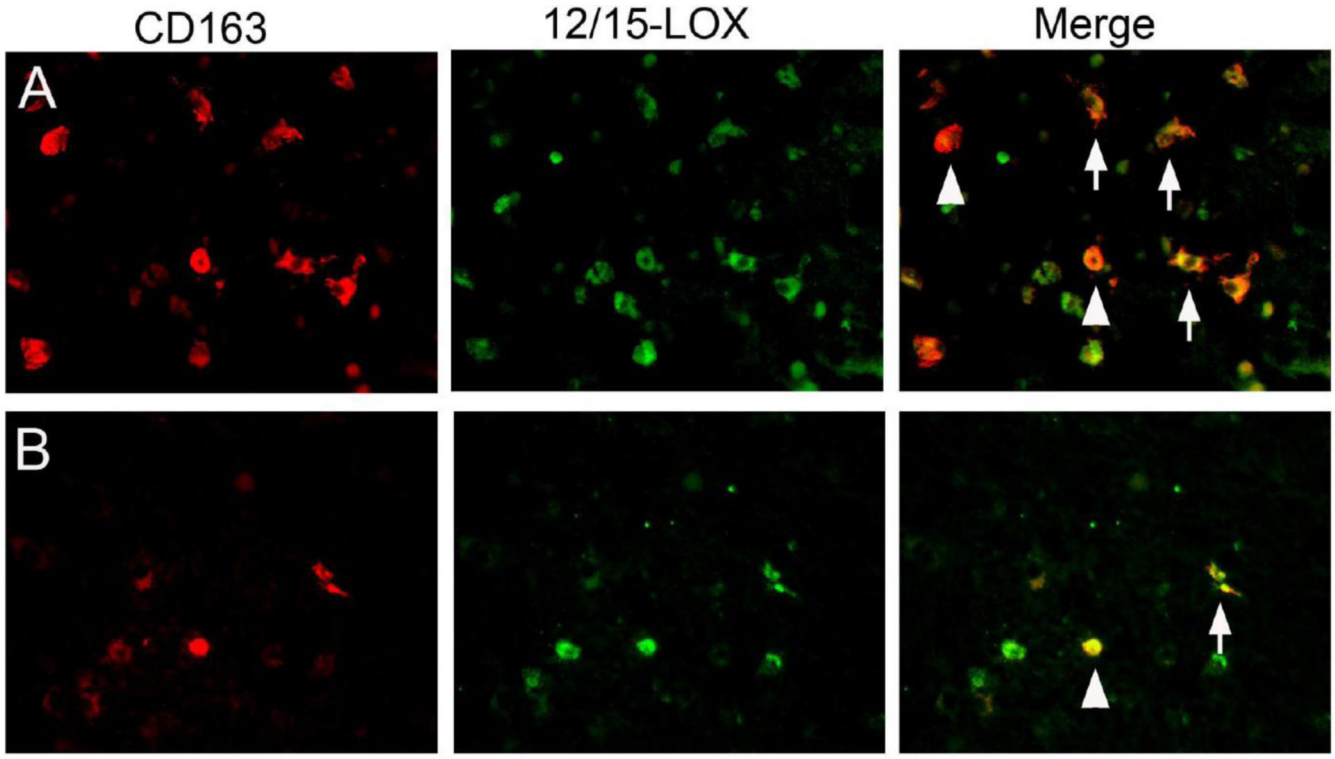

Fig. 6. Co-localization of *ALOX15* with the microglial/macrophage marker CD163

Depending on disease state, various cell types can show increased *ALOX15*. Shown here in A and B are two examples of brain tissue from infants with periventricular leukomalacia, where ALOX15 (12/15-LOX) co-localizes with the microglial/macrophage marker CD163, suggesting a role in disease pathology. *ALOX15* in these brains was also increased in oligodendrocytes (courtesy of Dr. Robin Haynes, Children's Hospital Boston)

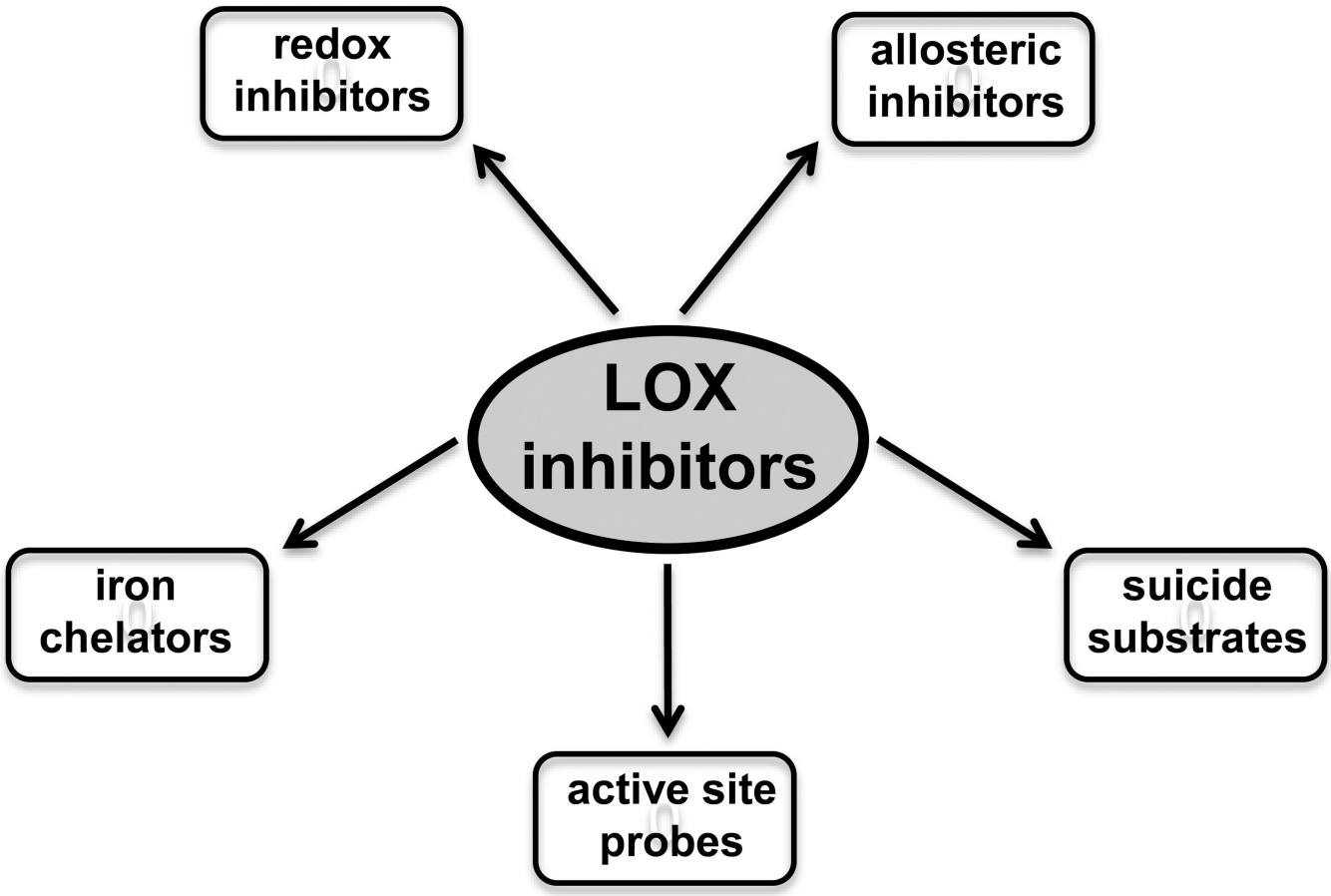

Fig. 7. Principal modes of action of lipoxygenase inhibitors

NDGA - nordihydroguaiaretic acid, HODE - hydroxy octadecadienoic acid, ETYA - 5,8,11,14-eicosatetraynoic acid, ODYA - 9,12-octadecadiynoic acid, OPP - 4-(2-oxapentadeca-4-yne)phenylpropanoic acid,

Table 1

Human ALOX genes and major expression sites of the corresponding LOX-isoforms

Human gene	Former name	Mouse gene	Former name	Major expression	References
ALOX15	12/15-LOX	alox15	Lc12-LOX*	eosinophils, bronchial epithelium	[19–21, 78]
ALOX15B	15-LOX2	alox15b	8-LOX	hair roots, skin, prostate	[22, 23]
ALOX12	pl12-LOX**	alox12	pl12-LOX	thrombocytes, skin	[24, 25, 92]
ALOX12B	12R-LOX	alox12b	12R-LOX	skin,	[26, 27]
ALOXE3	eLOX3	aloxe3	eLOX3	Skin	[28–30]
ALOX5	5-LOX	alox5	alox5	leukocytes, macrophages, dendritic cells	[114, 120, 138, 417]
pseudogene		alox12e	elox12	Skin	[18]

* lc – leukocyte-type,

** pl – platelet-type

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