DOI 10.1002/mnfr.200500131 Mol. Nutr. Food Res. 2005, *49*, 1014 – 1029

Review

The role of lipoxygenase-isoforms in atherogenesis

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Lipoxygenases (LOXs) form a heterogeneous family of lipid-peroxidizing enzymes, and several LOX-isoforms (12/15-LOX, 5-LOX) have been implicated in atherogenesis. However, the precise role of these enzymes is still a matter of discussion. 12/15-LOXs are capable of oxidizing lipoproteins (low-density lipoprotein (LDL), high-density lipoprotein (HDL)) to atherogenic forms, and functional inactivation of this enzyme in murine atherosclerosis models slows down lesion formation. In contrast, rabbits that overexpress this enzyme were protected from lesion formation when fed a lipid-rich diet. To contribute to this discussion, we recently investigated the impact of 12/15-LOX overexpression on *in vitro* foam cell formation. When 12/15-LOX-transfected J774 cells were incubated in culture with modified LDL, we found that intracellular lipid deposition was reduced in the transfected cells when compared with the corresponding control transfectants. This paper briefly summarizes the current status of knowledge on the biological activity of different LOX-isoforms in atherogenesis and will also provide novel experimental data characterizing the role of 12/15-LOX in cellular LDL modification and for *in vitro* foam cell formation.

Keywords: Eicosanoids / Gene expression / Inflammation / Modified lipoproteins / Oxidative stress

Received: August 2, 2005; accepted: August 4, 2005

1 Introduction

Atherosclerosis, the disease that gives rise to myocardial infarction, stroke, and vascular occlusive disease of the extremities, is the major cause of mortality in industrialized countries [1]. Risk factors for atherosclerosis have been identified by epidemiological studies [2], but the disease-initiating mechanisms remain largely elusive. The response to injury hypothesis [3, 4] emphasizes that atherosclerosis is a chronic fibro-proliferative disease of the arterial wall that is associated with aberrant immune reactions. In addition, the lipid oxidation hypothesis [5, 6] proposes that oxidized low-density lipoproteins (oxLDL) may trigger arterial wall injury and initiate foam cell formation. Since lipoxygenases (LOXs) are lipid-peroxidizing enzymes, which are capable of oxygenating LDL to an atherogenic form, they have been implicated in atherogenesis.

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Abbreviations: acLDL, acetylated low-density lipoprotein; **HDL**, high-density lipoprotein; **13-HODE**, 13-hydroxy-9*Z*,11*E*-octadecadienoic acid; **LDL**, low-density lipoprotein; **LOX**, lipoxygenase; **oxLDL**, oxidized low-density lipoprotein

1.1 The LOX family and LOX reaction

LOXs form a family of lipid-peroxidizing enzymes which catalyze the stereoselective oxygenation of polyunsaturated fatty acids containing a (1*Z*,4*Z*)-penta-1,4-dienoic system to the corresponding hydroperoxy derivatives [7]. They occur in plants [8], animals [9], and selected bacteria [10]. Most of our knowledge on LOX structure and function originates from studies on soybean LOX-isoforms. In several aspects (reaction mechanism, kinetic parameters, iron content, *etc.*) the soybean LOX-1 constitutes a suitable model for mammalian LOXs. However, other properties are quite different when plant and mammalian LOXs are compared. Thus, experimental data obtained with the soybean enzyme should be interpreted with care when conclusions to mammalian physiology are drawn.

LOXs catalyze peroxidation of polyenoic fatty acids using atmospheric oxygen as second substrate. Recently, it has been suggested that oxygen may not only constitute a LOX substrate but may also act as enzyme activator impacting the specific activity [11]. The mechanistic reasons for this reversible enzyme activation are rather complex and appear to involve direct interaction of oxygen with the catalytic nonheme iron. In principle, the LOX reaction consists of three consecutive steps (Fig. 1): (i) initial stereoselective hydrogen abstraction from a bisallylic methylene group

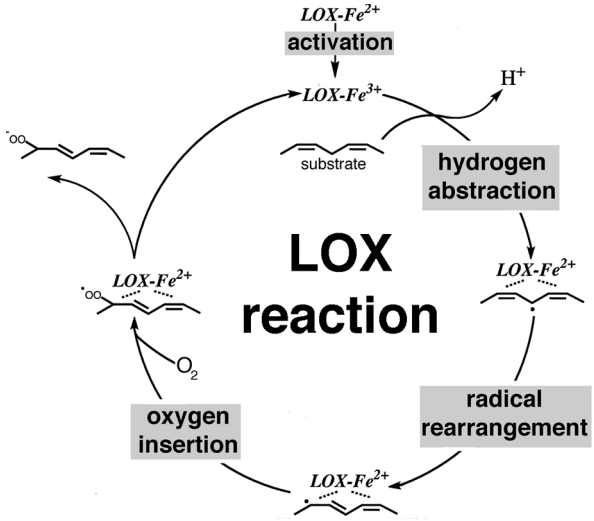

Figure 1. Scheme of LOX-reaction. Catalytically silent ferrous LOX must be activated and this activation process involves oxidation of the nonheme iron. LOX cycle itself consists of three consecutive steps. (i) Stereoselective abstraction of a hydrogen atom from a bisallylic methylene forming a carbon-centered fatty acid radical. Hydrogen atom is abstracted as proton and the ferric LOX is reduced by taking up the resulting electron. (ii) Radical rearrangement that is accompanied by *Z,E*-diene conjugation. (iii) Stereospecific insertion of molecular dioxygen at C-1 or C-4 of the pentadienyl system forming a fatty acid hydroperoxide radical. This oxygen-centered radical is subsequently reduced to the corresponding anion and the ferrous LOX is oxidized to the ferric form to close the catalytic cycle.

forming a carbon-centered fatty acid radical. Hydrogen abstraction is the rate-limiting step of the overall reaction. Formally, hydrogen is abstracted as proton and the electron is transferred to the ferric LOX. (ii) Radical rearrangement, which is accompanied by *Z,E*-diene conjugation. (iii) Stereoselective insertion of molecular dioxygen at C-1 or C-4 of the pentadienyl system forming an oxygen-centered fatty acid hydroperoxy radical. This radical intermediate is reduced to the corresponding anion and the enzyme is oxidized back to the ferric form. It should be stressed that the radical intermediates usually remain enzyme bound, which explains the high product specificity (formation of a single product isomer) of most mammalian LOXs. However, under certain reaction conditions (extreme pH, low oxygen concentration) the LOXs may serve as source of free lipid radicals [12]. In such cases, the enzymes form complex mixture of stereorandom oxygenation products.

Mammalian LOXs form a heterogeneous enzyme family. Completion of the human genome project indicated the existence of six functional LOX genes [13]. In the murine genome seven functional LOX genes have been identified. According to their phylogenetic relatedness mammalian LOXs may be classified into four different subfamilies

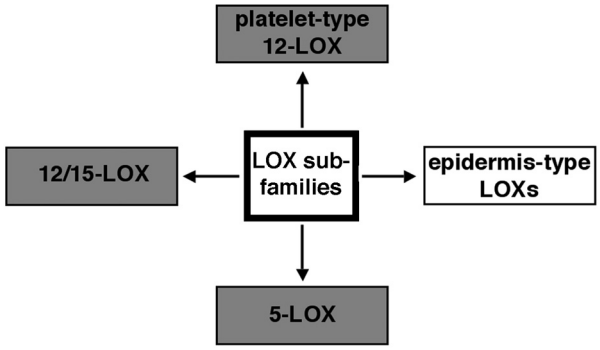

Figure 2. Subclassification of mammalian LOX-isoforms. Historically, LOXs were classified with respect to their positional specificity of arachidonic acid oxygenation. Unfortunately, the growing diversity of LOX-isoforms made this arachidonic acid-based nomenclature rather confusing, and thus different classification principles were recently applied. According to their phylogenetic relatedness, mammalian LOX can be subclassified in four distinct families [9]. Isoforms on gray background have been implicated in cardiovascular diseases. Role of the epidermis-type LOXs has not been investigated.

(Fig. 2): (i) 5-LOXs, (ii) 12/15-LOXs, (iii) platelet-type 12-LOXs, and (iv) epidermal-type LOXs [9]. For most LOX-isoforms, the biological activity is far from clear. According to the concept of the arachidonic acid cascade, LOXs are involved in the biosynthesis of lipid hormones, such as leukotrienes [14], lipoxins [15], hepoxilins [16], and other hydroxylated fatty acid derivatives, which exhibit a large array of biological activities. In addition, LOXs have been implicated in cell physiological processes not directly related to eicosanoid metabolism. 5-LOXs are involved in the leukotriene biosynthesis [14, 17]. Leukotriene antagonists and leukotriene synthesis inhibitors have been developed as antiasthmatics and some of them are available for prescription use. Under certain conditions the 5-LOX is also expressed in cells, which are not related to anaphylactic and/or inflammatory reactions [18]. Moreover, the intranuclear localization of the enzyme [19] in various cell types may suggest that the 5-LOX and/or 5-LOX products might be involved in regulation of gene expression.

12/15-LOXs have been implicated in cell development and differentiation. During the maturation of rabbit reticulocytes, this enzyme initiates a cytosolic breakdown cascade of the remaining mitochondria during reticulocyte/erythrocyte transition [20]. Similar mechanisms involving 12/15-LOXs have been suggested for the differentiation of eye lens cell [21]. The murine 12/15-LOX plays an important role in bone metabolism. Crossbreeding experiments with 12/15-LOX knockout mice indicated improvement of bone density and strength in animals lacking the enzyme, and pharmacological intervention studies with 12/15-LOX inhibitors confirmed these data in two rodent models of osteoporosis [22]. Whether similar effects can be observed in

humans remain to be investigated in the future. Human peripheral monocytes do not express a 12/15-LOX but in macrophages, which develop from peripheral monocytes in the presence of interleukin-4, the enzyme can be detected in large amounts [23]. Thus, 12/15-LOX may be important for monocyte–macrophage transitions or for macrophage function. More recent investigations on this topic (oligonucleotide-based microarray studies) suggested that interleukin-4 might contribute to switch peripheral monocytes into a resolving phenotype, suggesting a role for 12/15-LOXs in inflammatory resolution [24].

The platelet-type 12-LOX was the first mammalian LOX discovered back in 1974 [25]. Because of its preferential detection in platelets of various species, the enzyme was implicated in platelet function. In fact, platelet 12-LOX knockout mice exhibit an increased sensitivity for ADP-induced aggregation, suggesting a modulator role of the enzyme in blood clotting [26]. In rabbits, the platelet aggregating response toward platelet activating factor may also involve the 12-LOX pathway [27]. Moreover, the platelet-type 12-LOX has been implicated in radical-mediated engulfment of viruses and bacteria [28], and thus the enzyme may be relevant for the immune function of thrombocytes. It should be stressed that expression of platelet-type 12-LOX is not restricted to platelets [29, 30]. The enzyme also occurs in various types of epithelial cells and several parts of the brain. Although its biological role in these tissues is still unclear, there is evidence for involvement in regulation of epidermal water homeostasis [31], skin [32, 33] and prostate diseases [34], and neurodegenerative disorders [35].

The biological roles of the epidermal-type LOXs are quite unclear. There are no knockout mice available at the moment and isoform-specific inhibitors have not been developed so far. The human epidermal-type 15-LOX (15-LOX2) has also been related to skin disease [36] and prostate cancer [37, 38] but the mechanisms are still under discussion.

1.2 LOX structure

Although soybean LOX-1 was already crystallized in 1947, it took 46 years to solve its crystal structure [39]. The first 3-D structure of a mammalian LOX (rabbit 12/15-LOX) was reported in 1997 [40]. Comparison of these crystal structures indicated strong similarities between these isoforms despite considerable differences in the amino acid sequence. All LOX-isoforms have a two-domain structure (Fig. 3). For the rabbit enzyme, the small N-terminal domain comprises 115 amino acids and is composed of eight β-sheets. This domain is similar in sequence, size, and structure to C-terminal β-barrel domains of mammalian lipases, which contributes to localize the enzyme at the surface of complex lipid–protein assemblies (biomembranes

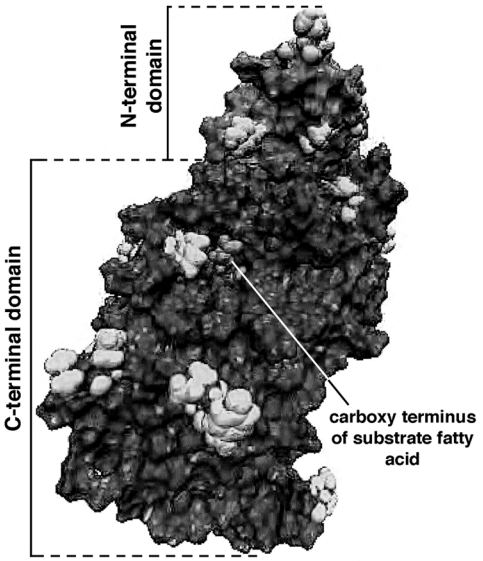

Figure 3. 3-D structure of rabbit 12/15-LOX. 3-D structure of the rabbit 12/15-LOX-arachidonic acid complex was modeled on the basis of the X-ray coordinates of 12/15-LOX crystals [40]. Small N-terminal β-barrel domain and the large catalytic subunit are indicated. Carboxylic end of a fatty acid substrate, which was modeled into the substrate-binding pocket, is also shown. Methyl end of the substrate is buried deeply inside the enzyme molecule. Surface exposed hydrophobic amino acids (bride residues) surround the entrance into the substrate-binding pocket. Most of these amino acids have been implicated in membrane binding of the enzyme as indicated by site-directed mutagenesis [120].

or lipoproteins). The β-barrel domain shares a 1600 Å^2 interface with the larger C-terminal domain, which consists of 18 helices interrupted once by a small β-sheet subdomain. The core of the C-terminal domain is formed by two long helices, which contain four of the five ligands of the catalytic nonheme iron. In the soybean LOX-1 water (or a hydroxyl) occupies the sixth ligand position and this ligand may be displaced when fatty acid substrates are bound. The substrate-binding cleft of 12/15-LOXs appears to be a boot-shaped cavity, which is directly accessible from the surface of the protein (Fig. 3). It is surrounded by a number of surface exposed hydrophobic amino acids, which are important for the membrane-binding activity of the enzyme [41]. The bottom of this pocket is defined by a triad of amino acids (Phe353, Ile418, and Ile593) and these structural elements constitute sequence determinants for the positional specificity of 12/15-LOXs [42]. Mutagenesis experiments and modeling studies on enzyme – substrate complexes suggested that the positively charged Arg403 might interact with the carboxylic group of the fatty acid substrate [43].

1.3 Oxidation hypothesis of atherosclerosis

Hypercholesterolemia, in particular increased LDL cholesterol levels, is a major risk factor for atherosclerosis. How-

ever, LDL *per se* might not be atherogenic since the peripheral metabolism of LDL lipids is well regulated. Detailed investigations on the mechanism of peripheral lipid metabolism indicated that cellular uptake of native LDL *via* the LDL-receptor pathway does usually not lead to intracellular lipid deposition. In fact, an increase in the intracellular cholesterol concentration reduces the cell surface density of LDL receptors and attenuates intracellular *de novo* synthesis of cholesterol. Thus, in principle, no excessive lipid uptake is possible. However, macrophages are capable of accumulating large amounts of cholesterol derivatives in the cytosol in form of lipid droplets and these droplets impart to the cells a foamy, vacuolated appearance [44]. Formation of such lipid-laden foam cells is induced *via* excessive uptake of modified LDL *via* scavenger receptor-mediated pathways. The oxidation hypothesis [5, 6] of foam cell formation proposes that *in vivo* oxygenation LDL constituents may be the major modifying process. Oxidized LDL species certainly occur in atherosclerotic lesions and in the peripheral blood stream and LDL oxidation products exhibit a complex array of proatherogenic activities [45, 46]. In animal atherosclerosis model, primary intervention studies with low molecular weight antioxidants revealed protective effects but in humans the situation appears to be more complex (Steinberg, this issue).

1.4 Involvement of LOX-isoforms in atherogenesis

Among the four mammalian LOX subfamilies, three (12/15-LOX, 5-LOX, platelet-type 12-LOX) have been related to cardiovascular diseases (Fig. 2) but the pathophysiological mechanisms of their involvement are quite distinct. In the mid 1980s, when eicosanoid research became a major focus of scientific interest, LOXs have been related to the pathogenesis of atherosclerosis [47]. Initially, research was focused on 12-LOXs since their stable reaction product (12*S*,5*Z*,8*Z*,10*E*,14*Z*)-12-hydroxy-5,8,10,14-eicosatetraenoic acid (12S-HETE) induced smooth muscle cell migration [48, 49]. The first direct evidence for the involvement of 12/15-LOXs in atherogenesis was provided in the late 1980s when activity assays suggested expression of 12/15-LOXs in atherosclerotic lesions [50, 51]. Unfortunately, at that time no mechanistic concept for the involvement of 12/15-LOX in atherogenesis was available. This situation changed when the oxidation hypothesis of atherosclerosis [5] became more popular. Based on the scattered experimental data available at that time, the oxidation hypothesis introduced a unifying mechanistic concept that has been serving as conceptional guide for designing research strategies for more than 2 decades. These strategies involved experiments in molecular and cellular model systems but were particularly focused on obtaining data in animal atherosclerosis models (overexpressing transgenic animals as well as knockout mice) and in humans.

1.5 Proatherogenic activity of various LOX-isoforms

1.5.1 12/15-LOXs

Oxidative modification converts LDL to an atherogenic form, which is rapidly taken up by macrophages or smooth muscle cells *via* scavenger receptor-mediated pathways [5, 6]. 12/15-LOXs are lipid-peroxidizing enzymes and thus, according to the oxidation hypothesis, they may contribute *in vivo* to LDL oxidation. Several lines of experimental evidence have been accumulated during the past 20 years, which suggest a proatherogenic activity of 12/15-LOXs.

(i) In reconstituted molecular model systems, 12/15-LOXs are capable of oxidizing LDL to an atherogenic form. This has been shown initially for the soybean LOX-1 [52] but later on also for various mammalian isoforms [53 – 55]. LOX catalyzed LDL oxidation may proceed *via* direct attack of the enzyme on LDL ester lipids or indirectly, *via* oxygenation of free fatty acids, which have previously been liberated from the ester lipid fraction. The hydroperoxy fatty acids formed may then initiate radical chain reactions leading to cooxidative modification of apolipoprotein B. *In vitro*, mammalian 12/15-LOXs appear to be most effective in catalyzing LDL oxidation when related to their arachidonic acid oxygenase activity (Table 1). In contrast, the human 5-LOX and the human platelet-type 12-LOX exhibit lower LDL oxidizing activities. These data are plausible since 12/15-LOX have been reported to oxygenate ester lipids [56, 57]. However, the relatively high LDL oxygenase activity of the human 5-LOX (30% when compared with the rabbit 12/15-LOX) is somewhat surprising since, according to our current understanding, this enzyme does not accept ester lipids as substrate. It may be possible that the free fatty acids present in the LDL particle may constitute the substrates for this enzyme. The hydroperoxy fatty acids formed in this primary reaction may then initiate secondary oxidations, which proceed independent of the primary LOX reaction. Pretreatment of LDL with LOX renders the lipoprotein prone to copper catalyzed oxidation [58, 59]. It was suggested that the enzyme might provide small amounts of hydroperoxides, which can be utilized by copper ions to decompose the endogenous antioxidants normally protecting LDL from metal catalyzed oxidation. Short-term incubation (up to 30 min) of the purified native rabbit 12/15-LOX with human LDL led to a specific pattern of oxygenation products [55]. In contrast, after long-term incubation a more complex mixture of oxygenated lipids was analyzed [54, 55]. These alterations in the product specificity can be explained by the fact that specific hydroperoxy lipids formed by the LOX reaction may undergo isomerization or may induce nonenzymatic secondary reactions rendering the overall product pattern more unspecific.

(ii) High-density lipoprotein (HDL) acts antiatherogenic since it enables reverse cholesterol transport [60]. These

Table 1. Relative LDL oxidizing activities of different LOX-isoforms. LDL oxygenase activity of the different LOX-isoforms was performed as follows: human LDL (0.52 mg protein/mL) was incubated with various mammalian LOX preparations in 0.1 M phosphate buffer for 15 min at room temperature. After the incubation period, sodium borohydride was added to reduce the hydroperoxy lipids formed to the corresponding hydroxy derivatives, and the lipids were extracted. Hydrolyzed lipid extracts (alkaline hydrolysis) were analyzed by RP-HPLC as described in Section 2. Arachidonate oxygenase activities of the various LOX preparations were assayed by HPLC quantitation of the LOX products formed from arachidonic acid during a 15-min incubation period. Native rabbit 12/15-LOX, the recombinant porcine 12/15-LOX, and the recombinant human platelet 12-LOX (crude enzyme preparations, ammonium sulfate precipitate) were assayed in 1 mL 0.1 M phosphate buffer, pH 7.4 containing 100 μM arachidonic acid as substrate. Recombinant human 5-LOX was tested in 1 mL 0.1 M phosphate buffer, pH 7.4 containing 0.1 mM ATP, 0.1 mM EDTA, 2 mM $CaCl_2$, 12 mg/mL phosphatidylcholine, and 20 μM arachidonic acid as substrate (optimized assay conditions). After incubation, the hydroperoxy fatty acids formed were reduced, 1 mL of methanol was added, and aliquots were directly injected to RP-HPLC

LOX-isoform	LDL oxygenase/ AA oxygenase ratio	Relative ratio of LDL oxygenase/ AA oxygenase
Rabbit 12/15-LOX	3.79×10^{-3}	100
Porcine 12/15-LOX	1.86×10^{-3}	49
Human platelet 12-LOX	0.64×10^{-3}	17
Human 5-LOX	1.10×10^{-3}	29

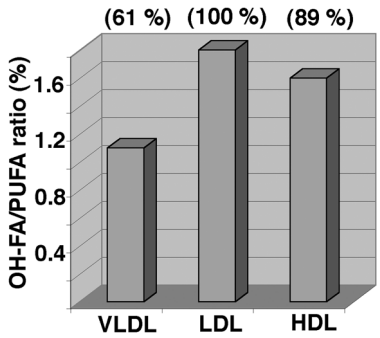

Figure 4. Oxidation of various lipoproteins by the rabbit 12/15-LOX. Human VLDL (236 nmol polyenoic fatty acids/sample), human LDL (255 nmol polyenoic fatty acids/sample), and human HDL (250 nmol polyenoic fatty acids/sample) were incubated with the rabbit 12/15-LOX (5.6 nkat/mL arachidonate oxygenase activity) for 15 min at room temperature. After incubation, sodium borohydride was added to reduce the hydroperoxy lipids formed to the corresponding hydroxy derivatives and the lipids were extracted [109]. Hydrolyzed lipid extracts (alkaline hydrolysis) were analyzed by RP-HPLC as described in Section 2 (LOX activity assays) to quantitate the hydroxy fatty acid content of the lipoprotein lipids. This value was then related to the amounts of nonoxidized polyenoic fatty acids (determination of the OH-FA/PUFA-ratio).

lipoproteins take up free cholesterol from peripheral cells to carry it to the liver where it is excreted as bile acids. HDL particles are also susceptible to oxidation [61, 62]. In fact, oxidative modification induces physicochemical alterations, which impact the biological activities. Oxidized HDL has an impaired cholesterol-accepting capacity [63, 64], and thus may not act antiatherogenic any more. When given to macrophage cultures, oxidized HDL increases the intracellular content of free cholesterol and also acts cytotoxic [65, 66]. Mammalian LOXs are capable of oxidizing HDL. In fact, the LDL- and HDL-oxidizing activity of the rabbit 12/15-LOX is comparable when the concentrations of the substrate lipoproteins in the assay system were normalized to a similar polyenoic fatty acid content (Fig. 4).

(iii) Peritoneal macrophages prepared from mice, in which the 12/15-LOX gene was functionally silenced, exhibited impaired LDL oxidizing capabilities [67]. On the other hand, fibroblasts transfected with 12/15-LOX oxidized LDL more strongly than corresponding controls [68]. When cultured murine macrophages transfected with the porcine 12/15-LOX were incubated with human LDL, oxidative modification of the lipoprotein was induced and a specific pattern of LOX products was detected in the LDL ester lipids [69]. Later on, it was reported that the LDL receptor-related protein was required for this reaction [70]. These data indicated that intracellular expression of a LOX might not be sufficient for an increased LDL oxidizing capacity of a cell. Other requirements must also be met.

(iv) Somatic gene transfer of the human 12/15-LOX gene to rabbit arteries led to the appearance of oxidized LDL epitopes in the transfected areas [71]. In these regions 12/15-LOX and oxLDL epitopes were colocalized. The contra-lateral arteries, which were mock-transfected, did not show any signs of oxLDL epitopes.

(v) To explore in more detail the *in vivo* impact of 12/15-LOX in animal atherosclerosis models transgenic mice were created (gain of function strategy), which overexpress the enzyme under the control of the preproendothelin promoter [72]. Screening of different tissues for transgene expression indicated high-level expression of 12/15-LOX in lung, heart, and aorta. When these animals were crossed with LDL-receptor knockout mice, the resulting animals turned out to be more susceptible to experimental atherosclerosis [73]. For the interpretation of these data one should keep in mind that 12/15-LOX is usually not expressed in the heart and in vascular endothelium. Whether this ectopic LOX expression may influence the pathophysiology of plaque formation is difficult to estimate at the moment.

(vi) An inverse approach (loss of function strategy) was taken when 12/15-LOX knockout mice [74, 75] were

crossed with apo-E- or LDL-receptor deficient mice. After several generations of backcrossing into Black6 genetic background, the double knockouts were put on a lipid-rich diet and significant reduction of lesion formation was observed [74, 75]. The 12/15-LOX$^{-/-}$-apo-E$^{-/-}$ mice have diminished plasma IgG autoantibodies against oxidized LDL epitopes, suggesting that 12/15-LOX might be involved in systemic LDL oxidation *in vivo*. An even more pronounced protection from lesion formation was observed when the animals were kept for a longer time period on a normal chow-diet. These data strongly suggest that in these animal atherosclerosis models the murine 12/15-LOX exhibits proatherogenic activities.

The mouse 12/15-LOX is expressed in various cell types including peritoneal macrophages, adipocytes, cardiomyocytes, and in the vasculature. To explore, which cellular source of the enzyme is important for atherogenesis, bone marrow was transplanted from 12/15-LO$^{-/-}$/apoE$^{-/-}$ mice into 12/15-LO$^{+/+}$/apoE$^{-/-}$ apoE mice and *vice versa* [76]. Deficiency of 12/15-LOX in bone marrow cells protected 12/15-LO$^{+/+}$/apoE$^{-/-}$ mice fed a Western-type diet from atherosclerosis to a similar extent as complete absence of the enzyme. Interestingly, the plasma levels of 8,12-iso-prostanes (measure of systemic lipid peroxidation) remained elevated after bone marrow transplantation, suggesting that bone marrow cell 12/15-LOX may not largely contribute to systemic lipid peroxidation. 12/15-LO$^{-/-}$/apoE$^{-/-}$ mice regained the severity of atherosclerotic lesion typical of apoE$^{-/-}$ mice after replacement of their bone marrow cells with bone marrow from 12/15-LO$^{+/+}$/apoE$^{-/-}$ mice. Moreover, peritoneal macrophages obtained from wild-type but not from 12/15-LOX deficient mice caused endothelial activation when incubated with native LDL. These data suggested that macrophage 12/15-LOX plays an important role in the development of atherosclerosis by promoting endothelial dysfunction [76].

(vii) In cholesterol-fed rabbits, a 12/15-LOX inhibitor slows down the formation of atherosclerotic lesions by inhibiting monocyte/macrophage enrichment in the vessel wall [77, 78]. *In vitro*, this compound inhibited the rabbit 15-LOX at submicromolar concentrations but did not affect fatty acid oxygenase activity of the human platelet 12-LOX, the rat 5-LOX, the ovine cyclooxygenase-1, and the human cyclooxygenase-2. The compound did neither significantly alter the plasma cholesterol concentration nor the animals' lipoprotein profile. Unfortunately, no direct proof for the *in vivo* inhibition of the 12/15-LOX has been presented.

Taken together, these findings strongly suggest a proatherogenic activity of 12/15-LOXs in various rodent atherosclerosis models. Whether these findings can be extrapolated to humans remains to be explored in the future. Unfortunately, recent studies on the presence or absence of 12/15-LOX in various stages of human atherosclerotic lesions did not reveal major indications for significant lesional expression of the enzyme [79]. The second point that needs to be addressed in the future is the question by which mechanisms 12/15-LOX prevent lesion formation *in vivo*.

Although there is evidence for a role of the enzyme in systemic LDL oxidation (oxidation hypothesis of atherosclerosis), alternative mechanisms may also be involved. For instance, the enzyme may be involved in the biosynthesis of compounds, which act as signaling molecules initiating proatherogenic event (cell migration, cell proliferation, leukocyte adhesion, vasoconstriction, platelet aggregation, inflammatory reaction, *etc.*). Moreover, intracellular catalytic activity of 12/15-LOX may regulate expression of redox-sensitive genes by increasing the intracellular peroxide tone. A selection of such LDL-unrelated proatherogenic mechanisms has been discussed before [80].

1.5.2 5-LOXs

For a long time atherosclerosis has been considered a hyperproliferative disease that involves dysregulations in local and systemic lipid metabolism. However, even 20 years ago an inflammatory component had been implicated in lesion formation, and the impact of antiinflammatory drugs was tested in animal atherosclerosis models [81 – 84]. Today atherosclerosis is regarded as inflammatory disorder, which is initiated by internal microinjuries of the arteries [85, 86]. Hypertension, hemodynamic turbulences, vascular infections, or chemical substances induce such microinjuries and initially, formation of an atherosclerotic lesion may be considered a repair response. Since inflammation appears to be involved in atherogenesis, it was predicted that inflammatory enzymes, receptors, and signaling molecules might be present in atherosclerotic lesions. Leukotrienes are key mediators in inflammatory reactions [14, 17], and thus lesional expression of leukotriene synthesizing enzymes was likely. Although expression of 5-LOX is not restricted to inflammatory cells, leukocytes were shown to be a rich source of lesional 5-LOX. In fact, the enzyme was detected not only in foamy macrophages of human lesions [79] but also in different cell types of murine atherosclerotic specimens [87].

Interestingly, a major site of 5-LOX expression is the vascular adventia [88]. In humans, the number of transcripts of both, 5-LOX and LT-receptors, appears to expand during lesion progression [79] suggesting a causal relation between 5-LOX expression and atherogenesis. In mice, a genetic locus was identified that blocks development of atherosclerosis despite extreme hyperlipidemia [89]. To identify genes with potential impact on atherogenesis, atheroresistant CAST mice were crossed with atherosusceptible Black6

animals yielding the congenic mouse strain CON6, which exhibits a similarly pronounced atheroresistance as the parent CAST strain. Importantly, CON6 mice retained the atheroresistance gene locus of the CAST animals and genotyping of the two strains identified the 5-LOX gene immediately underneath the linkage peak [87]. Moreover, bone marrow transplantation suggested the importance of leukocytes in the atheroresistance of CON6 mice [87]. Thus, the leukocyte 5-LOX emerged as an attractive candidate gene that could contribute to atheroresistance in CAST and CON6 mice. To address this hypothesis, 5-LOX expression was compared between atheroresistant (CON6) and atherosusceptible (Black6) mouse strains, and it was found that CON6 mice exhibited five-fold lower expression levels of both, 5-LOX mRNA and protein than the corresponding Black6 controls [87]. Comparison of the genomic 5-LOX sequences suggested two nucleotide exchanges in the 5-LOX coding region, which resulted at the protein level in two point mutations. Ile645 of Black6 mice was exchanged to Val (I645V), and Val646 was mutated to Ile (V646I). These amino acid exchanges are rather conservative, and thus no major functional consequences were initially expected. To explore whether these amino acid exchanges may be of functional relevance, the wild-type human 5-LOX (I645 + V646) and the three relevant mutants (I645V + V646, I645 + V646I, and I645V + V646I) were expressed as recombinant nonfusion protein in *E. coli* and characterized with respect to important properties (specific activity, LOX activity, positional specificity, and substrate affinity). It was found that each single mutant (I645V + V646, I645 + V646I) as well as the double mutant (I645V + V646I) exhibited reduced specific LOX activities but retained all other enzyme properties [90]. These data suggest that atheroresistant CON6 mice express a catalytically less active 5-LOX. It should be stressed at this point that all mutants created were catalytically active but their specific activity was different. This is an important observation since the original amino acid sequence of the murine 5-LOX contained an Ile at position 646 and the recombinant wild-type enzyme exhibited a measurable 5-LOX activity [91].

When apo-E knockout mice were crossed with 5-LOX deficient, the resulting double knockouts did not show significant differences in lesion formation when compared with 5-LOX expressing controls [88]. However, 5-LO deficiency markedly attenuated inflammation and neoangiogenesis in the vascular wall and reduced the formation of aortic aneurysms. These data do not confirm the hypothesis that the extent of lesion formation in apo-E deficient mice might be related to 5-LOX expression but they link the 5-LOX pathway to hyperlipidemia-induced inflammatory reactions in the vessel wall and to the pathogenesis of aortic aneurysms.

To explore a possible involvement of the 5-LOX pathway in human atherogenesis, genetic polymorphisms in the promoter of the 5-LOX gene (number of the tandem Sp1-binding motifs 5′-GGGCGG-3′) were studied [92]. These structural data were then correlated to the carotid-artery intimamedia thickness as suitable parameter for atherosclerotic alterations of the vessel wall and to general markers of inflammation in a randomly sampled cohort of 470 healthy volunteers. Variants of the normal 5-LOX genotype were found in 6.0% of the individuals included in cohort. Intimamedia thickness was significantly ($p < 0.001$) increased among the carriers of two variant alleles. The extent of significance of this increase in intimamedia thickness was similar to that associated with diabetes, which is one of the strongest cardiovascular risk factor. Moreover, the average plasma level of C-reactive protein (general marker of inflammation) was increased by a factor of 2 among the carriers of two variant alleles. These data clearly relate the 5-LOX genotype to the pathogenesis of human atherosclerosis.

1.5.3 Platelet-type 12-LOXs

The platelet-type 12-LOX is highly expressed in circulating thrombocytes and appears to modulate the biological functions of these cells [13, 26, 93]. This enzyme converts free arachidonic acid to (12*S*,5*Z*,8*Z*,10*E*,14*Z*)-12-hydro(pero)xy-5,8,10,14-eicosatetraenoic acid (12S-H(p)ETE) but does not accept C-18 polyenoic fatty acids as substrate. Moreover, the enzyme rarely oxygenates ester lipids, and thus may not be involved in LDL modification. As circulating blood cells, thrombocytes may also play a role in atherogenesis but the precise mechanisms are far from clear. 12S-HETE, the primary product of platelet 12-LOX, induces migration of smooth muscle cells [48, 49], which clearly is a proatherogenic process. Collagen and collagen-related protein (CRP) acutely induce 12SH(p)ETE biosynthesis in blood platelets [93]. Using specific inhibitors of intracellular signal transduction it was found that this upregulation involves activation of src-tyrosine kinases, PI3-kinases, and increased intracellular calcium concentration. Another interesting finding, which links the thrombocytic 12-LOX to atherogenesis, is its sensitivity toward platelet endothelial cells adhesion molecule (PECAM-1). Although the biological function of PECAM-1 has not been studied in detail it is involved in platelet–endothelium interaction, which clearly is important for atherosclerosis [93].

1.6 Antiatherosclerotic activity of 12/15-LOX

To establish a nonmurine model of 12/15-LOX overexpression, transgenic rabbits were created that overexpress the human 12/15-LOX in peripheral monocyte, but not in liver, heart, kidney, lung, or other tissues [94]. The expression level of the enzyme in monocytes prepared from transgenic animals was more than 20-fold higher than of corresponding controls. When these transgenic rabbits were fed a

lipid-rich Western-type diet, development of atherosclerotic lesions in the aorta was significantly ($p < 0.05$) reduced by about 50% [95]. In a second series of experiments a similar trend was observed (19% reduction) but here the difference was not significant any more. When the LOX overexpressing rabbits were crossed with Watanabe rabbits, an even more pronounced reduction (about 66%, $p < 0.05$) of the lesional area was measured. These data suggested that monocytes/macrophage overexpression of 12/15-LOX protected rabbits from atherosclerosis in two different rabbit atherosclerosis models.

Transient experimental anemia induces in rabbits a long-lasting systemic overexpression of 12/15-LOX [96]. To test whether or not such systemic overexpression of the enzyme may impact diet-induced atherogenesis, a mild experimental anemia was induced in female New Zealand White rabbits.

After the hematopoietic parameters had normalized, these animals and age-matched controls were fed a lipid-rich Western-type diet for 10 wk. Comparative determination (12/15-LOX overexpressing *vs*. control animals) of a variety plasma parameters as well as histological inspections of major organs did not reveal any indications for major organ malfunction, and the plasma lipid status was also similar in the two groups. However, quantitative analysis of arterial lipid deposition revealed that 12/15-LOX overexpressing rabbits accumulated significantly less free cholesterol, cholesteryl linoleate, and cholesteryl oleate in the thoracic aorta than the corresponding controls [97]. These data suggest that transient experimental anemia, which is accompanied by long-lasting overexpression of 12/15-LOX protects cholesterol-fed rabbits from aortic lipid deposition.

12/15-LOX may be regarded as lipid-catabolizing enzymes. In immature red blood cells the enzyme contributes to the maturational breakdown of mitochondria by oxidizing their membrane lipids [20, 21]. In cucumber seedling the enzyme has been implicated in utilization of storage lipids, which serve as major source for energy and carbon equivalents for the growing seedling during germination [8]. If the enzyme exhibits such a lipid-catabolizing activity during foam cell formation, an antiatherogenic activity in early stages of atherogenesis would be plausible. To test the impact of 12/15-LOX overexpression on foam cell formation an *in vitro* foam cell model was established, which was based on uptake of acetylated LDL by murine macrophages (J774 cells). These cells, which normally do not express the 12/15-LOX were transfected with a mammalian expression plasmid containing the cDNA of the porcine 12/15-LOX and a noncoding control sequence [69]. In this system it was found that 12/15-LOX-transfectants were protected from intracellular lipid deposition [98]. This effect was related to an attenuated uptake of modified LDL (impaired expression of scavenger receptor A) and accelerated intracellular lipid metabolism. These results suggest that the role of 12/15-LOX in atherogenesis might not be restricted to oxidative modification of LDL. In addition, overexpression of the enzyme may also impact the intracellular lipid metabolism, may alter the expression pattern of redox-sensitive genes, and may also modify lipid export.

Involvement of 12/15-LOX in eicosanoid biosynthesis may be another possibility to explain its antiatherogenic activity. 12/15-LOXs have been implicated in the biosynthesis of various lipid mediators, which exhibit a variety of biological activities [99]. An important class of 12/15-LOX derived eicosanoids are the lipoxins [15]. These polyhydroxylated arachidonic acid derivatives have been identified as mediators of inflammatory resolution [100, 101]. Since inflammation is an inherent process of atherogenesis, resolving-mediators may counteract or even reverse the formation of atherosclerotic lesions. 13-Hydroxy-9*Z*,11*E*-octadecadienoic acid (13-HODE), the major oxygenation product of linoleic acid *via* the 15-LOX pathway, has chemorepellent activities for various blood cells [51, 102]. Since cell adhesion to the vascular endothelium is an important event in early atherosclerosis, 13-HODE may be regarded as an antiatherogenic compound. Although the mechanistic reasons for this activity are not completely understood, 13-HODE may regulate integrin/ligand binding [103]. 13-HODE has also been reported to stimulate vascular prostacyclin production [104] and to inhibit thromboxane formation by blood platelets [105]. In rats 13-HODE induces a decrease in diastolic blood pressure after pretreatment of the animals with histamine or bradykinin [106]. This hypotensive effect may be due to a release of CGRP-like substances from capsaicin-sensitive nerves. Moreover, the major 12/15-LOX metabolite of arachidonic acid, 15-H(p)ETE, inhibits the procoagulant activity of endothelial cell treated with various cytokines [107], which may also be considered an antiatherogenic effect. Taken together these data suggest that eicosanoid formation (15-HETE, 13-HODE, lipoxins) *via* the 12/15-LOX pathway may contribute to the observed antiatherogenic effects of 12/15-LOX overexpression in different rabbit atherosclerosis models.

2 Materials and methods

2.1 Preparation of human LDL and lipoprotein modification

LDL was isolated from EDTA-plasma of healthy volunteers by sequential floating ultracentrifugation [108]. It was acetylated by incubating freshly prepared human LDL (10 mg) in 3.4 mL of saturated sodium acetate solution on ice. Then 15 mg of acetic acid anhydride was added in small portions (2 mg) over a time period of 60 min under constant

stirring. The mixture was further incubated for 30 min and the acetylated low-density lipoprotein (acLDL) solution was dialyzed overnight against PBS containing 0.3 mM EDTA.

2.2 Cell preparation, cell culture, and maintenance

12/15-LOX-transfected J774 cells and the corresponding mock-transfectants were kindly provided by Dr. Yoshimoto (Kanazawa University, Japan). The cells were cultured at 37°C with 5% CO_2 in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 mg/mL streptomycin sulfate. For the preparation of murine peritoneal macrophages, mice were injected with small amounts of a thioglycolate solution into the peritoneal cavity. The next day the animals were sacrificed by diethyl ether inhalation. The peritoneal cavity was rinsed twice with 5 – 8 mL of sterile PBS. The cells were spun down, resuspended in cell culture medium, and plated into 5 cm petri dishes. Macrophages were allowed to adhere for 2 h and nonadherent cells were washed away. The adhering macrophages were then conditioned for foam cell formation as described in Section 2.3.

2.3 *In vitro* foam cell formation

Murine macrophages (12/15-LOX-transfected and mock-transfected J774 cells, peritoneal macrophages of normal B6 mice and of 12/15-LOX deficient animals backcrossed to the same genetic background) were cultured as described in Section 2.2. Twenty-four hours before the experiment the fetal calf serum was removed from the culture medium. Immediately before the experiment the medium was exchanged, acLDL (50 μg/mL culture medium) was added, and the cells were incubated for 24 h. After this the medium was removed, the cells were harvested, and washed three times with PBS. The intracellular lipids were extracted [109], the solvent was evaporated, the residues were reconstituted in 0.1 mL of 2-propanol, and 0.3 mL of ACN was added. Aliquots of this solution were analyzed by RP-HPLC to quantitate the cellular content of free cholesterol and cholesterol esters (cholesteryl arachidonate, cholesteryl linoleate, and cholesteryl oleate). HPLC was performed on a Nucleosil column-C18 column (Macherey/Nagel, KS-system, 250 mm × 4 mm, 5 μm particle size) and compounds were eluted at 45°C with the solvent system ACN/2-propanol (75:25; v/v) at a flow rate of 1 mL/min. The absorbance at 210 nm was recorded.

2.4 LOX activity assay

To assay the LOX activity of 12/15-LOX- and mock-transfected J774 cells we incubated the cells with arachidonic acid, and the resulting LOX products were analyzed by HPLC. In detail, $1-5 \times 10^6$ cells were resuspended in 0.5 mL of PBS and 0.1 mM (final concentration) of arachidonic acid was added. The cells were lyzed by sonication (Labsonic-U 2000 needle-tip sonifier; Braun, Melsungen, Germany; two times 5 s at full power) and the mixture was incubated for 10 min at 25°C. The hydroperoxy lipids formed were reduced to the corresponding hydroxy compounds by addition of 0.1 mL of saturated sodium borohydride solution (dry methanol). The sample was acidified with 50 μL of glacial acetic acid and 0.6 mL of ice-cold methanol was added to adjust a methanol concentration of 50%. The sample was kept on ice for 10 min and protein precipitate was removed by centrifugation. Aliquots of the clear supernatant were directly injected to HPLC. Analysis was performed on a Nucleosil column-C18 column (Macherey/Nagel, KS-system, 250 mm × 4 mm, 5 μm particle size) and the LOX products were eluted isocratically using a solvent system of methanol/water/acetic acid (80:20:0.1, by vol) at a flow rate of 1 mL/min. The absorbance at 235 nm was recorded.

2.5 Cellular LDL oxidation

12/15-LOX-transfected and mock-transfected J774 cells were incubated for 24 h with native human LDL (350 μg/mL). The cells were removed from the plates by scraping and lipids were extracted in the cell suspension as described previously [107]. In selected experiments, the cells and the culture medium were extracted separately. The lipid extracts were hydrolyzed under alkaline conditions and the resulting mixture of free fatty acid was analyzed for hydroxylated (recording the absorbance at 235 nm) and nonoxidized polyenoic fatty acids (recording the absorbance at 210 nm) as described for the activity assays.

2.6 Intracellular proteolysis

For quantitation of the intracellular proteolysis the cells were harvested, washed, resuspended in PBS containing 0.1% Triton X-100 and 0.5 mM DTT, and then homogenized by gentle sonication using a microtip Braun sonifier (Braun, Melsungen, Germany). The crude homogenates were used as enzyme source. The assay mixture was 50 mM Tris-HCl buffer, pH 7.8 (1 mL) containing 20 mM KCl, 5 mM $MgCl_2$, 0.1 mM DTT, and 10 μM of sLLVY-7-amino-4-methylcoumarin (AMC) as substrate. After addition of 0.1 mL cell lysate (about 15 μg protein) the mixture was incubated at 37°C for 30 min, the reaction was stopped with 1.8 mL of 0.2 M glycine buffer, pH 10, and fluorescence of the liberated AMC was measured on a Shimadzu RF-5001 PC spectrofluorimeter (excitation 365 nm, emission 460 nm). Scale was calibrated with 2 mM AMC in DMSO.

3 Results

3.1 Involvement of cellular 12/15-LOX in oxidative modification of LDL

Fibroblasts do not express 12/15-LOX at significant levels. When these cells were transfected with the human enzyme they exhibited an increased LDL oxidizing capacity when compared with nontransfected counterparts [68]. Surprisingly, not all transfected clones, which overexpress the enzyme, showed such increased LDL oxidizing capability. These data suggested that, in addition to the expression of the enzyme, other factors and/or processes might be important for oxidation of extracellular LDL by intracellular LOXs. J774 murine macrophages do not express 12/15-LOX. When J774 cells, which were stably transfected with the porcine 12/15-LOX, were incubated with human LDL they oxidize the lipoprotein more efficiently than the corresponding mock-transfectants [69, 70]. More detailed analysis of the structure of LDL oxygenation products indicated a specific product pattern of oxidized LDL lipids, suggesting that they may be formed by direct LOX–LDL interaction. Later on, it was found that this specific LDL oxidation required expression of the LDL-receptor-related protein [67] since silencing of this cell surface protein prevented specific oxidation of LDL lipids.

When we repeated these experiments using the same transfectants (generously provided by Dr. Yoshimoto) we could not confirm the experimental data. In contrast, we found that both the 12/15-LOX-transfected cells and the corresponding controls (mock-transfectants) inhibited oxidative LDL modification induced by the culture medium. To quantitate LDL oxidation we determined the hydroxy fatty acid content of the cell membrane and LDL lipids, and related it to the amount of nonoxidized polyenoic fatty acids (OH-FA/PUFA-ratio). The OH-FA/PUFA ratio of human LDL prepared from various healthy donors ($n = 5$) varied between 0.2 and 0.4%, suggesting that about 2–4 out of 1000 polyenoic fatty acid residues were present as hydroxylated derivatives. When LDL was incubated in cell culture medium for 20 h the OH-FA/PUFA ratio went up to about 1.2% (Fig. 5A), indicating medium induced LDL oxidation during the incubation period. However, when the incubation was performed in the presence of cells the OH-FA/PUFA ratio of the LDL lipids ratio was hardly altered. There was no difference in the degree of LDL oxidation when 12/15-LOX-transfected and mock-transfected cells were compared (Fig. 5A). These data suggest that under our experimental conditions, J774 cells appear to protect extracellular LDL from oxidative modification regardless whether they express 15-LOX or not. Similar protective effects of cells on medium catalyzed LDL oxidation have been reported before, but the mechanistic reasons remain elusive [110]. One possibility to explain the lacking differences between 12/15-LOX- and mock-transfected cells was the assumption that the 12/15-LOX expressed in the transfectants was catalytically inactive. To rule out this possibility we performed activity assays incubating the cells with exogenous arachidonic acid. From Fig. 5B it can be seen that the 12/15-LOX-transfected cells convert this substrate to large amounts of the 12/15-LOX product 12-HETE. In contrast, culture medium and the mock-controls were unable to catalyze this reaction. These data indicated the enzymatic activity of the transgenic enzyme on exogenous substrate but there was still the possibility that the LOX might not react on endogenous lipids. LOXs are expressed as catalytically silent proteins and require activation to become enzymatically active [111]. Such activation is induced when cells are exposed to large amounts of exogenous polyenoic fatty acids. Thus, our finding that large amounts of LOX products are formed from exogenous arachidonic acid does not prove its intracellular activity on endogenous lipids. To test this activity we analyzed the degree of oxidation (OH-FA/PUFA ratio) of the membrane lipids in 12/15-LOX- and mock-transfected cells and found a two-fold increase in the steady state concentration of hydroxylated fatty acids. The membranes of 12/15-LOX-transfectants contained about 4% of oxygenated fatty acids in their membrane lipids. To provide evidence for the LOX origin of these oxidation products we determined the 13-HODE/9-HODE ratio. 12/15-LOXs convert linoleic acid to a specific pattern of hydroxy linoleic acid isomers (HODE) with strong preference of 13-HODE. In contrast, a 1:1 ratio of 13-HODE and 9-HODE is usually formed by nonenzymatic oxidation. Predominant formation of 13-HODE over the corresponding 9-isomer may thus be regarded as indicator for endogenous LOX reaction. The 13-HODE/9-HODE ratio of the oxidized fatty acids found in the membranes of 12/15-LOX-transfected cells was 2.3 indicating a strong preponderance of 13-HODE (Fig. 5C). In contrast, a 1:1 ratio was observed for the membrane lipids of the mock-transfected cells. These data proved the intracellular activity of the transfected enzyme on membrane lipids under our experimental conditions.

3.2 Impact of 12/15-LOX expression on *in vitro* foam cell formation

The physiological role of 12/15-LOX is certainly not its possible contribution to LDL oxidation. Instead, it might be related to intracellular lipid turnover. The enzyme has been implicated in the maturational breakdown of biomembranes [20, 21] and in the utilization of storage lipids during plant germination [8]. Thus, it may be considered a lipid-catabolizing enzyme. We recently reported that transfection of J774 cells with the porcine 12/15-LOX protected the cells from excessive lipid deposition when incubated with acetylated LDL [98], suggesting an atheroprotective activity of

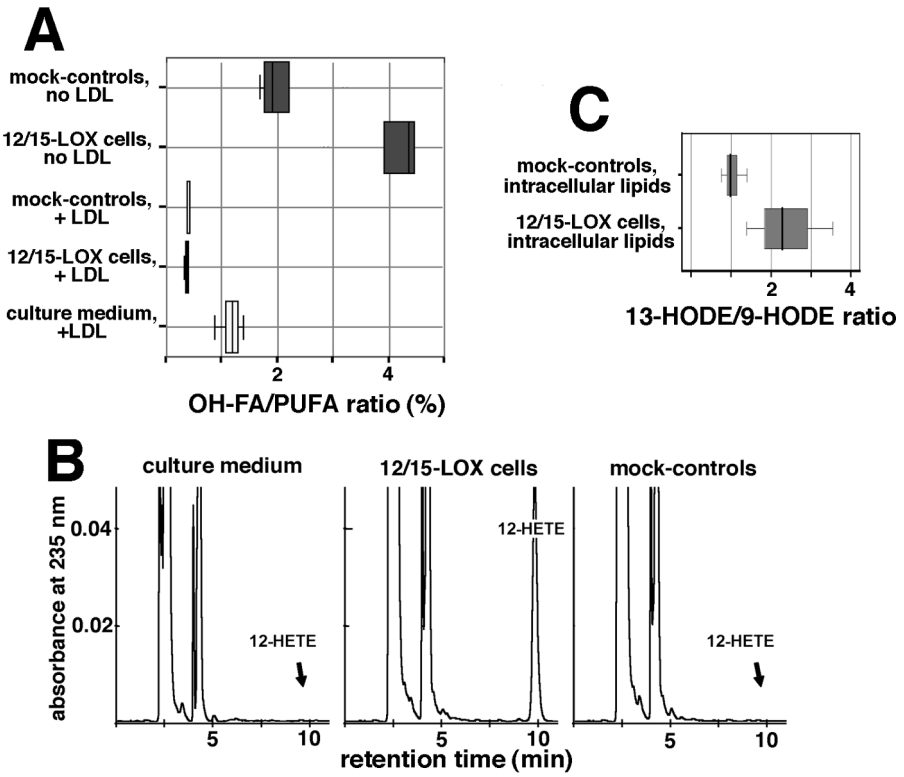

Figure 5. Oxidative modification of human LDL by transfected J774 cells. (A) Quantitation of LDL oxidation induced by 12/15-LOX-transfected and mock-transfected J774 cells. Cellular LDL oxidation was performed as described in Section 2 and the hydroxy fatty acid/polyenoic fatty acid ratio (OH-FA/PUFA ratio) of the LDL lipids was determined. Two independent sets of experiments ($n = 5$ in each set) with almost identical results were carried out but only one experiment is shown. (B) Activity assays of 12/15-LOX-transfected and mock-transfected J774 cells. Activity assays are described in Section 2. Only in 12/15-LOX-transfected cells, significant amounts of LOX products were formed. (C) Isomeric composition of hydroxy fatty acids isolated from the intracellular lipids prepared from 12/15-LOX-transfected and mock-transfected cells. In the mock-transfectants, we observed a 1 : 1 ratio of 13-HODE and 9-HODE and these data suggested nonenzymatic lipid peroxidation processes as metabolic origin. In the 12/15-LOX transfectants we found a strong preponderance of 13-HODE, suggesting involvement of 12/15-LOX in the biosynthesis of oxidized PUFAs. 13-HODE/9-HODE ratio was determined by sequential RP- (see activity assays in Section 2) and normal-phase HPLC. Normal-phase HPLC was carried out on a Zorbax-SIL column (250 mm × 4 mm, 5 μm particle size) with a solvent system of n-hexane/2-propanol/acetic acid (100/2/0.1, by vol) at a flow rate of 1 mL/min.

the enzyme. Mechanistic studies indicated that 12/15-LOX-transfectants express lower levels of scavenger receptor A, suggesting a less effective acLDL uptake. Moreover, we found that the intracellular metabolism of the internalized LDL lipids was accelerated. To test whether this may also be true for the proteolytic capacity of the cells we compared 12/15-LOX-transfectants and their mock-controls with respect to this enzymatic activity. From Fig. 6 it can be seen that LOX-transfected cells exhibit a higher proteolytic activity during the time-course of foam cell formation. Although the proteolytic activities of various cell batches varied considerably (four independent experiments), the overall difference between 12/15-LOX- and mock-transfectants was statistically significant ($p = 0.006$, paired t-test).

Our results that 12/15-LOX-transfected cells were protected from lipid deposition during *in vitro* foam cell formation contrast the findings of Huo *et al.*, who reported that peritoneal macrophages prepared from 12/15-LOX deficient and 12/15-LOX expressing mice accumulated similar amounts of intracellular lipids when exposed to acLDL [76]. In that cellular model expression of 12/15-LOX appears to be irrelevant for foam cell formation. The mechanistic reasons for these conflicting data are unclear but there were considerable differences in experimental designs of the two studies (see Section 4). These methodological differences included the way of macrophage preparation, the conditions of foam cell formation (starving period), and the method used for quantitation of intracellular lipid deposition (oil red staining *vs*. HPLC quantitation of intracellular cholesterol esters). To test the possible relevance of these methodological differences we repeated the experiment with peritoneal macrophages under exactly the same experimental condition used for the transfected J774

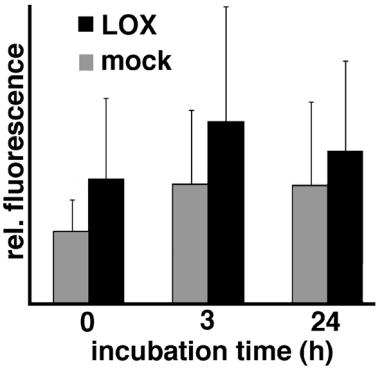

Figure 6. Proteolytic capacity of cells during *in vitro* foam cell formation. Foam cell assays were carried out with mock- and 12/15-LOX-transfected cells as described in Section 2. After the times indicated, the cells were washed, sonicated, and the proteolytic capacity was determined using a fluorescence-labeled proteolysis substrate. Four independent experiments were performed and all data summarized in the figure. Means ± SD are given. Despite the large variability between the different experiments, a highly significant difference (p-value of 0.0067) between LOX- and mock-transfected cells was observed using the paired Student's t-test (12 pairs of data).

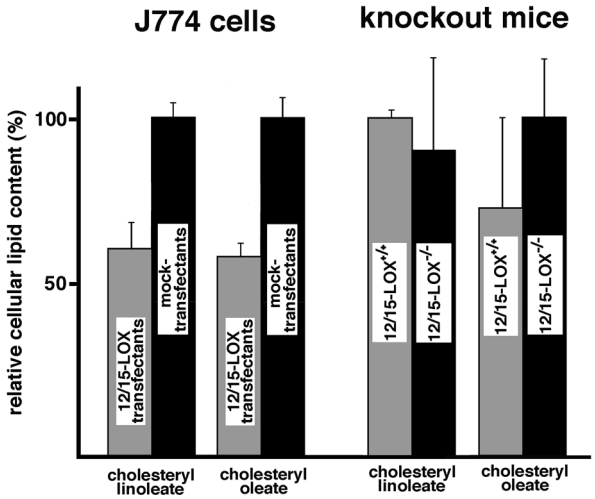

Figure 7. *In vitro* foam cell formation by transfected J774 cells and peritoneal macrophages prepared from normal and 12/15-LOX deficient mice. Peritoneal macrophages were prepared from normal and 12/15-LOX deficient mice (see Section 2). After adherence, the cells and the transfected J774 cells were used for *in vitro* foam cell formation as described in Section 2. Relative cholesterol ester contents of the cells were analyzed by HPLC and related to the cellular protein. Highest level for each cellular system was set 100% (J774 cells, $n = 4$; peritoneal macrophages, $n = 8$).

cells. As indicated in Fig. 7, 12/15-LOX expressing J774 cells accumulated about 40% less intracellular cholesterol esters than the corresponding controls and this difference was statistically significant ($p < 0.01$). In contrast, there was no significant difference in intracellular lipid deposition between 12/15-LOX expressing peritoneal macrophages and the corresponding cells prepared from 12/15-LOX knockout mice. The absolute values of intracellular cholesterol ester deposition in the different cell preparations are summarized in Table 2. It can be seen that in murine peritoneal macrophages, 12/15-LOX expression does not impact foam cell formation, which confirms the finding of Huo *et al.* However, in J774 cells, expression of 12/15-LOX did impact this process under strictly comparable experimental conditions. These data not only clearly demonstrate a different role of 12/15-LOX expression on foam cell formation, depending on the cellular model, but also indicate that under our experimental conditions peritoneal macrophages accumulate more intracellular lipids than J774 cells. It remains to be tested, which of these two model systems is more appropriate to mirror *in vivo* foam cell formation in developing atherosclerotic lesion.

Table 2. Absolute amounts of intracellular cholesterol esters after *in vitro* foam cell formation by transfected J774 cells and murine peritoneal macrophages. Peritoneal macrophages prepared from normal and 12/15-LOX deficient mice on one hand and 12/15-LOX transfected J774 and corresponding mock-transfectants on the other were used for *in vitro* foam cell formation. Absolute amounts of intracellular cholesterol esters (sum of cholesterol linoleate and cholesterol oleate) were quantitated by HPLC. Means of four (J774 cells) and eight (peritoneal macrophages) different experiments are given. SDs of the data are indicated in Fig. 7

LOX expression	Transfected J774 cells Chol-linoleate + chol-oleate, nmol/mg cellular protein		Peritoneal macrophages Chol-linoleate + chol-oleate, nmol/mg cellular protein	
+LOX	62.9	$p < 0.01$	147.4	n.s.
−LOX	108.9		149.3	

4 Discussion

Cellular oxidation of extracellular LDL is a complex process, which not only involves a variety of enzymatic and nonenzymatic redox reactions, but also other metabolic processes, such as intercompartmental lipid transfer, anabolic and catabolic lipid metabolism and cellular export of metabolites. There are also multiple ways of how an intracellular 12/15-LOX is capable of oxidizing extracellular LDL [112]. This metabolic complexity can hardly be controlled even in relatively simple *in vitro* systems. Minor variations in cell culture and incubation conditions may cause major alterations in the cellular metabolism and these changes may lead to conflicting experimental data. Our finding that J774 cells exhibit protective effects on medium-catalyzed LDL oxidation (Fig. 5) is rendering the situation even more complex.

These data indicate that cells might not just play a prooxidative role but may also protect from oxidative modification. Thus, the net effect of a cell preparation depends on the equilibrium of metabolic pro and antioxidative events and disturbance of this steady state may confer pro- or antioxidative properties to the cells. Although the mechanism of the antioxidative protection we observed has not been clarified, it might be speculated that cells release antioxidative compounds that protect LDL from oxidative modification by medium constituents. Another possibility to explain our data would be a selective cellular uptake of modified LDL. Such uptake would lower the OH-FA/PUFA ratio of extracellular LDL, and thus simulate antioxidative protection. Under our experimental conditions we found that about 7 – 15% of the LDL present in the cell culture medium was taken up during the incubation period. Whether this cellular uptake was selective for oxidized LDL has not been studied.

One of the possible mechanisms, by which a 12/15-LOX expressing cells mediate oxidative modification of extracellular LDL, is transfer of oxidized lipids from biomembranes to LDL particles. Intracellular 12/15-LOX is capable of oxidizing membrane lipids and our data on 12/15-LOX-transfected J774 cells indicated the presence of specifically oxidized PUFAs in the cellular membranes (Fig. 5). These oxidized lipids may then be transferred to LDL when the particle is transiently bound at the cell surface. Membrane binding of LDL may take place *via* direct lipid/lipid interaction or, more likely, is mediated by specific LDL-binding proteins. LDL-receptor-related protein [113] is such an LDL-binding surface receptor, and functional silencing of this protein prevented the transfer of specific 12/15-LOX products to extracellular LDL [70]. Thus, in this cellular model LDL-receptor-related protein is an essential part of the LDL oxidizing machinery and its absence prevents LDL oxidation. These data indicate that expression of a functional 12/15-LOX in a cellular system may not be sufficient for cellular LDL oxidation.

Foam cell formation is an essential step in atherogenesis [3 – 5, 44]. Although this process has extensively been studied in the past, the *in vivo* mechanisms are still unclear. Here we compared the impact of 12/15-LOX in two *in vitro* foam cell models and obtained conflicting data. Intracellular lipid deposition in transfected J774 cells was strongly attenuated by 12/15-LOX expression. In contrast, murine peritoneal macrophages accumulated similar amounts of cholesterol esters regardless whether they expressed the enzyme or not (Fig. 7, Table 2). Thus, two similar *in vitro* models yielded conflicting experimental data. The question which of these models is more appropriate to mirror the *in vivo* conditions cannot be answered at the moment. Peritoneal macrophages are native cells, and thus they might be preferred over a permanent cell line (J774 cells). However, working with native macrophages we experienced a high degree of variability (Fig. 7). This observation was not only related to interindividual differences (several individuals are required for a single set of experiments), but also to the way of macrophage preparation (eliciting agent, duration of eliciting period). The high degree of variability might mask subtle differences between normal and 12/15-LOX knockout macrophages, which might have shown up in a more reproducible experimental system. Moreover, although peritoneal and lesional macrophages are of joint origin there are certainly phenotypic differences between the two cell types. It might even be possible that lesional macrophages are more similar to J774 cells than to peritoneal macrophages. More detailed studies, which should include microarray characterization of the cellular gene expression patterns, are required to decide whether J774 cells, peritoneal macrophages, or even peripheral monocytes are the most appropriate model system to study *in vivo* foam cell formation.

Other points, which should be considered when discussing the conflicting results, are the different experimental approaches. Direct comparison of functional data obtained with “loss of function” (knockout mice) and “gain of function” (transfection studies or overexpressing transgenics) strategies might be dangerous. Overexpression of an enzyme in a cell type, which normally does not express this protein, is likely to alter the cellular phenotype. In fact, when we compared the gene expression pattern of 12/15-LOX-transfected J774 cells with corresponding mock-controls we found severe differences. More importantly, we did not observe inverse alterations when similar experiments were carried out with peritoneal macrophages obtained from 12/15-LOX deficient and 12/15-LOX expressing animals. Thus, “gain of function” strategies may not necessarily induce inverse alteration than “loss of function” experiments. In normal cell most metabolic events are gradually regulated and this regulatory homeostasis is certainly disturbed by genetic manipulation of any kind. In fact, high-level expression of an enzyme under the control of artificial regulatory elements (cell transfection) is likely to create a different intracellular environment than subtle up-regulation of the corresponding gene product.

On the other hand, complete functional silencing has certainly other biological consequences than gradual down-regulation or pharmacological inhibition.

As indicated above, pro- and antiatherogenic effects of the 12/15-LOX have been reported. In all murine atherosclerosis models a proatherogenic activity was found [73 – 76]. However, in several rabbit models antiatherogenic effects were observed. These data suggest species-specific differences in the biological activity of the enzyme. Since both the rabbit and the murine 12/15-LOX exhibit a comparable

LDL oxidase activity, such inverse biological function cannot be explained on the basis of the oxidation hypothesis. However, if the LOX products as lipid signaling molecules are important for atherogenesis (arachidonic acid cascade) such inverse effects become plausible. The murine 12/15-LOX converts arachidonic acid predominantly to 12S-H(p)ETE [91]. In contrast, the major eicosanoid formed by the rabbit enzyme is 15S-H(p)ETE [114]. The human 12/15-LOX oxidizes arachidonic acid predominantly to 15S-H(p)ETE [115] and thus, with respect to the positional specificity, it is more similar to the rabbit enzyme.

An alternative explanation for the conflicting data obtained in the different animal atherosclerosis might be a different biological dynamic of the enzyme. At early stages of foam cell formation, the enzymatic activity may be restricted to the intracellular compartment. At these stages, 12/15-LOX may counteract intracellular lipid deposition by accelerating the breakdown of internalized lipids. It has been reported before that oxidized cholesterol esters are better substrates for cytosolic cholesterol ester hydrolases [116], which liberate free cholesterol and fatty acids. The fatty acids may then be utilized *via* β-oxidation and free cholesterol may be exported by reverse cholesterol transport. If this scenario were true, it would explain the antiatherogenic effect of the enzyme. At later stages of plaque development, when foam cell formation is already advanced, the LOX reaction may run out of control. In these stages LOX substrates accumulate intracellularly, and under such suboptimal reaction conditions the enzyme might produce large amounts of oxidizing radicals [12]. In other words, if the cells are not capable any more to control this oxidation reaction, the enzyme might contribute to extracellular LDL oxidation *via* the formation of oxidizing radicals. Thus, 12/15-LOX may alter its biological activity during the time-course of lesion formation. A switch like that would explain the inverse biological activities observed in different atherosclerosis models.

If the biological activity of 12/15-LOX is species-dependent (proatherogenic in mice, antiatherogenic in rabbits), the question about its role in humans arises. Immunohistochemical staining, *in situ* hybridization [117], and the detection of a specific share among the oxygenation products of human lesions suggested expression of the enzyme during the time-course of lesion development [118, 119]. However, the recent failure to detect significant amounts of 12/15-LOX transcripts in human lesions of various stages challenged this hypothesis. However, if one assumes expression of the enzyme at early stage of human atherogenesis it remains unclear whether it may act pro- or antiatherogenic.

One way to address this question experimentally is to search for individuals, which, owing to genetic polymorphisms, show reduced expression of the enzyme or express enzyme species with lower specific activities. If such individuals exist, one may search for possible correlations of these polymorphisms with cardiovascular disorders. Such experiments are currently underway in different laboratories around the world and the results will contribute to answer the question, which role 12/15-LOXs may play in human atherogenesis.

5 References

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