Absence of 12/15-Lipoxygenase Expression Decreases Lipid Peroxidation and Atherogenesis in Apolipoprotein E–Deficient Mice

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Background—The enzyme 12/15-lipoxygenase (12/15-LO) has been implicated in the oxidative modification of LDL. In a murine model, we tested the hypothesis that deletion of 12/15-LO decreases atherogenesis by reducing oxidant stress, as measured by 2 indices of lipid peroxidation: isoprostane generation and autoantibody formation to malondialdehyde (MDA)-LDL, an epitope of LDL formed as a result of oxidative modification.

Methods and Results—12/15-LO–deficient (12/15-LO<sup>−/−</sup>) mice were crossed with apolipoprotein E–deficient (apoE<sup>−/−</sup>) mice. At 10 weeks of age, atherosclerotic lesion initiation was significantly delayed in the double-knockout mice. The rate of lesion progression was diminished at 8 and 12 months, and even at 15 months, lesion size was reduced 50% (P<0.0005) compared with control apoE<sup>−/−</sup> mice. The urinary and plasma levels of the specific isoprostane 8,12-iso-iPF<sub>2α</sub>-VI, as well as IgG autoantibodies against MDA-LDL, were significantly reduced in the double-deficient mice in parallel with decreased atherosclerosis at all time points from 10 weeks to 15 months of age compared with apoE<sup>−/−</sup> controls.

Conclusions—Enzymatic action of 12/15-LO contributes significantly to atherosclerotic lesion initiation and propagation in this murine model. Strong positive correlations exist between lesion size, isoprostane levels, and MDA-LDL autoantibodies, providing in vivo evidence for an enzymatic (12/15-LO) component to lipid peroxidation and atherogenesis. (Circulation. 2001;103:2277-2282.)

Key Words: atherosclerosis ■ enzymes ■ lipoxygenase ■ lipids

Atherosclerotic lesions develop in a characteristic fashion, first presenting as fatty streaks, consisting of lipid-rich macrophages (called foam cells) in the intima, beneath an intact endothelial cell layer. According to the oxidation hypothesis of atherosclerosis, oxidation of LDL plays a key role in the early pathogenic events, leading to foam-cell formation and the fatty streak. Products of oxidized LDL (oxLDL) are chemotactic for monocytes, promote endothelial cell binding of monocytes, and once the monocytes have been recruited into the arterial intima, inhibit the motility of macrophages. Macrophages can both initiate the oxidation of LDL and take up oxLDL in an unregulated manner, leading to foam-cell formation. There is now considerable evidence to support the presence of oxLDL in vivo, at least in animal models (reviewed in Reference 3). Many enzyme systems or nonenzymatic oxidative mechanisms have been demonstrated to mediate oxidation in vitro. The mechanism(s) responsible for oxidation of LDL in vivo, however, remain to be fully defined.

There is now considerable evidence to support a role for 12/15-lipoxygenase (12/15-LO) as important for mediating LDL oxidation and promoting atherogenesis. In vitro, the enzyme can initiate oxidation of LDL, and lipoxygenase inhibitors can greatly diminish the ability of macrophages to oxidatively modify LDL. When LDL preparations are incubated with 15-LO–transfected fibroblasts, they become seeded with hydroperoxides and develop biological properties of oxLDL, including display of oxidation-specific epitopes recognized by antibodies to oxLDL. Rabbit and human atherosclerotic lesions contain 15-LO mRNA/protein, stereospecific 15-LO reaction products can be demonstrated in lesions, and a specific 15-LO inhibitor reduced atherogenesis in rabbits. The most convincing data to date come from our recent studies in which disruption of 12/15-LO gene expression greatly diminished atherosclerosis in 15-week-old apolipoprotein E–deficient (apoE<sup>−/−</sup>) mice. Those studies, however, did not look at very early or long-term effects of 12/15-LO deletion on lesion progression.
nor were mechanisms explored by which LO deletion retarded atherogenesis.

Here, we examine the impact on atherogenesis of 12/15-LO deletion in apoE−/− mice over various time points up to 15 months of age. To relate these changes to measures of lipid peroxidation, we measured autoantibody titers to malondialdehyde (MDA)-LDL, an epitope of oxLDL generated when LDL undergoes oxidative modification that has previously been shown to be increased in apoE−/− mice and to correlate with the extent of atherosclerosis in LDL-receptor−/− mice.

We also made a more direct measure of in vivo lipid peroxidation. Isoprostanes (iPs), free radical–catalyzed products of arachidonic acid that localize to macrophages and vascular smooth muscle cells in human atherosclerotic plaque, are increased in a variety of syndromes putatively associated with oxidant stress and thus thought to reflect lipid peroxidation in vivo. Given that plasma and urinary iPs are inversely correlated with aortic lesion area in vitamin E–fed apoE−/− mice and that 12/15-LO gene disruption also inhibits atherogenesis in apoE−/− mice, we sought to test the hypothesis that 12/15-LO–abolished expression would reduce oxidant stress in apoE−/− mice, as evidenced by decreased lipid peroxidation in parallel with decreased extent of atherosclerosis.

Methods

Generation of 12/15-LO−/−/ApoE−/− Double-Knockout Mice

12/15-LO–deficient (12/15-LO−/−) mice were crossbred with apoE−/− mice (backcrossed 7 and 6 times, respectively, to the C57BL/6 background). Mice were genotyped either by PCR analysis (apoE) or Southern blot analysis (12/15-LO) as described previously. The mice were fed a normal mouse-chow diet (5015; Purina Mills). All animal procedures were carried out in accordance with institutional and NIH guidelines.

Tissue Preparation for Morphometric Determination of Atherosclerosis

Mice were anesthetized and bled via cardiac puncture. The aorta and its main branches were dissected from the aortic valve to the iliac bifurcation, and en face preparations/images were prepared as described.

Lipid and Lipoprotein Analysis

Plasma triglyceride and total cholesterol levels were analyzed as described. Lipoprotein profiles were determined by fast protein liquid chromatography gel filtration (Pharmacia LKB Biotechnology) on 2 Superox 6 columns in series.

Determination of Autoantibody Titers Against OxLDL Epitopes

The titers of IgG autoantibodies against MDA-modified LDL were determined in individual plasma samples (1:500 dilution) by sensitive chemiluminescence detection. The amount of IgG bound to the MDA-LDL antigen was detected with alkaline phosphatase–labeled anti-mouse IgG. Data are expressed as relative light units (RLU)/100 ms. All analyses were performed in single assays, and the intra-assay coefficient of variation was 8% to 10%.

IP Analysis

8,12-iso-iP−/−VI was measured by stable dilution isotope gas chromatography/mass spectrometry assay with [2H]−8,12-iso-iP−/− VI internal standard using peak ratios (interassay/intra-assay variabilities ±4% to 5%). Urine samples were collected in the presence of 0.1% butylated hydroxytoluene and stored at −80°C until analysis. An aliquot was stored for creatinine measurement. Blood samples were obtained via right ventricular puncture from animals fasted overnight as previously described.

Immunohistochemistry

Immunostaining of aortic root sections (8 μm) for detection of macrophages was carried out as described previously. Detection of T lymphocytes was performed similarly with goat anti-rat CD4, CD8 polyclonal antibodies (20 μg/mL; ATCC). Smooth muscle cell α-actin expression was examined by an avidin-biotin–horseradish peroxidase method (Vector Laboratories) with a primary mouse anti-human α-actin monoclonal antibody conjugated with FITC (Sigma Chemical Co) and a secondary biotinylated goat anti-FITC antibody (Sigma).

Statistical Analysis

Initial analyses were performed by Student’s t test. If the data did not fit the constraints of this parametric test, data were analyzed with Kruskal-Wallis ANOVA or the Mann-Whitney U test. A value of P<0.05 was considered significant. Correlations between autoantibody titers, iPs, and extent of lesions were determined by linear regression analysis with Instat 2.01 software (Instat Computer Software). Data are presented as mean±SEM.

Results


The present study was designed to explore lesion development throughout the lifespan of 12/15-LO−/− mice bred into apoE−/− mice fed a normal chow diet. Groups of mice with the respective genotypes were euthanized at 10 weeks and 8, 12, and 15 months of age. To investigate any effects of the 12/15-LO gene disruption on atherogenesis independent of the apoE-knockout phenotype, mice deficient in 12/15-LO (12/15-LO−/−/apoE+/−) were euthanized at 8, 15, and 24 months of age.

Total and HDL cholesterol levels were similar for all animals on the apoE−/− background at all time points irrespective of their 12/15-LO genotype (Table). Values of total cholesterol as well as the HDL fractions were similar to values normally found in apoE−/− mice on a regular chow diet. Total and HDL cholesterol levels of the 12/15-LO−/−+/ apoE−/− mice were similar to those found in C57BL/6 mice on a normal chow diet. Triglyceride levels were not significantly different between apoE−/− mice homozygous (12/15-LO−/−/−), heterozygous (12/15-LO−/−/−), or wild-type for 12/15-LO (data not shown). These data indicate that any effects of 12/15-LO deletion on atherogenesis are independent of changes in plasma lipoprotein levels.

By an en face method, the extent of atherosclerotic surface area was quantified in the aorta starting directly distal to the aortic valve and ending ∼1 cm into the common iliac arteries. Because lesions commonly develop at branching points of blood vessels, we included approximately the first centimeter of major branching arteries, ie, the brachiocephalic trunk, the left common carotid and subclavian arteries,
and the renal arteries. At 10 weeks, lesions had started to develop in the 12/15-LO<sup>+/−</sup>/apoE<sup>−/−</sup> mice (1263±643 μm<sup>2</sup>; n=7), and the average atherosclerotic lesion size in the 12/15-LO<sup>+/−</sup>/apoE<sup>−/−</sup> control was 4099±1738 μm<sup>2</sup> (n=13; Figure 1A). Because lesions were observed in only 6 of 13 mice of this genotype at this early time, the SD was high. In sharp contrast, only one of ten 12/15-LO<sup>+/−</sup>/apoE<sup>−/−</sup> mice had a small detectable atherosclerotic lesion. The observed differences were statistically significant by non-parametric testing (P=0.045).

After 8 months on mouse chow, atherosclerotic lesions had developed in all mice. The average size of atherosclerotic lesions in the 12/15-LO<sup>+/−</sup>/apoE<sup>−/−</sup> mice (66 009±5587 μm<sup>2</sup>; n=12) was significantly less than that found in 12/15-LO<sup>+/−</sup>/apoE<sup>−/−</sup> mice (95 126±7342 μm<sup>2</sup>; n=11; P=0.0044) and 12/15-LO<sup>+/−</sup>/apoE<sup>−/−</sup> mice (90 870±6144 μm<sup>2</sup>; n=12; P=0.0067). An absolute increment in total lesion area occurred in all 3 groups, but the 12/15-LO<sup>/−</sup> mice still had the smallest lesion areas at 1 year of age (Figure 1B). The final time point for lesion determination was 15 months, because apoE<sup>−/−</sup> mice usually fail to thrive and often die around this age. Even close to the end of their natural life span, the average size of atherosclerotic lesions in the 12/15-LO<sup>/−</sup>/apoE<sup>−/−</sup> mice (116 203±6756 μm<sup>2</sup>; n=13) was significantly less than in 12/15-LO<sup>+/−</sup>/apoE<sup>−/−</sup> mice (258 696±24 374 μm<sup>2</sup>; n=6; P=0.0015) and 12/15-LO<sup>/−</sup>/apoE<sup>−/−</sup> mice (236 249±19 037 μm<sup>2</sup>; n=10; P=0.0005) (Figure 2). In the absence of hypercholesterolemia, wild-type C57BL/6 mice with homozygous 12/15-LO gene disruption (12/15-LO<sup>/−</sup>/apoE<sup>−/−</sup>) had no detectable atherosclerotic lesions in their aortas when examined at 8 months (n=5), 15 months (n=9), or even 2 years of age (n=6).

Immunohistochemistry was carried out on aortic root sections of 8-, 12-, and 15-month-old mice. Although lesions were consistently smaller in the double-knockout mice, there were no significant alterations in lesion composition of macrophages and CD4- or CD8-positive lymphocytes compared with 12/15-LO–expressing mice, and smooth muscle α-actin expression was reduced in a variable manner at lesion sites in both groups of mice (data not shown).

**Figure 1.** Extent of atherosclerosis in en face aorta preparations from apoE<sup>−/−</sup> mice, wild-type (12/15-LO<sup>+/+</sup>) or heterozygously (12/15-LO<sup>+/−</sup>) or homozygously (12/15-LO<sup>/−</sup>) deficient for 12/15-LO. Each point represents lesion area from an individual animal, and bars depict average. A, At 10 weeks, differences in lesion area are significant between 12/15-LO<sup>+/−</sup>/apoE<sup>−/−</sup> and 12/15-LO<sup>+/−</sup>/apoE<sup>−/−</sup> mice (P=0.045; Kruskal-Wallis ANOVA). B, At later time points, average lesion areas are never significantly different between 12/15-LO<sup>+/−</sup>/apoE<sup>−/−</sup> and 12/15-LO<sup>/−</sup>/apoE<sup>−/−</sup> mice, but mean lesion areas in 12/15-LO<sup>/−</sup>/apoE<sup>−/−</sup> mice are always significantly smaller than in 12/15-LO heterozygous or wild-type mice.

**Figure 2.** En face aorta preparations from respective genotypes representative of mean lesion areas observed in these animals fed a normal mouse chow for 8 and 15 months, respectively.
nine; n=6) and 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> mice (0.535±0.205 ng/mg creatinine; n=6). This reduction could be observed at all time points. Plasma 8,12-iso-iPF<sub>2</sub>-VI was also reduced in the 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> mice compared with 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> and 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> mice. The differences were sustained throughout the study (Figure 3A). The correlation of urinary or plasma levels of 8,12-iso-iPF<sub>2</sub>-VI with the extent of lesion development was highly significant (r=0.84, P=0.03 for urinary 8,12-iso-iPF<sub>2</sub>-VI and r=0.86, P=0.009 for plasma 8,12-iso-iPF<sub>2</sub>-VI; Figure 3C).

**Correlation of MDA-LDL Autoantibodies With Lesion Size in 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup>, 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup>, and 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> Mice**

Individual plasma samples for MDA-LDL autoantibody titer measurements were obtained from mice at 10 weeks and 8, 12, and 15 months of age. At 10 weeks, MDA-LDL IgG autoantibody titers were reduced in 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> mice (2008±675 RLU/100 ms) and 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> mice (2392±361 RLU/100 ms) compared with 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> mice (4453±367 RLU/100 ms). This reduction in MDA-LDL autoantibody titers could be observed in the homozygous 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> mice at all time points (Figure 3B).

Titors of MDA-LDL IgG correlated significantly with extent of atherosclerotic lesion area (Figure 3D) and with the 8,12-iso-iPF<sub>2</sub>-VI in plasma and urine (Figure 4). These correlations were also sustained within each sex (data not shown).

**Discussion**

There has been substantial indirect evidence to support a role of 12/15-LO in atherogenesis, and we recently provided direct and specific evidence for the involvement of 12/15-LO in vivo.14 The present studies addressed the kinetics of lesion initiation and progression in the 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> mouse model. If 12/15-LO were influencing atherogenesis by promoting lipid peroxidation, then one might anticipate a decrement of oxidant stress with deletion of 12/15-LO, which correlates with the decrease in atherogenesis. To examine this hypothesis, we determined the extent of atherosclerotic lesion size and correlated this with 2 distinct proposed markers of oxidant stress, 8,12-iso-iPF<sub>2</sub>-VI and MDA-LDL IgG autoantibodies.

In the absence of any significant differences in cholesterol and triglyceride levels, no significant atherosclerotic lesions were observed in 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> mice at 10 weeks of age on a normal chow diet, whereas lesion development was already initiated in 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> and 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> mice.
apoE<sup>−/−</sup> control mice. These observations suggest that although lesion development is eventually initiated in the 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> mice, the lesion progress at a slower rate than observed in 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> and 12/15-LO<sup>−/−</sup>/apoE<sup>−/−</sup> mice. This finding is concordant with data from studies showing that predominantly stereospecific arachidonic and linoleate metabolites are found in early atherosclerotic lesions in humans and rabbits. Quantification of lesion areas in en face preparations depicts the spread of lesions along the vessel, but not the thickening and maturation of individual lesions. Thus, an increment in lesion surface as quantified via this methodology primarily reflects the development of new lesions. The concept that 12/15-LO is involved in early lesion development is corroborated by our finding that lesion development in 12/15-LO<sup>−/−</sup> mice is occurring at a slower pace throughout their life span on an apoE<sup>−/−</sup> background.

Markers that are increased in direct response to oxidative modification of LDL are likely to reflect local oxidant stress within the vascular wall. iPs and plasma oxLDL antibodies are such candidates. iPs are stable prostaglandin isomers that are generated during oxidative modification of arachidonic acid via a free radical–catalyzed mechanism. They have emerged as increasingly promising markers for oxidant stress and lipid peroxidation in vivo (eg, in atherosclerosis). In addition, antioxidant treatment with vitamin E significantly reduced iP generation in aortic tissue (as well as in plasma and urine) in parallel with a reduction in atherosclerotic lesion development in apoE<sup>−/−</sup> mice. The present study also indicates a highly significant correlation of 8,12-iso-iPF<sub>2α,VI</sub>, the most abundant of many F<sub>2</sub>-iPs, with atherosclerotic lesion stage in apoE<sup>−/−</sup> mice and also shows diminished 8,12-iso-iPF<sub>2α,VI</sub> levels in parallel with decreased atherosclerotic lesion formation in mice lacking 12/15-LO. This double-knockout model is the first to provide specific evidence for a crucial role of a lipoxgenase pathway in atherogenesis, and the data presented in this study strongly support the hypothesis that decreased oxidant stress is the most likely cause for diminished lesion development in these animals. Once 12/15-LO inserts a hydroperoxide in arachidonic or linoleic acid, it would then render that modified fatty acid more susceptible to further lipid peroxidation, which by propagation would start a chain reaction. This, in turn, could lead to the increased generation of specific iPs. The exact mechanisms linking an enzymatic pathway (12/15-LO) with nonenzymatic lipid peroxidation and generation of iPs, however, are unknown at present.

Gene transfer of apoE3 into hypercholesterolemic mice decreases established atherosclerosis, with a concomitant reduction in iPs. It is feasible that iPs play a direct role in lesion development in addition to reflecting lesion burden. iPs activate membrane prostanoid receptors and have recently been shown to activate peroxisome proliferator–activated receptors (PPARs), which in turn could play a major role in atherogenesis through complex and interrelated pathways. Thus, an interesting scenario of 12/15-LO involvement in lipid peroxidation and intracellular signaling is beginning to emerge.

Autoantibodies to epitopes of oxidatively modified LDL are found in plasma and lesions, with elevated titers of these antibodies frequently associated with increased vascular disease and antioxidant treatment of various hypercholesterolemic animal models reducing lesion progression and antibody titers (reviewed in Reference 3). As was the case with 8,12-iso-iPF<sub>2α,VI</sub>, we found significant correlation between the titers of autoantibodies to MDA-LDL and atherosclerotic lesion development in the present study. This finding further strengthens the contention that the mechanisms by which 12/15-LO mediates atherogenicity is by promoting lipid peroxidation and the generation of oxLDL.

Because both plasma and urine levels of iP correlate significantly with atherosclerotic lesion development, it seems justified to assume that iP generation is an independent marker for atherogenesis, even though iP generation increases with age. In a similar manner, autoantibody titers to MDA-LDL also increase with age even in C57BL/6 mice, although the absolute increase in titers with age is much greater in LDL-receptor<sup>−/−</sup> hypercholesterolemic mice. At present, data to support the oxidation hypothesis in humans are more limited.

In summary, the present study provides in vivo evidence linking 12/15-LO expression and lipid peroxidation to the initiation and propagation of atherosclerotic lesions in apoE<sup>−/−</sup> mice. It also supports the proposed use of F<sub>2</sub>-iPs and antibodies against oxLDL epitopes as potentially useful techniques for identifying populations that may have enhanced rates of lipid peroxidation and thus cardiovascular risk.

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