12/15-Lipoxygenase Gene Disruption Attenuates Atherogenesis in LDL Receptor–Deficient Mice

Jacob George, MD; Arnon Afek, MD; Aviva Shaish, PhD; Hana Levkovitz, BA; Nira Bloom, BA; Tillmann Cyrus, MD; Lei Zhao, MD, PhD; Colin D. Funk, PhD; Eliott Sigal, MD; Dror Harats, MD

Background—Human 15-lipoxygenase (LO) and its murine analogue 12/15-LO are capable of directly oxidizing esterified fatty acids in lipoproteins and phospholipids. Because these oxidized products possess atherogenic properties, it was suggested that LOs may be involved in enhancing atherogenesis. Previous in vivo tests of the role of LOs in atherogenesis animal models, however, have yielded conflicting results.

Methods and Results—Aiming to study the role of the 12/15-LO in murine atherogenesis, we crossed LDL-receptor–deficient mice (LDL-R−/−) with 12/15-LO–knockout mice and evaluated plaque formation 3 to 18 weeks after initiation of a high-fat diet. Atherosclerotic lesions were considerably reduced in the LDL-R/12/15-LO–double-knockout mice compared with LDL-R−/− mice at 3, 9, 12, and 18 weeks, at the aortic root as well as throughout the aorta. The cellular composition of plaques from mice deficient in 12/15-LO did not differ with respect to macrophage and T-lymphocyte content compared with plaques from 12/15-LO littermates.

Conclusions—12/15-LO plays a dominant role in promoting atherogenesis in LDL-R−/− mice. (Circulation. 2001;104:1646-1650.)

Key Words: lipoxygenase ■ atherosclerosis ■ lipoproteins ■ oxidation ■ cells

Oxidative processes have been shown to play a dominant role in the initiation and progression of atherosclerosis in animal models.1,2 Several lines of evidence support this contention. Oxidation-specific epitopes have been detected within the atherosclerotic plaque. In addition, in vitro studies have demonstrated that oxidized LDL (oxLDL) rather than native LDL is capable of promoting smooth muscle cell migration and endothelial, lymphocyte, and macrophage activation, properties that may explain its proatherogenic properties (reviewed by Heinecke3). Probably the most significant support for the oxidation hypothesis derives from studies showing that antioxidant administration reduces atherogenesis in various animal models.2 Two recent studies suggest that administration of vitamin E may be capable of reducing coronary heart disease events in humans, although in 3 other studies, it was found to be ineffective.6–8 Indirect results have incriminated several candidates in promoting oxidative modification, such as NADPH oxidase, myeloperoxidase, some cytochrome P450 members, the mitochondrial electron transport system, xanthine oxidase, and lipoxygenases (LOs) (reviewed in Reference 3). In contrast to the relatively strong evidence for the role of oxLDL in atherosclerosis, however, there is little or no evidence regarding the systems that oxidize LDL in vivo.

LOs are a family of non–heme iron–containing dioxygenases that insert molecular oxygen into polyenoic fatty acids.9 This family includes the human and rabbit 15-LOs, as well as the porcine, rat, and murine leukocyte-type 12-LOs, all of which can directly oxidize esterified fatty acids in lipoproteins and phospholipids.10 The latter enzymes are also called 12/15-LO, because they are capable of producing both 12-hydroperoxy-eicosatetraenoic acid (12-HPETE) and 15-HPETE from arachidonic acid. There is a close structural resemblance between 15-LO and 12/15-LO, because minor alterations in amino acid sequence can account for the species differences in oxygen insertional specificity.11

The involvement of LO in atherosclerosis is supported by indirect, as well as direct, in vivo observations. 15-LO was found to be present within atherosclerotic plaques of humans and rabbits, colocalizing with oxidation epitopes.12 The powerful data supporting involvement of LO in atherogenesis, however, come from animal studies. Initial observations have shown that overexpression of 15-LO in rabbit arteries was associated with increased occurrence of oxLDL epitopes.13 We recently showed that overexpression of human 15-LO in endothelial cells of the vessel wall of LDL receptor–deficient (LDL-R−/−) mice via preproendothelin promoter resulted in acceleration of early atherosclerotic...
lesions. Two recent studies have demonstrated that pharmacological inhibition of 15-LO resulted in attenuation of atherosclerosis in hypercholesterolemic rabbits. Probably the most conclusive evidence for the role of 12/15-LO in atherosclerosis to date comes from the study by Cyrus et al showing that disruption of the 12/15-LO gene in apoE-knockout mice significantly retarded atherosclerosis initiation and progression. Data from Shen et al, however, indicate that overexpression of 15-LO in rabbit macrophages is associated with reduced atherogenesis, thus questioning the proatherogenic role of 15-LO.

In the present study, we obtain further evidence for the role of 12/15-LO in murine atherogenesis by studying 12/15-LO gene disruption in LDL-R mice using 2 different types of high-fat diet in 2 laboratories (in Israel and the United States). The different experimental protocols of diet feeding are intended to induce either fatty streaks or more advanced lesions.

**Methods**

**Generation of LDL-R−/12/15-LO−/−-Double-Knockout Mice**

LDL-R-deficient mice were obtained from the Jackson Laboratory (Bar Harbor, Me). The generation of 12/15-LO−/− and LDL-R−/− mice was previously described. LDL-R−/− mice on a hybrid C57BL/6×129/Sv (Israeli colony) or backcrossed to C57BL/6 (7 times) (University of Pennsylvania colony) background were crossbred with LDL-R−/− mice. Mice were genotyped either by polymerase chain reaction analysis (LDL-R−/−) or Southern blot analysis (12/15-LO−/−) as described. All mice were kept on a 12-hour light/12-hour dark cycle, and food and water were available ad libitum. At the age of 11 to 12 weeks, LDL-R/12/15-LO−/− mice and LDL-R−/− littermates, were fed a Western-type diet. Mice were euthanized 3 to 18 weeks after initiation of the diet, and their hearts and aortas were removed for evaluation of the extent of atherosclerosis. Plasma was obtained for assessment of the lipid profile in all mice.

**Diets**

1. The Paigen-type diet contained 1.25% cholesterol, 7.5% casein, and 0.5% (wt/wt) sodium cholate (Harlan, Teklad Premier Laboratory Diets).
2. The Western-type diet contained 42% of calories from fat, 43% from carbohydrates, and 15% from protein (TD 96125, Harlan Teklad).

**Lipid Profile**

Total plasma cholesterol and triglyceride levels were determined with an automated enzymatic technique (Boehringer Mannheim). HDL cholesterol levels were determined by HDL cholesterol reagent (Sigma Chemical Co).

**Lipoprotein Oxidation**

Blood for lipoprotein isolation was collected in EDTA (1 mg/mL) from 3 pools from 3 mice in each group after 12 hours of fasting. LDL (density 1.019 to 1.063 g/L) was isolated from the plasma after density adjustment with KBr, by preparative ultracentrifugation at 50,000 rpm/min for 22 hours with a type 50 rotor. LDL preparations were washed by ultracentrifugation, dialyzed against 0.15 mol/L EDTA (pH 7.4), passed through an Acrodisc filter (0.22-μm pore size) to remove aggregates, and stored under nitrogen in the dark. Copper-oxidation of LDL was performed by incubation of postdialysis LDL (1 mg protein/mL in EDTA-free PBS) with copper sulfate (10 μmol/L) for 24 hours at 37°C. Lipoprotein oxidation was confirmed by analysis of thiobarbituric acid-reactive substances (TBARS).

**Assessment of Atherosclerosis in the Aortic Sinus**

Atherosclerotic fatty-streak lesions were quantified by measurement of the lesion size in the aortic sinus as previously described. The heart and upper section of the aorta were removed from the animals, and the peripheral fat was cleaned carefully. The upper section was embedded in O.C.T. compound (Miles Inc) and frozen. Every other section (10 μm thick) throughout the aortic sinus (400 μm) was taken for analysis. Sections were evaluated for fatty-streak lesions after staining with oil red O. Lesion areas per section were counted by use of a grid by an observer unfamiliar with the tested specimen.

**Sudan IV Staining of Aortic Lesions**

The aortas were dissected from the aortic arch to the iliac bifurcation and washed for 1 hour in PBS (pH 7.4) and 0.5 mmol/L EDTA on a rotating table. The aorta was then fixed with a formal succrose (4% paraformaldehyde, 5% sucrose, 20 mmol/L butylated hydroxytoluene, and 2 mmol/L EDTA, pH 7.4) overnight. The adventitial fat was trimmed from the aorta under a microscope and opened longitudinally, rinsed briefly in 70% ethanol, immersed for 6 minutes in a filtered solution of Sudan IV (Sigma Chemical Co) in 35% ethanol and 50% aceton for 10 minutes, and destained in 80% ethanol. The Sudan IV-stained aortas were assessed for lesion area by morphometry as previously described.

**Immunohistochemical Analysis of Aortic Sinus Sections**

Immunohistochemical stainings were performed as described with the following primary antibodies: rat anti-mouse CD3 clone: 17A2 (PharMingen), rat anti-mouse VCAM-1 (clone 429 MVCMAC, PharMingen), and rat anti-mouse Mac-1 clone M1/70 (Boehringer Mannheim). Slides were dried under airstream for 45 minutes, fixed in acetone for 5 minutes in −20°C, and rinsed 3 times for 5 minutes in Tris-buffered saline (TBS) containing 0.1% BSA and 0.05% Tween-20 (TBS-Tween-20). To reduce background signals, a 15-minute incubation with 10% nonimmune goat serum was followed by a 30-minute incubation with CAS (Zymed Laboratory) at room temperature. After blocking, the various primary antibodies diluted 1:50 in TBS-Tween, biotinylated second antibody, and streptavidin peroxidase conjugate (a kit from Zymed Laboratory) were added. The results of the Mac-1 and CD3 cells are expressed as positive nuclei/total plaque nuclei.

**Statistical Analysis**

Differences between groups with respect to lipid levels and plaque size and composition were made with a 2-sided Student’s t test. A value of P<0.05 was considered statistically significant.

**Results**

LDL-R-deficient mice maintain relatively moderate hypercholesterolemia and do not develop significant atherosclerosis on feeding a normal chow diet. Feeding a Paigen diet facilitates atherosclerosis concomitant with the elevation of cholesterol levels. The LDL-R-deficient mouse model, similar to the apoE-knockout model, displays features that indicate the involvement of oxidation in enhanced atherogenesis.

LDL-R−/−/12/15-LO−/− mice, like LDL-R−/− littermates, were healthy and fertile, gaining weight in a similar manner regardless of the diet provided.

After 3 weeks and 9 weeks on the high-fat diet, LDL-R−/−/12/15-LO−/− double-knockout mice and LDL-R−/− controls (12/15-LO−/−) maintained similar levels of cholesterol and triglycerides (Table).
Mean cholesterol and triglyceride levels in mice fed the Western diet for 12 weeks (3 pools of 3 mice each) were not different between LDL-R<sup>−/−</sup>/12/15-LO<sup>−/−</sup> (1445±171 and 308±59 mg/dL, respectively) and LDL-R<sup>−/−</sup>/12/15-LO<sup>+/−</sup> (1503±195 and 341±25 mg/dL, respectively) mice. Mean cholesterol and triglyceride levels in mice fed the Western diet for 18 weeks (3 pools of 3 mice each) did not differ significantly between LDL-R<sup>−/−</sup>/12/15-LO<sup>−/−</sup> (946±90 and 313±39 mg/dL, respectively) and LDL-R<sup>−/−</sup>/12/15-LO<sup>+/−</sup> (933±91 and 280±26 mg/dL, respectively) mice.

Pooled plasma LDL from LDL-R<sup>−/−</sup>/12/15-LO<sup>−/−</sup> mice displayed no significant difference in susceptibility to in vitro oxidation compared with plasma from LDL-R<sup>−/−</sup>/12/15-LO<sup>+/−</sup> mice (mean of 91.6 minutes in the former compared with 84.2 minutes in the latter; *P=0.93).

LDL-R<sup>−/−</sup>/12/15-LO<sup>−/−</sup>–double-knockout mice fed a high-fat diet for 3 weeks exhibited significantly early atherosclerotic lesions (9100±1700 μm²) compared with LDL-R<sup>−/−</sup>/12/15-LO<sup>+/−</sup> mice (25 200±3000 μm²; **P<0.005) at the aortic root. After 9 weeks of high-fat diet supplementation, atherosclerotic lesions were also reduced in LDL-R<sup>−/−</sup>/12/15-LO<sup>−/−</sup> mice (51 000±9000 μm²) compared with control mice (225 000±31 000 μm²; *P<0.001; Figure 1).

To further confirm the results of aortic root atherogenesis, we assessed the extent of atherosclerosis throughout the aorta by en face methodology. Because our previous observations indicated that after 3 weeks on a high-fat diet, no quantifiable lesions were observed throughout the aorta, lesions were evaluated after 9, 12, and 18 weeks of diet. Indeed, aortic surface lesion coverage was significantly reduced in LDL-R<sup>−/−</sup>/12/15-LO<sup>−/−</sup> mice at all time points versus LDL-R<sup>−/−</sup>/12/15-LO<sup>+/−</sup> control mice.

Mice fed a Western-type high-fat diet were evaluated in an additional study for atherosclerotic lesion coverage of the aorta. Twelve as well as 18 weeks of feeding with a Western diet resulted in a significant attenuation in the extent of plaque formation in the double-knockout compared with LDL-R<sup>−/−</sup> mice (Figure 2).

To investigate whether 12/15-LO gene disruption influenced plaque cellular composition in addition to plaque size, we made a quantitative assessment of macrophage and lymphocyte numbers. Early atherosclerotic lesions (after 3 weeks of Paigen diet) from LDL-R<sup>−/−</sup>/12/15-LO<sup>−/−</sup> mice showed a macrophage content (83.7±5.9%) similar to that in early lesions from LDL-R<sup>−/−</sup>/12/15-LO<sup>+/−</sup> mice (87.3±5.1%; Figure 3). More advanced lesions (9 weeks of high-fat diet) from 12/15-LO<sup>−/−</sup> mice were also found to exhibit a content of macrophages (82.5±5.5%) similar to that of control mice (73.5±6.6%). No differences were observed in the lymphocyte (CD3<sup>+</sup>) content between lesions of LDL-R<sup>−/−</sup>/12/15-LO<sup>−/−</sup> mice fed a Paigen diet for 3 weeks (3.0±1.1%) and control mice (2.6±1.0%; Figure 3) or 9 weeks (4.1±1.1% and 3.2±0.7%, respectively).

**Discussion**

In the present study, we address the role of 12/15-LO expression in atherogenesis. The optimal means of testing the hypothesis that this enzyme is proatherogenic is by cross-breeding experiments, identifying the effects of isolated gene disruptions. Indeed, Cyrus et al<sup>17</sup> recently provided compelling evidence of the role of 12/15-LO in murine atherogenesis by crossing apoE-knockout with 12/15-LO<sup>−/−</sup> mice, showing that early as well as advanced lesions were reduced. These observations are strongly supported by our recent observa-

**Table 1.** Effect of 12/15-LO gene disruption on aortic sinus atherosclerosis in LDL-R<sup>−/−</sup> mice. 12/15-LO<sup>−/−</sup> and 12/15-LO<sup>+/−</sup> LDL-R<sup>−/−</sup> mice were fed a Paigen diet for 3 and 9 weeks. At death, hearts were removed and sectioned at level of aortic sinus for evaluation of atherosclerotic lesion development after staining with oil red O (A and B). After high-fat diet for 9 weeks, aortas were removed, cleaned, and stained with Sudan IV as described in Methods for evaluation of aortic coverage (C). *P<0.01; **P<0.001; ***P<0.0001.

<table>
<thead>
<tr>
<th>Week</th>
<th>Total Cholesterol, mg/dL</th>
<th>Triglyceride, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>234±23</td>
<td>264±34</td>
</tr>
<tr>
<td>3</td>
<td>2460±112</td>
<td>2612±106</td>
</tr>
<tr>
<td>6</td>
<td>2348±123</td>
<td>2986±206</td>
</tr>
<tr>
<td>9</td>
<td>2689±119</td>
<td>3034±223</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Week</th>
<th>LDL-R&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>LDL-R&lt;sup&gt;+/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>12LO</td>
<td>12/15 LO&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>12/15 LO&lt;sup&gt;+/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>116±12</td>
<td>148±23</td>
</tr>
<tr>
<td>3</td>
<td>110±21</td>
<td>139±23</td>
</tr>
<tr>
<td>6</td>
<td>112±12</td>
<td>133±22</td>
</tr>
<tr>
<td>9</td>
<td>106±23</td>
<td>156±28</td>
</tr>
</tbody>
</table>

The mice were fed a high-cholesterol, high-fat diet. Animals (n=10 or 11) from each group were bled at 0, 3, 6, and 9 weeks in 2 independent studies. Plasma cholesterol and triglyceride concentrations were determined as described in Methods. Values are mean±SEM.
Frozen sections from LDL−/− and 12/15-LO−/−/LDL-R−/− mice (male and female) were fed a Western diet for 12 (n=13 to 16 mice/group) and 18 (n=11 to 12 mice/group) weeks. Extent of atherosclerosis was evaluated by calculating aortic surface coverage. *P<0.0001; **P=0.0004.

Figure 2. 12/15-LO gene disruption attenuates aortic lesion atherogenesis induced by 12 to 18 weeks of Western-type diet. 12/15-LO−/− and 12/15-LO−/−/LDL-R−/− mice (male and female) were fed a Western diet for 12 (n=13 to 16 mice/group) and 18 (n=11 to 12 mice/group) weeks. Extent of atherosclerosis was evaluated by calculating aortic surface coverage. *P<0.0001; **P=0.0004.

Figure 3. Plaque composition in LDL-R−/−/12/15-LO−/− mice. Frozen sections from LDL−/−/12/15-LO−/− and 12/15-LO−/− mice fed a Paigen diet for 3 and 9 weeks were subjected to immunostaining with anti–Mac 1 antibodies (to evaluate macrophage content; A) or with anti-CD3 antibodies (to assess lymphocyte content; B). Results represent mean±SD of ≥3 sections from 5 to 6 mice in each group.

Acknowledgment
This study was supported in part by NIH grant HL-53558 (Dr Funk).
References


12/15-Lipoxygenase Gene Disruption Attenuates Atherogenesis in LDL Receptor–Deficient Mice
Jacob George, Arnon Afek, Aviv Shaish, Hana Levkovitz, Nira Bloom, Tillmann Cyrus, Lei Zhao, Colin D. Funk, Eliott Sigal and Dror Harats

_Circulation_. 2001;104:1646-1650
doi: 10.1161/hc3901.095772

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/104/14/1646

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/