Basic Science Reports

Lipid Peroxidation and Platelet Activation in Murine Atherosclerosis

Tillmann Cyrus, MD; Lina X. Tang, BSc; Joshua Rokach, PhD; Garret A. FitzGerald, MD; Domenico Praticò, MD

Background—Lipid peroxidation and platelet activation are thought to be important contributors to the pathogenesis of atherosclerosis. The relevance of their interaction in vivo, however, is unknown.

Methods and Results—LDL receptor–deficient (LDLR−/−) mice on a high-fat diet developed extensive atherosclerosis and had increased urinary levels of 8,12-iso-isoprostane (iP) F2α-VI and 2,3-dinor-thromboxane (Tx) B2, markers of in vivo lipid peroxidation and platelet activation, respectively. Vitamin E supplementation suppressed 8,12-iso-iPF2α-VI biosynthesis and reduced atherosclerosis (65%) without having a significant effect on lipid levels or TxB2 biosynthesis. Addition of the platelet inhibitor indomethacin to vitamin E simultaneously suppressed 8,12-iso-iPF2α-VI and TxB2, significantly reduced soluble intercellular adhesion molecule-1 and monocyte chemoattractant protein-1, and remarkably, further reduced atherosclerosis (80%).

Conclusions—These results indicate that in vivo lipid peroxidation and platelet activation coexist in murine atherosclerosis and that lipid peroxidation does not contribute to platelet activation and reflects the oxidant component of the inflammatory response. Our findings suggest that oxidant stress and platelet activation represent 2 distinct therapeutic targets in atherosclerosis. We propose that a combination of antioxidants and platelet inhibitors might be rationally evaluated in the prevention of progression of human atherosclerosis. *(Circulation. 2001;104:1940-1945.)*

Key Words: atherosclerosis ■ free radicals ■ thromboxane ■ inflammation ■ antioxidants

Lipid peroxidation, in particular oxidative modification of LDL in vivo, is thought to play a functional role in atherogenesis.1,2 Evidence consistent with this hypothesis includes the presence of oxidized lipids in atherosclerotic lesions, the novel biological properties conferred on LDL by oxidation, and the reduction of murine atherosclerosis by structurally distinct antioxidants.3–6 Thromboxane (Tx)-dependent platelet activation in vivo is also considered an important component in the pathogenesis of the vascular complications of atherosclerosis.7 Support for this hypothesis comes from the efficacy of low-dose aspirin, which preferentially inhibits platelet cyclooxygenase (COX)-1, in protecting against cardiovascular disease.8–10 The relevance of platelets to the development and progression of atherosclerosis, however, is much less clearly understood. We have previously shown that isoprostane (iP) generation, a sensitive marker of in vivo lipid peroxidation,11 is increased in the atherosclerotic apolipoprotein E (apoE)–deficient (apoE−/−) mouse and that its suppression with vitamin E retards atherosclerosis.6 Recently, we also showed that Tx biosynthesis, a marker of in vivo platelet activation, is increased in 2 distinct murine models of atherosclerosis12 and that it is also of relevance to atherogenesis.13 Several reports have shown that in vivo platelet activation and oxidant stress may coincide in settings such as endotoxemia,14 ischemia-reperfusion injury,15,16 cigarette smoking,17,18 and human atherosclerosis.19–21 It is still unknown, however, whether platelet activation and lipid peroxidation contribute to each other or whether they represent 2 distinct therapeutic targets.

The present study was designed to explore their interaction in vivo in atherosclerosis using the LDL receptor–deficient (LDLR−/−) mouse on a high-fat diet. Using mass spectral analysis of urinary metabolites, we report increased generation of both 8,12-iso-iPF2α-VI and TxA2 during atherogenesis. Consistent with our previous results in apoE−/− mice, marked inhibition of lipid peroxidation by vitamin E retards atherogenesis; this occurs without inhibition of the elevated TxA2 biosynthesis in LDLR−/− mice. Coincidental suppression of Tx formation by indomethacin further augments the antiatherosclerotic effect of vitamin E.

Methods

Animals

LDLR−/− mice (backcrossed 10 times to C57BL/6 mice) were obtained from Jackson Laboratories (Bar Harbor, Me) at 6 weeks of

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age. All procedures and care of animals were approved by the Institutional Animal Care and Usage Committee of the University of Pennsylvania. After 2 weeks of acclimatization, they were fed a high-fat diet (normal chow supplemented with 0.15% cholesterol and 20% butterfat) for the entire study. At this time, animals were divided into 3 groups (n=14 each) and randomized to receive placebo, vitamin E (2 IU/g diet), or a combination of vitamin E (2 IU/g diet) plus indomethacin (6 mg/L). The animals receiving indomethacin alone have been described previously. Preliminary experiments demonstrated that the selected dose of indomethacin inhibited platelet cyclooxygenase activity ex vivo. Urine was collected in metabolic cages at 8, 16, and 26 weeks of age. Blood samples were obtained, as previously described, from animals fasted overnight by retro-orbital bleeding.

**Cox-1 Activity Ex Vivo**

Cox-1 activity ex vivo was assessed by measurement of serum TxB2. Blood was allowed to clot at 37°C for 1 hour as previously described. Serum was separated by centrifugation at 1000 rpm for 15 minutes and stored at ~80°C until analysis.

**Biochemical Analyses**

Serum TxB2, urinary 8,12-iso-IPF2α-VI, 2,3-dinor-TxB2, and 2,3-dinor-6-keto PGF1α were measured by stable dilution isotope gas chromatography/mass spectrometry assays, as previously described. Plasma cholesterol and triglyceride levels were determined enzymatically with Sigma reagents (Sigma Chemical Co). Levels of soluble intercellular adhesion molecule-1 (sICAM-1) and monocyte chemotactic protein-1 (MCP-1) were measured by ELISA kits (Endogen, Inc and R&D Systems, respectively).

**Platelet Aggregation Studies**

Platelet aggregation was studied as previously described. Arachidonic acid (100 µmol/L) was used as agent to induce an irreversible aggregation.

**Preparation of Mouse Aortas and Quantification of Atherosclerosis**

Mice were euthanized after the final blood collection. The aortic tree was perfused for 10 minutes with ice-cold PBS containing 20 µmol/L BHT and 2 mmol/L EDTA, pH 7.4, by inserting a cannula into the left ventricle and allowing free efflux from an incision in the vena cava. After removal of the surrounding adventitial fat tissue, the aorta was opened longitudinally from the aortic root to the iliac bifurcation, fixed in formal sucrose (4% paraformaldehyde, 5% sucrose, 20 µmol/L BHT, and 2 mmol/L EDTA, pH 7.4), then stained with Sudan IV. The extent of atherosclerosis was determined by the "en face" method. Quantification was performed by capturing images of aortas with a Dage-MTI 3CCD 3-chip color video camera connected to a Leica MZ12 dissection microscope, as previously described. Atherosclerosis was also quantified in the aortic root cross sections from fresh-frozen OCT-embedded hearts, as previously described. Briefly, alternate 10-µm frozen sections of the aortic root covering 300 µm of the proximal aorta, starting at the sinus, were fixed in aceton, rehydrated, and stained for atherosclerotic lesions with oil red O. Images were captured digitally with a video camera connected to a Leica microscope and analyzed by computerized image analysis (Image Pro Plus, Media Cybernetics). The acquisition of images and analysis of lesions was always performed in a blinded fashion.

**Immunohistochemistry**

Immunostaining of sections for macrophage content was performed as previously described. Briefly, the avidin-biotin–alkaline phosphatase method (Vector Laboratories and Boehringer Mannheim GmbH), using a rat monoclonal antibody to mouse macrophages (MOMA-2; Accurate Chem Sci Corp) diluted in PBS (1:30), was used. For immunostaining of smooth muscle cells, biotinylated mouse anti-human smooth muscle α-actin (Sigma Chemical Co) was used as primary antibody, followed by a FITC-conjugated secondary antibody. Antibody reactivity was detected with the Vectastain system (ABC Elite kit, Vector Laboratories, Inc) and developed with diaminobenzidine tetrahydrochloride (DAB) as substrate. Experiments in which equal amounts of nonimmune IgG were used revealed no immunostaining (data not shown).

**Statistical Analysis**

Results were expressed as mean±SEM. Total plasma cholesterol, triglycerides, serum TxB2, urinary 8,12-iso-IPF2α-VI, 2,3-dinor-TxB2, and 2,3-dinor-6-keto PGF1α and the extent of aortic atherosclerosis were analyzed by ANOVA and subsequently by Student’s unpaired t test, as indicated.

**Results**

Starting at 8 weeks of age, LDLR−/− mice were fed a high-fat diet for the entire study. Urine and blood samples were obtained at baseline and at 16 and 26 weeks of age. Body weight, total plasma cholesterol, and triglyceride levels were not different between animals at the beginning of the study (Table 1). LDLR−/− mice were randomized to receive placebo, vitamin E, or vitamin E plus indomethacin (n=14 animals for each group). The estimated average vitamin E intake for each mouse was ~8 IU/d. Assuming that each mouse drinks 3 to 4 mL water/d, the estimated daily intake of indomethacin was calculated to be ~10 to 20 ng. At the end of the study, i.e., at 26 weeks of age, in LDLR−/− mice on placebo there was a significant increase in plasma cholesterol, triglyceride levels, and body weight (Table 1). Excretion of 8,12-iso-IPF2α-VI, a predominant F2-IP in urine, was also increased in atherosclerotic lesions with oil red O. Images were captured digitally with a Dage-MTI 3CCD 3-chip color video camera connected to a Leica MZ12 dissection microscope, as previously described. Briefly, alternate 10-µm frozen sections of the aortic root covering 300 µm of the proximal aorta, starting at the sinus, were fixed in aceton, rehydrated, and stained for atherosclerotic lesions with oil red O. Images were captured digitally with a video camera connected to a Leica microscope and analyzed by computerized image analysis (Image Pro Plus, Media Cybernetics). The acquisition of images and analysis of lesions was always performed in a blinded fashion.

**TABLE 1. Body Weight, Plasma Cholesterol, Triglycerides, Vitamin E, Serum TxB2 levels, and Arachidonic Acid–Induced Platelet Aggregation in LDLR−/− Mice**

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Vitamin E</th>
<th>Indomethacin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight, g</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Base</td>
<td>20±1.5</td>
<td>21±2.0</td>
<td>22±2.0</td>
</tr>
<tr>
<td>Final</td>
<td>37.5±3*</td>
<td>36.8±2.5*</td>
<td>38.5±2.1*</td>
</tr>
<tr>
<td><strong>Cholesterol, mg/dL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Base</td>
<td>190±20</td>
<td>200±16</td>
<td>205±16</td>
</tr>
<tr>
<td>Final</td>
<td>1750±40*</td>
<td>1700±55*</td>
<td>1850±50*</td>
</tr>
<tr>
<td><strong>Triglycerides, mg/dL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Base</td>
<td>85±12</td>
<td>95±10</td>
<td>95±10</td>
</tr>
<tr>
<td>Final</td>
<td>800±45*</td>
<td>830±55*</td>
<td>850±55*</td>
</tr>
<tr>
<td><strong>Vitamin E, µmol/L</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Base</td>
<td>22±5</td>
<td>19±4.1</td>
<td>24±5</td>
</tr>
<tr>
<td>Final</td>
<td>20±4</td>
<td>91±8.5*</td>
<td>104±11*</td>
</tr>
<tr>
<td><strong>TxB2, ng/mL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Base</td>
<td>180±15</td>
<td>192±20</td>
<td>198±17</td>
</tr>
<tr>
<td>Final</td>
<td>195±10</td>
<td>13±2*</td>
<td>10±2.4*</td>
</tr>
</tbody>
</table>

**Platelet aggregation, LT%**

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Vitamin E</th>
<th>Indomethacin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base</td>
<td>80±15</td>
<td>82±4</td>
<td>8±2</td>
</tr>
</tbody>
</table>

Base indicates mice at 8 weeks of age; Final, after 18 weeks on high-fat diet; and LT, light transmission. At baseline, mice were randomized to receive placebo, vitamin E, or vitamin E plus indomethacin (n=14 animals for each group). Results are expressed as mean±SEM. *P<0.001 vs base or placebo.
sclerotic mice. This increment was already significant after 8 weeks on the high-fat diet (1.5±0.2 versus 3.3±0.2 ng/mg creatinine; P<0.001) and was further increased by the end of the study, ie, 26 weeks of age (Figure 1). A similar pattern was observed for urinary 2,3-dinor TxB₂, the major murine Tx metabolite,¹² and 2,3-dinor-6-keto PGF₁α, the prostaclin metabolite¹² (Figures 2 and 3). Thus, in LDLR⁻/⁻ mice, both lipid peroxidation and platelet activation increase with age as plasma cholesterol levels rise and atherosclerosis evolves. The elevation in 8,12-iso-IPF₂–VI and 2,3-dinor TxB₂ levels in relatively young LDLR⁻/⁻ mice (16 weeks old) is consistent with the hypothesis that in vivo, augmented lipid peroxidation and platelet activation antedate the presumed development of overt atherosclerosis.

A second group of animals was randomized to a high-fat diet supplemented with vitamin E. Compliance with vitamin E was evident from the rise in plasma levels by the end of the study (Table 1). Elevation of vitamin E was also evident when the values were normalized for cholesterol (data not shown). The administration of vitamin E did not affect the increase in body weight, plasma cholesterol, and triglycerides as the mice aged (Table 1). Furthermore, there was a corresponding reduction of 2,3-dinor-6-keto PGF₁α excretion by ≈60% (Figure 3). Indomethacin did not modify the suppressive effect of vitamin E on 8,12-iso-IPF₂–VI levels (Figure 1). Finally, a further reduction in circulating levels of sICAM-1 and MCP-1 was observed after indomethacin administration (Table 2). Because we did not find a correlation between these levels and 2,3-dinor TxB₂, it is plausible that this effect is not related to the suppression of Tx but rather to other anti-inflammatory properties of indomethacin, such as activation of peroxisome proliferator-activated receptors.²⁴

Mice were euthanized at the end of the study, and their aortas were analyzed for the extent of atherosclerosis. The atherosclerotic lesion area was first quantified by the en method. Extensive atherosclerotic lesions were observed throughout the aorta in untreated LDLR⁻/⁻ mice (Figure 4). Vitamin E reduced the lesion area by 65%. This reduction was inversely correlated with plasma levels of vitamin E (not

<table>
<thead>
<tr>
<th>TABLE 2. Circulating Levels of sICAM-1 and MCP-1 in LDLR⁻/⁻ Mice on a High-Fat Diet Receiving Placebo, Vitamin E, or Vitamin E Plus Indomethacin for 16 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
</tr>
<tr>
<td>sICAM-1, ng/mL</td>
</tr>
<tr>
<td>MCP-1, ng/mL</td>
</tr>
</tbody>
</table>

n=14 animals for each group. Results are expressed as mean±SEM. *P<0.02 vs placebo; †P<0.01 vs vitamin E alone.
shown). Coincident administration of indomethacin plus vitamin E further depressed lesion area by 80%, a decrement that was significantly greater than the one observed with vitamin E alone (Figures 4 and 5). Quantification of the atherosclerotic lesion area was also performed by section analysis of the proximal aorta in the first 300 μm of this region, starting at the aortic sinus and evaluating 10 alternate 10-μm sections. Consistent with the en face data in the aorta, supplementation of the diet with vitamin E resulted in a significant reduction in lesion size compared with the control group (155 667±12 754 vs 289 687±21 341 μm²/sec, P<0.001). Combined treatment with indomethacin and vitamin E led to an even further reduction of the atherosclerotic lesion area in the aortic sinus sections (110 830±16 480 μm²/sec, P<0.0001). Aortic root cross sections from all groups were immunostained with MOMA 2 and anti–human smooth muscle α-actin (Figure 6). Lesions from all mice studied consisted mainly of foamy macrophages, which stained positive for MOMA-2 and matched the lesion areas identified by staining with oil red O. Quantitative computer-assisted image analysis of immunostained serial cross sections of aortic root, however, revealed a significant reduction of macrophage-derived foam cells in the lesions of vitamin E–treated mice compared with the placebo group. This number was further reduced when sections from the group that received the combination treatment were analyzed (Figure 6). Staining of cells in these lesion areas was always negative for smooth muscle α-actin (Figure 6).

**Discussion**

The present studies demonstrate that LDLR−/− mice on a high-fat diet develop increased lipid peroxidation, as reflected by iP formation; marked platelet activation, as reflected by Tx biosynthesis; and systemic signs of inflammation, as shown by circulating levels of sICAM-1 and MCP-1. All of these developments coincide with the appearance of atherosclerotic lesions in the aorta.

We showed previously that iP levels are increased in a distinct model of atherogenesis, the apoE−/− mouse, and that inhibition of its generation by vitamin E coincided with a reduction of atherosclerosis.6 We now confirm and extend this observation in the LDLR−/− mice on a high-fat diet. In the present study, we show that lipid peroxidation is increased at a very early stage of the disease process and that an antioxidant regimen of vitamin E retards atherogenesis, consistent with the functional importance of oxidant stress in atherogenesis in this model as well. Supplementation with vitamin E has yielded conflicting results in human atherosclerosis, however, in which treatment typically would start after the disease is established.25 By contrast, in our studies, vitamin E was always administered at the very early stages of atherosclerosis.

LDLR−/− mice on a high-fat diet also have increased sICAM-1 and MCP-1, consistent with the hypothesis that this is a complex inflammatory disease.26 As in human hypercholesterolemia, we found that both cytokines are increased in atherosclerotic LDLR−/− mice. It is known that both molecules play a central role in the recruitment of monocyte/macrophages to atherosclerotic plaques, a step considered to be of pivotal importance in the development of atherosclerosis.27 Interestingly, expression and synthesis of these mediators is regulated by redox-sensitive mechanism(s),28,29 which may explain their discordant relationship with 8,12-iso-iPF2α-VI and 2,3-dinor TxB2 and their inverse correlation with plasma levels of vitamin E. In our study, a dosage of vitamin E that did not influence lipid levels reduced the extension of

![Figure 4. Aortic lesion areas of atherosclerotic LDLR−/− mice by en face preparation. Photomicrographs of representative Sudan IV–stained aortas of LDLR−/− mice on a high-fat diet receiving placebo, vitamin E, or vitamin E plus indomethacin at 26 weeks of age.](http://circ.ahajournals.org/Download)

![Figure 5. Percentage of total aortic atherosclerotic lesion areas in LDLR−/− mice on high-fat diet receiving placebo, vitamin E, or vitamin E plus indomethacin at 26 weeks of age. *P<0.001 vs placebo; **P<0.05 vs vitamin E alone.](http://circ.ahajournals.org/Download)
atherosclerosis, the monocyte-macrophage component of the aortic lesions, and circulating levels of these molecules.

Adding indomethacin to vitamin E further delayed atherogenesis. Although the efficacy of platelet-inhibitory drugs in the secondary prevention of cardiovascular diseases has been established,7 little information is available on the impact of Tx inhibition on atherogenesis. In general, most of the studies did not address the possibility that platelets activated in the circulation might release products that modulate lesion progression. Among them, Tx is the major product of COX-1 and is an amplifying signal for activation by more potent primary platelet agonists, such as thrombin.30 Although serum Tx production reflects the platelet maximal capacity to produce this compound, quantitative analyses of urinary metabolites, such as 2,3-dinor TxB2, have been developed and widely used as a sensitive and specific approach to the noninvasive assessment of total Tx biosynthesis in vivo.12,15

Recently, we showed that reduction of Tx biosynthesis into a functionally important range (95%) retarded atherogenesis in LDLR+/− mouse.13 consistent with the effects of a Tx receptor (TP) antagonist in apoE−/− mice.31

In addition to the conventional Tx and prostaglandin endoperoxide ligands, iPs may act as incidental ligands for the TP.11,32 They also exert a wide range of biological effects in vitro, including platelet activation, vasoconstriction, mitogenesis, adhesive interactions, and induction of tissue factor expression,33 all of which may have relevance to atherogenesis. In the present study, we demonstrated that despite suppression of 8,12-iso-iPF2α-VI, vitamin E had no effect on Tx generation in the LDLR−/− atherosclerotic mouse. Our findings suggest that lipid peroxidation products, including iPs, contribute minimally, if at all, to platelet activation during atherogenesis in this model and raise the possibility that vitamin E might have influenced atherogenesis by other mechanisms related to its antioxidant effect, such as inflammation. Thus, we observed that vitamin E further suppressed both atherogenesis and circulating inflammatory cytokine levels when combined with indomethacin. In summary, these observations suggest that oxidant stress and platelet activation represent distinct therapeutic targets in atherogenesis. A combination of antioxidants and platelet inhibitors might rationally be evaluated in the prevention of plaque progression in human atherosclerosis.

Acknowledgments

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ian Heart Association (03021N). Dr FitzGerald is the Robinette Foundation Professor of Cardiovascular Medicine.

References

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