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Cyclooxygenase-1 Deficiency in Bone Marrow Cells Increases Early Atherosclerosis in Apolipoprotein E– and Low-Density Lipoprotein Receptor–Null Mice

Vladimir R. Babaev, PhD; Lei Ding, BA; Jeff Reese, MD; Jason D. Morrow, MD; Matthew D. Breyer, MD; Sudhansu K. Dey, PhD; Sergio Fazio, MD, PhD; MacRae F. Linton, MD

**Background**—Cyclooxygenase-1 (COX-1) has been implicated in the pathogenesis of atherothrombosis and is expressed by the major cell types of atherosclerotic lesions. COX-1–mediated platelet thromboxane (TX) production has been proposed to promote both early atherosclerosis and thrombosis. Here, we examined the impact of COX-1 deficiency in bone marrow–derived cells on early atherogenesis in the mouse.

**Methods and Results**—LDL receptor (LDLR)−/− and apolipoprotein E (apoE)−/− recipient mice were lethally irradiated and transplanted with COX-1−/− bone marrow. Mice reconstituted with COX-1−/− marrow had nearly complete (99.7%) loss of platelet TXA2 and significantly suppressed levels of macrophage and urinary TXA2 metabolites. Serum lipid levels and lipoprotein distributions did not differ between recipients reconstituted with COX-1+/+ and COX-1−/− marrow. Surprisingly, the extent of atherosclerotic lesions in both LDLR−/− and apoE−/− mice reconstituted with COX-1−/− marrow was increased significantly compared with control mice transplanted with COX-1+/+ marrow. Peritoneal macrophages isolated from LDLR−/− mice reconstituted with COX-1−/− marrow had increased lipopolysaccharide-induced levels of COX-2 mRNA and protein expression. Fetal liver cell transplantation studies revealed a 30% increase in atherosclerosis in COX-1−/−→LDLR−/− mice compared with COX-1+/+→LDLR−/− mice, whereas the extent of atherosclerosis was unchanged in COX-1−/−/COX-2−/−→LDLR−/− mice.

**Conclusions**—COX-1 deficiency in bone marrow–derived cells worsens early atherosclerosis in apoE−/− and LDLR−/− mice despite virtual elimination of platelet TX production. These data demonstrate that platelet TX production does not aggravate early atherosclerotic lesion formation and that upregulation of COX-2 expression in COX-1−/− macrophages is proatherogenic. (*Circulation*. 2006;113:108-117.)

**Key Words:** platelets ■ macrophages ■ cyclooxygenase ■ prostaglandins ■ thromboxane

Eicosanoids are lipid-derived autacoids implicated in a large number of pathological processes, including inflammation and atherosclerosis. Prostaglandins are generated by cyclooxygenase (COX) through conversion of arachidonic acid to prostaglandin H2, which is further catalyzed by the distinct synthases to 5 major bioactive prostaglandins (e.g., PGE2, PGF2α, PGD2, and TXA2). COX exists as 2 isoforms, COX-1 and COX-2, encoded by 2 separate genes.1,2 COX-1 is constitutively expressed in every major cell type involved in the pathogenesis of atherothrombosis, including vascular cells, macrophages, and platelets. In contrast, COX-2 is induced rapidly at sites of inflammation and is expressed in atherosclerotic lesions.3,4 TXA2 is a potent stimulus of platelet aggregation, vasoconstriction, and vascular proliferation.5 Platelet TXA2 production, a COX-1–mediated process, is viewed as promoting atherothrombosis. In contrast, prostacyclin (PGI2) is a potent vasodilator and platelet inhibitor and is viewed as being antiatherothrombotic.5 The balance between TXA2 and PGI2 has been proposed to be an important determinant in the pathogenesis of atherosclerosis and thrombosis resulting in cardiovascular events.

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Nonselective inhibition of COX has been reported to reduce atherosclerosis both in cholesterol-fed rabbits6 and in murine models of atherosclerosis.7,8 COX-1 has been proposed to promote the development of atherosclerosis in LDL receptor (LDLR)−/− mice.8 On the basis of studies with a COX-1 inhibitor, Belton et al9 have implicated COX-1–mediated platelet thromboxane (TX) A2 generation in promoting the early stages of atherosclerotic lesion formation. In
humans, a low dose of aspirin (100 mg/d) significantly decreases the relative risk and total frequency of cardiovascular events. The beneficial effects of aspirin in reducing cardiovascular events have been largely attributed to inhibition of COX-1–mediated platelet TXA2 production.11,12

Genetic deficiency in the TXA2 receptor suppresses vascular proliferation and platelet activation in an arterial injury–induced model of neointimal hyperplasia in mice and inhibits atherosclerosis in apolipoprotein E (apoE)–/– mice.13 Similarly, the administration of the TXA2 antagonist S-18886 suppresses the development of atherosclerotic lesions in apoE–/– mice.15,16 However, aspirin alone was more effective in TX inhibition, with only slight effects on the development of atherosclerosis, compared with S-18886, which suggests that a mechanism independent of platelet-derived TX is responsible for suppression of the development of atherosclerosis.15 Because TX is abundantly expressed by platelets, macrophages, endothelial cells, and smooth muscle cells, the impact of TX production by different vascular cells on atherogenesis may vary depending on the stage of atherosclerotic lesion formation.

To examine the impact of COX-1 gene expression by bone marrow–derived cells on the development of atherosclerosis, LDLR+/– and apoE–/– mice were lethally irradiated and transplanted with COX-1–/– or COX-1+/+ marrow. In recipient mice reconstituted with COX-1–/– bone marrow–derived cells, platelet TXA2 production was virtually eliminated, and production of urinary and macrophage TXA2 metabolites was significantly reduced. Surprisingly, mice reconstituted with COX-1–/– bone marrow had increased atherosclerotic lesions in the absence of significant differences in serum lipids. These data demonstrate that COX-1–mediated platelet TXA2 production is not a major driving force in early atherosclerotic lesion formation.

**Methods**

**Animal Procedures**

COX-1 null mice17 were crossed onto the C57BL/6 background (sixth backcross) and mated with apoE–/– mice on the C57BL/6 background (from Jackson Laboratories Inc, Bar Harbor, Me) to obtain COX-1+/–, apoE–/– bone marrow donors. All recipient LDLR+/– and apoE–/– mice were on the C57BL/6 background and maintained in microisolator cages on a rodent chow diet containing 4.5% fat (PMI No. 5010). Animal care and experimental procedures were performed according to the regulations of Vanderbilt University’s Animal Care Committee.

**Bone Marrow Transplantation**

Recipient LDLR+/– and apoE–/– mice were lethally irradiated (9 Gy) from a cesium gamma source and transplanted with 5X10^6 bone marrow cells as described previously.18

**Serum Lipids and Lipoprotein Distribution Analysis**

The serum total cholesterol and triglyceride levels were determined on samples obtained from mice fasted for 4 hours as described previously.19 Fast performance liquid chromatography was performed on an HPLC system model 600 (Waters) with a Superox 6 column (Pharmacia).

**Platelet and Urinary Prostaglandin Metabolites**

To measure TXA2 metabolite levels, blood samples, peritoneal macrophages, or freshly isolated bone marrow cells were incubated in DMEM media containing 0.5% BSA, 5 μmol/L A23187 Ca2+ ionophore (Calbiochem), and 2 μmol/L arachidonic acid at 37°C for 30 or 120 minutes, respectively. Twenty-four-hour urine samples were purified by solid-phase extraction. Prostaglandins and prostaglandin metabolites were measured by gas chromatography/mass spectrometry with SCIEX API III+ triple quadrupole (Applied Biosystems) as described previously.20

**Flow Cytometric Analysis**

Blood cells were blocked with anti-FcγRIII/III antibodies, incubated with anti-mouse CD3, CD4, and CD8 antibodies labeled with allophycocyanin, fluorescein isothiocyanate, and phycoerythrin, respectively (BD Pharmingen, La Jolla, Calif), and 7AAD (7-aminoactinomycin D). Cells were then fixed and analyzed by 4-color flow cytometry with the Becton Dickinson fluorescent-activated cell sorter (Becton Dickinson).

**Immunocytochemistry**

Serial 5-μm cryosections of the proximal aorta were incubated with monoclonal rat antibodies to mouse glycoprotein Ib, CD41 (BD Pharmingen, San Diego, Calif) specific for platelets,23 or macrophages (MOMA-2; Accurate Chemical & Scientific Corp, Westbury, NY). The sections were treated with goat biotinylated antibodies to rat IgG (PharMingen), incubated with avidin-biotin complex labeled alkaline phosphatase (Vector Laboratories), and visualized with Fast Red TR/Naphthol AS-NX substrate (Sigma). A nonimmune rat serum was used as a negative control.

**Western Blotting**

Peritoneal macrophages were stimulated (50 ng/mL lipopolysaccharide [LPS]) for 5 hours and lysed in the presence of protease inhibitor (Roche Diagnostics). Extracted proteins were analyzed with rabbit antibodies to murine COX-2 (Cayman Chemical, Ann Arbor, Mich), β-actin (Abcam, Inc, Cambridge, Mass), and horseradish peroxidase–conjugated goat anti-rabbit antibodies (Upstate Cell Signaling, Lake Placid, NY).

**RNA Isolation and Real-Time Polymerase Chain Reaction**

Total RNA was isolated from peritoneal macrophages with Trizol reagent (Life Technologies, Inc) and purified with an RNA Easy kit (Qiagen). Relative quantification of the target mRNA was performed with the ABI Prism 7700 Sequence Detection System (Applied Biosystems) and normalized with 18S ribosomal RNA as an endogenous control. Primers and probes for 18S and COX-2 were obtained from Applied Biosystems.

**Statistical Analysis**

Statistical differences in mean serum lipids and aortic lesion areas between the groups were determined by Student t test, the Mann-Whitney rank sum test, and a 1-way ANOVA tests with SigmaStat version 2 software (SPSS Inc).

**Results**

**Generation of Chimeric Mice With COX-1–/– Bone Marrow–Derived Cells**

To evaluate the impact of COX-1 expression by bone marrow cells on atherosclerosis, 7-week-old male LDLR+/– mice were lethally irradiated and transplanted with male COX-1+/+ (n = 14, control group) or COX-1–/– (n = 17; experimental group) marrow. After 4 weeks on a
normal chow diet, they were fed a Western diet for 8 weeks. In a separate set of experiments, 8-week-old female apoE−/− mice were lethally irradiated, transplanted with COX-1−/−/apoE−/− (n=13) or COX-1−/−/apoE−/− (n=13) bone marrow cells, and fed a normal chow diet for 9 weeks. Eight weeks after transplantation, peritoneal macrophages isolated from recipient mice reconstituted with COX-1−/− marrow did not express COX-1, as demonstrated by real-time polymerase chain reaction analysis (data not shown), verifying their change in genotype.

Bone Marrow and Platelet and Urinary Eicosanoids
The impact of COX-1 deficiency on secretion of eicosanoids by freshly harvested bone marrow stimulated with the A23187 Ca2+ ionophore (5 µmol/L) was examined in vitro. Compared with bone marrow cells from wild-type mice, bone marrow cells from COX-1−/− mice had significantly lower levels of all types of prostanoids examined, including PGE2n (0.90±0.11 versus 0.30±0.02 ng/mL; P<0.001), 2,3-dinor 6-keto PGF1α (0.027±0.003 versus 0.008±0.003 ng/mL; P<0.001), PGE2 (2.78±0.21 versus 1.19±0.10 ng/mL; P<0.001), TXB2 (2.27±0.23 versus 0.13±0.01 ng/mL; P<0.001), and PGD2 (5.36±0.29 versus 2.49±0.21 ng/mL; P<0.001). Next, we evaluated the impact of elimination of COX-1 expression in bone marrow–derived cells on Ca2+ ionophore–stimulated platelet TXA2 production. After stimulation with 5 µmol/L A23187, production of platelet TXA2 metabolite TXB2 was virtually eliminated (99.7% reduction) in COX-1−/−→LDLR−/− mice compared with COX-1−/−→LDLR−/− mice (0.3±0.1 versus 139.4±15.8 ng/mL; P<0.001). The reduction in platelet TXB2 production was similar to the 99.5% reduction in platelet TXB2 production seen in COX-1−/−→LDLR−/− mice, the urinary excretions of both of the TX metabolites 11 dehydro- and 2,3-dinor-TXB2 were reduced to a similar extent, 79% and 74%, respectively (Figure 1A). In contrast, levels of the urinary prostacyclin metabolite 2,3-dinor-6-keto-PGF1α, PGE-M, and isoprostane were not altered compared with the levels of these metabolites in mice reconstituted with wild-type marrow (Figure 1B). Thus, COX-1 expression in bone marrow–derived cells is responsible for ~75% of systemic TX production.

Serum Lipids and Lipoprotein Distribution
There were no significant differences in serum lipid levels between LDLR−/− recipient mice reconstituted with COX-1−/− and COX-1−/− marrow on either the chow or the Western diets (Table 1). Similarly, serum lipid levels in COX-1−/−/apoE−/−→apoE−/− mice did not differ from control COX-1−/−/apoE−/−→apoE−/− mice with the exception that their triglycerides were higher at week 11 of the experiment (Table 2). Analysis of the serum samples by size exclusion chromatography revealed an accumulation of cholesterol in the VLDL, LDL, and IDL fractions in both LDLR−/− and apoE−/− recipients, with no differences between control and experi-

| TABLE 1. Total Serum Cholesterol and Triglyceride Levels in Male LDLR−/− Mice Reconstituted With COX-1+/+ or COX-1−/− Bone Marrow |
|----------------|----------------|----------------
| Serum Lipids | Baseline | 4 Weeks on Western Diet | 8 Weeks on Western Diet |
| COX-1−/−→LDLR−/− (n=14) | | | |
| Cholesterol | 215±7 | 607±44 | 671±41 |
| Triglycerides | 108±5 | 220±17 | 210±19 |
| COX-1−/−→LDLR−/− (n=17) | | | |
| Cholesterol | 227±8 | 667±49 | 748±19 |
| Triglycerides | 98±6 | 190±18 | 231±14 |

Values are in mg/dL (mean±SEM). The number of animals in each group is indicated by n. Differences were not significant between groups at any time point.
ment groups in either experiment (Figures 2A and 2B). These data indicate that COX-1 deficiency in marrow-derived cells does not affect plasma lipids in either LDLR−/− or apoE−/− recipient mice.

Quantification of Atherosclerotic Lesions
The extent of atherosclerotic lesions in the proximal aortas of LDLR−/− recipients reconstituted with COX-1−/− marrow cells was significantly greater than for COX-1+/−→LDLR−/− (124 196±27 631 versus 61 566±1610 μm²; P<0.03, Figure 3A). There was a statistically nonsignificant trend for increased atherosclerosis in the distal aortas of COX-1−/−→LDLR−/− mice compared with control mice (Figure 3B). Similarly, COX-1+/−/apoE−/−→apoE−/− mice had larger atherosclerotic lesions in their proximal aortas (39 463±3090 versus 28 352±2791 μm²; P<0.02) and their distal aortas (0.20±0.01% versus 0.18±0.01%; P<0.05) analyzed en face (Figures 4C and 4D) than control COX-1+/−/apoE−/−→apoE−/− mice. Thus, COX-1 deficiency in bone marrow–derived cells accelerates development of atherosclerotic lesions.

Glycoprotein Ibα Staining in Atherosclerotic Lesions
All groups of recipient mice developed moderate fatty streak lesions, consisting predominantly of macrophage-derived foam cells stained by the anti-macrophage antibody MOMA-2 (Figures 4A and 4B). Serial sections from the proximal aorta of apoE−/− recipients were stained with the antibody to platelet glycoprotein Ibα, CD41. Interestingly, the atherosclerotic lesions of the COX-1+/−/apoE−/−→apoE−/− control mice appeared to contain substantially more platelets, as indicated by CD41 staining, than COX-1+/−/apoE−/−→apoE−/− mice (Figures 4C and 4D). Quantitative image analysis of these sections demonstrated that the COX-1+/−/apoE−/−→apoE−/− mice had a 2-fold greater percentage of their lesion area staining for CD41 than apoE−/− mice reconstituted with COX-1−/− marrow (Figure 4F).

CD4/CD8 T-Cell Phenotype
Because COX-1 has been reported to impact T-cell development,24 we studied whether the impact of COX-1 deficiency affected the proportion of CD4 and CD8 peripheral blood T-cell subsets analyzed by 4-color staining flow cytometry. In COX-1 null control mice, there was a significant decrease in CD8 and an increase in CD4 T-cells compared with wild-type mice (38.6±1.1% and 57.4±1.5% versus 44.0±1.0% and 50.5±1.5%; P=0.016 and P=0.023, respectively). In contrast, LDLR−/− recipients reconstituted with COX-1−/− marrow had no significant differences in the number or the proportion of CD8+ and CD4+ cells compared with mice transplanted with COX-1+/− marrow (41.9±3.4% and 54.3±3.4% versus 41±1.1% and 55.5±0.9%; P=0.82 and P=0.75, respectively).

Macrophage Prostaglandins and COX-2 Expression
To analyze how COX-1 deficiency affects macrophage prostanoid production, peritoneal macrophages were isolated from LDLR−/− recipients reconstituted with COX-1+/− and COX-1−/− marrow. In response to A23187 5 μmol/L, peritoneal macrophages derived from COX-1+/−→LDLR−/− mice maintained similar levels of PGF2α, PGE2, and PGD2 production, whereas synthesis of PGI2 and TXA2 metabolites was reduced by 62% and 16%, respectively, compared with control COX-1+/−→LDLR−/− macrophages (Figure 5).

Previous studies have indicated that COX-2 expression may be increased in COX-1−/−-deficient cells.25 Therefore, we examined the expression of COX-2 in COX-1−/− macrophages at baseline and in response to LPS. Peritoneal macrophages were isolated from LDLR−/− recipients after 8 weeks of the Western diet. The macrophages isolated from COX-1+/−→LDLR−/− mice had increased

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**TABLE 2. Total Serum Cholesterol and Triglyceride Levels in Female apoE−/− Mice Reconstituted With COX-1+/−/apoE−/− or COX-1−/−/apoE−/− Bone Marrow**

<table>
<thead>
<tr>
<th>Serum Lipids</th>
<th>Baseline, 7 Weeks</th>
<th>Chow Diet, 11 Weeks</th>
<th>Chow Diet, 16 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-1+/−/apoE−/−→apoE−/− (n=13)</td>
<td>371±11</td>
<td>380±12</td>
<td>360±19</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Triglycerides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COX-1−/−/apoE−/−→apoE−/− (n=13)</td>
<td>363±10</td>
<td>397±16</td>
<td>389±18</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Triglycerides</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are in mg/dL (mean±SEM).

*P<0.05 compared with control COX1+/−/apoE−/−→apoE−/− group at this time point.

Figure 2. Lipoprotein distribution in LDLR−/− recipient mice reconstituted with COX-1+/− (●) or COX-1−/− (○) bone marrow (A) and apoE−/− recipients reconstituted with COX-1+/−/apoE−/− (●) or COX-1−/−/apoE−/− (○) marrow (B). Data are represented as an average (n=3) of the percent of total cholesterol per fraction. Fractions 14 to 17 contain VLDL; fractions 18 to 24 are IDL/LDL; and fractions 25 to 30 contain HDL. Fractions 31 to 40 include nonlipoprotein-associated proteins.
basal and LPS-stimulated levels of COX-2 expression as analyzed by real-time quantitative polymerase chain reaction analysis (Figure 6A) compared with macrophages isolated from COX-1+/−→LDLR−/− mice. To verify these findings, peritoneal macrophages were isolated from COX-1−/− and wild-type mice and incubated with LPS. Again, COX-1−/− macrophages responded to LPS activation with significantly higher (195%) levels of COX-2 gene activation than wild-type macrophages (Figure 6B). Liver X receptor-α (LXRα) agonist, TO-901317, to downregulate COX-2 expression in COX-1-deficient macrophages. The LXRα agonist significantly reduced COX-2 gene expression by 45% and 40% in COX-1−/− and wild-type macrophages, respectively (Figure 6B). In addition, COX-2 protein expression levels were analyzed in LPS-stimulated peritoneal macrophages from COX-1−/− and wild-type mice. Macrophages from COX-1−/− mice had significantly increased (18%) levels of COX-2 protein expression compared with macrophages from wild-type mice (Figures 6C and 6D).

**Generation of LDLR−/− Mice Null for Expression of Both COX-1 and COX-2 Genes in Bone Marrow–Derived Cells**

We have previously reported that elimination of COX-2 expression in bone marrow–derived cells reduces atherosclerosis in LDLR−/− and C57BL/6 mice, which supports a proatherogenic role for macrophage COX-2 in early atherogenesis. To further examine the hypothesis that compensatory upregulation of COX-2 in macrophages may have contributed to the increased atherosclerosis in COX-1+/−→LDLR−/− mice, we generated LDLR−/− mice with bone marrow–derived cells null for both COX-1 and COX-2. Because the double knockout of both COX-1 and COX-2 genes is lethal in mice soon after birth, we used the approach of fetal liver cell (FLC) transplantation. Briefly, 8-week-old female LDLR−/− mice were lethally irradiated and transplanted with female COX-1−/− (n=20), COX-1−/− (n=13), or COX-1−/−/COX-2−/− (n=13) FLCs. After 6 weeks on a chow diet, the recipient mice were fed the Western diet for 8 weeks. There were no differences in plasma lipid levels between these groups of mice (Table 3); however, mice transplanted with COX-1−/− FLCs had bigger (30%) atherosclerotic lesions in the distal aortas analyzed en face (0.26±0.01) than control mice reconstituted with COX-1+/− (0.20±0.02; P<0.05) or mice with COX-1−/−/COX-2−/− (0.20±0.02) FLCs (Figure 7). Thus, deletion of both COX-1−/−/COX-2−/− in bone marrow–derived cells eliminated the increase in atherosclerosis seen in COX-1+/−→LDLR−/− mice, which supports the hypothesis that the observed increase in macrophage COX-2 expression contributed to the increase in atherosclerosis in the COX-1−/−→LDLR−/− mice.

**Discussion**

COX-1 is expressed by all cell types involved in the pathogenesis of atherothrombosis, including endothelial cells, vascular smooth muscle cells, macrophages, and platelets. COX-1 has been reported to promote atheroscle-
rosis in murine models, and COX-1–mediated platelet TXA₂ production has been proposed to be responsible for the proatherogenic effects of COX-1 in early lesion formation. However, the relative impact of COX-1 expression by vascular cells and cells of hematopoietic origin on atherogenesis remains unclear. Therefore, we examined the impact of COX-1 expression by bone marrow–derived cells on early atherosclerotic lesion formation by generating both LDLR⁻/⁻/⁻/⁻ and apoE⁻/⁻/⁻/⁻ mice chimeric for COX-1 expression by bone marrow–derived cells. Platelet production of TXA₂ was virtually eliminated and the urinary...
TABLE 3. Total Serum Cholesterol and Triglyceride Levels in Female LDLR−/− Mice Reconstituted With Wild-Type, COX-1−/−, or COX-1−/−/COX-2−/− FLCs

<table>
<thead>
<tr>
<th>Serum Lipids</th>
<th>Baseline, 6 Weeks</th>
<th>4 Weeks on Western Diet</th>
<th>8 Weeks on Western Diet</th>
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<tr>
<td></td>
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<td></td>
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<tr>
<td>COX-1−/−/COX-2−/− → LDLR−/− (n=20)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>216±3</td>
<td>763±19</td>
<td>753±35</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>72±3</td>
<td>178±11</td>
<td>296±18</td>
</tr>
<tr>
<td>COX-1−/− → LDLR−/− (n=13)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>218±6</td>
<td>761±36</td>
<td>743±44</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>71±2</td>
<td>172±17</td>
<td>279±24</td>
</tr>
<tr>
<td>COX-1−/−/COX-2−/− → LDLR−/− (n=13)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>214±5</td>
<td>752±37</td>
<td>734±50</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>75±5</td>
<td>180±20</td>
<td>289±27</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM in mg/dL. The number of animals in each group is indicated by n. The differences are not significant between the groups at any time point.

TXA2 metabolites were dramatically reduced in the mice reconstituted with COX-1−/− bone marrow. Surprisingly, both LDLR−/− and apoE−/− recipient mice reconstituted with COX-1−/− bone marrow cells had significantly increased atherosclerotic lesion area compared with control mice transplanted with COX-1+/− marrow, in the absence of significant differences in serum lipids or lipoprotein profiles. These results demonstrate that COX-1−/− mediated platelet TX production is not a significant driving force in the development of early atherosclerotic lesion formation in LDLR−/− or apoE−/− mice. Furthermore, the net effect of the loss of COX-1−/− mediated eicosanoid production from bone marrow-derived cells on early lesion formation is proatherogenic. Hence, any possible beneficial effects of the loss of platelet TX in early atherosclerosis may be outweighed either by the loss of production of antiatherogenic COX-1−/− mediated eicosanoids from bone marrow-derived cells, production of proatherogenic COX-1−/− mediated eicosanoids by vascular cells, or a proatherogenic effect that is mediated by compensatory upregulation of COX-2 expression in macrophages. We provide strong support for a proatherogenic role of COX-2 expression by demonstrating that elimination of both COX-1 and COX-2 in bone marrow-derived cells of LDLR-deficient mice eliminates the increase in atherosclerosis attributable to deficiency of COX-1 in bone marrow-derived cells (Figure 7).

In the present studies, elimination of COX-1 expression in bone marrow-derived cells in apoE−/− and LDLR−/− mice resulted in virtually complete elimination of platelet TX. This proves that platelet TXA2 production is entirely COX-1−/− mediated and that the recipient mice were completely reconstituted with donor bone marrow. Urinary TX excretion was reduced by 74% to 79% in the mice reconstituted with COX-1−/− bone marrow (Figure 1), which indicates that platelets and bone marrow-derived cells are responsible for the vast majority of urinary TXB2. Given the rather small impact of COX-1 deficiency on macrophage TXB2 production (Figure 5), it is likely that platelets are responsible for the production of the majority of systemic TX production, as reflected by urinary TXB2. In contrast, urinary F2 isoprostanes, the prostacyclin metabolite 2,3-dinor 6-keto PGF1α, and PGE-M did not differ between control and experimental mice (Figure 1), which indicates that COX-1 expression in bone marrow-derived cells is not a significant contributor to the systemic production of F2 isoprostanes or prostacyclin. Furthermore, COX-1 expression in bone marrow-derived cells does not significantly affect plasma lipid or lipoprotein metabolism (Figure 2; Tables 1 and 2).

TX is widely held to promote atherothrombosis, because it promotes activation and aggregation of platelets and is a potent vasoconstrictor. Treatment of mice with a TX receptor antagonist and genetic elimination of the receptor significantly diminished atherogenesis. In contrast, prostacyclin inhibits platelet aggregation and is a potent vasodilator. The balance between prostacyclin and TX has been proposed to be an important determinant of both the development of atherosclerosis and thrombosis resulting in cardiovascular events. The beneficial effect of aspirin in reducing cardiovascular events is largely attributed to its ability to reduce platelet aggregation by inhibiting COX-1−/− mediated platelet TX production. Recently, the increased risk of cardiovascular events seen with COX-2 inhibitors has been attributed to a tilting of this balance, which results from reductions in PGI2 without an impact on platelet TX production. However, this paradigm has been challenged recently on the basis of studies by McAdam et al demonstrating COX-2−/− mediated production of PGI2 and nonplatelet-mediated TXA2 in smokers. The results of the present study do not address the issue of whether the balance between PGI2 and TX is a critical determinant of thrombosis and cardiovascular events. However, given our finding of increased early atherogenesis in the face of a dramatic reduction in urinary TX with no change in urinary PGI2, the present results indicate that the balance between TX and PGI2 is not a critical determinant of early atherosclerotic lesion formation.

![Figure 7](image-url)
COX-1 has been proposed to promote the development of atherosclerosis in LDLR−/− mice on the basis of the observation that the nonselective inhibitor indomethacin significantly reduced atherosclerosis, whereas the selective COX-2 inhibitor nimesulide produced a nonsignificant trend for a reduction in lesion area. Belton et al have implicated COX-1 in promoting the early stages of lesion formation, because treatment of apoE−/− mice fed a 1% cholesterol diet with the selective COX-1 inhibitor SC-560 for 8 weeks dramatically reduced atherosclerotic lesion formation, whereas SC560 did not induce regression or inhibit progression of established lesions. Although SC-560 did not prevent platelet adhesion in these studies, Belton et al suggested that the most likely explanation for the reduction in atherosclerosis was inhibition of platelet activation due to inhibition of COX-1–mediated platelet TXA2 generation. Consistent with the results of Belton et al, we have found that treatment of male LDLR−/− mice fed a Western diet with the COX-1 inhibitor SC-560 caused dramatic reductions both in urinary TX and in the extent of atherosclerosis compared with vehicle-treated mice (M.F.L., unpublished results). However, the present results demonstrate that platelet TX production is not a dominant driver of early atherosclerotic lesion formation, because elimination of COX-1 in bone marrow–derived cells results in increased atherosclerosis in 2 murine models (Figure 3) despite complete elimination of platelet TXA2 production. Therefore, the ability of SC-560 to reduce early atherosclerotic lesion formation is likely to be independent of its effect on inhibition of platelet TXA2 production. Interestingly, Cayatte et al have proposed that TXA2 receptor (TP) agonists other than TX, such as F2 isoprostanes and HETE, are responsible for TP-mediated promotion of atherosclerosis, on the basis of a finding that a TX receptor antagonist reduced atherosclerosis in apoE-deficient mice, whereas aspirin, which completely inhibited platelet TX production, did not affect the extent of atherosclerosis. Given that levels of F2 isoprostanes were not influenced by the elimination of COX-1 in bone marrow–derived cells (Figure 1), the possibility that a difference in this alternative TP agonist may have contributed to TP-mediated promotion of atherosclerosis in the present studies seems unlikely.

Our results demonstrating increased atherosclerosis in apoE−/− and LDLR−/− mice reconstituted with COX-1−/− bone marrow suggest that COX-1 expression by bone marrow cells is on balance antiatherogenic. Given the key role of monocyte recruitment and macrophage foam cell formation in early lesion formation, we examined the impact of COX-1 deficiency on peritoneal macrophage eicosanoid production after stimulation with a calcium ionophore. Interestingly, COX-1−/− macrophage production of PG12 was reduced dramatically (62%), whereas TXB2 production was only slightly reduced (16%) and the levels of PGF2α, PGE2, and PGD2 were unchanged compared with COX-1+/+ macrophages (Figure 5). PG12 exhibits protective properties by inhibiting platelet aggregation and smooth muscle cell proliferation.13 Macrophage PG12 reduces cholesterol uptake and foam cell formation but does not interfere with cholesterol export.33 In addition, PGI2 analogues dose-dependently inhibited production of inflammatory cytokines (tumor necrosis factor-α, interleukin-1β, and granulocyte-macrophage colony–stimulating factor) by human alveolar macrophages.34,35 Thus, the suppression of PGI2 production in COX-1−/− macrophages in the present studies may accelerate the development of atherosclerosis by promoting foam cell formation and the production of inflammatory cytokines. COX-1 deficiency has been reported to impact T-cell development; however, we did not see a change in CD4/CD8 phenotype in T cells from mice reconstituted with COX-1−/− compared with COX-1+/+ bone marrow. Therefore, an impact of COX-1 expression on atherosclerosis mediated by T cells seems unlikely. A significant contribution of donor-derived COX-1−/− endothelial cells or smooth muscle cells to the development of atherosclerosis is unlikely in the present studies, because we have previously found that the contribution of bone marrow–derived endothelial cells and smooth muscle cells to atherosclerotic lesions is minimal (<2%) under similar conditions of early lesion development.

Compensatory upregulation of the alternate COX isoform has been reported in macrophages isolated from COX-1−/− and COX-2−/− deficient mice.28 COX-2 compensation also occurs in the absence of COX-1 in a uterine preparation for implantation.37 In the present studies, peritoneal macrophages isolated from mice reconstituted with COX-1−/− marrow expressed significantly higher levels of basal and LPS-mediated COX-2. We have previously reported results of pharmacological and genetic studies that support a proatherogenic role for macrophage COX-2 expression in early atherosclerotic lesion formation.7,38 Hence, a compensatory increase in COX-2 expression in COX-1−/− macrophages may have contributed to the increase in atherosclerosis. Recent reports demonstrated that activation of the LXRα pathway inhibits LPS- and cytokine-induced macrophage expression of a number of inflammatory genes, including COX-2.28 To test whether this LXRα pathway for regulation of COX-2 expression was operative in COX-1 null cells, we incubated macrophages with LPS alone or with the LXRα agonist TO-901317. COX-1−/− macrophages responded with a significantly higher activation of COX-2 gene, but the LXRα agonist efficiently suppressed this upregulated COX-2 gene expression, which demonstrates that this LXRα-mediated regulatory pathway is intact in COX-1 null cells (Figure 6B). To examine the hypothesis that COX-2 expression by COX-1 null macrophages contributed to the increased atherosclerosis in COX-1−/−→LDLR−/− mice, we used an FLC transplantation approach to generate mice with COX-1−/−/COX-2−/− bone marrow–derived cells. Interestingly, the 30% increase in atherosclerosis seen in LDLR−/− mice reconstituted with COX-1−/− FLCs was not seen in the LDLR−/− mice transplanted with COX-1−/−/COX-2−/− FLCs (Figure 7), which provides strong support for the hypothesis that the observed upregulation of COX-2 in COX-1−/− macro-
phages contributed significantly to the observed increase in atherosclerosis.

In summary, elimination of the COX-1 gene expression from bone marrow–derived cells significantly accelerates early atherosclerotic lesion formation in both LDLR knockout and apoe-/- recipient mice, despite virtually complete elimination of platelet TX. Furthermore, we provide evidence for compensatory upregulation of COX-2 in macrophages, as well as evidence that COX-2 expression contributes significantly to the increased atherosclerosis in mice reconstituted with COX-1 null bone marrow–derived cells. These results challenge current paradigms suggesting that COX-1–mediated platelet TX production and the balance of TX and PGI2 are critical determinants of atherosclerotic lesion formation.

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Disclosures
None.

References
Cyclooxygenase-1 (COX-1) has been implicated in the pathogenesis of atherosclerosis and thrombosis. The ability of aspirin to reduce cardiovascular events is widely attributed to the inhibition of COX-1–mediated platelet thromboxane (TX). Furthermore, inhibition of COX-1 has been reported to reduce atherosclerosis in mice, and COX-1–mediated platelet TX has been proposed to promote atherosclerosis. To examine the impact of COX-1 deficiency in bone marrow–derived cells (BMDCs) on early atherogenesis, LDL receptor (LDLR)−/− and apolipoprotein E (apoE)−/− mice were lethally irradiated and transplanted with COX-1+/− marrow. Mice reconstituted with COX-1+/− marrow had nearly complete (99.7%) loss of platelet TXA2 production and no changes in urinary prostacyclin metabolites. Surprisingly, the extent of atherosclerotic lesions in both types of mice reconstituted with COX-1+/− BMDCs was significantly increased compared with control mice transplanted with COX-1+/+ marrow. Thus, COX-1 deficiency in BMDCs worsens early atherosclerosis in mice despite elimination of platelet TX, which suggests that the balance between prostacyclin and thromboxane is not a critical determinant of atherogenesis. Expression of COX-2 was increased in COX-1+/− peritoneal macrophages, which suggests that compensatory upregulation of macrophage COX-2 may underlie the increased atherosclerosis. To test this hypothesis, LDLR−/− mice were reconstituted with BMDC double-knockout (2KO) for both COX-1 and COX-2. Interestingly, reconstitution of LDLR−/− mice with 2KO BMDCs eliminated the increase in atherosclerosis seen in mice with COX-1−/− BMDCs. These data demonstrate that platelet TX production does not promote early atherogenesis and that upregulation of COX-2 expression in COX-1−/− macrophages is proatherogenic. The roles of COX-1 and COX-2 in atherosclerosis are complex and warrant further investigation.