Cyclooxygenase Isoforms and Platelet Vessel Wall Interactions in the Apolipoprotein E Knockout Mouse Model of Atherosclerosis

Orina A. Belton, PhD; Angela Duffy, PhD; Sinead Toomey, BSc; Desmond J. Fitzgerald, MD

Background—Cyclooxygenase (COX) activity is induced in human atherosclerosis, and the products formed may modify the disease directly or through an effect on platelets. We examined the role of COX-1 and -2 on platelet vessel wall interactions and development of atherosclerosis in a murine model.

Methods and Results—Apolipoprotein E–deficient (apoE−/−) mice fed a 1% cholesterol diet were treated with a selective COX-1 inhibitor (SC-560), a selective COX-2 inhibitor (SC-236), or vehicle. Urinary prostacyclin and thromboxane metabolites (2,3-dinor-6-keto-PGF1α and 2,3-dinor-TXB2) were increased in the apoE−/− knockout mouse. There was also induction of both COX isoforms in the vascular lesions formed, which stained for CD41, a platelet-specific marker, and for CD40L. Selective inhibition of COX-2 had no effect on lesion formation and, despite selective reduction in prostacyclin generation, had no effect on platelet activity, as measured by thromboxane formation or platelet deposition. Selective inhibition of COX-1 reduced 2,3-dinor-TXB2 generation and lesion formation. However, platelet deposition on the vessel wall persisted, with well-defined monolayers seen. There was also persistent expression of the macrophage marker CD68 and increased expression of the cell death protein Bax. In contrast to lesion development, the selective COX-1 inhibitor had no effect on the regression of evolving lesions.

Conclusions—COX-1 plays an important role in the early stages of lesion development in the apoE−/− knockout model of atherosclerosis, preventing gross lesion formation in the face of continued vascular injury and inflammation. Despite the inhibition of prostacyclin, COX-2 inhibition had no effect on lesion development or platelet–vessel wall interactions. (Circulation. 2003;108:3017-3023.)

Key Words: atherosclerosis • platelets • apoptosis

The generation of prostaglandins, specifically thromboxane (TXA2) and prostacyclin (PGI2), is grossly abnormal in patients with atherosclerosis.1 Prostaglandin generation is catalyzed by the enzyme cyclooxygenase (COX), and the increase in PGI2 reflects in part the induction of 2 COX isoforms, COX-1 and COX-2, in the vessel wall.2,3 On the other hand, much of the increase in TXA2 in atherosclerosis is derived from platelets4 and therefore is largely generated by COX-1, the only isoform in platelets.5 This is consistent with and indeed is evidence for enhanced platelet activity in patients with atherosclerosis.6,7 Prostaglandins may influence the development of atherosclerosis by modulating the inflammatory response,8 the expression of metalloproteinases,9 and the growth of cells implicated in the process, such as vascular smooth muscle cells.10 Alternatively, prostaglandins may influence the process indirectly by modifying platelet activity. TXA2 is a potent platelet activator, whereas PGI2 is a potent platelet inhibitor, and through their effects on platelet activity, these 2 COX products may influence the development of arterial thrombosis.11 Modulation of platelet activity may also influence the development of atherosclerosis, because platelet–vessel wall interactions contribute to the evolution of lesions. Thus, suppression of platelet adhesion attenuates the development of atherosclerosis in a murine model.12 COX-2 inhibitors selectively inhibit prostacyclin formation13,14 and consequently may enhance platelet activity. Such an effect by contributing to the development of atherosclerosis or the initial steps of arterial thrombosis may explain the increase in myocardial infarction reported in patients treated with the COX-2 inhibitor rofecoxib (VIGOR).15

In this study, we examine the contribution of COX-1 and COX-2 to the development of atherosclerosis and address whether they influence platelet function, in particular platelet–vessel wall interactions.

Methods

Animals

Homozygous apolipoprotein E–deficient (apoE−/−) mice on a C57BL/6J background (C57BL/6J-Apoem1Unc), tenth generation back-
crossed from 129/B6 F1 heterozygous to C57BL6) and wild-type littermate C57BL/6J controls were purchased from Jackson Laboratory (Bar Harbor, Maine). SC-236 (4-[5-(4-chlorophenyl)-3-(trifluoroethyl)-1H-pyrazol-1yl] benzenesulfonamide) and SC-560 (5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole) were kind gifts of Dr Peter Isakson (Pharmacia Corporation, Piscataway, NJ).

Wild-type and homozygous apoE<sup>−/−</sup> mice used for the studies were bred in house. At 40 days of age, wild-type mice were randomized to normal chow (0.5% total fat and 0.022% cholesterol, n=20) or 1.0% cholesterol-supplemented chow (normal chow supplemented with 15.8% fat and 1.0% cholesterol; SDS) (n=20). Homozygous apoE<sup>−/−</sup> offspring were randomized at 40 days to receive normal chow (n=20), 1.0% cholesterol diet (n=20), 1.0% cholesterol diet with SC-236 6 mg/kg daily (n=20), or 1% cholesterol diet with SC-560 15 mg/kg daily (n=20).

For regression studies, apoE<sup>−/−</sup> mice were fed a 1% cholesterol diet for 8 weeks (n=30) and were maintained on 1% cholesterol diet alone for an additional 8 weeks (n=10) or 1% cholesterol diet plus SC-560 15 mg/kg for an additional 8 weeks (n=10). All animals were killed by CO₂ inhalation.

Aortic tissue sections for immunohistochemistry analysis were placed in 10% formal saline (n=15) for each group. Tissue samples for the measurement of prostaglandin production ex vivo were placed in PBS (n=5). Urine was collected from all animal groups in metabolic cages 24 hours before euthanasia.

Animal care and treatment were conducted in conformity with institutional guidelines in compliance with international laws and policies. A license and permission for the study were obtained from the Department of Health.

**Prostaglandin Analysis**

*Vascular PGE₂*

After dissection of aorta, a segment of tissue was placed in 500 µL PBS for 45 minutes at 37°C, and the supernatant was removed and analyzed for PGE₂ by enzyme immunoassay (Assay Designs Inc). Protein concentrations of tissue segments were determined using the BioRad DC Protein Assay (BioRad).

*Urinary Prostaglandin Metabolite Excretion*

Twenty-four-hour urine samples were purified using solid-phase extraction, as described previously. Samples were spiked with 1 ng/mL deuterated (H₄) 2,3-dinor-6-keto-PGF₁α and O₁₈-labeled 2,3-dinor-TXB, and analyzed by reverse-phase HPLC tandem mass spectrometry (Sciex API III+ triple quadrupole, Applied Biosystems) operated in the negative-ion mode and using multiple-reaction monitoring.

**Immunohistochemistry**

The aortas were perfused in situ through a needle inserted into the left ventricle with PBS for 3 minutes and formal saline (0.9% NaCl and 10% formaldehyde) for 5 minutes. The aorta from aortic arch was removed and fixed in formal saline. Vessels were paraffin embedded (Shandon Citadell 200), and 50 serial flip-flop sections (5
to 8 μm) were cut transversely from the aortic arch (Leitz 1512, Wetzlar GMBH). The sections were incubated in primary antibody against COX-1, COX-2, Bax, CD68, CD40L (all goat polyclonal, Santa Cruz) or CD41 (rat monoclonal, Pharmingen). After washing in PBS, the slides were incubated in the secondary biotinylated antibody and the immunocomplex visualized using the diaminobenzidine chromagen (ABC Complex, Vectastain Elite kit, Vector Labs). Intensity of COX-1, COX-2, CD41, CD40L, Bax, and CD68 staining was measured by determining maximum density of DAB-stained cells using Image Pro Plus 4.0 software (MediaCybernetics). Sections (n = 15 from each animal) from the aortic arch were used to quantify atherosclerosis (5 animals from each group). The analysis was performed by the same investigator blinded to the study groups. Lesion areas were quantified and expressed as the percentage of total vessel area (lesion area/total vessel area = percentage lesion per total vessel area) using Image Pro Plus 4.0 software.

Statistical Analysis
The data were expressed as mean±SEM. For comparison between groups, the data were analyzed by ANOVA followed by paired or unpaired Student’s t tests, as appropriate, or, in instances where data distribution deviated from normality, using the Kruskal-Wallis nonparametric ANOVA and subsequent Mann-Whitney U test.

Results

COX Isoform Expression
COX-1 was the major COX isoform detected by immunohistochemistry in wild-type animals fed either diet and in apoE−/− animals fed a normal chow. In apoE−/− animals fed the high-cholesterol chow, there was an increase in COX-1 staining that was most pronounced in atherosclerotic lesions (Figure 1). Although a small level of COX-2 expression was detected in wild-type animals and in apoE−/− animals fed a normal chow, this was markedly increased in apoE−/− animals fed a high-cholesterol diet, largely localized to atherosclerotic lesions (Figure 1).

Vascular PGE2 Generation
PGE2 production from mouse aortas was determined ex vivo. There was no significant effect on diet on PGE2 in wild-type mice (2740±639 pg/mg normal chow versus 2331±721 pg/mg protein chow supplemented with cholesterol, n=5). In apoE−/− mice fed a normal chow, the levels were increased compared with wild-type mice (3910±878 versus 2331±721 pg/mg protein, n=5, P<0.05). The generation of PGE2 in these animals was reduced by ASA 200 μmol/L and by the COX-2 inhibitor NS-398 1 μmol/L (to 1512±463 and 2406±725 pg/mg protein, respectively). In apoE−/− mice fed a 1% cholesterol diet, the PGE2 levels were increased additionally (to 5623±875 pg/mg protein, n=5, P<0.05). Oral administration of SC-236 to apoE−/− mice fed a 1% cholesterol diet reduced vascular PGE2 levels (from 5623±875 pg/mg to 4086±770 pg/mg), which were unchanged by incubation with NS-398 (3486±380 ng/mg) but reduced by ASA 200 μmol/L (1815±567 ng/mg). Oral administration of SC-560 reduced vascular PGE2 levels (to 1871±360 mg/mg, P<0.01), and no additional reduction was seen with ASA 200 μmol/L (1288±346 pg/mg). These data suggest that both COX-1 and COX-2 contribute to the increased vascular PGE2 generation in the aorta of apoE−/− mice on a 1% cholesterol diet, with a major contribution from COX-1.

Urine was obtained 24 hours before euthanasia for determination of 2,3-dinor-TXB2 and 2,3-dinor-6-keto-PGF1α, principle metabolites of TXA2 and PGI2, respectively. Urinary 2,3-dinor-TXB2 was unaltered in wild-type animals fed a high-cholesterol chow compared with those fed a normal chow (34.2±13.6 versus 51.2±12.7 ng/mg creatinine, n=10) or in apoE−/− animals fed a normal chow (56.9±22.9 mg/mg creatinine, n=10). In apoE−/− animals fed a 1% cholesterol chow, there was an increase in urinary 2,3-dinor-TXB2 (Figure 2). This increase was unaltered by administration of the COX-2–selective inhibitor SC-236 but was abolished by the COX-1–selective inhibitor SC-560 (Figure 2). These data suggest that the COX-1 isoform is largely responsible for the increase in TXB2 generation in apoE−/− animals with atherosclerosis.

Urinary 2,3-dinor-6-keto-PGF1α was similar in wild-type mice fed a normal chow or high-cholesterol chow and in apoE−/− animals fed a normal chow (6.2±1.3 versus 6.1±1.9 mg/mg creatinine, n=10). In apoE−/− animals fed high-cholesterol chow, there was an increase in urinary 2,3-dinor-6-
keto-PGF₁α compared with those that received a normal diet (11.9±0.7 versus 4.8±0.8 ng/mg, P<0.05) (Figure 2). Administration of SC-236 abolished the increase in urinary 2,3-dinor-6-keto-PGF₁α (to 5.1±0.6 ng/mg creatinine, n=10, P<0.01). SC-560 also reduced urinary 2,3-dinor-6-keto-PGF₁α (to 8.0±0.6 ng/mg creatinine, n=10, P<0.05), although not to the same extent as SC-236. Thus, as in human atherosclerosis, both COX-1 and COX-2 contribute to the increase in PGI₂ generation seen in the apoE<sup>−/−</sup> mouse model.

**Quantification of Lesion Formation**

In apoE<sup>−/−</sup> animals fed a high-cholesterol chow, approximately 30% of the total vessel area developed atherosclerotic lesions at 8 weeks. Administration of SC-236 had no effect on atherosclerosis in these animals (25.9±4.6% versus 27.9±1.8% total area). However, administration of SC-560 markedly reduced lesion formation (Figure 3) in apoE<sup>−/−</sup> animals fed a high-cholesterol chow (1.7±1.56%, n=10, P<0.01). To examine the effects of the COX-1 inhibitor on progression or regression of established lesions, apoE<sup>−/−</sup> animals administered a cholesterol chow were randomized after 8 weeks to SC-560 or vehicle and continued on the high-cholesterol chow for an additional 8 weeks. The progression in lesion development (to 44±2.5%) was unaffected by the COX-1 inhibitor (52±2.5%) (Figure 3).

**CD41, CD40L, Bax, and CD68 Expression**

There was marked vascular staining for CD41 in the apoE<sup>−/−</sup> animals fed a high-cholesterol chow for 8 weeks that was localized to the lesions, providing evidence for platelet deposition (Figure 4). CD41 staining was similar in animals treated with the COX-2 selective inhibitor. Despite the absence of lesions in apoE<sup>−/−</sup> animals fed a high-cholesterol chow treated with SC-560, there was increased CD41 staining compared with apoE<sup>−/−</sup> animals fed a normal diet or wild-type animals. This was seen as a distinct layer of staining...
overlying the endothelium (Figure 4). Administration of SC-560 to apoE−/− animals after 8 weeks of a high-cholesterol chow diet did not influence the degree of CD41 staining (nor lesion progression, as outlined above).

There was marked expression of CD40L in apoE−/− animals fed a 1% cholesterol chow in the presence and absence of SC-236 compared with apoE−/− animals fed a normal diet. Similarly, despite the absence of gross lesions in animals treated with SC-560, there was increased CD40L staining compared with apoE−/− animals fed a normal diet or wild-type animals (Table). Furthermore, macrophage infiltration, detected by the surface marker CD68, was increased in apoE−/− animals fed a high-cholesterol chow and was unchanged by either SC-236 or SC-560 (Table).

There was increased expression of Bax, a marker of apoptosis, in apoE−/− animals fed a high-cholesterol chow compared with wild-type animals on the same diet (Figure 5). Bax expression was also increased in apoE−/− animals fed a high-cholesterol chow when treated with the COX-1 inhibitor despite the absence of gross lesions. Bax expression was similar in both regression groups, suggesting that treatment with the COX-1 inhibitor did not directly induce Bax.

**Discussion**

The profile of prostaglandin generation and expression of cyclooxygenases are remarkably similar in the apoE−/− murine and human atherosclerosis. In both, there is an increase in the biosynthesis of TXA2 and PGI2, with induction of COX-1 and COX-2 in the lesions. The increase in TXA2 in the murine model was unaffected by administration of the COX-2 selective inhibitor SC-236 but was prevented by the COX-1 inhibitor SC-560. This is consistent with previous data but provides more direct evidence that, as in humans, COX-1 is responsible for the increase in TXA2. In contrast,

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<th>CD68 and CD40L Expression in Wild-Type and ApoE−/− Animals Treated With the Selective COX-2 Inhibitor SC236 and COX-1 Inhibitor SC560 for 8 Weeks</th>
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*P<0.05 vs ApoE normal diet.
†P<0.05 vs ApoE 1% diet.
both the COX-1 and the COX-2 inhibitors suppressed urinary 2,3-dinor-6-keto-PGF\(_{1\alpha}\). The findings are consistent with both COX isoforms, contributing to the enhanced PGI\(_2\) generation, although the reduction with the COX-1 inhibitor may also reflect suppression of lesion formation and consequently COX-2 expression (Figure 1). Disruption of apoE alone had no effect on TXA\(_2\) formation, although vascular PGE\(_2\) was increased. There was a marked increase when the diet in apoE\(^{-/-}\) mice was supplemented with 1% cholesterol, a diet that results in a remarkable increase in serum cholesterol. The source of the TXA\(_2\) is unknown, but as in humans, it may be attributable to an increase in the activity of platelets, where COX-1 is the only isoform and TXA\(_2\) the principle product. Platelet activation may arise as a consequence of vascular injury induced by the high plasma cholesterol, and indeed the lesions developing in the model stained strongly for CD41, a highly abundant, platelet-specific protein.

There is experimental evidence that the increased activity of COX-2 contributes to the pathogenesis of atherosclerosis. Pharmacological inhibition of COX-2 has been reported to suppress lesion formation by approximately 30%.\(^{16,17}\) This may reflect COX-2 expression in monocytes/macrophages recruited to the lesions.\(^{9,16}\) Thus, replacement of these cells in mice with COX-2\(^{-/-}\) cells (by engrafting COX-2\(^{-/-}\) hepatic stem cells after marrow ablation) reduces lesion formation. On the other hand, Rott et al\(^{18}\) reported an increase in lesion formation in animals receiving a specific COX-2 inhibitor. This could be explained by selective suppression of PGI\(_2\) and enhanced platelet activation, because selective disruption of the IP (PGI\(_2\)) receptor enhances intimal hyperplasia after carotid injury.\(^{11}\) In our experiments, a COX-2 inhibitor administered at a dose that selectively reduced PGI\(_2\) and preserved TXA\(_2\) formation had no effect on lesion formation. It has been speculated that selective inhibition of PGI\(_2\) in the face of continued TXA\(_2\) formation by selective COX-2 inhibitors may increase platelet activity and the risk of thrombosis. In our experiments, there was no increase in platelet activity, measured either as deposition of platelets on vascular lesions or enhanced TXA\(_2\) formation, a marker of platelet activation in vivo.\(^{19,20}\)

In contrast to COX-2, there was evidence that COX-1 contributed to lesion development. Inhibition of COX-1 with SC-560 at a dose that prevented the increase in TXA\(_2\) formation markedly attenuated lesion development. Similar results have been reported with a selective TXA\(_2\)/prostaglandin endoperoxide antagonist S18886 and with aspirin in apoE\(^{-/-}\) and LDLR\(^{-/-}\) models.\(^{8,21,22}\) However, SC-560 did not induce regression or prevent the progression of lesions that had already developed, implicating COX-1 in the earlier stages of lesion formation.

It should be emphasized that although there were no gross lesions in COX-1-treated animals, the vessel wall was not normal. There was persistent macrophage accumulation in apoE\(^{-/-}\) mice despite administration of the selective COX-1 inhibitor and the absence of lesion formation. There was also persistent platelet deposition, seen as a monolayer adherent to

Figure 5. Immunohistochemistry staining for Bax in wild-type and apoE\(^{-/-}\) animals fed a 1% cholesterol diet on no treatment or coadministered SC-560 for 8 weeks. Also shown are apoE\(^{-/-}\) animals that, after a 1% cholesterol diet for 8 weeks, were treated for an additional 8 weeks with SC-560 while maintained on the diet. The staining shown at \(\times 40\) magnification is representative of 5 experiments. The intensity of staining was quantified using Image Pro Plus software. Mean±SEM. *\(P<0.05\) vs wild-type mice fed a normal diet; +\(P<0.05\) vs apoE\(^{-/-}\) mice fed a 1% cholesterol diet.
the endothelial surface and vascular expression of CD40L, albeit reduced compared with untreated apoE−/− animals on the cholesterol-supplemented diet. CD40L is a ligand for CD40, which is expressed on T-lymphocytes in atherosclerotic plaque and activates monocyte expression of cytokines. The protein is released by platelets even where platelet cyclooxygenase is inhibited by aspirin. Studies based on the neutralization by an antibody or disruption of gene demonstrate that CD40L contributes to lesion development in the apoE−/− model, although not at the early stages. There was also induction of Bax protein in the vascular smooth muscle cells. Bax is a cytosolic protein that is induced and relocates to mitochondria early in apoptosis. Bax expression has been reported along with other evidence of apoptosis in experimental and human atherosclerosis.

The most likely explanation for the effect of the COX-1 inhibitor is inhibition of platelet activity. Massberg at al. have reported that vascular lesions in the apoE−/− mouse stain for proteins secreted by platelets. Chronic suppression of platelet adhesion to the vessel wall markedly suppressed the development of atherosclerosis. The results suggest a model where platelet adhesion at a site of vascular damage results in platelet activation, a known consequence of the interaction between von Willebrand factor and glycoprotein Ib, and the release of platelet proteins. Several of these could contribute to the development of murine atherosclerosis, including growth factors (platelet-derived growth factor and transforming growth factor β), cytokines (interleukin-1α and interleukin-6), and other proteins that contribute to inflammation, such as CD40L. Although the COX-1 inhibitor used did not prevent platelet adhesion in our experiments, by preventing platelet TXA2 generation, it may have interrupted the subsequent steps of platelet activation, secretion, and aggregation. A role for platelets may be confined to the early stages of lesion development in the apoE−/− knockout model of atherosclerosis, preventing gross lesion formation in the face of continued vascular injury. Selective COX-2 inhibition did not affect lesion development or platelet–vessel wall interactions despite inhibition of PGI2 generation.

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