Cyclooxygenase-2 in Endothelial and Vascular Smooth Muscle Cells Restrains Atherogenesis in Hyperlipidemic Mice

Soon Yew Tang, PhD; James Monslow, PhD; Leslie Todd, BS; John Lawson, MS; Ellen Puré, PhD; Garret A. FitzGerald, MD


**Methods and Results**—In the present study, selective depletion of COX-2 in vascular smooth muscle cells and endothelial cells depressed biosynthesis of prostaglandin I\(_2\) and prostaglandin E\(_2\), elevated blood pressure, and accelerated atherogenesis in Ldlr knockout mice. Deletion of COX-2 in vascular smooth muscle cells and endothelial cells coincided with an increase in COX-2 expression in lesional macrophages and increased biosynthesis of thromboxane. Increased accumulation of less organized intimal collagen, laminin, \(\alpha\)-smooth muscle actin, and matrix-rich fibrosis was also apparent in lesions of the mutants.

**Conclusions**—Although atherogenesis is accelerated in global COX-2 knockouts, consistent with evidence of risk transformation during chronic nonsteroidal anti-inflammatory drug administration, this masks the contrasting effects of enzyme depletion in macrophages versus vascular smooth muscle cells and endothelial cells. Targeting delivery of COX-2 inhibitors to macrophages may conserve their efficacy while limiting cardiovascular risk. (*Circulation*. 2014;129:1761-1769.)

**Key Words:** atherogenesis ■ cyclooxygenase ■ endothelial cell ■ prostaglandin ■ vascular smooth muscle

**Clinical Perspective on p 1769**

Cells tend to make 1 or 2 dominant COX products, often with contrasting biological effects, such as the divergent effects of platelet COX-1–derived thromboxane A\(_2\) (TxA\(_2\)) and COX-2–derived endothelial PGI\(_2\) on platelet activation.\(^2,10\) Activated macrophages make predominantly TxA\(_2\) and prostaglandin E\(_2\) (PGE\(_2\)), and ligation of the TxA\(_2\) receptor or the E prostanoiid receptor-3 fosters atherogenesis.\(^11,12\) Consistent with these observations, deletion of myeloid cell COX-2 restrains atherogenesis, an effect most likely attributable to macrophage gene deficiency because COX-2 is not expressed in mature platelets and is expressed minimally in dendritic cells and neutrophils.\(^11\) Given the contrasting effects of global nonsteroidal anti-inflammatory drugs (NSAIDs) designed specifically to inhibit cyclooxygenase-2 (COX-2) relieve pain and inflammation but expose patients to a cardiovascular hazard comprising myocardial infarction and stroke, hypertension, heart failure, arrhythmogenesis, and sudden cardiac death.\(^1\) These effects are attributable to suppression of COX-2–derived cardioprotective prostaglandins, particularly prostacyclin (prostaglandin I\(_2\) [PGI\(_2\)]) in the vasculature and in cardiomyocytes.\(^2\) More controversial has been the potential impact of COX-2 inhibition on atherosclerosis. Extended dosing with COX-2 inhibitors in 3 placebo-controlled trials was associated with emergence of a detectable increase in cardiovascular events in patients initially selected to be at low risk of heart disease.\(^3-5\) Consistent with this observation, deletion of the PGI\(_2\) receptor fosters initiation and early development of atherogenesis in hyperlipidemic mice,\(^5,7\) offering a potential mechanism for risk transformation during chronic drug exposure. However, experiments with inhibitors of COX-2 and conventional COX-2 knockout (KO) mice showed accelerated, delayed, or no effect on atherosclerosis.\(^3\) Because this confusion may have reflected the failure to characterize the pharmacological specificity of enzyme inhibition and the diverse consequences of missing COX-2 during development, we induced global deletion of COX-2 postnatally. In these mice, atherogenesis is accelerated,\(^9\) consistent with the result in prostacyclin receptor KO mice.

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and macrophage COX-2 deletion on disease evolution and the causative implication of enzyme inhibition in vascular cells in other aspects of the NSAID-related cardiovascular hazard, we sought to elucidate the impact of COX-2 in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) on atherosclerosis.

**Methods**

**Materials**

All reagents used were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise stated.

**Generation of Vascular COX-2 KO Hyperlipidemic Mice**

Endothelial cell COX-2 knockout (Tie2Cre/COX-2−/−; EC KO), vascular smooth muscle cell COX-2 knockout (SM22Cre/COX-2−/−; VSMC KO), and endothelial/vascular smooth muscle cell double COX-2 knockout (Tie2Cre;SM22Cre/COX-2−/−; E/V DKO) mouse lines were generated as described. These mouse lines were crossed with Ldlr−/− mice fully backcrossed onto the C57BL/6 background (The Jackson Laboratory, Bar Harbor, ME). All KO s were genotyped with tail DNA to confirm the presence of Cre germline leakage. COX-2 F/F/Ldlr

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**Animals**

In all experiments, COX-2−/− deficient transgenic mice were compared with appropriate strain-, age-, and sex-matched control animals. Single nucleotide polymorphism analyses showed that EC KO, VSMC KO, and E/V DKO mouse lines for the atherosclerosis study achieved at least 90.5% to 92.5% purity on the C57BL/6 background. Mice of both sexes were fed a high-fat diet (HFD; 21.2% fat, 0.2% cholesterol; TD.88137, Harlan Teklad, Madison, WI) from 8 weeks of age for 3 and 6 months. Mice were weighed before and after the HFD feeding. All animals in this study were housed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Pennsylvania. All experimental protocols were approved by Institutional Animal Care and Use Committee.

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

Mice were transferred after HFD feeding to new cages without food from 8 am to noon. Water was provided ad libitum. All mice were euthanized between noon and 4 pm by CO2 overexposure. The extent of atherosclerosis (Phase 3 Imaging Systems, Glen Mills, PA) was determined by the en face method and by assessment of aortic root lesion burden, as described previously.

**Blood Pressure Measurement**

Systolic blood pressure was measured in conscious mice with the use of a computerized noninvasive tail-cuff system (Visitech Systems, Apex, NC), as described. Blood pressure was recorded once each day from 8 am to 11 am for 5 to 7 consecutive days after 3 days of training. Average systolic blood pressure was reported.

**Mass Spectrometric Analysis of Prostanoids**

Urinary prostanoid metabolites were measured by liquid chromatography/mass spectrometry as described. Such measurements provide a noninvasive, time-integrated measurement of systemic prostanoid biosynthesis, reflective of short-term minor alterations in product formation and of vascular stimulation. Briefly, mouse urine samples were collected with the use of metabolic cages over a 15-hour period (6 pm to 9 am). Systemic production of PGI2, PGE2, and TxA2, was determined by quantifying their major urinary metabolites: 2, 3-dinor 6-keto PGF1α (PGF-M); 7-hydroxy-5, 11-diketotetranorprostone-1, 16-dioic acid (PG-E-M); 11, 15-dixo-9, 5-tetranorprostan-1, 20-dioic acid (tetrnor PGD-M); and 2, 3-dinor TxB2 (Tx-M), respectively. Results were normalized with creatinine.

**Immunohistochemical Examination of Lesion Morphology**

Mouse hearts were embedded in OCT, and 10-μm serial sections of the aortic root were cut and mounted on Superfrost Plus slides (Fisher Scientific) for analysis of lesion morphology. Samples were fixed in acetone for 15 minutes at −20°C. Before treatment with the first antibody, sections were consecutively treated to block endogenous peroxidase (3% H2O2, for 15 minutes) with 10% normal serum blocking solution (dependent on host of secondary antibody, in 1% BSA/PBS for 15 minutes) and endogenous biotin (streptavidin-biotin blocking kit, SP-2002, Vector Laboratories). Sections were then incubated with the desired primary antibody in blocking solution overnight at 4°C. Samples were individually stained for collagen type I (1 μg/mL, 1310-01, Southern Biotech), laminin (2 μg/mL, L9393, Sigma), α-smooth muscle actin (α-SMA; 12.3 μg/mL, F3777, Sigma), vascular cell adhesion molecule-1 (VCAM-1; 10 μg/mL, 553331, BD Bioscience), COX-1 (1 μg/mL, 160109, Cayman Chemicals), COX-2 (1 μg/mL, 160106, Cayman Chemicals), CD11b (5 μg/mL, 557395, BD Bioscience), and CD11c (5 μg/mL, 553800, BD Bioscience), all with isotype-matched controls. Where required, sections were then incubated with biotinylated-IgG secondary antibody (specific to host of primary antibody, all 1 μg/mL, Vector Laboratories) diluted in 1% bovine serum albumin/phosphate-buffered saline (BSA/PBS) for 1 hour at room temperature. Sections were then incubated with streptavidin/horseradish peroxidase (1 μg/mL, 016-030-084, Jackson Immunoresearch) diluted in 1% BSA/PBS for 30 minutes at room temperature. Slides were equilibrated in sterile H2O for 5 minutes at room temperature, then developed with the use of the DAB substrate kit (K3468, Dako) according to the manufacturer’s protocol. Samples were counterstained with hematoxylin, dehydrated, and mounted in Cytoseal-60 (12-547, Fisher Scientific). Isotype-matched controls were performed in parallel and showed negligible staining in all cases.

**COX-2 CD11b Stepwise Double Staining**

Ten-micrometer frozen sections were left to air-dry for 5 minutes at room temperature. Samples were then fixed in acetone for 15 minutes at −20°C. Before treatment with the first antibody, samples were consecutively treated to block endogenous peroxidase (3% H2O2, for 15 minutes) with 10% normal serum blocking solution (in 1% BSA/PBS for 15 minutes) and endogenous biotin (streptavidin/biotin blocking kit, SP-2002, Vector Laboratories). The COX-2 primary antibody (1 μg/mL, 160106, Cayman Chemicals) or COX-2 IgG control antibody (1 μg/mL, 011-000-003, Jackson Immunoresearch) diluted in blocking solution was then added, and samples were incubated overnight at 4°C. After three 5-minute washes with 0.05% Tween-20/0.1% PBS, samples were incubated with biotinylated goat anti-rabbit IgG secondary antibody (1 μg/mL, BA-1000, Vector Laboratories) diluted in 1% BSA/PBS for 1 hour at room temperature. Three more washes were performed before samples were next incubated with streptavidin/alkaline phosphatase (1 μg/mL, 016-050-084, Jackson Immunoresearch) diluted in 1% BSA/PBS for 30 minutes at room temperature. Samples were again washed 3 times before being equilibrated in 0.1 mol/L Tris-HCl (pH 8.2) for 5 minutes at room temperature, then developed with the use of the Vector Red substrate kit (SK-5100, Vector Laboratories) according to the manufacturer’s protocol. Before double staining with CD11b, samples were once again treated with the streptavidin/biotin blocking kit. Biotinylated CD11b primary antibody (5 μg/mL, 557395, BD Pharmingen) or biotinylated IgG control (5 μg/mL, 13-4031, eBioscience) diluted in 1% BSA/PBS was then added, and samples were incubated overnight at 4°C. After 3 washes, samples were incubated with streptavidin/horseradish...
peroxidase (1 μg/mL, 016-030-084, Jackson Immunoresearch) diluted in 1% BSA/PBS for 1 hour at room temperature. After 3 more washes, samples were equilibrated in sterile H2O for 5 minutes at room temperature and developed with the use of the DAB substrate kit (SK-4100, Vector Laboratories) according to the manufacturer’s protocol. Samples were then counterstained with hematoxylin, dehydrated, and mounted in Cytoseal-60 (12-547, Fisher Scientific).

Second Harmonic Generation Analysis of Fibrillar Collagen Structure

Ten-micrometer aortic root frozen sections were left to air-dry for 5 minutes at room temperature before second harmonic generation (SHG) microscopy analysis of fibrillar collagen structure was performed. Samples were then fixed in acetone for 15 minutes at −20°C and submerged in PBS in a tissue culture dish. SHG images of the fibrillar collagen and background tissue autofluorescence images were captured at ×10 magnification with the use of a Prairie Technologies Ultima 2-Photon Microscope system (Middleton, WI). Images were taken with an excitation wavelength of 910 nm and captured through emission filters of 457 to 487 nm (SHG signal) and 525 to 570 nm (autofluorescence). The SHG and autofluorescence signals were pseudocolored in green and red, respectively, for observation of structure and morphology. Quantification of fibrillar collagen content, intensity, and organization was calculated with the use of Fiji Image Analysis software. First, the fibrillar collagen signal was isolated by subtracting the background autofluorescence signal from the original SHG image. Collagen content (area and intensity) was then quantified as percentage of total lesion area and total integrated density of the collagen signal, respectively. To measure fibrillar collagen organization, the Directionality macro (within Fiji) was first used to generate a fast Fourier transform powerplot of the fibrillar collagen signal. An ellipse was then superimposed over the positive signal generated within each powerplot, and the aspect ratio of the ellipse for each lesion was calculated. An aspect ratio with a value closer to 0 indicated random orientation, and that closer to 1 indicated oriented collagen fibers. For each lesion, 6 sections (equally spaced over the entire aortic root, ≈350 μm) were analyzed for fibrillar collagen content, intensity, and organization.

Statistical Analysis

For data analyzed by ANOVA, the Holm-Sidak or Dunnett posttest was used to compare the differences between the means only if the ANOVA returned a P<0.05. Sample sizes were based on variability of the test measurement and the desire to detect a minimal 10% difference in the variables assessed with (α=0.05 and power (1-β)=0.8.

Results

Aortic COX-2 Expression Is Modulated by Diet and Gene Deletion

Expression of COX-2 in the aortic arch and thoracic aorta of WT mice increased with the HFD-fed compared with normal chow–fed animals (Figure IA in the online-only Data Supplement). Vascular tissue–specific COX-2 KOs on a chow diet revealed significant reductions in aortic COX-2 expression compared with WT mice (Figure IB and IC in the online-only Data Supplement) and after HFD feeding for both 3 and 6 months (Figure IF and IG in the online-only Data Supplement). Although data from female mice at 6 months of HFD are illustrated, regardless of sex or study duration, COX-1 expression in aortic arch or thoracic aorta was not significantly altered in the mutants on a chow diet (Figure ID and IE in the online-only Data Supplement) or a HFD (Figure IH and II in the online-only Data Supplement).

Deletion of COX-2 in Vascular Cells Modulates Prostaglandin Biosynthesis in Mice on a HFD

There were no significant effects of genotype or sex on plasma cholesterol, triglycerides, glucose, or weight gain at different times in mice on a HFD. Thereafter, we focused our analysis on males at 3 months and on females at 6 months of a HFD because the extent of their atherosclerotic lesions was similar and their lesional morphology was not advanced to the point at which genotype-dependent effects are often undetectable. Deletion of COX-2 in vascular cells generally depressed biosynthesis of PGI2 (Figure IA and IE) and PGE2 (Figure IB and IF), as reflected by their urinary metabolites. However, this was not observed with EC deletion alone in female mice. This may reflect the imperfect matching of lesional development between males at 3 months and females at 6 months on a HFD. Biosynthesis of PGI2, was depressed by COX-2 deletion only in males (Figure IC). By contrast, thromboxane biosynthesis was increased in the single vascular mutants in both sexes and in the female compound mutants (Figure ID and IH). However, it was depressed in the compound male mutants.

Vascular COX-2 Depletion Elevates Systolic Blood Pressure and Restains Atherogenesis

Although systolic blood pressure was not altered in single EC or VSMC KO male mice after 3 months on a HFD, a significant elevation was observed in the compound E/V mutants compared with WT mice (WT versus E/V DKO, 114±1 versus 124±2 mm Hg, P=0.0002; Figure 2A). For female mice fed a HFD for 6 months, systolic blood pressure was also significantly elevated in both the compound mutants and those lacking COX-2 in VSMCs (WT versus VSMC KO and E/V DKO, 111±2 versus 121±2 versus 120±3 mm Hg, respectively; P=0.0001; Figure 2B). Atherosclerotic lesion burden was increased by deletion of COX-2 in vascular cells in both males at 3 months (WT versus EC, VSMC, and E/V DKO, 3.72±0.4% versus 7.17±0.7%, 5.19±0.3%, and 4.73±0.6%, respectively, P<0.0001; Figure 3A) and females at 6 months (10.15±0.7% versus 13.36±0.6%, 11.76±0.8%, and 13.19±0.6%, respectively, P<0.0003; Figure 3B) on a HFD. As lesions became advanced in males at 6 and 11 months on a HFD (Figure II in the online-only Data Supplement), these genotype-dependent changes were lost.

Lesional Morphology Consequent to COX-2 Deletion in Vascular KOs

Consistent with the lesion burden analyzed by the en face method, cross-sectional analysis of aortic root samples in female mice on a HFD for 6 months revealed an increase in the total lesion area in both single and double COX-2 KOs compared with WT mice (Figure 4A). Morphological analysis revealed fibrosis with increased accumulation of collagen and matrix-rich fibrotic areas detected by laminin staining in the COX-2 mutants compared with WT mice (Figure 5A and 5B). However, SHG 2-photon microscopy showed minimal changes to collagen content and collagen signal intensity between WT and E/V DKO lesions (Figure IIIA and IIIB in the online-only Data Supplement) and a more random orientation and less organized structure of lesional collagen fibrils.
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in the DKO, as revealed by the fast Fourier transform–based analyses (Figure 4B). Representative SHG-collagen images of WT and E/V DKO are shown (Figure 4C and 4D).

α-SMA staining, a marker of differentiated VSMCs, was increased in both single and compound COX-2 KO lesions (Figure 5C). Necrotic cores in COX-2 KOs did not differ significantly in size compared with those in WTs (Figure 5A through 5C). However, only single COX-2 KOs showed an upregulation of VCAM-1 for activated VSMCs (Figure 5D).

Discussion

Nine placebo-controlled trials of COX-2–selective NSAIDs (rofecoxib, celecoxib, and valdecoxib) have revealed a cardiovascular risk of myocardial infarction, hypertension, stroke, and heart failure.23,24 These risks are explicable in terms of the suppression of cardioprotective products of COX-2, particularly PGI₂. In mice, inhibition or deletion of COX-2–dependent PGI₂ formation augments the response to thrombogenic stimuli, elevates systemic and pulmonary blood pressure, disrupts vascular remodeling, and predisposes the animals to cardiac failure and arrhythmogenesis.12,14,18,25

Three of these placebo-controlled trials were performed in patients selected to be at low demographic risk of cardiovascular disease.3–5 Despite this, an increase in cardiovascular
events became detectable with extended dosing with either celecoxib or rofecoxib for >1 year. The time course of emergent risk would be consistent with a drug effect on atherogenesis, such as was observed in prostacyclin receptor–deficient mice. However, the results of studies of COX-2 deletion or inhibition in hyperlipidemic mice have been conflicting. This may reflect the systemic consequences of COX-2 deficiency in utero in the case of the conventional knockouts and of a failure to characterize the actual biochemical selectivity for COX-2 inhibition of the pharmacological regimens used. This is an important point because COX-1 inhibition or deletion attenuates atherogenesis.26,27 More recently, we generated mice in which global COX-2 deletion was accomplished postnatally, and in this case atherogenesis was accelerated in both sexes when they were crossed into apolipoprotein E–deficient mice.9

We have reported previously that both EC and VSMC COX-2 contribute substantially to systemic PGI₂ formation under physiological conditions in normolipidemic mice, as reflected by urinary PGI-M.10 In the present study, we extended these observations, showing that on a hyperlipidemic background, biosynthesis of both PGI2 and PGE₂ was derived in both sexes substantially from COX-2 in vascular cells and that the enzyme contributed to PGD2 biosynthesis in males. Expression of COX-2 was upregulated in both the aortic arch and thoracic aorta of littermate control mice fed a HFD, consistent with the increase in urinary PGI-M in apolipoprotein E–deficient and LdlR-deficient mice.28

We have previously reported that COX-2 in EC and VSMC restrains atherogenesis, which was quantified by analysis of en face preparations from male mice fed a high-fat diet for 3 or 6 months. Representative en face preparations are shown (bottom). Lesion area tended to increase in male or female cyclooxygenase-2 mutants fed a HFD for 3 or 6 months, respectively (A and B). One-way ANOVA revealed a significant effect of genotype (male, P=0.001; female, P=0.003) on lesion progression. Holm–Sidak multiple comparison tests were used to test significant differences between wild-type (WT) and cyclooxygenase-2 knockouts (KOs). Data are mean±SEM. **P<0.01, ****P<0.0001; n=18 to 22 per genotype. EC indicates endothelial cell; E/V DKO, endothelial/vascular smooth muscle cell double cyclooxygenase-2 knockout; and VSMC, vascular smooth muscle cell.
Platelet- and neutrophil–vessel wall interactions during the development of atherosclerosis are reflected by the urinary thromboxane metabolite TxA-M.28,29 TxA2 is also a dominant product of activated macrophages. Theoretically, cells outside the vascular compartment (glial cells and glomeruli, for example) have the capacity to generate thromboxane and may contribute to urinary TxA-M. Mice lacking the prostacyclin receptor fed a HFD excrete higher levels of TxA-M in both sexes.6 Moreover, previous studies have shown that COX-2–dependent formation of PGI2 in the vasculature restrains TxA2 biosynthesis.13,28 In the present study, urinary TxA-M was increased in COX-2 mutants fed a HFD, which perhaps reflects removal of a regulatory constraint on myeloid cell activation by suppression of the biosynthesis of PGI2.30 This phenomenon was not observed in male E/V DKO s, in which urinary TxA-M was depressed. Whether this reflects a feedback response to the differential impact of male sex hormones on thromboxane receptor expression31 that would be more exaggerated in the compound mutant or some other mechanism is unknown.

Previous studies in which COX-2 is deleted in a cell-specific fashion have highlighted the importance of prostaglandins in regulation of expression of their biosynthetic enzymes. Thus, deletion of COX-2 in cardiomyocytes results in COX-2 upregulation in cardiac fibroblasts, with a shift in the dominant enzyme product from PGI2 in the former to PGF2α in the latter.14 Similarly, COX-2 deletion in plaque macrophages was associated with enzyme upregulation in VSMCs, implying a shift from TxA2 and PGE2 as the dominant product in the former to PGI2 in the latter.13 In both cases, the shift in product formation may have contributed to the resultant phenotype: cardiac fibrosis and arrhythmogenesis in the heart14 and attenuated atherogenesis in the vasculature.13 In the present study, we observed increased expression of COX-2 in lesional...
macrophages of the mice in which COX-2 had been deleted in vascular cells, consistent with both the reduction in PGI-M and increased Tx-M excretion and the accelerated atherogenesis observed in these mutants.

We compared lesions of roughly similar size (at 3 months on a HFD in males and 6 months in females) and at a maturity level at which the impact of genotype was likely to be apparent. The COX-2 mutants developed more fibrotic lesions than did WT controls, as reflected by staining for collagen and laminin. This may be attributable to suppression of PGI formation. COX-2–derived PGI, an important negative regulator of the collagen synthesis that contributes to arterial stiffness,32 the accumulation of collagen and laminin in the atherosclerotic lesions can presumably be formed by the migration of VSMCs from the media, although the origin of the VSMCs is debatable.33 Interestingly, unlike previously reported proliferative/activated VSMCs (decrease in α-SMA and increase in VCAM-1 staining) in atherosclerotic lesions,9,13,34,35 subsets of lesional cells in the single mutants differed with respect to elevated expression of α-SMA (a marker of differentiated VSMCs) or of VCAM-1 (a marker of proliferative VSMCs).35,36 However, a proportion of these cells at discrete locations were stained positive for both α-SMA and VCAM-1. In contrast, VSMCs in the double E/V DKO resembled the differentiated phenotype that displays a uniform α-SMA immunoreactivity and minimal VCAM-1. These findings suggest that VCAM-1 expression is dependent on COX-2 in the vascular cells and that the impact of COX-2 can be compensated in the single mutants but not when the enzyme is completely depleted in vascular cells. Mature differentiated VSMCs retain the capacity for phenotypic plasticity.33 For example, in a rat model of balloon injury of carotid artery, medial VSMCs redifferentiate after endothelial injury and migrate to the intima, where they proliferate and secrete extracellular matrix components, such as collagen fibrils and elastin.37 In the present study, the proliferative VSMCs in mature neointima (after 14 days of endothelial injury) gradually redifferentiate and attain a contractile-like phenotype, as observed in the media.

Whereas immunohistochemical analysis clearly revealed an increase in lesional collagen content, SHG 2-photon microscopy analyses indicated that lesion size, fibrillar collagen content, and intensity were apparently unaltered between WT and E/V DKO. This disparity could be explained by the limitation of the SHG microscope in detecting the finer fibrillar collagen of lower-order structures. In addition to higher-order collagen structures, the antibody recognized the epitopes of fine collagen I structures, thus giving the higher signal. Despite this, we observed changes in the orientation of fibrillar collagens that accumulated in the atherosclerotic lesions. Specifically, fibrillar collagens in COX-2 E/V DKO lesions were significantly more random or less organized than those in WT lesions.

COX-2–derived prostanoids contribute to blood pressure control by regulating vascular tone and renal sodium transport,38 consistent with the risk of COX inhibitors in causing hypertension.39,40 Indeed, we have shown previously that vascular deletion of COX-2 renders mice susceptible to dietary salt–induced hypertension.41 In the present study, vascular COX-2 mutants on a hyperlipidemic background show a time-dependent increase in systolic blood pressure when placed on a HFD. This hypertensive phenotype is consistent with suppressed biosynthesis of PGI, or PGE,41–44: deletion of either prostacyclin receptor or E prostanoid-2 results in susceptibility to dietary salt–induced hypertension.45

A picture thus emerges that products of COX-2 may restrain or accelerate atherogenesis depending on the cell type in which they are formed. Global postnatal deletion of COX-2 may most closely resemble the consequences of systemic administration of COX-2 inhibitors, and, indeed, the acceleration of atherogenesis in these mice is consistent with the apparent gradual cardiovascular risk transformation observed.
in placebo-controlled, randomized trials of NSAIDs specific for inhibition of COX-2.21 However, this masks divergent effects of macrophage COX-2–derived TxA2, fostering disease and PGI2, derived from COX-2 in ECs and VSMCs acting as restraint. These observations and others28–31 suggest that targeting inhibitors of COX-2 selectively to the macrophage13 may shift fundamentally the balance of cardiovascular efficacy and risk for NSAIDs.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Placebo-controlled trials of cyclooxygenase-2 (COX-2)–selective nonsteroidal anti-inflammatory drugs have revealed higher cardiovascular risks associated with myocardial infarction, hypertension, heart failure, and stroke consequent to the suppression of cardioprotective prostanoids, particularly prostacyclin. We have reported previously that global postnatal deletion of COX-2 accelerates atherogenesis and that deletion of the receptor for prostaglandin I2 leads to initiation and early development of atherosclerosis in mice. Because of the contrasting biological impact of products of COX-2 and their varied predominance in cells during disease evolution, we have attempted to address the role of COX-2 in a tissue-specific manner. We have reported that deletion of COX-2 in myeloid cells retarded atherogenesis and that deletion in T cells had a minimal effect on lesion burden. In the present study, when mice were placed on a high-fat diet, deletion of COX-2 in endothelial or vascular smooth muscle cells or both increased systolic blood pressure and accelerated atherogenesis coincident with suppression of prostaglandin I2 biosynthesis. Suppression of COX-2 removes a constraint on enzyme expression in lesional macrophages, reflecting the interplay of the enzyme in cells relevant to the disease. Having a better understanding of the cell-specific biology of COX-2 deletion, we may advance the prospect for cell-targeted nonsteroidal anti-inflammatory drug delivery and minimize the cardiovascular adverse effects of these drugs.
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SUPPLEMENTAL MATERIAL

Cyclooxygenase-2 in Endothelial and Vascular Smooth Muscle Cells Restains Atherogenesis in Hyperlipidemic Mice

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Supplemental Methods

Preparation of Mouse Aortic Arch and Thoracic Aorta for Real-Time PCR Analysis of Gene Expression

Briefly, mouse aortic tree was perfused with ice-cold PBS dissolved in UltraPure™ DEPC-treated water to minimize degradation of RNA. Mouse aorta was cleaned of adventitial fat and dissected out into aortic arch and thoracic aorta. The aortas were immediately stored separately in RNALater® solution (Ambion, Austin, TX) at 4°C. After 24h, the aortas were transferred to -80°C for storage until analyses. RNA was extracted using TRIzol® Reagent (Life Tehcnologies, Grand Island, NY) and RNeasy Kit (Qiagen, Valencia, CA) following manufacturer’s protocol. Concentration and quality of extracted RNA from aortas were measured using NanoDrop® 1000 (Thermo Scientific, Wilmington, DE) and reverse-transcribed into cDNA using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Quantitative real time PCR was performed using Taqman Gene Expression Assays for Cox-1 (Mm00477214_m1), Cox-2 (Mm00478374_m1), Col1a1 (Mm00801666_g1), and Col1a2 (Mm00483885_m1) using an ABI Prism 7900HT real-time PCR system in a 384 well plate. Results were normalized with GAPDH (Mm99999915_g1).
S. Figure 1

(A) Relative mRNA levels for COX-2 in different conditions.

(B) AA-COX-2 levels under Chow diet.

(C) TA-COX-2 levels under Chow diet.

(D) AA-COX-1 levels under Chow diet.

(E) TA-COX-1 levels under Chow diet.

(F) AA-COX-2 levels under High fat diet.

(G) TA-COX-2 levels under High fat diet.

(H) AA-COX-1 levels under High fat diet.

(I) TA-COX-1 levels under High fat diet.
S. Figure 2
S. Figure 3
S. Figure 4
Supplemental Figure Legends

Supplemental Figure 1. High fat diet up-regulates expression of COX-2 in aortic arch and thoracic aorta. Aortic arch (AA) and thoracic aorta (TA) were dissected and RNA was extracted from WT and COX-2 KO mice on chow (6 months) or HFD (3 and 6 months). COX-2 transcripts were examined by real-time PCR as described in the Methods. A, A trend toward up-regulation of COX-2 in AA and TA after HFD feeding was observed in WT mice. COX-2 mRNA expression levels reflect vascular tissue specific COX-2 deficient mutants both on chow (B and C) or HFD (F and G). The expression levels of COX-1 in AA and TA were minimally altered in WT and COX-2 mutants on chow (D and E) or HFD feeding (H and I). Aortic COX-2 mRNA was increased in mice fed a HFD. Data are means ± SEMs. *p< 0.05, **p< 0.01, n=2-4 per group.

Supplemental Figure 2. Vascular cell COX-2 fails to modify atherogenesis in male mice after extended periods on a HFD. Aortic atherosclerotic lesion burden, represented by the percentage of lesion area to total aortic area, was quantified by en face analysis of aortas from mice fed HFD for 6 or 11 months. One-way ANOVA (Kruskal-Wallis test) showed no significant effects of genotype on lesion progression compared to WT. Data are means ± SEMs. n=18-22 (6 months) or 10-13 (11 months) per genotype.

Supplemental Figure 3. Vascular cell COX-2 deletion has minimal impact on lesional collagen content and intensity, and collagen gene expression in female mice on HFD. A and B, using second harmonic generation two-photon microscopy, quantification of collagen content and intensity were performed in 22 WT and 21 E/V DKO lesions from female mice on a HFD for 6 months. Data are means ± SEMs (Mann-Whitney test, two-tailed, p> 0.05). C, Thoracic
aortas were dissected and RNA was extracted from WT and COX-2 KO female mice fed 6 months HFD. Col1a1 and Col1a2 mRNA levels were examined by real-time PCR as described in the Methods. One-way ANOVA (Kruskal-Wallis test) showed no significant effect of genotype on collagen mRNA levels in thoracic aortas of KOs compared to WT, P> 0.05. Data are means ± SEMs. n=3 per genotype.

Supplemental Figure 4. Morphometric consequences of vascular COX-2 deletion on lesion development. Lesion morphology in aortic roots from female mice fed 6 months HFD were analyzed. Quantification of immunohistochemical staining of CD11c (A), CD11b (B), COX-1 (C) and COX-2 (D) from WT and KOs are shown in parallel with their representative aortic root sections. One-way ANOVA (Kruskal-Wallis test) showed a significant effect of genotype on lesional COX-2 positive cells (P=0.01). Dunnett’s multiple comparison tests were used to test significant differences between WT and COX-2 KOs. Despite a trend towards up regulation in staining for CD11c, CD11b, and COX-1 in COX-2 deficient mutants, no significant differences were detected. Data are means ± SEMs. n=3-5 (COX-1 and CD11c), n= 4-12 (CD11b and COX-2). L- lumen, m- media, nc- necrotic core.