COX-2–Derived Prostacyclin Modulates Vascular Remodeling

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Abstract—Suppression of prostacyclin (PGI2) biosynthesis may explain the increased incidence of myocardial infarction and stroke which has been observed in placebo controlled trials of cyclooxygenase (COX)-2 inhibitors. Herein, we examine if COX-2–derived PGI2 might condition the response of the vasculature to sustained physiologic stress in experimental models that retain endothelial integrity. Deletion of the PGI2 receptor (IP) or suppression of PGI2 with the selective COX-2 inhibitor, nimesulide, both augment intimal hyperplasia while preserving luminal geometry in mouse models of transplant arteriosclerosis or flow-induced vascular remodeling. Moreover, nimesulide or IP deletion augments the reduction in blood flow caused by common carotid artery ligation in wild-type mice. Generation of both thromboxane (Tx)A2 and the isoprostane, 8, 12–iso iPF2\(\alpha\)-VI, are increased in the setting of flow reduction and the latter increases further on administration of nimesulide. Deletion of the Tx\(\alpha\) receptor (TP) reduces the hyperplastic response to nimesulide and carotid ligation, despite further augmentation of TP ligand production. Suppression of COX-2–derived PGI2 or deletion of IP profoundly influences the architectural response of the vasculature to hemodynamic stress. Mechanism based vascular remodeling may interact with a predisposition to hypertension and atherosclerosis in contributing to the gradual transformation of cardiovascular risk during extended periods of treatment with selective inhibitors of COX-2. (Circ Res. 2005;96:1240-1247.)

Key Words: vascular remodeling • cyclooxygenase • oxidant stress • arteriosclerosis • prostaglandins

Cyclooxygenase (COX)-2 is the dominant source of prostaglandins (PGs) which mediate pain and inflammation, while COX-1 catalyzes the formation of PGs that subserve housekeeping functions, such as the maintenance of gastrointestinal (GI) integrity. Controlled trials of 2 specific inhibitors of COX-2,\(^1\) demonstrate that they cause significantly fewer serious GI adverse events than nonspecific, traditional nonsteroidal anti-inflammatory drugs (tNSAIDs) in patients with arthritis. Recently, rofecoxib was withdrawn from the market because of an increased incidence of myocardial infarction and stroke in the Adenomatous Polyp Prevention on VIOXX (APPROVe) trial. This hazard became increasingly apparent after only 18 months in these patients, who were initially at low apparent risk of cardiovascular disease.\(^2\) A similar excess in cardiovascular events has recently been reported with a third COX-2 inhibitor, celecoxib, again in a trial designed to prevent colonic adenomas.\(^3\) COX-2 inhibitors suppress biosynthesis of PGI2 without concomitant inhibition of thromboxane (Tx)A2,\(^4\) which derives predominantly from platelet COX-1.\(^5\) Suppression of this pathway would afford a mechanism by which the hazard of drug-induced thrombosis would relate to the patient’s underlying risk of cardiovascular disease.\(^6\) Indeed, this is consistent with evidence of a cardiovascular hazard in 2 placebo-controlled trials of valdecoxib, another member of the class, in patients undergoing coronary artery bypass grafting,\(^7\) a setting of hemostatic activation.\(^8\) Valdecoxib has now also been withdrawn from the market.

Given the implications for extended dosing with these drugs in patients at low risk of cardiovascular disease, interest has focused on the “latent period” before emergence of such a risk, such as the 18 months before the incidence of heart attack and stroke started to increase in patients treated with rofecoxib in the APPROVe study. Among the factors which might have contributed to acceleration of cardiovascular risk during that time are elevation of blood pressure\(^9\) and acceleration of atherogenesis,\(^10,11\) both of which may result from deletion of the receptor for PGI2 (the IP) in genetically predisposed strains of mice. Indeed, blood pressure was elevated by 3 to 4 mm Hg as early as 1 month in patients receiving rofecoxib in APPROVe.\(^2\) Aside from initiation and early development of atherogenesis, little is known about how suppression of COX-2 dependent PGI2 might condition the response of the vasculature to sustained stress, such as a rise in blood pressure. In this study, we examine the effect of disrupting PGI2 action in mouse models of transplant arteriosclerosis...
sclerosis and vascular remodeling which preserve the integrity of the endothelium, either by removing the IP or by administration of nimesulide, a nonproprietary but commercially available COX-2 inhibitor which confers COX-2 selectivity comparable to that of celecoxib.\textsuperscript{12} We report that either deletion of the IP or administration of a COX-2 inhibitor induces vascular hyperplasia and remodeling of the vasculature while maintaining luminal geometry. Such perturbation of the relationship between vascular hemodynamics and structure may interact with hypertension\textsuperscript{9,13} and atherosclerosis\textsuperscript{10,11} to contribute to an emerging cardiovascular hazard in patients initially at low cardiovascular risk during extended therapy with selective inhibitors of COX-2.

## Materials and Methods

### Animals

All animal studies were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. Male C57BL/6J mice, aged 4 to 6 weeks, were used for the flow-reduction models. The parental background strain of the male, 4 to 6 week-old donor mice in the transplant studies was also C57BL/6J (H2b). The background of the recipient mouse was C3H (H2k) (Taconic Farms Inc, Germantown, NY). Donor mice were homozygous for deletion of the IP (IPKO), as previously described\textsuperscript{14} or homozygous littermate controls. Wild-type (WT) littermate controls were also used for studies involving mice deficient in the Tx receptor (TPKOs).\textsuperscript{15}

Urine was collected over a 24 hour period in metabolic cages (Nalgene Labware) with vehicle or nimesulide allowed ad libitum. Urinary levels of prostanooid metabolites were measured by stable isotope dilution methods as previously described. The PGI\textsubscript{2} metabolite, 2, 3-dinor-6-keto PGF\textsubscript{1\alpha} (PGI-M) was measured by gas chromatography/mass spectrometry (GC/MS)\textsuperscript{16} and 2, 3 dinor TxB\textsubscript{2}, the Tx metabolite (Tx-M) was measured by reverse phase (C18) HPLC/MS/MS.\textsuperscript{16} The isoprostane, 8, 12-iso-IP\textsubscript{2} was measured by GC/MS, as described.\textsuperscript{17} A urine aliquot (0.1 mL) was used for measurement of creatinine by an automated colorimetric assay (Sigma Chemical Co., St Louis, Mo).

### Transplant Arteriosclerosis

Common carotid artery transplantations were performed as previously described.\textsuperscript{18,19} Briefly, the recipient mouse was prepared for transplantation via a midline cervical incision. The right common carotid artery (RC) was mobilized and divided midway from the innominate artery to the carotid bifurcation. The proximal and distal arteries were fashioned into arterial cuffs by passing the artery through autoclaved nylon tubing (0.63 mm O.D. and 0.5 mm I.D.), evverting the arterial ends over the tubing and securing with a 9 to 0 nylon tie. The donor mouse was operated on via a similar cervical midline incision: The full length of the RC was harvested between 8 to 10 minutes. No heparin or antibiotics were used at any time.

Grafts were harvested at 3 and 6 weeks after transplant. Graft harvest was undertaken by exposure of the donor artery, then median sternotomy and left ventricular puncture and an infusion of a 0.9% NaCl solution at 4°C. The donor carotid artery was then removed. The proximal half was placed in 4% phosphate-buffered formaldehyde (pH 7.2) and the distal half placed in OTC medium (Triangle Biomedical Sciences, Durham, NC) and rapidly frozen in liquid nitrogen. Five separate sections 10 µm apart were sectioned from different layers of the carotid artery. Sections were examined via a Sony 3CCD camera and television monitor and a Nikon microscope with images digitized with ImagePro analysis software (Media Cybernetics). The intima was defined as the region between the lumen and internal elastic lamina and the media as the region between the internal and external elastic lamina.

### Flow Dependent Vascular Remodeling

The left external carotid (EC) artery ligation (moderate) was performed as previously described.\textsuperscript{20} Briefly, the distal left common carotid artery (LC) artery and its bifurcation into the external and internal carotid were exposed using blunt dissection. 9-0 nylon sutures (USSC Sutures) were used to ligate the EC artery, incisions were closed (7-0 polypropylene) and mice were left to recover. Severe reduction in flow was achieved by ligating the LC artery just proximal to the external/internal carotid artery bifurcation. Five µm cross sections were used from parallel regions of RC and LC until reaching a parallel point at the RC artery branch point with the right subclavian artery. Although different permutations of this model of severe flow reduction may result in formation of neointima,\textsuperscript{21} dependent on the section of carotid artery analyzed\textsuperscript{13} and mouse strain,\textsuperscript{24,25} this was not observed in the present studies of severe flow reduction with complete LC ligation, unless the mice were also

![Figure 1. Luminal geometry is preserved through pronounced neointima formation in arteries transplanted from IPKO mice. Histological analysis (A) and quantitative morphometry revealed that both luminal area (B) and total vessel area (C) were significantly reduced in WT transplants (white bars) (P < 0.05) from 3 to 6 weeks after transplant. Lumen area in IPKO arteries (black bars) was comparable to WT arteries by 6 weeks. This involved pronounced and progressive neointimal proliferation (D) that exceeded what was observed in WT transplants (P < 0.05). Despite a divergent response to stress, the percent stenosis was comparable in the 2 groups 6 weeks after transplant (E) (bar = 100 µm).](http://circres.ahajournals.org/doi/abs/10.1161/CIRCRESAHA.118.314116)
treated with a COX-2 inhibitor (vide infra). Mice that developed thrombosis were excluded from the analysis. Mice were administered nimesulide (Sigma) in drinking water at a concentration of 40 mg/L containing 0.7% ethanol (changed tri-weekly) to achieve selective inhibition of COX-2, as previously described26 for 4 weeks. Subsequently, arterial ligation were performed in mice, and treatment was resumed with vehicle or nimesulide. 4 weeks after the ligation (a total of 8 weeks, receiving nimesulide) urine was collected from individual mice and frozen at -20° for analysis.

**Semiquantitative RT-PCR**

RC and LC were harvested, flash frozen, and pulverized to a fine powder. Total RNA was extracted using Trizol (Life Technologies). 500 ng RNA from individual common carotid arteries was reverse transcribed (RT), as described27. The RT reaction (2 μL) was amplified using Taq DNA polymerase (Boehringer Mannheim) and primers to murine COX-2 cDNA (sense: 5'-CCGGGTGCTGGGG-GAAGA)-3'; antisense: 5'-GTGGCTGTGGTGAAGCCTGGGA-3'). The PCR profile was set at 94°C melting, 55°C annealing, and 72°C extension for 2 minutes and semiquantitation was optimized to 27 cycles. The amplified transcripts were visualized on 1.5% agarose gels with the use of ethidium bromide.

**Immunohistochemistry and Immunoblotting**

Mice were exsanguinated via intracardial injection of PBS. Common carotid arteries were dissected and rapidly embedded for frozen cross-sectioning. Following a fixation step of 20 minutes in 70% ethanol, a quenching step of endogenous peroxidases, and an overnight block with goat serum, an antimurine COX-2 pAb (1:500, Cayman Biochemicals) was incubated overnight at 4°C. Subsequently, COX-2 immunostaining of frozen cross sections was performed using an ABC amplification method and DAB detection assay (Vector Laboratories). No staining occurred in sections incubated with secondary antibody alone. Immunoblotting for HO-1 was performed using a polyclonal antibody (Stressgen) and detected by a chemiluminescent method (ECL, Amersham).

**Blood Flow**

Blood flow was measured in RC or LC at their midpoint, using an ultrasonic flow probe (0.5 PSB-series probe, Transonic Systems Inc). Despite severe conditions of flow restriction, with cessation of flow distal to the ligation, a net flow pulse was retained across the flow probe after 1 week of ligation. Blood flow was recorded for 1 minute and a 30-second interval was used to determine and analyze peak blood flow using the PowerLab data acquisition system (ADInstruments) and Chart 4.0 software.

**Statistical Analysis**

Statistical analyses were performed using a computerized software package (GraphPad Prism version 3.02). The results presented are represented as mean±SEM. Experimental and control groups were analyzed using analysis of variance followed by posttest as indicated.
Results

Prostacyclin Receptor Deletion and Transplant Arteriosclerosis

Arteries allogenically transplanted from WT C57BL/6 mice to the histoincompatible C3H background exhibited the typical arteriopathy of rejection (Figure 1A, top panel) which progressed over time. Quantitative analysis of arterial geometry in transplanted arteries revealed a significant reduction in lumen area that occurred from 3 to 6 weeks after transplant (Figure 1B). Similarly, total vessel size quantified as cross-sectional area, declined in WT transplants from 3 to 6 weeks (Figure 1C). These reductions in vessel caliber were accompanied by neointimal proliferation 3 weeks after transplantation, as expected (Figure 1D). Neointimal proliferation and a reduction in total vessel area increased stenosis from 3 to 6 weeks after transplantation (Figure 1E). Deletion of the IP modulated progression of the arteriopathy (Figure 1A, bottom panel). Lumen area and total vessel area from transplanted IPKO arteries remained stable from 3 to 6 weeks (Figure 1B and 1C). However, the proliferative response was greater at 3 weeks in arteries lacking the IP and continued neointimal growth was evident 6 weeks after transplantation, a time when proliferation in the WT transplants had stabilized (Figure 1D). Despite these disparate effects on neointimal proliferation, encroachment on luminal area by 6 weeks was comparable between WT and IPKO arteries (Figure 1E). The blood flow–dependent regulation of COX-2 expression we observe in vivo is consistent with observations in cultured endothelial cells exposed to laminar shear stress.27

COX-2 Inhibition and Flow-Induced Vascular Remodeling

We next examined the consequences of suppressing COX-2 dependent PGI2 biosynthesis following surgically induced chronic flow reduction. Left EC ligation results in a moderate reduction in blood flow along the LC and a consequent reduction in LC luminal diameter, endothelial dysfunction, and an increase in cell death.28 After 7 days, expression of COX-2 mRNA was increased in response to the reduction in flow in the LC compared with the contralateral RC in wild-type mice (Figure 2A). Correspondingly, when the reduction in flow was severe, the increase in expression of COX-2 was more pronounced (Figure 2A). Immunohistochemical analysis revealed an increase in COX-2 expression in LC relative to RC that persisted after 4 weeks of severe flow reduction (Figure 2B). Interestingly, both nonligated RC and LC also exhibited detectable expression of COX-2 (data not shown).

Chronic administration of the COX-2 inhibitor, nimesulide, to wild-type mice depressed urinary PGI-M (Figure 2C) as expected,5 but did not effect either the internal vascular geometry, as measured by lumen diameter (Figure 2D i) or external structure, as reflected by the external elastic lamina (EEL) diameter (Figure 2D ii) of RCs or LCs obtained from control mice. Nimesulide depressed PGI-M to a similar degree under conditions of moderate and severe reduction in flow (Figure 2C). However, inhibition of COX-2 failed to alter the flow dependent reduction in vessel size, whether quantified as reductions in luminal (Figure 2D i), or EEL diameter (Figure 2D ii). This contrasted with a marked augmentation effect of neointimal proliferation by nimesulide under conditions of severe flow reduction (Figure 3A and 3B). IPKO mice undergoing severe flow reduction exhibited a reduction in lumen diameter comparable to wild-type mice, but a disproportionate change in EEL diameter, suggestive of vascular wall hypertrophy, a response analogous to that of neointimal growth observed during COX-2 inhibition. In contrast, TPKOs on a regimen of nimesulide exhibited proportional changes in lumen and EEL diameter (Figure 2D i, ii). Nimesulide also caused the reduction in blood flow in the LC that was comparable to IPKOs, either when compared with vehicle treated (Figure 4A and 4B) or contralateral controls (Figure 4C). Thus, consistent with the effects of IP deletion in transplant arteriosclerosis, suppression of PGI2 biosynthesis with nimesulide, under conditions of severe flow restriction, resulted in neointimal proliferation and compensatory vascular remodeling that preserved luminal geometry.

Events indicative of a predisposition to proproliferative signaling cascades were apparent under severe, but control (vehicle) conditions of flow reduction. Platelet activation, as reflected by Tx-M excretion, occurred during complete LC ligation under control conditions (Figure 5A). Inhibition of COX-2 with nimesulide failed to suppress Tx-M excretion under control conditions (Figure 5A), as expected.26 Oxidant stress, as reflected by urinary 8, 12-iso iPF2alpha-VI (Figure 5B),
was markedly augmented by nimesulide following severe flow reduction. Given that both Tx and isoprostanes can ligate the TP, the experiments were repeated in TPKOs. Although Tx and isoprostane generation in TPKOs given nimesulide was further augmented, (Figure 5A and 5B), the proliferative response (quantified as an increase in wall area) to severe flow reduction was markedly reduced in TPKO’s (Figure 5C). Moreover, IPKOs subjected to severe flow reduction also exhibited vascular wall hyperplasia, reminiscent of the response in wild-type mice administered nimesulide.

Discussion

COX-2 is a major source of PGI₂ biosynthesis in humans.⁴ Depression of PGI₂, without a concomitant inhibition of Tx, has been suggested as a mechanism by which selective inhibitors of COX-2 might predispose individuals to cardiovascular hazard.⁴,⁵ The emergence of this risk in placebo controlled clinical trials involving three COX-2 inhibitors is compatible with such a class based effect. Deletion of the IP predisposes mice to an exaggerated response to thrombotic stimuli¹¹ and both modulates blood pressure³⁰–³² and accelerates atherogenesis in genetically predisposed animals.¹⁰,¹¹ Deletion of the IP also modulates the response to vascular injury, resulting in a hyperproliferative response which encroaches on luminal integrity.¹⁴ However, little is known about how this pathway might modulate vascular structure and integrity when hemodynamic stress is sustained in the presence of intact endothelium, such as might occur in hypertension.

We sought to address this question using both genetic and pharmacological disruption of the pathway in 2 models of physiologic stress in which endothelial integrity is preserved. Allogenic transplantation of the carotid artery results in a proliferative, progressively occlusive response, a model of human transplant arteriosclerosis. Deletion of the IP exacerbated intimal hyperplasia. Although luminal encroachment was similar in IPKO and WT arteries 6 weeks after transplantation, this was achieved through markedly distinct structural rearrangements. Both a reduction in total vessel caliber and intimal hyperplasia accounted for the luminal narrowing in WT transplants. In contrast, luminal narrowing in IPKO transplants occurs primarily via an augmented neointimal proliferative response without a change in total vessel caliber. A similar response, hyperplasia with remodeling, was seen when arteries were subject to hemodynamic stress by severe reduction in flow in the ligation models. Here, a COX-2 selective regimen of nimesulide suppressed PGI₂ biosynthesis, but not Tx formation, in control mice. However, although nimesulide and deletion of IP both exacerbated the decline in pulsatile carotid blood flow in the more severe ligation model and resulted in marked vascular hyperplasia, they did not further modify the reduction in luminal diameter, which occurred consequent to arterial ligation. The reduction in flow with nimesulide and IPKOs may have reduced the shear stress to which the vessel was subjected. Indeed, low shear
acts as a stimulus to vascular smooth muscle cell proliferation.34–36 These results, congruent with those obtained with IP deletion in the transplant model, contrast with the exacerbation of the hyperplastic response to wire vascular injury in IPKOs.14 Here, substantial luminal encroachment was observed; the phenotype was rescued by coincidental deletion of the TP. Thus, retention of endothelial integrity favors preservation of luminal diameter.

While nimesulide did not influence Tx biosynthesis in the control mice, Tx formation in the setting of severe flow reduction was exacerbated. Such a COX-1 dependent source of Tx formation may reflect platelet activation in the face of hemodynamic perturbation; this has previously been demonstrated under conditions of both injury and flow reduction in common carotid arteries of rabbits.37 Exacerbation of a Tx response may in part be caused by the removal of PGII as a constraint on platelets, which we previously demonstrated in the context of vascular injury.14 Here under conditions of flow reduction, suppression of PGII by nimesulide or disruption of its activity by IP deletion may have magnified the effects of hemodynamic-induced Tx formation. PGII acts as a constraint on oxidant stress, both in occlusion/reperfusion injury38 and as atherogenesis proceeds in genetically predisposed mice.10 Moreover, inhibition of COX-2 markedly augmented a marker of oxidant stress, 8, 12-iso iPF2α−VI in the setting of severe flow reduction. Given that both isoprostanes and TxA2 can activate the TP39 and that coincidental deletion of the TP largely rescued the hyperplastic response to vascular injury in IPKOs,14 we examined vascular remodeling in TPKOs. Here, we found that hyperplasia induced by nimesulide in the setting of severe flow reduction was markedly diminished. This was despite a further augmentation in both TP ligands when the TP was deleted. This apparent compensatory increase in ligand generation is consistent with recent observations we have made in atherosclerotic mice. Here, Tx biosynthesis increases with atherogenesis and is further increased by a structurally distinct COX-2 inhibitor. Analogous to the experience with TP deletion, addition of a TP antagonist to the COX-2 inhibitor results in a marked augmentation of Tx biosynthesis and plaque destabilization.33

Figure 5. Vascular wall hyperplasia caused by COX-2 inhibition in the setting of severe flow reduction is mediated via the TP. A, Four weeks after ligation, severe reduction in flow results in an increase in urinary Tx-M in both untreated (severe vs control; n=10, *P<0.01) and nimesulide treated (severe/nimesulide vs control; n=10, †P<0.01) mice. This increment was further augmented significantly (TPKO vs severe/nimesulide and severe/vehicle; n=7 †P<0.01) in TPKOs. B, A similar pattern was seen with urinary 8, 12-iso iPF2α−VI, where generation of the isoprostane increased in the face of severe flow reduction only in mice treated with the COX-2 inhibitor (severe/nimesulide vs severe/vehicle; n=9, 7 respectively; †P<0.05). Again, isoprostane generation was further augmented in nimesulide treated mice lacking the TP (severe/nimesulide/TPKO vs severe/vehicle; n=7, 7 respectively; †P<0.01). C, Vascular hyperplasia in remodeled LC quantified here as wall area was increased significantly by severe flow reduction with nimesulide (n=9, P<0.05) and also IP deletion (n=6: P<0.05), but diminished by TP deletion treated with the COX-2 inhibitor. All analysis performed in mice, which underwent vehicle/nimesulide treatment for 8 weeks, while some mice underwent a 4-week flow reduction in the 8-week interval. Statistical significance was determined in A and B by one-way ANOVA and Bonferroni’s Multiple Comparison Test and in (C) by unpaired 2-tailed Student t test.
tory role for mediators derived from endothelium, such as NO. Evidence for such redundancy derives from eNOS KOs in which flow mediated vasodilatation is preserved by enhanced release of vasodilator prostaglandins, such as PGI₂ and endothelial hyperpolarizing factor.40,41

Vascular modeling occurs in both hypertension42–44 and atherosclerosis45 and deletion of the IP results in a rise in blood pressure (Francois and Coffmann unpublished observations; 2004) and accelerates atherogenesis46,47 in genetically prone mice. Here, we show a distinct impact on the remodeling response to hemodynamic stress, again mediated, at least in part, by removal of a biological constraint on TP ligands. These consequences of PGI₂ suppression, elevation of blood pressure, acceleration of atherogenesis and modulation of the vascular response to hemodynamic stress, may converge to alter vascular architecture and elevate cardiovascular risk during extended dosing with selective inhibitors COX-2.

Acknowledgments

Supported in part by grants from the National Institutes of Health (T32-HL07439, HL 62250 and HL 54500). G.A.F. is the Robinette Foundation Professor of Cardiovascular Medicine and the Elmer Bobst Professor of Pharmacology.

References


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Circ Res. 2005;96:1240-1247; originally published online May 19, 2005;
doi: 10.1161/01.RES.0000170888.11669.28
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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