Prostacyclin Synthase Gene Transfer Modulates Cyclooxygenase-2–Derived Prostanoid Synthesis and Inhibits Neointimal Formation in Rat Balloon-Injured Arteries

Michiharu Yamada, Yasushi Numaguchi, Kenji Okumura, Mitsunori Harada, Keiji Naruse, Hideo Matsui, Takayuki Ito, Tetsuo Hayakawa

Abstract—Previous studies have shown that prostacyclin (PGI₂) synthase (PCS) gene transfer inhibits neointimal formation in balloon-injured arteries. However, the role of each cyclooxygenase (COX) isoform in this healing mechanism remains unknown. We hypothesized that overexpression of PCS may modulate COX-2–mediated prostaglandin (PG) metabolism. That is to say, excessive PGH₂ derived from COX-2 after balloon injury may be converted into PGI₂ rather than PGE₂ or thromboxane (TX) A₂ by overexpressed PCS. We examined the expression of COX isoforms and evaluated the role of COX-2 with regard to the effects of PCS gene transfer by using 4-(4-cyclohexyl-2-methyloxazol-5-yl)-2-fluorobenzenesulfonyamide (JTE-522), a selective COX-2 inhibitor. Rats were divided into 4 groups in conjunction with PCS gene transfer and JTE-522 treatment. The PCS gene (30 μg) was transfected into rat balloon-injured arteries by a lipotransfection method. JTE-522 (30 mg/kg per day) was administered for 14 days after balloon injury. Immunohistochemical analysis demonstrated marked COX-2 expression on the neointima. PCS gene transfer markedly inhibited neointimal formation, but JTE-522 reversed this beneficial effect. PCS gene transfer augmented PGI₂ production and decreased PGE₂ production without affecting TXA₂ production, but JTE-522 inhibited this increase in PGI₂ production. In conclusion, PCS gene transfer modulated COX-2–mediated prostanoid synthesis and inhibited neointimal formation after balloon injury. (Arterioscler Thromb Vasc Biol. 2002;22:256-262.)

Key Words: prostacyclin ■ cyclooxygenase-2 ■ gene therapy ■ restenosis ■ balloon injury

Cyclooxygenase (COX) is the key enzyme that regulates the amount and duration of prostaglandin (PG) production in vessels. Recently, the role of COX in cardiovascular diseases such as atherosclerosis has been studied extensively. COX converts arachidonic acid into PGG₂, and then into PGH₂. PGH₂ is metabolized by specific isomerases to prostanoids such as prostacyclin (PGI₂), thromboxane (TX) A₂, and PGE₂, which exert a variety of biological actions involved in the maintenance of vascular homeostasis. There are 2 isoforms of the enzyme, COX-1 and COX-2, and they are encoded by distinct genes. COX-1 is constitutively expressed in most tissues, whereas COX-2 is an inducible isoform and almost undetectable in physiological conditions. COX-2 is induced by interleukin-1β, tumor necrosis factor-α, bacterial lipopolysaccharide, growth factors, phorbol esters, and other agents.

In normal vessels, the endothelium is enriched with COX activity, and the constitutive isoform of COX (COX-1) determines PG production in the endothelium. Once the barrier function of the endothelium is disrupted by an injury, such as angioplasty, COX-1 activity may decrease, and in turn, to compensate for the loss of COX-1 function, COX-2 expression is rapidly induced to an excessive level, resulting in an imbalance of PGs among PGH₂, PGE₂, PGI₂, and TXA₂. In that case, the vessel wall tends to be vasoconstrictive and thrombogenic, which might, in turn, lead to atherosclerosis. Prostacyclin is generated by vessel endothelium and vascular smooth muscle cells (VSMCs). PGI₂ is a potential vasodilator, which inhibits smooth muscle cell proliferation and platelet aggregation via cAMP and also modulates cholesterol turnover. Thus, PGI₂ may play an important role in modulating the vascular response to injury. We have recently shown that prostacyclin synthase (PCS) gene transfer inhibits neointimal formation in rat balloon-injured arteries; however, the role of each COX isoform in this healing mechanism after arterial injury remains unknown. Pritchard et al have reported that the smooth muscle cells of arteries express COX-2 mRNA and COX-2 protein after balloon injury. Moreover, there is evidence that COX-2 is expressed on VSMCs in human atherosclerotic lesions. Previous studies have indicated that there may be a specific link between the accumulation of PGE₂ and the induction of COX-2. We hypothesized that COX-2–derived excessive PGH₂ could be converted into PGI₂ rather than PGE₂ and TXA₂ by the

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From Internal Medicine II (M.Y., K.O., M.H., H.M., T.H.), Physiology II (K.N.), and the Department of Health Sciences (T.I.), Nagoya University School of Medicine, Nagoya, Japan, and the Departments of Surgery and Pathology (Y.N.), Children’s Hospital, Harvard Medical School, Boston, Mass.
Correspondence to Kenji Okumura, MD, Internal Medicine II, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8550, Japan. E-mail kenji@med.nagoya-u.ac.jp
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256
overexpression of PCS. We carried out immunohistochemical analysis to clarify the distribution of COX isoforms in rat arterial walls after balloon injury and evaluated the involvement of COX-2 by using 4-[(4-cyclohexyl-2-methyloxazol-5-yl)-2-fluorobenzenesulfonyl]amide (JTE-522), a selective COX-2 inhibitor, after PCS gene transfer.

Methods

Preparation of PCS Plasmid DNA

The plasmids encoding the PCS gene used in the present study have been described in detail previously. Briefly, a polymerase chain reaction–amplified PCS gene was cloned into pTarget (Promega), a cytomegalovirus (CMV) enhancer/promoter and simian virus 40 polyadenylation signal–driven mammalian expression vector, yielding pCMV-PCS. The functional character of these plasmids, such as protein expression level in vitro, has been confirmed in our previous reports.

Gene Delivery In Vivo and Inhibition of COX-2

Male Sprague-Dawley rats weighing 350 to 400 g were obtained from Japan SLC, Inc (Nagoya, Japan). We followed the National Institutes of Health guidelines regarding care and use of the animals. The in vivo gene transfer into rat carotid arteries was performed as previously described. Rats were anesthetized with sodium pentobarbital (50 mg/kg IP). After a midcervical incision, the right common carotid artery and its bifurcation were exposed. To prevent acute thrombosis during the procedure, heparin sodium (200 IU/kg) was intravenously injected 5 minutes before the balloon injury. The right common carotid artery was balloon-injured 3 times with a 2F Fogarty catheter (Baxter Healthcare) inserted through the external carotid artery as previously described by Clowes et al. To attain a constant degree of vessel wall injury for each of the animals, we kept the diameter of the balloon and the resistance during withdrawal constant and the same for each of the animals. The sham-operation involved simple ligation of the right external carotid arteries without balloon injury. Two vascular clips were placed at the distal end and in the middle of the injured arterial segment. A 24-gauge cannula was introduced into the common carotid artery via the external carotid artery. The lumen of the injured segment between the 2 clips was washed with PBS. pCMV-PCS (30 μg) with 100 μL Lipoctectamine Plus reagent (GIBCO-BRL) in a total volume of 300 μL was instilled into the lumen for 30 minutes, and then the catheter was carefully withdrawn, and the blood flow was restored. After confirmation of the carotid arterial flow, the wound was sutured. All procedures were performed under sterile conditions. In a prior study, the extent of endothelial denudation was confirmed at 2 days after balloon injury by Evans blue staining. We confirmed that the present plasmid vector system could transfer target genes into the injured arteries by using a plasmid carrying the lacZ gene (pCMV-lacZ) based on X-Gal staining, and the transgene efficiency was 8.5% in our previous reports. JTE-522 was suspended in 0.5% carboxymethylcellulose sodium (CMC-Na) solution. JTE-522 (30 mg/kg) or vehicle (0.5% CMC- Na) was administered orally at a volume of 5 mL/kg with gastric gavage starting 30 minutes before the injury and continuing for 14 days. The balloon-injured rats were divided into 4 groups in conjunction with PCS gene transfer and COX-2 inhibitor treatment as follows: (1) vehicle (injured group), (2) pCMV-PCS plus vehicle (pPCS group), (3) JTE-522 (JTE-522 group), and (4) pCMV-PCS plus JTE-522 (pPCS+JTE-522 group). All groups had 6 rats. Systolic blood pressure and heart rate were measured before and 14 days after balloon injury by the tail-cuff method with a sphygmomanometer (BP98A, Softron). The urinary level of 6-keto-PGF_1alpha was measured with a radioimmunoassay kit (NEN).

Immunohistochemistry

To evaluate COX-1 and COX-2 expression in balloon-injured arteries (n = 6) and uninjured arteries from sham-operated animals (n = 6), the rats were killed at 3, 7, and 14 days after balloon injury, and the right carotid arteries were perfusion-fixed with 2.5% phosphate-buffered glutaraldehyde and harvested under anesthesia with sodium pentobarbital. Immunohistochemical labeling was carried out on adjacent tissue sections. The excited carotid arteries were paraffin-embedded, cut into 4-μm cross sections, and mounted on glass slides. These sections were incubated in 0.3% hydrogen peroxide methanol for 30 minutes to block endogenous peroxidase activity. The nonspecific binding of rabbit serum was prevented by preincubating the sections with 0.1% normal goat serum. The sections were sequentially incubated at 4°C overnight with polyclonal rabbit anti-murine COX-1 antibody (catalogue No. 160109, Cayman Chemical Co) at a concentration of 1:200 or polyclonal rabbit anti-murine COX-2 antibody (catalogue No. 160106, Cayman Chemical Co) at a concentration of 1:200. After they were washed with PBS, the sections were incubated with biotinylated goat anti-rabbit IgG-conjugated horseradish peroxidase (Dako Japan Co) for an additional 60 minutes at room temperature. Each incubation was followed by a wash in PBS. Staining was visualized with chromogen, 0.06% 3,3′-diaminobenzidine/5% hydrogen peroxide in 0.05 mol/L Tris-HCl (pH 7.6), and hematoxylin for a counterstain. Control sections were incubated with nonimmune rabbit IgG at a concentration of 1:200.

Prostanoid Production in Rat Carotid Artery

The injured carotid arteries of each group were resected 7 days after balloon injury. The arteries were cut into 5-mm lengths, washed with PBS, and incubated in 1 mL of PBS (pH 7.4) at 37°C for 45 minutes. The levels of 6-keto-PGF_1alpha, TXB_2, and PGE_2 in the medium were measured to evaluate local PG1, TXA2, and PGE production, respectively, with radioimmunoassay kits (NEN). Moreover, we similarly examined the effect of COX-1 and COX-2 blockade in the injured and PCS gene-transfected arteries (n = 6 each) by using indomethacin (Sigma), a nonselective COX inhibitor. Indomethacin (5 mg/kg) was suspended in 0.5% CMC-Na solution and administered orally at a volume of 5 mL/kg with gastric gavage once daily for 7 days after balloon injury.

Quantification of Neointimal Formation

Fourteen days after balloon injury, 6 cross sections from each carotid artery were stained with hematoxylin and eosin and examined morphometrically with a computerized digital image analysis system (NIH image) in a blind manner. The areas within the internal elastic lamina (EEL area), the internal elastic lamina (IEL area), and the luminal area were measured. Other areas were calculated as follows: medial area = EEL area – IEL area; neointimal area = IEL area – luminal area; neointima-to-media (IM) ratio = neointimal area/medial area.

Statistical Analysis

All data are expressed as the mean ± SEM. Statistical analysis for multiple comparisons among the groups used 1-way ANOVA followed by the Bonferroni test. A value of P < 0.05 was considered statistically significant.

Results

COX Isoform Expression in Rat Balloon-Injured and Uninjured Arterial Vessel Wall

Fourteen days after balloon injury, sections of the injured and uninjured arterial segments were analyzed for COX-1 and COX-2 protein by immunohistochemical analysis (n = 6 each). In the balloon-injured arteries, there was diffuse expression of COX-2 protein on the neointima and adventitia (Figure 1D and 1F), whereas COX-1 expression was not detectable on the neointima (Figure 1C and 1E). Interestingly, COX-1 and COX-2 colocalized with regenerated endothelial cells (inset, Figure 1E and 1F). In contrast, in the uninjured control arteries, there was constitutive expression of COX-1 protein in the endothelium and adventitia (Figure 1A), whereas there was no detectable COX-2 except in the adventitia (Figure 1B).
Time Course of COX-1 and COX-2 Expression

We examined the time course of COX-1 and COX-2 expression after balloon injury by immunohistochemistry. COX-2 was expressed in the exposed subendothelium and in the adventitia by 3 days after injury (Figure 2B), it was clearly evident in the neointimal and adventitial areas by 7 days (Figure 2C), and it was widely expressed in the same areas by 14 days (Figure 2D). However, COX-1 expression was not detectable in the neointima except for the regenerated endothelium at any time after injury.

Relative Influence of PCS Gene Transfer and COX-1 and COX-2 Blockade on Prostanoid Production in Rat Carotid Arteries

We evaluated the production of PGI₂ in the arterial vessels by determining the 6-keto-PGF₁α levels 7 days after injury and found the levels to be significantly lower in balloon-injured vessels than the uninjured vessels. In contrast, the levels were significantly higher in pCMV-PCS–transfected vessels than in the injured vessels. The PCS gene transfer restored the 6-keto-PGF₁α levels to values even higher than those of the uninjured vessels; however, this effect of pCMV-PCS gene transfer was markedly suppressed with either indomethacin or JTE-522 (n = 6 in each group, Figure 3A). We evaluated TXA₂, the counterpart of derivatives from PGH₂ in the same samples, by determining the levels of TXB₂. The TXB₂ production levels were higher in the injured vessels than in the uninjured vessels, whereas there was no significant difference in the TXB₂ levels between the injured and pCMV-PCS–transfected vessels. Thus, balloon-injured carotid arteries transfected with the PCS gene were able to produce a larger amount of PGI₂ without any changes in their ability to produce TXA₂. JTE-522 had no effect on TXB₂ production (n = 6 in each group, Figure 3B). Moreover, PGE₂ levels, which are thought to reflect COX-2 activity in tissue, were measured in the same samples. There was no significant difference in the PGE₂ levels between the injured and uninjured vessels, whereas the levels in pCMV-PCS–transfected vessels were significantly lower than the levels in the injured vessels. JTE-522 and indomethacin also markedly suppressed PGE₂ production levels (n = 6 in each group, Figure 3C).

Systemic Effects of PCS Gene Transfer and JTE-522 Administration

Fourteen days after injury, systolic blood pressures for the test rats were 129±7, 124±2, 129±4, and 129±3 mm Hg in the injured, pPCS, JTE-522, and pPCS/JTE-522 groups.
respectively (n=6 each, P=NS). Regarding heart rate, no significant difference was observed among the 4 groups.

The baseline levels of urinary 6-keto-PGF\textsubscript{1α} were not significantly different among the 4 groups. Seven days after balloon injury, the urinary levels of 6-keto-PGF\textsubscript{1α} did not significantly change in any of the groups (data not shown). Thus, local delivery of pCMV-PCS to the carotid artery and systemic administration of JTE-522 had no effect on the systemic production of 6-keto-PGF\textsubscript{1α}.

**Effects of PCS Gene Transfer and JTE-522 on Neointimal Formation**

Figure 4 shows representative histological photomicrography of common carotid arteries 14 days after the injury. Figure 5 shows the morphometric analysis of these arteries. Morphometric analysis showed that the neointimal area in the pPCS group was reduced by 47.4% and that the I/M ratio was reduced by 45.5%, which provided larger luminal areas compared with the injured group 14 days after the injury. However, these beneficial effects of PCS gene transfer were reversed by JTE-522. There was no significant difference in the EEL area or medial area among the 4 groups.

**Discussion**

In the present study, we have shown that there is a marked expression of COX-2 on the neointima and adventitia in balloon-injured rat carotid arteries, whereas there is less expression of COX-1 on the neointima as observed during immunohistochemical analysis. Increased COX-2 expression was observed by 3 days after injury and was sustained up to 14 days. Furthermore, we observed significant inhibition of neointimal formation (47.4% and 45.5% reduction in the neointimal area and the I/M ratio, respectively) at 14 days after injury by single PCS gene transfer, but this beneficial effect was reversed by the COX-2 inhibitor in this animal model of restenosis.

We used JTE-522 as a selective COX-2 inhibitor. JTE-522 is selective for COX-2 at a daily dose of 0.3 to 30 mg/kg, and it does not cause severe gastric lesions at oral doses up to 300 mg/kg in rats.\textsuperscript{27} We administered JTE-522 (30 mg/kg per day)
orally with gastric gavage for 14 days. This relatively high dose of JTE-522 was selected because optimal inhibition of COX-2 in the vessel walls was insufficient with a dose <10 mg/kg per day in our prior study. At this dose, we did not observe any systemic side effects derived from the administration of JTE-522.

We have shown the time course of COX isoform expression and localization in balloon-injured arteries by immunohistochemical analysis. Pritchard et al.\(^1^8\) have reported that smooth muscle cells of rat arteries express COX-2 mRNA and COX-2 protein during 14 days after balloon injury by Northern and Western blot analysis. We could demonstrate COX-2 expression in the neointima and adventitia up to 14 days after balloon injury. The present results are consistent with those of Pritchard et al.

We did not observe a significant reduction in neointimal formation after arterial injury by JTE-522 at a dose of 30 mg/kg per day. Although we obtained an inhibition of neointimal formation after balloon injury by PCS gene transfer, this effect was completely reversed by JTE-522 treatment. These findings demonstrate that the effects of PCS gene transfer in injured arteries are functionally concerned with COX-2 expression. In terms of PG production, PGI\(_2\) production decreased significantly after balloon injury, whereas PCS gene transfer increased to more than the levels found in uninjured vessels. This effect of PCS gene transfer was reversed by either JTE-522 or indomethacin to the levels found in balloon-injured arteries (Figure 3A). PGI\(_2\) synthesis in the balloon-injured arteries that were transfected with the PCS gene appears to be tightly coupled with the COX-2 protein expression that increased after balloon injury in vivo. PGE\(_2\) enhances platelet aggregation\(^2^8,^2^9\) and chemotaxis of leukocytes, it increases vascular permeability\(^3^0,^3^1\) and it can inhibit in vitro cholesterol ester hydrolase activity, thus leading to lipid deposition in the arterial walls.\(^3^2\) Therefore, an increased release of PGE\(_2\) may promote the development of atherosclerosis.\(^1^0\) PGE\(_2\) production, which is thought to reflect COX-2 activity,\(^2^2,^2^3\) was not significantly changed by

![Figure 5](image_url). Morphometric analysis of rat carotid arteries 14 days after injury. Neointimal area (A) and I/M ratio (B) of the pPCS group were significantly reduced, and luminal area (C) was significantly enlarged in the pPCS group compared with the injured group, but these effects were reversed by JTE-522 treatment. At 14 days, there was no significant difference in medial area (D) or EEL area (E) among the 4 groups. Data are mean±SEM (n=6 each). *P<0.01 vs injured; †P<0.01 vs pPCS.
balloon injury. JTE-522 and indomethacin markedly suppressed PGE₂ production in the injured arteries (Figure 3C), whereas JTE-522 had no effect on PGE₂ production in the uninjured arteries in our preliminary study (data not shown). These results suggest that in normal vessels, COX-1 contributes to PGE₂ synthesis, whereas COX-2 mainly contributes in the injured arteries. Thus, the induction of COX-2 in the injured arteries may represent a compensatory mechanism that ensures the release of PGE₂ when the endothelium is damaged. In the present study, we found that PGE₂ production was significantly reduced by PCS gene transfer after balloon injury. This phenomenon may contribute to the reduction of neointimal formation after arterial injury. Although TXA₂ is mostly generated by platelets in normal vessels, it is produced in only very small amounts by endothelium and VSMCs. In the present study, TXA₂ production was significantly increased after balloon injury. Indomethacin markedly inhibited the increase of TXA₂ production in the injured vessels, whereas JTE-522 had little effect on it (Figure 3B). These findings suggest that in the injured arteries, COX-1 mainly contributes to the increase in TXA₂ synthesis. However, platelets that had adhered to the injured vessel walls may be responsible for this increase of TXA₂ production in the injured arteries, because platelet COX-1 is the major source of TXA₂. The balloon-injured arteries transfected with the PCS gene produced higher levels of PGH₂ than the balloon-injured arteries, downregulation of local PGI₂ production may occur through downregulation of COX-1 and PCS activity by disruption of endothelium. Moreover, this mechanical disruption of endothelium may induce COX-2 excessively to compensate for reduced COX-1 activity and result in the overproduction of PGH₂. Local PGE₂ production may not be affected because of a COX-2-mediated compensatory mechanism. This imbalance of PGs among PGH₂, PGI₂, PGE₂, and TXA₂ may be involved in the development of neointimal formation after balloon injury. However, in the balloon-injured vessels transfected with the PCS gene, excessive PGH₂ derived from the COX-2 pathway may be converted into PGI₂ rather than PGE₂ or TXA₂ by the overexpression of PCS. Thus, PCS gene transfer may modulate local PG metabolism.

**Figure 6.** Hypothesis regarding the pathway of PG production in the vessel wall. In the static state, COX-1-derived PGH₂ may be metabolized by specific isomerases to prostanoids such as PGI₂, TXA₂, and PGE₂, which maintain vascular homeostasis. In contrast, in balloon-injured vessels, downregulation of local PGI₂ production may occur through downregulation of COX-1 and PCS activity by disruption of endothelium. Moreover, this mechanical disruption of endothelium may induce COX-2 excessively to compensate for reduced COX-1 activity and result in the overproduction of PGH₂. Local PGE₂ production may not be affected because of a COX-2-mediated compensatory mechanism. This imbalance of PGs among PGH₂, PGI₂, PGE₂, and TXA₂ may be involved in the development of neointimal formation after balloon injury. However, in the balloon-injured vessels transfected with the PCS gene, excessive PGH₂ derived from the COX-2 pathway may be converted into PGI₂ rather than PGE₂ or TXA₂ by the overexpression of PCS. Thus, PCS gene transfer may modulate local PG metabolism.

**References**


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