Cyclooxygenase-1 and Bicistronic Cyclooxygenase-1/Prostacyclin Synthase Gene Transfer Protect Against Ischemic Cerebral Infarction

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Background—We tested the hypothesis that bicistronic cyclooxygenase-1 (COX-1)/prostacyclin synthase (PGIS) and COX-1 gene transfer reduce cerebral infarct volume by augmenting synthesis of protective prostaglandins.

Methods and Results—We infused into lateral ventricle of a rat stroke model recombinant adenoviruses (rAd) containing COX-1 (Adv-COX-1), COX-1 and PGIS (Adv-COX-1/PGIS), or Adv-PGK control vector, and we determined COX-1 and PGIS protein and eicosanoid levels and infarct volume. COX-1 and PGIS proteins were increased in a time-dependent manner. Adv-COX-1/PGIS infusion selectively augmented prostacyclin levels, with reduction of other eicosanoids in ischemic cortex and a significant reduction of infarct volume, even when the rAd was administered 5 hours after ischemia. Infusion of Adv-COX-1 also increased prostacyclin, suppressed leukotriene levels, and achieved a similar degree of cerebral protection. Its neuroprotection was abrogated by treatment with a selective COX-1 inhibitor.


Key Words: prostaglandins ■ genes ■ stroke ■ ischemia ■ gene therapy

Eicosanoids comprise a group of bioactive compounds derived from arachidonate metabolism via 3 major enzymatic pathways: cyclooxygenase (COX), lipoxygenase, and cytochrome P450 oxygenase. COX and lipoxygenase metabolites play diverse roles in brain damage.1,2 Thromboxane A2, prostaglandin (PG) E2, and leukotrienes (LT) contribute to postischemic cerebral blood flow reduction, brain edema, inflammation, and neuronal damage, whereas prostacyclin protects against postischemic brain injury. Prostacyclin is a potent vasodilator and inhibitor of platelet aggregation, leukocyte activation, and leukocyte-endothelial interactions.3 Its synthesis is catalyzed by 3 successive enzymatic reactions: liberation of arachidonic acid from membrane phospholipids by activated cytosolic phospholipase A2;2 conversion of arachidonic acid to PGH2 by COX (also known as prostaglandin H synthase);3 and conversion of PGH2 to PGI2 by prostacyclin (PGI2) synthase (PGIS).4 Two COX isoforms are involved in PGI2 synthesis in endothelial cells.5 COX-1 is constitutively expressed and plays an important physiological role, whereas COX-2 is inducible and plays diverse physiological and pathophysiological roles. COX-1 and PGI2 undergo suicidal inactivation during catalysis, thereby restraining the quantity of PGI2 synthesis.6,7 Intravenous PGI2 administration was shown to prevent ischemic brain damage in experimental studies,8 but controlled clinical trials did not confirm its beneficial effect.9 Stable PGI2 analogs administered directly into brain ventricles exert neuroprotective actions by increasing blood flow, by reducing platelet aggregation, and by direct neuron protection.10 Because PGI2 acts in a paracrine manner, we consider that gene transfer may provide a more physiologically relevant augmentation of PGI2 production. To this end, we have recently shown by combined COX-1 and PGI2 gene transfer that it is possible to augment PGI2 selectively without a concurrent increase in other prostanooids.11 We postulate that adenovirus-mediated transfer of bicistronic COX-1/PGIS would lead to a selective augmentation of PGI2 levels and significant neuroprotection. We have previously shown that COX-1 gene transfer in...
endothelial cells is also capable of increasing prostacyclin synthesis. In vivo injection of adenovirus-COX-1 (Adv-COX-1) into carotid arteries increases PGIs levels and prevents thrombus formation in a porcine model. We reasoned that COX-1 gene transfer also protects against cerebral infarction.

To test these hypotheses, we evaluated the effects of Adv-COX-1 and Adv-COX-1/PGIS versus a control vector (Adv-PGK) on eicosanoid levels in ischemic brain and on infarct volume in a rat ischemic stroke model.

Methods

Stroke Model

The rat focal cerebral ischemia-reperfusion model was described previously. In brief, right middle cerebral artery (MCA) of male Long-Evans rats was reversibly ligated under a stereomicroscope. Both common carotid arteries were then occluded using nontraumatic aneurysm clips. After 60 minutes of ischemia, arterial occlusion was released, and restoration of blood flow was verified. In this model, ischemia for 60 minutes produced a large infarct confined to the right MCA cortex region with >90% regional blood flow reduction. Rats were euthanized by decapitation under anesthesia. The brain was quickly removed, and the cerebral cortex was dissected in ice-cold saline for 5 minutes, and dissected coronally into 2-mm slices using a Jacobovitz brain slicer (Zivic-Miller), which were incubated in PBS (pH 7.4) containing 2% 3,3,5-triphenyltetrazolium chloride at 37°C for 30 minutes and then stored in 10% neutral-buffered formalin. The cross-sectional area of infarction in the right MCA territory for each brain slice was measured with a Zeiss IBAS image analyzer.

Western Blot Analysis

Analysis of COX-1 or PGIS proteins in cortex was performed as described previously, by a specific COX-1 antibody (Cayman) or a PGIS antibody kindly provided by Dr. K-H. Ruan (University of Texas–Houston Medical School). Protein bands were visualized by an enhanced chemiluminescence system (Pierce).

Preparation of Replication-Defective Recombinant Adenoviral Vectors

The procedure was previously described. We constructed in the replication-defective recombinant adenoviral (rAd) vector human phosphoglycerate kinase (PGK) promoter to drive COX-1 expression (Adv-PGK-COX-1 or Adv-COX-1); 2 separate PGK promoters (bicistronic) to drive COX-1 and PGIS, respectively (Adv-PGK-COX-1/PGIS or Adv-COX-1/PGIS); and a PGK alone to serve as control (Adv-PGK). Replication-defective rAd vectors were generated by homologous recombination and amplified in 293 cells as described previously. rAd stocks were prepared by CsCl gradient centrifugation, aliquoted, and stored at −80°C. Viral titers were determined by a plaque-assay method. Two hundred ninety-three cells were infected with serially diluted viral preparations and then overlaid with low melting-point agarose after infection. Numbers of plaques formed were counted within 2 weeks.

Measurements of Brain Tissue Eicosanoids by Enzyme Immunoassay

Cortex was homogenized gently in 1 mL ice-cold buffer (0.05 mol/L Tris at pH 7.0, 0.1 mol/L NaCl, 0.02 mol/L EDTA) and centrifuged at 55 000g for 1 hour. The supernatant was acidified and passed through a Sep-Pak C18 cartridge. Eicosanoids were eluted with 100% methanol, dried under nitrogen gas, redissolved in a small amount of buffer, and analyzed using enzyme immunoassay kits: PGE2, 6-keto-PGF1α, TXB2, and LTB4 kits from R&D System Ins and PGD2 and LTC4 kits from Cayman Chemical Co.

Intraventricular Infusion of Adenoviral Constructs

Based on our preliminary data, which suggest that Adv-COX-1 at 10⁸ plaque-forming units (pfu)/10 μL and Adv-COX-1/PGIS at 10⁷ pfu/10 μL were optimal, we infused rAd at these 2 titers intraventricularly throughout the study. Anesthetized rats were placed in a stereotaxic apparatus, and 10 μL of rAd was infused into the right lateral ventricle at a rate of 5 μL/min at the following coordinates: A, 3.0 mm caudal to bregma; R, 3.5 mm lateral to midline; and V, 3.0 mm ventral to dural surface. Periodic confirmation of proper placement of the needle was performed with infusion of fast green. To ascertain the transgene expression at the region of rAd infusion, we infused recombinant adenoviruses containing a green fluorescent protein (GFP) gene into the right lateral ventricle and examined the GFP expression in 8 coronal slices of right cortex 72 hours later. GFP was visualized in the lining ependymal cells and cells surrounding the right ventricular region in all 8 coronal brain slices. GFP was not visualized in the left ventricle. These results confirm the uptake and expression of rAd by ependymal and surrounding cells.

Measurement of Infarct Volume

The infarct volume in the right MCA territory was measured as described. Rats were euthanized, and the cortex was carefully removed, cooled in ice-cold saline for 5 minutes, and dissected coronally into 2-mm slices using a Jacobovitz brain slicer (Zivic-Miller), which were incubated in PBS (pH 7.4) containing 2% 3,3,5-triphenyltetrazolium chloride at 37°C for 30 minutes and then stored in 10% neutral-buffered formalin. The cross-sectional area of infarction in the right MCA territory for each brain slice was measured with a Zeiss IBAS image analyzer.

Statistical Analysis

ANOVA was used to compare the temporal expression of infarct volume and levels of eicosanoid. The level of differences among groups was analyzed by Fisher’s protected t tests (GB-STAT 5.0.4, Dynamic Microsystems Inc). P<0.05 was considered statistically significant.

Results

Intraventricular Infusion of Adv-COX-1 and Adv-COX-1/PGIS Increased COX-1 and PGIS Protein and Prostanoid Levels in Rat Brain

To evaluate the efficiency of gene expression of adenoviral administration via the ventricular route, we infused 10 μL of Adv-COX-1 or Adv-COX-1/PGIS into the right lateral ventricle of normal rats and determined COX-1 and PGIS protein levels 12 to 168 hours after administration. Compared with Adv-PGK control, Adv-COX-1 augmented COX-1 protein levels in a time-dependent manner (Figure 1A). Maximal augmentation was noted at 72 hours after administration. COX-1 protein levels were augmented by Adv-COX-1/PGIS also in a time-dependent manner, and the level peaked at 48 hours (Figure 1B). We next administered Adv-COX-1, Adv-COX-1/PGIS, or Adv-PGK 72 hours before ischemia and determined COX-1 proteins in the ischemic brain tissue. COX-1 proteins were increased more than 5-fold by Adv-COX-1 and 2-fold by Adv-COX-1/PGIS versus the Adv-PGK or untransduced ischemic control (Figure 1C). PGIS levels were increased by Adv-COX-1/PGIS also in a time-dependent manner, and the level peaked at 48 hours (Figure 1D). PGIS levels were increased by Adv-COX-1/PGIS-treated rats. COX-2 proteins were undetectable in any of the brain tissues (Figure 1D). PGIS levels were increased by Adv-COX-1/PGIS (Figure 2). Surprisingly, PGIS levels were also increased by Adv-COX-1, albeit to a lesser extent than by Adv-COX-1/PGIS (Figure 2).
Several key eicosanoids in brain tissues were measured at 72 hours after adenoviral infusion in normal rats. Adv-COX-1 increased ipsilateral PGE$_2$, 6-keto-PGF$_{1\alpha}$, PGD$_2$, and TXB$_2$ levels accompanied by reduced LTB$_4$ (Figure 3A). Adv-COX-1/PGIS significantly increased the level of 6-keto-PGF$_{1\alpha}$ and TXB$_2$ in normal brain tissues (Figure 3B).

**Adv-COX-1 Administration Increased Prostacyclin and Reduced Infarct Volume**

Results from previous studies indicate that transfer of COX-1 cDNA alone increases prostacyclin. We therefore evaluated the effects of Adv-COX-1 administration on prostacyclin and other eicosanoid levels and infarct volume. Intraventricular Adv-COX-1 administration 72 hours before MCA occlusion increased 6-keto-PGF$_{1\alpha}$ levels and other COX metabolites, including PGE$_2$ and PGD$_2$, in ischemic brain when compared with Adv-PGK administration (Figure 4A). TXB$_2$ levels were not significantly altered, whereas LTB$_4$ and LTC$_4$ were significantly reduced. The infarct volume in rats receiving Adv-COX-1 72 hours before ischemia was significantly reduced (Figure 4B). We next performed a time course experiment, wherein Adv-COX-1 was administered at reducing intervals from the onset of ischemia. The infarct volume was significantly reduced when Adv-COX-1 was administered at 24 hours, at the onset of ischemia, and, importantly, at 5 hours after ischemia (Figure 4B). The infarct volume was not significantly reduced when Adv-COX-1 was administered at 24 hours after ischemia (Figure 4B).

**Combined COX-1/PGIS Gene Transfer Selectively Augmented Brain Prostacyclin Levels and Reduced Infarct Volume**

We have recently shown in cell transfection experiments that concurrent COX-1 and PGIS expressions by using a bicistronic COX-1/PGIS vector selectively augmented PGI$_2$ synthesis. To determine whether the selective prostacyclin

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**Figure 1.** Western blot analysis. COX-1 protein levels in brain tissues of rats receiving Adv-PGK vector, Adv-PGK-COX-1, or Adv-PGK-COX-1/PGIS. A and B, Time course of COX-1 protein levels in brain tissues transduced with Adv-PGK control (C) at a representative time point and Adv-PGK-COX-1 or Adv-PGK-COX-1/PGIS at 12 to 168 hours after treatment. The lower panel shows densitometry of blots. The error bars are mean±SD of 3 experiments. *P<0.05; **P<0.01.

**Figure 2.** Western blot analysis of PGIS proteins in nonischemic rat brain tissues transduced with adenoviral vectors. The densitometric comparison is shown in the lower panel. Bar is mean±SD of 3 experiments. *P<0.05; **P<0.01.

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increase occurred in vivo, we intraventricularly infused Adv-COX-1/PGIS (10^7 pfu) 72 hours before MCA occlusion and measured eicosanoid levels in ischemic cortex 24 hours after ischemia/reperfusion. Compared with normal brains transduced with Adv-PGK control vectors, the Adv-PGK–transduced ischemic brains had a significantly higher level of PGE2 (P<0.01), PGD2 (P<0.01), TXB2 (P<0.01), LTB4 (P<0.01), and LTC4 (P<0.01) (Figure 5A versus 3A or 3B). The 6-keto-PGF1α level was higher in Adv-PGK–transduced ischemic brain, but the difference was not statistically significant. The eicosanoid levels in Adv-PGK–transduced ischemic brain tissue are comparable with those of ischemic brain without adenoviral transduction. The 6-keto-PGF1α level in Adv-COX-1/PGIS–transduced ischemic brain was increased by 3-fold over that of Adv-PGK control (Figure 5A). In contrast, PGE2, PGD2, TXB2, LTB4, and LTC4 levels were all significantly reduced in Adv-COX-1/PGIS–transduced ischemic brain tissues (Figure 5A). These results are consistent with selective augmentation of PGI2 productions by shunting PGH2 into the PGIS pathway.11 The ischemia-induced infarct volume was significantly lower in rats receiving Adv-COX-1/PGIS 72 hours before ischemia than in rats receiving Adv-PGK under an identical experimental protocol (Figure 5B). Results from time course experiments revealed that the infarct volume was significantly reduced when Adv-COX-1/PGIS was administered at −24 hours, at the onset of ischemia, and at 5 hours after ischemia (Figure 5B). Similar to Adv-COX-1 treatment, Adv-COX-1/PGIS was no longer effective in controlling cerebral infarction when administered 24 hours after ischemia/reperfusion (Figure 5B).

**Protective Effect of COX-1 Gene Transfer Was Abrogated by a Selective COX-1 Inhibitor**

To confirm the role that COX-1 plays in protection against ischemia-induced infarct, we treated rats with a selective COX-1 inhibitor, SC560 (2 mg/kg) intraperitoneally after reperfusion. SC560 significantly reduced all the COX-1 metabolites and increased LTB4 and LTC4 in Adv-COX-1–transduced ischemic brain tissues (Figure 6A). It also reduced COX-1 metabolites except TXB2 and increased LTB4 and LTC4 in Adv-COX-1/PGIS–transduced tissues (Figure 6B). It is to be noted that the 6-keto-PGF1α level in SC560-treated Adv-COX-1/PGIS–transduced tissues was significantly higher than that in SC560-treated Adv-COX-1–transduced tissues (Figure 6B versus 6A) and was close to that in Adv-COX-1–transduced tissues without SC560 treatment (Figure 6B versus 4A). SC560 treatment increased the infarct
volume in Adv-PGK–treated rats and abrogated the protective effect of Adv-COX-1 (Figure 7). By contrast, it did not reverse the protective effect of Adv-COX-1/PGIS (Figure 7).

Discussion

Results from this study indicate that intraventricular infusion of Adv-COX-1 or Adv-COX-1/PGIS is effective in augmenting COX-1 and/or PGIS expression in brain tissues and in reducing neuronal death caused by ischemic injury. Analysis of eicosanoids in ischemic cortex after gene transfer sheds light on how these 2 types of gene transfer confer protection against infarction. Adv-COX-1 administration causes a broad increase in COX-derived metabolites—notably PGI2, PGE2, and PGD2—whereas Adv-COX-1/PGIS increases selectively PGI2 in the ischemic brain tissue. Augmented PGI2 levels by both types of gene transfer probably account for the protective actions, as PGI2 is a potent inhibitor of platelet aggregation, vasoconstriction, and monocyte activation. Reduction in LTB4 and LTC4 levels in both types of gene transfer probably contribute to the protective effect because these 2 lipoxygenase metabolites are potent mediators of vasoconstriction and inflammation and are implicated in mediating brain infarction.11–13 Overexpression of COX-1 results in the production of a large quantity of PGH2, which is converted to final products by the downstream-specific enzymes such as PGI2, PGD2, PGE2, and thromboxane A2 synthases. These prostaglandin-specific enzymes are present in cells in brain. However, the exact types of cells that express these enzymes and are responsible for synthesis of their respective prostanoids is not entirely clear. Transfer of COX-1/PGIS increases the expression of an equivalent level of COX-1 and PGIS, and this co-overexpression leads to the conversion of a majority of PGH2 produced by COX-1 though the PGIS pathway.14 PGH2 available for other terminal enzymes appears to be reduced resulting in production of lower levels of other prostanoids. The reasons for reduced LTB4 and LTC4 production are unclear. Our data suggest that this may be caused by a negative regulation by the overproduced prostanoids such
as PGI₂. The crosstalk between COX and lipoxygenase pathway will require further investigation.

The important role of COX-1 in ischemic neuroprotection is supported by the demonstration that a selective COX-1 inhibitor abrogated the neuroprotective action of Adv-COX-1. It is interesting to note that the selective COX-1 inhibitor increased the infarct volume in nonviral-transfected and Adv-PGK-treated rats. The ischemic brain COX-1 protein level was increased over the basal level (Figure 1C), and this COX-1 level was constant for at least 48 hours after ischemia/reperfusion (data not shown). Our selective COX-1 inhibitor data are thus consistent with a vital role that COX-1 plays in controlling ischemic brain damage. Because the level of expression of this housekeeping gene in ischemic brain is relatively low, however, it may not produce a sufficient amount of neuroprotective prostaglandins. Augmentation of COX-1 by gene transfer increases its expression and the synthesis of PGI₂, thereby protecting the brain tissue from further damage. Neuroprotection augmented by COX-1 gene transfer was abrogated by COX-1 inhibition. This reversal of protection may be attributed to reduction of prostacyclin to the basal ischemic level and was equivalent to that in Adv-COX-1–transduced tissues without SC560 treatment. It is likely that this level of PGI₂ may still be adequate for protecting brain tissues from ischemic damage. It is intriguing that SC560 did not inhibit 6-keto-PGF₁α levels in Adv-COX-1– and Adv-COX-1/PGIS–transduced tissues to a similar extent. A higher 6-keto-PGF₁α level in Adv-COX-1/PGIS–transduced tissue than in Adv-COX-1–transduced tissue is not caused by a differential induction of COX-2, as neither viral construct had a significant effect on COX-2 level (Figure 1D). We suspect that the different 6-keto-PGF₁α level may be owing to a higher level of PGIS in Adv-COX-1/PGIS–transduced tissue than in Adv-COX-1–transduced tissue, resulting in higher capacity for converting residual COX-1–derived PGH₂ to prostacyclin. Further studies are needed to test this hypothesis.

We have evaluated the effects of Adv-COX-1 and Adv-COX-1/PGIS infusion on cerebral infarct volume at several time points before and after the 60-minute MCA ischemia. A significant reduction in the infarct volume was noted only at preischemic time points but also at 5 hours after ischemia. These results suggest multiple actions of prostaglandins on the pathophysiological events in ischemia-induced neuronal death. Neuronal death after ischemia can be divided into an early phase owing to excitotoxicity, and a delayed phase owing to inflammation and apoptosis. Because Adv-COX-1 and Adv-COX-1/PGIS administered 5 hours after ischemia remain effective in controlling ischemic brain damage, it is
likely that neuroprotective prostaglandins generated via the COX-1 or COX-1/PGIS pathway after ischemia are still capable of curtailing inflammation and apoptosis, thereby controlling neuronal damage. Loss of protective effects by gene transfer at 24 hours after ischemia is consistent with irreversible neuronal damage after the tissue has been under ischemic insults for 24 hours. Our findings have important therapeutic implications. COX-1–based gene transfer has a potential for treating human ischemic stroke when the therapeutic genes are administered at the reversible phase of neuronal damage. Results from our experiments suggest that “the window of therapeutic opportunity” during the reversible phase of ischemic neuronal damage is between 5 hours and 24 hours after cerebrovascular occlusion has occurred. It will be important to determine the maximal time point that gene transfer remains efficacious after ischemia.

Figure 6. Effect of SC560 on Adv-COX-1–treated (A) and Adv-COX-1/PGIS–treated (B) ischemic brain eicosanoid levels. Adenoviral constructs were administered 72 hours before ischemia. SC560 (2 mg/kg) was administered intraperitoneally after reperfusion, and infarct volume was determined 24 hours after ischemia/reperfusion. Difference in brain eicosanoid levels between rats with and without SC560 treatment is statistically significant for each eicosanoid (A, \( P < 0.05 \)) and is also significant (\( P < 0.05 \)) in Figure 6B, except TXB2.

Figure 7. Effect SC560 on brain infarct volume with and without adenoviral transduction. The experimental protocol was identical to that described in Figure 6. Bar is mean ± SD of 3 experiments. \(* P < 0.01\). The infarct size of Adv-COX-1–transduced rats was significantly smaller than that of Adv-PGK–transduced rats (\( P < 0.05 \)), which was reversed by SC560 treatment.

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