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.

Conclusions—COX-1/PGIS and COX-1 gene transfer reduce cerebral infarct volume by augmenting prostacyclin and suppressing leukotriene productions. COX-1–based gene transfer has potential for treating ischemic stroke.

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:1 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 PGIS gene transfer that it is possible to augment PGI₂ selectively without a concurrent increase in other prostanoids.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 PGI\(_2\) 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 \( \approx90\% \) regional blood flow reduction. Rats were euthanized by decapitation under anesthesia. The brain was quickly removed, and the cerebral cortex was isolated and immediately frozen in liquid nitrogen. All procedures were approved by the institutional Animal Studies Committee in accordance with the Public Health Service Guide for the Care and Use of Laboratory Animals.

**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 described. We constructed in the replication-defective recombinant adenoviral (rAd) vector a 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 \(-80^\circ\)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.

**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 Jacobowitz brain slicer (Zivic-Miller), which were incubated in PBS (pH 7.4) containing 2\% 2,3,5-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 \( \mu \)L 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 over the Adv-PGK control, Adv-COX-1 augmented COX-1 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 over the Adv-PGK control, Adv-COX-1 augmented COX-1 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 over the Adv-PGK control, Adv-COX-1 augmented COX-1 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).
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.$^{11}$ To determine whether the selective prostacyclin
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 were 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. 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.
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. When compared with Adv-PGK control, Adv-COX-1–administered brain tissues have a higher PGD₂ and PGE₂, whereas Adv-COX-1/PGIS–administered tissues have a lower PGD₂ and PGE₂. These 2 metabolites have been implicated in several pathophysiological processes, but their roles in ischemic brain damage remain controversial. Further studies are needed to determine whether changes in their levels by COX-1 gene transfer contribute to neuroprotection.

Our findings suggest that Adv-COX-1 or Adv-COX-1/PGIS infused into lateral ventricle is taken up and expressed in cells close to the ischemic region. The exact cell types involved in transgene expression are not yet characterized. Preliminary work suggests that ependymal cells and surrounding cells, possibly microglial cells, are capable of expressing the transgenes. Overexpressions of COX-1 with or without a concurrent PGIS overexpression in those cells cause changes in prostanoid levels that are consistent with those in cultured cells.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 PG\(_I_2\). 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 PG\(_I_2\), 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 PG\(_I_2\) may still be adequate for protecting brain tissues from ischemic damage. It is intriguing that SC560 did not inhibit 6-keto-PGF\(_{1\alpha}\) levels in Adv-COX-1– and Adv-COX-1/PGIS–transduced tissues to a similar extent. A higher 6-keto-PGF\(_{1\alpha}\) 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-PGF\(_{1\alpha}\) 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 PGH\(_2\) 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 not 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.

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References


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. \(\star P<0.01\). 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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