Gene Transfer of Inducible Nitric Oxide Synthase Impairs Relaxation in Human and Rabbit Cerebral Arteries

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Background and Purpose—These studies evaluated whether gene transfer of inducible nitric oxide synthase (iNOS) is a sufficient stimulus to produce vascular dysfunction in cerebral arteries.

Methods—Intracranial (pial) arteries were dissected from human brain tissue obtained during elective surgery. Isolated human arteries were incubated in vitro with adenovirus containing iNOS (AdiNOS) or a nonexpressive transgene (control, AdBglIII) (500 μL, 3×10⁹ plaque-forming units per milliliter), and vascular function was examined 24 hours later. In anesthetized rabbits, AdiNOS or AdBglIII (300 μL 1×10⁹) was injected into the cisterna magna. Three days later, the basilar artery was removed, and reactivity was examined ex vivo.

Results—In submaximally precontracted vessels, we observed impairment of NO-dependent relaxation in human cerebral arteries after gene transfer of iNOS. Maximum relaxation to bradykinin (1 μmol/L, an endothelium-dependent agonist) was 77±11% (mean±SE) after AdBglII and 31±22% (P<0.05) after AdiNOS. After AdiNOS, responses to nitroprusside (an endothelium-independent NO donor) also were impaired. Responses to both nitroprusside and bradykinin were improved by aminoguanidine (300 μmol/L), an inhibitor of iNOS. AdiNOS produced no change in vasoconstrictor responses to U46619. In basilar arteries from rabbits examined in vitro after gene transfer in vivo, responses to histamine, serotonin, and nitroprusside all were similar after AdiNOS or AdBglIII. In contrast, relaxation to acetylcholine was significantly depressed after AdiNOS. Maximum relaxation to acetylcholine (10 μmol/L) was 90±3% after AdBglII and 68±5% (P<0.05) after AdiNOS. Relaxation of arteries after AdiNOS was improved by aminoguanidine.

Conclusions—These studies suggest that expression of iNOS may impair NO-dependent relaxation in both human and rabbit cerebral arteries. (Stroke. 2002;33:2292-2296.)

Key Words: acetylcholine ■ adenoviruses ■ bradykinin ■ nitric oxide ■ nitric oxide synthase ■ superoxides

Inducible nitric oxide synthase (iNOS) is not present in normal blood vessels but is expressed in blood vessels during atherosclerosis, diabetes, endotoxemia, and after stroke. Although expression of iNOS in blood vessels is associated with vascular dysfunction in vascular disease, whether iNOS is directly involved with mechanisms of impairment is not clear. Several studies have used genetically targeted mice deficient in expression of iNOS to implicate iNOS in mechanisms of vascular dysfunction. Other studies, however, have used iNOS-deficient mice and gene transfer of iNOS and suggest that iNOS protects vascular function and inhibits hyperplasia and restenosis. In the present study we used adenovirus-mediated gene transfer to produce selective expression of iNOS in cerebral arteries to test the hypothesis that iNOS impairs vascular function.

Although iNOS is expressed in the central nervous system under pathological conditions, relatively few studies have examined effects of iNOS on cerebral vascular function, and none, to our knowledge, have examined human cerebral arteries. Bacterial lipopolysaccharide (LPS; endotoxin) induces expression of iNOS in multiple cell types in the central nervous system including astrocytes and neurons as well as blood vessels.Cerebral arterioles dilate in response to LPS by a mechanism that is mediated, at least in part, by iNOS. In addition, some studies suggest that iNOS contributes to chronic vasospasm after subarachnoid hemorrhage. Thus, iNOS may modulate vascular tone when it is expressed in cerebral vessels.

In a previous study we found that gene transfer of iNOS in vitro produced impaired contraction and relaxation in carotid arteries from rabbits. The first goal of the present study was to test effects of gene transfer of iNOS on vasomotor function in human cerebral arteries in vitro. The second goal was to examine effects of gene transfer of iNOS in vivo on function in basilar arteries from rabbits. We hypothesized that expression-
ing iNOS in cerebral arteries would impair vasomotor function.

**Materials and Methods**

**Adenovirus Vectors**

Two replication-deficient adenoviruses were used: (1) AdCMViNOS (AdiNOS) was constructed with the use of cDNA for mouse iNOS, and (2) AdCMVBglII (AdBglII), a null virus, was constructed with no functional transgene to serve as a control for nonspecific adenovirus effects. Adenovirus vectors were obtained from the Vector Core Laboratory of University of Iowa and stored at −80 °C until used.

**Human Vessels**

Specimens of human cerebral tissue that would otherwise have been discarded as waste were obtained with permission from patients undergoing elective surgery for intractable seizures at University of Iowa Health Care. All patients had seizure disorder involving mesial temporal lobe structures (eg, amygdala and hippocampus). The lateral neocortex showed no evidence of epileptic activity and was resected for the purpose of gaining access to the targeted mesial structures. Khurana et al performed a systematic evaluation of similar vessels obtained during resection of cerebral tissue from patients with seizures and found no evidence for morphological or histological abnormalities in these vessels. Because all treatments (vehicle, AdBglII, and AdiNOS) were applied to arteries from each patient, and no group of patients was compared with another group, it is unlikely that differences between patients account for differences between AdiNOS and AdBglII.

In the operating department, block sections of the anterior temporal lobe neocortex were resected and placed in cold (4 °C) oxygenated Krebs’ solution (in mmol/L: NaCl 118.3, KCl 4.7, NaH2PO4 1.2, NaHCO3 25, MgSO4 1.2, glucose 11, and CaCl2 2.5). In the laboratory, intracranial (pial) arteries (~150 to 300 μm in diameter) were dissected from brain specimens and stripped of connective tissue. Arteries were then cut into segments (~2 mm in length) for incubation with adenovirus or vehicle.

Arteries were incubated at 37 °C in a chamber aerated with 95% O2 and 5% CO2 in tissue culture medium (Dulbecco’s modified Eagle’s medium) containing 100 U/mL of penicillin and 100 μg/mL streptomycin. Vessel segments were incubated with tissue culture medium alone (vehicle) or with AdiNOS or AdBglII (3 × 109 plaque-forming units [pfu]). After 24-hour incubation with virus or vehicle, arteries were suspended on stainless steel hooks in organ baths for measurements of isometric tension. Preliminary studies suggested that arteries incubated in vehicle produced normal vasomotor function after incubation for 24 hours (data not shown). Optimal resting tension for cerebral arteries of this size was determined to be 0.25 g. U46619, a thromboxane A2 analogue, was used for contraction. Bradykinin produces NO-mediated relaxation of human pial arteries. Thus, bradykinin was used to measure endothelium-dependent relaxation. Nitroprusside was used as an endothelium-independent vasodilator.

**Animals**

Male New Zealand White rabbits (weight, 2.5 to 3.0 kg) were anesthetized with 5 mg/kg IM of xylazine and 50 mg/kg IM of ketamine. A 25-gauge needle was aseptically inserted into the cisterna magna as previously described. Then 150 μL of cerebrospinal fluid was withdrawn and replaced with 150 μL of adenoviral suspension (AdCMViNOS or AdCMVBglII in PBS with 3% sucrose, 1 × 1010 pfu/mL).

Three days after injection of vectors, rabbits were killed with sodium pentobarbital (50 mg/kg) into the marginal ear vein following exsanguination. The basilar artery was quickly removed and the cisterna magna was opened. The basilar artery was cut into 4 rings (~3 mm in length). All procedures followed institutional guidelines as approved by the Animal Care and Use Committee.

We were able to obtain human pial arteries from discarded tissue after elective surgery, but basilar arteries from humans were not available. Because of their small size, pial arteries from rabbits would have to have been studied by other methods in vitro. Although we realize that studying basilar arteries from rabbits and pial arteries from humans is not a perfect comparison, we suggest that it is a good initial step toward establishing relevance in humans.

**Vasomotor Function**

Rings of basilar arteries were mounted on stainless steel hooks in organ baths for measurements of isometric tension. Tension was increased stepwise to an optimal resting tension (0.5 g) and was periodically adjusted to the desired level during a 60-minute equilibration period. Vascular rings were then contracted twice with 60 mmol/L KCl and rinsed 3 times after each contraction. After a brief resting period, concentration-response curves for histamine (10−9 to 10−3 mol/L) and serotonin (10−9 to 10−3 mol/L) were measured. Concentration-response curves for acetylcholine (10−9 to 10−3 mol/L) and nitroprusside (10−9 to 10−7 mol/L) were measured after precontraction of vessels with an EC50 dose of histamine.

**Immunohistochemistry**

Basilar arteries were frozen, cut into sections (8 μm), and mounted on Superfrost/Plus slides (Fisher Scientific). The sections were incubated with normal horse serum for 1 hour. The sections were incubated 1 hour at 24 °C with rabbit polyclonal antibody to iNOS (Upstate Biotechnology Inc). Slides were washed and subjected to the streptavidin–biotin technique for visualization with the use of the Vectastain ABC-AP kit (Vector Laboratories). Sections were postfixed with 2% paraformaldehyde for 15 minutes and counterstained with nuclear fast red (Vector Laboratories) for 1 hour.

**Chemicals**

Acetylcholine chloride, histamine, serotonin (5-hydroxytryptamine), bradykinin, aminoguanidine, and sodium nitroprusside were obtained from Sigma Chemical Co and dissolved in saline.

**Statistical Analysis**

All data are expressed as mean±SEM. Comparisons were performed with the use of an independent ANOVA (1-way ANOVA) to test for difference among treatment groups, followed by the Tukey-Kramer multiple comparison test. Differences were considered to be significant at P<0.05.

**Results**

**Vasomotor Responses of Arteries From Humans**

Effects of AdiNOS were examined in human cerebral arteries. Contraction of intracranial arteries to U46619 (30 mmol/L) was similar in all treatment groups, reaching a maximum of 0.6±0.3, 0.6±0.2, and 0.7±0.2 g after vehicle, AdBglII, or AdiNOS, respectively. Vasorelaxation in response to bradykinin was significantly impaired after AdiNOS (Figure 1, left). Aminoguanidine (300 μmol/L) tended to improve relaxation (Figure 1, middle), and improvement was significant at the 1-μmol/L concentration of bradykinin. Responses to nitroprusside, an endothelium-independent NO donor, also were impaired in human intracranial small arteries after AdiNOS (Figure 1, right), and impairment tended to be improved by aminoguanidine (data not shown).

**Vasomotor Responses of Arteries From Rabbits**

Contractile responses of the basilar artery to KCl (40 mmol/L) were 0.6±0.1 g after AdBglII and 0.5±0.1 g after AdiNOS. Contractile responses to histamine were similar after either AdiNOS or AdBglII (Figure 2, left), and...
responses to serotonin tended to be increased after AdiNOS (Figure 2, right). Thus, adenovirus-mediated gene transfer of iNOS did not impair constriction in basilar arteries from rabbits.

Relaxation to acetylcholine was impaired in basilar arteries after transfection with AdiNOS compared with normal vessels or those with AdBglII (Figure 3, left). Relaxation to acetylcholine was improved by aminoguanidine (300 μmol/L) in the iNOS-transfected vessels (Figure 3, left). Aminoguanidine did not alter relaxation to acetylcholine in normal or BglII-treated vessels (data not shown). Maximum relaxation of vessels to nitroprusside reached 100% in normal vessels or after AdiNOS or AdBglII and was not altered by pretreatment with aminoguanidine (Figure 3, right).

**Immunohistochemistry**

Rings of arteries were analyzed histochemically for expression of iNOS. After injection of AdiNOS into cerebrospinal fluid, positive staining for iNOS was noted in adventitia of basilar arteries from rabbits but was not detectable in vascular muscle or endothelium (Figure 4). We did not observe any staining for iNOS in normal (not shown) or AdBglII-treated vessels (Figure 4). Positive staining for iNOS was noted in adventitia and endothelium after AdiNOS in vitro in human cerebral arteries (data not shown).

**Discussion**

The principal new finding in this study is that gene transfer of iNOS produces impairment of NO-dependent relaxation in cerebral arteries. This finding is important because iNOS is expressed in cerebral vessels and may contribute to impaired cerebral vascular function during conditions such as subarachnoid hemorrhage or ischemia.9–11,31 Studies were performed in both rabbit and human cerebral arteries. Although some differences were observed between human and rabbit preparations, gene transfer of iNOS inhibited relaxation in both species.

After gene transfer of iNOS in rabbits in vivo, iNOS is expressed in the central nervous system during inflammation and cerebral vascular disorders,9–11,31,32 relatively little is known about effects of iNOS on vasomotor function in cerebral blood vessels, especially in humans. Previous studies suggest that iNOS may contribute to vasoconstriction after subarachnoid hemorrhage,12,24,25 disruption of the blood-brain barrier during meningitis,33 and vasodilatation in response to LPS.23 Thus, previous studies suggest that iNOS may produce vasoconstriction or vasodilation. Impairment of NO-mediated relaxation by iNOS, as found in the present study, is consistent with a role for iNOS in vasospasm, but a direct link remains to be established.
Our results suggest that iNOS, at levels of expression produced by gene transfer, is a sufficient stimulus to impair NO-dependent relaxation. It is difficult to know whether levels of expression of iNOS achieved after gene transfer are comparable to levels that occur in disease states. Preliminary studies suggest that endogenous expression of iNOS during diabetes contributes to impairment of endothelium-dependent relaxation. Thus, pathophysiological levels of iNOS are sufficient to impair relaxation.

**Effects of iNOS on Vasoconstriction**

In previous studies, we reported that iNOS impairs contractile responses in carotid arteries from mice and rabbits. In contrast, rabbit and human cerebral arteries responded normally to vasoconstrictors after AdiNOS in the present study. Different levels of expression of iNOS in cerebral vessels in the present study and in carotid arteries in the previous studies may account for different effects on vasoconstriction. Differences in localization of iNOS may also contribute to different effects on constrictor responses.

**Effects of iNOS on Vasorelaxation**

NO-dependent vasorelaxation was impaired after gene transfer of iNOS. After AdiNOS, responses to bradykinin in human intracranial arteries and to acetylcholine in rabbit basilar arteries were impaired. These results, in cerebral arteries after gene transfer of iNOS in vivo and in vitro, are consistent with our previous findings in carotid arteries from rabbits after gene transfer of iNOS in vitro. Improved vasorelaxation in the presence of aminoguanidine suggests that iNOS mediates the impairment. Thus, iNOS is a sufficient stimulus to impair NO-dependent relaxation in cerebral arteries as well as in extracranial arteries.

Responses to nitroprusside, an endothelium-independent dilator, were different in cerebral arteries from rabbits and humans. Basilar arteries from rabbits relaxed completely to nitroprusside after gene transfer of iNOS, but responses of human arteries were modestly impaired. One difference between the 2 sets of experiments is that gene transfer of iNOS was performed in vitro in human arteries and in vivo in rabbit arteries. When gene transfer of iNOS was performed in vitro in rabbit carotid arteries in our earlier study, responses to nitroprusside were impaired. Thus, endothelium-dependent and -independent relaxation is consistently impaired after gene transfer of iNOS to arteries in vitro. The difference between present results in rabbit basilar and human intracranial arteries, therefore, may be a result of a difference between in vitro and in vivo gene transfer approaches.

A major difference between the in vitro and in vivo approaches used in these experiments is that gene transfer in vitro produces expression of iNOS in both endothelium and adventitia, whereas gene transfer in vivo produces expression of iNOS only in adventitia in basilar arteries. These data suggest that expression of iNOS in adventitia alone is a sufficient stimulus to impair responses to NO from eNOS, but impairment of responses to NO from nitroprusside may require expression of iNOS in both endothelium and adventitia. We cannot discount the possibility that different responses to nitroprusside after in vitro and in vivo gene transfer are simply a dosing effect. Expression of iNOS in adventitia plus endothelium may be higher and may produce more NO than expression of iNOS in adventitia alone. It is intriguing, however, to consider the possibility that expression of iNOS in different vascular cells, or layers, in blood vessels produces different effects on vasomotor function. Different effects of iNOS, as a result of differential cellular localization within the wall of blood vessels, is a novel concept that may provide some insight into differences in the literature regarding effects of iNOS under various conditions.

**Implications**

The use of cerebral blood vessels from humans for studies of vascular biology is important. Arteries can be harvested from resected brain tissue from humans and used for successful gene transfer experiments, as reported previously. An ultimate goal of gene transfer is to develop techniques with applications for gene therapy in humans. Most gene transfer studies to date have used animal models, which may or may not have direct applicability to human therapeutics. Although the present study addresses basic questions regarding a pathophysiological role of iNOS, the potential for extension of this approach to transfer genes for therapeutic applications is intriguing.
In summary, this study demonstrates that expression of iNOS in cerebral arteries can impair vasorelaxation to NO. Because iNOS is expressed in blood vessels during many cardiovascular diseases, vascular effects of iNOS may have implications for several pathological conditions. Our results suggest that therapy designed to reduce effects of iNOS may be beneficial in cerebral vascular disease characterized by endothelial dysfunction.

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