Expression and Function of Recombinant S1179D Endothelial Nitric Oxide Synthase in Canine Cerebral Arteries

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Background and Purpose—Bovine endothelial nitric oxide synthase (eNOS) is phosphorylated directly by the protein kinase Akt at serine 1179. Mutation of this residue to the negatively charged aspartate (S1179DeNOS) increases nitric oxide (NO) production constitutively in the absence of agonist stimulus. The present study was designed to determine the effect of mutant S1179DeNOS gene expression on vasomotor function of canine cerebral arteries.

Methods—Isolated basilar and middle cerebral arteries were exposed ex vivo (30 minutes at 37°C) to an adenoviral vector (10^10 plaque-forming units per milliliter) encoding the S1179DeNOS gene (AdCMVS1179DeNOS), the wild-type eNOS gene (AdCMVeNOS), or the green fluorescent protein (GFP) reporter gene (AdCMVGFP). Twenty-four hours after transduction, arteries were suspended in an organ chamber for isometric force recording, and levels of cGMP were measured by radioimmunoassay.

Results—Transgene protein expression was detected mainly in the vascular adventitia. In AdCMVS1179DeNOS-transduced arteries, basal levels of cGMP were significantly elevated compared with those in control (nontransduced), AdCMVGFP-, or AdCMVeNOS-transduced vessels (n=8; P<0.01). The elevation of cGMP was abolished by a NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), or by incubation in the calcium-free medium in the presence of calcium chelators. In AdCMVS1179DeNOS-transduced arteries, contractions to endothelin-1 (10^-10 to 10^-8 mol/L) were significantly reduced compared with those in control and AdCMVGFP-transduced arteries (n=7; P<0.05). The vasoconstrictor effect of endothelin-1 was restored in the presence of the NOS inhibitor L-NAME.

Conclusions—Our results suggest that in cerebral arteries, expression of recombinant S1179DeNOS increases basal production of NO and inhibits the vasoconstrictor effect of endothelin-1. This effect may have therapeutic application in prevention and treatment of cerebrovascular diseases. (Stroke. 2002;33:1071-1076.)

Key Words: cerebral arteries | gene transfer | nitric oxide | dogs

Nitric oxide (NO), produced by endothelial nitric oxide synthase (eNOS), is a potent vasodilator and plays an essential role in the regulation of vascular tone. Previous studies demonstrated that the recombinant eNOS gene can be transferred with the use of adenoviral vectors into the adventitial layer of cerebral and peripheral arteries. The expression of eNOS gene in the adventitia can augment the vasodilator effect of bradykinin and inhibit the vasoconstrictor effect of endothelin-1 (ET-1) in canine cerebral arteries.

Recently it was demonstrated that the serine/threonine kinase Akt specifically phosphorylates eNOS (at serines 1179 and 1177 for bovine and human eNOS, respectively). Phosphorylation on this residue is associated with an increase in NO production and activation of the enzyme at lower calcium/calmodulin concentrations than nonphosphorylated eNOS. Mutation of serine 1179 to aspartate (S1179DeNOS), to mimic the negative charge afforded by phosphate, increased NO production in the absence of stimulation by agonists, whereas mutation to alanine (S1179A) blocked Akt-dependent eNOS phosphorylation and NO release. The present study was designed to determine the effect of recombinant S1179DeNOS gene expression on the vasomotor function of canine cerebral arteries.

Materials and Methods

Construction, Propagation, and Purification of Adenoviral Vectors

Adenoviral vectors encoding green fluorescent protein (GFP), wild-type eNOS, the S1179D mutant eNOS, and inducible NOS (iNOS) were used. Construction of these vectors is described elsewhere. S1179DeNOS and GFP vectors were provided by Dr William C. Sessa (Yale University, New Haven, Conn), and iNOS vector was supplied by Dr Imre Kovesdi (GenVec, Inc, Gaithersburg, Md). Each...
insert was driven by a cytomegalovirus promoter. The adenovirus constructs (except iNOS) contained GFP sequence. Virus stocks were propagated in 293 cells and purified as previously described.13

Gene Transfer
All procedures were in accordance with Institutional Animal Care and Use Committee guidelines of Mayo Clinic. Rings (4 mm long) of basilar arteries were taken from mongrel dogs (weight, 18 to 27 kg) anesthetized with 30 mg/kg of intravenous thiopental. To remove blood, arterial rings were gently rinsed with cold (4°C) modified Krebs-Ringer bicarbonate solution (control solution; composition [mmol/L]: NaCl 118.3, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25.0, calcium-ethylenediaminetetraacetic acid 0.026, and glucose 11.1). Loose perivascular tissue was removed carefully, and rings were cut (4 mm long). Rings were randomly assigned for gene transfer and were transduced with adenoviral vectors (107 plaque-forming units [PFU]/mL for control, GFP, and S1179DeNOS and 109 PFU/mL for iNOS) in 100 µL minimal essential medium (MEM; containing 0.1% BSA, 100 U/mL penicillin, and 100 µg/mL streptomycin) for 30 minutes at 37°C. The viral titer and incubation time were considered to be optimal for ex vivo gene transfer on the basis of results of our previous studies.4,5 The rings were then transferred to fresh MEM and incubated for 24 hours at 37°C in a CO2 incubator (5% CO2; Forma Scientific, Inc). The next day the rings were suspended for isometric force recording in an organ chamber. Nontransduced arteries used as control for certain experiments were incubated in MEM alone for 24 hours.

Western Blot Analysis of Recombinant eNOS
Soluble proteins were extracted by mincing and homogenizing tissues in lysis buffer (pH 7.5) containing 50 mmol/L Tris-HCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 0.1% SDS, 0.1% deoxycholate, 1% Igepal, and a 1000-fold dilution of a mammalian protease inhibitor cocktail (all from Sigma). The homogenate was rotated for 1 hour at 4°C and then centrifuged (12 000g) for 5 minutes. After centrifugation, the supernatant was collected, and the total protein concentration was measured by a DC Protein Assay Kit (Bio-Rad). Prestained protein markers (Bio-Rad) and 150 µg of sample were loaded into precast 15% SDS/PAGE gels (Bio-Rad). The resolved proteins were transferred to 0.2-µm nitrocellulose membrane on a semidry electrophoretic transfer cell (Bio-Rad) for 1 hour at 37°C. The resolved proteins were transferred to 0.2-µm nitrocellulose membrane on a semidry electrophoretic transfer cell (Bio-Rad) for 1 hour at 37°C. Blots were blocked overnight (4°C) with 5% nonfat dry milk and incubated with monoclonal anti-eNOS antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, Calif) followed by peroxidase-conjugated streptavidin. After washing, bands were visualized by enhanced chemiluminescence (Amersham).

Immunohistochemical Analysis of Gene Expression
Recombinant eNOS protein expression was shown by immunohistochemical staining of transduced arterial rings that were frozen in O.C.T. compound (Sakura Torrance). Five-micrometer cross sections were cut and dried onto slides for 45 minutes (37°C). Sections were then fixed in cold acetone, and endogenous peroxidase was blocked with the use of 0.1% sodium azide/0.3% hydrogen peroxide. Nonspecific protein binding was blocked with a 30-minute incubation in 10% normal rabbit serum/0.5% Tween 20 in phosphate-buffered 0.5 mol/L saline. Sections were then incubated for 1 hour with a monoclonal antibody (2.2 µg/mL) raised against monoclonal anti-eNOS antibody (1:500 dilution, Transduction). The secondary antibody was biotinylated rabbit anti-mouse IgG, followed by peroxidase-conjugated streptavidin. Color development utilized 3-aminio-9-ethylcarbazole and hematoxylin counterstaining. For control studies, specificity of eNOS immunolabeling was examined by the following 3 methods: (1) omission of the primary antibody in the incubation medium, (2) eNOS immunostaining of AdCMVGFPS-transduced vessels, and (3) immunostaining of AdCMV51179DeNOS-transduced vessels with an isotype-matched nonrelevant antibody.

Analysis of Vascular Reactivity
Twenty-four hours after gene transfer, rings were connected to an isometric force-displacement transducer (Grass FT03; Grass Instrument Co) and suspended in an organ chamber filled with 25 mL of control solution (37°C, pH 7.4) aerated with 94% O2/6% CO2. Isometric tension was recorded continuously. The rings were allowed to stabilize at a resting tension of 0.2 to 0.4 g for 1 hour. Each ring was then gradually stretched to the optimal point of its length-tension curve (approximately 3.0 g) as determined by the contraction to 105 mol/L of uridine 5'-triphosphate (UTP).16 All experiments were conducted in the presence of 10-5 mol/L indo- methacin to eliminate the possible influence of endogenous cyclooxygenase. The incubation time with indomethacin or N4-nitro-l-arginine methyl ester (L-NAME) was 30 or 15 minutes, respectively. To evaluate relaxation responses, the rings were contracted with median effective concentration (EC50) of UTP (3×10-6 to 3×10-3 mol/L) before the addition of agonists. Concentration-response curves were obtained in a cumulative fashion. Several rings prepared from the same artery were studied in parallel. The relaxations were expressed as a percentage of maximal relaxations induced by 3×10-4 mol/L papaverine.

Measurement of cGMP
A radioimmunoassay technique was used to determine the levels of cGMP, as reported previously.17 Rings were initially incubated in MEM in a CO2 incubator at 37°C for 30 minutes. L-NAME (3×10-4 mol/L) was added to MEM containing certain rings for 30 minutes to inhibit eNOS enzymatic activity. After 30 minutes, rings were incubated another 30 minutes in 3-isobutyl-1-methylxanthine (IBMX, 10-5 mol/L; Sigma) to inhibit the degradation of cyclic nucleotides by phosphodiesterases. Then the rings were removed from the medium and quickly frozen in liquid nitrogen. After homogenization, cGMP levels were measured by a cGMP radioimmunoassay kit (Amersham). Protein assay was conducted by DC Protein Assay Kit (Bio-Rad).

To determine the effect of calcium in the production of cGMP, both an extracellular calcium chelator (EGTA, 10-5 mol/L) and an intracellular calcium chelator (BAPTA-AM, 2×10-5 mol/L; Calbiochem) were added to the calcium-free medium, and arteries were incubated for 60 minutes at 37°C.4

Drugs
The following pharmacological agents were used: bradykinin, L-NAME, indomethacin, papaverine hydrochloride, UTP (Sigma), diethylammonium (Z)-1-(N, N-diethylaminodiazene-1-ium-1,2-diolate (DEA-NONOate; Cayman Chemical), and ET-1 (Phoenix Pharmaceuticals, Inc). Drugs were dissolved in distilled water, and volumes of <0.15 mL were added to the organ chambers. Solutions of DEA-NONOate in the highest concentration were prepared in 1.5 mol/L Tris (pH 8.8). Concentrations of all drugs are expressed as the final moles per liter concentration in the control solution.

Statistical Analysis
The results of this study are expressed as mean±SEM; n refers to the number of dogs. An unpaired Student’s t test was used to detect significant differences when 2 groups were compared. Factorial ANOVA followed by Bonferroni/Dunn post hoc test was used to detect significant differences in multiple comparisons. Half-maximal effective concentrations (EC50) were calculated by nonlinear regression and expressed as −log mol/L. The concentration-response curves were analyzed by repeated-measures ANOVA followed by Bonferroni/Dunn post hoc test. Statistical significance was accepted at a level of P<0.05.

Results
Expression and Localization of Recombinant Protein
Expression of recombinant GFP or eNOS protein was confirmed in AdCMV51179DeNOS-transduced basilar arteries by Western blot analysis (Figure 1, top; data not shown for
expression of GFP). Immunohistochemistry showed that recombinant S1179DeNOS was localized predominantly in the adventitial layer (Figure 1, bottom). Some endothelial cells were also stained positively when arteries were transduced with AdCMVS1179DeNOS. However, AdCMVGFP-transduced vessels did not show eNOS immunoreactivity (data not shown).

Effects of Recombinant Proteins on Vasomotor Function
In AdCMVeNOS-transduced basilar arteries, endothelium-dependent relaxations to bradykinin (10^{-10} to 10^{-6} mol/L) were significantly augmented compared with control or AdCMVGFP-transduced arteries, as we previously reported (Table). However, in AdCMVS1179DeNOS-transduced arteries, relaxations to bradykinin were not altered compared with control or AdCMVGFP-transduced arteries (P=NS; Figure 2, top). Endothelium-independent relaxations to DEA-NONOate were identical in control, AdCMVGFP-, and AdCMVS1179DeNOS-transduced arteries (P=NS; Figure 2, bottom).

Concentration-Dependent Relaxation to Bradykinin

<table>
<thead>
<tr>
<th></th>
<th>EC_{50}</th>
<th>Maximum Relaxation,</th>
<th>100% Relaxation,</th>
<th>n</th>
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<tr>
<td></td>
<td>-log mol/L</td>
<td>%</td>
<td>g</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.24±0.13</td>
<td>88.7±3.1</td>
<td>3.86±0.20</td>
<td>7</td>
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<td>GFP 10^{10} PFU/mL</td>
<td>8.34±0.11</td>
<td>74.6±1.2</td>
<td>3.71±0.22</td>
<td>7</td>
</tr>
<tr>
<td>AdCMVeNOS 10^{10} PFU/mL</td>
<td>8.70±0.12*</td>
<td>81.2±2.4</td>
<td>4.07±0.25</td>
<td>7</td>
</tr>
</tbody>
</table>

*Significant difference compared with control or GFP group.

AdCMVS1179DeNOS-transduced arteries (P=NS; Figure 2, bottom).

Contractions to ET-1 (10^{-10} to 10^{-8} mol/L) in AdCMVS1179DeNOS-transduced arteries were significantly reduced compared with those in control and AdCMVGFP-transduced arteries (P<0.05). The vasoconstrictor effect of ET-1 was restored in the presence of a NOS inhibitor, L-NAME (3×10^{-4} mol/L; P<0.01; Figure 3). In contrast, contractile responses to UTP were not different in control, AdCMVGFP-, and AdCMVS1179DeNOS-transduced arteries (P=NS; Figure 4).

Effects of Recombinant Proteins on Intracellular cGMP Levels
In AdCMVS1179DeNOS-transduced arteries, basal levels of cGMP were significantly elevated compared with those in control, AdCMVGFP-, and AdCMVeNOS-transduced arteries (P<0.01), and the elevation was abolished by a NOS inhibitor, L-NAME (3×10^{-4} mol/L; P<0.01; Figure 5, top). By incubation in the calcium-free medium in the presence of
calcium chelators EGTA (10^{-3} mol/L) and BAPTA-AM (2 \times 10^{-3} mol/L), levels of cGMP were significantly reduced in AdCMVS1179DeNOS-transduced arteries (P<0.01) but not different in AdCMViNOS-transduced arteries (P=NS; Figure 5, bottom).

**Discussion**

This is the first study to examine the vasomotor function of cerebral arteries expressing recombinant S1179DeNOS gene. The major new findings are as follows: (1) expression of S1179DeNOS appears to increase basal production of NO in cerebrovascular wall, leading to (2) increased formation of cGMP and (3) attenuation of the vasoconstrictor effect of ET-1. These effects are most likely due to constitutively high enzymatic activity of S1179DeNOS.

In a series of previous studies we demonstrated that ex vivo adenovirus-mediated gene transfer resulted in expression of recombinant protein in adventitial fibroblasts of cerebral arteries.3–6 This observation was confirmed in the present study. In AdCMVS1179DeNOS-transduced arteries we detected high expression of recombinant protein by Western analysis. Further morphological analysis demonstrated expression of recombinant S1179DeNOS, mostly in adventitia.

Numerous studies described the effect of recombinant eNOS or neuronal NOS (nNOS) expression on vasomotor function of cerebral and peripheral arteries.3–10,18,19 Expression of these 2 NOS isoforms consistently augmented endothelium-dependent relaxations to different agonists, including bradykinin, and in some experiments selectively attenuated the vasoconstrictor effect of ET-1.4–6 On the basis of these observations, we first examined the effect of recombinant S1179DeNOS on endothelium-dependent relaxations to bradykinin. In the present study we confirmed our previous
findings and demonstrated that expression of recombinant wild-type eNOS augments endothelium-dependent relaxations to bradykinin. In contrast, expression of S1179DeNOS did not affect relaxation to bradykinin, suggesting that activation of recombinant enzyme is not affected by signal transduction involving stimulation of adventitial receptors for bradykinin and subsequent increase in intracellular calcium. It has been postulated that higher sensitivity of S1179DeNOS to calcium could be responsible for maximal activation of recombinant enzyme at subphysiological concentrations of calcium. In that scenario, S1179DeNOS would have constitutively high enzymatic activity and would be insensitive to increase in intracellular calcium induced by bradykinin or ET-1. Activation of S1179DeNOS with very low concentration of calcium offers the best explanation for the inability of bradykinin and ET-1 to further increase production and release of NO in S1179DeNOS-transduced arteries.

Expression of recombinant S1179DeNOS did not affect relaxations to the NO donor DEA-NONOate. This finding is in agreement with the fact that S1179DeNOS did not affect relaxations induced by endogenous NO released from the endothelium in response to bradykinin. It appears that despite higher basal formation of cGMP in vascular wall, sensitivity of guanylate cyclase to NO and reactivity of smooth muscle cells to NO are not changed in S1179DeNOS arteries.

Expression of S1179DeNOS selectively reduced the vasoconstrictor effect of ET-1. This was reversed in the presence of the NOS inhibitor L-NAME, strongly suggesting that activity of recombinant enzyme is responsible for increased basal level of NO and activation of vasodilator mechanisms that could attenuate ET-1–induced contraction of smooth muscle cells. This finding is consistent with the previously reported ability of recombinant eNOS to reduce contractions to ET-1. The mechanisms underlying reduced contractions to ET-1 in AdCMVS1179DeNOS-transduced arteries can be explained by functional antagonism between cGMP and ET-1. However, unlike in cerebral arteries expressing wild-type recombinant eNOS in adventitia, we could not detect ET-1–induced relaxations in arteries expressing S1179DeNOS (M. Akiyama, MD, et al, unpublished data, 2001). It is known that activation of ET-1 receptors on adventitial fibroblasts is coupled to increase in intracellular calcium. This is the mechanism responsible for activation of recombinant eNOS in adventitia of transduced arteries. Mimicking the phosphorylation of serine 1179 by mutation of eNOS to bradykinin. In contrast, expression of wild-type eNOS augments endothelium-dependent relaxations to bradykinin. However, these measurements were performed in the presence of $10^{-4}$ mol/L L-arginine, whereas in the present study transduced arteries were not supplemented with L-arginine. This may explain the fact that in the present study we could not detect a significant increase in cGMP levels in AdCMVeNOS-transduced arteries.

Measurements of cGMP were performed in parallel with same titers of AdCMVGFP, AdCMVeNOS, and AdCMVS1179DeNOS. Obtained results are consistent with idea that serine 1179 mutant enzyme is constitutively active and produces higher amount of NO than wild-type eNOS. A NOS inhibitor, L-NAME, prevented cGMP increase induced by S1179DeNOS, confirming that it is due to enzymatic activity of NOS. We confirmed that the cGMP increase in arteries transduced by iNOS is calcium independent. In contrast, incubation of AdCMVS1179DeNOS-transduced arteries in medium without calcium significantly reduced cGMP levels, suggesting that enzymatic activity of mutated enzyme is dependent on the presence of calcium. This finding is consistent with the demonstrated higher sensitivity of S1179DeNOS activity to intracellular calcium. It is tempting to speculate that mutation of eNOS may offer a therapeutic gene producing NO at a level that is significantly higher than that of wild-type eNOS. Besides the application of recombinant S1179DeNOS in prevention and/or treatment of conditions associated with vasoconstriction due to high production of ET-1 (eg, cerebral vasospasm), it is difficult to predict future therapeutic utilization of this gene. Previous studies demonstrated that NO is a downstream signal molecule of angiogenic growth factors. Whether higher basal production of NO in arteries expressing S1179DeNOS may stimulate angiogenesis is unknown, but this is certainly an area of investigation that deserves further attention.

Our findings suggest that in isolated cerebral arteries S1179DeNOS has the ability to produce a relatively high amount of NO in the absence of shear stress or physiological agonists. Further in vivo studies are needed to characterize exact conditions and vascular diseases that may benefit from gene delivery and expression of S1179DeNOS.

Acknowledgments

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References


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