Adventitia-dependent relaxations of canine basilar arteries transduced with recombinant eNOS gene

MASATO TSUTSUI,1 HISASHI ONOUE,1 YASUHIKO IIDA,1 LESLIE SMITH,1 TIMOTHY O'BRIEN,2 AND ZVONIMIR S. KATUSIC1

Departments of 1Anesthesiology and Pharmacology and of 2Endocrinology and Metabolism, Mayo Clinic, Rochester, Minnesota 55905

Tsutsui, Masato, Hisashi Onoue, Yasuhiro Iida, Les-lie Smith, Timothy O'Brien, and Zvonimir S. Katusic. Adventitia-dependent relaxations of canine basilar arteries transduced with recombinant eNOS gene. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1846–H1852, 1999.—We recently reported that expression of recombinant endothelial nitric oxide (NO) synthase (eNOS) gene in adventitial fibroblasts restores NO formation in canine cerebral arteries without endothelium in response to bradykinin ex vivo and in vivo. The present study was designed to further characterize the stimuli that can activate recombinant eNOS enzyme expressed in the adventitia of cerebral arteries. To stimulate recombinant eNOS, we used serum (0.1–10%), substance P (10⁻¹¹–3 x 10⁻⁹ M), and ANG II (10⁻⁷–10⁻⁵ M) because they increase intracellular calcium concentrations in fibroblasts. Endothelium-denuded segments of canine basilar arteries were incubated with an adenoviral vector encoding β-galactosidase gene or eNOS gene for 30 min at 37°C. After 24 h, vasomotor activity and cGMP formation in eNOS or β-galacto-sidase arteries were examined by isometric force recording and by radioimmunoassay, respectively. In control arteries and β-galactosidase gene-transduced arteries, serum caused concentration-dependent contractions, whereas in recombi-nant eNOS gene-transduced arteries, serum produced concentration-dependent relaxations. Substance P and ANG II had no effect on vascular tone in control and β-galactosidase arteries but caused concentration-dependent relaxations as well as a significant increase in cGMP levels in eNOS arteries. These relaxations were blocked by the NOS inhibitor N⁶-nitro-l-arginine methyl ester. Chemical treatment or mechanical inactivation of adventitial function significantly attenuated substance P-induced relaxations and ANG II-induced relaxations. These findings demonstrate that serum, substance P, and ANG II cause adventitia-dependent relaxations in cerebral arteries expressing the recombinant eNOS gene. This mechanism of vasodilatation may have beneficial effects in the prevention and treatment of vascular disorders characterized by the diminished bioavailability of NO, such as cerebral vasospasm.

angiotensin II; bradykinin; cerebral arteries; gene transfer; nitric oxide; serum; endothelial nitric oxide synthase

IN OUR PREVIOUS STUDIES WE constructed recombinant adenovirus encoding cDNA for endothelial nitric oxide synthase (eNOS) driven by the cytomegalovirus promoter and examined expression and function of recombinant eNOS gene in canine cerebral and peripheral arteries ex vivo (6, 21, 26) and in vivo (5). We used immunohistochemical, biochemical, and pharmacologi-cal analyses to demonstrate that the efficiency of adenovirus-mediated gene transfer ex vivo was much higher in cerebral arteries than in coronary or femoral arteries (6, 21, 26). Transgene expression in canine cerebral arteries was detected mostly in adventitia with moderate levels of expression in endothelial cells after ex vivo gene transfer (6, 21, 26). Intravascular administration of vectors may not be feasible in cere-bral blood vessels in vivo, because this approach requires interruption of cerebral blood flow and may damage the blood-brain barrier (10, 22). However, we demonstrated that functional expression of recombi-nant eNOS gene in adventitia of canine cerebral arter-ies can be achieved by administration of vectors into cerebrospinal fluid via the cisterna magna (5). Immuno-gold labeling and electron microscopy indicated that expression of recombinant eNOS protein was limited to adventitial fibroblasts. eNOS-transduced arteries with intact endothelium exhibited a calcium-dependent in-crease in cGMP production and an augmentation of bradykinin-induced relaxations. Interestingly, perivas-cular eNOS gene delivery caused significant concentra-tion-dependent relaxations to bradykinin, as well as an increase in cGMP formation even after endothelium removal (5, 26). In this study, we hypothesized that elevation of intracellular calcium concentrations in adventitial fibroblasts may elicit activation of recombi-nant eNOS enzyme and increase formation of nitric oxide. A review of the existing literature indicated that serum (19), substance P (9, 20), ANG II (9, 15), and bradykinin (3, 9) mobilize cellular free calcium in fibroblasts. Therefore, we decided to characterize the effects of recombinant eNOS gene expression in adventitia on vasomotor reactivity to these stimuli.

MATERIALS AND METHODS

Adenoviral vectors. Construction, propagation, purification, and evaluation of adenoviral vectors containing the eNOS gene used in the present study were described in detail previously (6). In brief, a replication-defective recombinant adenovirus vector encoding a bovine eNOS gene, driven by cytomegalovirus immediate-early promoter, was generated through homologous recombination. A full-length serotype 5 wild adenovirus was made replication deficient by the deletion of the early region 1 (E1), followed by the insertion of the DNA sequence encoding bovine aortic endothelial cell eNOS (generously provided by Dr. David G. Harrison, Emory Univer-sity, Atlanta, GA). The shuttle vector pACCMvPPlPA was a kind gift of Dr. Robert Gerard (University of Texas Southwest-ern Medical Center, Dallas, TX). A recombinant adenoviral
vector encoding β-galactosidase reporter gene driven by a cytomegalovirus promoter, which was used in all experiments as a control, was a kind gift of Dr. James M. Wilson (University of Pennsylvania, Philadelphia, PA).

Gene transfer. All procedures were in accordance with Institutional Animal Care and Use Committee guidelines of the Mayo Clinic. Rings (3 mm long) of basilar arteries were taken from anesthetized mongrel dogs (wt 18–27 kg, 30 mg/kg pentobarbital sodium iv). Arterial rings were gently rinsed with Krebs-Ringer bicarbonate solution composed of (in mM) 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 15.0 NaHCO₃, 0.0026 calcium EDTA, and 11.1 glucose to remove blood. All experiments were performed on arteries without endothelium, which was mechanically removed. The surfaces of needles (19–22 gauge) were made rough with sandpaper, and the needles were fixed for denudation in a dish filled with Krebs-Ringer bicarbonate solution. Endothelium removal was accomplished by sliding an arterial segment over the needle with two pairs of fine forceps under a microscope (5, 21, 26). After this procedure, the rings were randomly assigned for gene transfer. Arterial rings were transduced with an adenoviral vector in Eagle’s minimum essential medium (MEM) (with Earle’s salts, containing 0.1% BSA, 100 U/ml penicillin, and 100 mg/ml streptomycin) for 30 min at 37°C and then transferred to MEM and incubated for 24 h at 37°C in a CO₂ incubator (5% CO₂-95% air) (Forma Scientific, Marietta, OH) (24). Control arteries (nontransduced) were incubated in MEM for 24 h in the same manner.

To disrupt function of the adventitial layer, the outer surface of arterial rings was treated with 100% ethanol (contact time = 2–3 s). For mechanical disruption, a stainless steel wire was inserted into the lumen while the adventitial side was rubbed by gentle rolling on paper towels soaked with Krebs-Ringer bicarbonate solution. Adventitial dysfunction was confirmed by significant attenuation of relaxations induced by perivascular nerve stimulation with nicotine (10⁻⁵ M; Fig. 3A) (25). Integrity of underlying smooth muscle cells

| Table 1. Forskolin-induced relaxations in control, β-galactosidase gene-transduced, and eNOS gene-transduced canine basilar arteries without endothelium |
|---------------------------------|----------------|-----------------|-----------|
|                                 | EC₅₀, -log mol/l | Maximal Relaxations, % | n         |
| Control                         | 7.1 ± 0.1        | 94.8 ± 1.7      | 5         |
| β-Galactosidase                 | 7.2 ± 0.1        | 94.4 ± 1.4      | 5         |
| eNOS                            | 7.2 ± 0.1        | 91.5 ± 2.8      | 5         |

Data are means ± SE; n = no. of dogs. Neither β-galactosidase gene nor endothelial nitric oxide synthase (eNOS) gene expression affected relaxations to forskolin (10⁻⁵–10⁻⁶ M). Arterial rings taken from the same dogs were studied in parallel. EC₅₀, median effective concentration. Maximal relaxations, percentage of maximal relaxation induced by papaverine (3 × 10⁻⁴ M; 100% = 6.1 ± 0.6, 5.0 ± 0.5, and 5.2 ± 0.5 g for control, β-galactosidase, and eNOS, respectively). Statistical evaluation of data was performed by 1-way repeated-measure ANOVA.

Fig. 1. Effect of serum (A), substance P (B), or ANG II (C) on vascular tone in canine basilar arteries without endothelium transduced with the recombinant endothelial nitric oxide synthase (eNOS) gene. Relaxations were obtained during submaximal contractions induced by uridine 5’-triphosphate (UTP). Data are means ± SE and are percentage of maximal relaxation induced by papaverine (3 × 10⁻⁴ M; 100% = 4.0 ± 0.5, 3.4 ± 0.3, and 3.6 ± 0.2 g (A); 5.2 ± 0.5, 4.4 ± 0.4, and 3.7 ± 0.3 g (B); and 5.1 ± 0.4, 4.5 ± 0.3, and 4.9 ± 0.3 g (C) for control, β-galactosidase (β-Gal), and eNOS, respectively).
was confirmed by comparable contractions of smooth muscle induced with uridine 5'-triphosphate (UTP, Fig. 3B; Ref. 26).

Vascular reactivity. Twenty-four hours after gene transfer, arterial rings were connected to isometric force-displacement transducers (Grass Instruments, Quincy, MA), suspended in organ chambers filled with 25 ml of Krebs-Ringer bicarbonate solution (pH 7.4, 37°), and gassed with 94% O2-6% CO2. Isometric force was recorded continuously and arteries were allowed to stabilize for 1 h. The rings were then stretched progressively to optimal force (-3 g), determined by repeated stimulation with 10^{-6} M UTP (1). Concentration-response curves to serum (10^{-11}-10^{-5} M), ANG II (10^{-7}-10^{-5} M), or bradykinin (10^{-11}-3 \times 10^{-9} M) were cumulatively obtained during submaximal contractions with median effective concentration (EC_{50}) of UTP (10^{-6}-4 \times 10^{-5} M). To inhibit cyclooxygenase activity, we performed all experiments in the presence of indomethacin (10^{-5} M). The incubation time for indomethacin or N^G-nitro-L-arginine methyl ester (L-NAME; 3 \times 10^{-4} M) was 30 or 15 min, respectively. The relaxations were expressed as a percentage of maximal relaxations induced by papaverine (3 \times 10^{-4} M). In all experiments, arterial rings taken from the same dogs were studied in parallel.

Intracellular cGMP levels. A radioimmunoassay technique was used to determine the levels of cGMP, as reported previously (27). Indomethacin (10^{-5} M) and 3-isobutyl-1-methylxanthine (10^{-3} M) were added to the incubation medium 24 h after gene transfer for 30 min at 37°C to inhibit cyclooxygenase activity and the degradation of cGMP by phosphodiesterases, respectively. During the last 2 min of the 30-min incubation, certain rings were stimulated with either substance P (10^{-9} M) or ANG II (10^{-5} M), and rings were then removed from the medium and quickly frozen in liquid nitrogen. After homogenization, cGMP levels were measured by a cGMP RIA kit (Amersham, Arlington Heights, IL). Total protein levels were determined by Lowry’s method (12). Arterial rings taken from the same dogs were studied in parallel.

Drugs. The following agents were used: substance P, ANG II, bradykinin, nicotine, indomethacin, UTP, papaverine hydrochloride, L-NAME, phenylephrine bitartrate (Sigma, St. Louis, MO), fetal bovine serum, MEM, and penicillin-streptomycin (GIBCO BRL, Grand Island, NY). Indomethacin was dissolved with equal molar concentrations of Na_{2}CO_{3}. All concentrations are expressed as final molar concentration in medium or solution.

Statistical analysis. The results are expressed as means ± SE. In each set of experiments, n refers to the number of animals studied. Statistical evaluation of the data was performed by ANOVA, followed by Bonferroni-Dunn post hoc test (13). P < 0.05 was considered to be statistically significant.

RESULTS

Effect of serum, substance P, and ANG II. In control (nontransduced) and β-galactosidase gene-transduced basilar arteries without endothelium, low concentrations of serum (0.1–1%) caused concentration-dependent contractions (Fig. 1A). These contractions returned to initial vascular tone during cumulative addition of higher serum concentrations (3–10%) (Fig. 1A). In contrast, in recombinant eNOS gene-transduced arteries, 3–10% serum produced significant con-
Substance P (10^{-11}-3 \times 10^{-9} \text{ M}; \text{Fig. 1B}) and ANG II (10^{-7}-10^{-5} \text{ M}; \text{Fig. 1C}) had little effect on vascular tone in control and β-galactosidase arteries. However, these compounds caused significant concentration-dependent relaxations in eNOS arteries (Fig. 1, B and C; P < 0.05). On the other hand, relaxations induced by forskolin, a direct activator of adenylate cyclase, were identical among control, β-galactosidase, and eNOS arteries without endothelium (Table 1). These relaxations to...
serum (Fig. 2A), substance P (Fig. 2B), and ANG II (Fig. 2C) were blocked by the NOS inhibitor L-NAME (3 × 10⁻⁴ M; P < 0.05).

Effect of chemical or mechanical disruption of adventitia. To determine whether substance P-, ANG II-, or bradykinin-elicited relaxations in eNOS arteries without endothelium are adventitia dependent, the effect of chemical (ethanol treatment) or mechanical (adventitial rubbing) disruption of adventitia was examined. In ethanol-treated eNOS arteries, nicotine-induced perivascular nerve-dependent relaxations (25) were inhibited significantly, suggesting disruption of adventitial function (both P < 0.05; Fig. 3A). Contractions induced by UTP, an activator of smooth muscle cells (28), were comparable among control arteries and arteries exposed to ethanol or mechanical disruption of adventitia, which indicates integrity of underlying smooth muscle cell function (Fig. 3B). In chemically (Fig. 4A) or mechanically (Fig. 4B) treated eNOS arteries, substance P-induced relaxations were significantly attenuated (P < 0.05). ANG II- and bradykinin-induced relaxations were similarly inhibited after disruption of adventitia (Fig. 4, C and D, Table 2; P < 0.05).

cGMP levels. Stimulation with substance P (10⁻⁹ M) and ANG II (10⁻⁵ M) significantly increased cGMP production in eNOS-transduced arteries without endothelium but not in β-galactosidase-transduced arteries (Fig. 5).

Table 2. Effect of chemical or mechanical disruption of adventitial cell function on bradykinin-induced relaxations in eNOS arteries without endothelium

<table>
<thead>
<tr>
<th>Bradykinin, −log M</th>
<th>eNOS</th>
<th>eNOS + chemical treatment</th>
<th>eNOS + mechanical treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>0 ± 0</td>
<td>4.2 ± 1.5</td>
<td>28.5 ± 7.3</td>
</tr>
<tr>
<td>10.5</td>
<td>9.2 ± 2.0</td>
<td>24.8 ± 7.4</td>
<td>7.7 ± 0.7</td>
</tr>
<tr>
<td>10</td>
<td>3.8 ± 0.9</td>
<td>7.5 ± 0.6</td>
<td>7.8 ± 0.8</td>
</tr>
<tr>
<td>9</td>
<td>2.8 ± 1.1</td>
<td>7.2 ± 1.1</td>
<td>7.3 ± 2.0</td>
</tr>
<tr>
<td>8.5</td>
<td>0.8 ± 0.8</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as percentage of relaxation; n = 3 dogs. Chemical and mechanical treatments significantly inhibited bradykinin-induced relaxations in eNOS arteries, both P < 0.05.

DISCUSSION

Our previous studies demonstrated that genetically modified adventitial fibroblasts transduced with a recombinant eNOS gene restore the production of nitric oxide in arteries without endothelium. This supports a novel concept in vascular biology that fibroblasts in adventitia may play a role in the regulation of vascular tone after successful transfer and expression of a recombinant eNOS gene (26). The major new findings of the present study are that serum, substance P, and ANG II may activate recombinant eNOS expressed in basilar artery adventitia and cause adventitia-dependent relaxations via enhanced local production of nitric oxide.

Serum is vasoactive and causes contraction of isolated canine cerebral arteries (24) by increasing intracellular concentrations of calcium in smooth muscle cells (19). In the present study, recombinant eNOS gene expression reverses the vasoconstrictor effect of serum and produces vasodilatation. Substance P and ANG II did not affect vascular tone in control or β-galactosidase arteries without endothelium. In contrast, these compounds caused significant concentration-dependent relaxations in eNOS-transduced arteries. These results are consistent with the hypothesis that an increase in cellular free calcium in fibroblasts by calcium mobilizing agents may activate a recombinant eNOS enzyme.
Relaxations induced by a direct activator of adenylate cyclase, forskolin (18, 23), were identical among control, β-galactosidase, and eNOS arteries without endothelium. Relaxations induced by nitric oxide donors also have been reported to be comparable among those arteries (21). These findings indicate that the expression of recombinant eNOS gene in adventitia augments only relaxations to stimuli that can activate production and release of endogenous nitric oxide.

The relaxations to serum, substance P, and ANG II were inhibited by the NOS inhibitor L-NAME. These findings suggested that expression of recombinant eNOS gene in adventitia may cause an increase in local formation of nitric oxide in response to serum and vasoactive peptides. The selectivity of L-NAME was demonstrated in our previous studies (7). L-NAME did not affect relaxations evoked by the nitric oxide donor 3-morpholinosydnonimine, and the inhibitory effect of L-NAME was corrected by L-arginine (7).

We next sought to determine whether the observed relaxations in eNOS arteries without endothelium are adventitia-dependent. It was very difficult to completely remove the adventitial layer because cerebral arteries lack external elastic lamina (30). Therefore, we examined the effect of chemical (ethanol treatment) or mechanical (adventitial rubbing) disruption of adventitial function. In chemically or mechanically treated eNOS arteries, nicotine-induced perivascular nerve-dependent relaxations (25) were markedly reduced. On the other hand, contractions induced by UTP, an activator of smooth muscle cells (28), were not affected. These results suggest that adventitial dysfunction without damage of underlying smooth muscle function was achieved by chemical and mechanical treatments employed in the present study. In ethanol-exposed and adventitia-rubbed eNOS arteries, substance P-, ANG II-, and bradykinin-induced relaxations were significantly reduced, which supported our conclusion that the relaxant effects of these compounds are dependent on the presence of intact adventitia.

cGMP, a major second messenger for nitric oxide, is generated by activation of soluble guanylate cyclase, and changes in cGMP levels reflect nitric oxide production (11, 16). Substance P and ANG II significantly increased cGMP production in eNOS-transduced arteries without endothelium, suggesting that nitric oxide formation can be restored by the expression of recombinant eNOS gene in adventitia. This conclusion is reinforced by the fact that fibroblasts express receptors for substance P, ANG II, and bradykinin (3, 15, 20). Most importantly, activation of these receptors is coupled to the increase in intracellular calcium concentration needed for activation of eNOS enzymatic activity (3, 15, 20). It is also important that substance P is produced in perivascular peptidergic nerves (2), whereas ANG II is generated in blood vessel wall (4, 8). High activity of angiotensin-converting enzyme, which transforms ANG I into ANG II and converts bioactive bradykinin to inactive peptide (29), has also been detected in vascular adventitia (31). Whether recombinant eNOS protein expressed in adventitia can be activated by locally released endogenous peptides in vivo remains to be determined.

In summary, the present study demonstrates that serum, substance P, ANG II, and bradykinin cause adventitia-dependent relaxations in canine cerebral arteries without endothelium transduced with recombinant eNOS gene. Elevation of cellular free calcium concentration in adventitial fibroblasts is the most likely mechanism responsible for activation of recombinant eNOS. We speculate that this novel mechanism of vasodilatation may have a beneficial effect in prevention and treatment of cerebrovascular disorders characterized by decreased bioavailability of nitric oxide.

The authors thank Janet Beckman for preparing the manuscript. This work was supported in part by National Heart, Lung, and Blood Institute Grant HL-53524, National Institute of Neurological Disorders and Stroke Grant NS-37491, funds from the Bruce and Ruth Rappaport Program in Vascular Biology, Mayo Clinic Molecular Medicine Program, and the Mayo Foundation.

Address for reprint requests and other correspondence: Z. S. Katusic, Depts. of Anesthesiology and Pharmacology, Mayo Clinic, 200 First St. SW, Rochester, MN 55905 (E-mail: katusic.zvonimir@mayo.edu).

Received 16 November 1998; accepted in final form 2 February 1999.

REFERENCES


