Recombinant Gene Transfer of Endothelial Nitric Oxide Synthase Augments Coronary Artery Relaxations During Hypoxia

David G. Cable, MD; Vincent J. Pompili, MD; Timothy O’Brien, MD; Hartzell V. Schaff, MD

Background—Coronary arteries respond to hypoxia with transient relaxations, which increases coronary blood flow, in part, by release of nitric oxide. We hypothesized that increased expression of nitric oxide synthase might further augment blood vessel relaxation during hypoxia. The present study examined the effect of adenovirus-mediated transfer of bovine endothelial nitric oxide synthase (eNOS) on hypoxia-induced transient relaxations in canine coronary arteries.

Methods and Results—Paired segments of coronary arteries were exposed to vehicle (phosphate-buffered saline with albumin) or an adenovirus encoding either E. coli β-galactosidase (Ad.CMV LacZ, viral control; 10¹⁰ pfu/mL) or eNOS (Ad.CMVeNOS; 10¹⁰ pfu/mL) for 2 hours at 37°C. Immunohistochemistry with a monoclonal antibody specific for eNOS documented both endothelial and adventitial expression in Ad.CMVeNOS arteries, whereas vehicle and viral controls demonstrated only constitutive expression. Levels of cGMP were increased 5-fold in Ad.CMVeNOS arteries compared with controls. In arteries exposed to Ad.CMVeNOS, maximum contraction to prostaglandin F₂α was reduced compared with viral controls, and this effect was eliminated by pretreatment with a competitive inhibitor of eNOS (N⁶-monomethyl-L-arginine, 10⁻³ mol/L). Hypoxia-induced transient relaxation (95% N₂-5% CO₂) in Ad.CMVeNOS arteries (45.2±8.8%, n=6) was augmented compared with vehicle (26.3±6.0%) or viral (27.2±7.1%) controls.

Conclusions—Adenovirus-mediated gene transfer of nitric oxide synthase reduces receptor-dependent contractions and augments hypoxia-induced relaxations in canine coronary arteries; this method of augmentation of NO production might be advantageous for reduction of coronary artery vasospasm. (Circulation. 1999;100[suppl II]:II-335–II-339.)

Key Words: arteries n genes n genetics n nitric oxide n hypoxia n β-galactosidase n ischemia

Coronary arteries respond to hypoxic insult with a transient relaxation followed by contraction. This transient relaxation may be a homeostatic mechanism to preserve or augment coronary perfusion. Previous investigations have documented endothelial release of both prostacyclin and nitric oxide during hypoxia.¹⁻⁴ Release of these vasoactive substances may explain, in part, the initial transient relaxation of isolated vessels during hypoxia. However, accumulation of cGMP, the common effector of nitric oxide, is impaired during hypoxia at the level of the membrane receptor, G-protein, or nitric oxide synthase.⁵⁻⁷ Therefore, augmentation of nitric oxide production, as with gene transfer of nitric oxide synthase, may not produce a biological effect during hypoxia.

Continued hypoxia induces coronary artery contractions, the mechanism of which remains undefined, although an endothelium-derived contracting factor has been postulated.⁸ Vessels mechanically denuded of endothelium produce only a mild relaxation on exposure to hypoxia. The addition of an endothelial source to the organ bath is associated with a return of hypoxia-induced contractions, which suggests a diffusible factor. However, all presently identified endothelium-dependent contracting factors have now been excluded as mediators. The ability to reverse or counter the actions of this contracting factor by augmentation of nitric oxide production has not been reported to date, but this property might be advantageous in reducing coronary artery vasospasm.

We have previously demonstrated a role of nitric oxide in hypoxic vasoactive responses,⁹ but the effect of overexpression of NOS is not known. Accordingly, we designed experiments to evaluate adenovirus-mediated gene transfer of bovine endothelial nitric oxide synthase (eNOS) to canine coronary arteries and the subsequent response to hypoxia.

Methods

Humane care was provided in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication

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Gene Transfer

Coronary arteries were obtained from purpose-bred, heartworm-free, adult canines and divided into 3- to 4-mm segments; great care was used to ensure that segments were similar in length in paired arteries. Arteries were exposed to vehicle (phosphate-buffered saline with albumin [PBSA]) or an adenovirus encoding *E. coli β*-galactosidase (Ad.CMVlacZ, 10<sup>10</sup> pfu/mL) or eNOS (Ad.CMVeNOS, 10<sup>9</sup> pfu/mL) for 2 hours at 37°C as previously described. The effect of gene transfer was evaluated after 24 hours of incubation in a 5% CO<sub>2</sub> incubator (Forma Scientific Inc).

Histology

Vessels were flash-frozen (<70°C), sectioned, and immersed in acetone (4°C) and 1% paraformaldehyde-EDTA. Goat serum (5%)–PBS–Tween 20 was used to bind nonspecific protein sites. An eNOS monoclonal antibody (5 μg/mL; 1:50 dilution; 60 minutes; Transduction Laboratory) incubation was followed by biotinylated rabbit anti-mouse F(ab')<sub>2</sub> (1:300; 20 minutes) secondary antibody and peroxidase-conjugated streptavidin (1:300, 20 minutes). Immunoreactivity of eNOS was documented by 3-amino-9-ethylcarbazole and hematoxylin counterstaining.

In Vitro Studies

Coronary arteries were then mounted in organ chambers containing crystalloid solution (pH 7.4, 37°C) aerated with 95% O<sub>2</sub>–5% CO<sub>2</sub>. Arteries were suspended between 2 stainless-steel clips connected to a strain gauge for the measurement of isometric force (Statham UC2, Gould Inc). After confirming the presence of endothelium, we allowed the segments to equilibrate for 30 minutes with indomethacin (10<sup>−5</sup> mol/L) to block endogenous cyclooxygenase activity, because hypoxia releases prostacyclin. Hypoxia was produced by aeration with a 95% N<sub>2</sub>–5% CO<sub>2</sub> mixture.

Histologic sections were stained with hematoxylin and eosin. Sections were examined under a microscope (Zeiss Axioskop) with an AxioVision 32-bit digital camera (Zeiss, Jena, Germany).

Statistical Analysis

Results

Coronary arteries were immunostained with a monoclonal antibody specific for eNOS. Arteries exposed to either dilution vehicle (PBSA) or control recombinant adenovirus (Ad.CMVlacZ) demonstrated constitutive expression only of eNOS within the endothelium (Figures 1A and 1B). In contrast, Ad.CMVeNOS arteries demonstrated high-level expression within the endothelium, but no novo adventitial expression also was present (Figure 1C).

In Vitro Studies

No significant difference was noted in the tension generated to a maximal concentration of KCl (80 mmol/L). Coronary arteries exposed to vehicle (PBSA) and viral (Ad.CMVlacZ) controls generated similar tensions with KCl addition (11.9±0.8 versus 11.8±0.6 g, respectively; n=6; P=0.66). Similar responses were noted in Ad.CMVeNOS arteries (10.6±0.7 g; P=0.44 versus PBSA, P=0.25 versus Ad.CMVlacZ).

In contrast, when a receptor-dependent contracting agent was used, significant differences were noted after recombinant transfer of eNOS (Figure 2). Prostaglandin F<sub>2α</sub> generated a maximum tension of 4.3±0.6 g in PBSA and 4.7±0.7 g in Ad.CMVlacZ arteries (n=6; P=0.98). A significant reduction in maximum force generated with prostaglandin F<sub>2α</sub> was noted in Ad.CMVeNOS arteries (2.7±0.5 g) when compared with viral (P=0.05 versus Ad.CMVlacZ) but not vehicle controls (P=0.07 versus PBSA).

We were careful to ensure that paired arterial rings were the same length; greater length could correlate with greater smooth muscle mass and, subsequent, greater contraction. To guard against this variable, the maximum tensions generated to prostaglandin F<sub>2α</sub> were also normalized to the KCl contractions. Prostaglandin F<sub>2α</sub> generated a normalized tension of 36.1±4.5% KCl in PBSA and 39.6±5.9% KCl in Ad.CMVlacZ arteries (n=6, P=0.89). A significant reduction in maximum force was again noted in Ad.CMVeNOS arteries (26.2±4.8% KCl) when compared with viral (P=0.05 versus Ad.CMVlacZ) and now also with vehicle controls (P=0.03 versus PBSA). This contraction differential could be eliminated by pretreatment with large concentrations of L-NMMA (10<sup>−3</sup> mol/L), a competitive inhibitor of nitric oxide synthase. After pretreatment with L-NMMA, all groups contracted similarly to prostaglandin F<sub>2α</sub>(PBSA 60.3±8.1% KCl versus Ad.CMVlacZ 58.8±19.9% KCl versus Ad.CMVeNOS 49.1±10.6% KCl).

Hypoxia

Hypoxia was generated in the organ chambers by changing the gas mixture from 95% O<sub>2</sub>–5% CO<sub>2</sub> to aeration with 95% N<sub>2</sub>–5% CO<sub>2</sub>. The composition of the buffered crystalloid solution at various times is noted in the Table 1. Coronary arteries were contracted with a submaximal concentration of prostaglandin F<sub>2α</sub> (10<sup>−6</sup> mol/L), thus permitting either relaxation or contraction. On switching to the hypoxic gas mixture, all vessels maximally relaxed within 5 minutes (Figure 3).
Vehicle (PBSA; 26.3±6.0% baseline; n=6) and virus (Ad.CMVlacZ; 27.2±7.1% baseline) controls had similar hypoxia-induced relaxations (P=0.46). A significant augmentation of hypoxia-induced relaxations was noted in Ad.CMVeNOS arteries (45.2±8.8% baseline; P=0.01 versus PBSA, P=0.03 versus Ad.CMVlacZ).

After the transient relaxations, sustained contractions were noted in all control and 60% of the Ad.CMVeNOS vessels. No significant difference existed in PBSA (128.6±23.4% contraction), Ad.CMVlacZ (116.8±22.1%, P=0.41 versus PBSA), and Ad.CMVeNOS (127.0±47.9%, P=0.70 versus PBSA, P=0.74 versus Ad.CMVlacZ) arteries with regard to amplitude of hypoxia-induced contractions.

cGMP Levels
To further confirm augmented nitric oxide production in Ad.CMVeNOS arteries and evaluate the release during hypoxia, basal and hypoxic levels of cGMP were measured (Figure 4). Basal cGMP was 571.6±138.5 pg/mg of protein in PBSA (n=5) and 499.2±236.8 pg/mg in Ad.CMVlacZ (P=0.73 versus PBSA) coronary arteries. A nearly 5-fold increase was seen in basal cGMP level in Ad.CMVeNOS (2441.2±1309.1 pg/mg; P=0.21 versus PBSA, P=0.14 versus Ad.CMVlacZ), although this did not reach statistical significance. After 2.5 minutes of hypoxia, vessels treated with PBSA (760.8±155.2 pg/g) and Ad.CMVlacZ (626.8±243.1 pg/mg) produced significantly less cGMP than vessels exposed to Ad.CMVeNOS (2048.2±889.0 pg/mg; P=0.11 versus PBSA, P=0.03 versus Ad.CMVlacZ). If hypoxia was continued for 5 minutes, similar results were obtained; PBSA (499.2±177.4 pg/g) and Ad.CMVlacZ (522.2±184.0 pg/mg) had lower cGMP levels than Ad.CMVeNOS (2048.2±889.0 pg/mg; P=0.11 versus PBSA, P=0.13 versus Ad.CMVlacZ) arteries, although this did not reach statistical significance.

<table>
<thead>
<tr>
<th>Composition of Crystalloid Solution</th>
<th>pH</th>
<th>PCO2, mm Hg</th>
<th>PO2, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>7.47±0.01</td>
<td>32.4±0.7</td>
<td>526±56</td>
</tr>
<tr>
<td>5-minute hypoxia</td>
<td>7.45±0.01</td>
<td>33.3±0.8</td>
<td>71±5*</td>
</tr>
<tr>
<td>10-minute hypoxia</td>
<td>7.45±0.01</td>
<td>33.4±1.2</td>
<td>56±3*</td>
</tr>
</tbody>
</table>

*P=0.004 vs normoxia.
The present study demonstrates the sequelae of eNOS overexpression in coronary arteries during hypoxia. Overexpression of eNOS in canine coronary arteries significantly augmented relaxations during hypoxia, and this was associated with elevated cGMP levels throughout the early period of hypoxia. However, overexpression of eNOS did not alter the amplitude of sustained contractions during continued hypoxia.

Complementary techniques confirmed overexpression of nitric oxide synthase after adenovirus-mediated recombinant gene transfer. A monoclonal antibody specific for constitutive eNOS demonstrated that the enzyme was present in larger amounts in vessels exposed to Ad.CMVeNOS compared with controls. In addition, marked expression of eNOS was noted in the adventitia of these vessels. The affinity of adventitial fibroblasts for adenovirus-mediated gene transfer of NOS has previously been described.14

Furthermore, receptor-dependent contractions were reduced in arteries exposed to Ad.CMVeNOS compared with control vessels, and this difference was reversed by pretreatment with L-NMMA, a competitive inhibitor of nitric oxide synthase.15 Finally, basal cGMP levels were elevated in Ad.CMVeNOS arteries compared with controls. The immunohistochemistry together with the specificity of L-NMMA reversal and basal cGMP levels imply the overexpression of functional nitric oxide synthase in Ad.CMVeNOS coronary arteries. This compilation of findings is similar to previous studies from our laboratory11,16–19 and from others14,20 that show increased NO release after adenovirus-mediated gene transfer of eNOS.

After confirmation of augmented and functional nitric oxide synthase in vessels exposed to Ad.CMVeNOS, the vasoactivity of these vessels was evaluated. Simply increasing eNOS activity might not augment vasorelaxation during hypoxia. Moderate hypoxia can impair stimulated production of cGMP, and Johns et al21 noted reduced receptor-dependent and -independent stimulated cGMP levels in rabbit pulmonary arteries during hypoxia. However, sodium nitroprusside was still capable of increasing cGMP levels during hypoxia, which suggests that the site at which hypoxia blocked stimulated cGMP production was proximal to activation of soluble guanylate cyclase.

However, in contrast, augmented basal cGMP levels with recombinant NOS overexpression were noted in the present study. No significant attenuation of these basal levels was noted during 5 minutes of hypoxia, the time period that correlated with vasorelaxation. These augmented basal cGMP levels were associated with augmented hypoxic vasoactivity; hypoxia-induced transient relaxations were increased 2-fold in Ad.CMVeNOS arteries. Although a role for nitric oxide in hypoxic vasoactive responses has previously been demonstrated,9 the present studies confirm the utility of eNOS gene transfer to augment responses under hypoxic conditions.

In addition, the present study attempted to inhibit the actions of the contracting factor by overexpression of eNOS. Coronary arteries exposed to Ad.CMVeNOS produced equivalent hypoxia-mediated contractions compared with controls, indicating that eNOS overexpression may not prevent hypoxic-induced vasospasm.

Although augmentation of eNOS expression did not prevent hypoxia-induced vasospasm, increased nitric oxide production might prevent or interrupt the pathophysiology of acute coronary syndromes. Reduced coronary artery blood flow may occur secondary to either a thrombotic occlusion or arterial vasospasm. The inciting, early thrombi are often nonocclusive aggregates of platelets.22 Evidence of arterial vasospasm has been demonstrated pathologically23 and angiographically.24,25 Overexpression of eNOS would be expected to inhibit platelet aggregation and activation while also increasing blood flow by vasodilation, thus preventing tissue hypoxia.

In addition to the potentiation of coronary hypoxia-induced relaxations demonstrated in this study, gene therapy with eNOS augments nitric oxide release from human saphenous vein11 and porcine coronary arteries,19 inhibits intimal hyperplasia in rabbit arteries,20 and augments relaxation16 and inhibits contractions17 of human radial arteries. Despite these potential benefits, concerns still exist regarding the clinical use of eNOS-based gene therapy.
Augmentation of nitric oxide by recombinant gene transfer of eNOS, although unproven to date, would be expected to increase the potent metabolite of nitric oxide, peroxynitrite. Peroxynitrite is capable of lipoprotein oxidation and tyrosine nitration\textsuperscript{26,27}; these effects may be cytotoxic at high concentrations.\textsuperscript{28} The reactive oxidant may arrest sodium ion transport and cause myocardial injury.\textsuperscript{29,30} In addition to the cytotoxic effects that eNOS overexpression may cause, difficulty with clinical use of the present adenoviral vectors remains. Current vectors may not be applicable to humans because of inadequate safeguards and the potential for immunological response to the virus that could negate any beneficial action of gene transfer.\textsuperscript{31} In conclusion, adenovirus-mediated transfer of nitric oxide synthase reduces receptor-dependent contractions and augments hypoxia-induced relaxations in canine coronary arteries.

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