Mechanisms of Inducible Nitric Oxide Synthase–Mediated Vascular Dysfunction


Objective—Inducible nitric oxide synthase (iNOS) is expressed in arteries during inflammation and may contribute to vascular dysfunction. Effects of gene transfer of iNOS to carotid arteries were examined in vitro in the absence of systemic inflammation to allow examination of mechanisms by which iNOS impairs contraction and relaxation.

Methods and Results—After gene transfer of iNOS with an adenovirus (AdiNOS), constrictor responses to phenylephrine (PE) and U46619 were impaired. After AdiNOS, inhibition of soluble guanylate cyclase (sGC) with 1H-[1,2,4]oxadiazolo-[4,3,2]quinoxalin-1-one (ODQ) reduced the EC50 for PE from 4.33±0.78 μmol/L to 1.15±0.43 μmol/L (mean±SEM). These results imply that iNOS impairs contraction by activation of the NO/cGMP pathway. Relaxation to acetylcholine (ACH) also was impaired after AdiNOS. Sepiapterin (300 μmol/L), the precursor for tetrahydrobiopterin (BH4), improved relaxation to ACh. Because BH4 is an essential cofactor for production of NO by both iNOS and endothelial nitric oxide synthase (eNOS), these results suggest that iNOS may reduce production of NO by eNOS by limiting availability of BH4. Next, we examined effects of expression of iNOS in endothelium and adventitia. Selective expression of iNOS in endothelium, but not adventitia, impaired contraction to phenylephrine and relaxation to acetylcholine.

Conclusions—We conclude that: (1) iNOS may impair contraction in part by activation of sGC; (2) iNOS impairs relaxation, at least in part, by limiting availability of BH4; and (3) expression of iNOS in endothelium may be a more important mediator of vascular dysfunction than expression of iNOS in adventitia. (Arterioscler Thromb Vasc Biol. 2005;25:0-0.)

Key Words: acetylcholine ■ adenovirus ■ gene transfer ■ sepiapterin

Inflammation may contribute to vasomotor dysfunction in several cardiovascular diseases, including atherosclerosis, hypertension, stroke, and diabetes.1-5 Mechanisms by which vasoconstriction and relaxation are altered during inflammation are not fully understood. Although systemic production of vasoactive peptides and cytokines contributes to effects of inflammation on vascular function, local expression of inflammatory mediators such as inducible nitric oxide synthase (iNOS) also may alter vascular function. The overall goal of these studies was to evaluate mechanisms of vascular dysfunction produced by iNOS. We used gene transfer of iNOS in vitro to evaluate effects of iNOS in the absence of systemic inflammation.

Endothelial NO synthase (eNOS) is the only NOS expressed in endothelium of normal vessels. During inflammation, blood vessels express iNOS as well as eNOS.6 iNOS may produce NO continuously, if substrate and cofactors are not limited, in contrast to the regulated production of NO by eNOS. Expression of iNOS contributes to vascular dysfunction,7-9 but mechanisms by which iNOS impairs contraction and relaxation of arteries are not clearly defined. Several mechanisms have been proposed by which iNOS may impair contractile responses.10-12 The first goal of this study was to test the hypothesis that high output of NO produced by iNOS opposes contraction by activation of soluble guanylate cyclase.

Availability of cofactors also modulates production of NO by eNOS. Tetrahydrobiopterin is an essential cofactor for production of NO by NOS enzymes.13,14 Therefore, the second goal of this study was to test the hypothesis that iNOS impairs endothelium-dependent relaxation, at least in part, by limiting availability of tetrahydrobiopterin, an essential cofactor for NO production by eNOS.

It is well-demonstrated that several vasoactive compounds are produced in endothelium and diffuse to vascular muscle to influence vasomotor tone.15,16 Recent evidence suggests that vasoactive compounds derived from adventitia also modulate tone in vascular muscle.17,18 Expression of iNOS in adventitia may contribute importantly to altered vascular function during inflammation and cardiovascular diseases.19,20 The third goal of this study was to test the hypothesis that expression of iNOS in either endothelium or adventitia is sufficient to produce vascular dysfunction. By using gene transfer in an ex vivo model, we were able to selectively express iNOS in either endothelium or adventitia.
Methods

Animals
All procedures followed institutional guidelines as approved by the Animal Care and Use Committee of the University of Iowa. Adult male New Zealand White rabbits (2.5 to 3.0 kg) were euthanized with Nembutal (150 mg/kg).

Adenovirus Vectors
Adenovirus cytomegalovirus iNOS (AdiNOS) was constructed using cDNA for mouse iNOS and the cytomegalovirus promoter, as described previously.22 To control for nonspecific effects of adenovirus, control viruses expressing either β-galactosidase (AdLacZ) or no transgene (AdBglII) were used for comparisons with AdiNOS. We have used both control viruses in previous studies and found no effects on vascular function.23 Adenovirus vectors were propagated and titered by the Vector Core of the University of Iowa and maintained at −80°C until used.

Gene Transfer
For simultaneous transfection of both endothelium and adventitia, carotid arteries were removed and placed in cold Krebs buffer. Rings of carotid artery were incubated with virus (3×10¹¹ pfu/mL) in minimal essential medium (MEM) with 1% penicillin/streptomycin for 24 hours before evaluation of vascular function. Previously, we observed transgene expression in both endothelium and adventitia of arterial rings after this protocol.9 Adenovirus vectors were propagated and titered by the Vector Core of the University of Iowa and maintained at −80°C until used.

For selective expression of iNOS in endothelium or adventitia, carotid arteries were exposed in anesthetized rabbits. Segments of artery were selected to avoid regions containing side branches. To transfect endothelium only, both ends of an arterial segment were ligated, and control or iNOS virus was introduced via a cannula into the lumen. The ligated arterial segment was then removed and incubated in MEM for 24 hours. To transfet adventitia, arteries were cannulated, filled with culture medium without virus, and ligated. The external surface of arteries was then exposed to virus in incubation medium.

For selective transfection of either endothelium or adventitia, viruses were diluted in MEM to a concentration of 3×10¹⁰ pfu/mL and arteries were incubated with virus for 3 to 4 hours at 37°C in an incubator. Ligated vessels were then trimmed, rinsed, and cut into 4 equal segments (~3 mm in length). All vessels were placed in fresh MEM and returned to the incubator for 18 to 20 hours.

Immunohistochemistry
Immunohistochemistry was performed in segments of carotid artery after gene transfer of iNOS. Vessel segments were frozen and cut into sections (~5-µm-thick). Frozen sections of vessels were placed on poly-L-lysine–coated slides and were allowed to dry at room temperature. Sections were fixed in acetone and 1% paraformaldehyde at 4°C for 5 minutes. Horse serum (5%) and 0.2% albumin were used for blocking nonspecific binding proteins for 20 minutes. Sections were then incubated with mouse IgG monoclonal antibody to murine iNOS (1:50; Transduction Laboratories) for 30 minutes. Some rings were treated with 1H-[1,2,4]oxadiazolo-[4,3-d]quinazolin-1-one (ODQ) (10 µmol/L), an inhibitor of soluble guanylate cyclase,24 for 30 minutes before a second application of phenylephrine. Other rings were treated with aminoguanidine (AG) (300 µmol/L) or l-N6-(1-iminoethyl)lysine dihydrochloride (100 µmol/L) inhibitors of iNOS,25 or sepiapterin (300 µmol/L), a precursor of tetrahydrobiopterin,26 for 30 minutes before examination of a second dose–response curve to acetylcholine.

Results
Selective Expression of iNOS After Gene Transfer
We used immunohistochemistry to confirm selective expression of iNOS in either endothelium or adventitia. Selective expression of iNOS in endothelium or adventitia was achieved using ex vivo techniques in the rabbit carotid artery when either the endothelium or adventitia was exposed to iNOS (Figure 1).

Effects of iNOS on Vasoconstriction
After simultaneous transfection of both endothelium and adventitia with either iNOS or a control virus, phenylephrine produced dose-dependent contraction in carotid arteries (Figure 2). The response to phenylephrine was impaired after transfection with AdiNOS (Figure 2). Addition of l-N6-(1-iminoethyl)lysine dihydrochloride, a relatively selective inhibitor of iNOS, to the AdiNOS-treated vessels restored contraction to normal (Figure 2).

Selective expression of iNOS in endothelium-impaired responses to phenylephrine (Figure 3A). In contrast, after selective expression of iNOS in adventitia, responses to phenylephrine were normal (Figure 3B).
Responses to phenylephrine after inhibition of soluble guanylate cyclase with ODQ increased responses to phenylephrine in iNOS-transfected arteries. EC50 for phenylephrine was 4.33 ± 0.78 (µmol/L) after AdiNOS and 1.15 ± 0.43 after AdiNOS plus ODQ (n=6, P<0.05). ODQ also tended to alter the response to phenylephrine after control virus. EC50 after AdlacZ was 2.47 ± 0.37 and for lacZ plus ODQ was 1.03 ± 0.37 (n=4, P>0.05).

**Effects of iNOS on Vasorelaxation**

After simultaneous transfection of both endothelium and adventitia with iNOS, responses to acetylcholine were impaired (Figure 4). Responses to nitroprusside also were impaired after transfection with iNOS (data not shown). Sepiapterin, a precursor of tetrahydrobiopterin, had no effect after gene transfer of BglII and improved, but did not restore, responses to acetylcholine after AdiNOS (Figure 4). Responses to sodium nitroprusside were not improved by sepiapterin (data not shown).

YC-1 produces vasorelaxation by activation of the sGC/NO pathway. Relaxation to YC-1 was similar after AdiNOS (EC50 = 70.6 ± 23.2 µmol/L) and AdlacZ (EC50 = 63.8 ± 10.7 µmol/L).

Selective expression of iNOS in endothelium inhibited relaxation to acetylcholine (Figure 5A, left). In contrast, selective expression of iNOS in adventitia did not alter responses to acetylcholine (Figure 5B). Aminoguanidine, an inhibitor of iNOS, improved relaxation to acetylcholine in arteries in which iNOS was expressed in endothelium (Figure 5C) and did not alter responses to acetylcholine in arteries with expression of iNOS in adventitia (Figure 5D). Responses to nitroprusside also were impaired by expression of iNOS in endothelium (Figure 6A), but not in adventitia (Figure 6B). In contrast, responses to papaverine, a non-NO–mediated vasodilator, were not impaired after gene transfer of iNOS to endothelium or adventitia (Figure 6C and 6D).

**Discussion**

One major finding in this study is that impairment of endothelium-dependent vasorelaxation by iNOS may be produced in part by depletion of tetrahydrobiopterin. When vessels were pretreated with sepiapterin, a precursor of tetrahydrobiopterin biosynthesis, impaired vasorelaxation produced by iNOS was improved. These data suggest that depletion of tetrahydrobiopterin, a cofactor of NOS, may impair vasorelaxation by limiting the ability of endothelial cells to produce NO.

A second finding is that a primary mechanism by which iNOS impairs contraction is activation of sGC. Although NO

Figure 2. After simultaneous gene transfer of iNOS to both endothelium and adventitia (AdiNOS), responses to phenylephrine were impaired (n=6; P<0.05 vs control [AdBgl-II]). Impaired responses after iNOS were improved by addition of L-N6-(1-iminoethyl)lysine dihydrochloride (L-NIL) to organ bath (n=6; P<0.05 vs iNOS).

Figure 3. Responses to phenylephrine after selective gene transfer of iNOS to endothelium (A; n=8) or adventitia (B; n=9) (*P<0.05 vs LacZ).

Figure 4. Responses to acetylcholine after gene transfer of iNOS to both endothelium and adventitia. A, Sepiapterin (Sep) had no effect after gene transfer of BglII. B, Impaired responses after iNOS were improved by sepiapterin in organ bath (n=6; P=0.05 vs iNOS).

Figure 5. Responses to acetylcholine after gene transfer of iNOS to endothelium (A; n=8) or adventitia (B; n=9) (*P<0.05 vs LacZ). Responses to acetylcholine after inhibition of iNOS with aminoguanidine (AG) after gene transfer of iNOS to endothelium (C; n=8) or adventitia (D; n=9) (*P<0.05 vs LacZ).
Mechanism of Impaired Contraction by iNOS

Previous studies suggest that iNOS is an important mediator of vascular dysfunction during inflammation and in cardiovascular disease. It is not surprising that NO produced by iNOS opposes contraction, other investigators have speculated that impaired contraction by iNOS may result from several mechanisms other than the NO/cGMP pathway. For example, the NO from iNOS may impair vasoconstriction by interfering with calcium signaling. Recent evidence suggests that peroxynitrite forms nonvasoactive oxidative products with catecholamines. Thus, impaired contraction by iNOS may be a result of reduced effectiveness of vasoconstrictors. Although current data do not rule out additional mechanisms of impairment, iNOS-mediated impairment of contraction was substantially improved by inhibition of sGC with ODQ. These results support the hypothesis that NO from iNOS activates sGC and inhibits contractile responses during inflammation.

ODQ also tended to improve responses (not statistically significant) of arteries under control conditions, as well as after gene transfer of iNOS. A possible explanation is that some iNOS may have been produced during incubation of the arteries. Relaxation to acetylcholine in vessels incubated ex vivo tends to be less than in vessels that are not incubated. We speculate that iNOS may contribute to reduction in this loss of responsiveness after incubation.

Effects of Selective Expression of iNOS on Contraction

NO from eNOS readily diffuses from endothelium to vascular muscle, and we predicted that NO from iNOS in either endothelium or adventitia would affect vasomotor tone. Thus, we anticipated that expression of iNOS in either endothelium or adventitia would impair vasoconstriction.

Diffusion of NO from adventitia to smooth muscle was demonstrated in previous studies using gene transfer of eNOS. Relaxation to calcium ionophore was observed after endothelial denudation in arteries after gene transfer of eNOS and was inhibited by an NOS inhibitor. In addition, receptor-mediated activation of eNOS in adventitia produced vasorelaxation with a concurrent rise in cGMP in vascular muscle, suggesting that NO can diffuse from adventitia to media. Thus, it was surprising to observe that expression of iNOS in adventitia, in contrast to endothelium, failed to impair vasoconstriction in this study. One explanation for the unexpected result may be that insufficient substrate or cofactors are available in adventitia to sustain iNOS activity at a level that would impair contraction. Alternatively, although immunohistochemistry suggests that our gene transfer method produced substantial expression of iNOS in adventitia, it is possible that the level of expression of iNOS in adventitia was not sufficient to impair contraction.

Previous studies reported that contractile function was impaired when iNOS was expressed in adventitia after lipopolysaccharide (LPS) in vitro and in vivo. An advantage of using gene transfer to express iNOS is that it allows us to examine effects that are specific to iNOS. A limitation, however, is that our technique cannot examine the complex interactive effects of iNOS with other inflammatory re-
sponses to endotoxin. Previous studies may have observed effects of peroxynitrite (described) or other interactive effects of NO rather than, or in addition to, direct effects of NO from iNOS in adventitia.

In a previous study, we administered AdiNOS intraluminally to carotid arteries in vivo, and expression of iNOS in endothelium had no effect on contraction.9 It is likely that administration of AdiNOS in vivo produced a lower level of expression of iNOS in endothelium, because vessels were occluded for only 20 minutes to allow transfection before restoration of blood flow. In the current study, gene transfer proceeded for at least 3 hours.

Effects of iNOS on Relaxation

Tetrahydrobiopterin is an essential cofactor for production of NO by NOS enzymes.13,14 In the absence of sufficient tetrahydrobiopterin, NOS enzymes may become “uncoupled,” leading to production of superoxide.33,34 Thus, normal relaxation produced by NO from eNOS is dependent on adequate levels of tetrahydrobiopterin in endothelial cells. Sepiapterin is a stable precursor for tetrahydrobiopterin.26 Effects of tetrahydrobiopterin per se, but not sepiapterin, are confounded by potential oxidation reactions.35 Therefore, sepiapterin is commonly used to supplement tetrahydrobiopterin. In previous studies, acute inhibition of eNOS in organ bath studies improved responses to acetylcholine,9,32 One explanation for improved relaxation with acute inhibition of iNOS may be that dihydrobiopterin is rapidly reduced by endothelial cells to replenish tetrahydrobiopterin for use by eNOS.

Consistent with previous studies,9,32 we found that NO-dependent relaxation is impaired after gene transfer of iNOS to carotid arteries. In a previous study, relaxation to 8-bromo-cGMP was not impaired after gene transfer of iNOS,9 suggesting that cGMP-dependent kinase and other downstream mechanisms of relaxation remain intact in the presence of iNOS. Thus, it is likely that impaired responses to acetylcholine are a result of impaired production of NO by eNOS or possibly impaired activation of sGC by NO from eNOS.

Previously, we proposed that expression of iNOS in endothelium may impair endothelium-dependent relaxation because of colocalization of iNOS and eNOS. Because NOS enzymes require the same substrate and cofactors, iNOS and eNOS in endothelium may compete to produce NO. Our results using selective gene transfer of iNOS indicate that expression of iNOS in endothelium, but not adventitia, inhibits NO-dependent relaxation, which is consistent with this hypothesis. Normal relaxation to papaverine after gene transfer of iNOS suggests that vascular muscle retains normal function, and that impairment of responses is selective for NO-dependent pathways.

Results using sodium nitroprusside are consistent with previous studies of gene transfer of iNOS in which responses to endothelium-independent NO donors were impaired.6,9,38 Effects of sepiapterin may explain in part limited production of NO by eNOS in the presence of iNOS, but sepiapterin restored only a portion of impaired relaxation. Vasodilatation in response to nitroprusside is independent of eNOS.39 The primary mechanism by which iNOS impairs not only eNOS-mediated relaxation but also NO-dependent, non–eNOS-dependent relaxation remains elusive. Because superoxide is often elevated in blood vessels in settings in which iNOS is expressed,6,9 one possibility is that oxidation reactions contribute to impaired NO-mediated relaxation. To date, pharmacological and genetic approaches have failed to define a role for superoxide in NO-dependent vascular dysfunction produced by iNOS.6,9,32,38

YC-1 produces vasorelaxation by enhancing activation of sGC.40 The finding that relaxation to YC-1 is preserved after AdiNOS suggests that downstream pathways after sGC that produce relaxation of smooth muscle are intact in the presence of iNOS. Thus, iNOS-mediated impairment of relaxation to acetylcholine must occur either at the level of NO production by eNOS or during diffusion of NO from endothelial cells to sGC in vascular muscle.

In summary, effects of ODQ suggest that iNOS impairs contraction by activation of sGC. Providing sepiapterin as a source to replenish tetrahydrobiopterin in endothelial cells improves responses to acetylcholine that are impaired by iNOS. Thus, one mechanism by which iNOS inhibits vasorelaxation may involve reduction of availability of tetrahydrobiopterin for NO production by eNOS. Finally, selective expression of iNOS in endothelium impaired both contraction and relaxation of arteries, but vasomotor function was normal when iNOS was expressed in adventitia. We conclude that iNOS impairs vascular function by multiple mechanisms, and that expression of iNOS in endothelium is a sufficient stimulus to impair vasomotor function.

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