In Vivo Gene Transfer of Inducible Nitric Oxide Synthase to Carotid Arteries From Hypercholesterolemic Rabbits

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Background and Purpose—Hypercholesterolemia is associated with endothelial dysfunction. Inducible nitric oxide synthase (iNOS) is upregulated in atherosclerotic vessels. However, its role in the regulation of vascular function is not completely understood. We examined the effect of adenovirus-mediated gene transfer of iNOS to the hypercholesterolemic rabbit carotid artery in vivo.

Methods—Rabbits were fed a high-cholesterol or chow diet for 10 weeks. Two doses (1 × 10^6 and 1 × 10^7) plaque-forming units [pfu/mL] of adenoviral vectors encoding iNOS (AdiNOS) or β-galactosidase (Adβgal) were luminally delivered to the carotid arteries from chow- and cholesterol-fed animals. Vascular reactivity and superoxide levels were assessed in Adβgal- and AdiNOS-transduced vessels from chow- and cholesterol-fed animals after 3 days.

Results—Endothelium-dependent vasorelaxation was impaired in the carotid artery from cholesterol-fed animals. In AdiNOS-transduced arteries, transgene expression was demonstrated by positive immunostaining in the endothelium. Transduction with low-dose (1 × 10^6 pfu/mL) AdiNOS did not affect vascular reactivity in arteries from chow- and cholesterol-fed animals. In contrast, high-dose (1 × 10^7 pfu/mL) AdiNOS significantly reduced endothelium-dependent relaxation in vessels from cholesterol- but not chow-fed rabbits. After both low- and high-dose iNOS gene transfer, levels of O_2^- were significantly (P<0.05) elevated in carotid arteries from cholesterol-fed animals. Incubation with an O_2^- scavenger did not reverse vascular dysfunction in these arteries.

Conclusions—Adenoviral-mediated overexpression of iNOS results in increased production of O_2^- in carotid arteries from cholesterol- but not chow-fed animals. High-dose AdiNOS gene transfer is associated with reduced endothelium-dependent and -independent relaxation in vessels from cholesterol-fed animals. (Stroke. 2003;34:1293-1298.)

Key Words: carotid arteries • gene transfer • nitric oxide synthase • reactive oxygen species • superoxides • rabbits

Unlike normal arteries, atherosclerotic vessels express the cytokine-inducible isoform of nitric oxide (NO) synthase (iNOS).1–6 In atherosclerotic lesions, iNOS expression has been detected in neointimal smooth muscle cells,4 activated macrophages,5,6 and foam cells.6 The consequences of this intramural activation of iNOS are unknown. Unlike the endothelial (eNOS) and neuronal (nNOS) isoforms of NOS, which produce modest amounts of NO, iNOS activity is associated with a much larger release of NO,7,8 which acts as a vasodilator agent9 and is known to limit leukocyte adhesion,10 platelet adhesion,11 and vascular smooth muscle cell proliferation in the setting of allograft arteriosclerosis and neointimal hyperplasia.12–14 Atherosclerosis is associated with endothelial dysfunction15 and increased production of NO and superoxide anion.17 The role of iNOS expression in this process is unclear. Increased NO generation from iNOS may preserve endothelial function in the setting of hypercholesterolemia, whereas increased superoxide generation may result in abnormal endothelium-dependent relaxation. The latter scenario results in increased generation of peroxynitrite,18 which is detected in atherosclerotic lesions.6,19 Peroxynitrite is a cytotoxic molecule that causes cell damage and injury.20 We and others have shown that eNOS gene transfer improves endothelial dysfunction in the setting of hypercholesterolemia, atherosclerosis, diabetes mellitus, hypertension, and subarachnoid hemorrhage.21–27 nNOS gene transfer has also been shown to improve cholesterol-induced endothelial dysfunction.28 While iNOS gene transfer has not been studied in this context, it may be an attractive candidate as lower viral titers may be used because of the high enzymatic activity of this isoform.

Thus, the role of NO derived from iNOS in endothelial dysfunction is unclear, and the effects of iNOS gene transfer on vascular function in hypercholesterolemia have not been studied. To address these issues, we used in vivo adenoviral-
mediated gene transfer of iNOS and evaluated vascular reactivity and superoxide production of carotid arteries in an animal model of hypercholesterolemia.

Materials and Methods

Construction, Propagation, and Purification of Adenoviral Vectors
A recombinant adenovirus encoding the iNOS gene driven by a cytomegalovirus promoter was generated as previously described. A recombinant adenoviral vector encoding the Escherichia coli β-galactosidase gene (Adβgal) driven by the cytomegalovirus promoter was used as a control.

Animals
Experimental protocols were approved by the Institutional Animal Care and Use Committee and were performed in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care. Thirty-six New Zealand White rabbits were used in these experiments. The animals were housed individually in a room with a 12-hour light/dark cycle. Control rabbits (n=18) were fed a standard diet, and cholesterol-fed animals (n=18) received a diet supplemented with 1.0% cholesterol (Purina Mills) for 4 weeks and 0.5% cholesterol for 6 weeks.

In Vivo Carotid Artery Gene Transfer
After 10 weeks, in vivo gene delivery was performed in the animals. Each group was subdivided into 2 subgroups. In subgroup 1, carotid arteries were exposed to AdiNOS or Adβgal on the contralateral side at 1×10^8 plaque-forming units (pfu/mL). Subgroup 2 was exposed to AdiNOS or Adβgal at 1×10^9 pfu/mL. The method of transduction of the vessel segment was the same as we previously described. Briefly, induction of anesthesia was obtained with an intramuscular injection of ketamine (65 mg/kg), xylazine (13 mg/kg), and acepromazine (22 mg/kg). The common carotid arteries were exposed bilaterally, and proximal and distal vascular clamps were applied. After insertion of an angiocatheter into the proximal part of the isolated segment, the solution containing the adenoviral vector was applied. After insertion of an angiocatheter into the proximal part of the isolated segment, the solution containing the adenoviral vector (100 µL of AdiNOS or Adβgal 1×10^10 or 1×10^9 pfu/mL concentration) was instilled intraluminally. After 20 minutes, vascular clamps were removed, flow was restored, and the animals were allowed to recover. Three days later, the carotid arteries were harvested, and the animal was killed. Each artery was divided into 4 rings, 1 of which was used for immunohistochemistry, 1 for the determination of superoxide production, and 2 for vascular reactivity studies.

Immunohistochemical Analysis of Gene Expression
Rings were fresh-frozen in O.C.T. compound (Miles, Inc), and 5-µm-thick sections were cut. After immersion fixation in acetone (4°C) and drying, the slide was incubated in 0.1% sodium azide/0.3% hydrogen peroxide and then in 10% rabbit serum/PBS-Tween 20. An iNOS monoclonal antibody (1:50, Transduction Laboratory) was applied for 2 hours at room temperature, followed by incubation with biotinylated rabbit anti-mouse F(ab’)2 secondary antibody (1:50, Dako) for 60 minutes and peroxidase-conjugated streptavidin (1:50, Dako) for 60 minutes. After a 30-second immersion in 0.1 mol/L sodium acetate buffer (pH 5.2), iNOS immunoreactivity was visualized with 3-amino-9-ethylcarbazole and hematoxylin counterstaining.

Detection of Vascular Superoxide Production
Superoxide anion production was measured by lucigenin chemiluminescence. Briefly, 5-mm carotid artery segments were equilibrated for 30 minutes at 37°C in a modified Krebs-HEPES buffer. Scintillation vials containing 2 mL Krebs-HEPES buffer with 5 µmol/L lucigenin were placed into a scintillation counter (LS 5000, Beckman Instruments Inc) switched to the out-of-coincidence mode. Background signals were recorded, and a vascular ring was then added to the vial. Photon counts were recorded every 2 minutes for 8 minutes, and the background was subtracted. In some experiments, vessels were preincubated with Tiron (10^-3 M/L) for 15 minutes at room temperature before addition of lucigenin. The vessels were dried for 24 hours at 90°C and weighed. The results were expressed as counts per minute per milligram dry weight.

Analyses of Vascular Reactivity
Rings (4 mm) from each carotid artery were used for assessing vascular reactivity. Rings were suspended in organ chambers filled with 25 mL of gassed (94% O2 and 6% CO2) modified Krebs-Ringer bicarbonate solution (pH 7.4, temperature 37°C, composition [mmol/L]: 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25.0 NaHCO3, 0.026 calcium sodium EDTA, and 11.1 glucose). The rings were stretched to the optimal point on the length-tension curve as determined by repeated exposure to 20 mmol/L KCl. The maximal contraction of each ring was determined by KCl 60 mmol/L. Submaximal contractions were obtained by precontracting vessels with phenylephrine 10^-4 to 10^-3 mol/L to approximately 50% of maximum contraction for subsequent dose-responses curves to vasodilators. Acetycholine (10^-5 to 10^-4 mol/L) was added cumulatively during submaximal contraction to phenylephrine. In some experiments, rings were incubated for 15 minutes with Tiron (10^-2 mol/L) before stimulation with acetycholine. Finally, concentration responses to DEA-NONOate (10^-10 to 10^-5 mol/L) were obtained.

Statistical Analysis
Data are presented as mean±SEM. Statistical analysis was performed by ANOVA to detect significant differences in multiple comparisons and by unpaired Student’s t test when 2 groups were compared. A value of P<0.05 was considered statistically significant.

Results

Localization of iNOS Expression
Analysis of iNOS expression was assessed in transduced vessels by immunohistochemistry. Vessels from chow- and cholesterol-fed animals transduced with AdiNOS 1×10^9 or 1×10^8 pfu/mL and harvested 3 days later showed transgene expression predominantly in the endothelium (Figure 1C to 1F). In contrast, there was no staining in Adβgal-transduced arteries (Figure 1A and 1B).

Detection of Vascular Superoxide Production
Basal O2^- levels were similar in Adβgal-transduced arteries from chow- and cholesterol-fed animals (Figure 2A and 2B). However, after AdiNOS 1×10^9 pfu/mL, O2^- production increased by 3-fold (P<0.05) in carotid rings from cholesterol-fed but not from chow-fed animals (Figure 2A). Arteries transduced with AdiNOS 1×10^9 pfu/mL showed similar results (Figure 2B). Increased O2^- in AdiNOS-transduced vessels from hypercholesterolemic animals was inhibited in the presence of Tiron (Figure 2C).

Effects of Hypercholesterolemia and iNOS Gene Transfer on Vascular Reactivity
Maximal contractions to phenylephrine were similar after low-dose Adβgal and AdiNOS in arteries from chow-fed (3.6±0.4 and 3.8±0.6 g, respectively) and hypercholesterolemic (3.4±0.4 and 3±0.4 g, respectively) animals. After gene transfer with high-dose Adβgal and AdiNOS, maximal contraction to phenylephrine was not impaired in any group (chow-fed group Adβgal, 4.6±0.6 g; chow-fed group AdiNOS, 3.2±0.2 g; cholesterol-fed group Adβgal, 4.1±0.4 g; cholesterol-fed group AdiNOS, 3.1±0.4 g).
During submaximal contraction with phenylephrine, reduced endothelium-dependent vasorelaxation to acetylcholine was detected in vessels from cholesterol-fed animals (Figure 3A). In contrast, vasorelaxation to DEA-NONOate was similar in both groups (Figure 3B).

Next we examined the effect of low-dose (10^8 pfu/mL) iNOS gene transfer on relaxation of carotid rings from chow- and cholesterol-fed animals. In both groups, relaxation to acetylcholine was similar in AdiNOS- and Adβgal-transduced carotid arteries (Figure 3A). Similar results were obtained when relaxation in response to DEA-NONOate was assessed in the same arteries (Figure 3B).

After high-dose (10^9 pfu/mL) iNOS gene transfer, endothelium-dependent and -independent relaxation was unaltered in vessels from chow-fed animals (Figures 4A and 5A). In contrast, transduction with high-dose iNOS resulted in reduced endothelium-dependent and -independent relaxation in cholesterol-fed animals (Figures 4B and 5B). Endothelium-dependent vasorelaxation to acetylcholine in this group was not restored after incubation with Tiron (Figure 4C).

**Discussion**

The present study provides new information concerning the functional effects of iNOS expression on vascular reactivity and superoxide production in vessels from hypercholesterolemic animals. Gene transfer of iNOS to the lumen of the rabbit carotid artery resulted in transgene expression predominantly in the endothelium after 3 days. iNOS gene transfer with an adenoviral titer of 10^9 pfu/mL did not exert any effect on relaxation in either chow- or cholesterol-fed animals. In contrast, luminal administration of 10^9 pfu/mL AdiNOS reduced endothelium-dependent and -independent relaxation in vessels from hypercholesterolemic but not chow-fed animals. In addition, gene transfer of iNOS to the hypercholesterolemic rabbit carotid artery resulted in a 2- to 3-fold increase in superoxide anion production. These data suggest that iNOS overexpression may contribute to the development of endothelial dysfunction in hypercholesterolemia.

The animal model used in the present study is one of high-cholesterol feeding of rabbits. Ten weeks of high-cholesterol feeding was associated with severe hypercholesterolemia and moderate hypertriglyceridemia in the absence of atherosclerotic plaques. Endothelium-dependent vasorelaxation to acetylcholine was impaired in these vessels, while relaxation to a NO donor was not affected. This pattern of vascular dysfunction confirms previous observations made by our group and other groups using this model.24,32,33

We sought to assess the effects of iNOS overexpression on vascular reactivity of vessels from chow- and cholesterol-fed animals. In this study, in vivo gene transfer with both low- and high-dose iNOS did not exert any effect on contractile
responses to phenylephrine in carotid arteries of either chow-fed or hypercholesterolemic animals. This is in contrast to previous findings in which higher doses of AdiNOS were used. In addition, endothelium-dependent and -independent relaxation was not affected in carotid arteries from chow-fed animals. In vessels from cholesterol-fed animals, low-dose iNOS did not influence endothelial dysfunction. In contrast, high-dose iNOS gene transfer to hypercholesterolemic animals reduced endothelium-dependent relaxation. The reason for the discrepancy between high-dose and low-dose effects in the hypercholesterolemic animals is unclear. Increased superoxide production from the endothelium has previously been proposed as a mechanism of reduced vascular responses in the context of nitrate tolerance. However, in our study increased superoxide in high-dose iNOS-transduced vessels from hypercholesterolemic animals does not seem directly involved in the reduced relaxation for at least 3 reasons. First, while O$_2^-$ levels were dose-independently increased after iNOS gene transfer, increased superoxide generation after low-dose iNOS gene transfer did not result in impaired endothelium-dependent relaxation. Second, administration of Tiron did not reverse endothelial dysfunction, ie, O$_2^-$ production was blunted, but relaxation to acetylcholine was still reduced. Third, endothelium-dependent relaxation seems to be resistant to elevation of superoxide levels in rabbit carotid artery. In this model, reduced soluble guanylate cyclase activity and cGMP elevations in response to vasodilator stimul}
not control vessels are unclear. iNOS can serve as a source of superoxide anion in the absence of an adequate supply of substrate and cofactors. A relative deficit of arginine and tetrahydrobiopterin has been described in atherosclerotic vessels.\textsuperscript{37,38} Whether reduced arginine and tetrahydrobiopterin availability may contribute to the observed increase in superoxide anion formation in our model remains unknown.

Inflammation is associated with abundant expression of iNOS within the vessel wall, which is thought to be responsible for impaired endothelium-dependent vasorelaxation.\textsuperscript{34} With gene transfer approaches, it is possible to modulate transgene expression in the target organ. In this study we demonstrated that gene transfer with relatively low doses of AdiNOS (1\texttimes{}10\textsuperscript{8} and 1\texttimes{}10\textsuperscript{9} pfu/mL) results in transgene expression without altering vascular reactivity in normal vessels. We extended this observation to the effects of iNOS gene transfer in a diseased state, hypercholesterolemia. With the use of AdiNOS at a dose of 1\times{}10\textsuperscript{9} pfu/mL, NO-dependent vasorelaxation was impaired in hypercholesterolemic but not normal blood vessels. Thus, iNOS gene transfer is associated with impaired NO-dependent vasorelaxation and increased production of superoxide anion in the setting of hypercholesterolemia. This contrasts with the effect of eNOS gene transfer, which improves endothelium-dependent relaxation in a variety of animal models.

While our study examined the effect of iNOS gene transfer on vascular reactivity, the role of iNOS in intimal hyperplasia and atherosclerosis lesion progression is very complex. Rabbits fed a high-cholesterol diet for 24 weeks plus an iNOS inhibitor demonstrated reduced progression of atherosclerotic lesions, suggesting a deleterious effect of iNOS expression on lesion progression.\textsuperscript{14} Furthermore, vascular injury in the iNOS knockout mouse results in less intimal hyperplasia,\textsuperscript{39} and iNOS and apolipoprotein E double-knockout mice have reduced atherosclerosis.\textsuperscript{40,41} These data suggest that iNOS may have deleterious effects on atherosclerotic plaque progression. In contrast, short-term iNOS expression after adenoviral-mediated gene transfer has been shown to inhibit intimal hyperplasia after mechanical injury and transplantation.\textsuperscript{12,13} Thus, short-term, high-level expression of iNOS inhibits intimal hyperplasia. The mechanism for this effect is unclear but may involve superoxide generation. A distinction may need to be drawn between the effects of iNOS in the chronic atherosclerotic process and acute overexpression in the setting of mechanical injury.

In summary, we have demonstrated that in vivo adenoviral-mediated gene transfer of iNOS to the carotid artery of the hypercholesterolemic rabbit results in increased superoxide production and reduced relaxation. In contrast, iNOS gene transfer does not affect superoxide generation and vascular relaxation in carotid arteries from chow-fed animals.

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