Gene-Targeted Mice Reveal a Critical Role for Inducible Nitric Oxide Synthase in Vascular Dysfunction During Diabetes

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Background and Purpose—Inducible nitric oxide synthase (iNOS) is a mediator of vascular dysfunction during inflammation. The purpose of this study was to test the hypothesis that vascular dysfunction during diabetes is dependent on expression of iNOS.

Methods—Diabetes was produced in mice with streptozotocin (150 mg/kg IP). After 4 to 6 months of diabetes, vasomotor function was examined in vitro in carotid arteries from mice with targeted disruption of the gene for iNOS (iNOS-deficient mice) and from normal, wild-type (WT) mice.

Results—Contractile responses of carotid arteries to U46619, a thromboxane A₂ analogue, were not altered by diabetes in WT mice. Responses to U46619 were increased in arteries from diabetic iNOS-deficient mice compared with diabetic WT and nondiabetic mice (iNOS-deficient and WT mice). These results indicate that expression of iNOS inhibits an increased vasoconstrictor response during diabetes. Arteries from nondiabetic WT mice relaxed 83 ± 2% (mean ± SE) in response to acetylcholine (1 μmol/L) compared with 58 ± 6% in arteries from diabetic WT mice (P < 0.05 versus nondiabetic mice). In contrast, relaxation of carotid arteries to acetylcholine was similar (81 ± 4% versus 76 ± 6%; P > 0.05) in iNOS-deficient mice under nondiabetic and diabetic conditions, respectively. Thus, diabetes produced impairment of endothelium-dependent relaxation in arteries from WT but not iNOS-deficient mice. Endothelium-independent relaxation in response to nitroprusside was similar in arteries from all mice.

Conclusions—These results provide the first direct evidence that impairment of endothelium-dependent relaxation during diabetes is dependent on expression of iNOS. (Stroke. 2003;34:2970-2974.)

Key Words: acetylcholine • endothelium-derived relaxing factor • nitric oxide • streptozotocin

Hyperglycemia and/or diabetes activates components of the inflammatory response in vascular tissue and plasma.¹⁻⁵ Mechanisms by which inflammation contributes to vascular dysfunction in diabetes are not well understood. Inducible nitric oxide synthase (iNOS) is expressed in blood vessels in response to inflammation,⁶⁻¹⁰ and recent evidence indicates that expression of iNOS occurs in blood vessels during diabetes.¹¹,¹² The functional significance of iNOS in vascular dysfunction during diabetes has not been defined.

Impaired endothelium-dependent relaxation is a hallmark of vascular dysfunction in experimental models of diabetes¹³,¹⁴ and in humans with both type I and type II diabetes mellitus.¹⁵⁻¹⁷ Endothelial nitric oxide synthase (eNOS) is a critical mediator of endothelium-dependent relaxation, but the role of iNOS in endothelial function is poorly understood. Recently, using gene transfer of iNOS to normal arteries, we and others found that iNOS impairs endothelium-dependent relaxation.¹⁸⁻²⁰ Because iNOS is expressed and endothelial dysfunction is present in arteries during diabetes, and because iNOS has the potential to impair endothelial function, we speculated that iNOS impairs endothelial function in diabetes.

The first goal of this study was to examine the hypothesis that impairment of endothelium-dependent relaxation in blood vessels during diabetes is dependent on expression of iNOS. Although it is known that vasoconstrictor responses are altered in diabetes,¹⁵,²¹⁻²⁶ mechanisms that account for these changes are not well defined. Expression of iNOS in blood vessels impairs contractile responses under some conditions.⁶,¹⁸,²⁷ Thus, the second goal of these studies was to test the hypothesis that iNOS inhibits vasoconstrictor responses during diabetes.

Materials and Methods

Animals
We examined diabetic and nondiabetic wild-type (WT) and iNOS-deficient mice. Mice with targeted disruption of the gene for iNOS were obtained initially from Dr John Mudgett.²⁸ Most of the WT mice were littermate controls. A few C57BL/6 mice, which are the background strain for the iNOS-deficient strain, were used as additional controls. We found no differences in responses of arteries from WT littermates and C57BL/6 mice in this or previous studies.⁶,²⁹ In the present study data from WT littermates and WT C57BL/6 mice were combined. All procedures followed were within...
institutional guidelines approved by the Animal Care and Use Committee at University of Iowa.

Diabetes
Male and female mice (aged 8 to 16 weeks) were randomly assigned to receive either streptozotocin (150 mg/kg IP) or vehicle (citrate). Repeated low doses of streptozotocin are sometimes used to produce diabetes in mice, but a previous study reported that the low-dose regimen failed to produce diabetes in this strain of iNOS-deficient mice. Our preliminary experiments indicated, however, that a single dose of 150 mg/kg of streptozotocin was effective in producing hyperglycemia in approximately 70% of iNOS-deficient and WT mice. Thus, we used a single high dose of streptozotocin in these studies. Mice that did not become diabetic after streptozotocin were used as nondiabetic controls.

Vascular Function
Vasomotor function of carotid arteries was examined in vitro 4 to 6 months after vehicle or streptozotocin by measurement of isometric tension, as described previously. Briefly, mice were anesthetized with pentobarbital (75 to 100 mg/kg IP), and carotid arteries were removed and immediately placed in oxygenated Krebs’ buffer. Vessels were then suspended between 2 triangular hooks in an organ bath and attached to a force transducer for measurement of isometric tension.

We examined dose-dependent contraction of carotid rings in response to the thromboxane A2 analogue U46619 and relaxation in response to acetylcholine (1 nmol/L to 3 μmol/L) or nitroprusside (1 mmol/L to 100 μmol/L) after submaximal precontraction with U46619. To compensate for any variability between groups in constritor responses to U46619, we preconstricted vessels to approximately 50% to 60% of maximum response to U46619 rather than using a single concentration of U46619 for all vessels. Responses of the carotid artery to acetylcholine are mediated by eNOS independent vasorelaxation.

Drugs
Streptozotocin, acetylcholine, and sodium nitroprusside were obtained from Sigma Chemical Co. Sodium citrate was obtained from Fisher Scientific. U46619 was obtained from Cayman Chemical. Streptozotocin, acetylcholine, and sodium nitroprusside were dissolved in ethanol and then diluted in normal saline. All other drugs were dissolved and diluted in normal saline. Concentrations are expressed as final concentration of each drug in the organ bath.

Statistical Analysis
Data are expressed as mean±SEM. Group differences were determined by ANOVA followed by the Tukey post hoc test to evaluate significant differences between means. P<0.05 was considered to be statistically significant. Relaxation to acetylcholine and sodium nitroprusside was expressed as percent relaxation from precontraction to U46619.

Results
Blood glucose concentrations of >13.8 mmol/L were used as the criterion for inclusion of mice in the diabetic groups. Blood glucose concentrations during diabetes were 23.9±0.9 mmol/L in WT mice and 27.2±2.2 mmol/L in iNOS-deficient mice. In nondiabetic mice, mean blood glucose levels were 6.5±0.2 mmol/L in WT mice and 6.6±0.5 mmol/L in iNOS-deficient mice.

Contraction of Carotid Arteries
We examined contraction of carotid arteries from diabetic and nondiabetic mice in response to U46619 (Figure 1). All arteries contracted in a dose-dependent manner in response to U46619. In WT mice, vasoconstriction in response to U46619 was similar in nondiabetic and diabetic groups (Figure 1, left). In contrast, contraction of the carotid artery in response to U46619 was enhanced in diabetic iNOS-deficient mice compared with nondiabetic iNOS-deficient mice (Figure 1, right).

Endothelium-Dependent Relaxation of Carotid Arteries
To study vasorelaxation, precontraction was matched in arteries from diabetic and nondiabetic mice of each genotype. Carotid arteries from nondiabetic WT mice relaxed in a dose-dependent fashion in response to acetylcholine (Figure 2).

Figure 1. Contraction responses to U46619 were similar in arteries from nondiabetic (n=10) and diabetic (n=6) WT mice (P>0.05; left). In iNOS-deficient mice (right), responses to U46619 were enhanced during diabetes (n=10) (*P<0.05 vs nondiabetic [n=16]).

Figure 2. Relaxation to acetylcholine was similar in nondiabetic WT (n=12) or iNOS-deficient (n=13) mice. In WT mice (left), diabetes produced impaired responses to acetylcholine (n=7; *P<0.05 vs nondiabetic). In contrast, responses to acetylcholine were not impaired during diabetes in arteries from iNOS-deficient mice (right) (n=13; P>0.05).
Differences in vasomotor responses in these studies therefore indicate differential effects of diabetes in arteries with and without expression of iNOS.

In nondiabetic mice, vasomotor responses are similar in arteries from WT and iNOS-deficient groups (Figure 3).

**Discussion**

There are 2 major new findings in this study. First, vasoconstrictor responses to U46619 are increased during diabetes in arteries from iNOS-deficient but not normal WT mice. Thus, 1 effect of iNOS during diabetes is to inhibit increases in vasoconstrictor responses. Second, relaxation of the carotid artery to acetylcholine is impaired during diabetes in WT but not iNOS-deficient mice. These results suggest that iNOS is an important mediator of endothelial dysfunction during diabetes. To our knowledge, these studies using iNOS-deficient mice provide the first direct evidence (for any blood vessel) that vasomotor dysfunction during diabetes is dependent on expression of iNOS.

**Endothelium-Independent Relaxation of Carotid Arteries**

Responses to nitroprusside, an endothelium-independent vasodilator, were similar in diabetic and nondiabetic animals from both WT and iNOS-deficient groups (Figure 3).

**Endothelial Dysfunction During Diabetes Is Dependent on Expression of iNOS**

Preliminary experiments revealed that consistent impairment of endothelial function in carotid arteries in WT mice did not occur until approximately 4 months after induction of diabetes. In many studies, and also our own preliminary data, endothelial dysfunction occurs after shorter durations of diabetes in aorta from rats and mice. To our knowledge, these are the first studies to examine the function of carotid arteries in WT mice during diabetes. In this study we chose a duration of diabetes that was long enough to produce substantial effects on vascular function in WT mice because we anticipated that the degree of dysfunction would be decreased in iNOS-deficient mice.

Diabetes produced impaired relaxation to acetylcholine in carotid arteries from WT mice but not in arteries from iNOS-deficient mice. Because impairment does not occur in the absence of iNOS, this study provides the first direct evidence that iNOS is a key mediator of endothelial dysfunction during diabetes. Although previous studies using aminoguanidine demonstrated improvement in vascular function in diabetes, those studies are limited and difficult to interpret because aminoguanidine has several known effects in high levels of NO.

Although iNOS impairs constrictor responses in some settings, and several findings in this study suggest that iNOS is expressed in diabetic vessels, our present data show only a tendency (not significant) toward less constriction in carotid arteries from WT mice during diabetes. One of several possibilities may explain this apparent paradox. Our first suggestion is that enhanced sensitivity to U46619 during diabetes, as was unmasked in NOS-deficient mice, counterbalances impaired contractility produced by iNOS during diabetes, with a net result of fairly normal constriction in WT mice. We can speculate about a few other explanations. It is possible that levels of expression of iNOS in carotid arteries during diabetes are insufficient to produce impairment of constriction in WT mice. In a previous study using gene transfer of iNOS, we provided data supporting the concept that effects of iNOS on vascular function are dose dependent. In that study we saw a more dramatic effect of iNOS in producing impairment of endothelium-dependent relaxation than in impairment of contraction. Another possibility is that localization of expression of iNOS may have a major influence on its impact on vascular function. For example, selective expression of iNOS in endothelium may have different effects than selective expression of iNOS in adventitia or vascular muscle on contraction and/or relaxation.
addition to inhibition of iNOS. For example, aminoguanidine inhibits the development of advanced glycation end products, which are thought to contribute to the pathophysiology of diabetes. Previous studies have emphasized the importance of aminoguanidine in decreasing advanced glycation end products, but the data also support a role for iNOS in diabetic vascular dysfunction.

Data in this study are consistent with previous studies in which gene transfer of iNOS to blood vessels impaired endothelium-dependent relaxation. Although gene transfer approaches offer several advantages for studying mechanisms by which iNOS alters vascular function, there are also limitations to this approach. For example, the spatial and temporal expression of iNOS may not be the same after gene transfer when expression of iNOS is under control of a viral, rather than the endogenous, promoter. Thus, it is important to examine effects of endogenous iNOS. The present study of vascular effects of iNOS during diabetes, using genetically modified mice that do not express iNOS, allowed us to study the role of endogenous iNOS.

One potential mechanism by which iNOS may impair endothelium-dependent relaxation involves the generation of superoxide. Endothelial dysfunction during diabetes is improved by scavengers of superoxide. NO enzymes, including iNOS, can produce superoxide in settings with limited availability of substrate or cofactors. For example, a recent study suggests that during hyperhomocysteinemias, superoxide is produced by both NAD(P)H-oxidase and iNOS. Furthermore, superoxide levels are elevated in arteries after gene transfer of iNOS. Thus, iNOS may impair endothelium-dependent relaxation during diabetes by producing increased levels of superoxide.

Summary and Conclusions

Results of this study indicate that iNOS has a dual role in vascular function during diabetes. First, iNOS inhibits increases in vasoconstrictor responses during diabetes. Second, the present findings provide direct evidence that impairment of endothelium-dependent relaxation, which may contribute to cardiovascular dysfunction during diabetes, is dependent on expression of iNOS.

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