Impairment of Dilator Responses of Cerebral Arterioles During Diabetes Mellitus
Role of Inducible NO Synthase

Jiro Kitayama, MD; Frank M. Faraci, PhD; Carol A. Gunnett, PhD; Donald D. Heistad, MD

Background and Purpose—During diabetes, expression of inducible nitric oxide synthase (iNOS) plays an important role in the development of endothelial dysfunction in extracranial blood vessels. Progression of vascular dysfunction after the onset of diabetes differs among vascular beds. In this study, the effects of hyperglycemia/diabetes on vasomotor function were examined in cerebral arterioles at 2 different times in control and iNOS-deficient mice and compared with the effects on carotid arteries.

Methods—Streptozotocin (150 mg/kg IP) was given to induce diabetes. The diameter of cerebral arterioles was measured through a cranial window in diabetic and nondiabetic mice in vivo. Vasomotor function of the carotid artery was examined in vitro.

Results—In diabetic mice, responses of the cerebral arterioles to acetylcholine (1 μmol/L) were normal after 3 weeks of diabetes but were significantly impaired after 5 to 6 weeks of diabetes (4±1% [mean±SEM] increase in diameter) compared with control mice (14±1; P=0.0002). Responses to sodium nitroprusside were similar in diabetic and nondiabetic mice at both time points. In contrast, the vasomotor function of the carotid artery was not affected after 5 to 6 weeks of diabetes. In diabetic iNOS-deficient mice, cerebral arteriolar vasomotor function was not impaired, even after 4 months of diabetes.

Conclusions—During diabetes, endothelial dysfunction of cerebral arterioles requires expression of iNOS and develops earlier than in carotid arteries. (Stroke. 2006;37:2129-2133.)

Key Words: endothelium • diabetes mellitus • cerebral circulation • microcirculation • carotid arteries

Hyperglycemia during diabetes mellitus is associated with endothelial dysfunction. Effects of the duration of hyperglycemia on the progression of vascular dysfunction have been studied in several vascular beds. In the rat aorta, impairment of endothelium-dependent vasodilatation appears 1 to 2 months after induction of diabetes with streptozotocin and may be related in part to the accumulation of advanced glycosylation end-products (AGEs). On the other hand, endothelial function in small resistance arterioles seems to be impaired earlier than in large arteries; eg, vascular dysfunction is observed in rat intestinal arterioles as early as 1 week after induction of diabetes. In other small vessels, such as cremaster muscle and mesenteric arterioles, vascular dysfunction develops within 1 month of diabetes. In another pathological condition, hyperhomocysteinemia, cerebral arterioles appear to be more susceptible than large arteries to endothelial dysfunction.

Recent evidence suggests that impaired endothelium-dependent relaxation during diabetes may be dependent, in part, on the expression of inducible nitric oxide synthase (iNOS). Some studies also reported increased expression and activity of iNOS in the heart soon after the onset of diabetes. We have reported that in the carotid artery, endothelial dysfunction is present in wild-type but not in iNOS-deficient mice after 4 to 6 months of diabetes.

The role of iNOS in the development of endothelial dysfunction in intracranial blood vessels during hyperglycemia is unclear. Thus, the overall goals of this study were (1) to examine the effects of short-term hyperglycemia on vasomotor function of cerebral arterioles and carotid arteries and (2) to test the hypothesis that the expression of iNOS plays an important role in the development of endothelial dysfunction of intracranial blood vessels during diabetes.

Materials and Methods

Animal Preparation
Mice with targeted disruption of the gene for iNOS were obtained from Jackson laboratory (Bar Harbor, Me). C57BL/6 mice, which are the background strain for iNOS-deficient (−/−) mice, were used as wild-type controls. Male C57BL/6 mice (n=54) and iNOS−/− mice (n=21) (8 to 14 weeks old) were allocated randomly to receive...
either streptozotocin (150 mg/kg IP) or vehicle (citrate buffer). A single dose of 150 mg/kg of streptozotocin was effective in producing severe hyperglycemia in ~70% of iNOS−/− mice. Mice that did not become diabetic after streptozotocin were used as nondiabetic controls.14 The mice were studied 3 weeks, 5 to 6 weeks, or 4 months after streptozotocin or vehicle injection. All procedures followed institutional guidelines approved by the Animal Care and Use Committee at the University of Iowa.

Cranial Window
Mice were anesthetized with pentobarbital sodium (75 to 90 mg/kg IP). A catheter in the femoral artery was used to measure arterial pressure and to obtain blood samples. Mice were ventilated mechanically with supplemental oxygen. The depth of anesthesia was evaluated by applying pressure to a paw or tail and by observing changes in heart rate and blood pressure. Additional anesthetic (~20 mg·kg⁻¹·h⁻¹) was administered when such changes occurred. Arterial blood gases were maintained within normal limits throughout each experiment (pH=7.34±0.01, PCO₂=38±1 mm Hg, and PO₂=130±4 mm Hg). Body temperature was maintained at 37°C using a heating pad.

A cranial window was made over the left parietal cortex, and a segment of pial arteriole (27±1-μm diameter) was exposed. After part of the dura was opened, the cranial window was suffused with artificial cerebrospinal fluid (temperature=37°C; ionic composition in mmol/L: 132 NaCl, 2.95 KCl, 1.71 CaCl₂, 0.65 MgCl₂, 24.6 NaHCO₃, and 3.69 d-glucose) that was bubbled continuously with appropriate gases to maintain normal levels of pH and PCO₂. The window was suffused with artificial cerebrospinal fluid at least 30 minutes before the experiment. In cerebrospinal fluid sampled from the cranial window, pH was 7.36±0.01, PCO₂ was 39±1 mm Hg, and PO₂ was 113±2 mm Hg.

The diameter of cerebral arterioles was recorded and measured with a microscope, video recorder, and dimension analyzer. The diameter of 1 arteriole per animal was measured under control conditions and during topical application of acetylcholine (1 and 10 μmol/L) and sodium nitroprusside (0.01 and 0.1 μmol/L). The vasodilators were suffused over the craniotomy for 5 minutes, and the internal diameter of the pial arteriole was measured. Topical application of these agents did not produce any changes in systemic arterial pressure or prolonged change in arteriolar diameter after suffusion of a drug was stopped.

Vascular Function In Vitro
Vasomotor function of diabetic and nondiabetic C57BL/6 common carotid arteries was examined in vitro by measurement of isometric tension, as described previously.16–19 In brief, the 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 suspended between 2 triangular hooks in an organ bath and attached to a force transducer for measurement of isometric tension.

We examined contraction of carotid rings in response to the thromboxane A₂ analog U46619 and relaxation in response to acetylcholine (0.1, 1, 10 μmol/L) and sodium nitroprusside (0.1 and 10 μmol/L) after submaximal precontraction with U46619 to ~50% to 60% of maximum response (~0.3 g in nondiabetic mice and 0.5 g in diabetic mice). Responses of the carotid artery to acetylcholine are mediated by endothelial NOS in normal mice.19 Nitroprusside was used to examine endothelium-independent vasorelaxation.

Drugs
U46619 was dissolved in ethanol and then diluted with normal saline. All other drugs were dissolved and diluted in normal saline. Concentrations are expressed as final concentration of each drug in the cranial window and the organ bath.

Statistical Analysis
All values are expressed as mean±SEM. Dilatation of pial arterioles to acetylcholine and sodium nitroprusside is expressed as the percent change from baseline diameter. Relaxation of the carotid artery to acetylcholine and sodium nitroprusside is expressed as the percent change from precontraction to U46619. Single comparisons were made with an unpaired t test, and multiple comparisons were made with a repeated-measures 1-way ANOVA. P<0.05 was considered to be statistically significant.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results
Blood glucose concentrations of >13.8 mmol/L (or 248 mg/dL) were used as the criterion for inclusion of mice in the diabetic group. In C57BL/6 mice, blood glucose concentrations of diabetic mice were 31±2.1, 29±2.4, and 30±3.1 mmol/L at 3 weeks, 5 to 6 weeks, and 4 months after induction of diabetes, respectively. Blood glucose concentrations of nondiabetic mice were 10±0.4, 8±1.2, and 7±0.4 mmol/L, respectively. In iNOS−/− mice, blood glucose concentrations were 29±2.4 and 27±5.5 mmol/L after 5 to 6 weeks and 4 months of diabetes and were 11±1.4 and 9±1.0 mmol/L in nondiabetic control mice, respectively. Body weights were significantly lower in diabetic than in nondiabetic mice at each time point in both C57BL/6 and iNOS−/− mice, but there were no significant differences between C57BL/6 and iNOS−/− mice.

Effects of 3 to 6 Weeks of Diabetes on Cerebral Vasodilatation in C57BL/6 Mice
In C57BL/6 mice, 3 weeks after induction of diabetes, the dilator responses of cerebral arterioles to acetylcholine and nitroprusside were similar to those of nondiabetic mice (Figure 1A). However, 5 to 6 weeks after induction of diabetes, acetylcholine (1 μmol/L) produced less dilatation in diabetic than in nondiabetic mice (P=0.002, Figure 1C). Nitroprusside produced similar dilator responses of cerebral arterioles in diabetic and nondiabetic mice (Figure 1B and 1D).

Cerebral Vasodilatation in iNOS−/− Mice
In iNOS−/− mice, acetylcholine produced similar dilator responses of cerebral arterioles after 5 to 6 weeks of diabetes and in nondiabetic mice (Figure 2A). Dilatation of cerebral arterioles to nitroprusside was also similar in diabetic and nondiabetic mice (Figure 2B). Thus, responses to acetylcholine in iNOS−/− mice were normal in the presence of diabetes.

Effects of 4 Months of Diabetes on Cerebral Vasodilatation
After 4 months of diabetes, dilator responses of cerebral arterioles to both high- and low-dose acetylcholine were significantly reduced in diabetic C57BL/6 mice compared with nondiabetic mice (Figure 1E). In contrast, acetylcholine-induced dilator responses were similar in diabetic and nondiabetic iNOS−/− mice (Figure 2C). Dilatation of cerebral arterioles to nitroprusside was similar in C57BL/6 and iNOS−/− mice in diabetic and nondiabetic mice (Figures 1F and 2D).

Responses of Carotid Arteries
We have shown previously that 4 to 6 months of diabetes produces endothelial dysfunction of carotid arteries in C57BL/6 mice.12 In this study, we evaluated the effects of 5 to 6 weeks of diabetes on acetylcholine-induced relaxation of carotid arterioles.
ies, to correspond to the time at which responses of intracra-
nial vessels are impaired (see previous sections). Relaxation
of carotid arteries was similar in diabetic and nondiabetic
C57BL/6 mice (Figure 3A). Relaxation of carotid arteries in
response to nitroprusside was also similar in diabetic and
nondiabetic mice (Figure 3B).

Discussion
There are 2 major new findings in this study. First, dilator
responses to acetylcholine are impaired earlier in cerebral
arterioles than in carotid arteries during diabetes. These
findings are concordant with a previous study that indicated
that cerebral arterioles also are more susceptible than the
aorta to endothelial dysfunction during hyperhomocysteine-
mia.9,10 Second, during diabetes, dilator responses of cerebral
arterioles to acetylcholine were impaired in wild-type but not
in iNOS−/− mice. Thus, development of endothelial dys-
function in cerebral arterioles during short-term diabetes/

hyperglycemia is associated, at least in part, with the expres-
sion of iNOS.

The duration of hyperglycemia/diabetes is an important
determinant of endothelial dysfunction. There is a delay after onset
of hyperglycemia to the development of endothelial dysfunction
in large arteries. In aortas from rats with streptozotocin-induced
diabetes, responses to acetylcholine were not altered after 1 to 2
weeks but were impaired after 8 weeks of diabetes.20 Altered
expression of endothelial NOS and iNOS may contribute to the
changes in vascular responses.21 In some small extracranial
vessels, such as mesenteric and cremaster arterioles, endothelial
function tends to be impaired at a relatively early stage of
diabetes.2–8 In the current study, although 3 weeks of hypergly-
cemia/diabetes were insufficient to alter cerebral vasoreactivity,
dilator responses to acetylcholine in wild-type mice were im-

Figure 1. Effects of diabetes on responses of cerebral arterioles
in C57BL/6 mice. A and B, Effects of 3 weeks of diabetes on
acetylcholine- and nitroprusside-induced dilatation (n=7 in each
group). Baseline diameters of cerebral arterioles in diabetic and
nondiabetic mice were 25.6±2.3 and 26.8±1.4 μm, respec-
tively. C and D. Effects of 5 to 6 weeks of diabetes on
acetylcholine- and nitroprusside-induced dilatation (n=8 in non-
diabetic and n=7 in diabetic mice). *P=0.0002 vs nondiabetic
mice. Baseline diameters of cerebral arterioles in diabetic and
nondiabetic mice were 28.0±1.8 and 27.4±2.4 μm, respec-
tively. E and F. Effects of 4 months of diabetes on acetylcholine-
and nitroprusside-induced dilatation (n=6 in each group).
*P<0.05 vs nondiabetic mice. Baseline diameters of cerebral
arterioles in diabetic and nondiabetic mice were 23.8±0.9 and
23.9±2.0 μm, respectively. Values are mean±SEM.

Figure 2. Effects of diabetes on responses of cerebral arterioles
in INOS-knockout mouse. A and B, Effects of 5 to 6 weeks of dia-
betes on acetylcholine- and nitroprusside-induced dilatation
(n=5 in nondiabetic and n=7 in diabetic mice). Baseline diana-
ters of cerebral arterioles in diabetic and nondiabetic mice were
23.8±1.1 and 27.8±2.3 μm, respectively. C and D. Effects of 4
months of diabetes on acetylcholine- and nitroprusside-induced
dilatation (n=5 in each group). Baseline diameters of cerebral
arterioles in diabetic and nondiabetic mice were 24.0±1.6 and
27.3±1.6 μm, respectively. Values are mean±SEM.

Figure 3. Effects of diabetes (5 to 6 weeks) on relaxation of
carotid arteries in C57BL/6 mice. Values are mean±SEM; n=7
in nondiabetic and n=6 in diabetic mice. *P<0.05 vs nondia-
betic mice.
paired in cerebral arterioles but not in carotid arteries after 5 to 6 weeks of hyperglycemia. These findings indicate that the endothelium in cerebral arterioles may be more susceptible than carotid arteries to hyperglycemia.

Dilator responses of cerebral arterioles to acetylcholine were preserved in iNOS–/– mice after 5 to 6 weeks of diabetes, in contrast to the effects in wild-type mice. Our recent study12 and others11 provide evidence that expression of iNOS during diabetes is associated with endothelial dysfunction of extracranial arteries. iNOS may be expressed soon after the onset of diabetes. In the rat heart, expression of iNOS is detectable 3 weeks after injection of streptozotocin.14 Perfusion of isolated rats hearts with a high-glucose solution for only 2 hours increases iNOS gene expression and the release of NO.15 These results support our findings that early impairment of cerebral arteriolar function may be mediated, at least in part, by expression of iNOS. A recent study also suggests that induction of iNOS is dependent on the duration of hyperglycemia and may contribute to endothelial dysfunction in rats with streptozotocin-induced diabetes.21

Several studies have examined mechanisms of endothelial dysfunction in extracranial arteries during diabetes. Possible mechanisms of endothelial dysfunction include activation of the diacylglycerol–protein kinase C pathway22 and enhanced polyol and hexosamine pathways.23 These mechanisms may lead to oxidative stress in blood vessels, quenching NO and producing impairment of endothelial function. In cerebral arterioles of rats with streptozotocin-induced diabetes, a cyclooxygenase-derived product appears to contribute to endothelial dysfunction, presumably from stimulation of the thromboxane A2/prostaglandin H2 receptor.24 In addition to these mechanisms, formation of AGEs, which are the terminal adducts of nonenzymatic glycosylation of proteins, appears to play an important role in impairment of vasomotor function. AGEs may lead to expression of iNOS during diabetes, as well as activation of NADPH oxidase, and may be associated with subsequent endothelial dysfunction.1,25–30

Potential mechanisms by which upregulation of iNOS may impair endothelium-dependent relaxation probably involve oxidative stress. Excessive amounts of NO generated by iNOS may react with superoxide to form peroxynitrite, which is a powerful pro-oxidant that can mediate cytotoxic effects of high glucose15 and may thereby induce endothelial dysfunction.31 Moreover, NOS enzymes, including iNOS, can produce superoxide when the availability of substrate or cofactors is limited (‘‘uncoupling’’).31,32 Superoxide levels are elevated in arteries after gene transfer of iNOS.33,34 Thus, iNOS may impair endothelium-dependent dilator responses during diabetes via oxidative damage from generation of superoxide or peroxynitrite.

We speculate that several mechanisms may contribute to the different time courses of vascular dysfunction between cerebral arterioles and carotid arteries. First, there may be differences in superoxide dismutases in large arteries and arterioles. In the rat aorta, in which responses to acetylcholine are impaired after 10 (but not 1 or 4) weeks of diabetes, expression and activity of manganese superoxide dismutase were decreased after 10 but not after 1 or 4 weeks of diabetes.35 On the other hand, the activity of an antioxidant enzyme (manganese superoxide dismutase) is higher in intracranial arteries than in carotid arteries.36 Second, extracellular superoxide dismutase and plasma catalase activities in the aorta appear to decrease during diabetes.37 Thus, impaired dismutation of superoxide may play an important role in the development of endothelial dysfunction during diabetes. Furthermore, cerebral vessels may have a different potential for generation of superoxide compared with extracranial arteries. Endothelial cells in cerebral arteries and the aorta appear to have different Nox homologs of NADPH oxidase and activation mechanisms.38 Finally, we cannot exclude the possibility that differences in the experimental approach (studies of vasomotor responses in vivo versus in vitro) may contribute in some way to the experimental findings.

In the present study, we observed earlier development of endothelial dysfunction in cerebral arterioles than in carotid arteries. These findings are not predictable on the basis of previous studies that indicated that in humans, intracranial arteries are more resistant than extracranial arteries to oxidative stress induced by hypercholesterolemia and that the activity of antioxidant enzymes is higher in intracranial arteries than in peripheral blood vessels.36 On the other hand, our results are consistent with a previous study that indicated that cerebral arterioles in mice are more susceptible than the aorta to endothelial dysfunction during hyperhomocysteinemia.9,10 Possibly, the small size of arteries in mice may enhance their susceptibility to oxidative damage, as observed in cerebral9,10 and mesenteric39 arteries during hyperhomocysteinemia. Thus, the apparent difference in time to develop endothelial dysfunction between intracranial and extracranial arteries may be related to species differences between mice and humans or, more likely, to distinctive characteristics of each disease.

In summary, our results indicate that endothelium-dependent dilator responses of cerebral arterioles are impaired at a relatively early stage of diabetes compared with those of the carotid artery. Endothelial dysfunction, however, was not observed in diabetic iNOS–/– mice. We conclude that that activation of iNOS may play an important role in the development of early cerebrovascular dysfunction after onset of streptozotocin-induced diabetes.

Acknowledgment
We thank Arlinda LaRose for secretarial assistance.

Sources of Funding
This work was supported by National Institutes of Health grants NS24621, HL62984, HL16066, HL38901, HL55006, and HL14388 and funds from the VA Medical Service, the American Heart Association (0575092N), and a Carver Trust Research Program of Excellence.

Disclosures
None.

References


Impairment of Dilator Responses of Cerebral Arterioles During Diabetes Mellitus: Role of Inducible NO Synthase

Jiro Kitayama, Frank M. Faraci, Carol A. Gunnett and Donald D. Heistad

Stroke. 2006;37:2129-2133; originally published online June 29, 2006;
doi: 10.1161/01.STR.0000231654.79017.df

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/37/8/2129

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/