Impaired Endothelium-Dependent Responses and Enhanced Influence of Rho-Kinase in Cerebral Arterioles in Type II Diabetes

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Background and Purpose—Although the incidence of type II diabetes is increasing, very little is known regarding vascular responses in the cerebral circulation in this disease. The goals of this study were to examine the role of superoxide in impaired endothelium-dependent responses and to examine the influence of Rho-kinase on vascular tone in the cerebral microcirculation in type II diabetes.

Methods—Diameter of cerebral arterioles (29±1 μm; mean±SE) was measured in vivo using a cranial window in anesthetized db/db and control mice.

Results—Dilatation of cerebral arterioles in response to acetylcholine (ACh; 1 and 10 μmol/L), but not to nitroprusside, was markedly reduced in db/db mice (eg, 10 μmol/L ACh produced 29±1% and 9±1% in control and db/db mice, respectively). Superoxide levels were increased (P<0.05) in cerebral arterioles from db/db mice (n=6) compared with controls (n=6). Vasodilation to ACh in db/db mice was restored to normal by polyethylene glycol-superoxide dismutase (100 U/mL). Y-27632 (1 to 100 μmol/L; a Rho-kinase inhibitor) produced modest vasodilatation in control mice but much greater responses in db/db mice. Nω-nitro-L-arginine (100 μmol/L; an inhibitor of NO synthase) significantly enhanced Y-27632–induced dilatation in control mice to similar levels as observed in db/db mice.

Conclusions—These findings provide the first evidence for superoxide-mediated impairment of endothelium-dependent responses of cerebral vessels in any model of type II diabetes. In addition, the influence of Rho-kinase on resting tone appears to be selectively enhanced in the cerebral microcirculation in this genetic model of type II diabetes. (Stroke. 2005;36:342-347.)

Key Words: cerebral circulation ■ diabetes mellitus, type II ■ reactive oxygen species

Type II diabetes is a major risk factor for carotid artery disease and stroke as well as Alzheimer disease.1–4 For example, patients with type II diabetes are at a 2- to 4-fold higher risk for stroke than patients without diabetes.1 Although type II diabetes accounts for ~90% of all cases of diabetes in humans,1 there is very little data regarding vascular function in type II diabetes in the cerebral circulation and even less known about mechanisms of vascular dysfunction in this disease.

Endothelium-dependent relaxation is impaired in peripheral blood vessels in type II diabetes in animal models and in patients.5–11 In contrast, very little is known regarding endothelium-dependent responses of cerebral vessels during type II diabetes.12,13 Because the incidence of type II diabetes is increasing and because cerebral vascular responses in type II diabetes have not been examined in great detail, the first goal of this study was to test whether dilatation of cerebral arterioles in response to endothelium-dependent and -independent agonists is altered in type II diabetes. The study of endothelial function seems particularly appropriate, because endothelial dysfunction has emerged as an independent predictor of clinical events.14

Several mechanisms have been proposed to account for impairment of endothelium-dependent relaxation in peripheral blood vessels in type II diabetes, including enhanced production of reactive oxygen species, particularly superoxide, and reduced bioactivity of endothelium-derived NO.5,6,8,9 Thus, a second goal of this study was to examine the importance of superoxide in impairment of endothelium-dependent responses of cerebral arterioles in type II diabetes.

Recently, alterations in Rho-kinase activity have been implicated in cerebral blood vessels in hypertension.15 Activity of Rho-kinase affects calcium sensitivity and may affect resting tone of cerebral blood vessels. In addition,
activity of Rho-kinase may be inhibited by NO.\textsuperscript{16–18} Thus, a third goal of this study was to examine whether the influence of Rho-kinase on vascular tone was altered in type II diabetes. We hypothesized that the influence of Rho-kinase would be increased in type II diabetes and that a reduction in NO bioavailability would result in enhanced Rho-kinase activity. To accomplish these goals, we examined responses of cerebral arterioles in \textit{db/db} mice, a genetic model of type II diabetes characterized by hyperinsulinemia, insulin-resistance, hyperglycemia, and obesity.\textsuperscript{19}

Methods

Experimental Preparation

Male \textit{db/db} mice (\(n=32\)) and their nondiabetic controls (C57BlKs; \(n=42\)) were obtained from The Jackson Laboratory (Bar Harbor, Me). Animals had access to food and water ad libitum and were housed in the Animal Care Unit. All experimental protocols were reviewed and approved by The University of Iowa Animal Care and Use Committee before the start of these studies.

Animals were anesthetized with pentobarbital sodium (75 to 90 mg/kg IP), supplemented at \(\sim 10\) to 20 mg/kg per hour. The trachea was cannulated, and the animals were ventilated mechanically with air and supplemental oxygen. A femoral artery was cannulated for measurement of systemic pressure, to sample arterial blood including for determination of glucose levels. Arterial pressure under anesthesia was similar in the 2 groups and averaged \(\sim 70\) mm Hg. Arterial blood gases were measured and were found to be similar in the 2 groups (arterial pH, 7.32 \pm 0.01).

A cranial window was made over the left parietal cortex, and a segment of a pial arteriole was exposed as described previously.\textsuperscript{20} All drugs were applied topically over the cerebral vessels. Application of vehicle did not affect vessel diameter.

Experimental Protocols

Five groups of animals were studied. In all groups, diameter of 1 arteriole per animal was measured under control conditions and during application of drugs. In the first group, changes in arteriolar diameter were measured in response to the endothelium-dependent dilator acetylcholine (ACh; 1 and 10 \(\mu\)mol/L) and to the endothelium-independent dilators nitroprusside (0.1 and 1 \(\mu\)mol/L) and papaverine (10 and 100 \(\mu\)mol/L). All vasodilators were applied cumulatively, and the sequence of each was tested in a random manner.

In the second group, we sought to determine whether superoxide contributes to the alterations in vascular responses in type II diabetes. Changes in arteriolar diameter were measured in response to ACh and nitroprusside. After the first application of agonists, a 30-minute recovery period was allowed, and application of ACh and nitroprusside was repeated in the presence of polyethylene glycol-superoxide dismutase (PEG-SOD; 100 U/mL) or vehicle (control). We have shown previously that this concentration of PEG-SOD is within that which is efficacious in reducing superoxide levels in blood vessels.\textsuperscript{20–22} The cranial window was treated with PEG-SOD or vehicle for 30 minutes before and during the second application of agonists.

In the third group, we sought to determine whether the influence of Rho-kinase on arteriolar tone is altered in type II diabetes. To accomplish this, we examined effects of Y-27632 (a Rho-kinase inhibitor)\textsuperscript{23} 1 to 100 \(\mu\)mol/L on baseline tone in control and \textit{db/db} mice.

In the fourth group, we sought to determine whether the influence of protein kinase C (PKC) on arteriolar tone and the relative contribution is altered during diabetes. We examined effect of calphostin C (a selective PKC inhibitor)\textsuperscript{15,24} 0.01 to 1 \(\mu\)mol/L on baseline diameter in control and \textit{db/db} mice.

In the fifth group, we determined whether inhibition of NO production results in an enhanced influence of Rho-kinase on vascular tone in control mice and thus provides a potential mechanistic link between impaired NO-mediated responses and enhanced Rho-kinase activity in \textit{db/db} mice. We examined the effect of 100 \(\mu\)mol/L \(N^\text{G}\)-nitro-L-arginine (L-NNA; a NO synthase inhibitor) or vehicle for 30 minutes before and during application of ACh and Y-27632.

Detection of Superoxide

Superoxide levels were evaluated in cerebral arterioles using hydroethidine-based confocal microscopy as described previously.\textsuperscript{20,22} Briefly, sections of cerebral arterioles from control and \textit{db/db} mice were frozen in optimal cutting temperature compound, sectioned (30 \(\mu\)m) onto glass slides, and incubated with hydroethidine (2 \(\mu\)mol/L) for 30 minutes. Positive staining (red fluorescence) for superoxide was determined with a Noran Odyssey laser scanning confocal microscope equipped with a krypton/argon laser. Fluorescence was detected with a 585-nm long-pass filter. Laser settings were identical for acquisition of images, and vessels from control and \textit{db/db} mice were processed and imaged in parallel. Relative increases in fluorescence were determined in a blinded manner using NIH Image software (v 1.62) and was normalized to the cross-sectional area of the vessel wall.

Drugs

ACh, calphostin C, L-NNA, papaverine, PEG-SOD, and sodium nitroprusside were obtained from Sigma Chemical Co, and all were dissolved in saline. Y-27632 was obtained from CalBioChem and dissolved in milli-Q water. Hydroethidine was obtained from Molecular Probes and was dissolved in dimethyl sulfoxide.

Statistical Analysis

Vascular responses are presented as a percent change in diameter compared with baseline. For comparison of vessel diameter under control conditions and during application of agonists as well as for comparison of superoxide levels, statistical analysis was performed with the use of paired or unpaired t tests as appropriate. All values are expressed as mean \pm SE. \(P<0.05\) was considered significant.

Results

Baseline Parameters

Body weight (43 \pm 1 versus 29 \pm 1 g) and blood glucose (570 \pm 39 versus 108 \pm 8, mg/dL) were greater \((P<0.05)\) in \textit{db/db} mice than in control mice, respectively. Mice used were of similar age (5 \(\pm 0.3\) months). Baseline arteriolar diameter was slightly less \((P<0.05)\) in \textit{db/db} mice.
Cerebral Dilator Responses in \( db/db \) Mice

ACh produced dilatation of cerebral arterioles in control mice and this response was markedly impaired in \( db/db \) mice (Figure 1). This finding indicates that endothelium-dependent dilatation of cerebral arterioles in response to ACh is impaired in \( db/db \) mice.

Nitroprusside (Figure 1) and papaverine (10 \( \mu \)mol/L: 33 ± 9 and 29 ± 5%; 100 \( \mu \)mol/L: 61 ± 5 and 54 ± 5% in control and \( db/db \) mice, respectively) produced dilatation of cerebral arterioles that was similar in both groups. Thus, impaired responses to ACh in \( db/db \) mice were selective.

Effect of PEG-SOD on Endothelium-Dependent Responses

In both control and \( db/db \) mice, ACh produced vasodilation that was reproducible. For example, in control mice, ACh dilated cerebral arterioles by 16 ± 1% and 35 ± 5% before suffusion of vehicle and 15 ± 3% and 36 ± 6%, respectively, after suffusion of vehicle. Vasodilator responses to ACh were similar (\( P > 0.05 \)) in the absence and presence of PEG-SOD in control mice (Figure 2, left). In contrast, dilatation of cerebral arterioles in response to ACh was restored to normal levels by PEG-SOD in \( db/db \) mice (Figure 2, right).

Consistent with results obtained with PEG-SOD, superoxide levels, as measured using hydroethidine, were increased (\( P < 0.05 \)) in cerebral arterioles from \( db/db \) mice as compared with control mice (Figure 3).

Effect of Inhibition of Rho-Kinase and PKC on Vascular Tone

Application of Y-27632 produced increases in diameter of cerebral arterioles in control mice (Figure 4). This response was augmented in \( db/db \) mice (Figure 4), suggesting that the influence of Rho-kinase on resting cerebral microvascular tone is increased in type II diabetes.

Application of calphostin C produced dilatation in cerebral arterioles in control mice (Figure 4) and this response was reduced in \( db/db \) mice. These findings suggest that the increased influence of Rho-kinase on basal tone is selectively increased in type II diabetes.

Effect of L-NNA on Responses to ACh and Y-27632

In time control experiments, ACh produced a reproducible response in the presence vehicle in control mice (data not shown). Vasodilatation to ACh was markedly inhibited by L-NNA (Figure 5).

Figure 2. Vasodilator responses to ACh in control (left, \( n = 3 \)) and \( db/db \) mice (right, \( n = 5 \)) before application of PEG-SOD (control: □) and after application of PEG-SOD (100 U/mL: ■). Vasodilatation in response to ACh was significantly enhanced in the presence of PEG-SOD in \( db/db \) mice. *\( P < 0.05 \) versus control response.

Figure 3. Representative confocal fluorescent sections of cerebral arterioles from control (top left, \( n = 6 \)) and \( db/db \) (top right, \( n = 6 \)) mice incubated with hydroethidine for detection of superoxide. Fluorescent intensity (relative units) expressed as a percentage of the vessel wall (bottom) was increased in \( db/db \) mice as compared with controls. *\( P < 0.05 \). Scale bar = 20 \( \mu \)m.

Figure 4. Effect of cumulative application of the Rho-kinase inhibitor Y-27632 (left) on cerebral arteriolar diameter in control (\( n = 7 \)) and \( db/db \) (\( n = 5 \)) mice. Y-27632 produced modest dilatation in control mice, whereas Y-27632 produced dilatation that was nearly 2- to 3-fold greater in \( db/db \) mice. Effect of cumulative application of the PKC inhibitor calphostin C (right) on cerebral arteriolar diameter in control (\( n = 5 \)) and \( db/db \) (\( n = 4 \)) mice. Dilatation in response to calphostin C was significantly impaired in \( db/db \) mice. *\( P < 0.05 \) versus control mice.
Y-27632 produced modest dilatation in cerebral arterioles of control mice (Figure 6). Vasodilatation to Y-27632 was markedly enhanced after L-NNA treatment (Figure 6). These findings suggest that inhibition of NO production is associated with an increased influence of Rho-kinase on resting tone.

**Discussion**

There are 3 major new findings of this study. First, dilatation of cerebral arterioles in response to the endothelium-dependent dilator ACh was markedly reduced in db/db mice as compared with controls. This effect appears to be selective for endothelium as vascular responses to the endothelium-independent dilators were similar in control and db/db mice. Second, application of PEG-SOD, a superoxide scavenger, normalized dilator responses to ACh in db/db mice but had no effect on dilator response to ACh in control mice. Consistent with these findings, we also found that superoxide levels in cerebral arterioles were increased in db/db mice as determined using hydroethidine. To our knowledge, this is the first evidence, implicating a role for superoxide in impaired endothelium-dependent responses of cerebral blood vessels in type II diabetes. Third, Y-27632 produced greater dilatation of cerebral arterioles in db/db mice compared with controls. This effect was mimicked in control mice after inhibition of nitric-oxide synthase with L-NNA, suggesting that reductions in NO bioavailability are associated with increases in Rho-kinase activity. In contrast, calphostin C produced dilatation of cerebral arterioles in control mice, which was significantly reduced in db/db mice. These findings suggest that the relative influence of Rho-kinase on basal tone is enhanced in type II diabetes.

**Cerebral Vascular Responses in Type II Diabetes**

Previous studies have demonstrated that endothelium-dependent responses of peripheral blood vessels are impaired in experimental models of type II diabetes. In terms of the cerebral circulation, most studies have focused on the effect of type I diabetes on vascular responses.25–26 Only recently have studies begun to examine the effect of type II diabetes on the cerebral circulation.12–13 We found that response to the endothelium-dependent dilator ACh was selectively impaired in db/db mice. While the present study was ongoing, it was reported that cerebral blood vessels in Zucker rats, a model of type II diabetes with hypertension, have impaired endothelium-dependent responses.12,13 The studies of the Zucker rat did not define a mechanism(s) that accounted for the impairment. Thus, this study is the first, to our knowledge, to provide insight into mechanisms that account for vascular dysfunction in the cerebral circulation in type II diabetes.

**Impaired Cerebral Vascular Responses in Type II Diabetes: Role of Superoxide**

We and others have shown that superoxide can impair endothelium-dependent responses of cerebral blood vessels.20,27–30 Thus, we examined the role of superoxide in impaired endothelium-dependent responses of cerebral arterioles and found that PEG-SOD restored responses to ACh in db/db mice to that observed in controls. In parallel with these results, we found that superoxide levels were increased in the cerebral arterioles as detected using hydroethidine-based confocal microscopy. Our results are consistent with previous findings, which suggested that superoxide contributes to impaired endothelium-dependent responses in peripheral blood vessels in type II diabetes,5,6,8,9,10 but are the first to implicate superoxide as a mediator of endothelial dysfunction in the cerebral circulation in type II diabetes. A recent study suggests that superoxide may also produce impaired endothelial function in cerebral arteries in a model of insulin resistance.31

**Influence of PKC and Rho-Kinase on Cerebral Vascular Tone**

Vascular tone is determined by many mechanisms, including the activity of both constrictor and dilator pathways. Rho-kinase and PKC have been suggested to play an
important role in regulation of vascular tone.\textsuperscript{15,16,23} Although relatively little is known, activity of Rho-kinase and PKC may influence cerebral vascular tone under normal conditions.\textsuperscript{15} This influence may change during disease states.

Recently, it has been suggested that NO may influence vascular contractility through inhibition of Rho-kinase.\textsuperscript{16,17} Increases in superoxide could reduce the inhibition of Rho-kinase by NO, thereby increasing calcium-sensitivity.\textsuperscript{16–18} Thus, in this study, we set out to determine the relative influence of Rho-kinase and PKC on cerebral vascular tone under normal conditions and in type II diabetes. We used Y-27632 to examine the influence of Rho-kinase on vascular tone in control and \textit{db/db} mice. Y-27632 is a competitive inhibitor of the ATP-binding site on Rho-kinase and we found that Y-27632 produced dilatation of cerebral arterioles in control mice, suggesting that activity of Rho-kinase influences vascular tone under basal conditions. Importantly, Y-27632 produced much greater dilatation of cerebral arterioles in \textit{db/db} mice.

Inhibition of NO production with L-NNA produced increases in Y-27632–induced vasodilatation in control mice. This change was analogous to the increased Y-27632–induced responses observed in \textit{db/db} mice, a model with impaired NO-mediated signaling in cerebral arterioles. These results are consistent with the emerging concept that NO may inhibit Rho-kinase activity.\textsuperscript{16–18} These findings also provide a potential mechanistic link between studies related to impairment of vascular responses by superoxide and increases in Rho-kinase activity in \textit{db/db} mice.

In parallel experiments, we examined the influence of PKC, which has also been implicated in calcium-sensitization of smooth muscle to vasoconstrictor stimuli.\textsuperscript{15} We used calphostin C, which has been shown previously to be an efficacious and selective inhibitor of PKC.\textsuperscript{24,32} Calphostin C produced vasodilatation under basal conditions, suggesting that activity of PKC is normally functionally important. In contrast, the influence of PKC appears to be reduced in type II diabetes because calphostin C produced less vasodilatation in \textit{db/db} mice. We speculate that the decreased influence of PKC may be a compensatory response to offset the increased influence of Rho-kinase to maintain resting vessel diameter at near normal levels in type II diabetes.

Conclusions

These findings provide the first evidence for superoxide-mediated impairment of endothelial function in cerebral blood vessels in a genetic model of type II diabetes. In addition, activity of Rho-kinase appears to be selectively enhanced in this model. We speculate that such functional changes in type II diabetes may predispose cerebral vessels to enhanced vasoconstriction and may contribute to increased risk for stroke.

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