Superoxide-NO interaction decreases flow- and agonist-induced dilations of coronary arterioles in Type 2 diabetes mellitus

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Bagi, Zsolt, Akos Koller, and Gabor Kaley. Superoxide-NO interaction decreases flow- and agonist-induced dilations of coronary arterioles in Type 2 diabetes mellitus. Am J Physiol Heart Circ Physiol 285: H1404–H1410, 2003. —Type 2 diabetes mellitus (T2-DM) markedly increases the incidence of ischemic heart disease (IHD) and, consequently, mortality. However, the underlying mechanisms leading to IHD in T2-DM are not completely understood. We hypothesized that in T2-DM the regulation of coronary microvascular resistance by local mechanisms is altered. Thus, in coronary arterioles (diameter: ~ 80 μm) isolated from male mice with T2-DM (C57Bl/KsJ-db/db) and control littersmates, responses to changes in intraluminal pressure, flow, and agonists with known mechanisms of action were studied. Increases in pressure (from 20 to 120 mmHg) resulted in similar myogenic responses of coronary arterioles of control and db/db mice, whereas dilations in response to cumulative concentrations of ACh and the nitric oxide (NO) donor NONOate were significantly decreased compared with those of control vessels. On the other hand, responses to adenosine were not different between vessels of control and db/db mice. Increases in flow (0–20 μl/min) resulted in dilations of control vessels (maximum: 38 ± 4%) that were inhibited by the NO synthase inhibitor Nω-nitro-l-arginine methyl ester (l-NAME). In contrast, arterioles of db/db mice exhibited greatly reduced dilations to flow (maximum: 4 ± 6%) that were unaffected by l-NAME. In carotid arteries of db/db mice, superoxide dismutase (SOD)-sensitive, enhanced superoxide production was detected by dihydroethydine staining and lucigenin enhanced chemiluminescence. Correspondingly, intraluminal administration of SOD significantly augmented flow-, ACh-, and NONOate-induced dilations of diabetic arterioles, and then flow- and ACh-induced responses could be inhibited by l-NAME. Collectively, these findings suggest that in T2-DM, due to an enhanced superoxide production, NO mediation of agonist- and flow-induced dilations of coronary arterioles is reduced. This alteration in the regulation of coronary microvascular resistance may contribute to the development of IHD in T2-DM.

Type 2 diabetes mellitus; coronary arteriole; flow-induced dilation; nitric oxide; superoxide

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peripheral (mesenteric, skeletal muscle, and adipose tissue) arterioles of Type 2 diabetic subjects, there is enhanced pressure-sensitive myogenic constriction, which may adversely affect dilator mechanisms (6, 20). Another important physiologically relevant mechanism that contributes to the regulation of coronary resistance is flow-dependent dilation (19). In this context, a previous study (7) of skeletal muscle arterioles from Type 2 diabetic obese Zucker rats has found decreased flow-dependent dilation.

On the basis of the above findings, we hypothesized that Type 2 DM significantly alters the regulation of coronary arteriolar function by local mechanisms. Thus, in the present study, we aimed to characterize the alterations in pressure-sensitive myogenic constriction, flow-dependent dilation, and agonist-induced responses of coronary arterioles of Type 2 diabetic db/db mice, an accepted model of human Type 2 DM.

METHODS

Animal model of Type 2 DM. To study the dysfunction of coronary arterioles in Type 2 DM, a mouse model of Type 2 DM was chosen. The genetically diabetic mouse (C57BL/KsJ-db/db) has a mutation on chromosome 4 that inhibits the expression of leptin receptors. The loss of functional leptin in obese and hyperglycemic, whereas heterozygous (control) mice cannot be distinguished morphologically or physiologically from normal mice (11). The syndrome of Type 2 DM in db/db mice is similar to Type 2 DM in adult humans, which is also characterized by obesity, insulin resistance/hyperinsulinemia, and hyperglycemia.

Experimental procedures and determination of serum glucose and insulin levels. Twelve- to fourteen-week-old male control (n = 20) and db/db (n = 20) mice (purchased from Jackson Laboratory) were used. Animals were fed standard chow and given tap water ad libitum. Mice were housed in the animal care facility at the New York Medical College approved by the American Association for the Accreditation of Laboratory Animal Care. The experimental protocol was approved by the New York Medical College Animal Care and Use Committee. Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Blood was collected from the femoral artery and centrifuged immediately. Serum was stored at –80°C for later biochemical assays. The heart and carotid arteries were excised and placed in a silicone-lined petri dish containing cold (0–4°C) physiological saline solution (PSS) composed of (in mmol/l) 110 NaCl, 5.0 KCl, 2.5 CaCl₂, 1.0 MgSO₄, 1.0 KH₂PO₄, 5.5 glucose, and 24.0 NaHCO₃ equilibrated with a gas mixture of 10% O₂ and 5% CO₂, balanced with nitrogen, at pH 7.4. Serum glucose concentrations were measured with commercial glucose assay kits (Sigma). Serum insulin levels were determined by mouse ELISA kits (ALPCO Diagnostic) using a microplate reader (Bio-Tec).

Isolation of coronary arterioles. With the use of microsurgery instruments and an operating microscope, a branch of the septal artery (–0.5 mm in length) running intramuscularly was isolated and transferred into an organ chamber containing two glass micropipettes filled with PSS. Vessels were cannulated on both ends, and micropipettes were connected with silicone tubing to an adjustable PSS reservoir. Inflow and outflow pressures were set to 80 mmHg and continuously measured by a pressure servo-controlled system (Living Systems Instrumentation). The temperature was set to 37°C by a temperature controller (Grant Instruments). The internal arteriolar diameter at the midpoint of the arteriolar segment was measured by videomicroscopy with a microangiometer (Texas Instruments). Changes in arteriolar diameter and intraluminal pressure were continuously recorded with the Biopac MP100 system connected to a computer and analyzed with AcqKnowledge data-acquisition software (Biopac Systems).

Coronary arteriolar responses to agonists. Cumulative concentrations of the endothelium-dependent dilator ACh (10⁻⁹–10⁻⁶ mol/l), the endothelium-independent nitric oxide (NO) donor NONOate (10⁻⁹–10⁻⁶ mol/l), and adenosine (10⁻⁶–10⁻⁴ mol/l) were used to test the function of endothelium and smooth muscle of arterioles.

Pressure-induced arteriolar responses. Basal coronary arteriolar tone was established at 80 mmHg. Changes in diameter of arterioles were then measured in response to step increases in intraluminal pressure from 20 to 120 mmHg. To obtain the passive diameter, arterioles were exposed to a Ca²⁺-free solution containing EGTA (10⁻⁴ mol/l) and 10⁻⁴ mol/l sodium nitroprusside (SNP). Myogenic tone was calculated as the difference between each pressure step and the passive diameter (AD; in Ca²⁺-containing PSS) and passive diameters (PD; in Ca²⁺-free PSS) as follows: (PD – AD/PD) × 100 and expressed as percent.

Flow/shear stress-induced arteriolar responses. Coronary arteriolar responses were obtained to step increases in intraluminal flow (0–20 μl/min). Intraluminal flow was established at a constant intravascular pressure (80 mmHg) by changing inflow and outflow pressures to an equal degree, but in opposite directions, to keep midpoint luminal pressure constant. Intraluminal flow was measured with a ball flowmeter (Omega Engineering). Step increases in flow were used, and each flow rate was maintained for 5 min to allow the vessel to reach a steady-state diameter. Flow-induced arteriolar responses were expressed as changes in arteriolar diameter as a percentage of the passive diameter at an intraluminal pressure of 80 mmHg. Wall shear stress (WSS) was calculated by the formula 4π(Q/r³), where r is the radius of the perfusate (0.007 Poise at 37°C), Q is the perfusate flow, and r is the vessel radius.

Endothelium removal and inhibition of endothelial NO synthesis in arterioles. The endothelium of the arterioles was removed by perfusion of the vessel with air, as described previously (15). Endothelium denudation was ascertained by the loss of dilation to ACh (10⁻⁷ mol/l) and maintained dilation to the NO donor NONOate (10⁻⁷ mol/l). To inhibit endothelial NO synthase (eNOS), isolated coronary arterioles were incubated with L-NAME (10⁻⁴ mol/l for 20 min under no-flow conditions). Quantification of vascular superoxide production by lucigenin-enhanced chemiluminescence assay. Vascular superoxide production was assessed from carotid arteries isolated from control and db/db mice by the lucigenin-enhanced chemiluminescence method according to a modified protocol of Mozaz et al. (26). A segment of the carotid arteries was removed from mice, cleared of connective tissue, immersed in PSS (37°C), and incubated for 60 min. The arteries were then placed in scintillation vials containing HEPES-buffered (10 mmol/l, pH 7.4) PSS and lucigenin (10 μmol/l, Calbiochem), and chemiluminescence was measured in a liquid scintillation counter (Beckman LS-6000IC). Scintillation counts were obtained for 20 min after the addition of vessels, and background-corrected values were normalized to tissue weight.

Detection of in situ vascular superoxide production by ethidium bromide fluorescence assay. Dihydroethydine (DHE), an oxidative fluorescent dye, was used to localize...
superoxide production according to a modified protocol of Frisbee et al. (6). Cells are permeable to DHE, which in the presence of superoxide is oxidized to fluorescent ethidium bromide (EB). EB is trapped by intercalation with DNA, and the number of fluorescent nuclei indicates the relative level of superoxide production. Thus carotid arteries isolated from control and db/db mice were transferred to chambers containing PSS and incubated for 60 min at 37°C. DHE (5 × 10^{-6} mol/l, Molecular Probes) was then added to the PSS and incubated further for 10 min, followed by 5 min of washing in cold PSS to remove the nonintercalated EB molecules. Frozen sections of vessels were then visualized by fluorescence microscopy (Olympus) and then stained with hematoxylin-eosin (HE). The separately obtained EB fluorescent and HE images were overlaid using computer image software, and the number of EB-stained fluorescent nuclei was then counted in five control vessels and five vessels from db/db mice.

Use of superoxide dismutase. Coronary arterioles of control and db/db mice were incubated in the presence of superoxide dismutase (SOD; 120 U/ml) for 30 min under zero-flow conditions; coronary arteriolar responses were then obtained again. In lucigenin-enhanced chemiluminescence assays, after control signals were obtained, carotid arteries were incubated in oxygenated PSS for an additional 30 min at 37°C in the presence of SOD, and assays were then performed again. In case of DHE staining, parallel experiments were carried out. Carotid arteries of control or db/db mice were incubated in oxygenated PSS for 30 min, and SOD or vehicle (as control) was then added to the separate solutions for an additional 30 min. EB fluorescent images of frozen sections of arteries were obtained.

Statistics. Data are expressed as means ± SE. Statistical analyses were performed by two-way ANOVA for repeated measures, followed by a Tukey post hoc test or Student’s t-test as appropriate. P < 0.05 was considered statistically significant.

RESULTS

At 12 wk of age, the body weight, serum glucose, and serum insulin of db/db mice were significantly increased compared with age-matched lean control mice (Table 1).

Pressure-induced arteriolar responses. In coronary arterioles isolated from control and db/db mice, there were no significant differences between the active (79 ± 7 and 77 ± 5 µm, respectively) and passive (in Ca^{2+}-free PSS, 99 ± 7 and 107 ± 7 µm) arteriolar diameters developed to 80-mmHg intraluminal pressure (Fig. 1A). Also, there was no significant difference in active and passive arteriolar diameters (Fig. 1A) and the calculated myogenic tone (Fig. 1B) developed to stepwise increases in intraluminal pressure from 20 to 120 mmHg in the two groups.

Table 1. Body weight, serum glucose, and serum insulin in control and db/db mice at 12 wk of age

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<tr>
<th></th>
<th>Control</th>
<th>db/db</th>
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<tr>
<td>Body weight, g</td>
<td>31.8 ± 0.7</td>
<td>49.2 ± 1.19*</td>
</tr>
<tr>
<td>Serum glucose, mmol/l</td>
<td>5.8 ± 0.6</td>
<td>22.9 ± 1.3*</td>
</tr>
<tr>
<td>Serum insulin, pmol/l</td>
<td>173 ± 38</td>
<td>2,673 ± 315*</td>
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Values are means ± SE; n = 15–18 control mice and 15–18 homozygous diabetic (db/db) mice. *P < 0.05 vs. control.

Flow/shear stress-dependent arteriolar dilation. Increases in WSS elicited by increases in intraluminal flow (from 0 to 20 µl/min) resulted in substantial dilations of coronary arteries isolated from control mice, responses that were significantly reduced in arterioles of db/db mice (Fig. 2A). Endothelium removal abolished flow-dependent dilation in control arterioles but did not affect arterioles of db/db mice (data not shown). Inhibition of NO synthesis by L-NAME decreased flow-dependent dilation in control arterioles but did not affect responses of diabetic arterioles (Fig. 2A). Flow-induced responses of arterioles were also obtained after intraluminal administration of SOD (120 U/ml). Intraluminal administration of SOD enhanced flow-induced dilation in coronary arterioles isolated from db/db mice (Fig. 2B), responses that were inhibited by L-NAME.

Agonist-induced arteriolar dilation. In coronary arterioles of db/db mice, dilations in response to cumulative doses of ACh (Fig. 3A) and the NO donor NONOate (Fig. 3B) were significantly decreased compared with those of control vessels. On the other hand, responses to adenosine were not different between vessels of control and db/db mice (Fig. 3C). Intraluminal administration of SOD enhanced ACh- and NONOate-induced dilation in coronary arterioles isolated from...
DB/db mice but did not affect adenosine-induced dilations (Fig. 3).

Quantification and in situ detection of vascular superoxide production. Vascular superoxide production was assessed in carotid arteries of control and DB/db mice by the lucigenin-enhanced chemiluminescence method. Summarized data show that there was an enhanced lucigenin chemiluminescence in carotid arteries of DB/db mice, which was inhibited by preincubation with SOD (Fig. 4A).

DHE, an oxidative fluorescent dye, was used to localize superoxide production in situ in the wall of carotid arteries of five control and five DB/db mice. A fluorescent photomicrograph of EB staining showed an increased number of fluorescence-labeled nuclei, indicating enhanced superoxide production, in diabetic carotid arteries (24 ± 6 nuclei/section) compared with controls (8 ± 4 nuclei/section), which was significantly reduced by preincubation with SOD (11 ± 4 nuclei/section; Fig. 4B). Overlaid EB- and HE-stained photomicrographs showed that the enhanced superoxide production was mainly localized in the endothelial and subendothelial layers of the vessels.

DISCUSSION

The new findings of the present study are that in coronary arterioles isolated from DB/db mice, a model for Type 2 DM, NO mediation of flow- and agonist-induced dilation is reduced, whereas pressure-induced myogenic tone is unaltered. The reduced NO mediation in diabetic arterioles is due to an increased vascular production of superoxide anions.

Clinical and experimental studies have demonstrated that in Type 2 DM, in addition to specific metabolic changes, altered vasodilator mechanisms of coronary vessels can lead to a mismatch of myocardial supply and demand (4, 21, 29), thereby provoking ischemic episodes. Recently, it has been reported that...
vasodilation to hypoxia of coronary microvessels from humans with DM is reduced due to impaired ATP-sensitive K+ channel activation (25). Furthermore, changes in local regulatory mechanisms, intrinsic to the vascular wall, such as pressure-sensitive myogenic and flow-sensitive endothelial mechanisms, have been also proposed to contribute to the decreased dilator capacity of skeletal vessels in Type 2 DM (6, 7).

In the present study, we found that in db/db mice at 12 wk of age, body weight, serum glucose, and serum insulin are significantly increased compared with age-matched lean control mice (Table 1). Similar changes have been observed in Type 2 DM in humans. Microvessels respond to an increase or decrease in transmural pressure by constriction and dilation, respectively. In Type 2 diabetic db/db mice, Lagaud et al. (20) found that in mesenteric arterioles, there is enhanced pressure-induced myogenic tone due to the upregulation and activation of smooth muscle protein kinase C. In obese Zucker rats, Frisbee et al. (7) also reported enhanced myogenic tone in skeletal muscle arterioles. Because coronary vascular resistance is influenced by myogenic reactivity and enhanced myogenic tone could adversely affect vasodilator function of arterioles, in the present study, responses to increases in intraluminal pressure were first obtained in coronary arterioles of db/db mice. We found that no significant difference between active and passive diameters of coronary arterioles of db/db and control mice developed to 80-mmHg intraluminal pressure. Moreover, changes in diameter and the calculated myogenic tone of arterioles in response to stepwise increases in intraluminal pressure from 20 to 120 mmHg were also not significantly different in the two groups (Fig. 1, A and B), indicating that in db/db mice enhanced myogenic constriction is unlikely to be responsible for the decreased vasodilation of coronary arterioles. On the other hand, the unaltered basal myogenic tone of coronary arterioles of diabetic mice may support the hypothesis of earlier observations: that in the diabetic state, basal coronary blood flow is unchanged (12, 35).

The endothelium continuously regulates vascular diameter by releasing vasodilator (NO, prostacyclin, etc.) and vasoconstrictor (thromboxane, endothelin, etc.) substances, thereby contributing to regulation of vascular resistance. One of the primary in vivo physiological stimuli for local regulation of arteriolar diameter is the presence of intraluminal blood flow (13, 15, 18). Increases in intraluminal flow elicit endothelium-dependent vasodilation via the release of vasodilator substances, such as NO and dilator prostaglandins (15). It has been reported that in vivo, in skeletal muscle microvessels, flow-mediated dilation was significantly reduced in Type 2 diabetic obese Zucker rats compared with controls (7). In the present study, we found that increases in WSS via increases in intraluminal flow elicited substantial dilations of coronary arterioles of control mice that were significantly reduced in arterioles of db/db mice (Fig. 2A). One of the key mechanisms of decreased flow-induced dilation could be the reduced synthesis and/or availability of endothelium-derived NO. We found that inhibition of NO synthesis or endothelium removal abolished flow-induced dilation in control arterioles but did not affect the responses of arterioles of db/db mice (Fig. 2A), supporting the hypothesis that the decreased flow-induced dila-
tion of coronary arteries of db/db mice is due to the decreased mediation of the response by endothelium-derived NO. On the other hand, Miura et al. (24) found that in human atrial coronary microvessels, flow-induced dilation is mediated by hydrogen peroxide. This finding suggests that flow-induced dilation might be differently regulated in various regions of the myocardium.

Recently, Lagaud et al. (20) and Pannirselvam et al. (30) demonstrated that in mesenteric arteries of db/db mice, dilations in response to ACh were reduced, suggesting impaired endothelium-dependent NO-mediated dilation. Similarly, in coronary arterioles of db/db mice, there is decreased dilation in response to ACh compared with responses of control arterioles (Fig. 3A). Although the above-mentioned authors found unaltered dilation in response to the NO donor SNP, we found decreased dilation in response to the NO donor NONOate in coronary arterioles of db/db mice (Fig. 3B), which may be due to different prevailing levels of ROS. Others have also found reduced endothelium-independent dilation to the NO donor SNP (22, 39) or glycercine trinitrate in the brachial artery of Type 2 diabetic patients (22, 39). In contrast, dilations to adenosine were not different between vessels of control and db/db mice (Fig. 3C). These observations suggest that in coronary arterioles of db/db mice, whereas endothelium-dependent responses and responses to NO are reduced, smooth muscle function may not be affected by the diabetic condition.

A reduced NO-mediated dilation could be due to decreased synthesis of NO if, for example, the eNOS substrate L-arginine were not available (31) or the level of tetrahydrobiopterin were reduced (36, 37), as suggested to occur both in Type 1 (2) and Type 2 DM (30, 33). However, our finding that the dilation in response to the NO donor NONOate was also decreased in coronary arterioles of db/db mice suggests that an alteration in NO synthesis is unlikely to be the main cause of the decreased dilation. One of the other possible mechanisms that could be responsible for the impaired dilation is a reduced bioavailability of NO due to its interaction with reactive oxygen species (ROS). Indeed, it has been proposed that oxidative stress contributes to the development of vascular complications in Type 2 DM (3, 8, 34). In diabetic patients, increased ROS production has been observed together with decreased levels of antioxidants such as ascorbic acid, vitamin E, and glutathione (1, 23). Studies of various animal models of Type 2 DM showed that administration of scavengers of ROS, such as SOD and catalase, improved endothelium-dependent arteriolar dilations (7, 30), suggesting an important role for elevated levels of ROS, which may interfere with NO. In addition, in obese Zucker rats, Frisbee et al. (7) found that enhanced levels of ROS resulted in increased myogenic tone in gracilis arterioles, suggesting a role for ROS in pressure-induced vascular tone in Type 2 DM. However, we found no differences in the myogenic reactivity of coronary arterioles in control and db/db mice.

In the present study, using two different methods (DHE staining and lucigenin enhanced chemiluminescence), we found enhanced superoxide production in carotid arteries of db/db mice compared with controls, which was significantly reduced by preincubation of the vessels with SOD (Fig. 4, A and B). The DHE staining of carotid arteries demonstrates enhanced vascular production of superoxide anions in both endothelium and subendothelial smooth muscle cell layers. Our functional studies showed that intraluminal administration of SOD, which reduced superoxide production in carotid arteries, enhanced flow-, ACh-, and NONOate-induced dilation in coronary arterioles of db/db mice, supporting the hypothesis that enhanced vascular production of superoxide anions in coronary arterioles interferes with the mediation by NO of flow- and agonist-induced dilation in arterioles of db/db mice (Figs. 2 and 3).

It has been suggested that the excess production of vascular superoxide may be derived from different ROS-producing systems, including NADPH oxidases, xanthine oxidase, and neuronal NO synthase or eNOS in the vascular wall itself (41). Recent investigations of DM have proposed a crucial role for vascular NAD(P)H oxidases in the enhanced production of ROS (9, 10). Regardless of the source of ROS (40, 42), it is likely that an excess production of ROS contributes importantly to the alterations in NO-dependent coronary microvascular responses in Type 2 DM. Moreover, recent investigations suggest that NO, released from the coronary endothelium, besides its pivotal role in the mediation of vasodilator mechanisms, has an important role in the regulation of cardiac metabolism by reducing cardiac oxygen consumption (43). The reduced availability of cardiac NO by ROS could result in an increased oxygen consumption, thereby further compromising the function of the diabetic heart.

In summary, the present study demonstrates that in coronary arterioles isolated form mice with Type 2 DM, flow- and agonist-induced dilations are reduced due to enhanced superoxide production, which interferes with the mediation of the responses by NO, whereas pressure-induced myogenic tone remains unaltered. Although there may be no significant differences in basal coronary blood flow in diabetic hearts, the decreased mediation by NO of flow- and agonist-induced dilations could result in a significant reduction of the dilator capacity of coronary microvessels, predisposing these hearts to ischemic episodes.

DISCLOSURES

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REFERENCES


