Microvascular dysfunction after transient high glucose is caused by superoxide-dependent reduction in the bioavailability of NO and BH4

Zsolt Bagi, Erika Toth, Akos Koller, and Gabor Kaley
Department of Physiology, New York Medical College, Valhalla, New York 10595; and Department of Pathophysiology, Semmelweis University, 1445 Budapest, Hungary

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Bagi, Zsolt, Erika Toth, Akos Koller, and Gabor Kaley. Microvascular dysfunction after transient high glucose is caused by superoxide-dependent reduction in the bioavailability of NO and BH4. Am J Physiol Heart Circ Physiol 287:H626–H633, 2004.—We hypothesized that transient high-glucose concentration interferes with mediation by nitric oxide (NO) of flow-induced dilation (FID) of arterioles due to enhanced production of superoxide. In isolated, pressurized (80 mmHg) rat gracilis muscle arterioles (∼130 μm) after transient high-glucose treatment (tHG; incubation with 30 mM glucose for 1 h), FID was reduced (maximum: 38 ± 4%; after tHG, 17 ± 3%), which was not further diminished by the NO synthase (NOS) inhibitor Nω-nitro-l-arginine methyl ester (l-NAME; 18 ± 2%). Correspondingly, an enhanced polyethylene-glycol-SOD (PEG-SOD)-sensitive superoxide production was detected after tHG in carotid arteries by dihydroethyldine (DHE) staining. Presence of PEG-SOD during tHG prevented the reduction of FID (41 ± 3%), which could be inhibited by l-NAME (20 ± 4%). Administration of PEG-SOD after tHG did not prevent the reduction of FID (22 ± 3%). Sepiapterin, a precursor of the NO synthase cofactor tetrahydrobiopterin (BH4), administered during tHG did not prevent the reduction of FID (maximum, 15 ± 5%); however, it restored FID when administered after tHG (32 ± 4%). Furthermore, inhibition of either glycolysis by 2-deoxyglucose or mitochondrial complex II by 2-thenoyltrifluoroacetone reduced the tHG-induced DHE-detectable enhanced superoxide production in carotid arteries and prevented FID reduction in arterioles (39 ± 5 and 35 ± 2%). Collectively, these findings suggest that in skeletal muscle arterioles, a transient elevation of glucose via its increased metabolism, elicits enhanced production of superoxide, which decreases the bioavailability of NO and the level of the NOS cofactor BH4, resulting in a reduction of FID mediated by NO.

arteriole; glycolysis; mitochondrial complex II; 2-deoxyglucose

EPIDEMIOLOGIC STUDIES (45, 45a) have shown that strict glycemic control delays the development of microangiopathy and related cardiovascular risks in patients with diabetes mellitus. These studies (45, 45a) suggest that even transient increases in plasma glucose concentrations play an important role in the development of microvascular dysfunction in diabetes. However, the underlying mechanisms leading to functional impairment of microvessels in response to transient hyperglycemia are still not fully elucidated.

One of the important physiological roles of microvessels is the local regulation of blood flow, hence tissue perfusion (26, 32), which is known to be affected in the early phase of diabetes (37). Previous studies (13, 14) in small arteries and arterioles of diabetic subjects have demonstrated that before the appearance of morphological changes, a vasomotor dysfunction of microvessels develops, affecting both smooth muscle- and endothelium-mediated regulatory mechanisms. In line with these findings, we and others (15, 46) have previously found that in arterioles of rats with streptozotocin-induced diabetes, the pressure-induced myogenic constriction is enhanced, whereas agonist- and flow-induced, endothelium-dependent dilations are reduced (3, 43). Previous studies have also revealed that in vessels of healthy subjects short-term exposure to high glucose concentrations results in reduced endothelium-dependent, agonist- (6, 38, 39) and forearm occlusion-induced dilations (24), and functional hyperemia (23), which suggests that a transient elevation of glucose concentrations could induce functional changes in microvessels as well. However, effects of a transient elevation of glucose concentration on intrinsic vasoregulation, such as pressure-induced myogenic constriction and wall shear stress-dependent dilation of arterioles as well as the possible underlying mechanisms, have not yet been elucidated.

Superoxide has been previously shown to have an important role in the functional impairment of diabetic vessels (17). It was demonstrated that in type 2 diabetes that an enhanced superoxide production resulted in enhanced myogenic constriction (15) and decreased arteriolar dilations induced by high glucose, due to the interaction with endothelium-derived nitric oxide (NO) (4, 6, 38, 39). However, the proposed role of superoxide-related oxidative stress in vascular dysfunction has not yet been supported by epidemiologic studies that failed to demonstrate a beneficial effect of antioxidant therapy (28, 29).

Data of animal studies are also controversial, because we and others (3, 18) previously found that acute in vitro administration of antioxidants, such as SOD, failed to improve the impaired vasomotor responses in streptozotocin diabetic rats. These findings, however, do not necessarily argue against the possible role of superoxide in vessels exposed to high glucose concentrations. We propose that vascular oxidative stress induced by high glucose could initiate multiple changes in regulatory pathways in arterioles, which are not restorable by antioxidants.

The aforementioned, seemingly diverse findings prompted us to test the hypothesis that in arterioles a transient elevation of glucose concentration elicits enhanced superoxide production, which by interfering with certain signaling pathways impairs the intrinsic vasomotor regulation of arterioles. Thus arteriolar vasomotor responses to increases in intraluminal pressure and flow were investigated before and after exposure to high glucose, and
the possible changes in cellular mechanisms were studied by using specific pharmacological probes administered either during or after exposure to high glucose.

MATERIALS AND METHODS

Experiments were carried out in male Wistar rats (n = 55, weighing ~300 g). Animals were housed in the animal care facility approved by the American Association for the Accreditation of Laboratory Animal Care. Animals were fed standard rat chow and drank tap water ad libitum. All protocols were approved by New York Medical College’s Animal Care and Use Committee. After overnight fasting, rats were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg), and segments of carotid artery and gracilis muscle were removed; animals were then euthanized by an additional injection of pentobarbital sodium (150 mg/kg).

Isolation of gracilis skeletal muscle arteriole. With the use of microsurgery instruments and an operating microscope, gracilis arterioles (~1.5 mm in length) were isolated (3, 27) and transferred into an organ chamber containing two glass micropipettes filled with physiological salt solution (PSS) composed of (in mM) 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% O2-5% CO2 balanced with nitrogen, at pH 7.4. In this study, 10% oxygen was used in the superfusion bath, because higher concentrations of oxygen may cause excess free radical production as suggested previously (35). Vessels were cannulated on both ends, and micropipettes were connected with silicone tubing to a adjustable PSS reservoir. Inflow and outflow pressures were set to 80 mmHg and measured by a pressure servocontrol system (Living Systems Instrumentation). Temperature was set at 37°C by a temperature controller (Grant Instruments). The internal diameters at the midpoint of the isolated arterioles were measured by videomicroscopy with a microangiometer (Texas Instruments). Changes in arteriolar diameter and intraluminal pressure were continuously recorded with a Biopac-MP100 system connected to a computer and analyzed with AcqKnowledge data acquisition software (Biopac Systems). Perfusion flow was measured with a ball flowmeter (Omega; Stamford, CT).

Experimental protocols. After a 1-h incubation period in normal PSS containing 5.5 mM glucose, control arteriolar responses to increases in pressure and flow were obtained. Intraluminal PSS was then changed to one containing 30 mM glucose for a 1-h period and then to one containing 5.5 mM glucose (normal PSS) for another 10 min. Arteriolar responses were then reassessed. In separate experiments, in addition to 5.5 mM glucose, 25 mM mannitol was added as a hyperosmotic control.

In protocol 1, after transient high-glucose treatment (tHG), arterioles were incubated with Nω-nitro-l-arginine methyl ester (L-NAME; 10^{-4} M for 20 min), an inhibitor of NO synthesis, and arteriolar responses were reassessed. In protocol 2, tHG was performed in the presence of polyethylene-glycol-SOD (PEG-SOD; 120 U/ml) (20, 34), and then arteriolar responses were obtained before and after additional incubation with L-NAME. In protocol 3, arterioles were treated with PEG-SOD after the tHG and arteriolar responses were observed again. In protocol 4, tHG was performed in the presence of sepiapterin (1 μM) (3, 34, 41, 44), a precursor of tetrahydrobiopterin (BH4), and arteriolar responses were obtained before and after incubation with L-NAME. In protocol 5, arterioles were treated with sepiapterin after tHG, and arteriolar responses were then reassessed. In protocol 6, tHG was performed in the presence of 2-deoxyglucose (2-DG; 30 mM), a competitive inhibitor of glycolysis (11), and arteriolar responses were reassessed. Finally, in protocol 7, during tHG, 2-thienyltrifluoroacetone (TTFA; 10 μM), an inhibitor of mitochondrial complex II (25, 33), was administered, and arteriolar responses were then obtained again. Protocols were summarized in Fig. 1A. Arteriolar responses to ACh (3 × 10^{-8} M) and sodium nitroprusside (SNP; 10^{-7} M) were also obtained to assess the viability of endothelium and smooth muscle cells.

Pressure-induced responses. Basal arteriolar tone was established at 80 mmHg. Changes in diameter of arterioles in response to stepwise increases in intraluminal pressure from 20 to 120 mmHg (n = 7 in each group), before and after transient elevation of glucose (tHG) or mannitol concentrations, in the absence or presence of Nω-nitro-l-arginine methyl ester L-NAME. Data are means ± SE; *significant difference (P < 0.05).

![Fig. 1. A: responses (resp) of arterioles investigated in several protocols. 2-DG, 2-deoxyglucose; TTFA, 2-thienyltrifluoroacetone; PEG-SOD, polyethylene-glycol-SOD. Changes in diameter (B) and myogenic tone (C) of isolated skeletal muscle arterioles in response to step increases in intraluminal pressure (20 to 120 mmHg) (n = 7 in each group), before and after transient elevation of glucose (tHG) or mannitol concentrations, in the absence or presence of Nω-nitro-l-arginine methyl ester L-NAME. Data are means ± SE; *significant difference (P < 0.05).](http://ajpheart.physiology.org/Downloadedfrom)
Each pressure step was maintained for 5–10 min to allow the vessel to reach a steady-state diameter. To obtain passive diameters, arterioles were exposed to Ca$_{2+}$-free PSS containing EGTA ($10^{-3}$ M) and SNP ($10^{-4}$ M), and pressure-induced responses were reassessed.

**Flow-induced dilations.** Changes in diameter of arterioles were assessed in response to step increases in intraluminal flow (from 0 to 45 $\mu$L/min) at a constant intravascular pressure (80 mmHg) (4, 27). Intraluminal flow was established at a constant intravascular pressure (80 mmHg) by changing the inflow and outflow pressures to an equal degree but in opposite directions to keep midpoint luminal pressure constant. Step increases in flow were used, and each flow rate was maintained for 5–10 min to allow the vessel to reach a steady-state diameter. Flow-induced arteriolar dilations were observed before and after tHG and specific interventions in the presence of normal PSS (containing 5.5 mM glucose).

Detection of superoxide by ethidium bromide in isolated carotid arteries. Dihydroethydine (DHE) was used to detect superoxide production in isolated carotid arteries as described earlier (4, 15). Briefly, cells are permeable to DHE, which in the presence of superoxide is oxidized to fluorescent ethidium bromide. Ethidium bromide is trapped by intercalation with DNA, and the number of fluorescent nuclei indicates the relative level of superoxide production. Accordingly, carotid arteries were removed from rats, cleared of connective tissue, immersed in normal PSS containing 5.5 mM glucose or PSS containing 30 mM glucose, and incubated for 60 min in the absence or presence of PEG-SOD (120 U/ml), sepiapterin (1 $\mu$M), 2-DG (30 mM), or TTFA (10 $\mu$M) at 37°C. DHE ($5 \times 10^{-6}$ M; Molecular Probes) was then added to the PSS for 10 min followed by being washed in normal PSS. Frozen sections of arteries were then visualized and photographed by a digital camera attached to a fluorescence microscope (Olympus). Also, the same sections were further stained with hematoxylin and eosin. Images obtained with ethidium bromide fluorescence and hematoxylin and eosin staining were overlaid by using image software (Photoshop 6.0), and the number of fluorescent nuclei was then counted in the same area of four sections in each group.

**Data analysis.** Drugs were added to the vessel chamber or into the lumen of arterioles, and final concentrations are reported. All salts and chemicals were obtained from Sigma-Aldrich unless otherwise mentioned. Solutions were prepared on the day of the experiment. Myogenic tone of arterioles was expressed at each pressure step, as a percentage of the passive arteriolar diameter (4, 21). Flow-induced arteriolar dilations were expressed as changes in arteriolar diameter as a percentage of the maximal dilation of the vessel, defined as the passive diameter at 80 mmHg intraluminal pressure in a Ca$_{2+}$-free PSS. Data are expressed as means ± SE. Statistical analyses were performed by two-way repeated-measures ANOVA, followed by Tukey’s post hoc test or Student’s t-test, as appropriate. $P < 0.05$ was considered statistically significant.

**RESULTS**

Active arteriolar tone developed in response to intraluminal pressure of 80 mmHg without the use of any vasoactive agent. After tHG, in the presence of normal PSS (containing 5.5 mM glucose), the basal arteriolar diameters were not significantly different (before tHG, 126 ± 7, after tHG, 122 ± 6; Table 1). The passive diameters obtained in the absence of extracellular Ca$_{2+}$ were also not significantly different (before tHG, 177 ± 7; after tHG, 171 ± 6 $\mu$m; Table 1). Basal arteriolar diameters at 80 mmHg were not significantly different after incubation with L-NAME, PEG-SOD, sepiapterin, 2-DG, or TTFA (Table 1).

Pressure-induced responses. There were no significant differences in arteriolar diameters developed to step increases in intraluminal pressure from 20 to 120 mmHg before and after tHG or mannitol exposure in the absence or presence of L-NAME (Fig. 1B). Also, the calculated myogenic tone of vessels of each group was not significantly different (Fig. 1C).

**Table 1. Effects of various treatments on vessel characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>tHG</th>
<th>tHG + SOD</th>
<th>tHG + SEP</th>
<th>tHG + 2-DG</th>
<th>tHG + TTFA</th>
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<tbody>
<tr>
<td>No.</td>
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<td>14</td>
<td>7</td>
<td>7</td>
<td>5</td>
<td>6</td>
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<tr>
<td>Passive diameter, μm</td>
<td>165±7</td>
<td>166±6</td>
<td>169±4</td>
<td>174±6</td>
<td>172±8</td>
<td>169±6</td>
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<tr>
<td>Active diameter, μm</td>
<td>126±7</td>
<td>130±6</td>
<td>129±5</td>
<td>134±6</td>
<td>128±3</td>
<td>131±4</td>
</tr>
<tr>
<td>Number of fluorescent nuclei/field unit</td>
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<td>41±4*</td>
<td>8±3</td>
<td>33±10*</td>
<td>14±5</td>
<td>17±9</td>
</tr>
</tbody>
</table>

Values are means ± SE. tHG, transient high-glucose treatment; SEP, sepiapterin; 2-DG, 2-deoxyglucose; TTFA, 2-thenoyltrifluoroacetone. *$P < 0.05$ vs. control.

![Flow-induced dilations of isolated skeletal muscle arterioles before and after tHG in the absence ($n = 9$ in each group, A) or presence of L-NAME ($n = 7$ in each group, B) or presence of mannitol. Data are means ± SE; *significant differences ($P < 0.05$).](AJP-Heart Circ Physiol • VOL 287 • AUGUST 2004 • www.ajpheart.org)
Flow-induced responses. Increases in intraluminal flow (0–45 μL/min) elicited substantial dilations of arterioles. After tHG (but not mannitol exposure) flow-induced dilations were significantly reduced (Fig. 2, A and B). Before tHG (control) L-NAME significantly reduced (by ~50%) flow-induced arteriolar dilations but had no significant effect on responses of arterioles after the tHG (Fig. 2B). Similar to our previous findings (3, 27), the remaining dilations in response to flow were abolished by additional administration of the cyclooxygenase inhibitor indomethacin in both conditions (not shown).

Intraluminal administration of PEG-SOD (120 U/ml) during tHG significantly mitigated the reduction in flow-induced arteriolar dilations (Fig. 3A) that remained sensitive to inhibition by L-NAME (PEG-SOD during tHG; maximum dilation, 41 ± 3%; after L-NAME, 20 ± 2%). In contrast, if PEG-SOD was administered after tHG, it failed to prevent the reduction in flow-induced dilation (Fig. 3A).

Intraluminal administration of sepiapterin, a precursor of the NO synthase (NOS) cofactor BH₄ (3, 34, 41, 44), did not prevent the reduction in flow-induced dilation of arterioles during tHG. However, sepiapterin administered after the tHG significantly enhanced flow-induced dilations, restoring them close to control (Fig. 3B), which were reduced by additional administration of L-NAME (sepiapterin after tHG maximum dilation, 32 ± 5%; after L-NAME, 16 ± 5%).

Reduction of flow-induced dilation as a result of tHG was prevented by 2-DG, a competitive inhibitor of glycolysis (11), and flow-induced dilation remained sensitive to L-NAME (2-DG during tHG; maximum dilation, 42 ± 5%; after L-NAME, 22 ± 4%).

Administration of TTFA, an inhibitor of mitochondrial respiratory chain complex II (25, 33), during tHG substantially moderated the reduction of flow-induced dilation of arterioles that was inhibitable by L-NAME (TTFA during tHG maximum dilation, 35 ± 3%; after L-NAME, 17 ± 6%).

Detection of superoxide by ethidium bromide in carotid arteries. After tHG, fluorescent digital images of ethidium bromide and hematoxylin and eosin-stained sections showed an enhanced number of fluorescent nuclei (Table 1), indicating an enhanced THG-induced superoxide production in carotid arteries compared with vessels exposed to normal PSS (Fig. 4, A–D). During tHG, the presence of sepiapterin did not affect the number of fluorescent nuclei that was, however, significantly reduced by the presence of PEG-SOD, 2-DG or TTFA (Fig. 4, E–H).

**DISCUSSION**

The main findings of this study are 1) that transient elevation of glucose concentrations (tHG) in isolated skeletal muscle arterioles of healthy rats resulted in reduction of NO mediation of flow-induced dilation; 2) the presence of the superoxide scavenger PEG-SOD during, but not after tHG, prevented the reduction of flow-induced arteriolar dilation; 3) sepiapterin (a precursor of the NOS cofactor BH₄) restored flow-induced dilation if administered after, but not during, tHG; and 4) presence of 2-DG, an inhibitor of glycolysis, or the presence of TTFA, an inhibitor of mitochondrial complex II, also prevented the tHG-induced substantial reduction in flow-induced dilation. Collectively, these findings suggest that a transient elevation of glucose concentrations via increased glucose metabolism elicits enhanced production of superoxide that reduces the bioavailability of NO and the level of the NOS cofactor BH₄, thereby eliciting a reduction in flow-induced arteriolar dilation.

Epidemiologic studies revealed that in diabetes mellitus, hyperglycemia plays an important role in the development of microvascular complications (45, 45a). In diabetes, tissue perfusion is impaired due to microvascular alterations, such as impaired regulation of arteriolar tone and morphological changes of the arteriolar wall (13, 37). Previous in vivo studies (6, 23, 38) have shown that in mesenteric arterioles of healthy rats, a transient elevation of glucose concentration elicited a reduction in NO mediation of ACh- and functional hyperemia-induced arteriolar dilations, suggesting a key role for high glucose concentration in the functional impairment of microvessels. However, the high glucose-induced changes in vasoregulatory mechanisms intrinsic to the microvascular wall and the underlying mechanisms have not yet been fully elucidated.
Effect of tHG on arteriolar myogenic tone. Previously, it has been found that in skeletal muscle arterioles of type 1 diabetic animals, the pressure-induced myogenic tone is enhanced due to the activation of PKC (46) or inhibition of smooth muscle KCa channels (15). In contrast, we found no significant changes in myogenic tone of isolated coronary arterioles of type 2 diabetic mice (4). After topical administration of high-concentration glucose, Bohlen et al. reported a decreased (7, 8) or unaltered (6) arteriolar diameter in rat mesenteric arterioles studied in vivo. Cipolla et al. (10) have found that in isolated cerebral arteries, administration of high glucose increased the diameter of arteries. Differences in findings might be due to the different types of vessels studied. Also, differences in basal arteriolar tone could be due to differences in duration of high-glucose exposure, regardless of whether the studies were performed in vivo or in vitro and whether intraluminal flow was present or not. Whatever the reasons, it seemed necessary to investigate the effects of transient high-glucose concentration on the myogenic tone of isolated skeletal muscle arterioles.

We have found that tHG did not significantly affect the basal tone of isolated skeletal muscle arterioles (Table 1). Also, increases in intraluminal pressure from 20 to 120 mmHg elicited similar diameter changes or induced the same magnitude of myogenic tone of arterioles before and after tHG (Fig. 1, B and C). Furthermore, the finding that before or after tHG, inhibition of NO synthesis did not significantly affect the arteriolar myogenic tone suggests a lack of NO modulation of arteriolar basal tone at zero flow condition. However, it is of note that in in vivo conditions, the continuous presence of static flow contributes to basal arteriolar NO production. This could explain why inhibition of NO production elicits constriction of arterioles in vivo (7, 8), but it does not affect the tone of isolated vessels (27).

Effect of tHG on NO mediation of flow-induced arteriolar dilation. It is well known that NO plays an important role in the moment-to-moment regulation of arteriolar resistance, primarily because endothelial NOS (eNOS) is activated by the presence of intraluminal flow (19, 27). The presence and magnitude of flow-induced dilation is an important factor in the local regulation of tissue blood flow by the endothelium (26) that may become compromised in diabetes mellitus (3, 4, 43). Indeed, Tribe et al. (43) in mesenteric microvessels of streptozotocin diabetic rats and we (3) in skeletal muscle arterioles, previously have found that the NO mediation of flow-induced dilation is reduced. Previous studies also demonstrated that in...
vessels of healthy subjects, short-term increases in plasma glucose concentration resulted in a reduction of functional (23) or forearm occlusion-induced (24) hyperemia. However, in these experiments, due to the methods employed, the effect of tHG on flow-induced dilation could not be assessed.

In the present study, we found that stepwise increases in intraluminal flow elicited substantial dilation of arterioles, which was significantly reduced after tHG (Fig. 2A). Before tHG, flow-induced dilation was significantly reduced by L-NAME, an inhibitor of NO synthesis (Fig. 2B), and completely abolished by the additional administration of indomethacin, an inhibitor of prostaglandin synthesis, suggesting both NO- and dilator prostaglandin mediation of flow-induced responses. In contrast, after tHG, L-NAME did not significantly affect flow-induced dilation (Fig. 2B), although in this condition, the glucose concentration in PSS was restored to control levels. These findings suggest that even a short-term elevation of glucose concentration impairs NO mediation of flow-induced arteriolar dilation.

Role of tHG-induced superoxide production. It has been proposed (13) that oxidative stress may be responsible for the development of microvascular dysfunction in diabetes mellitus. Even early investigations (16) have shown that high glucose-induced reduction in arterial dilatation is mitigated by antioxidants given in vitro. Also, in patients with diabetes, administration of the antioxidant vitamin C prevented decreases in methacholine-induced brachial artery dilations (5, 42). In contrast, epidemiologic and clinical studies failed to demonstrate any significant beneficial effect of antioxidant therapy in the prevention of diabetes-induced vascular complications (28, 29). For example, vitamin E supplementation for 8 wk did not improve the reduced ACh- and bradykinin-induced dilations of brachial arteries in type 2 diabetic patients (16). Similarly, Heygate et al. (18) found that in mesenteric arteries isolated from type 1 diabetic rats, administration of SOD failed to improve the attenuated ACh-induced dilation. Also, we have found that administration of SOD did not enhance the reduced flow-mediated dilation of arterioles isolated from rats with type 1 diabetes mellitus (3). These observations throw into doubt a direct role for oxidative stress in hyperglycemia-induced vascular dysfunction.

In the present study, however, we found that tHG enhanced DHE-detectable superoxide production in carotid artery segments, which was significantly reduced by coincubation with the membrane permeable PEG-SOD (Fig. 4E). Correspondingly, administration of PEG-SOD during tHG prevented the substantial reduction of flow-induced dilations, responses that were significantly reduced by L-NAME (Fig. 3A). These findings suggest that in arterioles, tHG induces an enhanced superoxide production that by interacting with NO released from endothelium to increases in flow/shear stress results in a reduced arteriolar dilation.

Consequences of interaction among superoxide, NO, and BH4. We then tested whether incubation of arterioles with PEG-SOD after tHG restores flow-induced dilation. We have found, however, that after tHG, the reduced flow-induced dilation was not affected by PEG-SOD (Fig. 3A). This observation is in accordance with our previous observations that in arterioles isolated from diabetic rats with chronic hyperglycemia, SOD failed to restore the impaired NO mediation of flow-induced dilation (3). On the basis of these findings, we propose that vascular oxidative stress induced by high glucose may initiate multiple changes in arteriolar function, leading to a sustained perturbation of vasomotor mechanisms that cannot be reversed by antioxidants.

Previously, it has been found that an adequate level of the eNOS cofactor BH4 is essential for NO synthesis (40, 47) and NO-mediated, flow-induced dilation of skeletal muscle arterioles (3). It is also well documented that BH4 is highly sensitive to oxygen and/or nitrogen free radicals and the level of the reduced form of BH4 is decreased in oxidative stress-related pathological conditions (12, 30, 31). Recent observations have shown that chronic BH4 treatment of diabetic rats augmented agonist-induced, NO-mediated dilations of aortas (36), and also in humans, oral glucose challenge induced a...
reduction in agonist-induced forearm blood flow that was restored by pretreatment with BH4 (22). Furthermore, Alp et al. (2) found that in type 1 diabetic mice, overexpression of GTP-cyclohydrolase I, a key enzyme of the BH4 biosynthesis, preserved endothelium-dependent vasodilations of aortas. Thus it seemed plausible that during tHG, an enhanced level of superoxide, in addition to the interaction with NO, reduces the level of BH4 resulting in a sustained reduction in NO-mediated arteriolar dilations. To test this hypothesis, arterioles were incubated with sepiapterin, a precursor of BH4, either during or after tHG. Coincubation with sepiapterin during tHG did not reduce DHE-detectable superoxide production in carotid arteries (Fig. 4F) and did not prevent the reduction in flow-induced dilation of arterioles (Fig. 3B). In contrast, if sepiapterin was administered after tHG, it restored flow-induced arteriolar dilation close to the control magnitude (Fig. 3B). We interpret these results to mean that during tHG, the enhanced level of superoxide not only interacts with NO released in response to flow but also reduces the level of BH4. Therefore, after returning to normal glucose concentrations, NO synthesis can be restored by sepiapterin but not by antioxidants. Thus we propose that tHG-induced superoxide production can reduce NO mediation of flow-induced dilation by interfering with both the metabolism and synthesis of NO.

Source of tHG-induced vascular superoxide production. Several major cellular pathways have been suggested to be altered by hyperglycemia-induced superoxide production, such as activation of polyol, hexosamine, and PKC pathways, and formation of advanced glycation end products (9). Interestingly, recent reports (25, 33) proposed that an enhanced level of mitochondrial superoxide, likely produced during the enhanced rate of glucose metabolism, might be uniquely responsible for all high glucose-related processes.

To test the hypothesis that during tHG an enhanced rate of vascular glucose metabolism is responsible for mitochondrial superoxide production, in the present study, vessels were exposed to tHG in the presence of inhibitors of either glycolysis or mitochondrial respiration. We found that in carotid arteries, either the presence of 2-DG (a competitive inhibitor of glycolysis) or the presence of TTFA (inhibitor of the mitochondrial complex II) significantly reduced the DHE-detectable, tHG-induced superoxide production (Fig. 4, G and H). Correspondingly, in skeletal muscle arterioles, the presence of 2-DG during tHG prevented the reduction of flow-induced dilation (Fig. 5A), which remained sensitive to NOH inhibition. Moreover, the presence of TTFA substantially moderated the tHG-induced reduction in flow-induced dilation of arterioles (Fig. 5A), which was also inhibitible by l-NAME.

We conclude from these results that during tHG, a higher rate of glucose metabolism (glycolysis and mitochondrial utilization) elicits enhanced production of superoxide in mitochondria of skeletal muscle arterioles. Our findings are the first to demonstrate in isolated vessels that inhibition of mitochondrial complex II (by TTFA) reduces high glucose-induced mitochondrial superoxide production, and our results complement previous studies (33) showing an important role of mitochondrial superoxide in hyperglycemia-induced PKC activation, intracellular advanced glycation end products formation, and sorbitol accumulation in aortic endothelial cells.

In summary, in the present study we have found that in isolated skeletal muscle arterioles, a transient elevation of glucose concentration resulted in a sustained reduction of NO-mediated, flow-induced dilations. We propose that transient elevations of glucose concentration by increasing glucose metabolism elicit an enhanced mitochondrial superoxide production. Increased vascular levels of superoxide reduce the bioavailability of endothelium-derived NO and the level of the eNOS cofactor BH4, thereby also reducing the synthesis of NO, when eNOS is stimulated by flow. Thus in the prevention and/or treatment of diabetic microvascular complications, restoration of both the bioavailability of NO and BH4 should be taken into consideration.

GRANTS

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