Contribution of polyol pathway to arteriolar dysfunction in hyperglycemia. Role of oxidative stress, reduced NO, and enhanced PGH₂/TXA₂ mediation

Erika Toth,¹ Anita Racz,¹ Janos Toth,¹,³ Pawel M. Kaminski,³ Michael S. Wolin,³ Zsolt Bagi,²,³ and Akos Koller¹,³

¹Department of Pathophysiology, Semmelweis University, Budapest, Hungary; ²Division of Clinical Physiology, Institute of Cardiology, University of Debrecen, Debrecen, Hungary; and ³Department of Physiology, New York Medical College, Valhalla, New York

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Toth E, Racz A, Toth J, Kaminski PM, Wolin MS, Bagi Z, Koller A. Contribution of polyol pathway to arteriolar dysfunction in hyperglycemia. Role of oxidative stress, reduced NO, and enhanced PGH₂/TXA₂ mediation. Am J Physiol Heart Circ Physiol 293: H3096–H3104, 2007. First published September 14, 2007; doi:10.1152/ajpheart.01335.2006.—Hyperglycemia increases glucose metabolism via the polyol pathway, which results in elevations of intracellular sorbitol concentration. Thus we hypothesized that elevated level of sorbitol contributes to the development of hyperglycemia-induced dysfunction of microvessels. In isolated, pressurized (80 mmHg) rat gracilis muscle arterioles (~150 μm), high glucose treatment (25 mM) induced reduction in flow-dependent dilation (from maximum of 39 ± 2% to 15 ± 1%), which was significantly mitigated by an aldose reductase inhibitor, zopolrestat (maximum 27 ± 2%). Increasing doses of sorbitol (10⁻¹⁰–10⁻⁴M) elicited dose-dependent constrictions (maximum 22 ± 3%), which were abolished by endothelium removal, a prostaglandin H₂/thromboxane A₂ (PGH₂/TXA₂) receptor (TP) antagonist SQ-29548, or superoxide dismutase (SOD) plus catalase (CAT). Incubation of arterioles with sorbitol (10⁻⁷M) reduced flow-dependent dilations (from maximum of 39 ± 2% to 20 ± 1.5%), which was not further affected by inhibition of nitric oxide synthase by Nω-nitro-L-arginine methyl ester but was prevented by SOD plus CAT and mitigated by SQ-29548. Nitric oxide donor sodium nitroprusside-induced (10⁻⁹–10⁻⁶ M) dilations were also decreased in a SQ-29548 and SOD plus CAT reversible manner, whereas adenosine dilations were not affected by sorbitol exposure. Sorbitol significantly increased arterial superoxide production detected by lucigenin-enhanced chemiluminescence, which was inhibited by SOD plus CAT. Sorbitol treatment also increased arterial formation of 3-nitrotyrosine. We suggest that hyperglycemia by elevating intracellular sorbitol induces oxidative stress, which interferes with nitric oxide bioavailability and promotes PGH₂/TXA₂ release, both of which affect regulation of vasomotor responses of arterioles. Thus increased activity of the polyol pathway may contribute to the development of microvascular dysfunction in diabetes mellitus.

aldose reductase; sorbitol; flow-dependent dilation; oxidative stress; prostaglandin H₂/thromboxane A₂; nitric oxide

DIABETES MELLITUS IS A COMPLEX metabolic disease, in which an abnormal glucose metabolism initiates multiple alterations in the cardiovascular system (17, 47, 61). Human and animal studies indicated a key role for hyperglycemia contributing to the development of microvascular dysfunction in diabetes mellitus (5, 22, 23, 51). In this context, it has been previously demonstrated that high glucose concentrations impair flow and agonist-induced brachial artery relaxation in diabetic patients (28, 43) and also in healthy subjects (7, 28, 43, 55). The pathological role of high glucose concentrations was further emphasized by studies on isolated arterioles showing that endothelium-dependent dilations in response to increases in perfusate flow are markedly reduced in the presence of high glucose concentrations, suggesting that flow-dependent regulation of tissue blood flow is altered in a high-glucose environment (5, 14). Several subsequent studies proposed an important role for vascular oxidative stress, interfering with the bioavailability of NO, as a contributing factor in reduced NO mediation of arteriolar responses.

Previous studies (46, 60), including our own observations (4, 5), have also revealed a potential role of enhanced intracellular glucose metabolism in development of the deleterious effects of high glucose on microvascular responses. High glucose concentrations promote glucose entry into the polyol pathway (21). In the first step of this pathway, glucose is metabolized to sorbitol by the aldose reductase, a rate-limiting step of the pathway. At physiological glucose concentrations, sorbitol synthesis through the polyol pathway represents a minor (<3%) fate, but at higher glucose levels, frequently encountered in which diabetes mellitus, 30–35% of the glucose could be converted to sorbitol (45). It is well established that tissues that do not require insulin for the intracellular transport of glucose (e.g., nerve, lens, retina, and kidney) show an accumulation of sorbitol due to hyperglycemia-induced activation of aldose reductase pathway (21). Because aldose reductase has been reported to be present in high amounts both in endothelial cells (31) and in vascular smooth muscle cells (49), it is likely that sorbitol concentration is elevated in the vascular wall during exposure to high-glucose conditions.

Early studies have found that inhibition of aldose reductase normalized sorbitol levels and restored the reduced acetylcholine-induced endothelium-dependent relaxations of isolated aortic rings in type 1 diabetic rabbits (52, 54), yet the direct effect of sorbitol, especially on resistance arterioles, remains to be documented. Thus we hypothesized that inhibition of aldose reductase during elevated glucose concentrations ameliorates impairment of vasomotor function of arteriolar endothelium and that an elevated level of sorbitol elicits endothelial dysfunction of arterioles.

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Thus in the present study aldose reductase inhibition and high sorbitol concentration were used to elucidate the mechanisms responsible for the hyperglycemia-induced impairment of flow-dependent dilation of isolated skeletal muscle arterioles, an endothelium-dependent response known to be importantly involved in the regulation of arteriolar resistance.

MATERIALS AND METHODS

In the present experiments, isolated skeletal muscle arterioles from male Wistar rats (n = 52, weighing ~350 g) were used, as described previously (5). Animals were fed standard rat chow, and they had free excess to tap water. After overnight fasting, under sodium pentobarbital (50 mg/kg ip) anesthesia the gracilis muscle was exposed by an incision of the skin and was removed. Then the animal was euthanized. A section of the muscle was dissected out and was placed and fixed in a silicone-lined Petri dish containing cold (0–4°C), calcium-free physiological salt solution (PSS) composed of (in mM) 110 NaCl, 5.0 KCl, 2.5 CaCl2, 1.0 MgSO4, 1.0 KH2PO4, 5.0 glucose, and 24.0 NaHCO3, equilibrated with a gas mixture of 10% O2 and 5% CO2, balanced with nitrogen, at pH 7.4. All protocols were approved by the Institutional Animal Care and Use Committee at New York Medical College (Valhalla, NY) and Semmelweis University (Budapest, Hungary). Rats were euthanized with an intraperitoneal injection of pentobarbital sodium (150 mg/kg).

Isolation of arterioles. With the use of microsurgery instruments and an operating microscope, a segment (1.5–2 mm in length) of the gracilis arteriole was isolated and transferred into an organ chamber containing two glass micropipettes filled with calcium-containing PSS composed of (in mM) 110 NaCl, 5.0 KCl, 2.5 CaCl2, 1.0 MgSO4, 1.0 KH2PO4, 5.0 glucose, and 24.0 NaHCO3, equilibrated with a gas mixture of 10% O2 and 5% CO2, balanced with nitrogen, at pH 7.4. After the vessel had been cannulated by the proximal micropipette and was fixed with suture, the inflow pressure was increased to 20 mmHg to clear the lumen from blood. Then the other end of the vessel was cannulated and fixed to the distal micropipette. Micropipettes were connected with silicone tubing to an adjustable PSS reservoir. Inflow and outflow pressures were set to 80 mmHg. Temperature was set at 37°C by a temperature controller (Grant Instruments). During a 1-h equilibration period, the vessel was allowed to reach an active diameter (5). 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. Step increases in flow were used (0–40 μl/min in 10-μl/min steps), and each flow rate was maintained for 5 min to allow the vessel to reach a steady-state diameter. Perfusion flow was measured with a ball flow meter (Omega, Stamford, CT) (2).

Agonists were added into the organ chamber, and at each concentration the peak arteriolar response was registered. All salts and chemicals were obtained from Sigma-Aldrich, except as otherwise mentioned. The aldose reductase inhibitor zopolrestat was obtained from Pfizer as a gift. Solutions were prepared on the day of the experiment. The internal arteriolar diameter was measured by video microscopy with a microangiometer. Changes in arteriolar diameter were continuously recorded with a chart recorder (Cole Parmer, Vernon Hills, IL).

Effect of high glucose treatment on vasomotor responses of arterioles. After obtaining flow-induced dilations under control conditions (5 mM glucose), arterioles were incubated in high glucose, administered extraluminally (25 mM glucose for 60 min), a method yielding similar results to intraluminal administration (5). Then changes in diameter in response to increases in intraluminal flow were obtained in the presence of zopolrestat (10 μM) both in control conditions and after high-glucose conditions. The concentration of zopolrestat was chosen on the basis of the manufacturer’s description, which was in consonance with previous studies (24).

Effect of high-sorbitol treatment on basal diameter and vasomotor of arterioles. To find an appropriate concentration for sorbitol treatment, which has an immediate effect on the basal tone, first changes in diameter of arterioles to increasing concentrations of extraluminal sorbitol (10−10−10−4 M) were obtained under control conditions and after removal of endothelium by air flow through the arteriolar lumen (32). The efficacy of endothelium removal was tested by a single dose of ACh (10−7 M), an endothelium-dependent vasodilator.

In another group of arterioles, after obtaining control responses to extraluminal sorbitol, arterioles were incubated with the prostaglandin H2 (PGH2/thromboxane A2 (TXA2) (TP) receptor antagonist SQ-29548 (10−6 M; Cayman Chemicals) for 20 min, and then responses to sorbitol were obtained again. This concentration of SQ-29548 was chosen on the basis of previous investigations by us and others (27, 63), in which the efficacy of TP receptor inhibition by SQ-29548 was confirmed by increasing doses of TP receptor agonist U-46619 (27). In separate experiments, sorbitol-induced changes in basal arteriolar diameter were also observed in the presence of 80 U/ml superoxide dismutase (SOD) or 120 U/ml catalase (CAT) in the PSS, as described previously (27).

On the basis of findings that a 10−7 M concentration of sorbitol elicited half-maximal decrease in basal diameter of arterioles, flow- and agonist-induced vasomotor responses were investigated after incubation and in the presence of 10−7 M sorbitol concentration. In these series of experiments, arteriolar responses to step increases in intraluminal flow, adenosine (Ado, 10−7–10−4 M), and the NO donor sodium nitroprusside (SNP, 10−9–10−6 M) were obtained first under control conditions. Then arterioles were incubated with sorbitol (10−7 M) for 30 min, and then vasomotor responses of arterioles were obtained again in the continuous presence of sorbitol.

In similar protocols, in the presence of sorbitol the effect of the NO synthase inhibitor N-nitro-l-arginine methyl ester (l-NNAME, 10−4 M, 30 min), the cyclooxygenase inhibitor indomethacin (Indo, 2.5 × 10−3 M, 30 min), or the free-radical scavengers SOD and CAT on vasomotor responses of arterioles were assessed. The effect of TP receptor inhibitor SQ-29548 (10−6 M, 20 min) on flow-induced dilations were also obtained after sorbitol incubation and in the presence of sorbitol and l-NNAME. Also, changes in diameter of arterioles were obtained in response to adenosine and SNP before and after incubation with 10−7 M sorbitol and in the presence of SOD/CAT. In addition, SNP-induced dilations were obtained in the presence of sorbitol and the TP receptor antagonist SQ-29548.

Detection of superoxide by lucigenin-enhanced chemiluminescence. Femoral arteries were removed from rats (n = 8), cleared of connective tissue, immersed in PSS or 10−5 M sorbitol containing PSS in the presence or absence of SOD (120 U/ml) and CAT (200 U/ml), and were oxygenated and incubated for 30 min at 37°C. In these studies, a higher concentration of sorbitol and SOD/CAT were used to account for the larger amount of vascular tissues of arteries (16). Then arteries were placed in scintillation vials containing HEPES-buffered (10−6 M/l; pH 7.4) PSS solution, and lucigenin (10−6 M/l; Calbiochem) chemiluminescence was measured in a liquid scintillation counter (Beckman LS-6000IC) as described previously (5, 57). Scintillation counts were obtained 20 min after addition of vessels, and background-corrected values were expressed.

Detection of superoxide by ethidium bromide fluorescence in femoral arteries. Femoral arteries were removed from rats and were immersed in PSS, 10−5 M sorbitol-containing PSS, or 10−5 M sorbitol and SOD (80 U/ml) plus CAT (120 U/ml)-containing PSS for 30 min. Then dihydroethidium (5 μM; Molecular Probes) was added to the vials and incubated for a further 10 min. After this incubation period, arteries were washed out in ice-cold PSS and immersed in an embedding medium (Tissue-Tek optimum cutting temperature compound; Sakura Finetek). Frozen sections of femoral arteries were visualized by a digital camera (Olympus DP12) attached to a fluorescence microscope (Olympus BX51). Intensity of fluorescence of the
arteriolar wall was measured and quantified by ImageJ software. Relative fluorescence intensity was counted by extracting the intensity of the background from a standard size of the arteriolar wall. Measurement was repeated five times, and the mean intensity and SE were calculated.

Detection of 3-nitrotyrosine formation. Femoral arteries were removed from rats, cleared of connective tissue, and immersed in PSS or \(10^{-5}\) M sorbitol-containing PSS. Another group of vessels was treated with peroxynitrite (1 μM; Calbiochem) to serve as a positive control. After a 30-min incubation period, 100 μl of sample buffer was added and vessels were homogenized. Aliquots were separated by electrophoresis on a 10% polyacrylamide gel at 125 V for 1 h and were transferred onto a polyvinyl difluoride membrane. Antibodies used for detection of 3-nitrotyrosine were obtained from Cayman Chemicals. Anti-β-actin (Abcam) was used for loading controls. Signals were measured with chemiluminescence and were visualized autoradiographically, as described previously (3). Optical density of distinct bands was measured and quantified with NIH Image software.

Data analysis. Data are expressed as means ± SE. Arteriolar responses 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 Ca²⁺-free PSS obtained at the end of each experiment (32). Statistical analysis was performed by two-way ANOVA and Tukey’s post hoc test. Values of \(P < 0.05\) were considered statistically significant.

RESULTS

In the present experiments, an active arteriolar tone developed in the presence of an intraluminal pressure of 80 mmHg, without the use of any vasoactive agent (active, 163 ± 12 μm; passive, 235 ± 14 μm; \(n = 22\)). The basal arteriolar diameter at 80 mmHg was not significantly affected by incubation with L-NAME, Indo, SOD, and CAT or endothelium removal. We have found that in isolated arterioles, high glucose concentration (25 mM) elicited transient dilations (~25%) likely due to the hyperosmolality effect of glucose, as shown by our previous study (35), and then the diameter returned close to the initial value (basal diameter, 169 ± 17 μm). The basal diameter of arterioles was not affected by the aldose reductase inhibitor zopolrestat (174 ± 15 and 160 ± 13 μm in the absence or presence of high glucose, respectively), whereas \(10^{-7}\) M sorbitol elicited a significant decrease in basal diameter at peak response (from 142 ± 12 to 131 ± 18 μm).

Effect of high glucose and zopolrestat on flow-dependent responses of arterioles. High-glucose treatment significantly reduced flow-induced dilation (from maximum of 39 ± 2% to 15 ± 1%). Zopolrestat did not change flow-induced dilation under control glucose conditions (maximum 41 ± 2%), whereas it significantly mitigated the decrease in flow-induced dilation in high-glucose-treated arterioles (maximum 27 ± 2%; Fig. 1).

Effect of sorbitol on basal arteriolar diameter. Increasing doses of sorbitol elicited significant decreases in arteriolar diameter (maximum 22 ± 3% at \(10^{-4}\) M). Sorbitol-induced constriction was abolished by endothelium removal (maximum 2 ± 2%) or by the TP receptor antagonist SQ-29548 (maximum 3 ± 1%). Incubation of arterioles with SOD/CAT also abolished sorbitol-induced decrease in basal diameter of arterioles (maximum 1 ± 2%; Fig. 2).

Effect of sorbitol on flow-dependent responses of arterioles. Under control conditions, before sorbitol incubation, increases in intraluminal flow (0–40 μl/min) elicited substantial dilations of isolated arterioles. In the presence of sorbitol, however, flow-dependent dilations were significantly reduced (maximum from 40 ± 1% to 19 ± 2%; Fig. 3A), which were not further affected by the NO synthase inhibitor L-NAME (maximum from 21 ± 1% to 20 ± 1%), whereas additional administration of cyclooxygenase inhibitor Indo abolished the remaining dilations (maximum 6 ± 2%; Fig. 3A). Also, presence of SQ-29548 mitigated the reduction in flow-dependent dilation, both in the presence and absence of L-NAME (Fig. 3). Presence of SOD/CAT significantly enhanced dilations to increases in flow in sorbitol-treated arterioles, the magnitude of which reached the control levels (maximum 34 ± 3%; Fig. 4A).

Effect of sorbitol on agonist-induced responses of arterioles. Arteriolar dilations to the higher concentrations of NO donor SNP were significantly reduced after sorbitol treatment, which were restored to control levels in the additional presence of SOD/CAT (Fig. 4B). Also, TP receptor blockade with SQ-29548 significantly increased the reduced dilations to \(10^{-6}\) M SNP in sorbitol-treated arterioles from 39 ± 6 to 43 ± 5% (\(P < 0.05\)). SNP-induced dilations were also augmented in the presence of SOD/CAT (maximum dilations to \(10^{-6}\) M SNP, 43 ± 5%; \(P < 0.05\)).

In the presence of sorbitol, adenosine-induced dilations were unaffected by L-NAME (maximum at \(10^{-5}\) M, 49 ± 4%) or Indo (maximum at \(10^{-5}\) M, 40 ± 6%) and also were not affected by SOD/CAT treatment (Fig. 4C).

Assessment of sorbitol-induced vascular oxidative stress. Production of reactive oxygen species such as superoxide was assessed in control and sorbitol-treated femoral arteries.
by the lucigenin enhanced chemiluminescence method (38). Summary data show that there was a significant increase in lucigenin chemiluminescence-detectable superoxide production in arteries treated with sorbitol, which was inhibited by incubation of vessels with SOD (120 U/ml) and CAT (200 U/ml) (Fig. 5A). Also, sorbitol-treated arterial sections showed significantly enhanced ethidium bromide fluorescence intensity compared with control, whereas sections from arteries treated with sorbitol and SOD/CAT exhibited significantly reduced fluorescence (Fig. 5, B and C).

Vascular peroxynitrite formation in control and sorbitol-treated femoral arteries was estimated by the detection of 3-nitrotyrosine content with Western immunoblot analysis. A distinct pattern of 3-nitrotyrosine-containing proteins was detected in femoral arteries. Densitometry of these blots revealed significant enhancement of 3-nitrotyrosine content on sorbitol treatment in certain, but not all, bands (differences are depicted in Fig. 6, A and B; in band a, ~50 kDa; b, ~30 kDa; and c, ~15 kDa).

DISCUSSION

There are several salient findings of present study. Aldose reductase inhibition mitigates the reduction in flow-dependent dilation as result of high glucose treatment, and elevated level of sorbitol 1) impairs flow- and agonist-induced dilations of isolated skeletal muscle arterioles that are mediated by NO, 2) enhances PGH2/TXA2 mediation of arteriolar responses, and 3) increases the production of reactive oxygen and nitrogen species in the vascular wall.

Role of high glucose and aldose reductase in the development of arteriolar dysfunction. Several mechanisms have been suggested to contribute to the development of microvascular dysfunction observed in diabetes mellitus (14, 17). However, it seems that the presence of hyperglycemia is primarily responsible for the impairment of microvascular function, especially those related to the vasomotor function of endothelium.

Earlier studies on ring preparation of large vessels demonstrated that the presence of high glucose concentrations impairs endothelium-dependent relaxation of conduit vessels (23, 52). However, less is known regarding the functional impairment of microvessels, such as arterioles known to be involved in the regulation of tissue blood flow. Previously, we and others have shown that flow-dependent dilation is reduced in a high-glucose environment (5, 50), likely due to the lack of NO mediation of the responses (5, 8, 14), and a possible role for enhanced production of reactive oxygen species by interfering...
with the bioavailability of NO has been proposed (17, 20). In our previous studies (4, 5), we have found that an increase in intracellular metabolism of glucose is important in the development of high-glucose-induced endothelial dysfunction. After entering the cells, glucose can be metabolized by the glycolytic, pentose phosphate, and/or polyol pathways. At physiological glucose concentrations, the activity of the polyol pathway is minimal, but at higher glucose levels, frequently encountered during diabetes mellitus, a substantial portion of the glucose enters the polyol pathway and is converted to sorbitol (21, 45).

Thus it was logical to hypothesize that activation of the polyol pathway may contribute to the development of microvascular dysfunction and that inhibition of aldose reductase enzyme (the first step in the polyol pathway, which converts glucose to sorbitol) reduces the high-glucose-induced impairments (2). First, we confirmed that high-glucose treatment reduces flow-induced dilations, as shown previously (5). Next, we have found that blocking the polyol pathway by using the aldose reductase inhibitor zopolrestat ameliorated the high-glucose-induced reduction of flow-induced dilation (Fig. 1). The finding that zopolrestat did not affect arteriolar responses in the presence of normal glucose concentrations supports the idea that activity of the polyol pathway is minimal in normoglycemia (Fig. 1). On the other hand, our findings suggest that the polyol pathway contributes to development of endothelial dysfunction of microvessels exposed to hyperglycemia.

**Role of high sorbitol in the development of arteriolar dysfunction.** Because aldose reductase produces sorbitol from glucose, the next logical step was to test the effects of elevated concentrations of sorbitol on the function of arterioles. We have found that increasing concentrations of sorbitol elicited by 10.220.33.5 on October 23, 2017 http://ajpheart.physiology.org/ Downloaded from

![Fig. 5. Summary data of lucigenin chemiluminescence to detect superoxide anion production in arteries treated with sorbitol (10^-7 M) before and after incubation with SOD/CAT (n = 8; A). Data are means ± SE. *P < 0.05 vs. control; #P < 0.05 vs. sorbitol-treated group. Representative fluorescent photomicrographs (representative of 3 separate experiments; B) and summary data of relative fluorescence intensity (RU; C) of ethidium bromide fluorescence in isolated femoral arteries treated with sorbitol (10^-7 M) before and after incubation of SOD/CAT are shown. Data are means ± SE. *P < 0.05 vs. control; #P < 0.05 vs. sorbitol-treated group.](#)

![Fig. 6. A: Western blot detection of 3-nitrotyrosine level of femoral arteries treated with sorbitol or with authentic peroxynitrite (PON). Blot represents 3 separate experiments. B: summary data of densitometric analysis of representative bands. Data are means ± SE. *P < 0.05 vs. control; #P < 0.05 vs. sorbitol-treated group.](#)
decreases in basal arteriolar diameter (Fig. 2). Sorbitol-induced constrictions of arterioles were eliminated by endothelium removal or inhibition of TP receptors, suggesting a role for enhanced production of PGH₂/TXA₂, extending previous findings obtained in large vessels, showing that hyperglycemia increases the generation of constrictor prostaglandins (53). It is important to note that sorbitol increased vasomotor tone in a concentration-dependent manner, suggesting that even low concentrations of sorbitol, in the long term, can have an adverse effect on the endothelial regulation of arteriolar tone, promoting the development of increased arteriolar resistance in diabetes mellitus.

Next, we investigated the effect of sorbitol on the vasomotor function of arterioles. A previous study (54) found that intracellular sorbitol concentrations in red blood cells of normal and diabetic rabbits were 4.7 ± 0.6 and 13 ± 2.7 nmol/ml, respectively. Moreover, in cultured human endothelial cells, sorbitol levels were found to be 0.04–0.12 nmol/10⁶ cells under control conditions (5 mM glucose) and 0.08–0.38 nmol/10⁶ cells under hyperglycemia (20 mM glucose) (34). The exact concentration of sorbitol in the arteriolar wall is not known, thus we have used a 10⁻⁷ M concentration of sorbitol because it elicited half-maximal constriction of arterioles.

Compared with control responses, incubation of arterioles with sorbitol significantly reduced flow-dependent dilations, which were not further affected by inhibition of NO synthase, whereas additional inhibition of prostaglandin synthesis by indomethacin eliminated flow-induced dilations. We have also found that TP-receptor blockade significantly ameliorated the decreased flow-induced arteriolar dilation either in the absence or presence of L-NAME (Fig. 3, A and B). These findings suggest that exposing arterioles to an elevated level of sorbitol not only results in an inhibition of NO mediation of flow-dependent dilation but also leads to an increased production of constrictor prostaglandins PGH₂/TXA₂. Thus we suggest that elevation of intracellular sorbitol leads to the activation of arachidonic acid metabolism, which results in an increased production of PGH₂/TXA₂ (Fig. 6C). These findings are in line with findings of Chang et al. (12) showing that aldose reductase inhibition reduced the elevated production of gliomerular prostaglandins in diabetic rats compared with that of normal rats. Keogh et al. (30) also found increased prostaglandin levels in glomeruli from diabetic rats, suggesting a possible mechanism linking increased polyol pathway activity and an increase in phospholipase A₂ activity, which in turn yields an increased prostaglandin production.

Potential cellular sources of constrictor prostaglandins.

Early studies using human umbilical vein endothelial cells (HUVEC) suggested that the endothelium of vessels produce TXA₂ (39) under physiological conditions. Interestingly, however, Buzzard et al. (9) found that endothelial cells from rabbit pulmonary artery do not express TXA₂ synthase. The controversy seemed to be resolved by showing the presence of adherent platelets in primary cultures of HUVEC by histochemistry (44). Thus it was concluded that platelets adhered to the endothelium are the source of vasoactive TXA₂. Nevertheless, many other studies suggest that the endothelium itself can be the source of TXA₂. For example, Catravas and co-workers (1) found that ACh caused a threefold increase in TXA₂ release from isolated intrapulmonary arteries but not from extrapulmonary arteries, indicating the importance of the vascular bed investigated. In addition, using immunofluorescence microscopy, Riendeau et al. (29) showed that TXA₂ synthase is widely distributed throughout the endothelium of the aorta in dogs.

Under pathological conditions, functional and morphological differences may develop even between arterial vessels from the same animal, but from different organs. For example, Tesfamariam et al. (53) found that TXA₂ and PGF₂α are released from the endothelium of rabbit aorta if the vessels were exposed to high, but not to normal, levels of glucose. Also, we have found that flow-induced dilation is impaired in coronary arterioles in hyperhomocysteinemia, primarily due to the lack of NO mediation (56), whereas in skeletal muscle arterioles the increases in intraluminal flow induce endothelium-dependent constriction, which can be inhibited by a PGH₂/TXA₂ receptor antagonist or the TXA₂ synthase inhibitor CGS-13080 (6). Because it is unlikely that platelets are present in some isolated arteriolar preparations and not in others, although the method of endothelium removal is the same, we suggest that endothelium of arterioles can be the source of constrictor prostaglandins. In addition, one can also hypothesize that the endothelium of vessels of different size, such as arteries and arterioles, may have different roles in producing TXA₂. Moreover, there could be differences in arterial and venous vessels, because we have shown that skeletal muscle venules (18) and lymphatic vessels (37) of the healthy rats produce constrictor prostaglandins, contributing to endothelium-dependent, agonist-induced responses.

Collectively, the data suggest that TXA₂ is likely produced by the endothelium of sorbitol-treated arterioles, but a potential role of adherent platelets cannot be excluded with great certainty due to the presence of methodological and pharmacological limitations.

Role of reactive oxygen and nitrogen species in high-sorbitol-induced arteriolar dysfunction.

On the basis of previous findings with high-glucose treatment of arterioles, we suspected that reactive oxygen species contribute to the development of arteriolar dysfunction as a result of sorbitol treatment. Indeed, we found that the presence of sorbitol increased, in a SOD/CAT-reversible manner, superoxide production detected by both lucigenin chemiluminescence and by ethidium bromide fluorescence (Fig. 5). Correspondingly, the presence of SOD/CAT enhanced flow-induced and SNP-evoked dilations (Figs. 4 and 5) in arterioles exposed to high sorbitol. The reversibility of NO-mediated arteriolar responses by the free-radical scavengers SOD/CAT suggests that NO synthesis remains intact in the presence of high sorbitol and that increased levels of reactive oxygen species interfere with the bioavailability of NO. Simultaneous presence of NO and superoxide is known to form peroxynitrite (62), which nitrates tyrosine residues of proteins (48). Indeed, we have found an increased tyrosine nitration of proteins in high-sorbitol-exposed vessels, suggesting that this reaction indeed took place during sorbitol incubation (Fig. 6). Interestingly, we have also found that TP-receptor blockade slightly but significantly ameliorated SNP-induced arteriolar dilation after sorbitol treatment both in the absence and presence of SOD/CAT. These findings indicate that in addition to oxidative stress, TP-receptor activation by sorbitol counteracts dilations to the NO donor SNP, likely by interfering with guanylate cyclase activation, as suggested recently by Arshad et al. (2).
Still, it is an intriguing question: what is the source of reactive oxygen species during exposure of vessels to elevated sorbitol concentration? Increased glucose metabolism may elicit an enhanced mitochondrial superoxide production (2). Also, aldose reductase and NO synthase share the same pool of NADPH, as an obligate cofactor (17). Because in hyperglycemia, the glucose flux through the polyol pathway is enhanced, the increased activity of aldose reductase may limit NO production by blunting NO synthase activity via depleting intracellular NADPH levels. These ideas are supported by previous findings showing that administration of the NO synthase inhibitor l-NAME increases sorbitol accumulation in the aorta of nondiabetic and diabetic rats (45), whereas treatment with l-arginine (a precursor of NO) or the NO donor nitroglycerin prevented sorbitol accumulation (45). Okuda et al. (42) showed that aldose reductase inhibition decreases glucose-mediated inhibition of NO production in cultured HUVEC. Also, NO donors cause a time- and concentration-dependent loss of catalytic activity of recombinant human placental aldose reductase. A site-directed mutation of aldose reductase in which the active-site amino acid is changed cannot be inactivated by NO donors, suggesting that NO may be an endogenous regulator of aldose reductase (11).

Increased activation of the polyol pathway may also increase the cytosolic NADH/NAD+ ratio. Furthermore, the metabolism of sorbitol to fructose increases cytosolic NADH, which is the substrate of NADH oxidase known to be an important source of reactive oxygen species production (25, 38, 59), especially in hyperglycemic conditions (25, 38). Future studies are needed to elucidate which of the above-mentioned mechanisms are activated, and their interactions, in elevated sorbitol conditions.

Interaction of reactive oxygen and nitrogen species with prostaglandin synthesis. The interaction between reactive oxygen and nitrogen species and prostaglandin metabolism and regulation of arteriolar tone has been a subject of several previous investigations. In our previous studies in skeletal muscle arterioles from normal rats (15) and also from type 2 diabetic mice (19), hydrogen peroxide-induced arteriolar contractions were completely abolished either by the TP receptor antagonist SQ-29548 or by the cyclooxygenase inhibitor Indo. Thus it is plausible that sorbitol-induced superoxide anion production results in an increased hydrogen peroxide level in the arterioles, which in turn facilitates constriction prostaglandin PGE2/TXA2 synthesis.

In this study, we have also found an enhanced 3-nitrotyrosine formation of the proteins in isolated vessels on sorbitol incubation (Fig. 6). We have measured 3-nitrotyrosine staining to detect formation of peroxynitrite by sorbitol treatment, providing evidence for the increased presence of superoxide, which forms peroxynitrite with NO. On the other hand, on the basis of previous studies, it seemed to be an attractive hypothesis that increased production of peroxynitrite inhibits PGJ2 synthesis by tyrosine nitration (48), yielding reduced PGJ2 synthesis and an enhanced level of nonmetabolized PGG2 (26), which in turn can activate TP receptors. Indeed, synthesis of PGJ2 by the aortic rings of diabetic rats was found to be decreased and inversely correlated with the sorbitol content of the sciatic nerves of the animals. Also, decreased PGJ2 synthesis was reversed by the administration of an aldose reductase inhibitor (58). In addition to skeletal muscle circulation, this pathway was shown to be involved in retinal, neural, and vascular damage in animals with type 1 diabetes mellitus (13, 40, 41). Yet the amount of peroxynitrite produced in response to sorbitol treatment and the consequent 3-nitrotyrosine-dependent inactivation of PGJ2 synthase remain unclear in the present study. Furthermore, it should be noted that after sorbitol incubation, Indo treatment reduced the remaining dilation, indicating a contribution of dilator prostaglandins in flow-induced arteriolar responses. Thus one can only speculate that even in the presence of inactivated (or partly inactivated) PGJ2 synthase, dilator prostaglandins other then PGI2, such as PGE2, still can be formed in response to flow. In microvessels, a possible role for PGE2 synthesis is supported by earlier findings, demonstrating a primary role of PGE2 in mediation of arteriolar responses (10). Earlier, we also found that PGE2 is a strong dilator of arterioles in vivo (33). Thus future investigations are needed to elucidate the vasomotor role of PGI2 and PGE2 in sorbitol-treated arterioles.

Collectively, the findings of the present study underscore the importance of polyol pathway activation under high-glucose conditions by showing that inhibition of aldose reductase mitigated high-glucose-induced arteriolar dysfunction and that elevated levels of sorbitol result in arteriolar dysfunction, a finding that may question the human consumption of sorbitol as a glucose substitute (36). The underlying mechanisms responsible for the polyol pathway activation-induced arteriolar dysfunction are the increased production of reactive oxygen and nitrogen species, which interfere with the mediation of vasomotor function by NO and contribute to the increased level of PGG2/TXA2, promoting thereby the development of increased arteriolar resistance in diabetes mellitus.

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