Diabetes-Induced Endothelial Dysfunction in Streptozotocin-Treated Rats: Role of Prostaglandin Endoperoxides and Free Radicals

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ABSTRACT

The vasoactive responses of renal arteries from diabetic and control rats were compared in vitro in arteriograph assemblies. Diabetes was established by an iv injection of streptozotocin (55 mg/kg) in Wistar-Kyoto rats. Endothelium-dependent relaxations mediated by nitric oxide (EDNO) were impaired in arteries from the diabetic rats: the impairment in endothelial function increased with duration of the diabetic state. After 6 and 16 wk, the concentrations of acetylcholine required to produce 50% relaxation increased with duration of the diabetic state. After 6 and 16 wk, the concentrations of acetylcholine required to produce 50% relaxation of norepinephrine preconstriction were 3.2 and 25 μM for arteries from diabetic rats and 0.4 μM in control arteries, representing 8- and 62-fold decreases in sensitivity to the endothelium-dependent vasodilator in the diabetic arteries. After 6 wk of diabetes, renal arteries also became 20-fold less sensitive to relaxation induced by histamine, another agonist that induces EDNO-mediated relaxations. The inhibition of EDNO production with l-Nω-nitroarginine produced greater impairments in acetylcholine relaxations in arteries from diabetic rats than from control rats. Relaxations in response to acetylcholine were impaired in arteries from diabetic rats because of increased production of factors that opposed the vasorelaxant effects of EDNO, rather than from decreased production of EDNO. Pretreatment of the diabetic arteries with the hydroxyl radical scavenger dimethylthiourea normalized relaxations in response to acetylcholine. The blockade of prostaglandin H2-

Diabetes mellitus represents a major risk factor for the development of cardiovascular disease (1,2). Diabetic vascular disease involving the conduit arteries predisposes to accelerated atherosclerosis, which is particularly prominent in the coronary circulation (3). Microvascular disease due to diabetes mellitus targets the renal and retinal circulations, producing diabetic nephropathy and retinopathy in upwards of half of subjects with diabetes (4). The mechanism(s) responsible for the production of diabetic vascular disease is incompletely understood (5-10). There is increasing evidence that hyperglycemia is critically important in the pathogenesis of diabetic vascular disease. Hyperglycemia triggers a cascade of functional and structural alterations in vascular cells, resulting in diabetes-induced vascular dysfunction (5,6,11,12). Endothelial cells serve as an initial target of diabetes-induced vascular dysfunction. Alterations in the vasoactive and antiatherosclerotic properties (13-16) of endothelial cells may both initiate and sustain diabetic vascular disease and its end-organ complications.

The goal of this study was to examine the vasoactive function of endothelial cells of arteries from the kidneys in an animal model for diabetes mellitus, namely streptozotocin (STZ)-induced diabetes in rats. The objectives were twofold: (1) to establish that endothelial dysfunction develops in intermediate-sized arterioles in the renal circulation and (2) to elucidate the potential mechanism(s) responsible for the endothelial dysfunction.

METHODS AND MATERIALS

Protocol for Inducing Diabetes

Male Wistar-Kyoto rats weighing 220 to 250 g, obtained from Harlan Laboratories (Boston, MA).
were randomized to receive an iv injection of STZ (55 mg/kg body wt) or vehicle (sodium citrate, 0.1 M; pH 4.5). Rats with a fasting blood glucose of more than 300 mg/dL 1 wk after the injection of STZ were considered diabetic (STZ-diabetes); vehicle-treated rats served as controls. All rats were maintained on standard Purina chow (St. Louis, MO); all procedures were in accordance with institutional guidelines. Rats were euthanized 6 to 24 wk after the injections; studies carried out at the time of euthanasia included determinations of serum creatinine, fasting glucose, hematocrit, and weight of the kidneys.

Vessel Preparation for In Vitro Studies

Rats were anesthetized with ether. The abdominal aorta was cannulated, and the kidneys were perfused in situ with chilled, heparinized saline at a pressure of 75 to 80 mm Hg. After the removal of all visible blood elements, the kidneys were removed and placed in chilled Krebs-Ringer bicarbonate containing (in millimolar concentrations): NaCl, 118; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 22; edetate calcium disodium, 0.026; and glucose, 11. Segments of distal interlobar arteries (lumen diameter, 100 to 250 μm), were carefully dissected from slices of kidneys and cannulated at each end with glass micropipettes (50- to 80-μm-diameter tips) secured in arteriograph chambers (Living Systems Instruments, Burlington, VT). The arteriographs were positioned on the stages of inverted microscopes equipped with a video camera to project the vessel image onto a television monitor. A videoelectronic dimension analyzer provided online digital display of vessel lumen diameter and wall thickness and DC signals of lumen diameter and luminal pressure, which were recorded. The arteries were allowed to equilibrate for 45 min while being perfused with Krebs solution (37°C and pH 7.4 ± 0.05) at a flow rate of 40 to 60 μL/min and at a pressure of 60 to 70 mm Hg. Lumenal perfusion was continued throughout all experiments. The maximum constriction to norepinephrine (0.1 to 1 μM) added to the extraluminal Krebs bathing solution was determined to occur at a distending pressure of 60 to 70 mm Hg. To remove endothelium, a branch of the interlobar artery was cannulated and perfused with chilled 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) followed by Krebs solution. A 1.5- to 2-mm segment of the artery was then prepared for study in the arteriograph. Removal of the endothelium was confirmed by the absence of relaxation in response to acetylcholine.

Study Protocol

To study relaxations, the arteries were first partially constricted (40 to 50% decrease in lumen diameter) with norepinephrine or phenylephrine (0.01 to 1 μM); after stabilization of the contraction, vasodilating agonists were added in a cumulative fashion (−9 to −4 log M). A tracing of an experiment depicting concentration-dependent relaxations induced by acetylcholine is presented in Figure 1. Drugs were added to the extraluminal bathing solution unless otherwise stated. For comparative analyses, relaxations, measured by increases in lumen diameter, are expressed as percentage of the increased tone (constriction) induced by norepinephrine; thus, complete relaxation of the norepinephrine contraction is shown as 0% and no relaxation is shown as 100% in the data figures. Endothelium-dependent relaxations were assessed by the responses to incremental concentrations of acetylcholine or histamine. Endothelium-independent relaxations were assessed by concentration-dependent responses to sodium nitroprusside and verapamil. When indicated, the arteries were preincubated with: (1) SQ 29548 (5 μM), an inhibitor of prostaglandin H₂-thromboxane A₂ (PGH₂-TxA₂) receptors (17); (2) N⁵-nitro-l-arginine (l-NA; 100 μM) or N⁵-nitro-l-arginine methyl ester (l-NAME; 100 μM); (3) 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7; 5 μM), an inhibitor of protein kinase C (18); (4) superoxide dismutase (SOD; from bovine erythrocytes, 3,570 U/mg of protein), a scavenger of superoxide anions; or (5) 1,3-dimethyl-2-thiourea (DMTU; 1 mM), a cell-permeable hydroxyl radical scavenger (19). Four arteries were examined from each animal to enable comparison of the responses to acetylcholine with and without inhibitor during a single exposure to acetylcholine.

![Figure 1](image-url)

Figure 1. Representative tracing showing changes in the lumen diameter of a renal interlobar artery when partially contracted with norepinephrine (NE; 0.3 μM) and then challenged with incremental concentrations of acetylcholine added to the bath solution. The response was allowed to stabilize before the addition of each incremental concentration of acetylcholine. Note that −6 log M acetylcholine produced essentially complete relaxation of the initial contraction, restoring lumen diameter to the original dimension noted before the addition of NE. Lumen pressure remained constant at 60 to 62 mm Hg during the experiment.
Drugs

Acetylcholine hydrochloride, L-norepinephrine, phenylephrine, sodium nitroprusside, histamine hydrochloride, H-7, SOD, l-NA, l-NAME, CHAPS, and STZ were obtained from Sigma Chemical Co. (St. Louis, MO). DMTU was purchased from Aldrich Chemical Co. (Milwaukee, MI). SQ 29548 was a gift from Squibb Research Institute (Princeton, NJ). Stock solutions of SQ 29548 were prepared in ethanol and stored at -20°C.

Statistical Analysis

Data are presented as the mean ± SE; N refers to the number of rats studied. The concentration of an agonist required to produce 50% relaxation of the norepinephrine precontraction was calculated for each experiment and expressed as negative log molar (pD2). Individual datum points, the area under the concentration-response curves, and pD2 values were analyzed. Statistical analysis of the data comparing responses in vessels obtained from control and diabetic rats was carried out by analysis of variance. If significant results were revealed by this analysis, then differences between group means were evaluated by the use of a t test for unpaired observations. The effects of inhibitors on endothelium-dependent responses were analyzed by comparing the response of the arteries in the presence and absence of the inhibitor; analysis of these data was carried out by the use of a t test for paired observations. A two-tailed value of P < 0.05 was considered to indicate a statistical difference.

RESULTS

STZ Induced a diabetic state in rats in a very reproducible fashion. Within 1 wk of injection, blood glucose levels exceeded 300 mg/dL in over 95% of the injected rats; fasting blood glucose remained elevated between weeks 6 to 24 after STZ injection (Table 1). In addition, STZ rats developed other manifestations of diabetes mellitus noted in humans (20), including enlargement of the kidneys and evidence of increased GFR, as reflected by a significantly lower serum creatinine. Renal enlargement was maximal after 6 wk of diabetes; absolute kidney weights were 2.46 ± 0.06 and 3.73 ± 0.14 g in control and 6-wk STZ rats, respectively (P < 0.005). Kidney weights, expressed as a percentage of total body weight, remained significantly elevated after 24 wk of diabetes; however, with the substantial weight loss of the STZ rats, absolute kidney weights were less in the STZ rats (2.51 ± 0.14 versus 2.77 ± 0.11 g in STZ and control rats, respectively; P < 0.05). The lumen diameter of the interlobar arteries selected for in vitro studies tended to increase in the STZ rats. Vessel enlargement in STZ was the result of dilation, rather than of vessel wall thickening. Wall thickness/lumen ratios for the arteries selected for studies were essentially identical in control and STZ rats studied at 6 to 24 wk after injection (Table 1). Systolic blood pressures measured by the tail cuff method 6 wk after injections were comparable in control and STZ rats (151 ± 6 versus 149 ± 5 mm Hg for control and STZ rats, respectively; N = 13). Blood hematocrits were similar in control and STZ rats at 6 wk (44.5 versus 44.8%, respectively), whereas at 24 wk after injection hematocrit values were higher in STZ rats (45 ± 0.8 versus 48 ± 0.5% in control and STZ rats, respectively; P < 0.05, N = 12).

Endothelium-Dependent Relaxations

Endothelium-dependent relaxations induced by acetylcholine (-9 to -4 log M) were impaired after 6 wk of hyperglycemia in renal arteries from diabetic rats (Figure 2). The impairment in relaxations in response to the muscarinic agonist became progressively greater with the duration of the diabetic state. The concentrations of acetylcholine required to effect a 50% relaxation of the preconstriction decreased

<table>
<thead>
<tr>
<th>Group (N)</th>
<th>Glucose (mg/dL)</th>
<th>Creatinine (mg/dL)</th>
<th>Weight (g)</th>
<th>Kidney Wt (% Total Body Wt)</th>
<th>Lumen (µm)</th>
<th>Wall Thickness: Lumen Ratiob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 6 (20)</td>
<td>160 ± 9</td>
<td>0.81 ± 0.04</td>
<td>320 ± 11</td>
<td>0.73 ± 0.02</td>
<td>190 ± 3</td>
<td>0.11 ± 0.005</td>
</tr>
<tr>
<td>Control 24 (12)</td>
<td>162 ± 8</td>
<td>0.83 ± 0.03</td>
<td>360 ± 12</td>
<td>0.77 ± 0.03</td>
<td>196 ± 6</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>DM 6 (16)</td>
<td>449 ± 26a</td>
<td>0.66 ± 0.04d</td>
<td>285 ± 12a</td>
<td>1.31 ± 0.05c</td>
<td>212 ± 5</td>
<td>0.11 ± 0.011</td>
</tr>
<tr>
<td>DM 16 (8)</td>
<td>529 ± 31a</td>
<td>0.61 ± 0.03d</td>
<td>240 ± 12c</td>
<td>1.24 ± 0.06c</td>
<td>236 ± 6</td>
<td>0.09 ± 0.006</td>
</tr>
<tr>
<td>DM 24 (12)</td>
<td>490 ± 26c</td>
<td>0.68 ± 0.09d</td>
<td>213 ± 16c</td>
<td>1.18 ± 0.04c</td>
<td>226 ± 4</td>
<td>0.11 ± 0.007</td>
</tr>
</tbody>
</table>

a Mean values ± SE. DM = diabetic rats; DM 6, 16, or 24 refers to diabetic rats studied after 6, 16, or 24 weeks of diabetes.
b Wall thickness divided by lumen diameter of arteries studied in vitro.

P < 0.005 versus control rat values.

P < 0.05 versus control rat values.
from 6.4 ± 0.2 (0.4 μM) in control arteries to 5.5 ± 0.2 (3.2 μM) and 4.6 ± 0.2 (25 μM) in interlobar arteries from diabetic rats after 6 and 16 wk, respectively, of diabetes (representing decreases of 8- and 62-fold in sensitivity of the diabetic arteries to acetylcholine). Relaxations induced by acetylcholine were comparable in arteries from 24- and 16-wk diabetic rats (pD₂, 4.4 ± 0.3). Relaxations in response to acetylcholine were abolished after the removal of the endothelium (maximum relaxations, <10%; N = 4) in both control and diabetic arteries. The nitric oxide synthase inhibitor L-NA (100 μM) produced greater inhibition of acetylcholine-mediated relaxation in diabetic than in control renal arteries (Figure 3); L-NA produced a 3-fold shift in pD₂ for control arteries and a more than 50-fold shift in arteries from 16-wk diabetic rats. L-NA (100 μM) produced decreases of 5% or less in basal lumen diameters of both control and diabetic arteries (N = 4 for each group).

To determine if the impairment in endothelium-mediated relaxations noted in arteries from diabetic rats was unique for acetylcholine, the responses to additional agonists were examined. Relaxations induced by histamine (Figure 4) were also impaired in renal arteries from diabetic rats. After 6 wk of diabetes, renal arteries from diabetic rats were 20-fold less sensitive to histamine than were arteries from control rats (pD₂ values were 5.0 ± 0.3 [10 μM] versus 6.3 ± 0.5 [0.5 μM] in control arteries; P < 0.05; N = 6). Prior removal of the endothelium decreased relaxations induced by histamine (−9 to −4 log M) to <10% in control and diabetic renal arteries (N = 4); pretreat-
ment with L-NA (100 µM) decreased the pD2 for histamine to 4.4 ± 0.3 (40 µM) and more than 4 (>100 µM) in control and diabetic renal arteries, respectively (P < 0.05 for treated and untreated arteries; N = 6 for each group). To compare the inhibition of histamine-induced relaxations produced by L-NA in control and diabetic arteries, ED75 values were compared. L-NA produced 80- and 70-fold decreases in sensitivity to histamine in arteries from control and diabetic rats, respectively.

Because assessments of endothelium-dependent relaxations require prior activation of the arteries with a constricting agonist, we compared the sensitivity of the arteries from control and diabetic rats to norepinephrine. The concentrations of norepinephrine required to decrease the lumen diameter by 40 to 50% were similar in arteries from control and 16- to 24-wk diabetic rats (6.86 ± 0.3 and 6.82 ± 0.3 -log M or 0.14 and 0.15 µM; N = 30 and 10, respectively; P < 0.9). Arteries from 6-wk diabetic rats required significantly less norepinephrine for partial activation (7.23 ± 0.4 -log M or 0.06 µM; N = 30; P < 0.005 versus control arteries); arteries from 6-wk diabetic rats incubated with L-NA required even less norepinephrine (7.32 ± 0.4 -log M or 0.04 µM; N = 12; P < 0.008 versus control arteries), whereas arteries from 16-wk diabetic rats required concentrations of norepinephrine similar to control rats for comparable activation (6.79 ± 0.3 -log M or 0.16 µM; N = 10; P < 0.8).

**Mechanism(s) Responsible for Diabetic Endothelial Dysfunction**

Additional experiments were performed to elucidate the possible mechanism(s) responsible for the impaired response of arteries from diabetic rats to acetylcholine. Relaxations induced by acetylcholine in diabetic renal arteries were improved after preincubation with the PGH2-TxA2 receptor antagonist SQ 29548 (5 µM) (Figure 5). The pD2 for acetylcholine increased from 5.3 ± 0.2 (5 µM) to 5.9 ± 0.2 (1.3 µM) in renal arteries from 6-wk diabetic rats (P < 0.05; N = 15) and from 6.4 ± 0.2 (0.4 µM) to 6.9 ± 0.4 (0.12 µM) (P < 0.1; N = 11) in renal arteries from control rats after treatment with SQ 29548. To check for the completeness of receptor blockade by SQ 29548, additional experiments were performed with 10 µM concentrations of the receptor inhibitor. The higher concentration of SQ 29548 failed to improve relaxations of diabetic arteries beyond that observed with the lower concentration. The pD2 values increased from 5.4 ± 0.3 (4 µM) to 6 ± 0.4 (1 µM) in diabetic arteries (N = 5; P < 0.05) and from 6.5 ± 0.2 (0.3 µM) to 6.6 ± 0.4 (0.25 µM) in control arteries (N = 5; P > 0.10). On the basis of recent observations by Tesfamariam and Cohen (21) that PGH2 may induce endothelial cell dysfunction via superoxide anion generation, experiments were carried out testing the effects of SOD on endothelium-dependent relaxations in arteries from control and diabetic rats. The addition of SOD (2.5 to 150 U/mL, final concentrations) to the lumen perfusate produced reversal of partial contractions of renal arteries induced by norepinephrine as well as by phenylephrine; data for experiments with norepinephrine are shown in Figure 6. Maximum relaxations were noted at SOD concentrations of 25 to 50 U/mL in both control and diabetic arteries; thus, only the data for the range from 2.5 to 50 U/mL are presented. Relaxations induced by SOD were significantly greater in diabetic arteries at all concentrations of SOD. Relaxations induced by SOD were inhibited by more than 80% by pretreatment of the arteries with L-NAME (100 µM; N = 6; P < 0.05) but were not affected (<10% inhibition) by pretreatment with indomethacin (5 µM) (N = 6; P > 0.1). The addition of SOD (150 U/mL) to the bath solution only had no effect on the lumen diameter of the precontracted arteries from control or diabetic rats (N = 6 each group). Similarly, lumen perfusion with SOD (50 U/mL) had no effect on the lumen diameter of control or diabetic renal arteries during basal (no precontraction) conditions (N = 4 each). SOD, added to the bath (150 U/mL) or lumen perfusate (50 U/mL) before norepinephrine activation of the arteries, failed to improve acetylcholine-mediated relaxations in arteries from 6-wk diabetic rats (Figure 7). It should be noted that higher concentrations of norepinephrine were required to activate arteries perfused with SOD in the lumen (legend to Figure 7). The preincubation of renal arteries with the hydroxyl...
radical scavenger DMTU markedly improved relaxations in response to acetylcholine in arteries from 6-wk diabetic rats but had no effect on arteries from control rats (Figure 8). The pD2 values increased from 5.9 ± 0.2 (1.3 μM) in untreated diabetic arteries to 7.8 ± 0.3 (0.16 μM) in arteries pretreated with DMTU (N = 12; P < 0.05). The pD2 for acetylcholine for control arteries before and after treatment with DMTU were 6.6 ± 0.2 (0.25 μM) and 6.5 ± 0.2 (0.31 μM) (N = 5). The concentrations of norepinephrine required for partial activation of control and diabetic arteries were not affected by the presence of DMTU or SQ 29548, as noted in the legends to the figures. Finally, the preincubation of renal arteries from 6-wk diabetic rats with the protein kinase C inhibitor H-7 (5 μM) did not improve relaxations in response to acetylcholine (pD2, 4.6 ± 0.3 and 4.7 ± 0.2 without and with H-7; N = 6).

Endothelium-Independent Relaxations

Relaxations induced by the calcium channel antagonist verapamil (−9 to −4 log M), were similar in renal arteries from control and 6-wk diabetic rats; the pD2 values ± SE were 6.15 ± 0.2 and 6.2 ± 0.1, and...
were slightly more sensitive. Whereas arteries from control rats to sodium nitroprusside (Figure 2) were more sensitive than arteries from control rats. The maximum relaxations were 99 and 98%, respectively (N = 6). Renal arteries from 6-wk diabetic rats were slightly more sensitive, whereas arteries from 16- to 24-wk diabetic rats were significantly less sensitive than arteries from control rats to sodium nitroprusside (Figure 9); the pD2 values for nitroprusside were 5.0 ± 0.2 and 5.9 ± 0.2 for arteries from 16- to 24-wk diabetic and control rats, respectively (P < 0.05; N = 6).

**DISCUSSION**

These experiments demonstrate that diabetes mellitus induced by STZ produces endothelial dysfunction in the renal arteries of rats. Other investigators have previously reported that diabetes mellitus impairs endothelium-dependent relaxations in the isolated aorta of diabetic rats and rabbits (11,12,22-25) and in the cerebral vessels of diabetic rats (26,27). In this study, endothelium-dependent relaxations induced by acetylcholine as well as by histamine were impaired in the interlobar arteries of diabetic rats after 6 wk of diabetes. Relaxations in response to acetylcholine were further impaired with the duration of the diabetic state (Figure 2). Relaxations induced by both acetylcholine and histamine were abolished by the prior removal of the endothelium, establishing the endothelium dependence of the relaxations. The inhibition of relaxations in response to both agonists that was noted after preincubation with L-NA suggests that the relaxations were mediated at least in part by L-arginine-derived nitric oxide (EDNO) (13). L-NA produced greater inhibition of relaxations in arteries from diabetic animals (Figure 3). This finding suggests that greater amounts of EDNO were produced in diabetic arteries in response to acetylcholine. If the diabetic arteries were in fact producing greater amounts of EDNO, why, then, were acetylcholine-induced relaxations impaired in the arteries from the diabetic rats?

The impairment in endothelium-dependent relaxation could arise from multiple mechanisms, including: (1) decreased production of EDNO; (2) increased inactivation of EDNO by endothelium-derived free radicals; (3) impaired diffusion of EDNO to underlying vascular smooth muscle; (4) decreased sensitivity of diabetic vascular smooth muscle to the vasorelaxant properties of EDNO; (5) increased production of an endothelium-derived constricting factor (EDCF); or (6) a combination of the above factors. The findings of this study provide no evidence for decreased production of EDNO in diabetic arteries. Rather, the evidence favors an increased production of EDNO in diabetic arteries. The enhancement in EDNO-mediated relaxations after the pretreatment of activated diabetic arteries with SOD and the abolition of the effect of SOD by L-NAME suggest that diabetic arteries are quite capable of producing and releasing normal if not increased amounts of EDNO (Figure 6). The experiments with SOD are inconclusive, however, in that SOD failed to improve relaxations of control and diabetic arteries in response to acetylcholine. A partial explanation for the failure of SOD to improve acetylcholine-induced relaxations may be the fact that when arteries were pretreated with SOD in the lumen perfusate before norepinephrine activation (as was the case with the acetylcholine study), higher concentrations of norepinephrine were required to achieve 40 to 50% decreases in the lumen diameter of the arteries (legend to Figure 7). In essence, any relaxation produced by SOD during activation of the artery is overwhelmed by additional norepinephrine and therefore achieves a stable activated state before the addition of acetylcholine. The higher concentrations of norepinephrine in the presence of SOD in the lumen may decrease the sensitivity of arteries to acetylcholine (relaxation). SOD, a large, water-soluble molecule, is unlikely to penetrate the vessel wall to enter the endothelial cells in any appreciable quantity. Thus, the failure of SOD to improve acetylcholine relaxations when added to the bath solution only may not be unexpected. The enhanced relaxations in response to acetylcholine noted in diabetic arteries after pretreatment of the vessel with the PGH2-TxA2 receptor antagonist SQ 29548 provide evidence that the vasorelaxant properties of EDNO are blunted by an increased production of endoperoxides (PGH2-TxA2). Diabetic arteries...
were fivefold more sensitive to acetylcholine after the inhibition of PGH₂-TxA₂ receptors. PGH₂ (or TxA₂) can oppose the vasorelaxant properties of EDNO by two mechanisms: (1) by acting as an EDCF and (2) by promoting the production of endothelium and cyclooxygenase-derived free radicals (21). The greater relaxations of norepinephrine-activated arteries from diabetic rats produced by SOD perfusion in this report (Figure 6) offer evidence that superoxide anions may serve to inactivate EDNO, as has been reported by others (28). Other investigators have reported that norepinephrine and phenylephrine stimulate increased production of both EDNO (29) and superoxide in the activation of renal arteries. The failure of SOD perfusion to improve relaxations in response to acetylcholine in renal arteries in the experiments presented here must be noted, however. Acetylcholine may activate greater production of EDNO-inactivating radicals, which either overwhelm and/or are inaccessible to SOD radical scavenging. The marked enhancement in relaxations in response to acetylcholine noted in diabetic but not in control arteries pretreated with the hydroxyl radical scavenger DMTU provides the strongest evidence in support of the potential inactivation of EDNO by free radicals, presumably hydroxyl radicals, in arteries from diabetic rats. Inactivation of EDNO by hydroxyl radicals has been described in cerebral resistance arteries by other investigators (30). DMTU has been demonstrated to scavenge hydroxyl radicals (19); an alternate mechanism by which DMTU may prevent hydroxyl radical effects is by binding "catalytic" trace metals required for the production of hydroxyl radicals (31). Direct proof of radical binding by DMTU is not provided in this study. We do have evidence that DMTU does not exert direct effects on vascular smooth muscle that lead to the enhanced relaxations noted in diabetic arteries. The preincubation of control and diabetic arteries with DMTU did not alter the sensitivity of either group of arteries to norepinephrine; the concentrations of norepinephrine required to produce 50% decreases in lumen diameters were the same with and without DMTU. DMTU does not affect relaxations in response to acetylcholine in control arteries (Figure 8), nor does DMTU alter nitroprusside-induced relaxations in rat mesenteric resistance arteries precontracted with norepinephrine or phenylephrine (32). Because both acetylcholine and nitroprusside relaxations are mediated by nitric oxide, this suggests that the increased relaxations noted only in the renal arteries from diabetic rats after pretreatment with DMTU do not arise from a nonspecific effect of the compound on vascular smooth muscle. We interpret this data as indirect evidence that diabetic renal arteries appear to produce increased quantities of endoperoxides and free radicals that oppose or inactivate the vasoactive properties of EDNO. The final responses of the diabetic vessels are dictated by balanced or unbalanced production of EDNO and opposing factors; with prolonged exposure to diabetes, vascular smooth muscle responsiveness to dilating and constricting factors may also become altered.

Several reports suggest that free radical production is increased in arteries from diabetic animals (33). Multiple pathways capable of producing the increased free radicals have been described, including lipid peroxidation (8), advanced glycosylation end-products (8,34), sorbitol-diacylglycerol metabolism (5), prostaglandin endoperoxide synthase (21), and more recently, nitric oxidase synthase (35).

Impaired diffusion of EDNO to the underlying vascular smooth muscle is not supported by findings from the experiments presented here. The acute reversibility of the impairment in endothelium-mediated relaxations by pretreatment of the diabetic arteries with inhibitors of PGH₂ receptors (SQ 29548) or with free radical scavengers, none of which alter the structure of the vessels, points away from EDNO diffusion limitations as an important etiologic mechanism responsible for diabetic endothelial dysfunction.

The potential role of decreased sensitivity of diabetic vascular smooth muscle to the vasorelaxant properties of EDNO in the production of diabetic endothelial dysfunction cannot be totally disregarded in view of the blunted responses to the nitrovasodilator sodium nitroprusside noted in arteries from rats exposed to prolonged hyperglycemia (Figure 9). These experiments suggest that there may be an element of insensitivity of vascular muscle to EDNO after prolonged exposure to the diabetic state. An alternate explanation for the decreased sensitivity of diabetic arteries to nitroprusside is the inactivation of nitric oxide (derived from nitroprusside) by hydroxyl radicals. However, during the early phases (i.e., after 6 wk) of diabetic mellitus in this animal model, there is at least normal if not increased sensitivity to nitric oxide.

There is evidence for an increased production of an EDCF (PGH₂ or TxA₂) in diabetic arteries as discussed above. Both of these prostaglandin endoperoxides can produce contraction of vascular smooth muscle and, thereby, may oppose the vasorelaxant effects of EDNO. The incomplete normalization of relaxations in response to acetylcholine in arteries from diabetic animals after the inhibition of PGH₂-TxA₂ receptors suggests that additional factors are operative in impairing EDNO-mediated relaxations in diabetic arteries. The evidence from this and other studies underscores the potential importance of increased free radical production with subsequent inactivation of EDNO in diabetic endothelial dysfunction (12). Tesfamariam and Cohen (12) recently re-
ported that free radicals mediate endothelial cell dysfunction caused by elevated glucose in the rabbit aorta. The impairment of endothelium-dependent relaxation in response to acetylcholine caused by exposure to elevated glucose was prevented by SOD, catalase, defereroxamine or allopurinol; the free radical scavengers restored abnormal acetylcholine relaxation without altering the increased levels of endothelium-derived immunoreactive prostanoids in the aortas. In addition, the impairment of acetylcholine relaxations in the aortas from alloxan-induced diabetic rabbits was restored to normal by SOD; endothelial dysfunction was not observed in diabetic animals treated with the antioxidant probucol.

Interactions between endothelium-derived free radicals, EDNO, and superoxide anions may be of considerable importance in diabetic endothelial dysfunction. Superoxide anions accelerate the breakdown of EDNO (and vice versa); superoxide anions also can react with nitric oxide to form peroxynitrite (36,37). Peroxynitrite itself may serve as a damaging radical and may also be further metabolized to the highly reactive hydroxyl radicals (38). Endothelium-dependent responses attributed to hydroxyl radicals have been described in the cerebral arteries of cats (39) and in the aorta of rats (40). EDNO serves important physiologic roles, including inhibiting platelet aggregation (13); inhibiting platelet, granulocyte, and monocyte adhesions to vessel walls (13); inhibiting proliferative responses (41,42); modulating vascular smooth muscle tone (13); and possibly serving as an antioxidant (43,44). Nitric oxide also may serve a critical role in autocrine and paracrine functions of endothelial cells by inhibiting cyclooxygenase, lipoxygenase, and epoxygenase activities (all iron-dependent enzymes) (43). The inactivation of EDNO may serve as a critical step in the genesis of diabetic vascular disease.

In summary, diabetes mellitus impairs endothelium-dependent relaxations in response to both acetylcholine and histamine in the renal arteries of rats. Diabetic endothelial dysfunction results in large part from the enhanced production of endothelium-derived free radicals, which inactivate or oppose the vasorelaxant properties of EDNO. Indirect evidence is presented for the increased production of both superoxide and hydroxyl radicals in diabetic arteries. Potential strategies to minimize diabetic endothelial dysfunction, in addition to optimizing blood glucose control, include the application of agents that combat the enhanced oxidative stress arising from multiple mechanisms that are operative in diabetes.

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