Short-term type 1 diabetes alters the mechanism of endothelium-dependent relaxation in the rat carotid artery

C. H. Leo,1,2 A. Joshi,2 and O. L. Woodman1

1School of Medical Sciences, Health Innovation Research Institute, RMIT University, Bundoora, Victoria, Australia; and 2Department of Pharmacology, University of Melbourne, Parkville, Victoria, Australia

Submitted 16 December 2009; accepted in final form 3 June 2010

Leo CH, Joshi A, Woodman OL. Short-term type 1 diabetes alters the mechanism of endothelium-dependent relaxation in the rat carotid artery. Am J Physiol Heart Circ Physiol 299: H502–H511, 2010. First published June 11, 2010; doi:10.1152/ajpheart.01197.2009.—The aim of the present study was to examine the effect of an early stage of streptozotocin-induced diabetes on the mechanism(s) of endothelium-dependent relaxation. Diabetes was induced by a single injection of streptozotocin (48 mg/kg iv), and the ACh-induced relaxation of rat carotid arteries was examined 6 wk later. A diabetes-induced increase in superoxide levels, determined by L-012-induced chemiluminescence, from carotid arteries was associated with endothelial nitric oxide (NO) synthase (eNOS) uncoupling and increased catalytic subunit of NADPH oxidase expression. The sensitivity and maximum response to ACh were similar in normal and diabetic rats despite a decrease in NO release detected by 4-amino-5-methylamino-2′,7′-difluorofluorescein. In normal rats, N-nitro-l-arginine (100 μM) plus 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (10 μM), to inhibit NO and soluble guanylate cyclase (sGC), respectively, abolished ACh-induced relaxation, whereas in diabetic rats, the maximum relaxation to ACh was attenuated (maximum relaxation: 25 ± 5%), but not abolished, by that treatment. The remaining ACh-induced relaxation was abolished by NO scavengers, cupric chloride (to degrade nitrosothiols), or blockers of endothelial K+ channels. Western blot analysis of the carotid arteries indicated that diabetes significantly increased the expression of eNOS but decreased the proportion of eNOS uncoupling as the dimer. These findings demonstrate that in early diabetes, ACh-induced relaxation is maintained but is resistant to NO inhibition. In early diabetes, nitrosothiol-mediated opening of K+ channels may act in conjunction with NO stimulation of sGC to maintain endothelium-dependent relaxation despite the increase in vascular superoxide levels.

endothelial nitric oxide synthase; nitric oxide; nitrosothiols; soluble

HYPERGLYCEMIA is a primary cause of diabetic vascular complications, the principal cause of morbidity and mortality in patients with diabetes. A hallmark of these vascular complications is the development of endothelial dysfunction, which is characterized by impaired endothelium-dependent relaxation in various vascular beds and in different models of diabetes (8) as well as in diabetic patients (16, 39). A number of studies (17, 21, 26, 32, 45) have demonstrated that incubation of isolated blood vessels in a high concentration of glucose (10, 34, 41, 42) or acute hyperglycemia by glucose loading in healthy human subjects (1) impairs ACh-induced endothelium-dependent relaxation in the microcirculation and macrocirculation. Thus, acute hyperglycemia causes a rapid impairment of endothelial function. In contrast, in streptozotocin (STZ)-induced diabetes, Pieper (32) reported a triphasic change in endothelial function: i.e., enhanced relaxation at 1 wk after STZ treatment, unaltered at 1–2 wk after STZ treatment, and impaired at 8 wk after STZ treatment, even though the animals were hyperglycemic at all of those times after STZ treatment. Furthermore, other studies (17, 21, 26, 32, 45) have demonstrated that endothelial dysfunction is not observed until at least after 7 wk of the initial STZ treatment. The inconsistency between the acute effect of hyperglycemia in vitro and in vivo, and the absence of impairment of relaxation in response to short-term hyperglycemia in vivo, led us to postulate that there may be changes in the mechanism of relaxation between 3 and 7 wk after STZ treatment to preserve endothelial function in the early stages of diabetes.

Endothelium-dependent relaxation is mediated by multiple factors, including nitric oxide (NO), prostacyclin, and an unidentified EDHF. The relative contribution of these various endothelium-derived factors to relaxation in the presence of diabetes-induced endothelial dysfunction remains controversial (8, 46). For example, endothelium-dependent relaxation is normally entirely mediated by NO in the aorta; however, in diabetes, there is an upregulation of non-NO vasodilator mediators, such as EDHF (27). In contrast, there is no compensation for diabetes-induced impairment of endothelium-dependent relaxation in the carotid artery (37).

Pieper and others (17, 21, 26, 32, 45) have clearly shown that endothelium-dependent relaxation is not impaired until at least 7 wk after STZ treatment; however, it remains unclear as to how endothelium-dependent relaxation is maintained at earlier stages of diabetes. Therefore, we sought to verify whether short-term diabetes results in the upregulation of compensatory mechanism(s) to preserve endothelium-dependent relaxation in response to hyperglycemia in the rat carotid artery.

METHODS

Animals. Male 8- to 10-wk-old Sprague-Dawley rats (University of Melbourne Animal Facility, Melbourne, Victoria, Australia) were randomly divided into two groups: normal and diabetic. Diabetes was induced by a single injection of STZ (48 mg/kg) into the rat tail vein after the rat had been fasted overnight. Control groups received an equivalent volume of the vehicle (0.1 M citrate buffer) alone. Six weeks after STZ or vehicle treatment, rats were euthanized with pentobarbitone sodium (325 mg/kg ip, Virbac, Melbourne, Victoria, Australia). Blood samples were obtained from the left ventricle by cardiac puncture, and glucose concentrations were measured using a one-touch glucometer (Roche, Sydney, New South Wales, Australia). The induction of diabetes was considered successful when the glucose level was >25 mM. All procedures involved were approved by the Animal Experimentation Ethics Committees of Melbourne and RMIT Universities and conformed with the Australian National Health and Medical Research Council guidelines.
Medical Research Council code of practice for the care and use of animals for scientific purposes.

**Myography experiments.** Carotid arteries were removed and placed in Krebs bicarbonate solution (118 mM NaCl, 4.7 mM KCl, 1.18 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 11.1 mM d-glucose, and 2.5 mM CaCl₂, pH 7.4) bubbled with 5% CO₂ in O₂ at 37°C. Arteries were cleared of fat and connective tissue and cut into 2- to 3-mm-long segments. Carotid artery rings were mounted in a myograph (model 610M, Danish Myo Technology, Aarhus, Denmark) containing Krebs solution. Passive tension was adjusted to 15 mN over 60 min and recorded on a chart recorder (model 3721, Yokogawa, Tokyo, Japan). Once resting tension had stabilized, the Krebs solution was replaced with an isotonic high K⁺ solution, in which K⁺ was replaced Na⁺ in the solution, for 20 min to induce maximum contraction. To assess the integrity of the endothelium, carotid artery rings were precontracted to 50% of the maximum response with phenylephrine (0.01–1 μM), and a single dose of ACh (10 μM) was used to relax the artery rings. ACh-induced relaxation was >80% of the precontracted tone in all cases, indicating that the endothelium was functionally intact. After further washouts and a return to basal tension, arteries were again precontracted to 50% of the maximum response using phenylephrine (0.01–1 μM) and/or the thromboxane receptor agonist 9,11-dideoxy-9α,11α-epoxymethanoprostaglandin F₂α (U-46619; 1–10 μM). The effect of the treatment on relaxant responses was examined by cumulative concentration-response curves to ACh (0.1 nM–10 μM) and sodium nitroprusside (SNP; 0.01 nM–10 μM). In addition, responses to ACh and SNP were examined after 20 min of incubation with different combinations of N-nitro-L-arginine (l-NNA; 100 μM or 1 mM), a nonselective NO synthase (NOS) inhibitor; indomethacin (10 μM), a nonselective cyclooxygenase inhibitor; 1H-[1,2,4]imidazolo[4,3-a]quinazolin-1-one (ODQ; 10 μM), an inhibitor of soluble guanylate cyclase (sGC); 3-(p-aminohippuril)-5-[(p-toluenesulfonyl)amino]-1H-pyrazole (ODQ, 100 μM), a NO scavenger; 1-tetramethylammonium-1-oxyl-3-oxide (carboxy-PTIO; 200 μM), a NO scavenger; 1-cysteine (1 mM), a NO scavenger; 1-[2-chlorophenyl]-(diphenyl)methyl]-1H-pyrazole (TRAM-34; 1 μM), a selective blocker of the intermediate-conductance Ca²⁺-activated K⁺ (IKCa) channel; apamin (1 μM), a small-conductance Ca²⁺-activated K⁺ (SKCa) channel inhibitor; and cupric chloride (10 μM or 30 μM, 30 min), an agent that causes the degradation of S-nitrosothiol (38).

**Western blot analysis.** Western blot analysis was performed as previously described (40) with the following modifications. Carotid arteries were homogenized in 150 μl of ice-cold lysis buffer [100 mM NaCl, 10 mM Tris, 2 mM EDTA, 0.5% (wt/vol) sodium deoxycholate, and 1% (vol/vol) Triton X-100, pH 7.4] with protease and phosphatase inhibitors cocktails (Roche), and the total protein concentration of the samples was quantified using a Bradford assay. Equal amounts of protein homogenate were subjected to SDS-PAGE and Western blot analysis with mouse primary antibodies (all diluted 1:1000 unless otherwise stated, overnight, at 4°C) against endothelial NOS (eNOS), inducible NOS (iNOS), caveolin-1 (BD Transduction Laboratories, Lexington, KY), and catalytic subunit of NADPH oxidase (Nox2; Abcam, Cambridge, UK, 1:2,000). To normalize for the amount of protein on the gels, proteins were reprobed with a loading control antibody (actin). All proteins were detected by enhanced chemiluminescence (Amersham, GE Healthcare, Sydney, New South Wales, Australia) after an incubation with anti-mouse secondary antibody (Millipore) for 1 h at room temperature (1:2,000). All protein bands were quantified by densitometry (Bio-Rad chemidoc; Sydney, New South Wales, Australia) and expressed as a ratio of the loading control. To investigate eNOS homodimer formation in the tissue, a nonboiled sample was resolved by 6% SDS-PAGE at 4°C (22), and membranes were probed and visualized as described above.

**Superoxide measurements.** Superoxide levels in the carotid arteries were measured by L-012-induced chemiluminescence assay as previously described (20) with the following modifications. The carotid artery was isolated, cleared of fat and connective tissue, and cut into 2- to 3-mm-long segments in Krebs-HEPES buffer [composed of (in mM) 99.90 NaCl, 4.7 KCl, 1.0 KH₂PO₄, 1.2 MgSO₄·7H₂O, 11.0 d-glucose, 25.0 NaHCO₃, 2.5 CaCl₂·2H₂O, and 20.0 Na-HEPES, pH 7.4]. Carotid arteries were incubated at 37°C for 60 min in Krebs-HEPES buffer either alone, in the presence of l-NNA (100 μM, a NOS inhibitor), or in the presence of apocynin (300 μM, a NAPDH oxidase inhibitor). Krebs-HEPES buffer (300 μl), containing L-012 (100 μM, Wako Pure Chemicals, Osaka, Japan) and the appropriate treatments were placed into a 96-well optiplate, which was loaded into a polarstar Optima plate reader (BMG Labtech, Melbourne, Victoria, Australia) to measure background photon emission at 37°C. After background counting had been completed, a single ring segment of the carotid artery was added to each well, and photon emission was recounted. Superoxide counts of the carotid artery were subtracted from background counts and normalized with dry tissue weight.

**NO measurements.** Intracellular levels of NO in the carotid artery were measured by 4-aminso-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate; Invitrogen, Sydney, New South Wales, Australia) after an incubation with anti-mouse secondary antibody (1:2,000). Strips of carotid arteries were loaded with DAF-FM diacetate (5 μM) for 60 min in Krebs buffer in either the absence or presence of l-NNA (100 μM) followed by exposure to ACh (10 μM) for 30 min. Background fluorescence was measured using an Optima plate reader (BMG Labtech) after excitation at 485 nm and emission at 520 nm. After being rinsed three times with Krebs buffer to remove excess probe, a single segment of the carotid artery was added to each well, and fluorescence intensity was recounted. NO counts of the carotid artery were subtracted from background fluorescence and normalized with dry tissue weight.

**Drugs used.** All drugs were purchased from Sigma-Aldrich (St. Louis, MO) except for ACh perchlorate (BDH Chemicals, Dorset, UK), ODQ, carboxy-PTIO, and U-46619 (Cayman Chemical, Ann Arbor, MI). All drugs were all dissolved in distilled water except for Hb (dissolved in Krebs solution), indomethacin (dissolved in 0.1 M sodium carbonate), l-NNA, (dissolved in 0.1 M sodium bicarbonate), ODQ, carboxy-PTIO, DAF-FM diacetate, apocynin, and TRAM-34 (dissolved in DMSO), and U-46619 (dissolved in absolute ethanol as a 1 mM stock solution; subsequent dilutions were in distilled water).

**Statistical analyses.** All results are expressed as means ± SE; n represents the number of animals per group. Concentration-response curves from rat isolated carotid arteries were computer fitted to a sigmoidal curve using nonlinear regression (Prism version 4.0, GraphPad Software, San Diego, CA) to calculate the sensitivity of each agonist (pEC₅₀). Maximum relaxation (Rmax) to ACh or SNP was measured as a percentage of precontraction to phenylephrine/U-46619. Group pEC₅₀ and Rmax values were compared via one-way ANOVA with a post hoc Dunnett’s test or Student’s unpaired t-test as appropriate. Results from Western blot analysis, DAF-FM, and L-012 were compared by either a Student’s unpaired t-test or one-way ANOVA with a post hoc Dunnett’s test as appropriate. P values of <0.05 were considered statistically significant.

**RESULTS**

**Body weights and blood glucose levels.** Body weights and blood glucose levels of the rats are shown in Table 1. Six weeks after treatment with STZ or vehicle, both groups of rats gained weight; however, the weight gained in normal rats was significantly greater than in diabetic rats (Table 1). Blood glucose levels of diabetic rats were significantly greater than those of normal rats (Table 1).

**Effect of diabetes on superoxide generation.** Superoxide generation in carotid arteries from normal and diabetic rats measured using L-012 chemiluminescence is shown in Fig. 1. Superoxide levels in carotid arteries from diabetic rats were significantly higher than those in normal rats. To examine whether uncoupled eNOS may contribute to superoxide pro-
conducted in the presence of indomethacin. The presence of contribution from a non-NO, nonprostanoid factor were all diabetic rats, further experiments to determine the potential did not affect endothelium-dependent relaxation in normal or diabetic rats.

Having established that cyclooxygenase inhibition the NOS inhibitor L-NNA was used. L-NNA significantly decreased superoxide release from diabetic but not normal arteries. Apocynin, a NADPH oxidase inhibitor, attenuated the generation of superoxide to comparable levels in normal and diabetic rats (Fig. 1).

Effect of diabetes on the relaxation to ACh and SNP. Relaxant responses to ACh and SNP in carotid arteries from rats treated with STZ or vehicle are shown in Fig. 2. The sensitivity and maximum relaxation to ACh and SNP were not significantly different between normal and diabetic rats (Table 2).

Effect of diabetes on the mechanism of relaxation to ACh and SNP. The relaxation to ACh in carotid arteries from normal and diabetic rats was not affected by indomethacin treatment (Fig. 2 and Table 2), suggesting that a cyclooxygenase product was not involved in endothelium-dependent relaxation in carotid arteries. L-NNA (100 μM) or ODQ abolished responses to ACh in normal arteries (Fig. 2A and Table 2) but not in diabetic arteries (Fig. 2B and Table 2). A 10-fold higher concentration of L-NNA did not cause a significantly greater reduction in the maximum relaxation to ACh [L-NNA (100 μM): Rmax = 34 ± 6% vs. L-NNA (1 mM): Rmax = 33 ± 4%, n = 7, P > 0.05].

The presence of indomethacin did not affect SNP-induced maximum relaxation in carotid arteries from normal or diabetic rats. NOS inhibition tended to increase the sensitivity to SNP in arteries from normal rats, although the change in pEC50 was not statistically significant, and in diabetic rats L-NNA treatment significantly shifted the SNP concentration-response curve to the left (Fig. 2D and Table 2). ODQ abolished the response to SNP in normal arteries (Fig. 2C and Table 2), whereas SNP-induced relaxation (Fig. 2D and Table 2) was attenuated but not abolished by ODQ in diabetic arteries.

Role of IKCa and SKCa in ACh- and SNP-induced relaxation in diabetes. Having established that cyclooxygenase inhibition did not affect endothelium-dependent relaxation in normal or diabetic rats, further experiments to determine the potential contribution from a non-NO, nonprostanoid factor were all conducted in the presence of indomethacin. The presence of TRAM-34 + apamin, blockers of IKCa and SKCa channels, respectively, significantly reduced the maximum relaxation to ACh in diabetic arteries (Rmax: 76 ± 4%) compared with normal arteries (Rmax: 94 ± 2%; Fig. 3 and Table 3). In arteries from diabetic rats, the presence of TRAM-34 + apamin abolished the residual ACh-induced relaxation, which was apparent after L-NNA + ODQ treatment.

There was similar relaxation to SNP in the presence of L-NNA + ODQ or L-NNA + ODQ + TRAM-34 + apamin in normal and diabetic arteries. TRAM-34 + apamin alone had no effect on SNP-induced relaxation in either normal or diabetic rats (Table 3).

Characterization of L-NNA-resistant relaxation in diabetes. The relaxation to ACh was abolished by indomethacin + L-NNA (100 μM and 1 mM) in arteries from normal rats but not diabetic rats. To test whether the resistance to NO inhibition in diabetic arteries may be due to an alternative source of NO such as nitrosothiols, NO scavengers and copper were used. The addition of Hb, a nonselective NO scavenger, abolished the residual ACh-induced relaxation after treatment with indomethacin + L-NNA (P < 0.05 by Dunnett’s test; Fig. 4 and Table 4). Similarly, the residual ACh-induced relaxation was significantly attenuated by the addition of either carboxy-PTIO or L-cysteine, which specifically scavenge NO• and NO−, respectively (Fig. 4 and Table 4). The presence of cupric chloride significantly decreased the sensitivity to ACh in diabetic arteries (indomethacin + cupric chloride: pEC50 = 6.71 ± 0.08 vs. indomethacin: pEC50 = 7.05 ± 0.12, n = 5, P < 0.05; Fig. 5). The addition of cupric chloride to indomethacin + L-NNA significantly reduced the residual ACh-induced relaxation (indomethacin + L-NNA + cupric chloride: Rmax = 9 ± 3% vs. indomethacin + L-NNA: Rmax = 34 ± 6%, n = 7, P < 0.05; Fig. 5). The presence of Hb + indomethacin alone significantly attenuated the ACh-induced relaxation in both normal and diabetic rats (Table 4).

Rmax to SNP in diabetic and normal arteries was not affected by L-NNA, Hb, or the combination of the two inhibitors (Table 4). The sensitivity to SNP was significantly increased by indomethacin + L-NNA and decreased by indomethacin + Hb or indomethacin + L-NNA + Hb in both normal and diabetic rats (Table 4).

Effect of diabetes on NO production detected by DAF-FM fluorescence. In normal arteries, stimulation with ACh caused a significant increase in NO, which was abolished by L-NNA.

Table 1. Body weights and blood glucose levels before and 6 wk after STZ or vehicle treatment of male Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight, g</th>
<th>Blood glucose level, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>69</td>
<td>236 ± 6</td>
</tr>
<tr>
<td>6 wk After Treatment</td>
<td>36</td>
<td>418 ± 14*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats/group. Shown is a comparison of rat body weights and blood glucose levels before and 6 wk after streptozotocin (STZ; 48 mg/kg) or vehicle treatment. ND, not determined. *Significantly different from rats before treatment (P < 0.05 by Dunnett’s test); †significantly different from normal rats 6 wk after STZ injection (P < 0.05 by Student’s unpaired t-test).

Fig. 1. Superoxide levels in the carotid artery from normal rats (open bars) and diabetic rats (solid bars) in the absence or presence of either N-nitro-L-arginine (L-NNA; 100 μM), a nitric oxide (NO) synthase (NOS) inhibitor, or apocynin (Apo; 300 μM), a NADPH oxidase inhibitor. Diabetic rats had significantly higher levels of superoxide production compared with normal rats. In the presence of L-NNA, superoxide levels were significantly decreased in diabetic arteries. NADPH oxidase appeared to be the major source of superoxide in both groups of rats. Results are shown as means ± SE; n = 7–13 experiments. *Significantly different from normal rats (P < 0.05 by Student’s unpaired t-test); #significantly different from control within the respective group (P < 0.05 by Student’s unpaired t-test).
Diabetes had no effect on the basal release of NO, but stimulation with ACh caused a significantly smaller increase compared with normal arteries. In the presence of L-NNA, the response to ACh was abolished in normal arteries but only attenuated in diabetic arteries, suggesting a non-NOS source of NO (Fig. 6).

**Effect of diabetes on the expression of iNOS, eNOS, Nox2, and caveolin-1 in the carotid artery.** In diabetes, the expression of eNOS and Nox2, which is the catalytic subunit of NADPH oxidase, were significantly increased, whereas caveolin-1 (11), which is a modulator of eNOS activity, was significantly decreased in diabetic rat carotid arteries compared with normal rats (Fig. 7, A, C, and D). Diabetes also significantly reduced the proportion of eNOS expressed as a dimer, suggesting that eNOS was uncoupled (Fig. 7B). Furthermore, there was no detectable expression of iNOS in rat carotid arteries from either group, indicating that this did not replace eNOS as a source of vascular NO production (data not shown).

**DISCUSSION**

This study demonstrated that, while 6 wk of diabetes caused a significant increase in vascular superoxide production, it did not affect the sensitivity or maximum response to ACh, but there were marked changes in the mechanism of endothelium-dependent relaxation. Diabetes caused a resistance of ACh-induced relaxation to NOS inhibition and also revealed some sensitivity of endothelium-dependent relaxation to inhibition by K^+ channel blockers in addition to the usual role for NO-induced stimulation of sGC. The NOS-independent component of the response to ACh could be attenuated by scavengers of NO• and NO•/H1002 and by the degradation of nitrosothiols with copper ions. Western blot analysis indicated that diabetes caused an increased expression of eNOS and decreased expression of caveolin-1 protein but that it also significantly reduced the proportion of eNOS expressed as a dimer, indicating that eNOS was uncoupled and could, in part, be producing superoxide instead of NO. In the early stages of STZ-induced diabetes, despite marked hyperglycemia, increased production of superoxide, and decreased NO synthesis detected by DAF-FM, endothelium-dependent relaxation was maintained possibly by a NOS-independent source of NO, such as nitrosothiols. In diabetes, the nitrosothiol-derived NO-mediated relaxation involves the opening of K^+ channels in addition to the activation of sGC.

In the present study, STZ-treated rats exhibited hyperglycemia and lower body weights than normal rats, demonstrating that diabetes was successfully induced. The observation that endothelium-dependent and -independent relaxation of the carotid arteries were not impaired 6 wk after STZ treatment is consistent with other studies (17, 21, 26, 32, 45) examining the
SNP-induced relaxation of carotid arteries from normal and absence (control) or presence of the same time, ACh (10^{-6} M) was maintained in the diabetic vessels, we demonstrated that endothelium-dependent relaxation induction even though the animals were hyperglycemic after STZ treatment. Although endothelium-dependent relaxation dysfunction was only observed at least 7 wk after diabetes time course of the vascular response to diabetes, as endothelial eNOS uncoupling and decreased NO synthesis in diabetic studies have shown that diabetes could also cause eNOS increased expression of Nox2 in carotid arteries, which could be due to vascular infiltration of macrophages (30). Previous • VOL 299 • AUGUST 2010 • www.ajpheart.org time course of the vascular response to diabetes, as endothelial dysfunction was only observed at least 7 wk after diabetes induction even though the animals were hyperglycemic after STZ treatment. Although endothelium-dependent relaxation was maintained in the diabetic vessels, we demonstrated that superoxide production detected by L-012 was increased and, at the same time, ACh (10 \mu M)-stimulated NO release detected by DAF-FM was decreased in diabetes. Nox2-dependent NADPH oxidase (5, 15) and uncoupled eNOS (18) have been shown to be among the major sources of vascular superoxide in disease, including in diabetes, and in this study the increased superoxide production in the diabetic endothelium and vascular smooth muscle cells (VSMCs) (6) was associated with the increased expression of Nox2 in carotid arteries, which could be due to vascular infiltration of macrophages (30). Previous studies have shown that diabetes could also cause eNOS uncoupling via multiple mechanisms, such as increased endothelial arginase activity (36) or depletion of the eNOS cofactor tetrahydrobiopterin by decreasing the expression of guanosine triphosphate cyclohydrolase I and/or dihydrofolate reductase (6, 35, 44). We observed a decreased in caveolin-1 expression and an increase in eNOS expression, but this was accompanied by a decreased proportion of eNOS as a dimer, resulting in eNOS uncoupling and decreased NO synthesis in diabetic carotid arteries, consistent with reported observations at a later stage of diabetes (45). Indeed, Zou et al. (47, 48) have demonstrated that the uncoupling of eNOS could be observed after peroxynitrite treatment for 5 min, suggesting a rapid effect of oxidant stress on eNOS dimerization (47, 48).

Despite eNOS uncoupling in diabetes, the amount of NO being produced was adequate to induce full relaxation, as observed in our functional experiments, where near-maximum relaxation was obtained with 10^{-6} M ACh in both normal and diabetic arteries. This could possibly be due to an increased sensitivity of sGC and/or the contribution of non-NOS-derived NO to endothelium-dependent relaxation in diabetes. Indeed, we observed that in normal rats, SNP-induced relaxation was abolished in the presence of ODQ, whereas in diabetic rats, there was significant relaxation, perhaps due to enhanced sGC activity. In addition, we observed that NO production detected by DAF-FM was resistant to NOS inhibition in diabetes, suggesting a role for a non-NOS-derived source of NO.

Although endothelium-dependent relaxation was not impaired in the diabetic arteries, we demonstrated that the mechanism of endothelium-dependent relaxation was altered. In normal carotid arteries, endothelium-dependent relaxation was solely mediated by NO acting on sGC, as ACh-induced relaxation of arteries from normal rats was abolished by L-NNA and/or ODQ but was unaffected by indomethacin or K_{Ca} channel blockers. In contrast, in diabetes, ACh-induced relaxation of the carotid arteries was in part resistant to the inhibition of NOS and/or sGC. The resistance of ACh responses to

<table>
<thead>
<tr>
<th></th>
<th>Normal Rats</th>
<th>Diabetic Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC_{50}</td>
<td>R_{max} (%)</td>
</tr>
<tr>
<td>ACh-induced relaxation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.26 ± 0.23</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>L-NNA</td>
<td>ND</td>
<td>4 ± 4*</td>
</tr>
<tr>
<td>ODQ</td>
<td>ND</td>
<td>4 ± 4*</td>
</tr>
<tr>
<td>Indo</td>
<td>7.12 ± 0.12</td>
<td>93 ± 2</td>
</tr>
<tr>
<td>SNP-induced relaxation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.23 ± 0.19</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>L-NNA</td>
<td>8.86 ± 0.24</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>ODQ</td>
<td>ND</td>
<td>4 ± 4*</td>
</tr>
<tr>
<td>Indo</td>
<td>8.37 ± 0.14</td>
<td>100 ± 1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 experiments in normal rats and 7 experiments in diabetic rats. Shown are comparisons of the sensitivity (pEC_{50}) and maximum relaxation (R_{max}) to ACh and sodium nitroprusside (SNP) in the absence (control) or presence of N-nitro-L-arginine (L-NNA; 100 \mu M), 1H-[1,2,4]oxadiazolo-[4,3-a]quinazolin-1-one (ODQ; 10 \mu M), or indomethacin (Indo; 10 \mu M) in endothelium-intact carotid arteries from normal and diabetic rats. *Significantly different from the corresponding control ACh/SNP-induced response in carotid arteries within the normal/diabetic group (P < 0.05 by Dunnett’s test); †significantly different from the normal group in carotid arteries within the respective inhibitor group (P < 0.05 by Student’s unpaired test).

Fig. 3. Cumulative concentration-response curves to ACh in the presence of Indo. Indo + l-NNA + ODQ. Indo + l-NNA + ODQ + TRAM-34 + apamin (Apa), or Indo + TRAM-34 + Apa in endothelium-intact carotid arteries isolated from normal rats (A) or diabetic rats (B). The relaxation response to ACh in the presence of Indo + l-NNA + ODQ was abolished in arteries from normal rats but not in diabetic rats, where significant relaxation was observed. Indo + TRAM-34 + Apa abolished ACh-induced relaxation in both groups. Results are shown as means ± SE; n = 5–10 experiments. pEC_{50} and R_{max} values determined from the data presented in these graphs are shown in Table 3.
NOS inhibition did not appear to be due to an inadequate inhibition of NOS, as there was no greater inhibitory effect with a 10-fold higher concentration of L-NNA. This suggested a possible upregulation of alternative mechanism(s) of endothelium-dependent relaxation. To investigate the possible role of prostanoids in endothelium-dependent relaxation, we used indomethacin, a nonselective cyclooxygenase inhibitor, but there was no evidence that a prostanoid made any contribution to endothelium-dependent relaxation in the carotid arteries. Alternative explanations for the resistance to NOS and sGC inhibition could be that diabetes stimulates the involvement of a non-NO, nonprostanoid factor (EDHF) to endothelium-dependent relaxation or that NO may act through a sGC-independent pathway, causing the opening of KCa channels. Therefore, to examine this possibility, we increased the NO inhibition by the addition of NO scavengers (Hb, carboxy-PTIO, and L-cysteine) and demonstrated that ACh-induced relaxation was abolished with the addition of NO scavengers in the diabetic rat. Hence, the ability of NO scavengers to abolish endothelium-dependent relaxation in the presence of indomethacin + L-NNA from diabetic arteries perhaps argues against a role for EDHF. Rather, it is possible that there is a non-NOS source of NO in diabetes, consistent with our observation that ACh-induced DAF-FM fluorescence in the presence of L-NNA was higher in diabetic arteries compared with normal arteries. Taken together, our data suggest that in diabetes, there is an additional action of NO acting through a sGC-independent pathway, causing the opening of IKC\textsubscript{a} and SKCa channels to induce relaxation.

We postulated that to maintain vascular relaxation in the carotid artery during the early stages of diabetes, NO derived from a NOS-independent source such as nitrosothiols may be involved. To address the role of nitrosothiols in modulating endothelium-dependent relaxation in diabetes, we used a nitrosothiol-degrading agent, cupric chloride (13, 14, 38), in the presence of L-NNA from diabetic arteries. Inhibition of endothelial KCa channels caused a reduction in endothelium-dependent relaxation in the presence of indomethacin + L-NNA + Cu\textsuperscript{2+} from diabetic arteries. Taken together, our data suggest that in diabetes, there is an additional action of NO acting through a sGC-independent pathway, causing the opening of IKC\textsubscript{a} and SKCa channels to induce relaxation.

Table 3. Effect of Indo, L-NNA, ODQ, TRAM-34, and apamin on ACh- and SNP-induced relaxation of carotid arteries from normal and diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Normal Rats</th>
<th></th>
<th>Diabetic Rats</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC\textsubscript{50}</td>
<td>R\textsubscript{max}, %</td>
<td>pEC\textsubscript{50}</td>
<td>R\textsubscript{max}, %</td>
</tr>
<tr>
<td>ACh-induced relaxation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indo</td>
<td>6.91 ± 0.15</td>
<td>95 ± 2</td>
<td>7.00 ± 0.12</td>
<td>94 ± 2</td>
</tr>
<tr>
<td>Indo + L-NNA + ODQ</td>
<td>ND</td>
<td>4 ± 4*</td>
<td>ND</td>
<td>25 ± 5*</td>
</tr>
<tr>
<td>Indo + TRAM-34 + apamin</td>
<td>6.70 ± 0.17</td>
<td>94 ± 2</td>
<td>6.51 ± 0.10</td>
<td>76 ± 4*†</td>
</tr>
<tr>
<td>Indo + L-NNA + ODQ + TRAM-34 + apamin</td>
<td>ND</td>
<td>3 ± 3*</td>
<td>ND</td>
<td>3 ± 3*</td>
</tr>
<tr>
<td>SNP-induced relaxation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indo</td>
<td>8.41 ± 0.20</td>
<td>101 ± 1</td>
<td>8.49 ± 0.17</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>Indo + L-NNA + ODQ</td>
<td>ND</td>
<td>44 ± 4*</td>
<td>ND</td>
<td>27 ± 5*</td>
</tr>
<tr>
<td>Indo + TRAM-34 + apamin</td>
<td>8.24 ± 0.18</td>
<td>100 ± 0</td>
<td>8.18 ± 0.10</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>Indo + L-NNA + ODQ + TRAM-34 + apamin</td>
<td>ND</td>
<td>40 ± 6*</td>
<td>ND</td>
<td>31 ± 5*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7 experiments in normal rats and 5 experiments in diabetic rats except for Indo alone in diabetic rats, where n = 10 experiments. Shown are comparisons of the sensitivity (pEC\textsubscript{50}) and R\textsubscript{max} to ACh and SNP in the presence of Indo (10 μM) alone or in the additional presence of L-NNA (100 μM) + ODQ (10 μM), TRAM-34 (1 μM) + apamin (1 μM), or L-NNA (100 μM) + ODQ (10 μM) + TRAM-34 (1 μM) + apamin (1 μM). *Significantly different from the corresponding control ACh/SNP-induced response in carotid arteries within the normal/diabetic group (P < 0.05 by Dunnett’s test); †significantly different from the normal group in carotid arteries within the respective inhibitor group (P < 0.05 by Student’s unpaired test).

To examine whether EDHF contributed to the endothelium-dependent relaxation of carotid arteries in diabetes, the combination of TRAM-34 and apamin was used to block IKC\textsubscript{a} and SKCa channels, respectively. Inhibition of endothelial KCa channels caused a reduction in endothelium-dependent relaxation in carotid arteries from diabetic, but not normal, rats, suggesting the involvement of the opening of endothelial KCa channels in relaxation during diabetes. While this supports the possible release of an EDHF, the observation could also be explained by NO causing relaxation through the activation of KCa channels. Therefore, to examine this possibility, we increased the NO inhibition by the addition of NO scavengers (Hb, carboxy-PTIO, and L-cysteine) and demonstrated that ACh-induced relaxation was abolished with the addition of NO scavengers in the diabetic rat. Hence, the ability of NO scavengers to abolish endothelium-dependent relaxation in the presence of indomethacin + L-NNA from diabetic arteries perhaps argues against a role for EDHF. Rather, it is possible that there is a non-NOS source of NO in diabetes, consistent with our observation that ACh-induced DAF-FM fluorescence in the presence of L-NNA was higher in diabetic arteries compared with normal arteries. Taken together, our data suggest that in diabetes, there is an additional action of NO acting through a sGC-independent pathway, causing the opening of IKC\textsubscript{a} and SKCa channels to induce relaxation.
relaxation in the presence of l-NAME (+ indomethacin) in the diabetic rat, further supporting the possibility that NO derived from nitrosothiols was responsible for maintaining vascular function. Indeed, nitrosothiols have been shown to release both NO* and NO− in response to several agonists, including Ach (2, 12, 28, 31), and to cause vascular relaxation through endothelial IKCa and SKCa channels (3). Our results suggest that in diabetes, as has previously been shown in hypertensive (24), where NO production/activity is impaired, nitrosothiol-derived NO may serve as a compensatory source of the vascular NO supply.

Previous studies (4, 29, 33) have suggested that NO may activate large-conductance KCa (BKCa) channels, which are found on VSMCs to cause relaxation, rather than opening endothelial IKCa and SKCa channels (3). Our results suggest that in diabetes, as has previously been shown in hypertensive (24), where NO production/activity is impaired, nitrosothiol-derived NO may serve as a compensatory source of the vascular NO supply.

Table 4. Effect of Indo, l-NAME, Hb, carboxy-PTIO, and l-cysteine on Ach- and SNP-induced relaxation of carotid arteries from normal and diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Normal Rats</th>
<th>Diabetic Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>pEC50</td>
</tr>
<tr>
<td>Ach-induced relaxation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indo</td>
<td>6</td>
<td>7.03 ± 0.26</td>
</tr>
<tr>
<td>Indo + l-NAME</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>Indo + Hb</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>Indo + l-NAME + Hb</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>Indo + l-NAME + carboxy-PTIO</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>Indo + l-NAME + l-cysteine</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>SNP-induced relaxation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indo</td>
<td>6</td>
<td>8.35 ± 0.11</td>
</tr>
<tr>
<td>Indo + l-NAME</td>
<td>6</td>
<td>8.84 ± 0.07*</td>
</tr>
<tr>
<td>Indo + Hb</td>
<td>6</td>
<td>7.46 ± 0.08*</td>
</tr>
<tr>
<td>Indo + l-NAME + Hb</td>
<td>6</td>
<td>7.86 ± 0.14*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of experiments. Shown are comparisons of the sensitivity (pEC50) and Rmax to Ach and SNP in the presence of Indo (10 μM) alone or in the additional presence of l-NAME (100 μM), hemoglobin (Hb; 20 μM), l-NAME (100 μM) + Hb (20 μM), l-NAME (100 μM) + carboxy-2-phenyl-4,5,5-tetramethylimidazoline-1-oxide (carboxy-PTIO; 200 μM), or l-NAME (100 μM) + l-cysteine (1 mM). *Significantly different from the corresponding control Ach/SNP-induced response in carotid arteries within the normal/diabetic group (P < 0.05 by Dunnett’s test); †significantly different from the normal group with Indo + l-NAME treatment (P < 0.05 by Student’s unpaired test).

Fig. 5. Cumulative concentration-response curves to Ach in the presence of Indo, cupric chloride (10 μM), Indo + l-NAME (100 μM), or Indo + l-NAME + cupric chloride in endothelium-intact carotid arteries isolated from diabetic rats. *pEC50 significantly different compared with Indo treatment (P < 0.05 by Student’s unpaired t-test); #Rmax significantly different compared with Indo + l-NAME. Results are shown as means ± SE; n = 5–7 experiments. See the text for pEC50 and Rmax values.

Fig. 6. NO levels in carotid arteries from normal rats (open bars) and diabetic rats (solid bars) in the absence or presence of either Ach (10 μM) or Ach (10 μM) + l-NAME (100 μM), a NOS inhibitor. Diabetic rats had significantly lower levels of NO production compared with normal rats. In the presence of l-NAME, NO levels were significantly increased in diabetic arteries. DAF-FM, 4-amino-5-methylamino-2′,7′-dihydrofluorescein. Results are shown as means ± SE; n = 7 experiments. *Significantly different from normal rats (P < 0.05 by Student’s unpaired t-test); †significantly different from Ach within the respective group (P < 0.05 by Dunnett’s test).
endothelium-dependent relaxation. Our study, at 6 wk of diabetes, also noted the increase in superoxide and decrease in NO production and eNOS expression/uncoupling, but endothelium-dependent relaxation was preserved. Taken together, these results suggest that hyperglycemia acutely increases oxidative stress but that nitrosothiols may serve as a compensatory vascular NO supply, which can maintain endothelium-dependent relaxation. We postulate that with prolonged diabetes the compensatory mechanisms may fail and endothelium-dependent relaxation is impaired. This is a likely explanation for the differences between our observations and those of Shi et al. (31), who also studied the effects of STZ-induced diabetes on the mechanism of endothelium-dependent relaxation in rat carotid arteries. Diabetes also significantly reduced the proportion of eNOS expressed as the dimer. Representative blots are shown for each corresponding graph. Results are shown as means ± SE; n = 6–7 experiments. *Significantly different from normal rats (P < 0.05 by Student’s unpaired t-test).

In conclusion, after 6 wk of STZ-induced diabetes, NO, not from NOS activity but perhaps from nitrosothiol stores, may be acting in part through a sGC-independent mechanism, particularly by the opening of IKCa and SKCa channels, to preserve endothelial function in the presence of diabetes-induced vascular oxidant stress. Therefore, these results suggest that the additional role of nitrosothiol at this early stage of diabetes in the rat carotid artery is an adaptive mechanism to counteract the inactivation of NO by superoxide to maintain vascular relaxation in response to hyperglycemia.

ACKNOWLEDGMENTS

The authors thank Priya Sivakumaran and Indrajeetsinh Rana for technical assistance.

GRANTS

C. H. Leo was a recipient of an RMIT University International Research Scholarship, and A. Joshi was a recipient of a Melbourne University International Fee Remission Scholarship.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).
REFERENCES


