

Reduced Vasorelaxant Effect of Carbon Monoxide in Diabetes and the Underlying Mechanisms

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Carbon monoxide (CO) is an endogenous gaseous factor that relaxes vascular tissues by acting on both the cGMP pathway and calcium-activated K⁺ (K_{Ca}) channels. Whether the vascular effect of CO is altered in diabetes had been unknown. It was found that the CO-induced relaxation of tail artery tissues from streptozotocin-induced diabetic rats was significantly decreased as compared with that of nondiabetic control rats. The blockade of the cGMP pathway with ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) completely abolished the CO-induced relaxation of diabetic tissues but only partially inhibited the CO effect in normal tissues. Single-channel conductance of K_{Ca} channels in diabetic smooth muscle cells (SMCs) was not different from that of normal SMCs. However, the sensitivity of K_{Ca} channels to CO in diabetic SMCs was significantly reduced. CO (10 μmol/l) induced an 81 ± 24% increase in the mean open probability of single K_{Ca} channels in normal SMCs but had no effect in diabetic SMCs. Long-term culture of normal vascular SMCs with 25 mmol/l glucose or 25 mmol/l 3-OMG (3-O-methylglucose) but not 25 mmol/l mannitol significantly reduced the sensitivity of K_{Ca} channels to CO. On the other hand, the sensitivity of K_{Ca} channels to CO was regained in diabetic SMCs that were cultured with 5 mmol/l glucose for a prolonged period. The decreased vasorelaxant effect of CO in diabetes represents a novel mechanism for the vascular complications of diabetes, which could be closely related to the glycation of K_{Ca} channels in diabetic vascular SMCs. *Diabetes* 50:166–174, 2001

The vasorelaxant effects of carbon monoxide (CO) have been demonstrated (1–4), and heme oxygenase (HO) that cleaves the heme ring to form biliverdin and CO has been located in many different types of vascular smooth muscles (5,6). The production of CO from vascular tissues has also been directly measured (7). These studies emphasize the importance of CO as an

endogenous vasorelaxant factor under physiological (8) or pathophysiological (9,10) conditions. The elevation of cellular cGMP levels and the opening of plasma membrane K⁺ channels are the main mechanisms that have been proposed to explain the vascular effects of CO. CO may increase cGMP content via its stimulatory interaction with the heme in the regulatory subunit of guanylyl cyclase. Increased cGMP would consequently decrease the intracellular Ca²⁺ concentration ([Ca²⁺]_i) in smooth muscle cells (SMCs) through the inhibition of inositol triphosphate formation, the activation of Ca²⁺-ATPase, and the inhibition of Ca²⁺ channels. The opening of K⁺ channels leads to membrane hyperpolarization, which in turn inhibits the agonist-induced increase in inositol triphosphate, reduces Ca²⁺ sensitivity and resting Ca²⁺ level, and relaxes SMCs (11). Our studies have demonstrated that CO directly enhanced the activity of the big-conductance calcium-activated K⁺ (K_{Ca}) channels in rat tail artery SMCs via a cGMP-independent mechanism (2,3). Whether the vascular effects of CO are mediated by cGMP or specific types of K⁺ channels or both depends on the vascular tissue types, the developmental stages of the tissue, and the animal species.

Vascular complications of diabetes are largely manifested as hypertension (12,13), peripheral vessel occlusion, atherosclerosis, retinopathy, and nephropathy (14). These vascular complications are responsible for most of the morbidity and mortality of patients with diabetes and have been reproduced in both insulinopenic (15) and insulin-resistant rats (16). Altered vascular sensitivity to different vasoconstricting substances, such as bradykinin (17), changed properties of ion channels (18), and abnormal calcium handling in diabetic vascular SMCs (19) are among many putative mechanisms for the vascular complications of diabetes. The involvement of CO in the etiology of diabetes has been implicated as CO upregulated, whereas nitric oxide downregulated insulin secretion from pancreatic islets (20). The altered metabolism of CO in cardiac tissues from streptozotocin-induced diabetic rats has also been shown recently (21). It was hypothesized that the vasorelaxing effects of CO may change in diabetes, constituting a novel mechanism for the diabetic vascular complications.

In the present study, the vasorelaxant effect of CO on tail artery tissues from streptozotocin-induced diabetic rats was examined and compared with that from normal control rats. The relative contributions of cGMP pathway and K_{Ca} channels to the putatively altered effect of CO on the contractility of diabetic vascular tissues were investigated. The direct effect of CO on single K_{Ca} channel activity was determined. The role of glycation of K_{Ca} channels in the altered effect of CO was further analyzed. Finally, the vascular responses to the endogenously generated CO in diabetic rat tail artery tissues were assayed. Our results provide evidence that corroborates the altered vascular effect of CO in diabetes and unravels the underlying cellular mechanisms.

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3-OMG, 3-O-methylglucose; BSA, bovine serum albumin; [Ca²⁺]_i, intracellular Ca²⁺ concentration; ChTX, charybdotoxin; CO, carbon monoxide; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HBSS, Hank's buffered saline solution; HO, heme oxygenase; K_{Ca}, calcium-activated K⁺; NPo, open probability with *N* representing the number of single channels in one patch; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PBS, phosphate-buffered saline; PHE, phenylephrine; SMC, smooth muscle cell; STZ, streptozotocin.

RESEARCH DESIGN AND METHODS

Animal model of diabetes. Adult male Sprague-Dawley rats weighing 150–180 g were maintained on standard rat diet and tap water ad libitum with 12-h light:dark cycles in a quiet environment. After the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg body wt), diabetes was induced by a single injection via the lateral tail vein or penis vein of streptozotocin (STZ) (60 mg/kg body wt) dissolved in sodium citrate buffer (pH 4.5) (19). Age-matched control rats were injected with an equal volume of vehicle (sodium citrate buffer). The STZ-injected rats were used in the present study 1 month after the induction of diabetes. Glycosuria was determined using Chemstrip (Boehringer Mannheim). Mean blood pressure of the anesthetized rats (sodium pentobarbital, 60 mg/kg i.p.) was determined through the right femoral artery by a pressure transducer connected to a Biopac system (Biopac System), and recorded by a computer. At the time of tail artery removal, 1.5-ml blood samples were collected from the rats in the fasted state. The measurement of blood levels of glucose and glycosylated hemoglobin was performed by Chemistry Laboratory at the Royal University Hospital, University of Saskatchewan.

The laboratory animal care guidelines (National Institutes of Health) were followed, and animal experimental protocols were approved by the University Committee on Animal Care and Supply of the University of Saskatchewan.

Measurement of isometric tension development of isolated rat tail artery tissues. The method for measurement of isometric tension development of isolated rat-tail artery tissues has been described previously (4,17,22). Briefly, tail arteries were isolated from rats. After cleaning connective tissues, the arteries were cut into helical strips (~1.5 cm in length). The vascular tissues were mounted in 10-ml organ baths filled with Krebs' bicarbonate solution (bubbled with 95% O₂ and 5% CO₂), which was composed of the following (in mmol/l): 115 NaCl, 5.4 KCl, 1.2 MgSO₄, 1.2 NaH₂PO₄, 25 NaHCO₃, 11 glucose, and 1.8 CaCl₂. The pH and osmolality of Krebs' solution were adjusted to 7.4 and 290 mOsm, respectively. The tail artery strip was mounted with one end immobilized and another end tied to a transducer. These strips were mechanically stretched to achieve a basal tension of ~0.7 g and were allowed to equilibrate for 1 h before the start of experiments. Because the vascular effect of CO was not dependent on the presence of an intact endothelium (4), endothelium was removed from vascular strips by a rubbing procedure. The absence of a functional endothelium was verified by the inability of acetylcholine (1 μmol/l) to induce relaxation of tail artery tissues as shown in our previous study (4,17). Concentration-response curves of the tissues to CO were obtained by accumulative addition of CO to the organ bath.

The isometric tension development was measured with FT 03 force displacement transducers (Grass Instruments). Data acquisition and analysis were accomplished using a Biopac system (Biopac System), including MP 100 WS acquisition units, TCI 100 amplifiers, Acknowledge software (3.01), universal modules, and a Macintosh computer.

Cell preparation. Single SMCs were dispersed enzymatically following our established procedure (23) with modifications. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg body weight). Tail arteries were isolated and connective tissue removed under a dissecting microscope. The arteries were cut open longitudinally and immersed in a Ca²⁺- and Mg²⁺-free Hanks' buffered saline solution (HBSS) (Gibco) at 4°C. The arterial strips were then processed in the following solutions at 37°C: 1) low-Ca²⁺ (0.2 mmol/l) HBSS containing collagenase/dispase (1.5 mg/ml, Boehringer Mannheim), elastase (0.5 mg/ml, type II, Sigma), trypsin inhibitor (1 mg/ml, Sigma) and bovine serum albumin (BSA) (2 mg/ml, Sigma) for 50 min; 2) Ca²⁺-free HBSS in which the tissue was rinsed twice; and 3) Ca²⁺-free HBSS with collagenase 1 mg/ml (type II, Sigma) and BSA 2 mg/ml for 20 min. Next, arterial pieces were transferred to a Ca²⁺-free HBSS at 4°C and triturated for 5 min. Calcium concentration of the incubating solution was gradually increased to 1.7 mmol/l. The dispersed cells were plated onto 35 mm Petri dishes in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing penicillin (100 U/ml, Sigma) and streptomycin (0.1 mg/ml, Sigma) and maintained at 4°C for at least 4 h. These freshly isolated cells were used in electrophysiological recording within 8–24 h of isolation.

In some experiments, the isolated cells were primarily cultured in DMEM containing 5% fetal calf serum (FCS) (Gibco) at 37°C. Glucose, 3-O-methylglucose, or mannitol was added to the culture medium at different concentrations, and the medium was changed every other day. The cells were used for the patch-clamp study 8–35 days after in culture.

Single-channel recording. The inside-out and outside-out configurations of the patch-clamp technique were used to record single K_{Ca} channel currents as described previously (2,3). Pipettes with a resistance of 6–8 MΩ were used and the seal resistance was usually >10 GΩ. Membrane patches with no more than 3 channels were used for experiments. Single-channel currents were filtered at 2 KHz (8-pole Bessel, -3 dB) and recorded with a 5 μs sampling interval in a gap-free mode. For each concentration of CO tested, at least 60 s of chan-

nel activity was recorded directly on a computer hard disk. The open probability (N_{po}), with *N* representing the number of single channels in one patch, and the unit amplitude of K_{Ca} channels were determined from all-point histograms with a Fetchan program (Axon Instruments). N_{po} of K_{Ca} channels was averaged over 2- to 5-min recordings to describe the changes in channel activity after different treatments. Membrane patches with unstable N_{po} over time were excluded from further analysis. A current level >50% of the unit channel current was considered to reflect a channel opening. The external surface of membrane patches was bathed in a solution containing the following (in mmol/l): 145 KCl, 10 HEPES, and 10 glucose. The internal surface of membrane patches was exposed to a solution containing the following (in mmol/l): 145 KCl, 10 HEPES, 1.2 MgCl₂, 10 glucose, 1 EGTA, and 0.5 μmol/l of free Ca²⁺, [Ca²⁺]_i. [Ca²⁺]_i of the recording solution was calculated using a computer program (EQCAL, Biosoft), which takes account of the individual impact of pH, EGTA, calcium, and magnesium on the final free-calcium concentration.

Osmolalities and pH of all solutions used for electrophysiological recordings were adjusted to 290 mOsm and 7.4, respectively. All electrophysiological experiments were carried out at room temperature.

Total cell protein preparation and Western immunoblot assay. Total cell proteins were prepared as described in our previous publication (24). Briefly, rat-tail arteries, aortae, mesenteric artery, and spleen were respectively homogenized with a polytron homogenizer in 0.5 ml of Tris-buffered saline (20 mmol/l Tris-HCl [pH 7.4], 0.25 mmol/l sucrose, and 1 mmol/l EDTA) containing protease inhibitor mixture (2 μl of 1 mol/l phenanthroline, 300 mmol/l iodoacetamide, 10 mmol/l phenylmethylsulfonyl fluoride, 1 mg/ml antipain, 1 mg/ml leupeptin, 1 mmol/l pepstatin A, and 1 mol/l benzamidine). The homogenate was centrifuged at 6000g for 15 min at 4°C to remove nuclei and undisturbed cells. Protein concentration was determined using a Bio-Rad protein assay solution with BSA as standard. For Western blot, total cell proteins were loaded and run on standard 7.5% SDS-polyacrylamide gel in Tris-glycine electrophoresis buffer (25 mmol/l Tris, 200 mmol/l glycine [pH 8.3], and 0.1% SDS). Proteins were transferred onto nitrocellulose membrane in 192 mmol/l glycine, 25 mmol/l Tris (pH 8.3), and 20% methanol at 100 V for 1.5 h in a water-cooled transfer apparatus. The membrane was blocked in a blocking buffer, phosphate-buffered saline (PBS) containing 3% nonfat milk at room temperature for 2 h. The membrane was then probed overnight at 4°C with monoclonal antibodies against HO-1 (1:300) and HO-2 (1:100) (Stressgen) in the blocking buffer. After washing five times in PBS, the membrane was subsequently incubated with goat anti-mouse IgG or goat anti-rabbit IgG (Bio-Rad) conjugated with horseradish peroxidase diluted 1:5000 in the blocking buffer for 2 h at room temperature. Bound antibodies were detected using chemiluminescent substrate kit (NEN Life Science Products).

Chemicals and data process. To prepare the CO solution, 20 ml of stock solution in a sealed glass tube was bubbled with a stream of CO (purity 99.9999%, Canadian Liquid Air) for 20 min under the pressure of 100 kPa at 37°C. Of this CO-saturated solution, 1 μl contains 30 ng of the gas (4). The stock solution of CO was prepared freshly before each experiment and then diluted immediately to the desired concentrations with bath solution. The estimated CO concentration was based on the solubility of CO at 37°C, the extent of dilution of the CO-saturated solution, and the assumption that the loss of the added CO from the bath solution at the time of experiments was negligible. Because the assumption was not strictly correct, the actual concentration of CO might be somewhat lower than the estimated concentration. During the experiments, the cells or vascular tissues were continuously superfused and a complete solution change in the recording chamber was accomplished within 30 s. The effects of CO on K_{Ca} channel currents were continuously recorded before and after the beginning of superfusing cells with a CO-containing bath solution. Usually, the stable effect of CO was observed within 1–3 min of CO application and correspondingly recorded.

Phenylephrine (PHE), acetylcholine, indomethacin, STZ, 3-OMG (3-O-methylglucose), and other chemicals were purchased from Sigma Chemical (St. Louis, MO). Charybdotoxin (ChTX), iberiotoxin, and apamin were from Alomone Labs (Jerusalem, Israel). ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) was from Tocris (Ballwin, MO).

The data were expressed as means ± SE. Concentration-response curves were analyzed using a computerized curve-fitting software (Microcal Origin, version 4.1, Microcal Software) to obtain EC₅₀. The comparison of EC₅₀ under different conditions was performed by analysis of variance followed by Student's *t* test in conjunction with the Newman-Keuls test when applicable. The significant difference between treatments was defined at a level of *P* < 0.05.

RESULTS

Severe diabetes developed in the STZ-treated diabetic rats. Compared with normal control rats, diabetic rats lost body weight, had glycosuria, and developed hyperglycemia with the

TABLE 1
Changes in the functional parameters in diabetic rats

	Controls	Diabetic rats	<i>P</i>
Blood pressure (mm Hg)	97.9 ± 7.9	103.3 ± 3.3	>0.05
<i>n</i>	6	6	
Glycated-Hb (%)	5.11 ± 0.2	11.07 ± 0.5	<0.01
<i>n</i>	7	7	
Osmolality (mOsm/l)	292 ± 4	341 ± 3	<0.01
<i>n</i>	10	10	
Blood glucose (mmol/l)	7.3 ± 0.9	32.3 ± 2.2	<0.01
<i>n</i>	7	7	
Serum [K ⁺] (mmol/l)	7.9 ± 0.7	6.4 ± 0.4	>0.05
<i>n</i>	7	7	
Serum [Na ⁺] (mmol/l)	137.8 ± 1.9	128.7 ± 1.3	<0.01
<i>n</i>	7	7	
Serum [Ca ²⁺] (mmol/l)	2.4 ± 0.04	2.1 ± 0.1	>0.05
<i>n</i>	7	7	
Body weight (gm)	357.6 ± 7.5	206 ± 15.9	<0.01
<i>n</i>	7	7	
Urine leucocyte	Positive	Positive	—
<i>n</i>	5/7	3/10	
Urine pH	6.7 ± 0.52	5.7 ± 1.8	>0.05
<i>n</i>	7	10	
Urine glucose (mmol/l)	<2.8	>55	—
<i>n</i>	7	10	
Urine ketones	Positive	Positive	—
<i>n</i>	0/7	4/10	
Urine erythrocyte	Positive	Positive	—
<i>n</i>	5/7	10/10	

fasting glucose concentration of plasma elevated to 32.2 ± 2.2 mmol/l (*n* = 10). The mean blood pressure and serum concentrations of K⁺ and Ca²⁺ were not different between diabetic rats and the controls. However, serum concentrations of Na⁺ and the appearance of blood cells and ketones in urine were higher in diabetic rats than in control rats (Table 1).

CO induced a concentration-dependent relaxation of the PHE-precontracted endothelium-free tail artery tissues (Fig. 1). This vasorelaxant effect of CO was significantly reduced in diabetic vascular tissues. EC₅₀ of the vasorelaxant effect of CO was 58 ± 24 μmol/l in normal tissues (*n* = 8) but 131 ± 38 μmol/l in diabetic tissues (*n* = 8, *P* < 0.05) (Fig. 1).

Whether the decreased CO effect on diabetic vascular tissues was due to the altered responsiveness of cGMP pathway or K_{Ca} channels in vascular SMCs was further examined by incubating vascular tissues with 10 μmol/l ODQ, a specific inhibitor for the soluble guanylyl cyclase. Without pretreating vascular tissues with ODQ, CO (300 μmol/l) induced a 60 ± 7% relaxation of normal vascular tissues (*n* = 8). In the presence of ODQ, CO only induced a 38 ± 8% relaxation of normal vascular tissues (*n* = 8). This represents a 63% inhibition of CO effect by ODQ (Fig. 2). Further prolonging the ODQ incubation time from 10 to 20 min (*n* = 4) or increasing the concentration of ODQ from 10 to 30 μmol/l (*n* = 4) did not induce additional inhibition of the CO effect (data not shown). In contrast, 10-min incubation of diabetic rat tail artery tissues with 10 μmol/l ODQ completely abolished the vasorelaxant effect of CO (Fig. 3).

Because CO relaxes normal rat tail arteries by stimulating the cGMP pathway, as well as high-conductance K_{Ca} channels (1–4), the altered functions of K_{Ca} channels in vascular SMCs would also affect the vascular effects of CO. Therefore, the

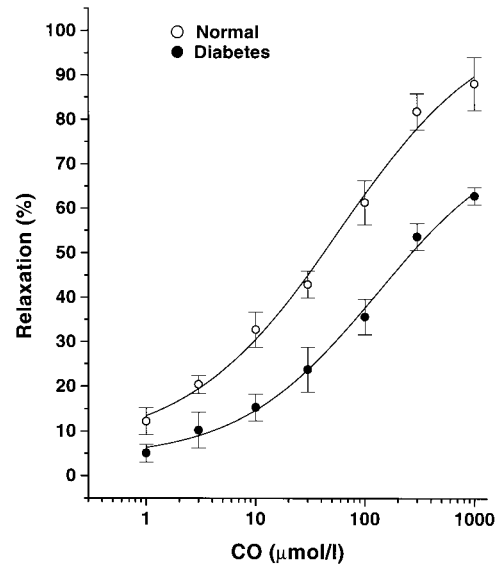


FIG. 1. The CO-induced concentration-dependent relaxation of normal and diabetic rat tail artery tissues. The vascular tissues were precontracted with phenylephrine (1 μmol/l). The CO-induced vasorelaxation was significantly reduced in diabetic vascular tissues. Each data point represents eight experiments.

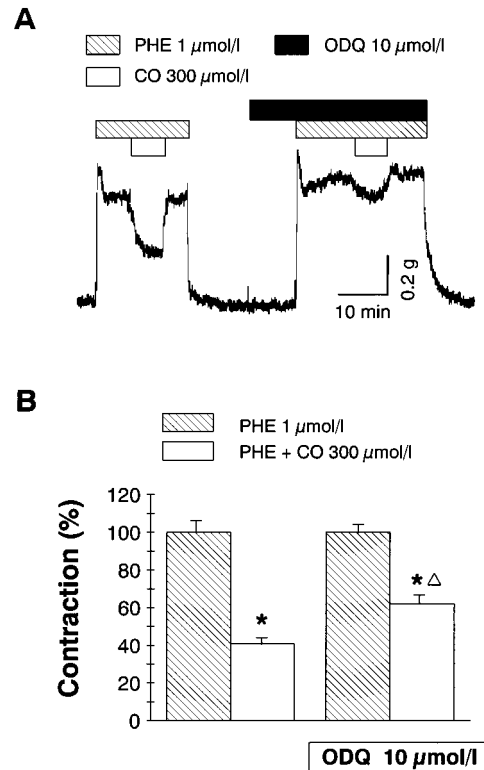


FIG. 2. The blockade of the cGMP-mediated signaling pathway reduced but not completely abolished the CO-induced relaxation of normal rat vascular tissues. The vascular tissues were precontracted with PHE (1 μmol/l). A: Actual tension development trace from one normal tail artery tissue. B: The CO-induced vasorelaxation was partially inhibited by ODQ in normal vascular tissues. *n* = 8 for each group. **P* < 0.05 between the groups with or without CO treatment; Δ *P* < 0.05 between the CO-treated groups in the absence or presence of ODQ.

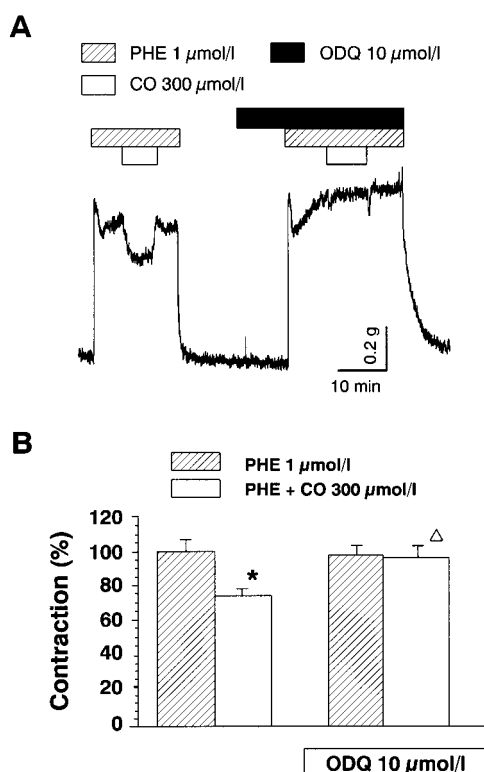


FIG. 3. The blockade of the cGMP-mediated signaling pathway completely abolished the CO-induced vasorelaxation in the vascular tissues of 1-month diabetic rats. The vascular tissues were precontracted with PHE (1 $\mu\text{mol/l}$). **A:** Actual tension development trace from one diabetic rat tail artery tissue. **B:** The CO-induced vasorelaxation was completely inhibited by ODQ in diabetic vascular tissues. $n = 8$ for each group. $*P < 0.05$ between the groups with or without CO treatment. $\Delta P < 0.05$ between the CO-treated groups in the absence or presence of ODQ.

subsequent study focused on the characteristics of K_{Ca} channels and their modulation by CO in diabetic tail artery SMCs.

As demonstrated in our previous report, in normal rat tail artery SMCs, a high-conductance K_{Ca} channel was also identified in diabetic rat tail artery SMCs. The characteristics of this K_{Ca} channel were not different among normal and diabetic rat tail artery SMCs (Fig. 4). With symmetric KCl (145 mmol/l) on both sides of the patch membrane, single-channel conductance was linearly related to membrane potentials over the range of -100 to $+60$ mV with no evidence of rectification. The single-channel conductances were 239 ± 8 pS ($n = 8$) in normal rat tail artery SMCs and 230 ± 6 pS ($n = 6$) in diabetic SMCs ($P > 0.05$). The NPo of K_{Ca} channels was decreased by ChTX (100 nmol/l) or iberiotoxin (100 nmol/l), but not by apamin (100 nmol/l) in both normal and diabetic tail artery SMCs (data not shown). Single-channel conductance of K_{Ca} channels was not modified by CO in either normal rat tail artery SMCs or diabetic SMCs. However, CO significantly increased the NPo of K_{Ca} channels in normal tail artery SMCs in a concentration-dependent manner (3–30 $\mu\text{mol/l}$) in both outside-out and inside-out patches. This stimulatory effect of CO was greatly reduced in diabetic artery SMCs (Fig. 5). For instance, the mean NPo over 3 min of recording was increased by CO (10 $\mu\text{mol/l}$) by $81 \pm 24\%$ in normal SMCs ($n = 6$, $P < 0.05$). At the same concentration, CO had no effect on the mean NPo of K_{Ca} channels in diabetic SMCs. When the concentration of

CO was increased to 30 $\mu\text{mol/l}$, the mean NPo of single K_{Ca} channels over 3 min of recording was increased by $173 \pm 14\%$ in normal SMCs ($n = 5$) but only by $48 \pm 30\%$ in diabetic SMCs ($n = 4$, $P < 0.05$ vs. the effect of CO on normal SMCs).

The decreased K_{Ca} channel sensitivity to CO might be explained by the altered glycation status of K_{Ca} channel proteins or hyperosmolality-induced structural and/or functional alterations in diabetic SMCs. To test the influence of glycation on K_{Ca} channels, tail artery SMCs were isolated and incubated in vitro under different conditions (Fig. 6). After culturing normal vascular SMCs for 8 days with 5 mmol/l glucose in the culture medium, the single-channel conductance and the sensitivity to ChTX or iberiotoxin of high-conductance K_{Ca} channels recorded in the cell-free patches were similar to those of freshly isolated normal SMCs. CO (10 $\mu\text{mol/l}$) increased the mean NPo over 3 min of the recording of the single K_{Ca} channels by $95 \pm 23\%$, taking the NPo level before the application of CO as 100% ($n = 4$, $P < 0.05$). After culturing SMCs isolated from diabetic rats for 8 days with 25 mmol/l glucose, high-conductance K_{Ca} channels had similar characteristics to those of freshly isolated diabetic SMCs. In these 8-day cultured diabetic cells, K_{Ca} channels were also not sensitive to CO, with the 3-min mean NPo showing no change after the application of CO (10 $\mu\text{mol/l}$) ($n = 4$). In another set of experiments, normal SMCs were cultured with 25 mmol/l glucose or 25 mmol/l 3-OMG for a period of 2–7 days. This culture scheme yielded inconsistent results. K_{Ca} channels in some cells were sensitive, but in others were insensitive to CO (data not shown). Interestingly, after 8 days in culture the sensitivity

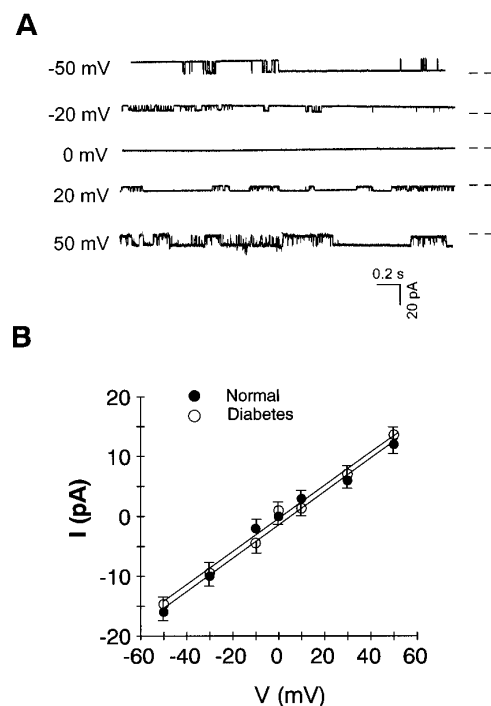


FIG. 4. Characterization of high-conductance K_{Ca} channels in diabetic tail artery SMCs. **A:** The actual single-channel current traces recorded at different membrane potentials from one inside-out patch of diabetic SMC. The dashed lines indicate the closed states. **B:** I-V relationships of single K_{Ca} channels recorded from inside-out patches of normal vascular SMCs or diabetic vascular SMCs. $n = 5$ for each data point. The single-channel conductance was determined by least-square linear regression analysis.

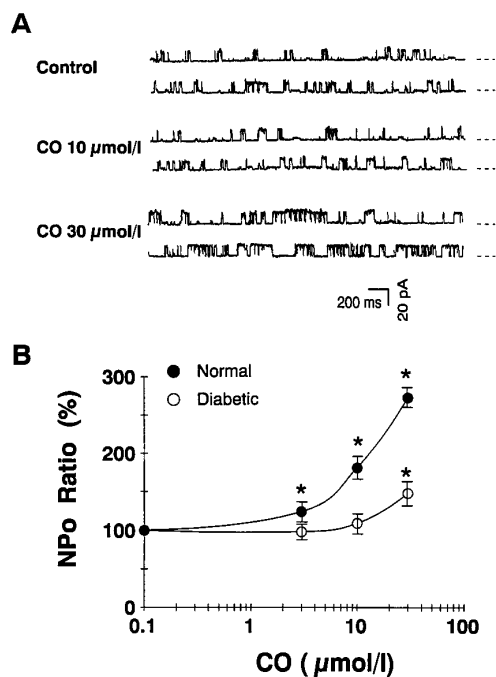


FIG. 5. The concentration-dependent effect of CO on single K_{Ca} channel currents in tail artery SMCs. **A:** The actual single-channel current traces recorded at different membrane potentials from one outside-out patch of a diabetic SMC. The dashed lines indicate the closed states. Membrane potential, +50 mV. **B:** The effect of CO on the NPo of single K_{Ca} channels recorded from outside-out patches of diabetic vascular SMCs was significantly decreased compared with those in normal vascular SMCs. * $P < 0.05$ before and after the application of CO in the same patch. $n = 4-8$ for each data point.

of K_{Ca} channels of normal SMCs to CO was consistently diminished. In these cells, CO (10 $\mu\text{mol/l}$) had no effect on the 3-min mean NPo of single K_{Ca} channels. In a similar previous study, a 10-day culture period was applied for the study of the influence of high concentrations of glucose or 3-OMG on the cellular functions of pericytes (25). Also shown in Fig. 6 is that culturing normal SMCs for 8 days with 25 mmol/l mannitol did not alter the effect of CO on K_{Ca} channels. A $73 \pm 9\%$ increase in the mean NPo of K_{Ca} channels in these cells was observed in the presence of CO (10 $\mu\text{mol/l}$) ($n = 4$, $P < 0.05$).

To examine whether deglycation of K_{Ca} channels of diabetic SMCs would regain the channel sensitivity to CO, tail artery SMCs from 1-month diabetic rats were cultured for different periods. After culturing diabetic SMCs for 8 days with 5 mmol/l glucose K_{Ca} channels in these cells (Group 5G/8D, $n = 5$) still lacked responsiveness to CO (Fig. 7). To avoid the insufficient nonenzymatic deglycation, further experiments were carried out to prolong the incubation period up to 35 days. The activity of K_{Ca} channels in these chronic deglycated diabetic SMCs was significantly increased by CO. As shown in Fig. 7, CO (10 $\mu\text{mol/l}$) increased the mean NPo over 3 min of recording of the single K_{Ca} channels in these cells (Group 5G/35D) by $62 \pm 8\%$ ($n = 5$, $P < 0.05$). The regained sensitivity to CO was unlikely to be related to the prolonged incubation period because CO (10 $\mu\text{mol/l}$) still had no effect on NPo of K_{Ca} channels in diabetic SMC cultured for 35 days but with 25 mmol/l glucose (Group 25G/35D, $n = 4$).

Whether the vascular responses to the endogenous CO were altered in diabetic vascular tissues was further studied

in the next series of experiments. Tail artery tissues were preincubated in the dark for 6 h with hemin (20 $\mu\text{mol/l}$). Hemin acts as the inducer for the expression of the inducible HO (HO-1) and as the substrate of HO to promote the endogenous CO production from vascular tissues, thus decreasing the PHE-induced vasoconstriction (4). In agreement with our previous study (4), 6-h incubation of tail artery tissues from normal rats or diabetic rats without hemin added did not alter the resting tension level or the PHE-induced concentration-dependent vasoconstriction (data not shown). The concentration-dependent vasoconstriction of normal tissues induced by PHE was significantly inhibited by hemin incubation (Fig. 8A). The EC_{50} of PHE effects was 0.24 ± 0.03 and 1.19 ± 0.12 $\mu\text{mol/l}$ without or with hemin incubation, respectively ($P < 0.05$). In contrast, the vasoconstrictive effect of PHE on diabetic tail artery tissues was not affected by 6-h hemin (20 $\mu\text{mol/l}$) incubation (Fig. 8B).

To determine whether the lack of relaxant effect of hemin on diabetic vascular tissues was due to the loss of expression of HO in diabetic tissues, Western blot assay was employed to examine the expression level of HO in normal and diabetic rat tissues. The expression of HO-1 proteins was hardly detected in normal rat vascular tissues but apparently visible in rat spleen. In 1-month diabetic tissues, the expression of HO-1 proteins was significantly induced in all vascular tissues

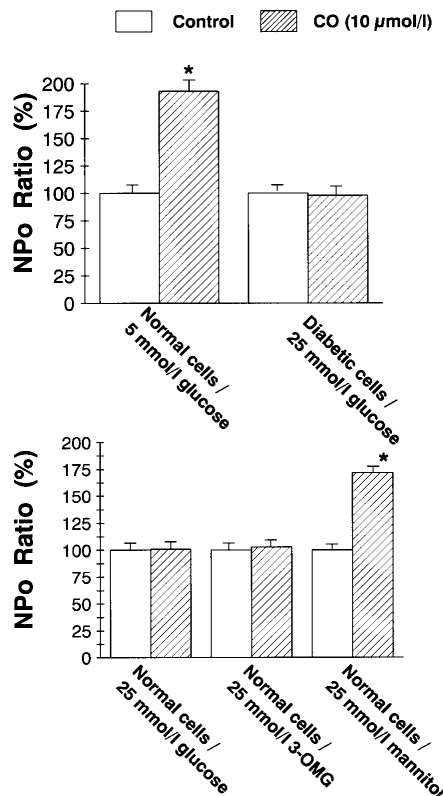


FIG. 6. The diminished effect of CO on high-conductance K_{Ca} channels is due to the glycation of K_{Ca} channel proteins. Tail artery SMCs from normal or diabetic rats were cultured under different conditions for 8 days with 5% FCS. Then, the changes in the mean NPo over 3 min of recording of single K_{Ca} channels in outside-out patches (membrane potential, +50 mV) before and after the application of CO were determined. * $P < 0.05$ before and after the application of CO to the same patch. $n = 3-5$ for each data point.

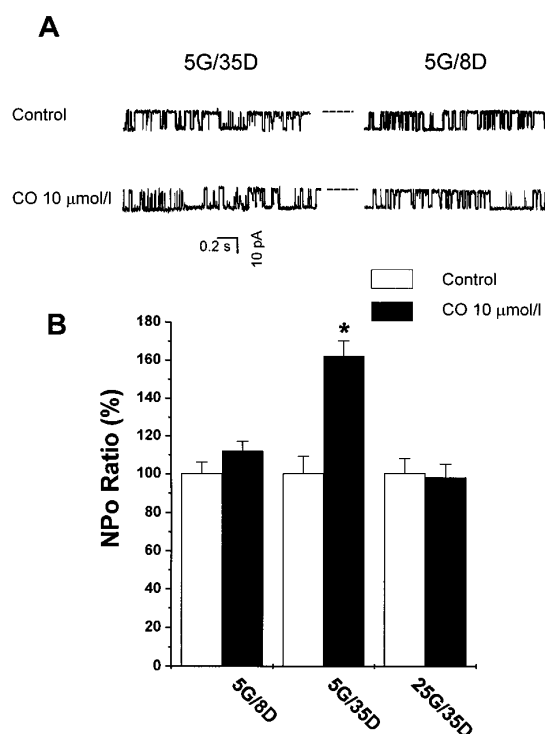


FIG. 7. Deglycation of K_{Ca} channel proteins in diabetic SMCs regained the sensitivity to CO. **A:** Representative single-channel recordings showing the regained K_{Ca} channel sensitivity to CO after 35 days of culture with 5 mmol/l glucose (left panel) and the insensitivity of K_{Ca} channels to CO after 8 days of culture (right panel). The dashed lines beside the original current traces denote the close states of K_{Ca} channels. **B:** Relative changes in the mean NPo over 3 min of recording of single K_{Ca} channels in outside-out patches before and after the application of CO. * $P < 0.05$ before and after the application of CO to the same patch. $n = 3-5$ for each data point. Membrane potential, +50 mV. The culture condition of each group was specified by concentration of glucose (G) in the medium and the culture duration in days (D).

tested. In contrast, there was no difference in the abundance levels of HO-1 proteins between normal and diabetic spleen tissues (Fig. 9). These results demonstrated that the induced HO-1 protein expression was not a nonspecific general tissue response to diabetes. Figure 10 illustrates the expression of constitutive HO (HO-2) protein in normal and diabetic rat tissues. Different from the expression pattern of HO-1, HO-2 proteins were detected in all normal rat tissues examined. More interestingly, the expression levels of HO-2 proteins were not altered in all diabetic vascular tissues ($P > 0.05$).

DISCUSSION

CO actively participates in the fine regulation of vascular contractilities under physiological conditions. Altered metabolism or vascular actions of CO in many cases are coupled to cardiovascular diseases. For instance, the increased endogenous production of CO was reported in chronic uremic patients (10). Increased HO-1 expression and activity in hypoxia, ischemia-reperfusion, and subarachnoid hemorrhage have also been reported (1). The application of the HO-inhibitor zinc protoporphyrin IX more significantly enhanced the blood pressure in SHR-SP rats than in normotensive control WKY rats (26). The mRNA levels of HO-2 in the aorta and kidney and of HO-1 in the ventricle were also significantly higher in SHR-SP than in WKY rats (26). Not only important

for the peripheral resistance regulation in genetically hypertensive rats, CO also plays an important role in regulating cardiac function. A recent study showed that the decreased endogenous CO production in SHR-SP may contribute to the cardiac hypertrophy (27). Furthermore, endogenous-produced CO has been shown to suppress the acute hypertension responses of conscious rats induced by various vasoconstrictive agents (28). Alm et al. (29) demonstrated the immunoreactivity of HO in the rat islets of Langerhans, including insulin-immunoreactive β -cells. After treatment with alloxan to induce diabetes, the immunoreactivity of HO in rat islet cells virtually disappeared. This study provided evidence for the pathophysiological role of the HO/CO system in the development of insulin-dependent diabetes, for this system appears to modulate the islet hormone secretion. In a recent study on type 1 and type 2 diabetic patients, it was found that the levels of exhaled CO were higher when compared with those in healthy subjects. There was a positive correlation between exhaled CO levels and the incidence of hyperglycemia in all subjects and the duration of diabetes (30). To date, the altered functions of CO or the expression pattern of HO in diabetic vascular tissues had not been reported. The STZ-induced experimental diabetes is a well-established animal model of diabetes, characterized with hyperglycemia and hypoinsulinemia (17-19). Using this diabetic animal model, we studied the vascular effects of CO in diabetes and the underlying mechanisms. Our results are summarized as the

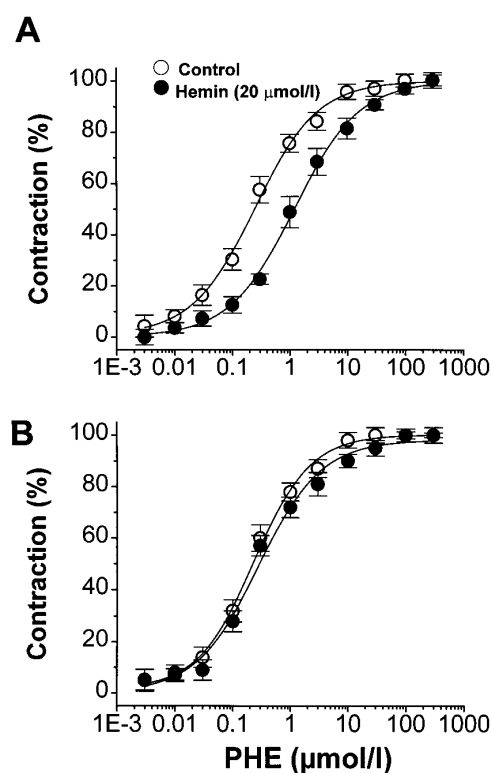


FIG. 8. The effects of hemin (20 $\mu\text{mol/l}$) incubation for 6 h on the phenylephrine (PHE)-induced constriction of tail artery tissues from normal or diabetic rats. **A:** The contractile response of normal vascular tissues to PHE was significantly reduced after incubating the tissues with hemin for 6 h. **B:** The contractile response of diabetic vascular tissues to PHE was not changed after incubating the tissues with hemin for 6 h. $n = 8$ per data point.

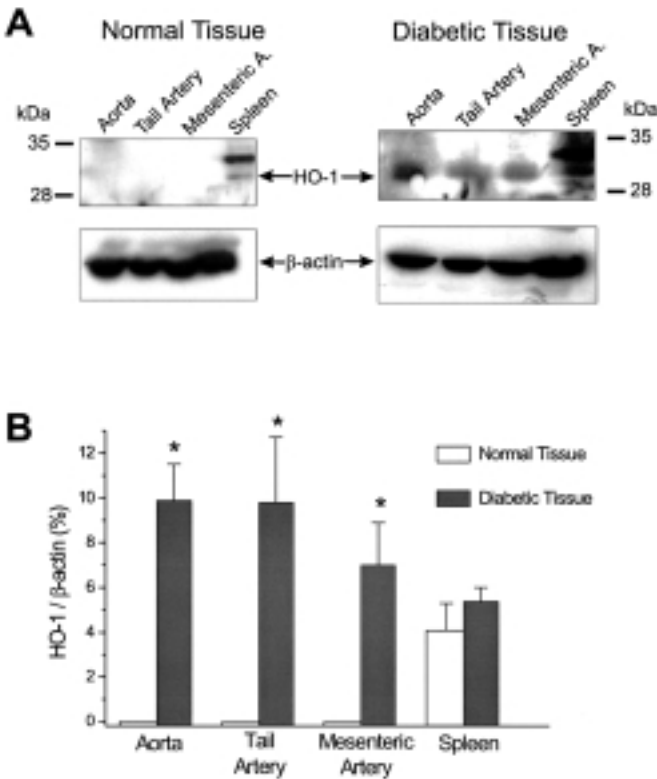


FIG. 9. Increased expression of HO-1 proteins in 1-month diabetic tissues determined by Western immunoblot assay. **A:** Representative immunoblots of rat aorta, tail artery, mesenteric artery, and spleen proteins (40 μg/lane). Except for two bands for the spleen microsomes, a single band of 32 kDa was identified in all vascular tissue microsomes, which corresponds to the known molecular mass of HO-1. β-actin proteins were detected as the housekeeping control. **B:** The relative abundance of HO-1 proteins in normal and 1-month diabetic tissues. The immunoblot gel images were scanned and digitized. The pixel densities of HO-1 proteins were then digitized and analyzed using software (UN-SCAN-IT; Silk Scientific). A, artery; *n* = 3 for each group; **P* < 0.01.

following: 1) The CO concentration-dependent relaxation of tail artery tissues from streptozotocin-induced diabetic rats was significantly decreased as compared with that of nondiabetic control rats; 2) the vasorelaxant effect of CO in diabetes was solely mediated by cGMP; 3) the sensitivity of K_{Ca} channels in diabetic vascular SMCs to CO was significantly reduced; 4) the glycation of K_{Ca} channel proteins may account for the reduced channel sensitivity to CO; and 5) the vasorelaxation induced by endogenous CO was also significantly reduced in diabetes although no reduction in the expression levels of HO was detected in diabetic vascular tissues. Taken together, our data demonstrated a decreased vasorelaxant function of CO in diabetes. This altered vascular effect of CO could be at least in part related to a diminished sensitivity of K_{Ca} channels of diabetic SMCs to CO.

The mechanisms for the reduced sensitivity of K_{Ca} channels to CO in diabetic SMCs were investigated. After chronically culturing normal tail artery SMCs with 25 mmol/l glucose or 25 mmol/l 3-OMG, the sensitivity of K_{Ca} channels in these SMCs to CO was consistently diminished. Because 3-OMG is a nonmetabolizable glucose analog, these results indicate that the glycation of K_{Ca} channels rather than the metabolism of glucose by cultured vascular SMCs might be the mecha-

nism for the altered K_{Ca} channel functionality in diabetes (25). On the other hand, chronically culturing normal SMCs with 25 mmol/l mannitol did not alter the effect of CO on K_{Ca} channels. The role played by the hyperosmolality in the diminished effect of CO on K_{Ca} channels in diabetes would thus be correspondingly minimized. If glycation of K_{Ca} channels was responsible for the decreased sensitivity to CO, deglycation of K_{Ca} channels should regain the channel sensitivity to CO. Following this line of thought, we cultured diabetic SMCs with 5 mmol/l glucose for different periods of culture time. A short culture duration (8 days) did not improve the sensitivity of K_{Ca} channels to CO in these cells. This result could underline the notion that the nonenzymatic deglycation of K_{Ca} channels would not be completed within the short incubation scheme. Even in the presence of high concentrations of certain deglycosylating enzymes, the deglycation of proteins was hardly completed in 10–20 days (31). In our preliminary experiments, the cell viability significantly deteriorated when the deglycation enzyme aminoguanidine (0.1 mol/l) was included in the culture medium (R.W., Z.W., L.W., S.T.H., R.P.-W., unpublished observations). Therefore, a prolonged cultured period beyond 8 days in the absence of deglycation enzyme became our choice for the purpose of deglycating K_{Ca} channels. As expected, culturing diabetic SMCs with 5 mmol/l glucose for ~5 weeks (35 days) restored the

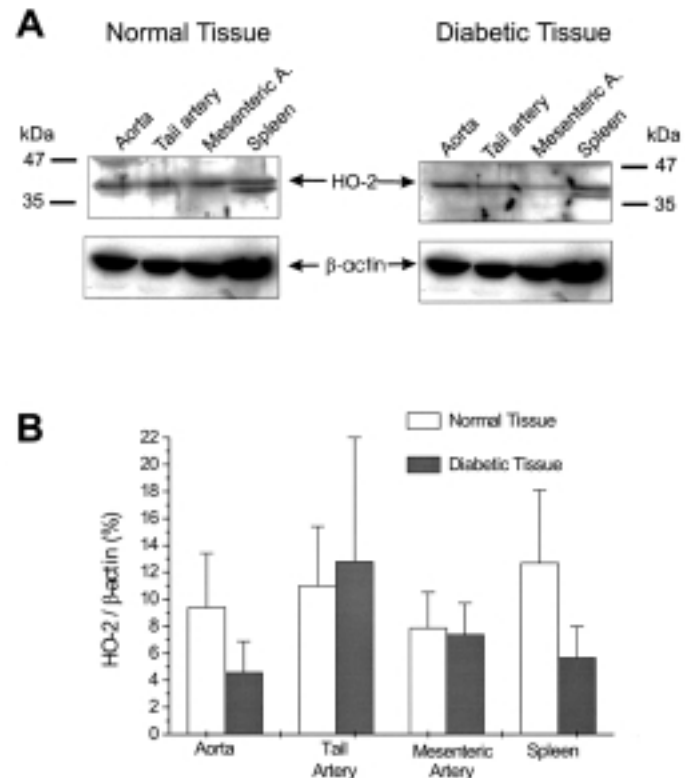


FIG. 10. Expression levels of HO-2 proteins in normal or 1-month diabetic tissues determined by Western blotting assay. **A:** Representative immunoblots of rat aorta, tail artery, mesenteric artery, and spleen proteins (40 μg/lane). The identified HO-2 bands were ~36 kDa, which corresponds to the known molecular mass of HO-2. β-actin proteins were detected as the housekeeping control. **B:** The relative abundance of HO-2 proteins in normal and 1-month diabetic tissues. The immunoblot gel images were scanned and digitized. The pixel densities of HO-2 proteins were then digitized and analyzed using software (UN-SCAN-IT). A, artery; *n* = 3 for each group; **P* < 0.05.

sensitivity of K_{Ca} channels to CO. The control experiment showed that K_{Ca} channels in diabetic SMCs cultured for the same period (35 days) but with 25 mmol/l glucose still exhibited significantly lower sensitivity to CO (Fig. 7). All aforementioned data supported the idea that glycation of K_{Ca} channels affected their responsiveness to CO. Future studies to isolate K_{Ca} channel proteins from diabetic SMCs would provide direct evidence confirming the glycation/deglycation status of these channel proteins under different conditions.

Glycation of proteins involves a complex series of reactions, including initial attachment of glucose to proteins by Schiffbase formation followed by Amadori rearrangement to generate stable ketoamine. It is known that hyperglycemia-induced protein glycation and lipid peroxidation are enhanced in diabetic subjects (32). Several mechanisms may account for the glycation-induced diminished sensitivity of K_{Ca} channels to CO. Glycation of K_{Ca} channels may produce steric hindrance for the exposure of the CO acting sites on the K_{Ca} channel proteins (33). Although the major glycation sites on proteins are asparagine, glutamine, and lysine (34,35), the glycation process will also affect the microenvironment of other amino acid residues in proteins, including histidine. An altered accessibility to the surface histidines has been shown in the glycated RNase A protein (34). CO mainly interacts with a histidine residue located on the extracellular domain of K_{Ca} channels (3). This interaction of CO with histidine may be impeded by the glycation of K_{Ca} channels. Glycation of channel proteins may lead to protein thiol oxidation, protein aggregation, and cross-linking (36). The glycation of the gap junction protein has been shown to alter the COOH-terminus arm, thus decreasing the channel permeability (36). In addition, the glycation of K_{Ca} channels may cause channel gate inflexibility as one of the consequences of the conformational changes, thus decreasing their open probability to be otherwise enhanced by CO. Glycation of ion channel proteins may facilitate the formation of advanced glycation end products. The latter would damage the structural integrity of channel proteins by targeting on histidine and other side chains (35).

Due to the presence of vasculopathy and/or neuropathy, minor trauma in diabetic patients will result in cutaneous ulceration and failure of wound healing, eventually necessitating lower-extremity amputations. Of diabetic patients, ~51% receive lower-extremity amputation (37). However, the nature of vasculopathy of diabetic lower extremities remains unclear. The anatomy of the rat tail vascular bed is suggestive of the study of distal blood circulation in human limbs and digits (38). The main artery supplying cutaneous blood flow to the rat tail is the tail artery. The function of this artery has been used as an assay to evaluate the impaired cutaneous vascular functions in hypertension (39) and other related peripheral vascular diseases (40). Therefore, the constrictive responses of rat tail artery smooth muscles to different vasoactive factors, including CO, may be relevant to the altered vascular functions in diabetic distal vasculopathy. Abnormal blood flow patterns in the diabetic foot unrelated to distal ischemia have been shown previously. In one study on insulin-dependent diabetic patients who had previous surgeries for diabetic foot infections, it was found that the most significant change in peripheral circulation was the loss of the normal triphasic pattern of arterial blood flow in lower limbs (41). The forward blood flow in systole was increased but the reversed flow in diastole decreased to the diabetic foot. Consequently, transcutaneous

venous oxygen tension was increased with vein distension (42,43). Using the STZ-induced diabetes as a model of type 1 diabetes and using the rat tail artery as a representative peripheral blood vessel, our study showed significant changes in vasoconstrictive properties of peripheral vascular tissues from diabetic rats. The altered properties of L-type calcium channels (18), decreased responsiveness to vasoactive bradykinin (17), abnormal calcium handling by diabetic SMCs (19), and decreased vasorelaxant effect of CO as demonstrated in the present study may all contribute to the abnormal regulation of peripheral resistance and altered peripheral blood flow patterns in diabetes.

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