Attenuated Agonist Evoked Vasoconstrictor Responses in the Perfused Mesenteric Vascular Bed of Streptozotocin Diabetic Rats

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We compared agonist-evoked responses in the perfused mesenteric vascular bed (MVB) of streptozotocin (STZ) diabetic Sprague-Dawley rats 2 and 14 weeks after induction of diabetes. Endothelin-1 (ET-1)-, methoxamine (MTX)-, and KCl-evoked vasoconstrictor responses were unchanged in 2-week-old diabetic rats. In contrast, both the sensitivity ($P < 0.01$) and the maximal vasoconstrictor responses ($P < 0.05$) to ET-1 were attenuated in 14-week-old diabetic rats, whereas endothelin plasma levels were increased ($P < 0.05$). Although no differences were observed in responses to KCl in either the 2- or 14-week-old diabetic groups, MTX-evoked maximal responses were attenuated in the 14-week-old group ($P < 0.01$). Changes in agonist-evoked responses in the 14-week-old diabetic group were unaffected by the protein kinase C (PKC) inhibitor, staurosporine, the phospholipase C (PLC) inhibitor, U73122, the calcium channel blocker, nifedipine, the calcium pump inhibitor, cyclopiazonic acid (CPA), or by endothelial denudation. Sodium fluoride (NaF), an activator of guanosine triphosphate binding proteins (G proteins) normalized the responses in the 14-week-old diabetic group. These data suggest that advanced stages of STZ are associated with alterations in G protein receptor coupling and/or activity leading to the attenuation of responses to vasoconstrictor agonists. [Exp Biol Med Vol. 226(10):940–946, 2001]

Key words: G protein; vascular smooth muscle; diabetes; mesenteric vascular bed

Insulin-dependent diabetes mellitus (IDDM) is associated with metabolic and cardiovascular abnormalities. Similarly, rats treated with streptozotocin (STZ) demonstrate profound hyperglycemia and reduced plasma insulin levels, along with altered responses to vasoconstrictor stimuli, leading to vascular complications characteristic of IDDM (1). The STZ rat also demonstrates other symptoms of uncontrolled human diabetes, including weight loss, hyperglycemia, polyuria, polydipsia, and hyperphagia. Thus, examining the vascular dysregulation in the STZ rat may provide insight into the development of vascular complications in human IDDM. Although alterations in vasoconstrictor and vasodilatory function in STZ rats have been demonstrated, the mechanisms underlying these changes are poorly understood. There may be decreases in receptor number coupled to alterations in second messenger systems (1) and/or alterations in the handling of intracellular calcium by vascular smooth muscle (VSM) (2, 3). Diabetes also has considerable effects on the vascular endothelium. Enhanced endothelium-dependent vasorelaxation is a feature of early diabetes, whereas at later stages, vasodilatation is diminished (4). Nitric oxide (NO), a key endothelial-derived vasodilatory factor, is focal in mediating these responses. The endothelium also produces another factor integral in modulating vascular responses, endothelin-1 (ET-1). At pathological levels, this peptide has profound vasoconstrictor effects and contributes to changes in blood flow in the diabetic vasculature (5). In this study we examined the effect of diabetes duration on vasoconstrictor responses to methoxamine (MTX), potassium chloride (KCl), and ET-1 in the perfused mesenteric vascular bed (MVB), a preparation representative of the resistance function of the circulation (6).

Materials and Methods

Plasma Glucose and Weight. At 9 weeks of age, male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada),kept under standard conditions in the animal housing facilities were treated with STZ (55 mg/kg) in citrate buffer (pH 4.5, 0.5 mg/ml i.p. = diabetic group) or with citrate buffer alone (control group). Fasting plasma glucose levels were assessed by the glucose oxidase method

This work was supported by grants-in-aid from the Canadian Institutes for Health Research and the Heart and Stroke Foundation of Saskatchewan. D.M. and R.L.H. are thankful to the Canadian Institutes for Health Research for their Doctoral Research Awards.

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Received June 11, 2001.
Accepted July 19, 2001.

1535-3702/01/22610-0940$15.00

(One Touch, Lifescan, Vancouver, British Columbia, Canada) 1 week post-treatment and at the time of sacrifice (2 and 14 weeks post-STZ) to verify hyperglycemia (>15 mM). All procedures were conducted in accordance with the guidelines of the University Animal Care Committee.

Assay Procedures. Plasma samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA), centrifuged at 2000*g* for 10 min, and stored at −70°C. ET-1 was measured using a commercially available radioimmunoassay (RIA) kit (Amersham, Oakville, Ontario, Canada) as previously described (7). One milliliter of sample was acidified with 2.0 M HCl and was centrifuged at 1000*g* for 5 min. The sample was loaded onto Amprep 500 mg C2 minicolumns (Amersham), washed with 0.1% trifluoroacetic acid (TFA), and then eluted with 80% acetonitrile and 0.1% TFA. ET-1 was detected using an [^{125}I] ET-1 RIA kit with negligible cross reactivity values to vasoactive peptides. However, the kit demonstrated considerable cross reactivity with other ET isopeptides. The percent recovery of ET-1 was determined to be about 80%.

Perfusion Measurements. The superior mesenteric artery was cannulated, and the MVB was detached from its intestinal borders and removed. The preparation was immediately perfused with a modified Krebs bicarbonate solution, as previously described (8). The cannulated MVB was then placed in a jacketed organ bath and was perfused via a peristaltic pump (Harvard Apparatus Peristaltic Pump) at a constant rate of 5 ml/min with the oxygenated (95% O_2/5% CO_2) 37°C Krebs solution. Vasoconstriction caused an increase in perfusion pressure (PP) that was measured (as mm Hg) with a strain gauge transducer (Beckman, Palo Alto, CA) placed into the circuit just prior to the MVB. A Grass Polygraph (Quincy, MA) then electronically integrated the pulsatile pressure signal.

Measurement of Agonist-Evoked Responses. In all protocols, an equilibration period of 30 min was allowed to stabilize the baseline. The vasoconstrictor responses to a Krebs solution containing KCl (20–120 mM) were completed first. After washout, bolus dose challenges to the α_1 agonist, MTX (1.0 μM–1.0 mM) were performed. ET-1 bolus dose (0.1 nM–10 μM) challenges were completed last. We have previously determined that the sequence of agonist exposure is not of consequence in regards to subsequent responses (9, 10); however, because of the long duration of effect, ET-1 challenges were always performed last.

In the second protocol, endothelial denudation was accomplished by repeated injection of air into the vascular bed as previously described (11). Briefly, this was undertaken as follows. The basal tone was increased via perfusion of a MTX (70 μM) Krebs buffer. Subsequently, the vasodilatory response to a maximal acetylcholine (ACh) concentration (1.0 ml of a 0.1 mM solution) was assessed. The MVB was disconnected from the apparatus and a syringe was then connected to the cannula through which air was injected. The MVB was then reconnected to the perfusion apparatus and an ACh challenge was repeated. Denudation was characterized as a 75% or greater reduction in the maximal response to ACh. Agonist-evoked responses were then conducted as in the first protocol.

In the third protocol, bolus agonist-evoked challenges were conducted in the 14-week-old control and diabetic rat MVB preparations exposed for 30 min to reagents known to inhibit essential components of the phosphatidylinositol signal transduction pathway. The agents used were the phospholipase C (PLC) inhibitor, U-73122 (1 μM), the protein kinase C (PKC) inhibitor, staurosporine (5 nM), the L-type calcium channel blocker, nifedipine, (3 μM), and the calcium pump blocker cyclopiazonic acid (CPA; 3 μM). The effects of guanosine triphosphate binding protein (G protein) stimulation were also assessed using sodium fluoride (NaF; 10 mM).

Drugs. ACh, CPA, MTX, NaF, U-73122, staurosporine, and nifedipine were all purchased from Sigma (Oakville, Ontario, Canada). ET-1 was obtained from American Peptide Co. (Sunnyvale, CA). All Krebs solution salts were of analytical grade and were obtained from BDH (Toronto, Ontario, Canada).

Statistical Analysis. Dose-response (DR) curves to each agonist were analyzed individually in each tissue and the results were expressed as increases in PP (mmHg) or the percentage of change in PP relative to respective control. The potency of agonist-evoked vasoconstriction was expressed as the negative logarithm of the concentration producing one-half the maximal response (EC_{50} value). Both maximal responses (E_{max}) and the EC_{50} values were expressed as the mean ± SEM. Comparison of mean values amongst various groups was performed by analysis of variance (ANOVA; Superanova program, SAS Institute, San Francisco, CA). Simultaneous multiple comparisons were examined by Scheffe's F-test.

Results

Metabolic Parameters. There were no significant differences in body weight at the time of STZ treatment. At sacrifice, the 2-week-old control animals were significantly heavier ($P < 0.01$) as were the14-week-old controls ($P < 0.001$). However, neither the 2- nor 14-week-old STZ groups demonstrated a significant change in weight. The mean blood glucose of 2-week-old STZ rats (25.2 ± 2.2 mM) was significantly greater than in controls (5.3 ± 0.3; $P < 0.001$). Similarly, blood glucose was elevated in the 14-week-old STZ rats (24.4 ± 1.2) relative to the control group (5.7 ± 0.4; $P < 0.001$). There were no significant differences in mean blood glucose values between the 2- and 14-week-old controls or the 2- and 14-week-old STZ-treated groups.

Agonist-Evoked Changes in Perfusion Pressure. Responses to MTX were not altered after 2 weeks of diabetes (Fig. 1A). However, at 14 weeks the E_{max} to MTX was significantly reduced with no significant shift in the EC_{50} values (Table I). No differences in either the E_{max} or the EC_{50} values were noted in ET-1-evoked responses for

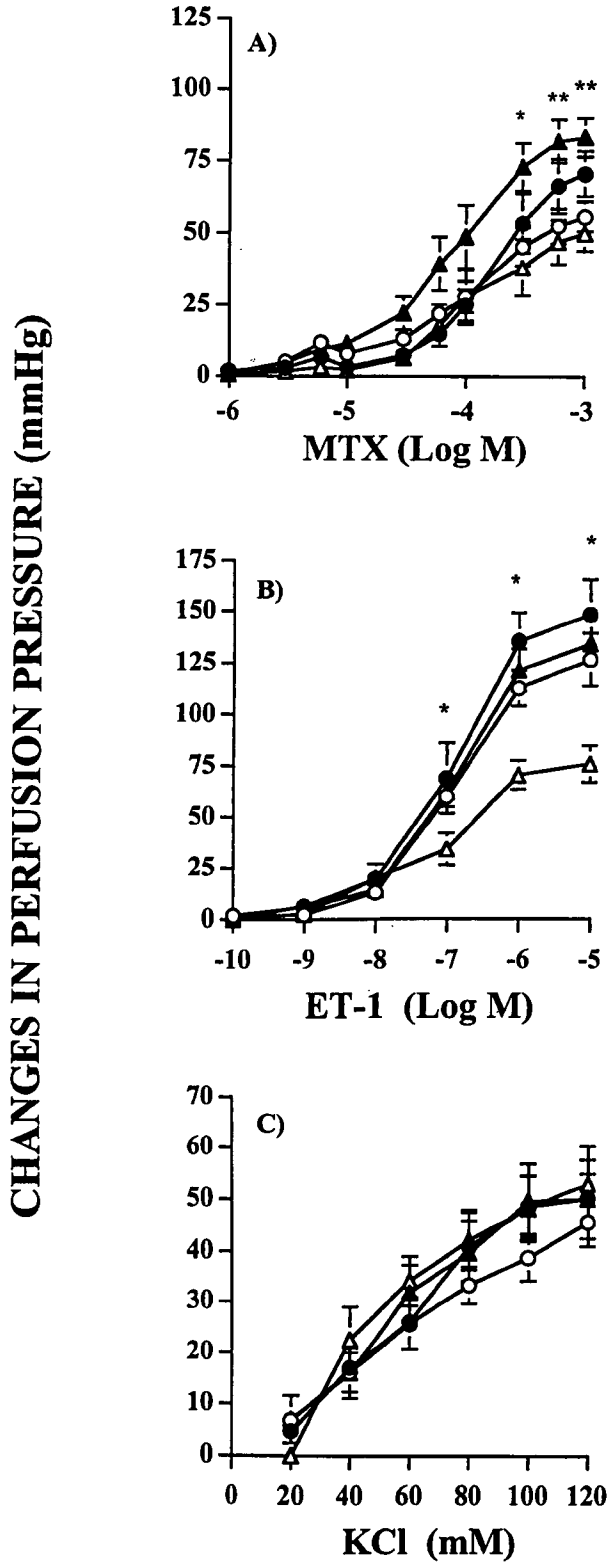

Figure 1. Concentration-perfusion pressure (mm Hg) response curves to MTX (1.0 μM–1.0 mM [represented as –log M]) (A), ET-1 (0.1 nM–10 μM [represented as –log M]) (B), and KCl (20 mM–120 mM) (C) in the perfused MVB of 2- and 14-week-old SD rats treated with STZ. ●, Two-week-old age matched controls; ○, 2-week-old STZ; ▲, 14-week-old age matched controls; and △, 14-week-old STZ. Each data point is a mean ± S.E.M. of 7 to 10 determinations performed using different MVB preparations. (*$P < 0.05$, **$P < 0.01$, 14-week-old STZ versus 14-week-old control).

2-week-old STZ diabetic rats (Table I). In contrast, the DR curve to ET-1 was shifted to the right and a decrease in E_{max} (Fig. 1B) was observed in the 14-week-old STZ treatment group ($P < 0.05$). No significant differences were observed in KCl responses for either the 2- or the 14-week-old diabetic group versus respective controls (Fig. 1C).

ET Plasma Levels. After 14 weeks of STZ-induced diabetes, ET plasma levels were increased to 1.79 ± 0.52 pM from 1.08 ± 0.05 pM ($P < 0.05$). However, after 2 weeks of STZ diabetes, there was actually a significant reduction in plasma ET levels from 1.11 ± 0.04 pM to 0.44 ± 0.19 pM ($P < 0.05$). Plasma samples were taken from seven 14-week-old control rats and from 10 rats for each of the other groups.

Effect of Endothelial Denudation. Endothelial denudation enhanced contractile responses to all agonists in all groups (Table I). However, endothelial denudation did not have differential effects on either control or diabetic rats when responses are expressed as a percentage of the PP generated in the nondenuded, respective control (Fig. 2). Responses in the endothelial intact preparations of the 14-week-old STZ group were also significantly lower than in their respective non-STZ controls ($P < 0.05$).

Effects of Phosphatidylinositol Pathway Regulators. Neither staurosporine, U-73122, nifedipine, nor CPA affected baseline PP. NaF did not significantly elevate the baseline PP in either group. All of the inhibitors attenuated maximal responses to MTX and ET-1 in 14-week-old control and diabetic rats with no effect on sensitivity (Table II). As with endothelial denudation, no differential effects were observed when responses were expressed as a percentage of PP generated in the respective control (Fig. 3). However, NaF restored responses to MTX and ET-1 to control levels in the 14-week-old diabetic group (Fig. 3E). NaF also enhanced the sensitivity to MTX evoked-responses in STZ rats relative to both the STZ-untreated control and to the NaF-exposed, non-STZ control, whereas ET-1 sensitivity was enhanced relative to the diabetic control (Table II).

Discussion

The present investigation confirms that agonist-evoked vasoconstrictor responses in the perfused MVB of STZ diabetic rats are dependent on the pre-existing duration of diabetes, and that STZ-induced diabetes ultimately desensitizes G protein-mediated responses. Fourteen weeks of diabetes profoundly attenuated the constrictor responses to both MTX and ET-1, whereas 2 weeks had no effect. No alterations in KCl responses were noted at either stage. Several explanations for decreased agonist-evoked vasoconstriction in STZ-induced diabetes have been proposed. Diminished contractile responses have been attributed to alterations in adrenoreceptor expression (12), decreased uptake and incorporation of amino acids into contractile proteins (13), and to alterations in post-receptor calcium handling and activity (14). The lack of change in KCl-evoked responses precludes alterations in calcium handling *per se* and sug-

Table I. Analyses of Concentration-Pressure Responses to Methoxamine (MTX), Endothelin-1 (ET-1), and Potassium Chloride (KCl) Depolarization in the Presence and Absence of Endothelium in the Perfused Mesenteric Vascular Bed (MVB) of Adult Male Sprague-Dawley Rats after 2 and 14 weeks of Streptozotocin (STZ) Diabetes

	2-week				14-week			
	Control		STZ		Control		STZ	
	EC_{50} (–log M)	E_{max} (mm Hg)	EC_{50} (–log M)	E_{max} (mm Hg)	EC_{50} (–log M)	E_{max} (mm Hg)	EC_{50} (–log M)	E_{max} (mm Hg)
Before endothelium denudation								
MTX	3.9 ± 0.1	70 ± 7	3.7 ± 0.1	59 ± 3	4.1 ± 0.2	86 ± 7	3.9 ± 0.2	49 ± 7[a]
ET-1	6.9 ± 0.4	149 ± 18	7.1 ± 0.2	130 ± 13	7.0 ± 0.3	135 ± 6	6.5 ± 0.2[b]	76 ± 9[b]
KCl	1.3 ± 0.03	51 ± 3	1.3 ± 0.04	47 ± 6	1.2 ± 0.03	52 ± 4	1.3 ± 0.1	54 ± 7
	(n = 10)		(n = 10)		(n = 7)		(n = 10)	
After endothelium denudation								
MTX	4.3 ± 0.1	97 ± 9[c]	4.1 ± 0.02	86 ± 6[c]	4.3 ± 0.1	110 ± 8[d]	4.2 ± 0.1	64 ± 7[a,d]
ET-1	7.0 ± 0.02	212 ± 12[c]	6.9 ± 0.1	191 ± 10[c]	6.9 ± 0.1	175 ± 14[c]	6.8 ± 0.1	97 ± 4[a,d]
KCl	1.3 ± 0.1	83 ± 10[d]	1.3 ± 0.1	74 ± 6[d]	1.3 ± 0.03	78 ± 9[d]	1.3 ± 0.1	79 ± 12[d]
	(n = 9)		(n = 10)		(n = 9)		(n = 9)	

Note. Each value shown is mean ± S.E.M. of n determinations (shown in parentheses) performed using differing MVB preparations.
[a] $P < 0.01$ vs 14-week control.
[b] $P < 0.05$ vs 14-week control.
[c] $P < 0.01$ vs respective, endothelium intact group.
[d] $P < 0.05$ vs respective, endothelium intact group.

gests that attenuated responses may be due to receptor or post-receptor alterations. This provides a common link between alterations in responses to ET-1 and MTX, as the attenuated responses both employ G protein-coupled receptors.

STZ diabetes has been demonstrated to induce dysregulation at the level of the receptor/G protein complex (15). It has been reported that sexual dysfunction in diabetic rats is a result of a defect in the vasoactive intestinal peptide receptor or the associated G protein (16). Whether STZ-induced attenuation of agonist-evoked vasoconstrictor responses is a result of receptor downregulation, dysregulation of G protein-receptor coupling or a combination of these factors remains to be explored. The ability of NaF to rescue both impaired MTX and ET-1 responses in the present study confirms the presence of a functionally relevant alteration at the receptor/G protein level. It has also been reported that prostanoid production is altered in a duration-dependent fashion in STZ diabetic rats (17, 18). Specifically, there appears to be a shift towards prostacylcin rather than thromboxane generation. This phenomenon may further contribute to the attenuation of vasoconstriction, although the functional evidence is not clear. Indeed, in our hands, indomethacin has never been shown to increase (or decrease) the responses to vasoconstrictor agonists in the perfused MVB (8, 19). This inability has also been demonstrated by others, using the same vascular bed preparation (20–22).

Vascular function, particularly in pathological states such as diabetes, may also rely on the bioactivity of ET-1. ET-1 is a powerful paracrine regulator of VSM tone (23). Although this isoform comprises the lion's share of circulating ETs, two other isoforms, ET-2 and ET-3, exist (24). There are also two receptor subtypes for endothelin, namely, ET_A (located only on VSM cells (VSMC) and ET_B (located on both endothelial cells and VSMC). Intravenous infusion of ET-1 causes an initial fall in blood pressure followed by a sustained increase. The initial fall occurs via ET-mediated release of vasodilatory factors from the endothelium as a consequence of activation of endothelial ET_B receptors (25). The subsequent pressor action of ET-1 is mediated by activation of ET_A receptors located on VSMC. Increased ET-1-induced vasodilatation has been observed in STZ diabetes, probably due to a decrease in ET-1 vasoconstrictor activity (26).

Both elevated and decreased plasma ET-1 levels have been reported in patients with IDDM (5). Duration-dependent alterations in plasma ET-1 levels have also been noted in STZ diabetic rats (5). In agreement, attenuated ET-1 plasma levels were found in rats with diabetes of not greater than 5 weeks (27), whereas plasma levels in older diabetic rats have been shown to be higher (26, 28). However, elevated plasma ET-1 levels have been noted as early as 1 week after STZ diabetes (26). Increased circulating ET levels could be either due to a damaged endothelium releasing more ET-1 (29) and/or due to decrease in NO-mediated inhibition of ET-1 release (30). Conversely, an increase in ET-1 plasma levels could be the result of an increase in NO bioactivity. Methodology for measuring ET-1, assay characteristics, and variability in the metabolic state likely contribute to these discrepancies (5). A key remaining question is: What are the factors responsible for the observed changes in ET-1 release and action in diabetes mellitus? It has been proposed that ET-1 plasma levels correlate directly

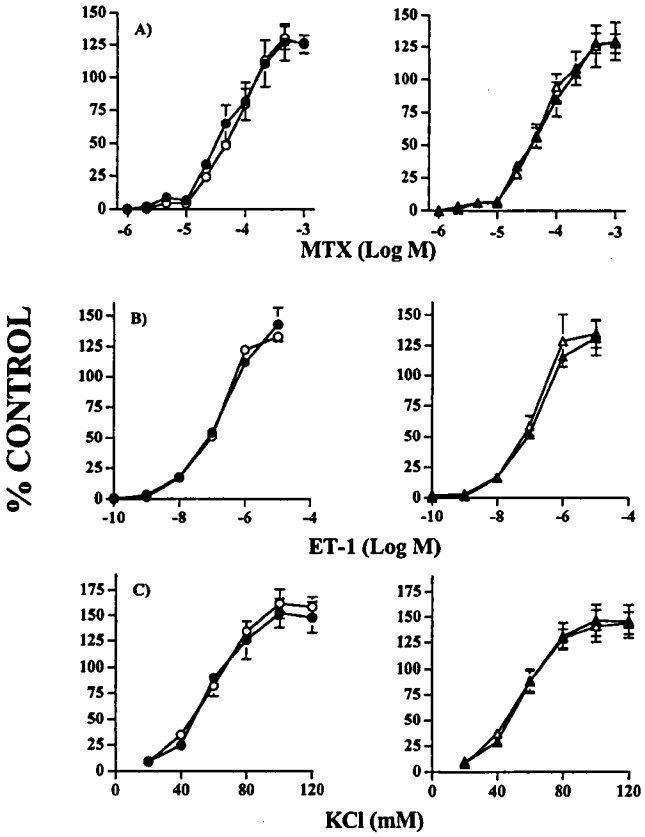

Figure 2. Concentration response curves in the MVB of endothelium-denuded preparations to MTX (1.0 μM–1.0 mM [represented as -log M]) (A), ET-1 (0.1 nM–10 μM [represented as -log M]) (B), and KCl (20 mM–120 mM) (C). Responses are represented as the percentage of perfusion pressure generated relative to the respective, nondenuded control. ●, Two-week-old age-matched controls; ○, 2-week-old STZ; ▲, 14-week-old age-matched controls; and △, 14-week-old STZ. Each data point is a mean ± S.E.M. of 7 to 10 determinations performed using different MVB preparations.

with hyperglycemia (26), although reports of attenuated plasma ET-1 levels in rats that have been diabetic for less than 5 weeks would seemingly suggest a more complex interaction. Interestingly, it has recently been demonstrated that restoration of metabolic control can indeed correct altered plasma ET-1 levels in diabetes mellitus (9, 31). Regardless of the direction of change, altered plasma ET-1 levels could contribute to diminished responses to MTX, since ET-1 is known to potentiate the responses to α adrenoceptor agonists (32).

The ability of fluoroaluminates to activate G proteins is well known (33). However, it has more recently been proposed that this effect may be attributable specifically to fluorides, as NaF has been suggested to activate G proteins in VSM (34, 35). Moreover, fluoroaluminates have been shown to have a stronger influence on PLC, whereas NaF may be more intimately involved with the G protein/L-type calcium channel complex (35). As nifedipine did not demonstrate any differential effect on vasoconstrictor responses in STZ rats, the ability of NaF to preferentially enhance responses in the diabetic rat further suggests a defect at the receptor/G protein level. It is also interesting to note that fluoroaluminates attenuated responses to phenylephrine in rabbit femoral arteries, whereas NaF did not (35). Likewise, in the present study, we have noted that NaF actually enhanced agonist-evoked responses and that this effect was particularly dramatic in STZ rats.

Our work demonstrates that duration-dependent alterations in vascular reactivity contribute to the state of cardiovascular dysregulation in diabetes. Particularly, it appears that diabetes may ultimately impart alterations at the receptor/G protein level. However, this effect may be vascular bed-specific because in the basilar artery diminished vasodilator response *per se* results in an enhanced contractile state (36). In general, although disparity regarding contractile responses to various agonists in STZ diabetic animals exists, there appears to be an increase in tension responses in conduit vessels in conjunction with a decreased PP response in resistance vessels (1). Given the limitations of studying contractility in conduit vessels as an indication of resistance, the perfused MVB, a preparation representative of the resistance function of the circulation (6), seems favorable. However, as this technique is inherently insensitive to small changes in reactivity, our study employed an

Table II. Analyses of Concentration-Perfusion Response Curves to MTX and ET-1 in the MVB of 14-Week STZ Diabetic Rats in the Presence of Signaling Pathway Inhibitors

	MTX						ET-1					
	(*n*)	Control		(*n*)	STZ		(*n*)	Control		(*n*)	STZ	
		EC_{50} (-log M)	E_{max} (mm Hg)		EC_{50} (-log M)	E_{max} (mm Hg)		EC_{50} (-log M)	E_{max} (mm Hg)		EC_{50} (-log M)	E_{max} (mm Hg)
Control	(7)	4.1 ± 0.2	86 ± 7	(10)	3.9 ± 0.2	49 ± 7	(7)	7.0 ± 0.3	135 ± 6	(10)	6.5 ± 0.2	76 ± 9
Staurosporine	(4)	4.1 ± 0.1	49 ± 9[a]	(5)	4.0 ± 0.1	23 ± 4[a,b]	(4)	7.1 ± 0.2	77 ± 4[a]	(5)	7.0 ± 0.2[c]	49 ± 8[a,b]
U-73122	(5)	4.1 ± 0.1	56 ± 3[a]	(5)	4.0 ± 0.1	31 ± 7[a,b]	(5)	7.2 ± 0.1	93 ± 10[a]	(5)	6.8 ± 0.5	62 ± 6[a,b]
Nifedipine	(4)	4.2 ± 0.04	40 ± 4[a]	(4)	4.1 ± 0.04	25 ± 3[a,b]	(4)	7.1 ± 0.1	86 ± 8[a]	(4)	6.7 ± 0.1	54 ± 8[a,b]
CPA	(5)	3.9 ± 0.2	17 ± 4[a]	(5)	4.0 ± 0.1	6 ± 1[a,b]	(5)	6.7 ± 0.4	21 ± 4[a]	(5)	6.8 ± 0.1	18 ± 1[a]
NaF	(5)	4.0 ± 0.1	98 ± 9[c]	(5)	4.5 ± 0.1[b,c]	89 ± 7[a]	(5)	7.2 ± 0.1	153 ± 13	(5)	7.2 ± 0.2[c]	134 ± 4[a]

Note. Each value shown is mean ± S.E.M. of *n* determinations (shown in parentheses) performed using different MVB preparations.
[a] $P < 0.01$ vs respective 14-week control.
[b] $P < 0.05$ vs control response in the presence of the inhibitor.
[c] $P < 0.05$ vs respective 14-week control.

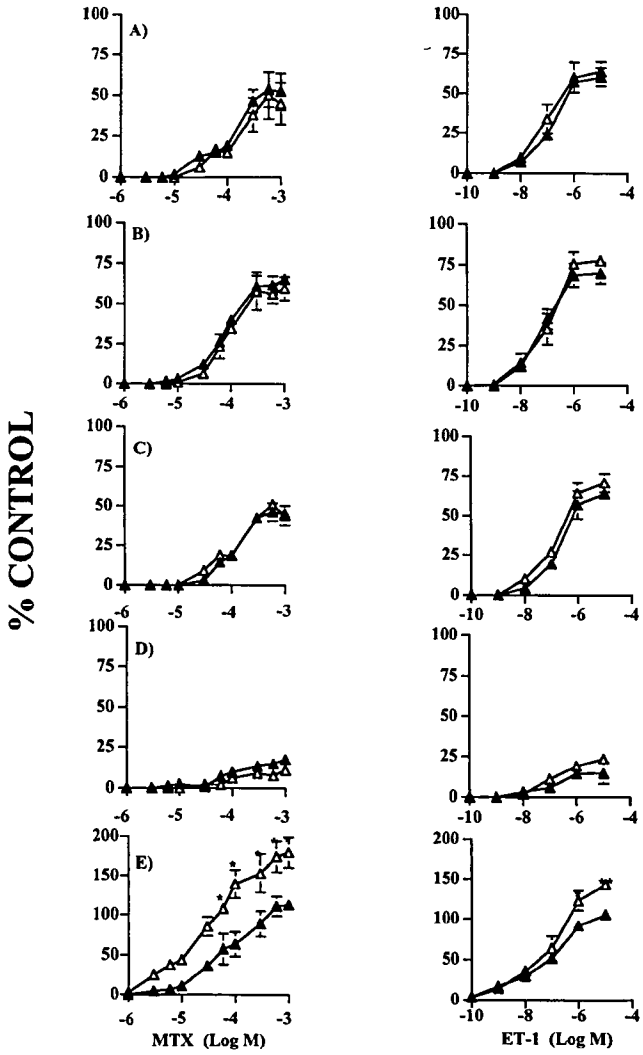

Figure 3. Concentration response curves to MTX (1.0 μM–1.0 mM [represented as –log M]; left panels) and ET-1 (0.1 nM–10 μM [represented as –log M]; right panels) in the perfused MVB of 14-week-old vehicle- or STZ-treated rats in the presence of staurosporine (5.0 nM) (A), U-73122 (1.0 μM) (B), nifedipine (3.0 μM) (C), cyclopiazonic acid (3.0 μM) (D), and sodium fluoride (10 mM) (E). Responses are represented as the percentage of perfusion pressure generated relative to the respective control. ●, Fourteen-week-old age-matched controls; △, 14-week-old STZ. Each data point shown is a mean ± S.E.M. of 4 to 5 separate determinations using different MVB preparations (*$P < 0.05$, **$P < 0.01$).

overt state of diabetes, which obviated vascular abnormalities and allowed for study of these changes. Indeed, there may be alterations, including deficiencies at other steps in the phosphatidylinositol signaling, that occur prior to 14 weeks. Nonetheless, clear evidence supporting alterations at the receptor/G protein level after 14 weeks of STZ-induced diabetes is provided.

We would like to thank Ms. D. Brown and Mr. B. Wilcox for their technical assistance.

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