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Enhanced Expression of Inducible Nitric Oxide Synthase May Be Responsible for Altered Vascular Reactivity in Streptozotocin-induced Diabetic Rats

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Growing evidence indicates that enhanced generation or actions of nitric oxide (NO) are implicated in the pathogenesis of hypertension in spontaneously hypertensive rats and diabetic nephropathy in streptozotocin (STZ)-induced diabetic rats. We investigated whether inducible nitric oxide synthase (iNOS) expression in STZ-induced diabetic rats is responsible for the alterations of vascular reactivity. Diabetic state was confirmed 28 days after injection of STZ (i.p) in rats by measuring blood glucose. In order to evaluate whether short term (4 weeks) diabetic state is related with altered vascular reactivity caused by iNOS expression, isometric tension experiments were performed. In addition, plasma nitrite/nitrate (NOx) levels and expression of iNOS in the lung and aorta of control and STZ-treated rats were compared by using Griess reagent and Western analysis, respectively. Results indicated that STZ-treated rats increased the maximal contractile response of the aorta to phenylephrine (PE), and high K^+, while the sensitivity remained unaltered. Endothelium-dependent relaxation, but not SNP-mediated relaxation, was reduced in STZ-treated rats. Plasma nitrite/nitrates are significantly increased in STZ-treated rats compared to controls. The malondialdehyde (MDA) contents of liver, serum, and aorta of diabetic rats were also significantly increased. Furthermore, nitrotyrosine, a specific foot print of peroxynitrite, was significantly increased in endothelial cells and smooth muscle layers in STZ-induced diabetic aorta. Taken together, the present findings indicate that enhanced release of NO by iNOS along with increased lipid peroxidation in diabetic conditions may be responsible, at least in part, for the augmented contractility, possibly through the modification of endothelial integrity or ecNOS activity of endothelium in STZ-diabetic rat aorta.

Key Words: Nitric oxide, Diabetes, Streptozotocin, Vascular smooth muscle, Peroxynitrite

INTRODUCTION

Impaired responses of cerebral arterioles to endothelium-dependent agonists may have an important implication for a mechanism by which diabetes contributes to stroke (William et al, 1991). Free radicals can participate in such reactions as the peroxidation of polysaturated fatty acids (Steel et al, 1991). Superoxide anion ($\cdot O_2^-$) from endothelial cells may favor development of arterial vasospasm not only by inactivating endothelium-derived relaxing factor (EDRF) (Gryglewski et al, 1986; Rubanyi & Vanhoutte, 1986) and inhibiting prostacyclin synthesis (Salmon et al, 1978; Gryglewski et al, 1986) but also by having a direct vasoconstrictor action on the vascular smooth muscle (Gryglewski et al, 1986; Rubanyi & Vanhoutte, 1986).

Now there is a strong evidence that nitric oxide (NO) contributes to the development of experimentally-induced diabetes (Seftel et al, 1997) as well as to an altered oxidant-antioxidant balance as a predisposing factor in animal models of diabetes (Uzel et al, 1987; Jongkind et al, 1989). Disturbances of micronutrient status may be a predisposing factor, but

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how these observations in animal models relate to the etiology of insulin-dependent diabetes mellitus (IDDM) or non insulin-dependent diabetes mellitus (NIDDM) is presently unclear. There is also an evidence which suggests that reactive oxygen species are involved (ROS) in diabetic complications in animal models and diabetic patients (Matkovics et al, 1982).

The present study was designed to investigate whether altered vascular reactivity in STZ-induced diabetic rat aorta is due to the enhanced expression of inducible nitric oxide synthase (iNOS). We found that peroxynitrite played a primary role in disrupting the endothelial integrity in STZ-induced diabetic rats and was responsible for the increased vascular contractile force to vasoconstrictors.

METHODS

Induction of diabetes

Male Wistar rats (16 wks old, 350～400 g) were randomly divided into two groups: diabetic and control. Each group consisted of 4～8 animals. The diabetic group received a single intraperitoneal injection of streptozotocin (60 mg/kg; dissolved in 0.01 M citrate buffer, pH 4.5) in order to induce diabetes. Age-matched control rats were injected with sodium citrate buffer alone. All animals were provided water ad libitum for 4 wks before sacrifice. Serum glucose levels were determined by glucose oxidase methods (Wendell & Nelson, 1986).

Preparation of tissues

Rats were weighed, anesthetized with diethyl ether, and the thoracic aorta was carefully excised and placed in oxygenated (95% O_2～5% CO_2) Krebs-Ringer bicarbonate solution. Each aorta was cleaned of loosely adhering fat and connective tissue and cut into rings of 5 mm length. Care was taken to ensure that the endothelial layer was not damaged during tissue preparation. Where indicated, the endothelium was removed by gently rubbing the intimal surface of the ring with a wooden rod. The composition of the Krebs-Ringer bicarbonate solution was as follows (in mM): 118.5 NaCl, 4.74 KCl, 2.5 $CaCl_2$, 1.18 $MgSO_4$, 1.18 KH_2PO_4, 24.9 $NaHCO_3$, 10.0 glucose, and 0.03 EDTA.

Contraction studies

Each ring was suspended by a pair of stainless hooks in a water-jacketed bath filled with 10 ml of Krebs-Ringer bicarbonate solution. A resting tension of 2 g was applied to the aortic rings, which were then allowed to equilibrate for 90 min before experimental procedures were initiated. Isometric tension was induced using a submaximal concentration of phenylephrine (PE, 10 nM～10 μM), and successful removal of the endothelium was confirmed by the inability of tissues to relax in response to acetylcholine (Chang et al, 1992).

Malondialdehyde content

Levels of malondialdehyde (MDA) were determined by the thiobarbituric acid (TBA) reaction (Yagi, 1975; Chang et al, 1993). Incubations were carried out in triplicate and were terminated by the addition of 0.5 ml of 50% TCA, followed by the addition of 1.0 ml of 0.67% TBA. Samples were then heated in a boiling water bath for 15 min, cooled to 4°C and centrifuged (4000 × g for 10 min). The optical density of the supernatant was determined at 535 nm, and the amount of MDA in the samples was calculated using a standard curve prepared with malondialdehyde bis (dimethyl acetal). The assay was linear over the range of 0.5 to 15 nmol of MDA. Samples that remained at 37°C did not contain detectable levels of MDA, indicating that no additional MDA was produced during the boiling procedure with TBA.

Nitrite/nitrate assays

The stable metabolic product of NO, nitrite/nitrate were determined in deproteinated plasma by Griess reagent (Green et al, 1982). All samples were kept at −70°C until analysis. To a 500 μl sample was added 100 μl 35% sulfosalicylic acid, and this was reacted for 30 min at room temperature for decomposition. For assay of nitrite and nitrate of samples, 200 μl sample containing 100 ml of 200 mM ammonium formate (including 100 mM HEPES, Sigma Chemical Co.) was reduced to nitrite at 37°C for 1 h by adding 100 μl nitrate reductase [E.Coli (ATCC-25922)], followed by centrifugation to precipitate nonreacting E.Coli for 5 min, and then the nitrite was quantified. Nitrite production was quantified by read-

ing optical density at 540 nm and calculated from the standard curve using $NaNO_2$.

Western analysis

The protein levels of iNOS was determined by Western analysis from the lung and aortic tissues of control and diabetic rats. Protein samples were electrophoretically size-separated with a discontinuous system, consisting of a 6% polyacrylamide resolving gel and 5% polyacrylamide stacking gel, and transferred to a nitrocellulose membrane at 20 V and 100 mA overnight. The membranes were washed, blocked, incubated with a 1 : 2500 dilution of monoclonal mouse-anti-iNOS antibody (Transduction Laboratories, Lexington, KY, USA), and then incubated with a horseradish peroxidase-labelled goat anti-mouse IgG (1 : 1000). The bound secondary antibody was detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). The protein levels of the iNOS were determined by analyzing the signals of Western blot autoradiograms using BioRad densitometer.

Immunohistochemical localization of nitrotyrosine

Tyrosine nitration, a specific 'foot print' of peroxynitrite formation, was detected in aorta sections by immunohistochemistry according to Cuzzocrea et al. (1998). Control and STZ-treated rat aorta was fixed in 10% buffered formalin and 8 μm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in phosphate buffered saline for 20 min. Non-specific adsorption was minimized by incubating the section in 2% normal goat serum in phosphate buffered saline for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin. The sections were then incubated overnight with 1 : 1000 dilution of primary anti-nitrotyrosine antibody or with control solutions. Controls included buffer alone or non specific purified rabbit IgG. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex.

Drugs

Drugs used were as follows. Streptozotocin, phenylephrine hydrochloride, acetylcholine, sodium nitroprusside, indomethacin, and ferricytochrome C were obtained from Sigma Chemical Co. (St. Louis, MO). Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex were obtained form Vector Laboratories (Burlingame, CA, USA). Primary anti-nitrotyrosine antibody was from Upstate Biotech (Saranac Lake, NY, USA). iNOS probe was obtained from Professor H.T. Chung at Wonkwang University.

Statistical analysis

All values are expressed as mean$\pm$SE. Contractile responses to each agonist were calculated as the increase in tension (g). Contents of MDA were expressed as nmol/mg wet weight/min. Statistical analysis of the data was performed by Student's unpaired t test for the comparison of two groups and one-way analysis of variance for the comparison of three or more groups. When F was significant, differences between individual groups were calculated with Turkey's test. Differences were considered statistically significant when $P<0.05$.

RESULTS

General features of experimental animals

The general features of the STZ-treated rats and their age-matched controls are shown in Table 1. The STZ-treated rats had significantly reduced body weights and elevated serum glucose levels compared with control animals at the time of sacrifice. The STZ-treated rats demonstrated other symptoms commonly associated with diabetes mellitus, including polyuria, diarrhea, and cataracts.

Table 1. General characteristics of control and STZ-diabetic rats

	Control	STZ-treated rats
Serum glucose (mg/dl)	133.3 ± 4.75^a $(4)^b$	549.6 ± 66.3* (6)
Body weight (g)	402 ± 4 (4)	324 ± 15* (6)

[a]: the mean$\pm$S.E.
[b]: number of experimental animal.
*: $p<0.05$ between control and STZ-treated rats.

Table 2. Maximum responses and pD_2 values to phenylephrine (PE) or KCl for control (C) and streptozotocin (STZ)-treated rats, with (+) and without (−) endothelium (E).

		C (+E)	STZ (+E)	C (−E)	STZ (−E)
PE	Maximum response (g)	1.18±0.9 (4)[c]	2.13±0.1[a] (6)	1.81±0.3 (3)	2.37±0.1[b] (6)
	pD_2 (M)	7.30±0.6[d] (4)	7.52±0.1 (6)	7.51±0.1 (3)	7.97±0.2 (7)
KCl	Maximum response (g)	1.05±0.07 (3)	2.12±0.04[a] (3)	N.D.	N.D.
	pD_2 (M)	1.70±0.16	1.69±0.11	N.D. (3)	N.D. (3)

[a]: P<0.05 between control and STZ with endothelium intact aorta.
[b]: P<0.05 between control and STZ with endothelium denuded aorta.
[c]: number of experiments.
[d]: the mean±S.E.
N.D.: not determined.

Agonist-induced contraction

The magnitude of the contractile response, but not the sensitivity, of aortas from STZ-treated animals to KCl (30~100 mM) and PE (10 nM~10 μM) was significantly greater than controls (Table 2). In control preparations, endothelium-denuded aortas showed from significantly (P<0.05) greater response than endothelium-intact ones in PE-induced contraction. For example, the maximum contractile force of endothelium-intact and denuded control preparations was 1.18 ±0.9 g and 1.81±0.3 g, respectively. Although the maximum contractile forces in aortas from STZ-treated preparations of endothelium-intact and denuded were not statistically different [the former was 2.13±0.1, the latter 2.37±0.1, (P>0.05)], which was, however, significantly greater (P<0.05) than endothelium-intact control preparations.

Agonist-induced relaxation

The magnitude of carbachol, but not sodium nitroprusside (SNP) induced relaxation of arterial rings contracted with PE, was significantly (P<0.05) reduced in endothelium intact aortas from STZ-treated animals as compared to controls (Fig. 1), indicating that endothelial intergity and/or production of EDRF by muscarinic agonist was impared in STZ-treated rats.

Plasma nitrate and nitrite

In order to know whether plasma NOx levels are increased in STZ-induced diabetic conditions, blood samples were collected and NOx contents were examined by Griess reagent. As shown in Fig. 2, the NOx levels were significantly (P<0.05) higher in STZ-treated diabetic rats at days 28 than in control rats.

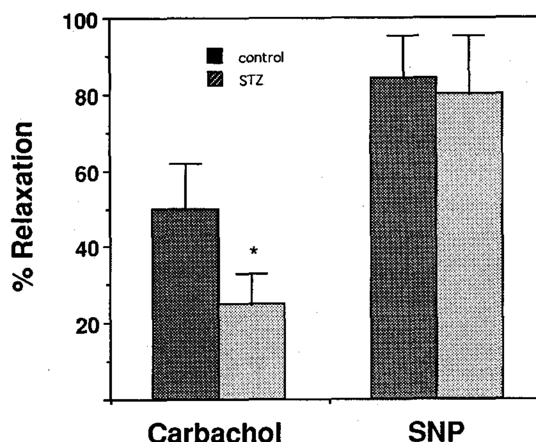

Fig. 1. Comparison of relaxation response to carbachol and SNP in aortic rings in control and STZ-treated rats. Rat thoracic aortic rings were precontracted submaximally with PE. Carbachol (1 μM) and SNP (10 nM) were added at the time of peak contractile response to PE (1 μM). Relaxation was expressed as percentage of contraction to PE. Each bar represents the mean±SE of 6 experiments. * Significantly greater than control values (p<0.05).

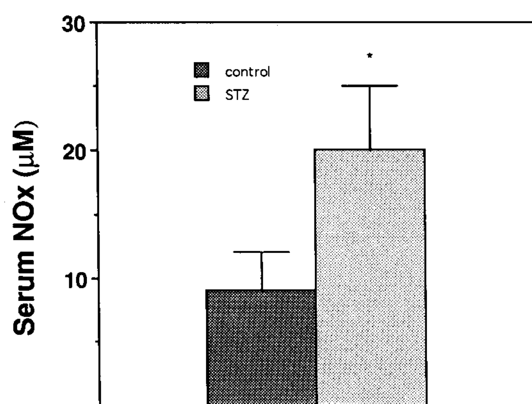

Fig. 2. Serum NOx leveles in STZ-induced diabetic and control rats. Blood was collected by cardiac puncture and samples were centrifuged and serum fraction was analyzed for its contents of nitrite/nitrate. Data represnt mean ±SE of 3 separate experiments. * significantly different at $p<0.05$.

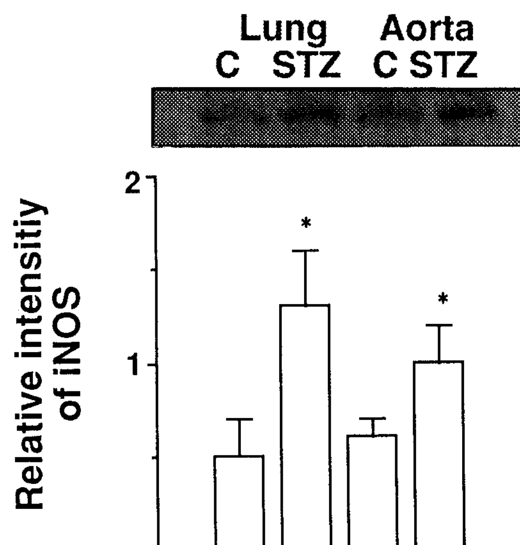

Fig. 3. Western blot analysis of iNOS protein expression in STZ-induced diabetic and control rat. Densitometic analysis shows in the lower pannel. Each point represents mean±SE of 3 separate experiments. * significantly different at $P<0.05$.

Expression of iNOS

To understand whether the increased levels of plasma NOx are due to the increased expression of iNOS, we compared the levels of iNOS expression in lung tissues from controls and STZ-induced diabetic rats. The inducible form of NOS was expressed in control rats as well as STZ-induced diabetic rats. However, the intensity of the iNOS expression was much stronger in STZ-treated tissues than in controls (Fig. 3).

Lipid peroxidation and $\cdot O_2^-$ *content*

In order to investigate whether there is enhanced lipid peroxidation in tissues from diabetic rats, we measured MDA, an indicator of lipid peroxidation in the liver, serum and aorta (Table 3), as well as $\cdot O_2^-$ levels in aorta from controls and diabetic rats (Fig. 4). The MDA content of the serum of diabetic rats was significantly ($P<0.05$) higher than that of controls. The MDA content of the liver and aorta of diabetic rats was also significantly ($P<0.05$) higher than that of controls.

Nitrotyrosine staining in STZ-induced diabetic aorta

Aortic sections were evaluated for the immunohistological staining for nitrotyrosine, a specific foot print of peroxynitrite. Staining for nitrotyrosine was

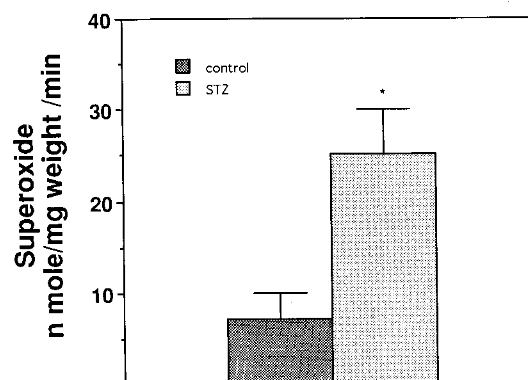

Fig. 4. Basal superoxide production in thoracic aorta from control and STZ-treated rat. Data represent the mean±SE of 4 experiments. * Significantly greater than control values ($p<0.05$).

absent in controls, but the staining of STZ-induced diabetic rats revealed a strong positive staining which appeared to be localized in endothelial and smooth muscle layers (Fig. 5). Although data were not presented here, electronmicroscopy examination showed that endothelial layer of STZ-induced diabetic rat aorta, but not that of controls, was damaged.

Table 3. MDA levels of serum, liver, and aorta in control and diabetic rats

	Control	Diabetes
Serum (n mole/10 μl)	3.6 ± 0.4^a $(3)^b$	7.1 ± 0.5* (5)
Liver (n mole/mg protein)	92.5 ± 16 (3)	255.2 ± 12* (5)
Aorta (n mole/mg wet weight)	5.7 ± 1.4 (3)	25.4 ± 3.2* (5)

[a]: the mean ± S.E.
[b]: number of experimental animal.
*: $P < 0.05$ between control and STZ-treated rats.

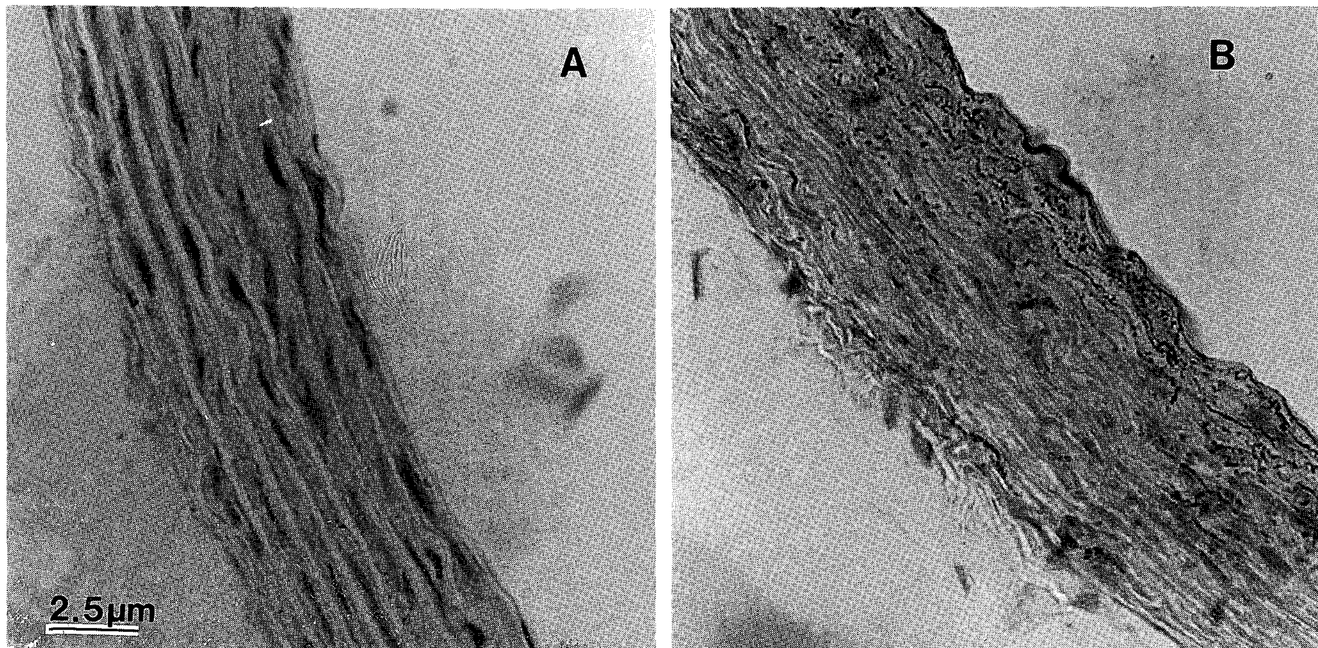

Fig. 5. Immunohistochemical localisation of nitrotyrosine in the aorta. Nitrotyrosine staining was absent in aorta from control rats (A). Nitrotyrosine was localized in the endothelial cells and smooth muscle layers in STZ-treated rat aorta (B).

DISCUSSION

The present results demonstrate that the magnitude of contractile response to PE and KCl was significantly enhanced in STZ-treated rat aorta without changing the sensitivity of the preparations to the agonists. These results are in accord with our previous report and others that diabetics and controls show no difference in the EC_{50} values for KCl and PE, while the maximum contractile responses to KCl and PE are significantly greater in diabetics than in controls (Head et al, 1987; White & Carrier, 1990; Masato et al, 1991; Chang et al, 1993). We demonstrated that the SNP-induced, endothelium-independent relaxation was not modified in diabetic rats. However, carbachol-induced, endothelium-dependent relaxation was significantly impaired in diabetic rats. This observation suggests that the increased maximal force of contraction in aortic preparations from diabetic rats was attributable to increased destruction of EDRF and/or decreased release or synthesis of EDRF possibly due to peroxynitrite (see below). The cGMP-pathway for relaxation in smooth muscle may not be impaired in diabetic aorta, since SNP-induced relaxation was not altered. It has been reported that $\cdot O_2^-$ causes endothelium-dependent vasoconstriction, possibly due to the inactivation of EDRF (Rubanyi & Vanhoutte, 1986; Gryglewski et al, 1986). $\cdot O_2^-$ may potentially

play an important role in the pathophysiology of complications associated with diabetes. We reported that $\cdot O_2^-$ content was significantly ($P<0.05$) augmented in diabetic rings compared with control ones (Chang et al, 1993). This was confirmed by others that the diabetic rat aorta released significantly more $\cdot O_2^-$ than control rat aorta (Pieper et al, 1992, Langenstroer & Pieper, 1992; Chang et al, 1993). As a consequence of $\cdot O_2^-$ reaction along with NO released from iNOS expression, peroxynitrite may be produced. Peroxynitrite is cytotoxic via a number of independent mechanisms. Its acute cytotoxic effects include initiation of lipid peroxidation and inactivation of a variety of enzymes such as membrane pumps and mitochondrial respiratory enzymes (Crow et al, 1995; Szabo et al, 1995). In this study, lipid peroxidation was assessed by measuring the tissue content of MDA, one of the end product of lipid peroxidation. There were some morphological changes in the endothelium of diabetic aorta, possibly due to the peroxynitrite production, since MDA and iNOS expression were significantly increased in the serum, liver, and aorta of diabetic rat.

In contrast to present results and others, Head et al. (1987) has provided equally compelling evidence for a decrease in contractile force of aorta from the diabetic rats. The reasons for this discrepancy are not clear, but may be related to the differences in the duration or severity of diabetes or to the release of endothelium-derived relaxation factor (EDRF/NO) in diabetes. It may be possible that the duration of diabetes influences the production of oxygen free radicals including $\cdot O_2^-$ and NO, which can partly account for the differences in muscle reactivity to agonists. Collectively, these inconsistent reports on vascular tone of diabetic aorta may suggest the potential importance of the quantity of ROS and argue against vascular tone of aorta on different duration of diabetes. Based on our present study, we speculate that the increased contractile response of aortas from diabetic rats may be related to changes in ecNOS activity and/or peroxynitrite formation which in turn results in reduced response to endothelium-dependent relaxation. It should be noted that whether the generation of large amounts of NO from iNOS expression in STZ-diabetic animals inhibits ecNOS activity by negative feed back mechanism in vascular smooth muscle is not known. There was a report that iNOS activity was decreased when inflammatory cytokines were allowed to express iNOS in human hepatocyts (Taylor et al, 1997).

In summary, our results suggest that the ability of STZ-induced diabetes to increase the contractility of aorta in response to vasoactive agonist, nonselectively, may be due to the increased expression of iNOS and $\cdot O_2^-$ production in various organs including vascular smooth muscle in diabetic conditions, since this feature is non-specific with respect to agonists.

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