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Impaired Endothelium-Dependent Relaxation is Mediated by Reduced Production of Nitric Oxide in the Streptozotocin-Induced Diabetic Rats

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To evaluate the involvement of nitric oxide production on the endothelium-dependent relaxation in diabetes, we have measured vascular and endothelial function and nitric oxide concentration, and the expression level of endothelial nitric oxide synthase in the streptozotocin-induced diabetic rats. Diabetic rats were induced by the injection of streptozotocin (50 mg/kg i.v.) in the Sprague-Dawley rats. Vasoconstrictor responses to norepinephrine (NE) showed that maximal contraction to norepinephrine (10^{-5} M) was significantly enhanced in the aorta of diabetic rats. Endothelium-dependent relaxation induced by acetylcholine was markedly impaired in the aorta of diabetic rats, these responses were little improved by the pretreatment with indomethacin. However, endothelium-independent relaxation induced by nitroprusside was not altered in the diabetic rats. Plasma nitrite and nitrate ($NO_2/_3$) levels in diabetic rats were significantly lower than in non-diabetic rats. Western blot analysis using a monoclonal antibody against endothelial cell nitric oxide synthase (eNOS) revealed that the protein level was lower in the aorta of diabetic rats than in non-diabetic rats. These data indicate that nitric oxide formation and eNOS expression is reduced in diabetes, and this would, in part, account for the impaired endothelium-dependent relaxation in the aorta of streptozotocin-induced diabetic rats.

Key Words: Streptozotocin-induced diabetic rats, Endothelium-dependent relaxation, Nitric oxide, Endothelial nitric oxide synthase

INTRODUCTION

Cardiovascular dysfunction and vascular injury are the leading causes of morbidity and mortality in diabetic patients (Garcia et al, 1974; Decker et al, 1978). Alteration in the production and action of endothelial cell products can contribute significantly to abnormal modulation of vascular tone in certain disease states (Rongen et al, 1994). Studies in experimental animals designed to investigate the mechanism of this dysfunction have implicated various factors including increased destruction by oxygen-derived free radicals (Langenstroer & Pieper, 1992; Pieper & Meier, 1992; Tesfamariam & Cohen, 1992; Diederich et al, 1994), release of an endothelium-derived constracting factor (Tesfamariam et al, 1989b; Mayhan et al, 1991), a decreased release or production of endothelium-derived relaxing factor (EDRF).

Nitric oxide (NO) release from the endothelium plays an important role in the regulation of vascular tone, inhibition of platelet and leukocyte aggregation and addhesion (Moncada et al, 1991). These properties suggest that the level of NO production by the endothelium may play a pivotal role in the regulation of vascular diseases. Endothelium-dependent vasodilation has been reported to be impaired in the early stages of insulin-dependent diabetic patients (Pieper & Gross, 1991; Calver et al, 1992; Kamata et al, 1992; Cohen, 1993; Ohnstone et al, 1993; McNally et

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al, 1994; Makimattila et al, 1996) and insulin-indepenent diabetic patients (McVeigh et al, 1992). Also vasodilator responses to agonists which release nitric oxide from endothelial cells are known to be impaired in both conduit (Pieper & Gross, 1988; Kamata et al, 1989; Cameron & Cotter, 1992) and resistance (Mayhan et al, 1991; Mayhan, 1992; Taylor et al, 1994; Ohishi & Carmines, 1995) vessels obtained from experimental diabetic animals.

Impaired agonist-stimulated Ca influx and NO production have been observed after exposure of rat or porcine endothelial cells to elevated glucose concentration (Salameh & Dhein, 1998). In vitro studies on human umbilical vein endothelial cells showed that an enhanced glucose flux inhibited NO production by blunting NOS activity although the type of NOS involved was not identified (Okuda et al, 1996). Endothelial dysfunction in fructose-fed rats and streptozotocin-diabetic rats may be due to a decreased synthesis of nitric oxide at least in the perfused kidney (Kamata & Yamashita, 1999). On the other hand, a number of studies have demonstrated increased NO release under conditions of hyperglycemia and diabetes (Sobrevia et al, 1996). Expression of mRNA and protein for eNOS was increased after culture with elevated glucose concentrations in human aortic endothelial cells for 5 days (Cosentino et al, 1997). Thus, there is some confusion in the scientific literature with regard to the role of NO/NOS in the induction of vascular dysfunction in diabetes. Also, there is little information available concerning measurement of endothelial nitric oxide synthase (eNOS) protein content in diabetes mellitus.

The aim of this study was to examine the involvement of nitric oxide on the impaired endothelium-dependent relaxation in the diabetic rats.

METHODS

Induction of diabetes

Male sprague-dawley rats (200~220 g) were divided randomly into non-diabetic and diabetic groups. All rats were housed in hanging cages and had access to food and water ad libitum. One group of rats was injected with streptozotocin (50 mg/kg i.v.) to induce diabetes. The second group of rats (non-diabetic rat) was injected with vehicle. Blood samples, for measurement of blood glucose concentration, were obtained at 10 weeks after injection of streptozotocin or vehicle, and on the day of the experiment.

Organ bath study

The thoracic aorta was carefully excised and excess connective tissue and fat were removed. In all instances, extreme care was taken not to touch the luminal surface or to stretch the preparation during the isolation so as not to damage the endothelium. Aortic segments were cut into rings and mounted on parallel wires in isolated tissue baths which were thermoregulated to 37°C. The medium consisted of a Tris-Tyrode's buffer containing (in mM) NaCl 158, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, glucose 6 and Tris base 6 maintained at pH 7.4 with 100% O_2. Rings were stretched to optimal resting tensions of 1.0 g. Isometric tension was measured using a Grass Model 7400 polygraph and FT03C force-displacement transducers. Each ring was equilibrated for 1 hr. At the plateau of tension development to norepinephrine, concentration-dependent relaxation to acetylcholine or nitroprusside was used to evaluate endothelium-dependent versus endothelium-independent vasodilation, respectively.

Nitric oxide measurements

Plasma (150 μl) was ultra-filtered by centrifugation at 12000 rpm for 30 min, using 10 kDa molecular weight filters (Ultrafree-MC, Millipore). NO assay was performed in a standard flat bottomed 96-well polystyrene microtitre plate containing 50 μl/well of standard or sample. The assay was blanked against phosphate-buffered saline (PBS). Fifty microlitres of nitrate reductase and β-NADPH were added to each well giving final concentration of 300 U/L and 25 μM, respectively. The plate was incubated at room temperature for 3 h. Excess β-NADPH was consumed by addition of 10 μl of PBS containing α-ketoglutaric acid (80 mM, final 4 mM), NH_4Cl (1 M, final 100 mM), and L-glutamic dehydrogenase (5 U/ml, final 500 U/L) followed by 10 min incubation at 37°C. The nitrite concentration was then measured by the addition of 50 μl each of Griess reagents 1 and 2, and the absorbance was read at 540 nm using a plate reader after 10 min incubation at room temperature.

Western blot analysis

Thoracic aortae were obtained from the control and the diabetic rats and homogenized on ice with a tissue homogenizer (Biospec Products Inc.) in the buffer composed of 50 mM Tris-HCl (pH 7.4), 2% SDS, 1 mM dithiothreitol, 1 μg/ml leupeptin, and 1 mM phenylmethylsulphonyl fluoride. The homogenized tissues were centrifuged at 10,000 g for 30 min and the supernant was stored at $-70^{\circ}C$ until further analysis. Aliquots of tissue homogenates were used for protein assay (Protein assay reagent, Bio-Rad, USA) and Western blot analysis. 50 μg of protein extracts obtained from the rat aortae were separated by 7.5% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane (Hybond ECL, Amersham, USA). Non-specific binding to the membrane was blocked by 5% nonfat dry milk in phosphate buffered saline-0.1% Tween 20 (PBS-T) for 2 hours at $25^{\circ}C$. The membrane blots were washed twice with PBS-T and then incubated with a mouse monoclonal antibody diluted 1 : 1500 (Transduction Laboratory, USA) which were raised against a 140 kDa peptide fragment corresponding to the amino acid sequence from 1,030 to 1,209 of the human endothelial nitric oxide synthase (eNOS) protein. The membranes were then incubated with a 1 : 1000 dilution of the rabbit anti-mouse Ig G conjugated to horseradish peroxidase (Amersham, USA). The signal strengths were analyzed using an ECL detection system (Amersham, USA) after autoradiography.

RESULTS

Characteristics of diabetic animals

A total of 13 diabetic and 10 non-diabetic rats were used for this study. Diabetic rats demonstrated significant elevation in serum glucose at 10 weeks after streptozotocin injection (50 mg/kg, i.v.). Blood glucose levels were elevated in diabetic rats (453.1 $\pm$ 14.9 mg/dl) compared to non-diabetic ones (107.0 $\pm$ 5.5 mg/dl). At the conclusion of the study, control rats weighed 444.3 $\pm$ 12.7 g while diabetic rats weighed 242.6 $\pm$ 12.6 g. Systolic blood pressure was elavated in diabetic rats (132.3 $\pm$ 2.8 mmHg) compared to non-diabetic ones (123.3 $\pm$ 3.3 mmHg)(Table 1).

Vascular reactivity studies

Norepinephrine induced the contraction in both control and diabetic aortic rings. Maximal contractile

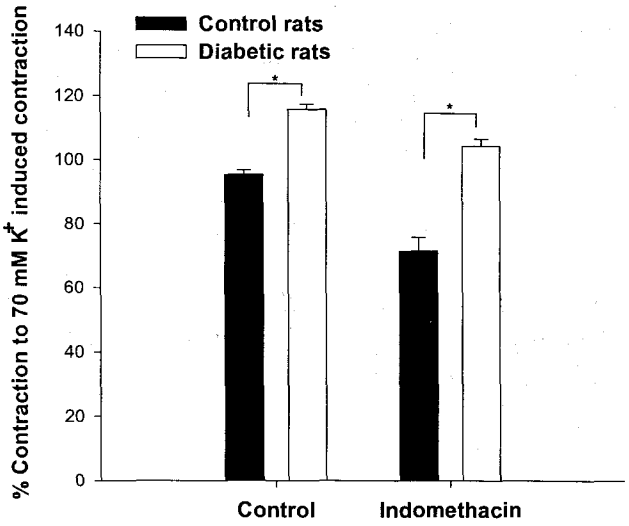

Fig. 1. Norepinephrine (10^{-5} M)-induced contraction in the absence and presence of indomethacin (2×10^{-6} M), an inhibitor of cyclooxygenase, in the aortic rings of control and diabetic rats. Results are presented as percentage contraction of the maximal vasocontraction induced by 70 mM K^{+}. *$P<0.05$ compared to control (n=4).

Table 1. General characteristics of streptozotocin-induced diabetic rats

Group	Control rats (n=10)	Diabetic rats (n=13)	Significance
Blood glucose (mg/dl)	107.0 $\pm$ 5.5	453.1 $\pm$ 14.9	$P<0.01$
Body weight (g)	444.3 $\pm$ 12.7	242.6 $\pm$ 12.6	$P<0.01$
Systolic blood pressure	123.3 $\pm$ 3.3	132.3 $\pm$ 2.8	$P<0.05$

*Systolic blood pressure was measured with tail-cuff method.

force by norepinephrine was expressed as percentage of 70 mM K^+-induced contraction. There was significant difference in the maximum tension development (95.5±1.3% and 115.7±1.7% for control and diabetic rats, respectively). Also, there was significant difference in tension development before and after indomethacin exposure in either control rings (95.5±1.3% to 71.6±4.3%, n=4 each) or in diabetic rings (115.7±1.7% to 104.3±2.2%, n=4 each)(Fig. 1).

Endothelium-dependent relaxation

Acetylcholine produced concentration-dependent relaxation in the precontracted rings with norepinephrine (10^{-5} M) of control and diabetic rats. The endothelium-dependent relaxation induced by acethylcholine was significantly attenuated in diabetic rings compared to non-diabetic control rings. Impaired endothelium-dependent relaxation was not improved by the pretreatment with indomethacin (2×10^{-6} M), an inhibitor of cyclooxygenase, in the aorta of diabetic rats (Fig. 2).

Endothelium-independent relaxation

Sodium nitroprusside is well known as endothelium-independent vasodilator or nitric oxide donor. Endothelium-independent relaxation induced by sodium nitroprusside is not impaired in the aortic rings of diabetic rats compared with non-diabetic rats (Fig. 3).

Plasma nitric oxide level in diabetes

In order to evaluate the effect of diabetes on plasma nitric oxide concentration, the level of nitrite (NO_2) and nitrate (NO_3) as the stable metabolite of nitric oxide was measured using modified griess reaction in the plasma of both rats. Plasma nitrite and nitrate in diabetic and non-diabetic rats were 2.9 ±0.1 μM and 4.2±0.3 μM, respectively. Plasma nitrite/ nitrate in diabetic rats was significantly decreased compared with that in non-diabetic rats (Fig. 4).

The expression of eNOS protein in diabietes

To evaluate the effect of diabetes on the protein expression for endothelial nitric oxide synthase, eNOS protein levels in aortic homogenate were measured using the western blots using monoclonal

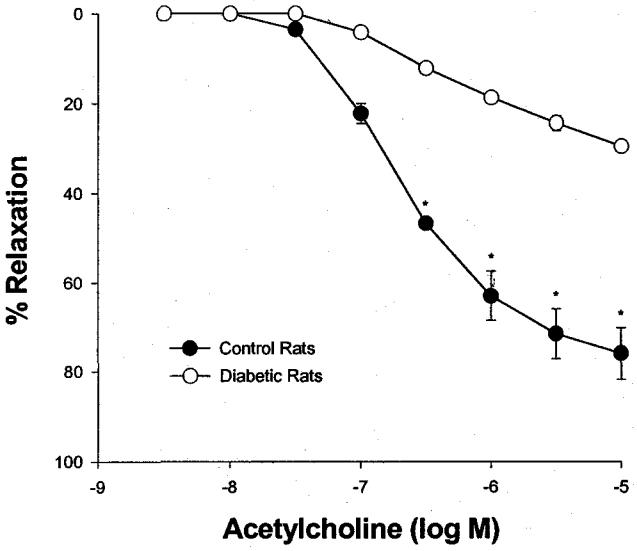

Fig. 2. Endothelium-dependent relaxation induced by acetylcholine in the presence of indomethacin (2×10^{-6} M, an inhibitor of cyclooxygenase) in the aorta of control (n=5) and diabetic rats (n=4). The results are presented as percentage relaxation of maximal contraction induced by norepinephrine (10^{-5} M).

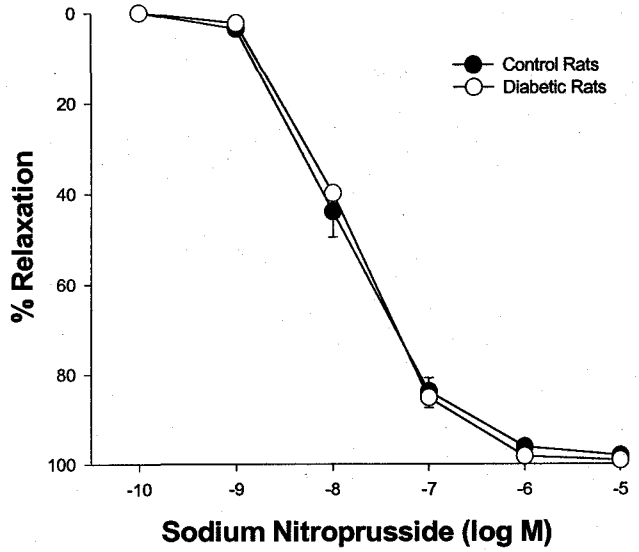

Fig. 3. Endothelium-independent relaxation induced by sodium nitrioprusside in the aortic rings of control and diabetic rats. The results are presented as percentage relaxation of maximal contraction induced by norepinephrine (10^{-5} M) in the aortic rings pretreated with L-nitroarginine methyl ester (10^{-4} M), an inhibitor of non-specific nitric oxide synthase.

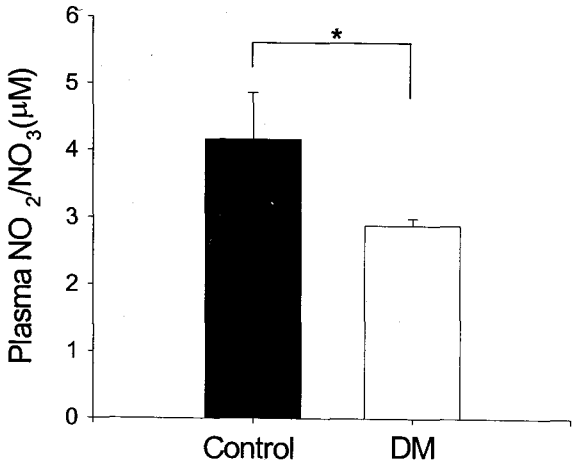

Fig. 4. Plasma nitrite/nitrate concentration in control and diabetic rats. After nitrate was converted to nitrite with nitrate reductase, nitrite/nitrate concentration was measured using modified Griess reaction. *P<0.05

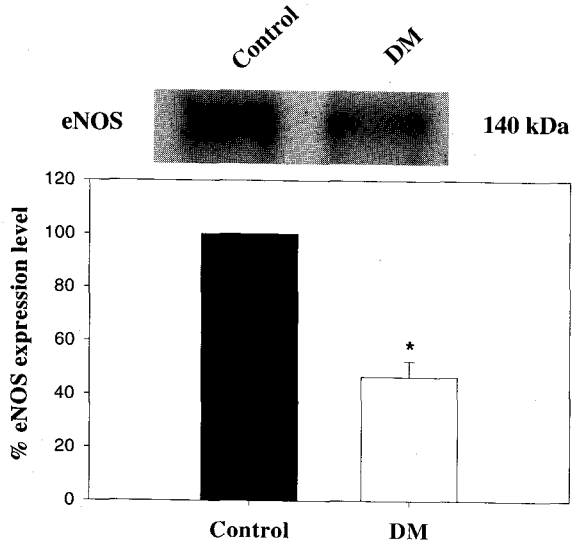

Fig. 5. Western blot analysis of aortic homogenates (50 μg of total protein) probed with a monoclonal antibody of endothelial nitric oxide synthase (n=4).

antibody and its level was analyzed using densitometry. As shown in Fig. 5, eNOS protein expression was significantly decreased in diabetic rats than in non-diabetic rats. The degree of eNOS protein expression in the aortic homogenate of diabetic rats was about 47±5.8% that of non-diabetic rats.

DISCUSSION

Nitric oxide is produced in the endothelium of normal vessels both in a basal release (Tesfamariam et al, 1989) and in response to a variety of endothelium-dependent vasodilator (Tesfamariam et al, 1989b; Martin et al, 1986). Endothelial nitric oxide synthase (eNOS) produces NO and citrulline from L-arginine in response to small and large perturbations in intracellular calcium. Production of nitric oxide from the constitutive nitric oxide synthase in endothelial cells is believed to contribute to the phenomenon known as endothelium-dependent relaxation of blood vessels. The conversion of the substrate, arginine, to form nitric oxide by this enzyme is dependent on the substrate concentration and the availability of cofactors such as calcium ion, calmodulin, and tetrahydrobiopterin and NADPH (Mlsch & Busse, 1991).

In the present study, to evaluate the endothelial dysfunction on the agonist induced contraction in diabetic rats, we measured the amplitude of norepinephrine-induced contraction and endothelium-dependent relaxation in the aortic ring of diabetic rats. The amplitude of NE-induced contraction in diabetic rats was greater than that in control rats. The contractions in the presence of indomethacin in diabetic rats were also greater than in control rats. This finding suggests that augmented agonist-induced contraction in the diabetic rats may be due to decreased release of endothelium-derived relaxing factor in the endothelial cells of diabetic rats.

Insulinopenic diabetes mellitus produces impaired endothelium-dependent relaxation in a variety of blood vessels (Pieper & Gross, 1991; Kamata et al, 1992; Cohen, 1993; Pieper & Peltier, 1995). In the present study, at the plateau of tension development to norepinephrine, concentration-dependent relaxation responses to acetylcholine or nitroprusside were conducted to evaluate endothelium-dependent versus endothelium-independent vasodilation, respectively. Endothelium-dependent relaxation induced by acetylcholine in the aortic rings pretreated with indomethacin was impaired significantly in diabetic rats. However, endothelium-independent relaxation induced by nitroprusside in the aortic rings pretreated with L-nitroarginine methyl ester was unaltered. These findings consistently indicate no impairment in relaxation vascular smooth muscle to nitrovasodilators suggesting no generalized impairment in the sensi-

tivity of diabetic vascular smooth muscle to activation of guanylate cyclase (Oyama et al, 1986; Mayhan, 1989; Tesfamariam et al, 1989; Kamata et al, 1992). Therefore, impaired endothelium-dependent relaxation in diabetic rats might be due to decreased release of nitric oxide in the endothelial cells of diabetic rats.

Several factors have been implicated in endothelial dysfunction. It has been suggested that elevated glucose, which exists in diabetes, may be a significant factor in the development of this dysfunction, since increases in glucose concentration either in vivo (Bohlen & Lash, 1993; Pieper et al, 1995) or in vitro (Tesfamariam & Cohen, 1992; Taylor & Poston, 1994) diminish endothelium-dependent vasodilation of normal blood vessels. By contrast, it is not known with certainty whether reductions in insulin levels which coincide with elevated glucose in the diabetic plays a direct role in regulating endothelium- dependent relaxation.

The influence of hyperglycemia and diabetes on the synthesis and release of NO by cells and tissues have been the subject of intense interest in recent years, with a number of studies suggesting either quenching of normally released NO or impaired NOS activity as possible mechanisms for these effects. Consistent with these reports are increases in mRNA and/or protein for eNOS after culture with elevated glucose concentrations in human aortic endothelial cells after 5 days (Consentino et al, 1997). In contrast, longer-term culture under high glucose conditions of human umbilical vein endothelial cells revealed no alteration in either mRNA or protein for eNOS (Mancusi et al, 1996). Recently, Chakravarthy et al (1998) reported that the reduction in the synthesis of the constitutively released NO by cultured endothelial cells, using an isolated amperometric NO sensor, which permitted the direct measurement of this gaseous molecule. This reduction in NO synthesis by glucose occurred in a dose-dependent manner, with the highest concentrations of glucose causing the most marked reduction in NO release. Metabolic competition for NADPH by aldorase reductase and NOS has been proposed as a potential mechanism of decreased NO production in diabetes (Stevens et al, 1995). Aldorase reductase consumption of NADPH may directly impair the activity of NOS, then NO production was decreased in diabetes.

The current investigation describes the reduction of NO release from rats which was treated with streptozotocin for 10 weeks. Significantly, we have also demonstrated the reduction in eNOS protein in rat aorta, suggesting a mechanism for the reduced synthesis of NO in diabetic rats. Decreased NO production in the diabetic rats may be due to decreased expression of eNOS in diabetic rats. These results indicate that chronic hyperglycemia in diabetic rats may play a significant role in the down-regulation of the eNOS protein. However, mechanism of down-regulation of eNOS protein by hyperglycemia was needed further evaluation.

From the above results, we suggest that decreased nitric oxide formation may be contributed to impaired endothelum-dependent relaxation in the streptozotocin-induced diabetic rats.

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