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Characterization of ET_B Receptor-mediated Relaxation in Precontracted Mesenteric Artery from Streptozotocin-induced Diabetic Rats

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Diabetes mellitus is associated with vascular complications, including an impairment of vascular function and alterations in the reactivity of blood vessels to vasoactive substances in various vasculature. In the present study, the authors have observed endothelin-B (ET_B) receptor agonist-induced relaxation in precontracted mesenteric arterial segments from streptozotocin (STZ)-induced diabetic rats, which was not shown from control rats or in other arterial segments from diabetic rats. Accordingly, the goal of this study was to investigate in what way STZ-induced diabetes altered reactivity of the mesenteric arterial bed and to examine the causal relaxation, if any, between this ET_B receptor-mediated relaxation and endothelial paracrine function, especially nitric oxide (NO) production. The relaxation induced by ET_B agonists was not observed in mesenteric arteries without endothelium. The relaxation to ET_B agonists was completely abolished by pretreatment with BQ788, but not by BQ610. Nω-nitro-L-arginine methyl ester and soluble guanylate cyclase inhibitors, methylene blue or LY83583 significantly attenuated the relaxant responses to ET_B agonists, respectively. When the expression of eNOS and iNOS was evaluated on agarose gel stained with ethidium bromide, the expression of eNOS mRNA in diabetic rats was significantly decreased, but the expression of iNOS was increased compared with control rats. Furthermore, the iNOS-like immunostaining was densely detected in the endothelium and slightly in the arterial smooth muscle of diabetic rats, but not in control rats. These observations suggest that ET_B receptor may not play a role in maintaining mesenteric vascular tone in normal situation. However, the alterations in ET_B receptor sensitivity were found in diabetic rats and lead to the ET_B agonist-induced vasorelaxation, which is closely related to NO production. In the state of increased vascular resistance of diabetic mesenteric vascular bed, enhanced NO production by activation of iNOS could lead to compensatory vasorelaxation to modulate adequate perfusion pressure to splanchnic area.

Key Words: ET_B receptors, Mesenteric artery, Relaxation, Streptozotocin, Diabetes, Nitric oxide

INTRODUCTION

Diabetes Mellitus is the most common serious metabolic disorder caused by altered insulin release and reactivity produced from the pancreas β-cell. Diabetes is associated with many vascular complications including atherosclerosis-induced hypertension, coronary heart disease, stroke and peripheral vascular disease (Lavy et al, 1973; Cohen et al, 1983; Wolf et al, 1983; Aboot et al, 1987), being the main factor to increase the adult mortality rate (Ruderman et al, 1992).

There have been extensive studies about the mechanism by which diabetes induces cardiovascular diseases but it is not yet definitely known. Some mechanisms demonstrated up to now are associated with the abnormal endothelium-dependent relaxation to vascular relaxing factor (Oyama et al, 1986; Meraji et al, 1987; Tesfamariam et al, 1989), and the altered regulation of vascular tone by the unbalanced production of endothelium-derived relaxing factors and contracting factors, causing changes in vascular reactivity (contraction and relaxation) (Furchgott & Vanhoutte, 1989; Moncada & Palmer, 1991) and in contractile response by abnormal calcium mobilization (Wang et al, 1998). In addition, some mechanisms are considered to be related to structural and morphological changes of endothelium (Colwell et al, 1979; Moore et al, 1985) including increased platelet adhesion and aggregation (Mayne et al, 1970; Heath et al, 1971; Sagel et al, 1975), the loss of endothelium, attenuation of cell junction, increased adhesiveness of neutrophils by altered specific adherence glycoprotein synthesis and dysfunction of endothelium

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ABBREVIATIONS: ET, endothelin; No, nitric oxide; STZ, streptozotocin; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide.

(Lorenzi, 1992), etc. Also, it has been reported that the attack rate of atherosclerosis was increased by the increased oxidized low density lipoprotein by blood lipid peroxidation (Makita et al, 1996), speculating endothelial dysfunction plays an important role in the development of diabetic vascular complications.

The endothelium releases endothelium-derived relaxing factor (EDRF) and endothelium-derived contracting factor (EDCF) in physiological and pathophysiological conditions and controls the tone of the underlying vascular smooth muscle and actively regulates vascular reactivity to various vascular activating factors (Furchgott & Vanhoutte, 1989). EDRFs comprise nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factor (EDHF) (Félétou & Vanhoutte, 1999). De Vriese et al (2000) reported impaired endothelial function to release these vascular activating factors was associated with the pathogenesis of diabetic vascular disease.

Among these various vascular activating factors, endothelin-1 (ET-1) was reported as a potent vasoconstrictor (Yanagisawa et al, 1988) isolated from the supernatant of cultured porcine endothelium. There have been many studies regarding its physiological role to regulate vascular tone and pathophysiological role (Levin et al, 1995). ETs are a family of 21-amino acid peptides and three isopeptides, ET-1, ET-2 and ET-3 have been identified (Itoh et al, 1988; Yanagisawa et al, 1988; Inoue et al, 1989). ETs have various biological roles in many tissues including vascular constriction and relaxation (Huggins et al, 1993).

Although there have been many reports about the roles of ETs on the development of diabetic vascular disease, there are still disputes. For example, clinical studies reported plasma ET-1 level was increased and vasoconstriction to ET-1 was attenuated in diabetic patients (Fulton et al, 1991; Hodgson & King, 1992). However, ET-1 level was decreased in porcine endothelium cultured in a hyperglycemic condition (Hattori et al, 1991) and ET-1-induced contraction was enhanced (Tammesild et al, 1992). ET-1 was also reported to be released from the endothelium by insulin (Yanagisawa et al, 1988), and inhibit insulin-stimulated glucose uptake (Chou et al, 1994), and induce insulin resistance (Juan et al, 1996; Ottosson-Seeberger et al, 1997). Based on these results, ET-1 is considered to participate in diabetic-induced vascular complications. Recently, there have been many studies about ET_B receptors such as selective down-regulation of ET_B receptors (Brothers et al, 2002) and changes in the density and localization of ET_B receptors (De Juan et al, 2000; Saito et al, 2000), attracting more attention to the related role of ET_B receptors in diabetes-induced vascular dysfunction.

In the present study, we observed ET_B receptor agonist-induced vasorelaxation in preconracted mesenteric arterial segments from streptozotocin (STZ)-induced diabetic rats, which was not shown from control rats or in other arterial segments from diabetic rats. Accordingly, the goal of this study was to clarify the mechanism by which STZ-induced diabetes altered reactivity of the mesenteric arterial bed and to examine the causal relaxation, if any, between this ET_B receptor-mediated vasorelaxation and endothelial paracrine function, especially NO production.

METHODS

Induction of diabetes

Male Sprague-Dawley rats (300~350 g) were treated with a single injection of streptozotocin (55 mg/kg, i.p.) in 0.1 M citrite buffer (pH 4.5). Age-matched control rats were treated with the vehicle (0.1 M citrite buffer, i.p.). All animals were allowed free access to food and water. One and 4 weeks after injection, the blood glucose levels of diabetic rats were measured with glucometer. Only rats displaying elevated blood glucose levels (200 mg/dl) were considered to be diabetic and control rats had normal blood glucose levels when tested at the same time.

Preparation of tissues

Rats were anesthetized with thiopental sodium (55 mg/kg, i.p.) and killed. Mesenteric arteries were carefully excised and placed in physiological salt solution (PSS). Each tissue was cleaned of adhering fat and connective tissue. All experiments were performed using PSS of the following composition (in mmol/L) : NaCl 130, $NaHCO_3$ 14.9, $MgSO_4$ 1.17, NaH_2PO_4 1.18, $CaCl_2$ 1.6, $NaHCO_3$ 14.9 and dextrose 5 mM. The solution was bubbled with 95% O_2-5% CO_2 to give a pH of 7.4 at 37°C.

Measurement of isometric tension

Mesenteric arteries were placed in a wax block containing oxygenated PSS and fat and connective tissue were removed. Arterial segments were cut into rings (2~3 mm) and mounted on parallel wires in 5 ml muscle chamber which were thermoregulated to 37°C. Rings were stretched to optimal resting tensions of 1.5 g. Isometric tension was measured using a Polygraphy (Grass Instrument Co., 7E) and force-displacement transducer (Grass Instrument Co., FT03). Following the equilibrated period for 90 minutes, the rings were exposed to 60 mM KCl to determine contractile function. To study the effects of antagonist, it was preincubated for 15 minutes. The relaxant responses to agonists were determined in vessels precontracted with 30 nM U46619. Concentration-responses curves were expressed as a percentage of the contractile response elicited by U46619.

Measurement of nitric oxide levels

Isolated mesenteric arteries were frozen in liquid nitrogen and homogenated by homogenizer (Brinkmann, Kinematiza CH-6010 KRIENS-LU) in 5 volumes of 0.1 M phosphate buffer (HEPES 100, sucrose 320, EDTA 0.1, dithiothreitol 1 mM). The homogenates were centrifuged (1,700 ×g, for 20 minutes) and protein quantity was measured with supernatants by Bio-Rad protein assay (Bradford, 1976). Griess reagent system kit (Promega) was used to measure nitrite/nitrate of plasma sample and tissue homogenate (50 μl). They were placed in 96 well and reacted with 50 μl sulfanilamide for 10 minutes at 25°C without light. And 0.1% N-1-naphthylethylenediamine 2HCl (50 μl) was added to each well. Ten minutes later, nitrite concentrations were determined at an optical density of 540 nm using Power Wave X340 (Bio-Tek Instruments, Inc.).

Immunohistochemistry

Isolated mesenteric arteries were placed in 0.01 M picric acid and 2% paraformaldehyde mixture (in 0.1 M sodium phosphate buffer) for fixation. Then, the sections were immersed in 7.5%, 15% and 30% sucrose solution (in 0.1 M sodium phosphate buffer) sequentially to dehydrate and frozen after embedding in OCT compound (Tissue-Tek, Miles Scientific Inc.). The sections were cut in 5 μm thick sections and mounted on poly-L-lysine coated slide glass and air-dried overnight. After fixation in cold aceton for 20 minutes, the sections were exposed to 0.3% hydrogen peroxide solution (Junsei Chemical Co.) for 20 minutes to inhibit the endogenous peroxidase activity and washed three times in phosphoric buffer solution (PBS) at 10-minutes interval. The sections were exposed to 2% bovine serum albumin (BSA, blocking antibody) for an hour to block nonspecific binding of antibody. And then eNOS antiserum (mouse monoclonal IgG_1 anti eNOS, Oncogene Research Products, 1 : 500) and iNOS antiserum (mouse monoclonal IgG_1 anti iNOS, Oncogene Research Products, 1 : 500) diluted in buffer (0.02 M PBS contained 0.05% BSA) were added to cover the sections. The sections were incubated at 4°C for overnight. They were then washed with PBS. Biotinylated goat anti-mouse IgG (Oncogene Research Products, 1 : 1,000) were applied onto the sections and incubated for two hours. After washing with PBS, the sections were incubated with avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain Elite ABC kit, Vector Laboratories, Inc.) for an hour. Peroxidase activity was visualized using a solution containing diaminobenzidine (DAB) substrate (Vector Laboratories, Inc.). The sections were dehydrated in graded alcohols and xylen and coverslipped with malinol (Muto Pure Chemicals Ltd.). Quantitative analysis of eNOS and iNOS were measured with image analysis system (Image-Proplus, Media Cybernetics, Silver Spring, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated with TRIzol reagent. RNA concentration was determined by measuring absorbance at 260 nm (A_{260}). The samples which showed A_{260}/A_{280} ratio from 1.5 to 2.0 RNA were reverse transcribed in 50 μl reaction mixture containing 100 U MMLV reverse transcriptase. The sense primer for eNOS was 5'-ATACCCTCAGTGCACAGGCT-3', and the antisense primer was 5'-TGATGGCTGAACGAAGATTG-3'. The sense primer for iNOS was 5'-GTGTTCCACCAGGAGATGTTG-3', and the antisense primer was 5'-CTCCTGCCCGCTGAGTTCGTC-3'. The sense primer for β-actin was 5'-TCATGAAGTGTGACGTTGACATCCGT-3', and the antisense primer was 5'-CCTAGAAGCATTTGCGGTGCACGATG-3'. Each 50 μl reaction mixture contained 1 μl of cDNA, 1 μl of each primer (100 pmol/L), 1 Unit of Taq DNA polymerase, 5 μl of 10× Taq polymerase buffer and optimal concentration of $MgCl_2$. The samples were placed onto a thermal cycler and preheated 1 minute at 94°C. Each cycle consisted of three periods: denaturation for 1 minute at 95°C, annealing for 1 minute at 55°C for iNOS, at 56°C for eNOS, and extension for 1 minute at 72°C. The PCR products were separated on a 2% agarose gel by electrophoresis. Quantitative analysis were measured with densitometer (GS-710, Bio-Rad Laboratories) and the quantity of each mRNA was expressed as a percentage to that of β-actin mRNA.

Statistics

Values are expressed as means±S.E.M. Results were statistically evaluated by Students' t-test for the differences between control and diabetic rats. $P<0.05$ was accepted as statistically significant.

RESULTS

Effects of diabetes induction in animals

There was no significant difference in STZ-preinjection weights of control and diabetic rats (Table 1). Four weeks postinjection, weights of control rats were increased (315± 9 g to 435±10 g), whereas diabetic rats showed a significant decrease in body weight (330±9 g to 215±6 g) ($P<0.01$). Control rats showed no significant difference in blood glucose level between STZ-preinjection and -postinjection. However, blood glucose level was markedly increased in diabetic rats at 4 weeks after STZ-injection (126±3 mg/dl to 615±17 mg/dl) ($P<0.01$).

ET_B receptor agonist-induced vasorelaxation

In mesenteric arteries, sarafotoxin and IRL 1620, ET_B receptor agonists, did not exert any effect on basal tone in both groups. However, these ET_B agonists induced relaxation in diabetic rats at low concentrations of 0.1~10 nM in the arterial segments precontracted with 30 nM U46619 which induced thromboxane A_2 and these relaxation disappeared from the concentration over 10 nM (Fig. 1A). After precontracting with phenylephrine, an α-adrenergic agonist, a similar trend of relaxation was observed (Fig. 1B).

We tested an identical experiment with thoracic aorta. Sarafotoxin S6c and IRL 1620 showed no effect on basal tone in thoracic aortic rings of both groups. However, being different from in the mesenteric artery, sarafotoxin S6c and IRL 1620 exerted no relaxation or weak contraction in diabetic rats (Fig. 2). Also, experiments with basilar artery and posterior cerebral artery showed similar results with thoracic aorta. Therefore, sarafotoxin S6c and IRL 1620-induced relaxations after pretreatment with U46619 showed tissue specificity.

Table 1. Body weight and blood glucose concentration in control and STZ-induced diabetic rats

	Control rats		STZ-treated rats	
	Before[+]	4 weeks[++]	Before[+]	4 weeks[++]
Body weight (g)	315±4 (20)	435±10 (20)	330±9 (23)	215±5** (23)
Blood glucose (mg/dl)	128±4 (20)	130±5 (20)	126±3 (23)	615±17** (23)

Values are expressed as means±S.E.M. Numbers in parentheses represent the number of experiments, respectively. **Value is significantly different from age-matched control ($P<0.01$). [+]Before: before vehicle or STZ. [++]4 weeks: 4 weeks after single infection of vehicle or STZ (55 mg/kg, i.p.).

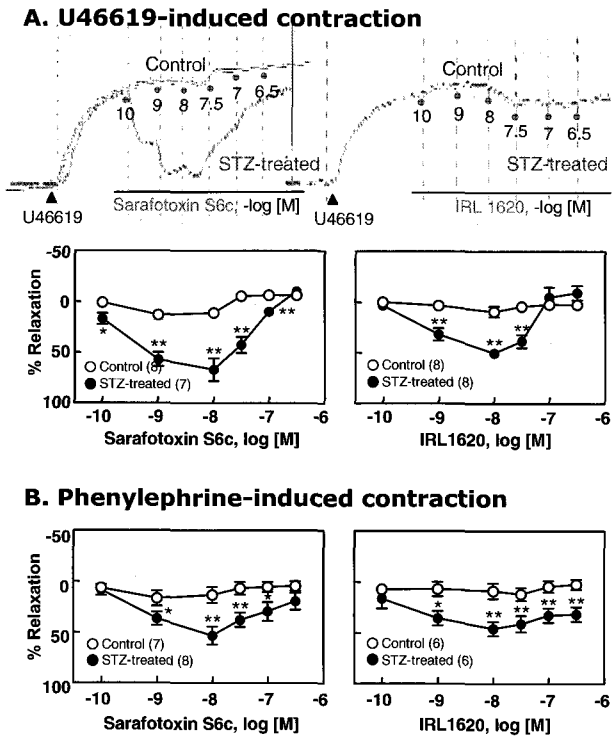

Fig. 1. ET_B receptor agonist-induced relaxation in the mesenteric arteries precontracted with 30 nM U46619, a thromboxane A_2 analogue (A) and phenylephrine, an a-adrenergic agonist from diabetic rats (B). Values are expressed as means±S.E.M. Numbers in parentheses represent the number of experiments. *P<0.05; **P<0.01 vs. control.

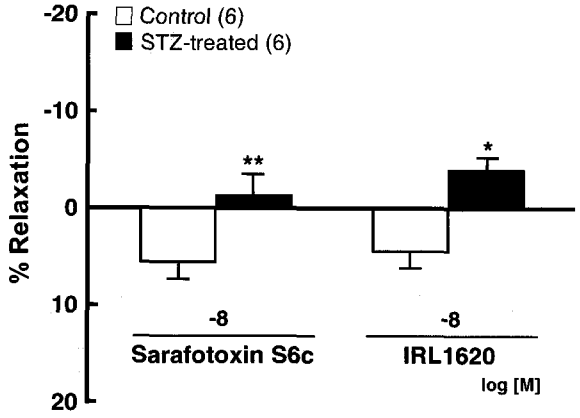

Fig. 2. ET_B receptor agonist-induced responses in thoracic aortic rings from control and diabetic rats. Each bar represents mean± S.E.M. The rings were precontracted with 10~30 nM U46619. *P< 0.05; **P<0.01 vs. control.

Characteristics of ET_B receptor agonist-induced relaxation

Effect of the endothelium on the relaxation: In this study, we tested if the activation of the endothelium played a role in ET_B receptor agonist-induced relaxation. The endothelium was removed by softly rubbing and then relaxations in response to sarafotoxin S6c and IRL 1620 were observed. Endothelium denudation was confirmed by the administration of acetylcholine (1 μM). Relaxation induced by ET_B receptor agonist was not observed in isolated mesenteric arteries without endothelium (Fig. 3).

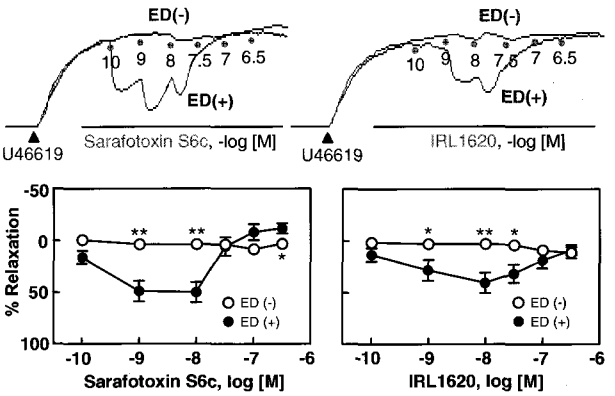

Fig. 3. Effect of endothelial denudation on ET_B receptor agonist-induced relaxation in isolated mesenteric arteries from diabetic rats. Values are expressed as means±S.E.M. of 6~7 experiments. Numbers in parentheses represent the number of experiments. *P<0.05; **P<0.01 vs. ED(+). ED(+) : intact endothelium; ED(−): without endothelium.

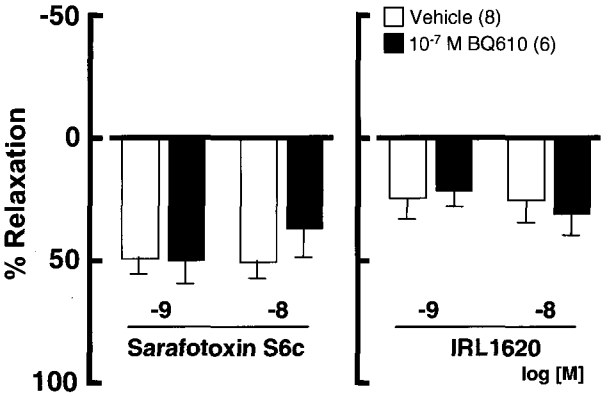

Fig. 4. Effect of BQ610, an ET_A receptor antagonist on sarafotoxin S6c- and IRL1620- induced relaxation in mesenteric arteries from diabetic rats. Values are expressed as means±S.E.M. Numbers in parentheses represent the number of experiments.

Blockade effect of ET receptor antagonists: To examine the contribution of ET receptor isopeptides in diabetic mesenteric artery, relaxations to sarafotoxin and IRL 1620 were observed after pretreatment with ET_A receptor antagonist, BQ610 and ET_B receptor antagonist, BQ788. The relaxations were not influenced by pretreatment with BQ610 (Fig. 4). However, ET_B receptor agonist-induced relaxation showed concentration-dependent attenuation by pretreatment with BQ788 and was abolished by 0.1 μM BQ788 (Fig. 5), demonstrating the relaxation in diabetic mesenteric artery is mediated by ET_B receptors.

Relation with endogenous prostaglandin: The pretreatment effect of indomethacin determined observed to

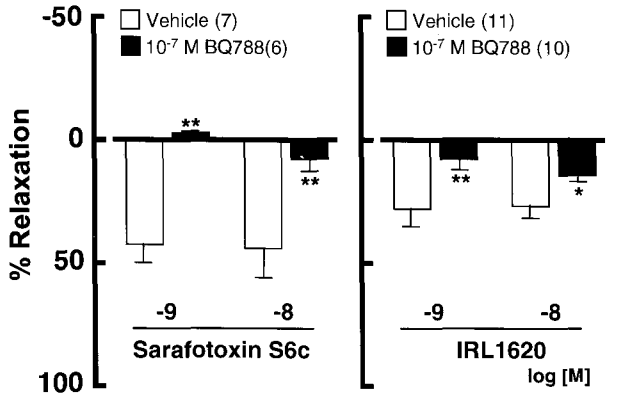

Fig. 5. Effect of BQ788, an ET_B receptor antagonist on sarafotoxin S6c- and IRL1620- induced relaxation in mesenteric arteries from diabetic rats. Values are expressed as means±S.E.M. Numbers in parentheses represent the number of experiments. *P<0.05; **P<0.01 vs. vehicle.

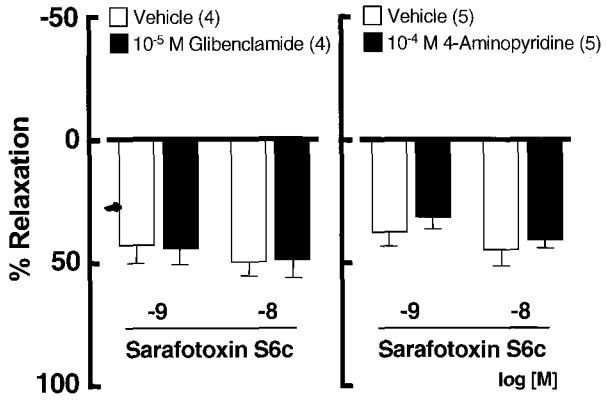

Fig. 7. Effect of K^+ channel blockers on sarafotoxin S6c-induced relaxation in mesenteric arteries from STZ-treated rats. Values are expressed as means±S.E.M. Numbers in parentheses represent the number of experiments.

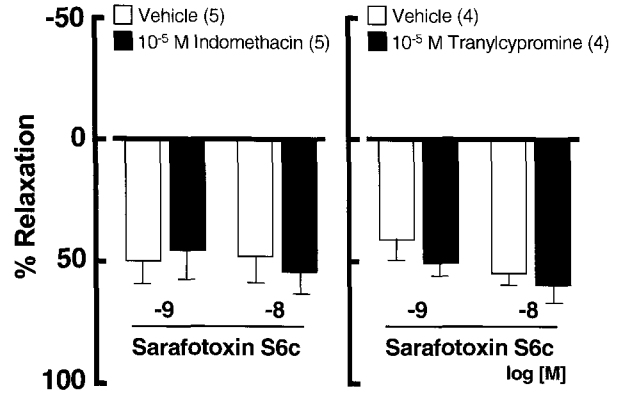

Fig. 6. Effect of prostaglandin synthesis inhibitors on sarafotoxin S6c-induced relaxation in mesenteric arteries from STZ-treated rats. Values are expressed as means±S.E.M. Numbers in parentheses represent the number of experiments.

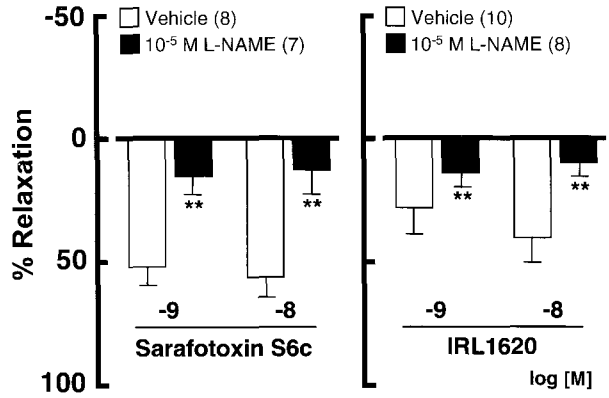

Fig. 8. Effect of L-NAME, an NO synthase inhibitor, on ET_B receptor agonist-induced relaxation in mesenteric arteries from STZ-treated rats. Values are expressed as means±S.E.M. Numbers in parentheses represent the number of experiments. **P<0.01 vs. vehicle.

investigate a possible mediation of endogenous prostaglandin to ET_B receptor agonist-induced relaxation. Pretreatment with 10 μM indomethacin (20 minutes) had no effect on the relaxation to sarafotoxin S6c and IRL 1620 (Fig. 6A), representing prostaglandin has no relation with ET_B receptor agonist-induced relaxation. Furthermore, the relaxation was not blocked by tranylcypromine (10 μM), a prostaglandin synthase inhibitor (Fig. 6B).

These results showed that endogenous prostaglandins, especially prostacyclin, played no role in the relaxation by sarafotoxin S6c and IRL 1620 in diabetic mesenteric artery.

Relation with K^+ channel activity: The endothelium causes vasorelaxation by producing EDRF like NO, and opening K^+ channel by the release of EDHF. To examine the possible involvement of endogenous EDHF in vasorelaxation in the mesenteric artery of diabetic rats, we used glibenclamide (10 μM), a K_{ATP} channel blocker and 4-amino-pyridine, an inhibitor of voltage-dependent K^+ channel, respectively. These K^+ channel blockers had no effect on the relaxation to sarafotoxin S6c (Fig. 7).

Blockade effect of NO synthase inhibitor on ETB receptor agonist-induced relaxation: Fig. 8 showed that ET_B receptor agonist-induced relaxation was inhibited or blocked by pretreatment with N-NAME (10 μM), a NO synthase inhibitor (P<0.01) (Fig. 8). In addition, soluble guanylate cyclase inhibitors, methylene blue and LY 83583 significantly attenuated the relaxant responses to sarafotoxin S6c (Fig. 9). These results suggested that endothelium-derived NO was closely related to the induction of ET_B receptor agonist-induced relaxation in diabetes. Thus, in the following experiments, we examined the production of NO and the distribution and expression of NO synthase in mesenteric artery to investigate the relation with NO produced in the endothelium.

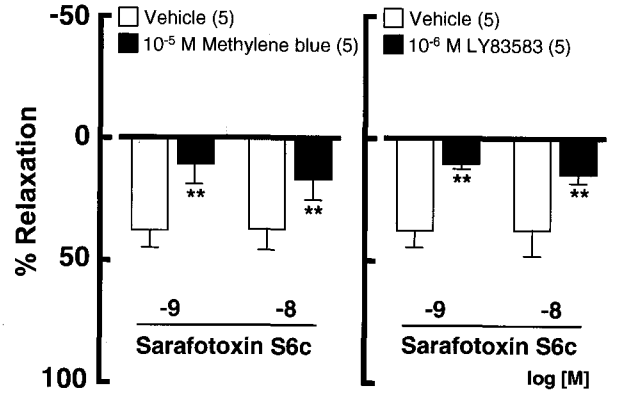

Fig. 9. Effect of methylene blue and LY83583, soluble guanylate cyclase inhibitors, on the sarafotoxin S6c-induced relaxation in mesenteric arteries from STZ-treated rats. Values are expressed as means±S.E.M. Numbers in parentheses represent the number of experiments. **P<0.01 vs. vehicle.

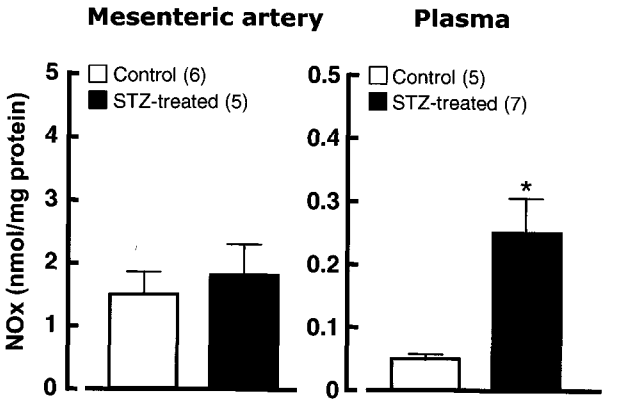

Fig. 10. Nitrite/nitrate (NOx) levels in the arterial homogenate and plasma. Values are expressed as means±S.E.M. Numbers in parentheses represent the number of experiments. *P<0.01 vs. control.

Changes of arterial and plasma NO levels by diabetes

In mesenteric arterial segments, nitrite/nitrate levels were 1.5±0.4 nmol/mg in control rats and increased to 1.8 ±0.4 nmol/mg in diabetic rats without a statistical significance (Fig. 10). Whereas the plasma nitrite/nitrate levels were 0.05±0.01 nmol/mg in control rats and significantly increase to 0.25±0.07 nmol/mg in diabetic rats (P <0.05).

Expression of NO synthase by immunohistochemistry

Fig. 11 showed eNOS immunostaining was detected abundantly in the endothelium of control rats, but not in diabetic rats. In contrast, whereas iNOS immunostaining was densely observed in the endothelium and slightly in the mesenteric arterial smooth muscle of diabetic rats, but not of control rats (Fig. 12).

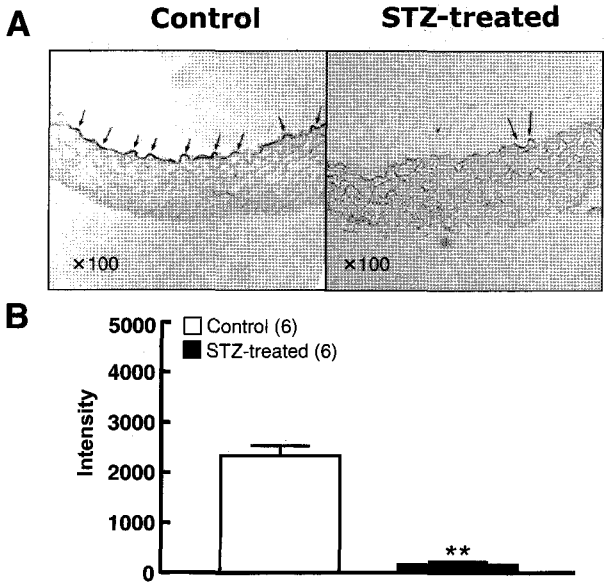

Fig. 11. (A) Visualization by immunohistochemistry of the eNOS expression in sections of the mesenteric arteries obtained from control (left) and STZ-treated rats (right). (B) Densitometric analysis of the immunohistochemical staining of eNOS expression. Values are expressed as means±S.E.M. Numbers in parentheses represent the number of experiments. **P<0.01 vs. control.

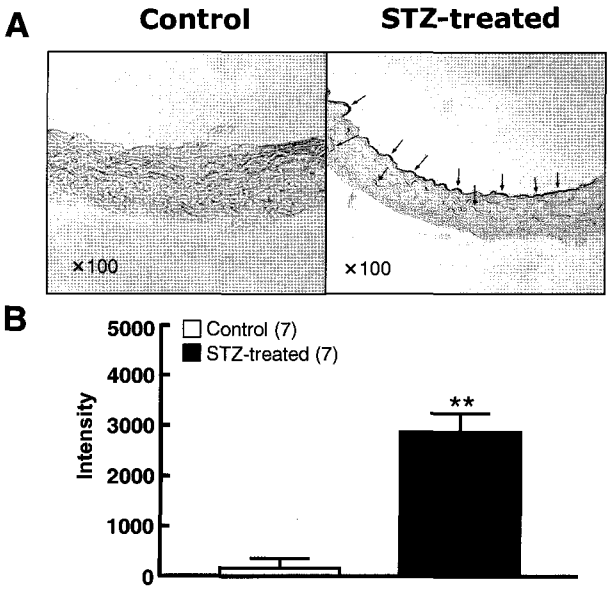

Fig. 12. (A) Visualization by immunohistochemistry of the iNOS expression in sections of the mesenteric arteries obtained from control (left) and STZ-treated rats (right). (B) Densitometric analysis of the immunohistochemical staining of iNOS expression. Values are expressed as means±S.E.M. Numbers in parentheses represent the number of experiments. **P<0.01 vs. control.

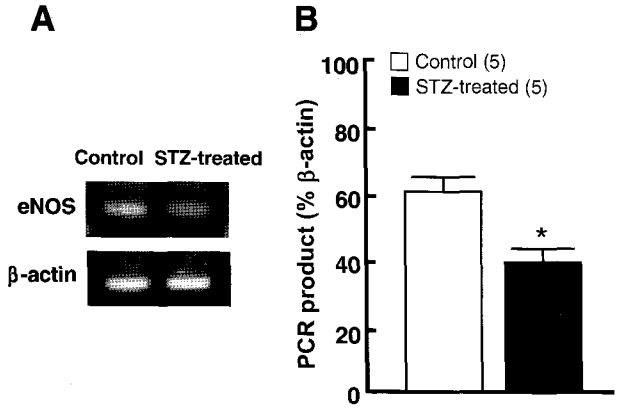

Fig. 13. Expression of eNOS mRNA by RT-PCR in mesenteric arteries from control and STZ-treated rats. (A) Ethidium bromide stained gel of RT-PCR products that reflects eNOS mRNA expression abundance. (B) The relative decrease in eNOS mRNA expression in mesenteric artery from STZ-treated rats. Results are expressed as means±S.E.M. *$P<0.05$ vs. control.

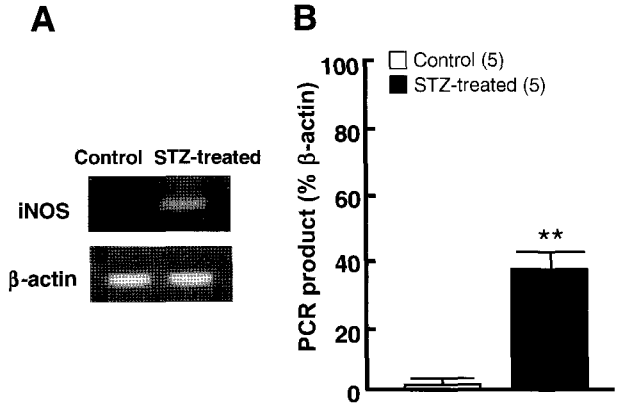

Fig. 14. Expression of iNOS mRNA by RT-PCR in mesenteric arteries from control and STZ-treated rats. (A) Ethidium bromide stained gel of RT-PCR products that reflects iNOS mRNA expression abundance. (B) The relative increase of iNOS mRNA expression in mesenteric artery from STZ-treated rats. Results are expressed as means±S.E.M. **$P< 0.01$ vs. control.

Expression of eNOS and iNOS mRNA by RT-PCR

When the expression of eNOS and iNOS mRNA was evaluated on agarose gels stained with ethidium bromide, the expression of eNOS mRNA in diabetic rats was significantly decreased compared with control rats ($P<0.05$). The expression of iNOS mRNA, however, was increased in diabetic rats ($P<0.01$) (Figs. 13 and 14).

DISCUSSION

To reveal pathophysiological mechanisms of vascular complications induced by diabetes mellitus, we set a hypothesis that ETs known as the most potent endogenous vasoconstrictor may play a key role in the regulation of vascular tone. The ET_B receptor agonists, sarafotoxin S6c and IRL 1620 induced concentration-dependent relaxation (Eglezos et al, 1993; Karaki et al, 1993) to the precontracted mesenteric artery with U46619 in diabetic rats. These relaxations were neither observed in control mesenteric artery, nor under basal tension in both control and diabetic rats.

In addition, this reaction was not observed in other vessels (e.g. thoracic aorta, basilar artery or posterior cerebral artery) of diabetic rats, confined in mesenteric artery. In this study, we concluded ET_B receptor agonist-induced relaxation in diabetic rats was closely related to diabetic vascular complications and the mechanisms were investigated.

Under basal tension, ET_B receptor agonist-induced relaxation was not observed in both control and diabetic rats, but in diabetic mesenteric artery precontracted with U46619. Thus, it could be concluded that ET_B receptor-mediated reaction was occurred as a compensatory regulation in the state of increased vascular resistance by diabetic complications. This conclusion was confirmed by the result that ET_B receptor agonist induced relaxation contracted with high concentration of K^+ (60 mM) and phenylephrine, an α-adrenergic agonist. Pretreatment with BQ788 showed no effect on the relaxation by sarafotoxin S6c and IRL 1620 in control rats. Thus, it was speculated that in control rats, ET_B receptors found on the endothelium might not be related to the regulation of vascular tone under basal tension or in case of the increased vascular tone by vascular contracting factors, otherwise the relaxation by ET_B receptors on the endothelium was in accord with the contraction by ET_B receptors on smooth muscle, showing no changes in vascular tone. But there is yet no explanation for this. Judging from the result of this study, ET_B receptor-induced relaxation was considered as a diabetes-induced pathophysiological phenomenon localized in specific tissues like mesenteric artery. In diabetic rats, ET_B receptor agonists-induced relaxation was not observed in isolated mesenteric artery without endothelium, indicating the relaxation resulted from endothelium dysfunction.

ET_A and ET_B receptors are known to exist in mammalia (Sakurai et al, 1990). ET_A receptors are found in smooth muscle and ET_B receptors in the endothelium and smooth muscle. ET_A receptors and ET_B receptors on smooth muscle induce contraction (Cardell et al, 1992; Martine et al, 1992), and ET_B receptors on the endothelium induce relaxation (Takayanagi et al, 1991; Martine et al, 1992; Allock et al, 1995). Based on these facts, we examined which receptor isoform mediated the relaxation by sarafotoxin S6c and IRL 1620. The relaxation didn't occur by pretreatment with BQ610, an ET_A receptor antagonist, whereas it was attenuated or blocked by pretreatment with BQ788, an ET_B receptors antagonist, suggesting the relaxations by sarafotoxin S6c and IRL 1620 were mediated by ET_B receptors on endothelium. It was supported by the fact that ET_B receptor agonist induced no relaxation in diabetic rats without endothelium. Moreover, it was reported that the expression of ET_B receptor mRNA in the retina was significantly increased in diabetic rats (Deng et al, 1999; Evans et al, 2000). Thus, it is assumed that ET_B receptor agonist-induced relaxation is caused by the increased expression of ET_B receptors by diabetes. However, it is not confirmed yet and more studies are needed about it.

Endothelium regulate vascular basal tone by releasing a variety of contracting and relaxing factors (Wheatcroft et al, 2003). Therefore, the present study was preferen-

tially focused on the relation between EDRF and ET_B receptor agonist-induced relaxation.

Kawai and Ohhashi (1991) reported the relaxation by $PGF_{2\alpha}$ was blocked by N^G-mono-methyl-L-arginine, a NO synthase inhibitor and thus the relaxation via NO-cyclic GMP pathway was associated with the production of prostaglandin. However, in the present study, ET_B receptor agonist-induced relaxation was not inhibited by indomethacin, indicating the production of endogenous prostaglandin by cyclooxygenase has no effect on the relaxation. This hypothesis was supported by the result that ET_B receptor agonist-induced relaxation was not affected by the pretreatment with tranylcypromine, prostacyclin synthase inhibitor (Weksler et al, 1977).

Although NO is the main vascular relaxing factor to cause endothelium-dependent relaxation, EDHF is also considered as an important factor to regulate vascular tension and reactivity in various vessels, especially in resistance vessels including small arteries (Félétou & Vanhoutte, 1999). However, EDHF does not seem related to ET_B receptor agonist-induced relaxation because ET_B receptor caused relaxation in the preparation contracted with 60 mM K^+. In this study, the relaxation was not inhibited by 4-aminopyridine, a voltage-dependent K^+ channel blocker and glibenclamide, an ATP-dependent K^+ channel blocker, which showed ET_B receptor agonist-induced relaxation in diabetes was not due to K^+ channel opening (Quandt, 1988; Rudy, 1988; Beech & Bolton, 1989).

ET_B receptor agonist-induced relaxation under the increased vascular tone during diabetes was blocked by L-NAME, a NO synthase inhibitor (Moore et al, 1990). Furthermore, methylene blue inhibited the relaxation by NO and the activity of soluble guanylate cyclase by nitrovasodilator (Gruetter et al, 1981; Griffith et al, 1985; Martin et al, 1985). LY 83583 was also known to inhibit the production of cyclic GMP by blocking soluble guanylate cyclase through NO oxidization (Malta et al, 1988). Considering the characteristics of L-NAME, methylene blue and LY83583, ET_B receptor agonist-induced relaxation seemed to be related with the production of NO or vascular cyclic GMP. NO is synthesized from the terminal guanidino-nitrogen atom of the amino acid L-arginine (Moncada et al, 1991). The produced NO binds to heme group of soluble guanylate cyclase present in vascular cytoplasm and cause the increase of cellular cyclic GMP (Rapoport & Murad, 1983), inducing the relaxation of vascular smooth muscle (Ignarro & Kadowitz, 1985).

These results suggest that the increased vascular tone during diabetes may activate ET_B receptors located on the endothelium as a compensatory reaction, which causes the increase of EDRF (NO) release from endothelium and cyclic GMP production from smooth muscle, leading to relaxation. Ikeda et al. (2001) reported that the up-regulation of the ET_B receptors observed in the diabetic adrenal gland may be a compensatory mechanism for maintaining adrenal blood flow. In addition, ET-1, which has an equal affinity to ET_A and ET_B receptors, induced vasorelaxation in diabetes (Ikeda et al, 2001). This result has been supported by many reports that the relaxation was due to the increased production of EDRF such as NO or prostacyclin (De Nucci et al, 1988; Tsukahara et al, 1994; Schillings et al, 1995; Gellai et al, 1996).

In the present study, plasma nitrite/nitrate levels were increased in diabetic rats. This result agreed with the previous report that renal NO production and urinary excretion of nitrite/nitrate was increased in diabetes. Jang et al. (1999) reported that the increased nitrite/nitrate production in diabetic tissues were due to the increased eNOS production (Sugimoto et al, 1998; Veelken et al, 2000), leaving arguments about the concerned NO synthase isoforms.

In this study, the expressions of eNOS and iNOS in mesenteric artery were observed by immunohistochemistry. Four isoforms of NO synthase were identified (Föstermann et al, 1991), but there have been many studies about two isoforms. One is constitutive NOS (cNOS) found from cerebellum (Bredt & Synder, 1990) or the endothelium. The other form is iNOS found from macrophages (Studhr et al, 1991), neutrophils (McCall et al, 1989), vascular smooth muscle cells (Busse & Mülschr, 1990) and the endothelium (Radomski et al, 1990).

Our immunohistochemical study showed eNOS distributed abundantly in the endothelium of control, but not in diabetic rats, whereas iNOS was densely detected in the endothelium and slightly in the mesenteric arterial smooth muscle of diabetic rats, but not of control rats. In addition, the expression of eNOS mRNA in diabetic rats was decreased (30%), but the expression of iNOS mRNA was significantly increased compared with control rats. Therefore, it could be concluded that ET_B receptor agonist-induced relaxation in the precontracted diabetic mesenteric artery was due to iNOS expression in the endothelium and smooth muscle cells. This result was different from the report that diabetes caused selective down-regulation of the density and sensitivity of ET_B receptor (Brothers et al, 2002). However, it was supported by the reports that ET_B receptor antagonists had effects on the progression of diabetic nephropathy (Hocher et al, 2001) and that the expression of ET_B receptor mRNA was increased in the mesenteric artery and thoracic aorta of the insulin resistant obese Zucker rats (Wu et al, 2000).

In conclusion, in the state of increased vascular resistance of diabetic mesenteric vascular bed, enhanced NO production by activation of iNOS leads to compensatory vasorelaxation to modulate adequate pressure to splanchnic area and ET_B receptor located on endothelium may play a pivotal role on this endothelial dysfunction.

REFERENCES

Aboot RD, Donahue RP, Macmahon SW, Reed DM, Yano K. Diabetes and the risk of stroke. *J Am Med Assoc* 257: 949–952, 1987

Allcock GH, Battistini B, Fournier A, Warner TD, Vane JR. Characterization of endothelin receptors mediating mechanical responses to the endothelins in the isolated stomach strip of the rat. *J Pharmacol Exp Ther* 275: 120–126, 1995

Beech DJ, Bolton TB. Properties of the cromakalim-induced potassium conductance in smooth muscle cells isolated from the rabbit portal vein. *Br J Pharmacol* 98: 851–864, 1989

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254, 1976

Bredt DS, Snyder SH. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci USA* 87: 682–685, 1990

Brothers TE, Grubbs AL, Zhang Y, Ergul A. Selective downregulation of endothelin-B receptors in diabetic African-American patients. *Ethn Dis* 12(S1): 46–50, 2002

Busse R, Mülsch A. Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett* 26: 87–90, 1990

Cardell LO, Uddman R, Edvinsson L. Evidence for multiple endothelin receptors in the guinea-pig pulmonary artery and trachea. *Br J Pharmacol* 105: 376–380, 1992

Chou YC, Perng JC, Juan CC, Jang SY, Kwok CF, Chen WL, Fong JC, Ho LT. Endothelin-1 inhibits insulin-stimulated glucose uptake in isolated rat adipocytes. *Biochem Biophys Res Commun* 202: 688–693, 1994

Cohen RA, Sheppherd JT, Vanhoutte PM. Inhibitory role of the endothelium in the response of isolated coronary arteries to platelets. *Science* 221: 273–274, 1983

Colwell JA, Halushka PV, Sargi KE, Lopesvirella MF, Sagel J. Vascular disease in diabetes. Pathophysiological mechanisms and therapy. *Arch Intern Med* 139: 225–230, 1979

De Juan JA, Moya FJ, Ripodas A, Bernal R, Fernandez-Cruz A, Fernandez-Durango R. Changes in the density and localization of endothelin receptors in the early stages of rat diabetic retinopathy and the effect of insulin treatment. *Diabetologia* 43: 773–785, 2000

Deng D, Evans T, Mukherjee K, Downey D, Chakrabarti S. Diabetes-induced vascular dysfunction in the retina: role of endothelins. *Diabetologia* 42: 1228–1234, 1999

De Nucci G, Thomas R, D'Orleans-Juste P, Antunes E, Walder C, Warner TD, Vane JR. Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc Natl Acad Sci USA* 85: 9797–9800, 1988

De Vriese AS, Verbeuren TJ, Van de Voorde J, Lameire NH, Vanhoutte PM. Endothelial dysfunction in diabetes. *Br J Pharmacol* 130: 963–974, 2000

Eglezos A, Cucchi P, Patacchini R, Quartara L, Maggi CA, Mizrahi J. Differential effects of BQ-123 against endothelin-1 and endothelin-3 on the rat vas deferens: evidence for an atypical endothelin receptor. *Br J Pharmacol* 109: 736–738, 1993

Evans T, Xi Deng D, Mukherjee K, Downey D, Chakrabarti S. Endothelins, their receptors, and retinal vascular dysfunction in galactose-fed rats. *Diabetes Res Clin Pract* 48: 75–85, 2000

Félétou M, Vanhoutte PM. The alternative: EDHF. *J Mo Cell Cardiol* 31: 15–22, 1999

Förstermann U, Schmidt HH, Pollock JS, Sheng H, Mitchell JA, Warner TD, Nakane M, Murad F. Isoforms of nitric oxide synthase. Characterization and purification from different cell types. *Biochem Pharmacol* 42: 1849–1857, 1991

Fulton DJ, Hodgson WC, Sikorski BW, King RG. Attenuated responses to endothelin-1, KCl and $CaCl_2$, but not noradrenaline, of aortae from rats with streptozotocin-induced diabetes mellitus. *Br J Pharmacol* 104: 928–932, 1991

Furchgott RF, Vanhoutte PM. Endothelium-derived relaxing and contracting factors. *FASEB J* 3: 2007–2018, 1989

Gellai M, Fletcher T, Pullen M, Nambi P. Evidence for the existence of endothelin-B receptor subtypes and their physiological roles in the rat. *Am J Physiol* 271: R254–R261, 1996

Griffith TM, Edwards DH, Lewis MJ, Henderson AH. Evidence that cyclic guanosine monophosphate (cGMP) mediates endothelium-dependent relaxation. *Eur J Pharmacol* 112: 195–202, 1985

Gruetter CA, Kadowitz PJ, Ignarro LJ. Methylene blue inhibits coronary arterial relaxation and guanylate cyclase activation by nitroglycerin, sodium nitrite, and amyl nitrite. *Can J Physiol Pharmacol* 59: 150–156, 1981

Hattori Y, Kasai K, Nakamura T, Emoto T, Shimoda SI. Effect of glucose and insulin on immunoreactive endothelin-1 release from cultured porcine aortic endothelial cells. *Metabolism* 40: 165–169, 1991

Heath H, Brigden WD, Canever JV. Platelet adhesiveness and aggregation in relation to diabetic retinopathy. *Diabetologia* 7: 308–315, 1971

Hocher B, Schwarz A, Reinbacher D, Jacobi J, Lun A, Priem F, Bauer C, Neumayer HH, Raschack M. Effects of endothelin receptor antagonists on the progression of diabetic nephropathy. *Nephron* 87: 161–169, 2001

Hodgson WC, King RG. Effects of glucose, insulin or aldose reductase inhibition on responses to endothelin-1 of aortic rings from streptozptocin-induced diabetic rats. *Br J Pharmacol* 106: 644–649, 1992

Huggins JP, Pelton JT, Miller RC. The structure and specificity of endothelin receptors: their importance in physiology and medicine. *Pharmacol Ther* 59: 55–123, 1993

Ignarro LJ, Kadowitz PJ. The pharmacological and physiological role of cyclic GMP in vascular smooth muscle relaxation. *Annu Rev Pharmacol Toxicol* 25: 171–191, 1985

Ikeda K, Wada Y, Sanematsu H, Foster HE Jr, Shin D, Weiss RM, Latifpour J. Regulatory effect of experimental diabetes on the expression of endothelin receptor subtypes and their gene transcripts in the rat adrenal gland. *J Endocrinol* 168: 163–175, 2001

Inoue A, Yanagisawa M, Kimura S, Yoshitoshi K, Miyauchi T, Goto K, Masaki T. The human endothelin family: Three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc Natl Acad Sci USA* 86: 2863–2867, 1989

Itoh Y, Yanagisawa M, Ohkubo S, Kimura C, Kosaka T, Inoue A, Ishida N, Mitsui Y, Onda H, Fujino M, Masaki T. Cloning and sequence analysis of cDNA encoding the precursor of a human endothelium-derived vasoconstrictor peptide, endothelin: identity of human and porcine endothelin. *FEBS Lett* 231: 440–444, 1988

Jang JK, Kang YJ, Seo HG, Seo SJ, Chang KC. Enhanced expression of inducible nitric oxide synthase may be responsible for altered vascular reactivity in streptozotocin-induced diabetic rats. *Korean J Physiol Pharmacol* 3: 375–382, 1999

Juan CC, Fang VS, Huang YJ, Kwok CF, Hsu YP, Ho LT. Endothelin-1 induces insulin resistance in conscious rats. *Biochem Biophys Res Commun* 227: 694–699, 1996

Karaki H, Sudjarwo SA, Hori M, Takai M, Urade Y, Okada T. Induction of endothelium-dependent relaxation in the rat aorta by IRL 1620, a novel and selective agonist at endothelin ET_B receptor. *Br J Pharmacol* 109: 486–490, 1993

Kawai Y, Ohhashi T. Prostaglandin F2α-induced endothelium-dependent relaxation in isolated monkey cerebral arteries. *Am J Physiol* 260: H1538–H1543, 1991

Lavy S, Melamed E, Cahane E, Carmon A. Hypertension and diabetes as risk factors in stroke patients. *Stroke* 4: 751–759, 1973

Levin ER. Endothelins. *N Engl J Med* 333: 356–363, 1995

Lorenzi M. Glucose toxicity in the vascular complications of diabetes: the cellular perspective. *Diabetes Metab Rev* 8: 85–103, 1992

Makita Z, Yanagisawa K, Kuwalima S, Bucala R, Vlassara H, Koike T. The role of advanced glycosylation end-products in the pathogenesis of atherosclerosis. *Nephrol Dial Transplant* 5(Suppl 11): 31–33, 1996

Malta E, Macdonald PS, Dusting GJ. Inhibition of vascular smooth muscle relaxation by LY83583. *Naunyn-Schmiedeberg's Arch Pharmacol* 337: 459–464, 1988

Martin W, Villani GM, Jothianandan D, Furchgott RF. Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J Pharmacol Exp Ther* 232: 708–716, 1985

Martine C, Gillian AG, Volker B, Bernd Michael L, Rolf O. The endothelin ET_B receptor mediates both vasodilation and vasoconstriction *in vivo*. *Biochem Biophys Res Commun* 186: 867–873, 1992

Mayne EE, Bridges JM, Weaver JA. Platelet adhesiveness, plasma fibrinogen and factor 8 levels in diabetes mellitus. *Diabetologia* 6: 436–440, 1970

McCall TB, Boughton-Smith NK, Palmer RM, Whittle BJ, Moncada S. Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. *Biochem J* 261: 293–296, 1989

Meraji S, Joyakody L, Senaratine MPJ, Thomson ABR, Kappagoda T. Endothelium-dependent relaxation in aorta of BB rat. *Diabetes* 36: 978–981, 1987

Moncada S, Palmer RM. Biosynthesis and actions of nitric oxide. *Semin Perinatol* 15: 16–19, 1991

Moore PK, al-Swayeh OA, Chong NW, Evans RA, Gibson A. L-NG-nitro arginine (L-NOARG), a novel, L-arginine-reversible

inhibitor of endothelium-dependent vasodilatation *in vitro*. *Br J Pharmacol* 99: 408–412, 1990

Moore SA, Bohlen HG, Miller BG, Evans AP. Cellular and vessel wall morphology of cerebral cortical arterioles after short-term diabetes in adult rats. *Blood Vessels* 22: 265–277, 1985

Ottosson-Seeberger A, Lundberg JM, Alvestrand A, Ahlborg G. Exogenous endothelin-1 causes peripheral insulin resistance in healthy humans. *Acta Physiol Scand* 161: 211–220, 1997

Oyama Y, Kawasaki H, Hattori Y, Kanno M. Attenuation of endothelium-dependent relaxation in aorta from diabetic rats. *Eur J Pharmacol* 131: 75–78, 1986

Quandt FN. Three kinetically distinct potassium channels in mouse neuroblastoma cells. *J Physiol* 395: 401–418, 1988

Radomski MW, Palmer RM, Moncada S. Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc Natl Acad Sci USA* 87: 10043–10047, 1990

Rapoport RM, Murad F. Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cGMP. *Circ Res* 52: 352–357, 1983

Ruderman NB, Williamson JR, Brownee M. Glucose and diabetic vascular disease. *FASEB J* 6: 2905–2914, 1992

Rudy B. Diversity and ubiquity of K channels. *Neuroscience* 25: 729–749, 1988

Sagel J, Colwell JA, Crook L, Laimins M. Increased platelet aggregation in early diabetes mellitus. *Ann Intern Med* 82: 733–738, 1975

Saito M, Wada Y, Ikeda K, Wang Z, Foster HE Jr, Smith SD, Weiss RM. Expression of endothelin receptor subtypes and their messenger RNAs in diabetic rat prostate: effect of insulin treatment. *Mol Cell Biochem* 210: 1–12, 2000

Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, Masaki T. Clonong of a cDNA encoding a non- isopeptide-selective subtype of the endothelin receptor. *Nature* 348: 732–735, 1990

Schilling L, Feger GI, Ehrenreich H, Wahl M. Endothelin-1-induced contraction and relaxation of isolated rat basilar artery: effect of the endothelium. *J Cardiovasc. Pharmacol* 26(Suppl 3): S197–S199, 1995

Stuehr DJ, Cho HJ, Kwon NS, Weise MF, Nathan CF. Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc Natl Acad Sci USA* 88: 7773–7777, 1991

Sugimoto H, Shikata K, Matsuda M, Kushiro M, Hayashi Y, Hiragushi K, Wada J, Makino H. Increased expression of endothelial cell nitric oxide synthase (ecNOS) in afferent and glomerular endothelial cells is involved in glomerular hyperfiltration of diabetic nephropathy. *Diabetologia* 41: 1426–1434, 1998

Takayanagi R, Kitazumi K, Takasaki C, Ohnaka K, Aimoto S, Tasaka K, Ohashi M, Nawata H. Presence of non-selective type of endothelin receptor on vascular endothelium and its linkage to vasodilation. *FEBS Lett* 282: 103–106, 1991

Tammesild PJ, Hodgson WC, King RG. Increased sensitivity to endothelin-1 in isolated Krebs'-perfused kidneys of streptozotocin-diabetic rats. *Clin Exp Pharmacol Physiol* 19: 261–265, 1992

Tesfamariam B, Jakubowski J, Cohen RA. Contraction of diabetic rabbit aorta due to endothelium-derived PGH_2/TXA_2. *Am J Physiol* 257: H1327–H1333, 1989

Tsukahara H, Ende H, Magazine HI, Bahou WF, Goligorsky MS. Molecular and functional characterization of the non-isopeptide-selective ET_B receptor in endothelial cells. Receptor coupling to nitric oxide synthase. *J Biol Chem* 269: 21778–21785, 1994

Veelken R, Hilgers KF, Hartner A, Haas A, Bohmer KP, Sterzel RB. Nitric oxide synthase isoforms and glomerular hyperfiltration in early diabetic nephropathy. *J Am Soc Nephrol* 11: 71–79, 2000

Wang R, Liu Y, Sauve R, Anand-Srivastava MB. Diabetes-related abnormal calcium mobilization in smooth muscle cells are induced by hyperosmolality. *Mol Cell Biochem* 183: 79–85, 1998

Weksler BB, Marcus AJ, Jaffe EA. Synthesis of prostaglandin I2 (prostacyclin) by cultured human and bovine endothelial cells. *Proc Natl Acad Sci USA* 74: 3922–3926, 1977

Wheatcroft SB, Williams IL, Shah AM, Kearney MT. Pathophysiological implications of insulin resistance on vascular endothelial function. *Diabet Med* 20: 255–268, 2003

Wolf PA, Kannel WB, Verter J. Current status of risk factors for stroke. *Neurol Clin* 1: 317–343, 1983

Wu SQ, Hopfner RL., McNeill JR, Wilson TW, Gopalakrishnan V. Altered paracrine effect of endothelin in blood vessels of the hyperinsulinemic, insulin resistant obese Zucker rat. *Cardiovasc Res* 45: 994–1000, 2000

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki TA. Novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332: 411–415. 1988