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Mechanisms underlying the attenuation of endothelium-dependent vasodilatation in the mesenteric arterial bed of the streptozotocin-induced diabetic rat

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1 Experiments were designed to investigate the mechanisms underlying the diabetes-related impairment of the vasodilatations of the perfused mesenteric arterial bed induced by acetylcholine (ACh) and K^+.

2 In streptozotocin (STZ)-diabetic rats, the ACh-induced endothelium-dependent vasodilatation was attenuated. The dose-response curves for ACh in control and diabetic rats were each shifted to the right by N^G-nitro-L-arginine (L-NOARG) and by isotonic high K^+ (60 mM). The ACh dose-response curves under isotonic high K^+ were not different between control and diabetic rats.

3 We also examined the vasodilatation induced by K^+, which is a putative endothelium-derived hyperpolarizing factor (EDHF). The mesenteric vasodilatation induced by a single administration of K^+ was greatly impaired in STZ-induced diabetic rats. Treatment with charybdotoxin plus apamin abolished the ACh-induced vasodilatation but enhanced the K^+-induced response in controls and diabetic rats. After pretreatment with ouabain plus $BaCl_2$, the ACh-induced vasodilatation was significantly impaired and the K^+-induced relaxation was abolished in both control and diabetic rats.

4 The impairment of the endothelium-dependent vasodilatation of the mesenteric arterial bed seen in STZ-induced diabetic rats may be largely due to a defective vascular response to EDHF. It is further suggested that K^+ is one of the endothelium-derived hyperpolarizing factors and that the vasodilatation response to K^+ is impaired in the mesenteric arterial bed from diabetic rats.

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Abbreviations: ACh, acetylcholine; EDHF, endothelium-derived hyperpolarizing factor; EDRF, endothelium-derived relaxing factor; HDL, high density lipoprotein; KHS, Krebs-Henseleit solution; LDL, low-density lipoprotein; L-NOARG, N^G-nitro-L-arginine; LPC, lysophosphatidylcholine; NO, nitric oxide; PGE_2, prostaglandin E_2; PGI_2, prostaglandin I_2; STZ, streptozotocin; TXB_2, thromboxane B_2

Introduction

The endothelium plays an important role in the control of vascular tone *via* the release of a number of vasodilator substances. These include endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980), now identified as nitric oxide (NO) (Palmer *et al*., 1987), prostaglandin I_2 (PGI_2) (Moncada *et al*., 1976) and endothelium-derived hyperpolarizing factor (EDHF) (Chen *et al*., 1988; Komori & Vanhoutte, 1990; Garland *et al*., 1995). Hyperpolarization may make an important contribution to vasodilatation, particularly in small resistance arteries, where a change in membrane potential of a few millivolts may cause a substantial change in vessel diameter (Nelson *et al*., 1990; Brayden & Nelson, 1992), and it has been reported that tissue perfusion is predominantly regulated by resistance arteries (i.e. small arteries with diameters of <500 μm) (Mulvany & Aalkjaer, 1990).

There are a number of candidates for EDHF. These include epoxyeicosatrienoic acids (EETs), which are cytochrome P-450 monooxygenase-arachidonic acid metabolites, and the endocannabinoid anandamide, which is also derived from arachidonic acid (Hecker *et al*., 1994; Campbell *et al*., 1996; Mombouli & Vanhoutte, 1997; Randall & Kendall, 1998; Fisslthaler *et al*., 1999). Recently, some interesting evidence has suggested that K^+ is also a candidate for EDHF, its action being exerted *via* its efflux through charybdotoxin- and apamin-selective K^+ channels on endothelial cells (Edwards *et al*., 1998). Whatever the exact chemical identity of EDHF, this factor elicits relaxation by activating K^+ channels, leading to hyperpolarization in smooth muscle cells (Taylor & Weston, 1988). Furthermore, it has been reported that the contribution of EDHF to endothelium-dependent relaxations increases as the vessel size decreases (Tomioka *et al*., 1999).

Diabetes mellitus is associated with vascular complications, including an impairment of vascular function and alterations in the reactivity of blood vessels to neurotransmitters in the macro- and microvasculature. There is an accumulating body of evidence to show that the relaxation responses induced in aortic strips by endothelium-dependent agents are weaker in streptozotocin (STZ)-induced diabetic rats than in normal controls (Oyama *et al*., 1986; Pieper & Gross, 1988; Kamata *et al*., 1989a,b; 1996; Poston & Taylor, 1995). A recent report suggested that an impairment of the endothelium-dependent hyperpolarization induced by ACh may be present in mesenteric arteries from STZ-induced diabetic rats (Fukao *et al*., 1997).

In the present study, the experiments were designed to investigate the mechanisms underlying the diabetes-related impairment of the acetylcholine (ACh)-induced endothelium-dependent vasodilatation of the perfused mesenteric arterial bed. We were especially interested in determining whether K^+

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is able to cause vasodilatation in the mesenteric arterial bed and, if so, how the K^+-induced vasodilatation might be altered in the diabetic state.

Methods

Animals and induction of diabetes mellitus

Male Wistar rats, 8 weeks old and 220–250 g in weight, received a single injection *via* the tail vein of STZ 66 mg kg^{-1}, dissolved in citrate buffer. Age-matched control rats were injected with the buffer alone. Food and water were available *ad libitum* to all animals. Blood pressure and heart rate were recorded by the tail-cuff method. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Science, Sports and Culture, Japan).

Measurement of plasma insulin, glucose, cholesterol and triglyceride

Plasma insulin was measured by radioimmunoassay (Amersham International plc., Buckinghamshire, U.K.). The concentration of glucose in the plasma was determined by the *O*-toluidine method. Plasma cholesterol levels and triglyceride levels were determined by the use of a commercially available enzyme kit (Wako Pure Chemical Co. Ltd., Osaka, Japan).

Preparation of the perfused mesenteric arterial bed

Ten weeks after treatment with STZ or buffer, rats were anaesthetized with ether and then given an intravenous injection of 1000 units kg^{-1} of heparin. Following this injection, the mesenteric arterial bed was rapidly dissected out and placed into modified Krebs-Henseleit solution (KHS, composition in mM: NaCl 118; KCl 4.7; $NaHCO_3$ 25; $CaCl_2$ 1.8; NaH_2PO_4 1.2; $MgSO_4$ 1.2; dextrose 11; 0.25% bovine serum albumin). The mesenteric artery and vein were tied off near the caecum, and the remaining intestine was then separated from the arterial bed along the intestinal wall. The mesenteric arterial bed was then perfused using the method described previously by us (Kamata & Makino, 1997; Makino & Kamata, 1998). Briefly, warm (37°C), oxygenated (95% O_2–5% CO_2) KHS was pumped into the mesenteric arterial bed, using a peristaltic pump operating at a rate of 5 ml min^{-1}, through a cannula inserted into the superior mesenteric artery. Vascular responses were detected as changes in perfusion pressure; this was monitored continuously by way of a pressure transducer (Nihon Kohden, Model AP2001, Tokyo, Japan) and recorded on a pen recorder.

In the first series of experiments, we examined the vasodilator responses of perfused mesenteric arterial beds to various agonists. Following a 60-min equilibration period, the perfusion circuit was transformed into a closed system by collecting the perfusate in a second bath and from thence recirculating it through the mesenteric arterial bed. The total volume of the closed system was 50 ml and agents were administered *via* the bath. In some preliminary experiments, the preparation of mesenteric arterial bed was constricted by perfusion with a solution containing 4×10^{-6} to 3×10^{-5} M methoxamine, which resulted in an increase in perfusion pressure of approximately 105–120 mmHg, and then maximally relaxed with a perfusion solution containing 10^{-6} M ACh, a response which confirmed the integrity of the endothelium in our preparation. In order to standardize the vasodilator responses obtained with different drugs, papaverine (10^{-4} M) was injected into each vascular bed and the resulting vasodilator response expressed as 100%. Each preparation received a different vasodilator agent. In each preparation, once the methoxamine-induced contraction had reached a plateau, vasodilator responses to a given agonist were elicited in a cumulative manner. To investigate the influence of N^G-nitro-L-arginine (L-NOARG), isotonic high K^+ (60 mM) or 10^{-5} M indomethacin on these agonist-induced responses, the mesenteric arterial bed was incubated in the appropriate solution for 30 min before the addition of methoxamine. To exclude the involvement of EDHF, some experiments were performed in which the preparation of the mesenteric arterial bed was depolarized with isotonic high K^+ (60 mM) in the presence of nicardipine (10^{-7} M); the mesenteric arteria bed was then constricted with methoxamine (4×10^{-6} to 3×10^{-5} M) (the starting pressure was adjusted to 105–120 mmHg in this preparation). Each preparation was used to test only one antagonist or isotonic high K^+ medium.

In a second series of experiments, we examined the effects of the application of K^+ (5 mM, so that the total K^+ concentration in the bath was 9.7 mM) in a single concentration-effect manner. In this series of experiments, propranolol (10^{-6} M), tetrodotoxin (10^{-6} M) and capsaicin (10^{-5} M) were used to exclude the influence of other relaxing factors.

Drugs

Streptozotocin, methoxamine hydrochloride, N^G-nitro-L-arginine (L-NOARG) indomethacin, nicardipine, propranolol, capsaicin, tetrodotoxin, papaverine hydrochloride, pinacidil and bovine serum albumin (Fraction V) were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetylcholine chloride was purchased from Daiichi Pharmaceutical Co. (Tokyo, Japan).

Statistical analysis

Data are expressed as the mean ± s.e.m. When appropriate, statistical differences were assessed by Dunnett's test for multiple comparisons after a one-way analysis of variance. Statistical comparisons between concentration-response curves were made by means of a two-way ANOVA with Bonferroni's correction performed *post hoc* to correct for multiple comparisons. $P<0.05$ was considered significant in both types of test.

Results

General characteristics

Haemodynamic variables and the plasma levels of glucose, insulin, cholesterol and triglyceride were measured at 10 weeks after the STZ injection (Table 1). By comparison with the age-matched controls: (i) both systolic blood pressure and heart rate were lower in STZ-diabetic rats; (ii) the concentration of glucose in the plasma was elevated at 10 weeks; (iii) plasma insulin levels were significantly lower in STZ-induced diabetes and (iv) plasma total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL) cholesterol and triglyceride levels were all significantly raised in STZ-induced diabetic rats.

Concentration-dependent vasodilator responses

The basal perfusion pressures in mesenteric arterial beds from STZ-induced diabetic rats and age-matched controls were 60.1 ± 1.0 mmHg, $n=10$, and 64.0 ± 1.7 mmHg, $n=10$, respectively ($P>0.05$). Perfusion with methoxamine (4×10^{-6} to 3×10^{-5} M) increased the perfusion pressures to 108.7 ± 4.3 mmHg (control, $n=10$), and 117.6 ± 5.3 mmHg (diabetic, $n=10$), respectively. Therefore, the initial perfusion pressure was adjusted to 105–120 mmHg in subsequent experiments under various experimental conditions.

In perfused mesenteric arterial beds preconstricted with methoxamine, infusion of cumulative concentrations of ACh (10^{-10} to 10^{-4} M) caused concentration-dependent vasodilatation. The vasodilatation induced by ACh was attenuated in STZ-diabetic rats (Figure 1). While the maximum vasodilatation was not different between age-matched controls and STZ-diabetic rats, these groups differed significantly in the sensitivity to ACh shown by their methoxamine-precontracted mesenteries (Table 2 and Figure 1, inset). Table 2 and Figure 2 show the effects of various treatments on the ACh-induced vasodilator responses. In both control and diabetic mesenteric arterial beds, incubation with the nitric oxide synthase inhibitor L-NOARG (10^{-4} M) had no effect on the maximum vasodilatation to ACh but analysis of the whole dose-response curves showed a significant right-ward shift for each group (control and diabetic). In the mesenteric arterial bed treated with L-NOARG (10^{-4} M), the concentration-response curve for ACh was significantly different between controls (log $EC_{50} = -7.81 \pm 0.08$ M) and diabetic rats (log $EC_{50} = -6.62 \pm 0.25$ M). In other preparations, pretreatment with isotonic high K^+ (60 mM) markedly reduced the vasodilatation induced by ACh in both groups and, under high K^+, the maximum response in diabetes was not different from that in the control group. Moreover, in the mesenteric arterial bed treated with isotonic high K^+, the concentration-response curves for ACh were comparable between controls (log $EC_{50} = -6.74 \pm 0.18$ M) and diabetic rats (log $EC_{50} = -6.57 \pm 0.06$ M). No change in the ACh-induced concentration-response curve was produced by preincubation with indomethacin (10^{-5} M) in either control or diabetic rats. The vasodilator responses of the mesenteric arterial beds to ACh in the presence of high K^+ were completely inhibited in the presence of L-NOARG (10^{-4} M) plus indomethacin (10^{-5} M) as shown in Figure 3.

Table 1 Haemodynamic variables and plasma contents in STZ-induced diabetic rats

Parameters	*Control (10)*	*Diabetic (9)*
Body weight (g)	502.3 ± 9.28	226.8 ± 9.31***
Blood pressure (mmHg)	151.8 ± 3.66	140.1 ± 2.94*
Heart rate (beats min^{-1})	378.5 ± 13.1	291.2 ± 13.6***
Plasma glucose (mg dl^{-1})	162.3 ± 5.78	609.9 ± 11.6***
Plasma insulin (ng mg^{-1})	2.93 ± 0.30	0.91 ± 0.13***
Plasma total cholesterol (mg dl^{-1})	199.9 ± 8.21	279.2 ± 15.9***
Plasma HDL cholesterol (mg dl^{-1})	77.4 ± 3.69	95.8 ± 4.92**
Plasma LDL cholesterol (mg dl^{-1})	122.5 ± 8.55	183.4 ± 13.7**
Plasma triglyceride (mg dl^{-1})	193.0 ± 13.3	914.6 ± 189.7**

Each value represents the mean ± s.e.mean. Values for determinations are shown within parentheses. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control.

Time course and magnitude of the vasodilatation induced by K^+

To clarify the characteristics of the K^+-induced vasodilatation, we examined its time course and magnitude. In the presence of propranolol (10^{-6} M), tetrodotoxin (10^{-6} M), capsaicin (10^{-5} M), L-NOARG (10^{-4} M) and indomethacin (10^{-5} M), K^+ (5 mM) caused a vasodilatation of the mesenteric arterial bed from age-matched controls, the peak vasodilatation (at about 3 min) being by $26.7 \pm 2.03\%$ ($n=4$). By contrast, the vasodilator response to this dose of K^+ (5 mM) was almost negligible in diabetic rats ($2.27 \pm 4.70\%$, $n=4$) (Figure 4). Preincubation with both charybdotoxin (10^{-7} M) plus apamin (10^{-7} M) significantly enhanced the 5 mM K^+-induced vasodilatation in mesenteric arterial beds from the age-matched controls and tended to enhance it in the diabetics. However, the vasodilatation induced by 5 mM K^+ was significantly attenuated, even reversal, in the presence of ouabain (10^{-3} M) plus $BaCl_2$ (5×10^{-4} M) (Figure 5). To make a comparison with this result, we also examined the effect of these antagonists on the ACh-induced vasodilatation in the presence of indomethacin and L-NOARG. In both age-

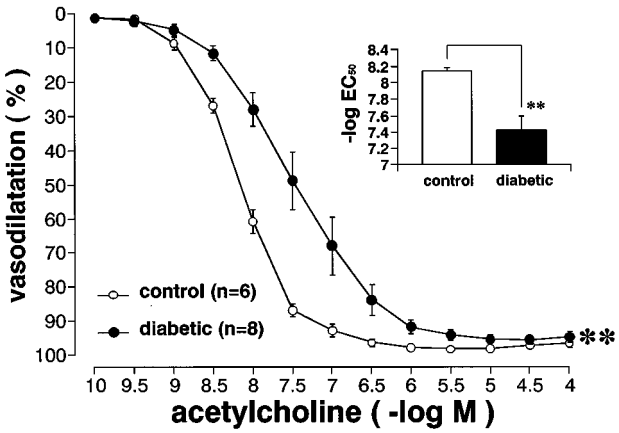

Figure 1 Concentration-response curve for ACh-induced vasodilatation of methoxamine (4×10^{-6} to 3×10^{-5} M)-preconstricted mesenteric arterial beds from age-matched controls and STZ-induced diabetic rats. Inset: −log EC_{50} values for control (C) and diabetic (D) groups. Each point or column represents the mean ± s.e.mean of 6–8 experiments. **$P<0.01$ for control vs diabetic mesenteric arterial beds.

Table 2 Maximal responses and EC_{50} values for ACh-induced vasodilatation in mesenteric arterial beds from age-matched control and STZ-induced diabetic rats

	Control		*Diabetic*	
Treatment	*Max. response (%)*	*Log EC_{50}*	*Max. response (%)*	*Log EC_{50}*
Untreated	98.4 ± 0.52	-8.14 ± 0.05	96.2 ± 1.2	-7.43 ± 0.17
L-NOARG	96.1 ± 0.78	-7.81 ± 0.08*	95.7 ± 1.2	-6.62 ± 0.25*
60 mM K^+	72.5 ± 7.6**	-6.74 ± 0.18***	83.3 ± 2.5***	-6.57 ± 0.06**
Indomethacin	100.0 ± 0.69	-8.19 ± 0.08	96.8 ± 1.0	-7.75 ± 0.23

L-NOARG, N^G-nitro-L-arginine (10^{-4} M), indomethacin (10^{-5}). Each value represents the mean ± s.e.mean of 5–10 experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs treated.

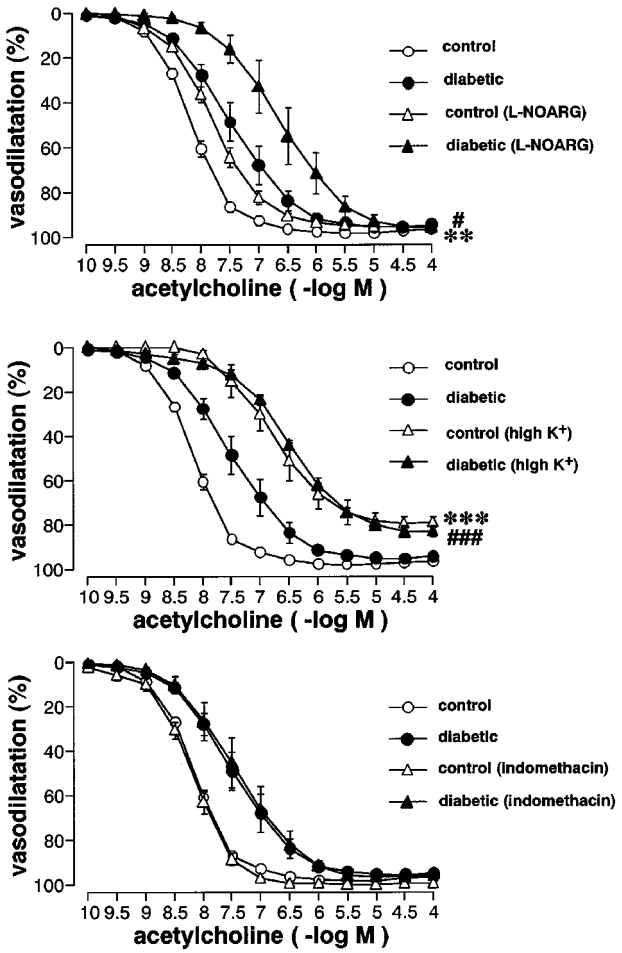

Figure 2 Effects of various agents on the concentration-response curves for ACh-induced vasodilatation of methoxamine (4×10^{-6} to 3×10^{-5} M)-preconstricted mesenteric arterial beds from age-matched controls and STZ-induced diabetic rats. Each point represents the mean$\pm$s.e.mean of 5–10 experiments. $^{**}P<0.01$, control rats vs control rats treated with L-NOARG (10^{-4} M). $^{***}P<0.001$, control rats vs control rats treated with high K^+ (60 mM). $^{\#}P<0.05$, STZ-diabetic rats vs STZ-diabetic rats treated with L-NOARG (10^{-4} M). $^{\#\#\#}P<0.001$, STZ-diabetic rats vs STZ-diabetic rats treated with high K^+ (60 mM).

matched control and diabetic rats, the ACh-induced vasodilatation was almost completely abolished by charybdotoxin plus apamin, while pretreatment with ouabain and $BaCl_2$ significantly reduced the ACh response in both groups (Figure 6). As can be seen in Figure 6b, there was no difference between control and diabetic mesenteric arterial beds in the response to ACh in the presence of ouabain plus $BaCl_2$.

The vasodilatations induced by 5 mM K^+ were not affected by the removal of the endothelium in both control and diabetic rats (Figure 7, upper panel). A relatively high concentration of Ba^{2+} (5×10^{-4} M) alone or ouabain (10^{-3} M) alone did not affect the K^+-induced vasodilatation in control rats, respectively (Figure 7, lower panel). Furthermore, a relatively low concentration of Ba^{2+} (5×10^{-5} M) plus ouabain (10^{-3} M) also did not change the vasodilatation induced by K^+.

To test the non-specific effect of a relatively high concentration of Ba^{2+} (5×10^{-4} M), we examined the effects of Ba^{2+} on the pinacidil-induced vasodilatation and methoxamine-induced vasoconstriction. As can be seen in Figure 8, no effects were found in both responses. The basal tone of the perfused mesenteric arterial beds from control rats were not significantly affected by ouabain (10^{-3} M) plus $BaCl_2$

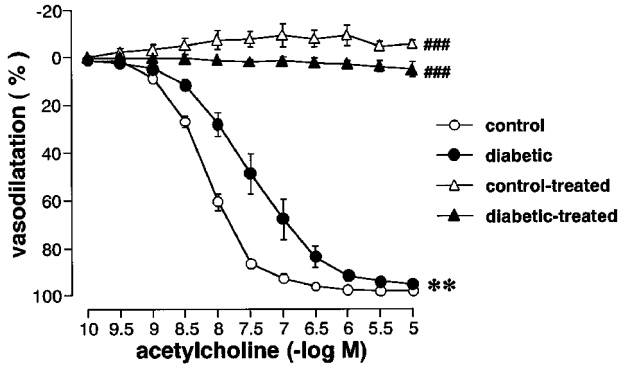

Figure 3 Effects of L-NOARG plus high K^+ (60 mM) plus indomethacin on the concentration-response curves for ACh-induced vasodilatation of methoxamine (4×10^{-6} to 3×10^{-5} M)-preconstricted mesenteric arterial beds from age-matched controls and STZ-induced diabetic rats, treated, L-NOARG (10^{-4} M) plus high K^+ (60 mM) plus indomethacin (10^{-5} M). Each point represents the mean$\pm$s.e.mean of 6–8 experiments. $^{**}P<0.01$, control vs diabetic rats. $^{\#\#\#}P<0.001$, untreated vs treated.

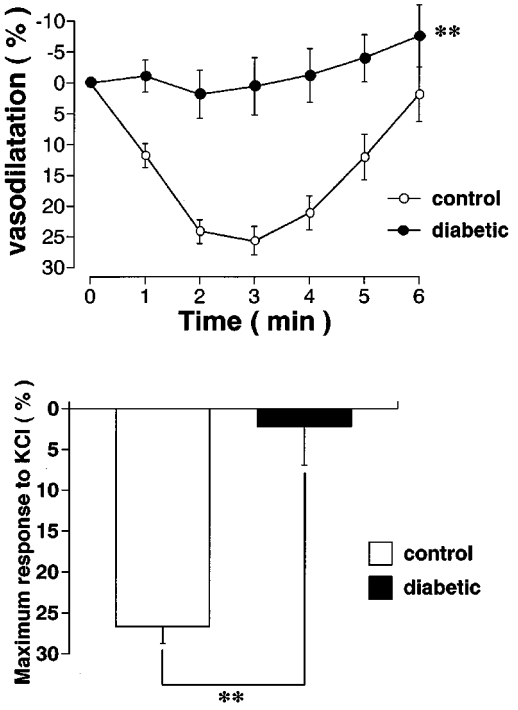

Figure 4 Time course and magnitude of vasodilator responses to K^+ in methoxamine (4×10^{-6} to 3×10^{-5} M)-preconstricted mesenteric arterial beds from age-matched controls and STZ-induced diabetic rats in presence of propranolol (10^{-6} M), tetrodotoxin (10^{-6} M), capsaicin (10^{-5} M), L-NOARG (10^{-4} M) and indomethacin (10^{-5} M). Maximum response is shown in the lower panel. Each point represents the mean$\pm$s.e.mean of six experiments. $^{**}P<0.001$ vs control.

(5×10^{-4} M) (untreated, 76.5$\pm$2.2 mmHg ($n=4$); treated, 84.5$\pm$6.1 mmHg ($n=4$) ($P>0.05$).

Discussion

The major conclusion to be drawn from the present study is that the impairment of the ACh-induced endothelium-dependent vasodilatation that is seen in mesenteric arterial beds from diabetic rats may be due largely to a defective

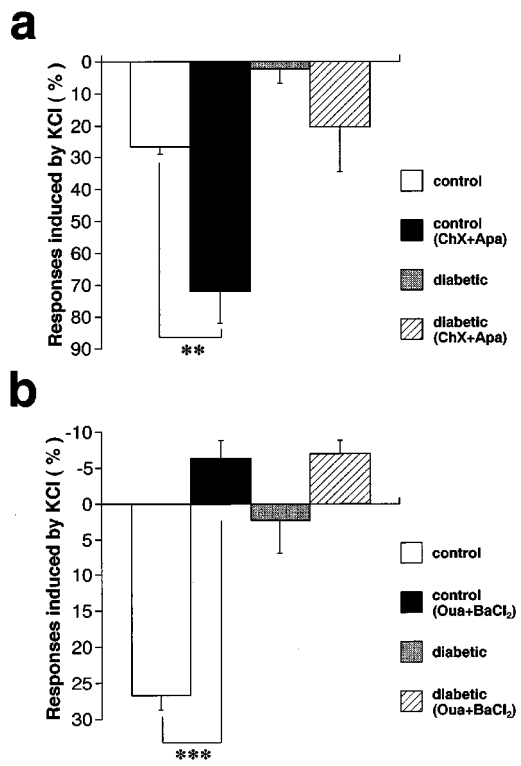

Figure 5 Effects of various agents on 5 mM K^+-induced vasodilatation in methoxamine (4×10^{-6} to 3×10^{-5} M)-preconstricted mesenteric arterial beds from age-matched controls and STZ-induced diabetic rats in presence of propranolol (10^{-6} M), tetrodotoxin (10^{-6} M), capsaicin (10^{-5} M), L-NOARG (10^{-4} M) and indomethacin (10^{-5} M). (**a**) effects of charybdotoxin (10^{-7} M) plus apamin (10^{-7} M) on K^+-induced vasodilatation; (**b**) effects of ouabain (10^{-3} M) plus $BaCl_2$ (5×10^{-4} M) on K^+-induced vasodilatation. Each column represents the mean±s.e.mean of four experiments. Note the different scales in (a) and (b). $^{**}P<0.01$, $^{***}P<0.001$ control vs after treatment with the indicated blockers. ChX; charybdotoxin (10^{-7} M), Apa; apamin (10^{-7} M), Oua; ouabain (10^{-3} M) and $BaCl_2$ (5×10^{-4} M).

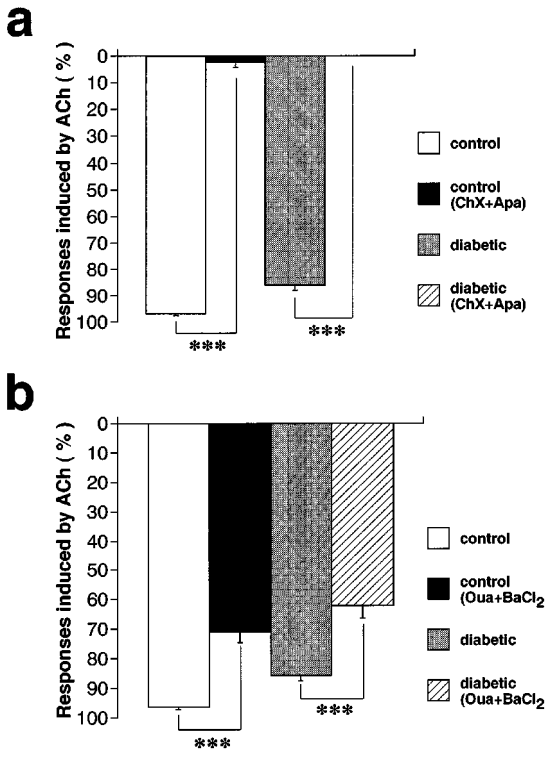

Figure 6 Effects of various agents on ACh-induced vasodilatation in methoxamine (4×10^{-6} to 3×10^{-5} M)-preconstricted mesenteric arterial beds from age-matched controls and STZ-induced diabetic rats in presence of L-NOARG (10^{-4} M) and indomethacin (10^{-5} M). (**a**) effects of charybdotoxin (10^{-7} M) plus apamin (10^{-7} M) on ACh (10^{-6} M)-induced vasodilatation; (**b**) effects of ouabain (10^{-3} M) plus $BaCl_2$ (5×10^{-4} M) on ACh (10^{-6} M)-induced vasodilatation. Each column represents the mean±s.e.mean of six experiments. $^{***}P<0.001$ control vs after treatment with the indicated blockers. ChX; charybdotoxin (10^{-7} M), Apa; apamin (10^{-7} M), Oua; ouabain (10^{-3} M) and $BaCl_2$ (5×10^{-4} M).

vascular response to K^+. In the present study, we also found that K^+ was able to induce a decrease in the perfusion pressure of the control mesenteric arterial bed and that this response was greatly attenuated in the diabetic state.

In the present study, our first finding was that the endothelium-dependent vasodilator response of the mesenteric arterial bed to ACh was attenuated in STZ-induced diabetic rats. PGI_2 has been found to contribute to endothelium-dependent relaxation in several isolated blood vessels and to contribute to vasodilatation in perfused organs (Vegesna & Diamond, 1986). It has been reported that the release from the mesenteric arterial bed of 6-keto-prostaglandin $F_{1\alpha}$ (a metabolite of PGI_2), thromboxane B_2 (TXB_2, a metabolite of thromboxane A_2) and PGE_2 is significantly greater in diabetic rats than in controls (Fujii *et al*., 1986; Koltai *et al*., 1997). However, in the present study, pretreatment with indomethacin had no effect on the ACh-induced dose-dependent vasodilatation in either control or diabetic rats, suggesting that prostanoids are not involved in the ACh-induced vasodilatation in mesenteric arterial beds.

In the analysis presented in this paragraph and the next, we focus on the residual vasodilatation seen under L-NOARG or high K^+. Under a high concentration of L-NOARG, the residual vasodilatation can be assumed to represent the component of the vasodilatation mediated by EDHF. On the other hand, the residual vasodilatation seen when the bed was fully depolarized by high K^+ (60 mM) can be assumed to represent the component mediated by NO. The ACh-induced vasodilatation in the presence of L-NOARG was significantly weaker in the diabetic mesenteric vessels than in the control mesenteric vessels, suggesting that the release or synthesis of EDHF in the diabetic state may be reduced by comparison with that in the controls. When the mesenteric arterial bed was fully depolarized with high K^+, the ACh dose-response curve was almost the same in controls and diabetic rats, suggesting that the NO-mediated component of the vasodilatation induced by ACh may be comparable between controls and diabetic rats. As shown in Table 1, there is a marked increase in plasma LDL concentration in the diabetic state, indicating that the impairment of the EDHF-mediated response may be, at least in part, due to the increase in plasma LDL. Indeed, one possible explanation for the decreased EDHF-mediated vasodilatation may be the following sequence of events: STZ-diabetes leads to an increase in plasma LDL cholesterol (Kamata *et al*., 1996) and a decrease in superoxide dismutase contents of blood vessels and activity (Kamata & Kobayashi, 1996; Kobayashi & Kamata, 1999); the decreased superoxide dismutase activity causes an increased accumulation of superoxide anions; the accumulated superoxide anions may oxidize LDL (Kobayashi & Kamata, 1999); the oxidized LDL may itself impair the EDHF-mediated response and/or the oxidized LDL may release lysophosphatidylcholine (LPC) which then attenuates the EDHF-mediated hyperpolarization (Fukao *et al*., 1995); the oxidized LDL or LPC may also inhibit Ca^{2+} influx into the

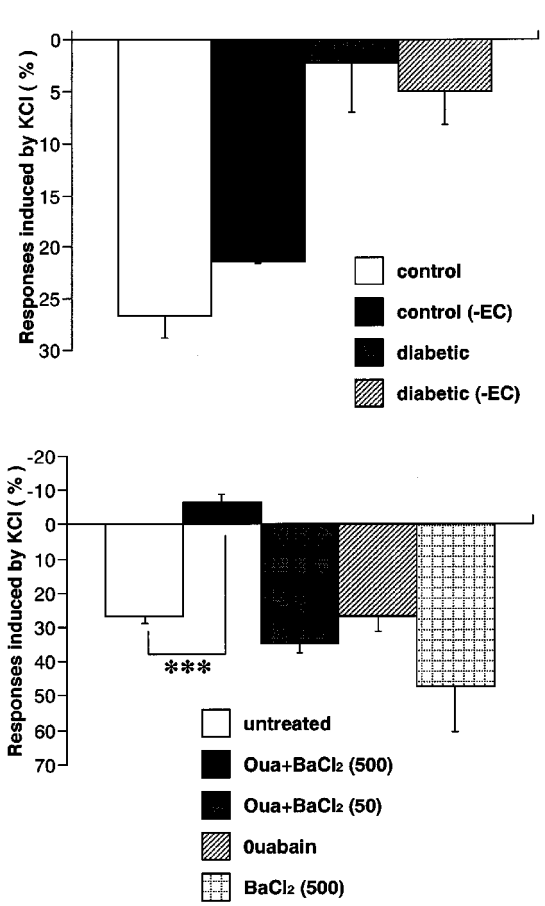

Figure 7 Effects of endothelium-removal and various agents on 5 mM K^+-induced vasodilatation in methoxamine (4×10^{-6} to 3×10^{-5} M)-preconstricted mesenteric arterial beds from age-matched controls and STZ-induced diabetic rats. Upper panel: effects of endothelium-removal (−EC) on K^+-induced vasodilatation in control and STZ-induced diabetic rats. Lower panel: effect of Oua; ouabain (10^{-3} M) plus $BaCl_2$ (5×10^{-4} M), ouabain (10^{-3} M) plus $BaCl_2$ (5×10^{-5} M), ouabain (10^{-3} M) alone, and $BaCl_2$ (5×10^{-4} M) alone on K^+-induced vasodilatation in control rats. Each column represents the mean±s.e.mean of six experiments. ***$P<0.001$, untreated vs treated with ouabain (10^{-3} M) plus $BaCl_2$ (5×10^{-4} M).

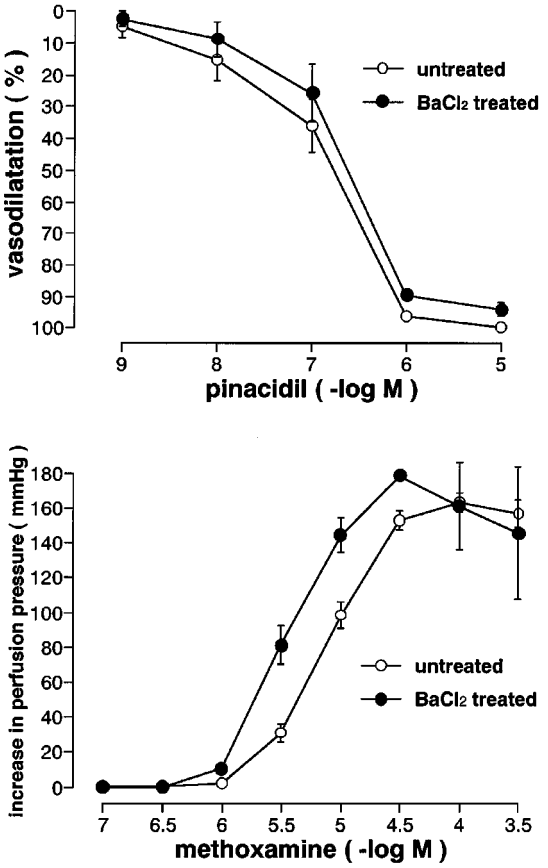

Figure 8 Effects of $BaCl_2$ on pinacidil-induced vasodilatation and methoxamine-induced vasoconstriction in mesenteric arterial beds from control rats. Each column represents the mean±s.e.mean of 7–8 experiments.

endothelium (Kamata & Nakajima, 1998), thus reducing the production or release of EDHF (since this requires an increase in intracellular Ca^{2+} in the endothelium, Chen & Suzuki, 1990). As a result, the ACh-induced vasodilatation in the presence of L-NOARG would be significantly impaired in the mesenteric arterial beds from diabetic rats (Figure 2).

Recently, it has been reported that K^+ is one candidate for EDHF in rat arteries (Edwards *et al*., 1998). The sequence would be that when ACh binds to its receptors on the endothelium, charybdotoxin- and apamin-sensitive K^+ channels in the endothelium are opened and K^+ efflux occurs into the myoendothelial space. The resulting increase in myoendothelial K^+ concentration hyperpolarizes and relaxes adjacent smooth muscle cells by activating Ba^{2+}-sensitive K^+ channels and ouabain-sensitive Na^+, K^+ ATPase. There are other candidates for EDHF, namely endocannabinoids, anadamide and epoxyeicosatrienoic acids (EETs), which are cytochrome-P450-monooxygenase-derived metabolites of arachidonic acid (Mombouli & Vanhoutte, 1997; Campbell *et al*., 1996; Hecker *et al*., 1994; Randall & Kendall, 1998; Fisslthaler *et al*., 1999). Furthermore, recent evidence suggests that direct heterocellular gap junctional communication between endothelium and smooth muscle may contribute to NO-independent relaxation (Chaytor *et al*., 1998; Hutcheson *et al*., 1999; Yamamoto *et al*., 1998; 1999). In the present study, we focused on K^+ as a possible candidate for EDHF. K^+ (5 mM) caused a vasodilatation of the mesenteric arterial bed from age-matched controls, the peak vasodilatation being by 26.7±2.03%, while the vasodilator response to this concentration of K^+ was almost negligible in diabetic rats. Although we do not know the precise identity of the mechanisms underlying the vasodilator response of the mesenteric arterial bed to K^+, this is the first report that K^+-induced vasodilatation is attenuated in the diabetic state.

Although incubating the mesenteric arterial bed with charybdotoxin (10^{-7} M) plus apamin (10^{-7} M) enhanced the 5 mM K^+-induced vasodilatation, the vasodilatation induced by ACh was completely abolished by these agents in both age-matched controls and diabetics, suggesting that an action of K^+ on smooth muscle may not be responsible for the ACh-induced vasodilatation. The vasodilatation induced by 5 mM K^+ was converted to a small contraction in controls and diabetics by treatment with ouabain (10^{-3} M) plus $BaCl_2$ (5×10^{-4} M). The result is consistent with recent evidence that the K^+-induced hyperpolarization and relaxation in rat mesenteric artery are significantly inhibited by ouabain plus Ba^{2+} (Edwards *et al*., 1998). For comparison with this result, we also examined the effect of these agents on the ACh-induced vasodilatation in the presence of indomethacin and L-NOARG. In both age-matched control and diabetic rats, the ACh-induced vasodilatation was almost abolished by charybdotoxin plus apamin and was also slightly, but significantly, inhibited by treatment with ouabain plus $BaCl_2$, suggesting

that not only charybdotoxin- and apamin-selective K^+ channels but also Ba^{2+}-sensitive K^+ channels and Na^+/K^+ ATPase may be, at least in part, responsible for the ACh-induced vasodilatation seen in the presence of indomethacin and L-NOARG. In our diabetic rats, the K^+-induced vasodilatation was very significantly weaker than in the controls, suggesting that the activities of Ba^{2+}-sensitive K^+ channels and Na^+/K^+ ATPase in the smooth muscle might be decreased. Indeed, as can be seen in Figure 6b, there was no difference between control and diabetic mesenteric arterial beds in the response to ACh in the presence of ouabain plus $BaCl_2$, suggesting that the defect in diabetes is in the response to K^+.

To examine the ACh- or K^+-induced vasodilatation in the mesenteric arterial beds, we used a high concentration of Ba^{2+}. The K^+-induced vasodilatation was not affected by a high concentration of Ba^{2+}, and the vasodilatation induced by pinacidil, a K^+-channel opener (Carlsen *et al*., 1981), and the vasoconstriction induced by methoxamine were not affected by the concentration of Ba^{2+}. These results suggest that a high concentration of Ba^{2+} alone has non-specific action of K^+-channel.

When we examined the K^+-induced vasodilatation in the perfused mesenteric arterial beds, the beds were treated with propranolol, tetrodotoxin, capsaicin, L-NOARG and indomethacin, suggesting that the vasodilatation induced by K^+ is not related to the release of noradrenaline, vasoactive intestinal polypeptide, calcitonin-gene related peptide, NO and prostaglandins. The vasodilator responses of the mesenteric arterial beds to ACh in the presence of high K^+ were completely inhibited in the presence of L-NOARG plus indomethacin and incubating the mesenteric arterial bed with indomethacin alone had no influence on the ACh-induced vasodilatation. These results strongly suggest that EDRF released from the endothelium of the mesenteric arterial bed may be NO and EDHFs.

In conclusion, our results suggest that the impairment of the endothelium-dependent vasodilatation of the mesenteric arterial bed seen in STZ-induced diabetic rats may be largely due to a defecting vascular response to K^+. It is further suggested that K^+ may be one of the EDHFs. Although the precise identity of the mechanisms underlying attenuation of the K^+-induced vasodilatation in the mesenteric arterial bed from diabetic rats is not yet clear, K^+ may play an important role in this aspect of the pathogenesis of diabetes mellitus.

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References

BRAYDEN, J.E. & NELSON, M.T. (1992). Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science,* **256,** 532–535.

CAMPBELL, W.B., GEBREMEDHIN, D., PRATT, P.F. & HARDER, D.R. (1996). Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ. Res.,* **78,** 415–423.

CARLSEN, J.E.E., KARDEL, T., HILDEN, T., TANGO, M., TRAP-JENSEN, J. (1981). Immediate central and peripheral haemodynamic effects of a new vasodilating agent pinacidil (P1134) in hypertensive man. *Clin. Physiol.,* **1,** 375–384.

CHAYTOR, A.T., EVANS, W.H. & GRIFFITH, T.M. (1998). Central role of heterocellular gap junctional communication in endothelium-dependent relaxations of rabbit iliac arteries. *J. Physiol. (Lond.),* **508,** 561–573.

CHEN, G. & SUZUKI, H. (1990). Calcium dependency of the endothelium-dependent hyperpolarization in smooth muscle cells of the rabbit carotid artery. *J. Physiol. (Lond.),* **421,** 521–534.

CHEN, G., SUZUKI, H. & WESTON, A.H. (1988). Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *Br. J. Pharmacol.,* **95,** 1165–1174.

EDWARDS, G., DORA, K.A., GARDENER, M.J., GARLAND, C.J. & WESTON, A.H. (1998). K^+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature,* **396,** 269–272.

FISSLTHALER, B., POPP, R., KISS, L., POTENTE, M., HARDER, D.R., FLEMING, I. & BUSSE, R. (1999). Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature,* **401,** 493–497.

FUJII, K., SOMA, M., HUANG, Y.-S., MANKU, M.S. & HORROBIN, D.F. (1986). Increased release of prostaglandins from the mesenteric vascular bed of diabetic animals: The effects of glucose and insulin. *Prostaglandins Leukotrienes Med.,* **24,** 151–161.

FUKAO, M., HATTORI, Y., KANNO, M., SAKUMA, I. & KITABATAKE, A. (1995). Evidence for selective inhibition by lysophosphatidylcholine of acetylcholine-induced endothelium-dependent hyperpolarization and relaxation in rat mesenteric artery. *Br. J. Pharmacol.,* **116,** 1541–1544.

FUKAO, M., HATTORI, Y., KANNO, M., SAKUMA, I. & KITABATAKE, A. (1997). Alteration in endothelium-dependent hyperpolarization and relaxation in mesenteric arteries from streptozotocin-induced diabetic rats. *Br. J. Pharmacol.,* **121,** 1383–1391.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature,* **288,** 373–376.

GARLAND, C.J., PLANE, F., KEMP, B.K. & COCKS, T.M. (1995). Endothelium-dependent hyperpolarization: a role in the control of vascular tone. *Trends Pharmacol. Sci.,* **16,** 23–30.

HECKER, M., BARA, A.T., BAUERSACHS, J. & BUSSE, R. (1994). Characterization of endothelium-derived hyperpolarizing factor as a cytochrome p450-derived arachidonic acid metabolite in mammals. *J. Physiol. (Lond.),* **481,** 407–414.

HUTCHESON, I.R., CHAYTOR, A.T., EVANS, W.H. & GRIFFITH, T.M. (1999). Nitric oxide-independent relaxations to acetylcholine and A23187 involve different routes of heterocellular communication: role of gap junctions and phospholipase A_2. *Circ. Res.,* **84,** 53–63.

KAMATA, K. & KOBAYASHI, T. (1996). Changes in superoxide dismutase mRNA expression by streptozotocin-induced diabetes. *Br. J. Pharmacol.,* **119,** 583–589.

KAMATA, K. & MAKINO, A. (1997). A comparative study on the rat aorta and mesenteric arterial bed of the possible role of nitric oxide in the desensitization of the vasoconstrictor response to an α_1-adrenoceptor agonist. *Br. J. Pharmacol.,* **120,** 1221–1226.

KAMATA, K. & NAKAJIMA, M. (1998). Ca^{2+} mobilization in the aortic endothelium in streptozotocin-induced diabetic and cholesterol-fed mice. *Br. J. Pharmacol.,* **123,** 1509–1516.

KAMATA, K., MIYATA, N. & KASUYA, Y. (1989a). Impairment of endothelium-dependent relaxation and changes in levels of cyclic GMP in aorta from streptozotocin-induced diabetic rats. *Br. J. Pharmacol.,* **97,** 614–618.

KAMATA, K., MIYATA, N. & KASUYA, Y. (1989b). Involvement of endothelial cells in relaxation and contraction responses of the aorta to isoproterenol in naive and streptozotocin-induced diabetic rats. *J. Pharmacol. Exp. Ther.,* **249,** 890–894.

KAMATA, K., SUGIURA, M., KOJIMA, S. & KASUYA, Y. (1996). Preservation of endothelium-dependent relaxation in cholesterol-fed and streptozotocin-induced diabetic mice by the chronic administration of cholestyramine. *Br. J. Pharmacol.,* **118,** 385–391.

KOBAYASHI, T. & KAMATA, K. (1999). Relationship among cholesterol, superoxide anion and endothelium-dependent relaxation in diabetic rats. *Eur. J. Pharmacol.,* **367,** 213–222.

KOLTAI, M.Z., HADHAZY, P., POSA, I., KOCSIS, E., WINKLER, G., ROSEN, P. & POGATSA, G. (1997). Characteristics of coronary endothelial dysfunction in experimental diabetes. *Cardiovasc. Res.,* **34,** 157–163.

KOMORI, K. & VANHOUTTE, P.M. (1990). Endothelium derived hyperpolarizing factor. *Blood Vessels,* **27,** 238–245.

MAKINO, A. & KAMATA, K. (1998). Elevated plasma endothelin-1 level in streptozotocin-induced diabetic rats and responsiveness of the mesenteric arterial beds to endothelin-1. *Br. J. Pharmacol.,* **123,** 1065–1072.

MOMBOULI, J.V. & VANHOUTTE, P.M. (1997). Endothelium-derived hyperpolarizing factor(s): updating the unknown. *Trends Pharmacol. Sci.,* **18,** 252–256.

MONCADA, S., GRYGLEWSKI, R., BUNTING, S. & VANE, J.R. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature,* **263,** 663–665.

MULVANY, M.J. & AALKJÆR, C. (1990). Structure and function of small arteries. *Physiol. Rev.,* **70,** 921–961.

NELSON, M.T., PATLAK, J.B., WORLEY, J.F. & STANDEN, N.B. (1990). Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *Am. J. Physiol.,* **259,** C3–C18.

OYAMA, Y., KAWASAKI, H., HATTORI, Y. & KANNO, M. (1986). Attenuation of endothelium-dependent relaxation in aorta from diabetic rats. *Eur. J. Pharmacol.,* **132,** 75–78.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature,* **327,** 524–526.

PIEPER, G.M. & GROSS, G.J. (1988). Oxygen free radical abolish endothelium-dependent relaxation in diabetic rat aorta. *Am. J. Physiol.,* **255,** H825–H833.

POSTON, L. & TAYLOR, PD. (1995). Endothelium-mediated vascular function in insulin-dependent diabetes mellitus. *Clin. Sci.,* **88,** 245–255.

RANDALL, M.D. & KENDALL, D.A. (1998). Endocannabinoids: a new class of vasoactive substances. *Trends Pharmacol. Sci.,* **19,** 55–58.

TAYLOR, S.G. & WESTON, A.H. (1988). Endothelium-derived hyperpolarizing factor: a new endogenous inhibitor from the vascular endothelium. *Trends Pharmacol. Sci.,* **9,** 272–274.

TOMIOKA, H., HATTORI, Y., FUKAO, M., SATO, A., LIU, M., SAKUMA, I., KITABATAKE, A. & KANNO, M. (1999). Relaxation in different-sized rat blood vessels mediated by endothelium-derived hyperpolarizing factor: importance of processes mediating precontractions. *J. Vesc. Res.,* **36,** 311–320.

VEGESNA, R.V.K. & DIAMOND, J. (1986). Elevation of cyclic AMP by prostacyclin is accompanied by relaxation of bovine coronary arteries and contraction of rabbit aortic rings. *Eur. J. Pharmacol.,* **128,** 25–31.

YAMAMOTO, Y., FUKUTA, H., NAKAHIRA, Y. & SUZUKI, H. (1998). Blockade by 18 β-glycyrrhetinic acid of intercellular electrical coupling in guinea-pig arterioles. *J. Physiol. (Lond.),* **511,** 501–508.

YAMAMOTO, Y., IMAEDA, K. & SUZUKI, H. (1999). Endothelium-dependent hyperpolarization and intercellular electrical coupling in guinea-pig mesenteric arterioles. *J. Physiol. (Lond.),* **514,** 505–513.

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