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Alterations in endothelium-dependent hyperpolarization and relaxation in mesenteric arteries from streptozotocin-induced diabetic rats

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1 The aim of this study was to determine whether endothelium-dependent hyperpolarization and relaxation are altered during experimental diabetes mellitus. Membrane potentials were recorded in mesenteric arteries from rats with streptozotocin-induced diabetes and age-matched controls. The resting membrane potentials were not significantly different between control and diabetic mesenteric arteries (-55.3 ± 0.5 vs -55.6 ± 0.4 mV). However, endothelium-dependent hyperpolarization produced by acetylcholine (ACh; 10^{-8}–10^{-5} M) was significantly diminished in amplitude in diabetic arteries compared with that in controls (maximum -10.4 ± 1.1 vs -17.2 ± 0.8 mV). Furthermore, the hyperpolarizing responses of diabetic arteries were more transient.

2 ACh-induced hyperpolarization observed in control and diabetic arteries remained unaltered even after treatment with 3×10^{-4} M N^G-nitro-L-arginine (L-NOARG), 10^{-5} M indomethacin or 60 u ml^{-1} superoxide dismutase.

3 Endothelium-dependent hyperpolarization with 10^{-6} M A23187, a calcium ionophore, was also decreased in diabetic arteries compared to controls (-8.3 ± 1.4 vs -18.0 ± 1.9 mV). However, endothelium-independent hyperpolarizing responses to 10^{-6} M pinacidil, a potassium channel opener, were similar in control and diabetic arteries (-20.0 ± 1.4 vs -19.2 ± 1.1 mV).

4 The altered endothelium-dependent hyperpolarizations in diabetic arteries were almost completely prevented by insulin therapy. Endothelium-dependent relaxations by ACh in the presence of 10^{-4} M L-NOARG and 10^{-5} M indomethacin in diabetic arteries were also reduced and more transient compared to controls.

5 These data indicate that endothelium-dependent hyperpolarization is reduced by diabetes, and this would, in part, account for the impaired endothelium-dependent relaxations in mesenteric arteries from diabetic rats.

Keywords: Diabetes mellitus; acetylcholine; membrane potential; hyperpolarization; vascular smooth muscle; vasodilatation

Introduction

Vascular deterioration is one of the complicating features of human and experimental diabetes (Garcia *et al*., 1974; Tomlinson *et al*., 1992). Some of the vascular changes in diabetes may be related to alterations in endothelial function (Ruderman & Haudenschild, 1984). In this regard, the vascular responsiveness to endothelium-dependent relaxant agents has been extensively investigated in animal models of diabetes mellitus. Impaired endothelium-dependent relaxations have been consistently demonstrated in blood vessels from streptozotocin-induced diabetic rats (Oyama *et al*., 1986; Pieper & Gross, 1988; Kamata *et al*., 1989), spontaneously diabetic BB rats (Meraji *et al*., 1987; Durante *et al*., 1988) and alloxan-induced diabetic rabbits (Tesfamariam & Cohen, 1992).

The endothelium-dependent relaxant responses to agents such as acetylcholine (ACh) are largely due to release of endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980). EDRF is now considered to be identical to nitric oxide (NO; Ignarro *et al*., 1987; Palmer *et al*., 1987) or to a related nitroso compound (Myers *et al*., 1990). Endothelial cells also mediate hyperpolarization of vascular smooth muscle cells in response to ACh (Chen & Suzuki, 1989; Feletou & Vanhoutte, 1988). Several lines of evidence strongly suggest that this hyperpolarizing response is generated by an endothelium-derived substance that is distinct from EDRF-NO (Bény & Brunet, 1988; Komori *et al*., 1988; Brayden, 1990). Thus, the existence of an endothelium-derived hyperpolarizing factor (EDHF) has been proposed by some investigators (Chen *et al*., 1991; Vanhoutte, 1989), though it remains unidentified. Membrane hyperpolarization of vascular smooth muscle cells by EDHF can contribute to arterial relaxation by blunting voltage-dependent mechanisms (Nelson *et al*., 1990). Whereas EDHF may provide a secondary system to NO in large arteries, EDHF appears to be a major determinant of vascular tone in small arteries (Garland *et al*., 1995). Recently, it has been shown that endothelium-dependent hyperpolarization is depressed in aorta from renal hypertensive rats (Van de Voorde *et al*., 1992) and in mesenteric artery from spontaneously hypertensive rats (Fujii *et al*., 1992). However, the effect of diabetic state on endothelium-dependent hyperpolarization of vascular smooth muscle has not yet been investigated.

The present study was designed to evaluate EDHF-mediated responses in mesenteric arteries from rats with streptozotocin-induced diabetes. Initially, we characterized ACh-induced hyperpolarization in rat mesenteric artery. Then, we determined whether endothelium-dependent hyperpolarization and the associated relaxation are altered in diabetes. A preliminary account of these data was presented to the First International Symposium devoted to endothelium-derived hyperpolarizing factor, in Vaux de Cernay, France (Fukao *et al*., 1996).

Methods

Induction of diabetes

Eight week old male Wistar rats (180–200 g) were divided randomly into two groups. The rats were lightly anaesthetized

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with diethyl ether and the diabetic group received a single injection of streptozotocin (45 mg kg^{-1}) dissolved in a citrate buffer solution into the tail vein. The control group received an equivalent volume of the citrate buffer solution alone. Control and diabetic rats were caged separately but housed in a similar environment. Both groups of animals were given the same diet and water *ad libitum*. Six diabetic rats were treated daily with subcutaneous injections of ultralente insulin (8 u day^{-1}; Novo Nordisk, Copenhagen, Denmark). Insulin therapy was begun 1 day after streptozotocin injection, and was continued up to the day before the animals were sacrificed.

On the day of the experiment, a blood sample was collected from the renal vein and serum glucose level was determined by Rapid Blood Analyzer Super using Uni-Kit (Chugai, Tokyo, Japan).

Membrane potential recording

Eight to 12 weeks after treatment with streptozotocin or the buffer, rats were anaesthetized with diethyl ether. The main branch of the superior mesenteric artery was carefully excised and placed on a plate containing oxygenated physiological salt solution (PSS) at room temperature. The arteries were then cleaned of adherent connective tissues, cut into rings of 3 mm length, and opened longitudinally. Care was taken to ensure that the endothelial layer was not damaged during processing of the tissue preparation. Where indicated, the endothelial cells was removed by gently rubbing the intimal surface of the vessel with a moistened cotton ball. The tissue was pinned down, intimal side upward, on the bottom of an organ chamber (capacity 3 ml), and superfused at a constant flow rate of 7 ml min^{-1} with PSS aerated with 95% O_2 and 5% CO_2. The temperature of perfusate was kept constant at 37°C. The composition of PSS was as follows (in mM); NaCl 118.2, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 10.0. After the preparations had been equilibrated for 60 min, glass microelectrodes filled with 3 M KCl (tip resistance 40–80 MΩ) were inserted into the smooth muscle cells from the intimal side. After a stable recording of membrane potential for at least 2 min had been obtained, changes in membrane potential produced by ACh, A23187, pinacidil and sodium nitroprusside (SNP) were recorded by use of a high-impedance amplifier (Nihon Kohden, MEZ-8201, Tokyo, Japan) from the continuous recordings. When N^G-nitro-L-arginine (L-NOARG) or indomethacin was used, each agent was applied 15 min before the addition of ACh. Superoxide dismutase (SOD) was applied 10 min before the addition of ACh. Electrical signals were continuously monitored on a oscilloscope (Nihon Kohden VC-10, Tokyo, Japan) and recorded on a chart recorder (Watanabe Sokki WR3101, Tokyo, Japan). Further details of the experimental procedure have been described elsewhere (Fukao *et al*., 1995).

Measurement of relaxant responses

Rat mesenteric arterial rings were prepared as described above. Each ring was suspended by a pair of stainless steel pins in a water-jacketed bath filled with 6 ml of normal PSS. The solution in the bath was gassed with 95% O_2 and 5% CO_2 (pH 7.4) and its temperature was maintained at 37°C. The rings were stretched until an optimal resting tension of 1.0 g was loaded and then allowed to equilibrate for at least 60 min. Force generation was monitored by an isometric transducer (Unique Medical UMTB-1, Tokyo, Japan) and a carrier amplifier (Nihon Kohden AP-621G, Tokyo, Japan). The output of the force transducer was registered on a pen recorder (Rikadenki R-64, Tokyo, Japan). After the equilibration period, the rings were exposed several times to high K^+ PSS (80 mM $[K^+]_0$) until reproducible contractile responses were obtained. High K^+ PSS was made by substituting NaCl with equimolar KCl. The vessels were precontracted with 10^{-7} M phenylephrine 15 min after the treatment with 10^{-4} M L-NOARG and 10^{-5} M indomethacin in order to exclude the involvement of EDRF-NO and prostanoids. After the contraction had reached a plateau level, ACh was applied in a cumulative manner. To examine the time course of relaxant response to ACh, the single concentration of ACh (10^{-6} and 10^{-5} M) was applied to the arteries precontracted with 10^{-7} M phenylphrine in the presence of 10^{-4} M L-NOARG and 10^{-5} M indomethacin. We estimated the ACh responses in the presence of L-NOARG and indomethacin as EDHF-mediated relaxations, since our preliminary experiments showed that L-NOARG- and indomethacin-resistant relaxations were markedly reduced in 20 mM K^+ PSS and completely abolished in 30 mM K^+ PSS. Relaxations were expressed as a percentage of the height of the contraction induced by phenylephrine.

Drugs

The following drugs were used: ACh chloride (Wako, Osaka, Japan), A23187 (Calbiochem, San Diego, CA, U.S.A.), pinacidil (Shionogi, Osaka, Japan), L-NOARG, indomethacin, SOD (from human erythrocytes; 2000–4000 u mg^{-1} protein), SNP, streptozotocin, tetraethylammonium (TEA), tetrabutylammonium (TBA), charybdotoxin (CTX), apamin and glibenclamide (Sigma Chemical, St. Louis, MO, U.S.A.). A23187 was prepared in dimethyl sulphoxide and diluted in ethanol. Indomethacin was prepared in 50 mM Tris. Pinacidil and L-NOARG were prepared in 0.2 N HCl and glibenclamide in 0.05 N NaOH. Other drugs were dissolved in distilled water. PSS was used for further dilution to the proper concentrations. Solvents used to dissolve drugs did not, themselves, affect electrical and mechanical responses at their final bath concentrations.

Statistical analysis

All values are expressed as means ± s.e.mean. Two-way analysis of variance (ANOVA) was used to compare concentration-response curves of hyperpolarization and relaxation between control and diabetic groups, followed by Scheffé's multiple comparison test. Other variables were compared by use of paired and unpaired Student's t test. Values of $P<0.05$ were accepted as indicating a significant difference.

Results

General characteristics

Eight to 12 weeks after injection, all rats treated with streptozotocin exhibited severe hyperglycaemia and their serum glucose levels (651 ± 14 mg dl^{-1}, $n=57$) were approximately 3.8 fold ($P<0.001$) higher than the levels of age-matched control animals (173 ± 4 mg dl^{-1}, $n=37$). The body weights of diabetic rats (159 ± 2 g, $n=68$) were significantly lower than those of control rats (348 ± 5 g, $n=59$, $P<0.001$). In addition, the wet weights of arterial preparations from diabetic rats (0.14 ± 0.01 mg mm^{-2}, $n=18$) were significantly lighter than those from control animals (0.17 ± 0.01 mg mm^{-2}, $n=17$, $P<0.01$). Serum glucose levels (169 ± 43 mg dl^{-1}, $n=6$) and the body weights (341 ± 13 g, $n=6$) in insulin treated diabetic rats were not significantly different from those of controls.

Characteristics of membrane hyperpolarization by ACh

As shown in Figures 1a and 2, ACh produced sustained hyperpolarization of the smooth muscle membrane in control rats. When the endothelium was removed, ACh did not produce significant changes in the resting membrane potentials (−0.2 ± 0.7 mV, $n=5$; Figure 1a). The hyperpolarizing response to ACh was reduced in 20 mM K^+ PSS and enhanced in 1 mM K^+ PSS (Figure 1b). TBA (5×10^{-4} M), a non-specific K^+ channel blocker, and apamin (3×10^{-7} M), a specific blocker of small conductance Ca^{2+}-activated K^+ channel, significantly inhibited ACh-induced hyperpolarization (Figure 1c). TEA, a relatively specific blocker of large conductance

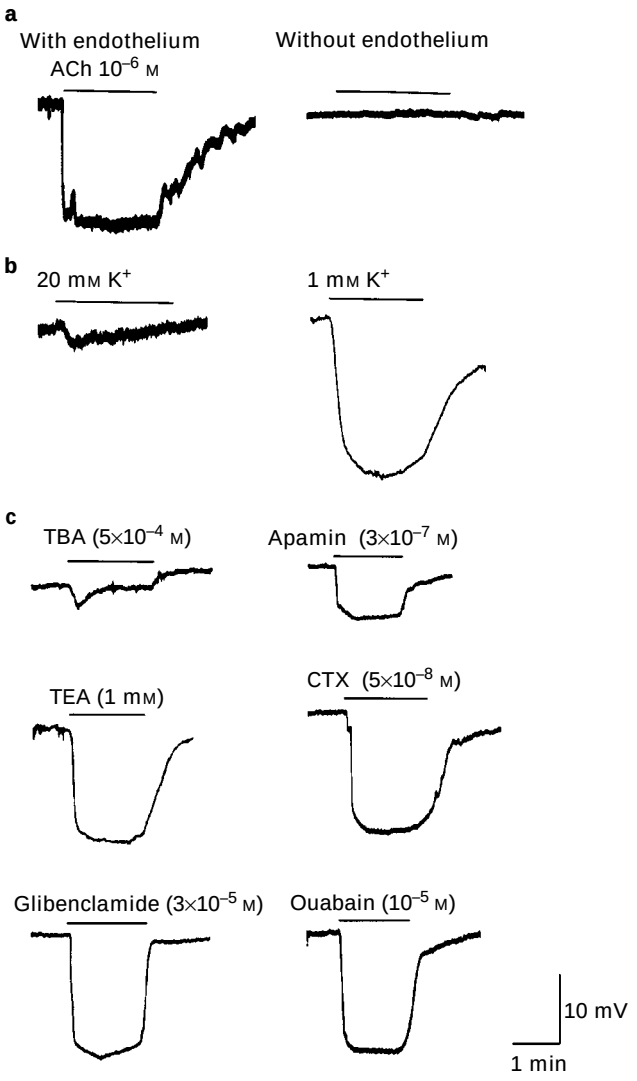

Figure 1 Effects of endothelium removal and various interventions on membrane hyperpolarization of smooth muscle cells induced by 10^{-6} M ACh in mesenteric arteries from control rats. (a) Effect of endothelium removal. (b) Effects of 20 mM K^+ and 1 mM K^+ solution. Concentrations of K^+ were modified by replacing NaCl with KCl. The membrane was depolarized by about 11 and 1 mV in the presence of 20 mM K^+ and 1 mM K^+ solution, respectively. (c) Effects of 5×10^{-4} M tetrabutylammonium (TBA), 3×10^{-7} M apamin, 1 mM tetraethylammonium (TEA), 5×10^{-8} M charybdotoxin (CTX), 3×10^{-5} M glibenclamide and 10^{-5} M ouabain. TBA, apamin and TEA depolarized the membrane by about 11, 3 and 3 mV, respectively. CTX, glibenclamide and ouabain had almost no effect on the membrane potential. ACh was applied during the period indicated by horizontal bars. All agents were applied to the bath 15–30 min before the addition of ACh.

Ca^{2+}-activated K^+ channels, inhibited ACh-induced hyperpolarization only at a high concentration (10 mM) (Figure 1c and Table 1). However, charybdotoxin (5×10^{-8} M), a specific blocker of large conductance Ca^{2+}-activated K^+ channels, glibenclamide (10^{-5} M), an ATP-sensitive K^+ channel blocker, and ouabain (10^{-5} M), a Na,K-ATPase inhibitor, were without effect on membrane hyperpolarization by ACh (Figure 1c). The summarized data are shown in Table 1.

Altered endothelium-dependent hyperpolarization in diabetic rats

The average resting membrane potential of smooth muscle cells in mesenteric arteries obtained from diabetic rats (-55.6 ± 0.4 mV, $n=83$) did not differ from that from age-matched control rats (-55.3 ± 0.5 mV, $n=80$). However, the peak amplitude of endothelium-dependent hyperpolarization by ACh was significantly diminished in diabetic arteries compared to controls (Figure 2). In addition, diabetic arteries exhibited a transient hyperpolarization with the membrane potential returning to near-basal levels despite continuation of the ACh infusion (Figure 2). The concentration-response curves for the peak amplitude of hyperpolarization induced by ACh in control and diabetic rat mesenteric arteries are shown in Figure 3.

Pretreatment with L-NOARG (3×10^{-4} M), a compound that inhibits the formation of NO from L-arginine, did not affect the hyperpolarizing response to ACh in either control or diabetic arteries (Figure 4). The hyperpolarizing responses of both control and diabetic arteries were also not affected by pretreatment with indomethacin (10^{-5} M), a cyclo-oxygenase inhibitor (Figure 4). Furthermore, pretreatment with SOD (60 u ml^{-1}), a specific scavenger of superoxide anions, had no effect on the hyperpolarizing effect of ACh in either control or diabetic arteries. Thus, the peak hyperpolarizing responses of control arteries to 10^{-6} M ACh were -16.3 ± 0.7 and -16.0 ± 1.0 mV ($n=3$) before and 10 min after exposure to 60 u ml^{-1} SOD, while the responses of diabetic arteries were -10.7 ± 1.5 and -11.0 ± 1.7 mV ($n=3$) before and after the treatment, respectively. In addition, the transient nature of ACh-induced hyperpolarization observed in diabetic arteries remained unchanged with L-NOARG, indomethacin or SOD.

A23187 (10^{-6} M) produced endothelium-dependent hyperpolarization in both control and diabetic arteries. Hyperpolarizations by A23187 were abolished by the removal of the endothelium (data not shown). The hyperpolarizing effect of A23187 in diabetic arteries was smaller in magnitude and was more transient than that in control arteries (Figure 5a). The summarized data are shown in Figure 5b.

Pinacidil, an ATP-sensitive K^+ channel opener, elicited endothelium-independent hyperpolarization of the smooth muscle membrane, which was virtually abolished by 10^{-5} M glibenclamide (data not shown). The hyperpolarizing responses to pinacidil (10^{-6} M) were identical between control and diabetic arteries (Figure 6a). The concentration-response curves for the peak amplitude of hyperpolarization by pinacidil in control and diabetic rat mesenteric arteries are shown in Figure 6b.

SNP, a cyclic GMP-generating substance, caused small hyperpolarization in a concentration-dependent manner in both control and diabetic arteries. Thus, SNP at 10^{-5} and 10^{-4} M hyperpolarized the membrane potential by -2.7 ± 0.4 ($n=6$) and -4.5 ± 0.8 mV ($n=6$) in control arteries and by -2.7 ± 0.3 ($n=3$) and -4.3 ± 0.3 mV ($n=3$) in diabetic arteries, respectively. The difference in SNP-induced hyperpolarization between control and diabetic arteries was not statistically significant.

Effect of insulin treatment

Treatment with insulin prevented the alterations in endothelium-dependent hyperpolarization observed in diabetes. Endothelium-dependent hyperpolarizations by ACh and A23187 were not decreased and were sustained in arteries from diabetic rats treated with insulin (Figure 7a). The summarized data of membrane hyperpolarization by ACh (10^{-6} M) and A23187 (10^{-6} M) in insulin-treated rats are shown in Figure 7b. Membrane hyperpolarizations induced by ACh and A23187 in insulin-treated rats were not significantly different from those in controls.

Decreased endothelium-dependent relaxation induced by ACh in diabetic rats

The concentration-response curves for the peak amplitude of endothelium-dependent relaxation by ACh in the presence of 10^{-4} M L-NOARG and 10^{-5} M indomethacin in control and diabetic rat mesenteric arteries are shown in Figure 8a. The peak amplitude of relaxation was significantly reduced in

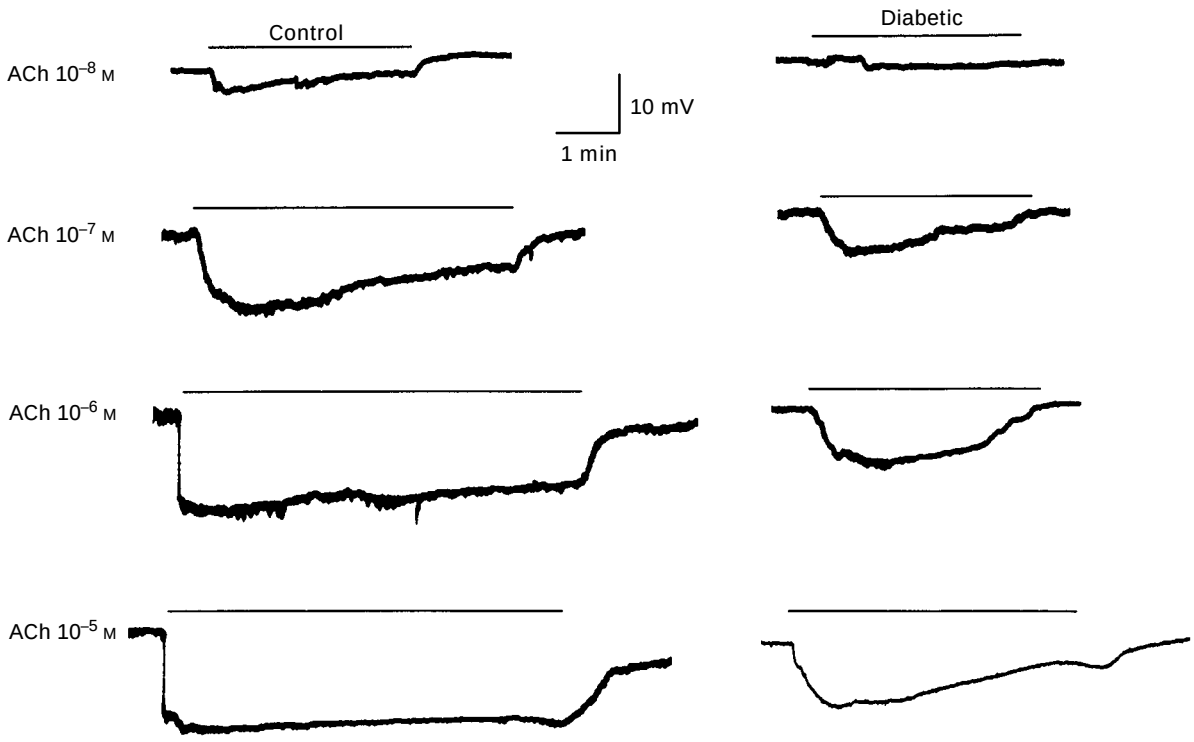

Figure 2 Actual recordings of the hyperpolarizing responses to ACh at various concentrations in mesenteric arteries from control and diabetic rats. ACh was applied during period indicated by horizontal bars. Recordings were from different preparations.

Table 1 Effects of K^+ channel blockers and other agents on resting membrane potential and membrane hyperpolarization by ACh (10^{-6} M)

	Changes in membrane potential (mV)			
	ACh (before)	*Treatment*	*ACh (after)*	n
$20K^+$	-16.5 ± 0.2	10.7 ± 1.5	-2.5 ± 0.5***	6
$1K^+$	-16.2 ± 0.3	0.6 ± 0.4	-29.3 ± 1.1***	6
TBA (5×10^{-4} M)	-15.8 ± 1.1	11.3 ± 3.3	-3.2 ± 0.9***	6
Apamin (3×10^{-7} M)	-15.2 ± 0.3	2.6 ± 0.6	-8.0 ± 1.1***	6
TEA (1 mM)	-15.0 ± 0.4	3.0 ± 0.3	-14.2 ± 0.7	5
TEA (10 mM)	-15.8 ± 1.4	8.8 ± 1.8	-5.3 ± 1.6***	6
CTX (5×10^{-8} M)	-16.7 ± 0.9	1.0 ± 1.0	-16.0 ± 1.2	6
Glibenclamide (3×10^{-5} M)	-17.5 ± 0.9	1.8 ± 0.7	-17.5 ± 0.9	6
Ouabain (10^{-5} M)	-17.2 ± 0.9	0.6 ± 0.8	-17.2 ± 0.9	5

***$P<0.001$, vs the respective ACh response before treatment.

diabetic rat mesenteric arteries compared with controls. Figure 8b shows the time courses of relaxations by 10^{-6} and 10^{-5} M ACh in control and diabetic rat mesenteric arteries. Diabetic arteries exhibited a reduced and more transient relaxation compared to controls.

Discussion

This study demonstrated that endothelium-dependent hyperpolarization produced by ACh is altered in mesenteric arteries from rats with streptozotocin-induced diabetes. First, the amplitude of the hyperpolarizing response was significantly diminished in diabetic arteries. Second, the hyperpolarization was of transient nature in diabetic arteries, while being sustained in controls. Endothelium-dependent relaxation in the presence of L-NOARG and indomethacin was also decreased and transient in diabetic arteries. The alterations in endothelium-dependent hyperpolarizations were nearly completely preserved by insulin therapy, suggesting that the altered hyperpolarizing responses are indeed due to diabetes. Thus, the results with insulin treatment essentially eliminate the possibility that the alterations observed in diabetic rats were a result of a direct effect of streptozotocin on the arteries.

Recent studies provide evidence that endothelium-dependent hyperpolarization by ACh may be mediated by EDHF (Chen *et al*., 1991; Feletou & Vanhoutte, 1988; Vanhoutte, 1989) and its ionic mechanism is likely to be an increase in outward current, attributable to an opening of K^+ channels located on arterial smooth muscle cells (Chen & Suzuki, 1989). Our results also suggest that the hyperpolarizing response to ACh in rat mesenteric arteries is mediated by an opening of K^+ channels. It was observed that ACh-induced hyperpolarization was reduced in high K^+ (20 mM) solution and enhanced in low K^+ (1 mM) solution, which will modulate the K^+ equilibrium potential of smooth muscle cells (Casteels & Kuriyama, 1966). The lack of effect of ACh on the membrane potential in high K^+ solution is in good agreement with previous results obtained by other investigators (Waldron & Garland, 1994). In addition, we found that TBA, a nonspecific K^+ channel blocker, almost completely eliminated ACh-induced hyperpolarization. Several types of K^+ channels have been shown to mediate EDHF responses in different arteries from different species (Garland *et al*., 1995). However, in our preparations, the K^+ channel, which is involved in membrane

hyperpolarization by EDHF, may be mainly a small conductance Ca^{2+}-activated K^+ channel, because the small conductance Ca^{2+}-activated K^+ channel specific blocker apamin significantly attenuated ACh-induced hyperpolarization. Our findings are in agreement with other experimental results in rat (Adeagbo & Triggle, 1993), rabbit (Murphy & Brayden, 1995) mesenteric and porcine coronary (Hecker *et al*., 1994) arteries. Large conductance Ca^{2+}-activated K^+ channels have been described in most types of vascular smooth muscle (Nelson, 1993) and also shown to be involved in EDHF responses (Chen *et al*., 1991; Eckman *et al*., 1992; Cowan *et al*., 1993). However, large conductance Ca^{2+}-activated K^+ channels are unlikely to contribute to membrane hyperpolarization in rat mesenteric arteries, because TEA (1 mM), a moderately selective inhibitor of the large conductance Ca^{2+}-activated K^+ channel, and CTX (5×10^{-8} M), a specific blocker of large conductance Ca^{2+}-activated K^+ channel, had no effect on membrane hyperpolarization by ACh. The blocking effect of the high concentration of TEA (10 mM) may be due to a nonspecific action on other K^+ channels and/or indirect ac-

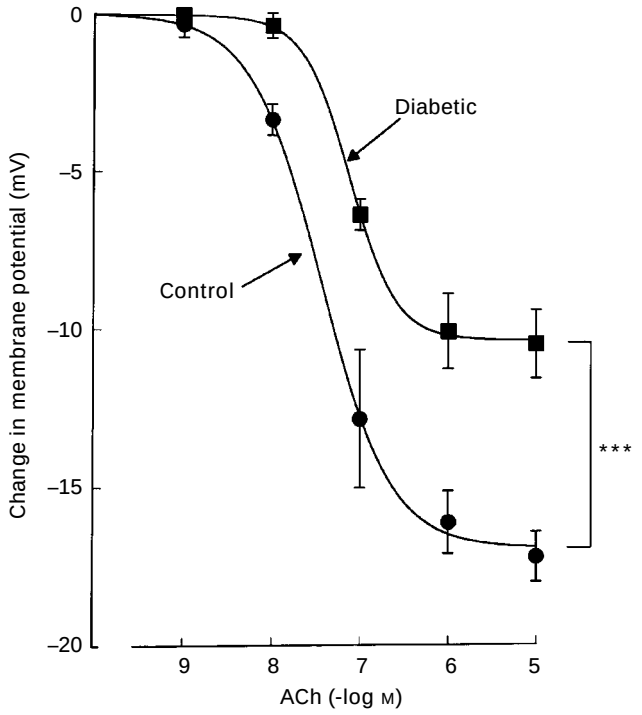

Figure 3 Concentration-response curves for ACh-induced hyperpolarization in mesenteric arteries with intact endothelium from control and diabetic rats. Data are means of 6–10 experiments; vertical lines show s.e.mean. ***$P<0.001$, vs respective control values.

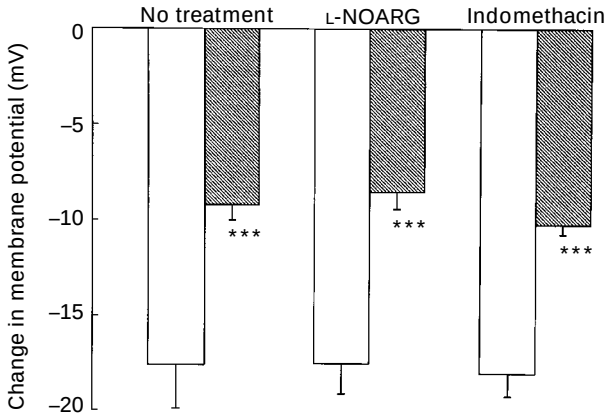

Figure 4 Effects of L-NOARG (3×10^{-4} M) and indomethacin (10^{-5} M) on hyperpolarization induced by ACh (10^{-6} M) in mesenteric arteries from control (open columns) and diabetic (hatched columns) rats. Data are means±s.e.mean of 5–10 experiments. ***$P<0.001$, vs respective control values.

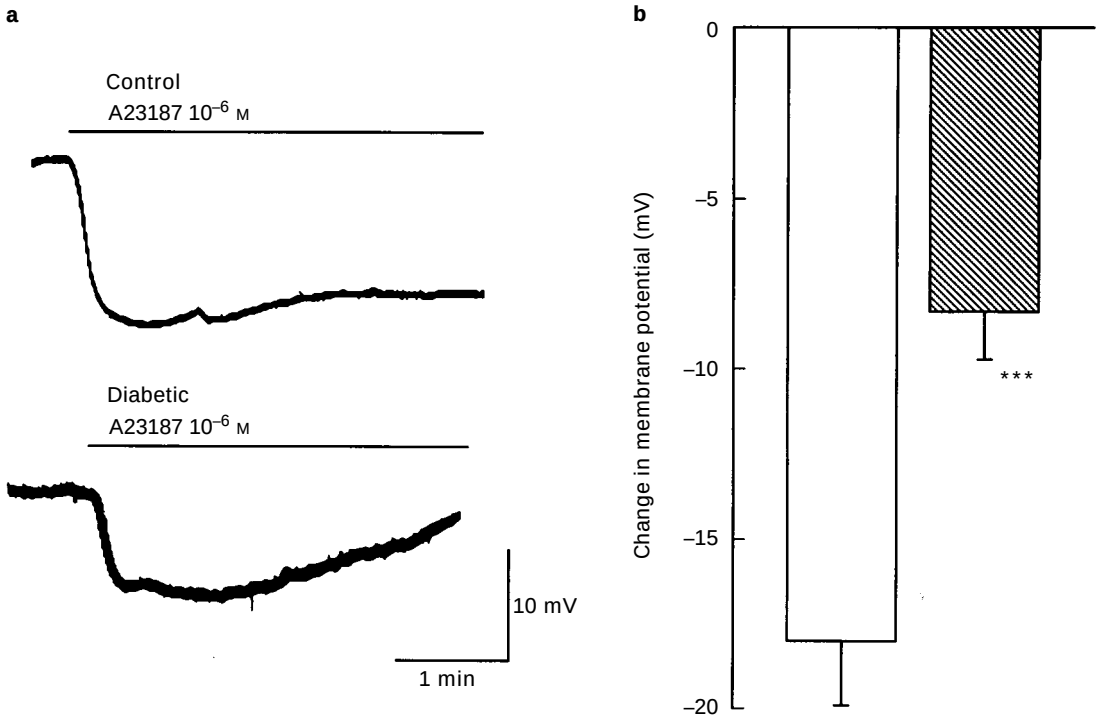

Figure 5 Effects of A23187 (10^{-6} M) on membrane potentials of smooth muscle cells in mesenteric arteries with intact endothelium from control and diabetic rats. (a) Actual recordings of the hyperpolarizing responses to A23187 in control and diabetic rats. (b) Summarized data of membrane hyperpolarization by A23187 in mesenteric arteries from control (open column) and diabetic (hatched column) rats. Data are means±s.e.mean of 6 experiments. ***$P<0.001$, vs corresponding control.

tions such as antagonism of muscarinic receptors (Cook & Haylett, 1985). Consistent with other many studies (Fujii *et al*., 1992; Garland & McPherson, 1992), glibenclamide, a specific blocker of ATP-sensitive K^+ channel, had no effect on the hyperpolarization, indicating a lack of involvement of ATP-sensitive K^+ channel in EDHF responses. Stimulation of Na,K-ATPase has been suggested to contribute to EDHF responses in canine coronary artery (Feletou & Vanhoutte, 1988). However, we found that ouabain, a Na,K-ATPase inhibitor, did not affect ACh-induced hyperpolarization. The same results have been obtained in rabbit ear artery (Suzuki, 1988). Small conductance Ca^{2+}-activated K^+ channels have

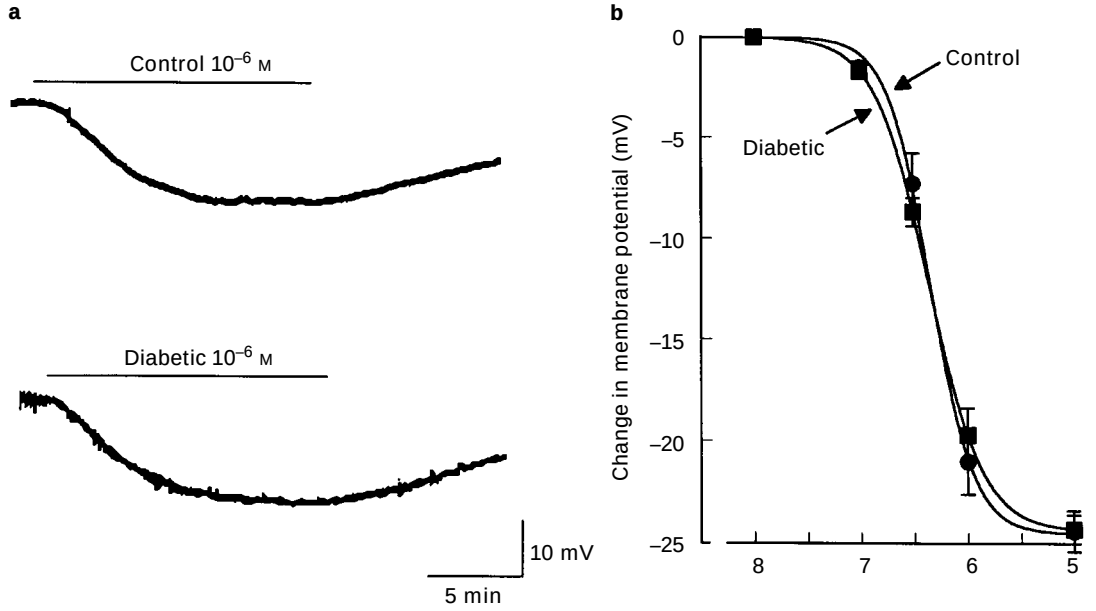

Figure 6 Effects of pinacidil on membrane potentials of smooth muscle cells in mesenteric arteries without endothelium from control and diabetic rats. (a) Actual recordings of the hyperpolarizing responses to pinacidil (10^{-6} M) in control and diabetic rats. (b) Concentration-response curves for pinacidil-induced hyperpolarization in mesenteric arteries from control and diabetic rats. Data are means of 6–10 experiments; vertical lines shows s.e.mean. The difference in concentration-response curves between control and diabetic arteries was not statistically significant.

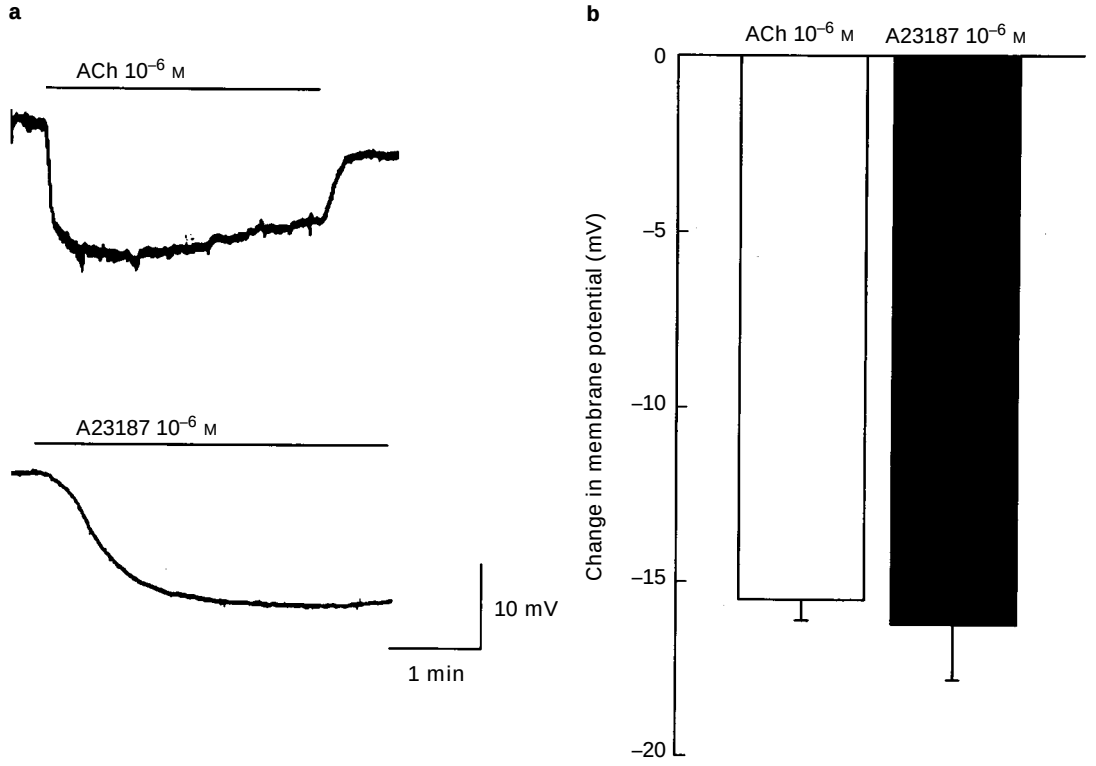

Figure 7 Effect of insulin treatment of diabetic rats on membrane hyperpolarization by ACh (10^{-6} M) and A23187 (10^{-6} M) in rat mesenteric artery. (a) Actual recordings of the hyperpolarizing responses to ACh and A23187 in insulin-treated diabetic rats. (b) Summarized data of membrane hyperpolarization by ACh and A23187 in mesenteric arteries from insulin-treated diabetic rats. Data are means ± s.e.mean of 6 experiments.

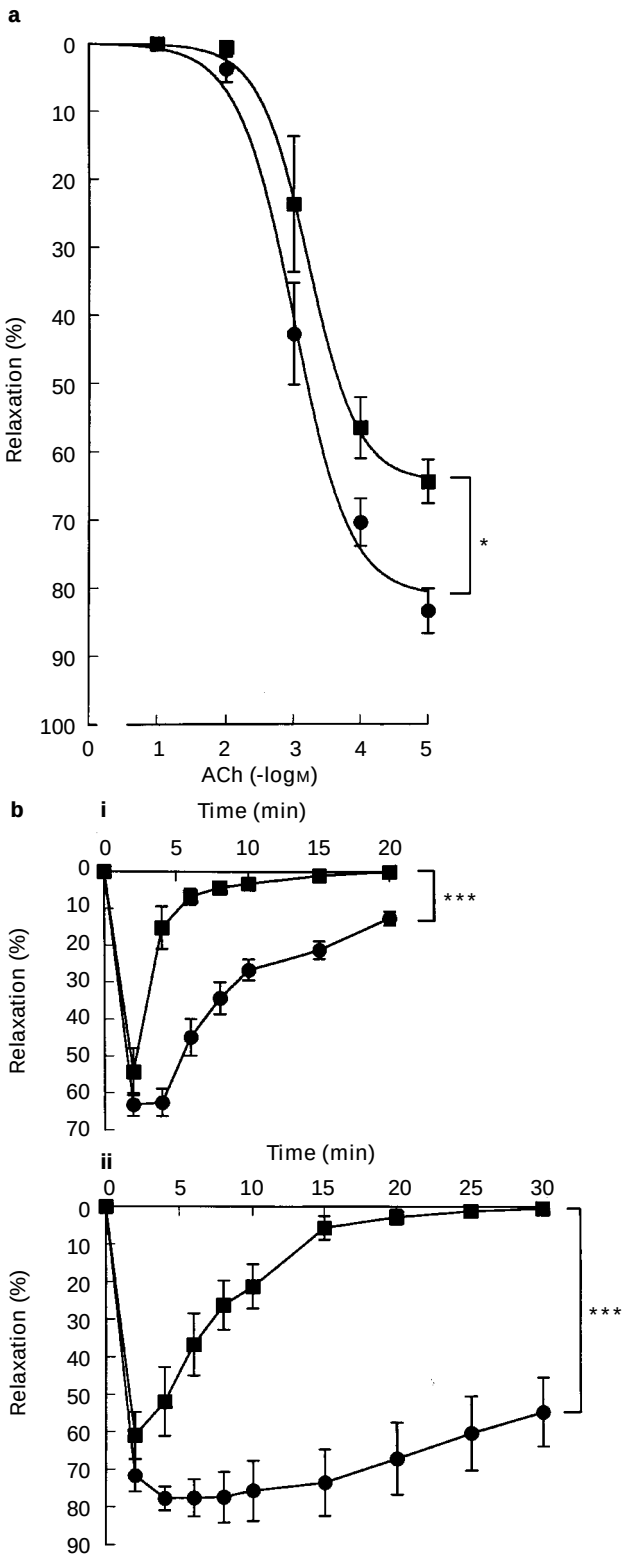

Figure 8 Relaxations produced by ACh in the presence of L-NOARG (10^{-4} M) and indomethacin (10^{-5} M) in control and diabetic rat mesenteric arterial rings precontracted with phenylephrine (10^{-7} M). L-NOARG and indomethacin were added to the bath 15 min before application of phenylephrine. (a) Concentration-response curves for ACh in control (●) and diabetic (■) rat mesenteric arteries. ACh was added to the bath cumulatively. The points shown are means of 7 experiments; vertical lines show s.e.mean. (b) Time courses of endothelium-dependent relaxations induced by ACh at (i) 10^{-6} and (ii) 10^{-5} M during contractions to phenylephrine (10^{-7} M) in control (●) and diabetic (■) rat mesenteric arterial rings. Data are means of 10 experiments; vertical lines show s.e.mean. Responses are expressed as % relaxation of phenylephrine-induced contraction. $*P<0.05$ and $***P<0.001$, vs corresponding control.

been shown to exist in both endothelium (Groschner *et al*., 1992) and vascular smooth muscle cells (Van Renterghem & Lazdunski, 1994). It is thus a possibility that apamin might have inhibited membrane hyperpolarization of endothelial cells and reduced the influx of Ca^{2+}, thereby reducing the release of EDHF and membrane hyperpolarization of smooth muscle cells. However, small conductance Ca^{2+}-activated K^+ channels appear to be on the vascular smooth muscle cells. Chen & Cheung (1992) have shown that the hyperpolarizing responses of endothelial cells to endothelium-dependent vasodilators such as ACh are not inhibited by apamin.

Even in mesenteric arteries from diabetic rats, ACh did not affect the membrane potential when the endothelium was absent. Thus, a direct depolarizing effect of ACh is unlikely to be a cause of decreased membrane hyperpolarization in diabetes. Since the resting membrane potentials were not different between control and diabetic arteries and the responsiveness of smooth muscle cells to pinacidil was preserved, it appears that the impaired hyperpolarizing responses to ACh are not due to the altered K^+ channels of smooth muscle cells in diabetic arteries. However, it should be noted that K^+ channels responsible for hyperpolarization by ACh are distinct from those activated by pinacidil.

The initial step of endothelium-dependent hyperpolarization would be activation of the receptor mechanism which is triggered by agonist-binding of muscarinic receptors in endothelial cells. The decreased hyperpolarizing response to ACh might reflect a reduced number of muscarinic receptors in endothelial cells during chronic diabetic conditions. Alternatively, coupling of muscarinic receptors to transduction mechanisms involved in the release of EDHF might be impaired in diabetes. However, disturbances of both muscarinic receptors and their coupling to transduction mechanisms are unlikely to be a cause of the diminished hyperpolarizing effect of ACh in diabetic mesenteric arteries. We found that the Ca^{2+} ionophore A23187, which is known to cause endothelium-dependent hyperpolarization by bypassing any receptor mechanisms (Chen & Suzuki, 1990), also produced a diminished hyperpolarizing response in diabetic mesenteric arteries.

NO has been shown to be involved in ACh-induced hyperpolarization of smooth muscle cells of guinea pig uterine (Tare *et al*., 1990), coronary (Parkington *et al*., 1993) and rabbit mesenteric (Murphy & Brayden, 1995) arteries. Increased production of oxygen-derived free radicals and decreased free radical scavenger systems have been described in diabetes (Wolff & Dean, 1987). A great deal of evidence strongly suggests that increased production of oxygen-derived free radicals could readily destroy EDRF-NO, thereby impairing endothelium-dependent relaxation in diabetic vessels (Pieper & Gross, 1988; Hattori *et al*., 1991; Tesfamariam & Cohen, 1992; Diederich *et al*., 1994). If endothelium-dependent hyperpolarization in rat mesenteric arteries is mediated by EDRF-NO, attenuated endothelium-dependent hyperpolarization in diabetic arteries could be explained by enhanced destruction of EDRF-NO. This does not appear to be the case, since pretreatment with L-NOARG, an inhibitor of NO synthase, did not affect ACh-induced hyperpolarization in either control or diabetic arteries. We (Fukao *et al*., 1997) and others (Chen *et al*., 1988) have confirmed that oxyhaemoglobin, which inhibits NO-mediated responses by scavenging NO, has no effect on ACh-induced hyperpolarization. In addition, there are several studies showing that exogenous NO is unable to produce hyperpolarization in blood vessels in which endothelium-dependent hyperpolarization is observed (Bény & Brunet, 1988; Brayden, 1990; Komori *et al*., 1988). In this study, to our surprise, SNP, a guanosine 3′:5′-cyclic monophosphate (cyclic GMP)-generating agent, caused hyperpolarization in a concentration-dependent manner. However, the extent of hyperpolarization produced by SNP was small and far less than that produced by ACh. Furthermore, SNP-induced hyperpolarization was blocked by glibenclamide (unpublished observations). Finally, SNP-induced hyperpolarization did not exhibit a difference between control and diabetic arteries. Hence, the

present findings are in agreement with the concept that endothelium-dependent membrane hyperpolarization cannot be explained by EDRF-NO but by another factor, presumably EDHF (Chen *et al*., 1991). We also found that pretreatment with SOD, a specific scavenger of superoxide anions, had no effect on ACh-induced hyperpolarization in either control or diabetic arteries, suggesting that superoxide anions or free-radicals derived from superoxide anions are not involved in decreased endothelium-dependent hyperpolarization in diabetic arteries.

There have been observed significant increases in prostanoids, including thromboxane A_2 and prostaglandin $F_{2\alpha}$ in diabetic vessels (Tesfamariam *et al*., 1989). However, it seems unlikely that an increased release of prostanoids is able to produce depolarization of the membrane potentials and thereby counteract endothelium-dependent hyperpolarization in diabetic arteries. In this study, pretreatment with indomethacin did not affect the hyperpolarizing response to ACh in either control or diabetic arteries, therefore, it appears unlikely that prostanoids are involved in this response. We cannot exclude the possibility that other endothelium-derived substances, such as endothelin, may produce membrane depolarization and reduce the hyperpolarizing response to ACh. Indeed, it has been shown that the plasma concentrations of endothelin-1 (Takahashi *et al*., 1990; Collier *et al*., 1992) and of big endothelin-1 (Tsunoda *et al*., 1991), a precursor of endothelin-1, are elevated in patients with diabetes mellitus. Furthermore, increased production of endothelin-1 from mesenteric arteries has been demonstrated in streptozotocin-induced diabetic rats (Takeda *et al*., 1991). However, we have found that the decreased response in diabetic arteries was not affected by BQ-123, a selective ET_A receptor antagonist, which has been shown to block the arterial smooth muscle depolarization by endothelin-1 (Nakashima & Vanhoutte, 1993) (unpublished observations).

Endothelium-dependent relaxation by ACh in the presence of L-NOARG and indomethacin, which is considered to be mediated exclusively by membrane hyperpolarization of vascular smooth muscle cells by EDHF, was also significantly impaired in diabetes. The maximum hyperpolarizing and relaxant responses of diabetic arteries to ACh were about 61 and 77% of the responses of controls, respectively. Hyperpolarization of the cellular membrane is generally accepted to decrease Ca^{2+} influx by closing voltage-dependent Ca^{2+} channels, thereby lowering intracellular Ca^{2+} levels and relaxing the tissue. It is, therefore, possible that the impairment of endothelium-dependent hyperpolarization might enhance voltage-dependent Ca^{2+} influx. Several studies have demonstrated that the vascular responsiveness to vasoactive agents which induce extracellular Ca^{2+}-dependent contraction is enhanced in diabetes (Abebe & MacLeod, 1990; White & Carrier, 1990). Such an increased responsiveness of diabetic vasculature could be exaggerated by impaired endothelium-dependent hyperpolarization. Altered vascular responses to vasoactive agents may be of particular importance to some manifestations of vascular dysfunction during diabetes. Endothelium-dependent hyperpolarization has been suggested to play a predominant role in the regulation of resistance small arteries. Thus, the findings of the present study may have important implications for mechanisms by which diabetes exhibits vascular dysfunction, because small vessel dysfunctions such as retinopathy, nephropathy and neuropathy are among the major complications in diabetes.

In conclusion, endothelium-dependent hyperpolarization, presumably mediated by EDHF, is impaired in mesenteric arteries from diabetic rats. The present results indicate that this impaired endothelium-dependent hyperpolarization could partly contribute to the altered endothelium-dependent relaxations in diabetic arteries. The impairment of EDRF-NO-mediated relaxations of diabetic blood vessels has been well established. Taken together, such endothelial cell dysfunctions may promote the development of vascular lesions in diabetes.

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References

ABEBE, W. & MACLEOD, K.M. (1990). Protein Kinase C-mediated contractile responses of arteries from diabetic rats. *Br. J. Pharmacol*., **101,** 465 – 471.

ADEAGBO, A.S.O. & TRIGGLE, C.R. (1993). Varying extracellular $[K^+]$: a functional approach to separating EDHF- and EDNO-related mechanisms in perfused rat mesenteric arterial bed. *J. Cardiovasc. Pharmacol*., **21,** 423 – 429.

BÉNY, J.L. & BRUNET, P.C. (1988). Neither nitric oxide nor nitroglycerin accounts for all the characteristics of endothelially mediated vasodilation of pig coronary arteries. *Blood Vessels*, **25,** 308 – 311.

BRAYDEN, J.E. (1990). Membrane hyperpolarization is a mechanism of endothelium-dependent cerebral vasodilation. *Am. J. Physiol*., **259,** H668 – H673.

CASTEELS, R. & KURIYAMA, H. (1966). Membrane potential and ion content in the smooth muscle of the guinea-pig's tanea coli at different external potassium concentrations. *J. Physiol*., **184,** 120 – 130.

CHEN, G. & CHEUNG, D.W. (1992). Characterization of acetylcholine-induced membrane hyperpolarization in endothelial cells. *Circ. Res*., **70,** 257 – 263.

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.

CHEN, G. & SUZUKI, H. (1989). Some electrical properties of the endothelium-dependent hyperpolarization recorded from rat arterial smooth muscle cells. *J. Physiol*., **410,** 91 – 106.

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

CHEN, G., YAMAMOTO, Y., MIWA, K. & SUZUKI, H. (1991). Hyperpolarization of arterial smooth muscle induced by endothelial humoral substances. *Am. J. Physiol*., **260,** H1888 – H1892.

COLLIER, A., LEACH, J.P., MCLELLAN, A., JARDINE, A., MORTON, J.J. & SMALL, M. (1992). Plasma endothelin like immunoreactivity levels in IDDM patients with micro albuminuria. *Diabetes Care*, **15,** 1038 – 1040.

COOK, N. & HAYLETT, D.G. (1985). Effect of apamin, quinin and neuromuscular blockers on calcium-activated potassium channels in guinea-pig hepatocytes. *J. Physiol*., **358,** 373 – 394.

COWAN, C.L., PALACINO, J.J., NAJIBI, S. & COHEN, R.A. (1993). Potassium channel-mediated relaxation to acetylcholine in rabbit arteries. *J. Pharmacol. Exp. Ther*., **266,** 1482 – 1489.

DIEDERICH, D., SKOPEC, J., DIEDERICH, A. & DAI, F.X. (1994). Endothelial dysfunction in mesenteric resistance arteries of diabetic rats: role of free radicals. *Am. J. Physiol*., **266,** H1153 – H1161.

DURANTE, W., SEN, A.K. & SUNAHARA, F.A. (1988). Impairment of endothelium-dependent relaxation in aortae from spontaneously diabetic rats. *Br. J. Pharmacol*., **94,** 463 – 468.

ECKMAN, C.L., FRANKOVICH, J.D. & KEEF, K.D. (1992). Comparison of the actions of acetylcholine and BRL 38227 in the guinea-pig coronary artery. *Br. J. Pharmacol*., **106,** 9 – 16.

FELETOU, M. & VANHOUTTE, P.M. (1988). Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *Br. J. Pharmacol*., **93,** 515 – 524.

FUJII, K., TOMINAGA, M., OHMORI, S., KOBAYASHI, K., KOGA, T., TAKATA, Y. & FUJISHIMA, M. (1992). Decreased endothelium-dependent hyperpolarization to acetylcholine in smooth muscle of the mesenteric artery of spontaneously hypertensive rats. *Circ. Res.*, **70,** 660 – 669.

FUKAO, M., HATTORI, Y., KANNO, M., SAKUMA, I. & KITABATAKE, A. (1995). Thapsigargin- and cyclopizonic acid-induced endothelium-dependent hyperpolarization in rat mesenteric artery. *Br. J. Pharmacol.*, **116,** 1541 – 1544.

FUKAO, M., HATTORI, Y., KANNO, M., SAKUMA, I. & KITABATAKE, A. (1996). EDHF responses in arteries from diabetic rats. In *Endothelium-Derived Hyperpolarizing Factor*. ed. Vanhoutte, P.M., pp. 263 – 270. Amsterdam: Hardwood Academic Publishers.

FUKAO, M., HATTORI, Y., KANNO., M., SAKUMA, I. & KITABATAKE, A. (1997). Sources of Ca^{2+} in relation to generation of acetylcholine-induced endothelium-dependent hyperpolarization in rat mesenteric artery. *Br. J. Pharmacol.*, **120,** 1328 – 1334.

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

GARCIA, M.J., MCNAMARA, P.M., GORDON, T. & KINNELL, W.B. (1974). Morbidity and mortality in diabetics in the Framingham population; sixteen-year follow-up study. *Diabetes*, **23,** 104 – 111.

GARLAND, J.G. & MCPHERSON, G.A. (1992). Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. *Br. J. Pharmacol.*, **105,** 429 – 435.

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 – 29.

GROSCHNER, K., GRAIER, W.F. & KUKOVETZ, W.R. (1992). Activation of a small-conductance Ca^{2+}-dependent K^+ channel contributes to bradykinin-induced stimulation of nitric oxide synthesis in pig aortic endothelial cells. *Biochem. Biophys. Acta*, **1137,** 162 – 170.

HATTORI, Y., KAWASAKI, H., ABE, K. & KANNO, M. (1991). Superoxide dismutase recovers altered endothelium-dependent relaxation in diabetic rat aorta. *Am. J. Physiol.*, **261,** H1086 – H1094.

HECKER, M., BARA, A.T., BAUERSACHES, J. & BUSSE, R. (1994). Characterization of endothelium-derived hyperpolarizing factor as a cytochrome P450-derived arachidonic acid metabolite in mammals. *J. Physiol.*, **481,** 407 – 414.

IGNARRO, L.J., BYRNS, R.E., BUGA, G.M. & WOODS, K.S. (1987). Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacological and chemical properties that are identical to those of nitric oxide radical. *Circ. Res.*, **61,** 866 – 879.

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

KOMORI, K., LONRENZ, R.R. & VANHOUTTE, P.M. (1988). Nitric oxide, ACh, and electrical and mechanical properties of canine arterial smooth muscle. *Am. J. Physiol.*, **255,** H207 – H212.

MERAJI, S., JAYAKODY, L., SENARANTE, M.J.P., THOMSON, A.B.R. & KAPPAGODA, T. (1987). Endothelium-dependent relaxation in aorta of BB rats. *Diabetes*, **36,** 978 – 981.

MURPHY, M.E. & BRAYDEN, J.E. (1995). Apamin-sensitive K^+ channels mediate an endothelium-dependent hyperpolarization in rabbit mesenteric arteries. *J. Physiol.*, **489,** 723 – 734.

MYERS, P.R., MINOR, R.L.JR., GUERRA, R.JR., BATES, J.N. & HARRISON, D.G. (1990). Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble *S*-nitrosocysteine than nitric oxide. *Nature*, **345,** 161 – 163.

NAKASHIMA, M. & VANHOUTTE, P.M. (1993). Endothelin-1 and -3 cause endothelium-dependent hyperpolarization in the rat mesenteric artery. *Am. J. Physiol.*, **265,** H2137 – H2141.

NELSON, M.T. (1993). Ca^{2+}-activated potassium channels and ATP-sensitive potassium channels as modulatores of vascular tone. *Trends Cardiovas. Med.*, **3,** 54 – 60.

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.*, **131,** 75 – 78.

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

PARKINGTON, H.C., TARE, M., TONTA, M.A. & COLEMAN, H.A. (1993). Stretch revealed three components in the hyperpolarization of guinea-pig coronary artery in response to acetylcholine. *J. Physiol.*, **465,** 459 – 476.

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

RUDERMAN, N.B. & HAUDENSCHILD, C. (1984). Diabetes as an atherogenic factor. *Prog. Cardiovasc. Res.*, **26,** 373 – 412.

SUZUKI, H. (1988). The electrogenic Na-K pump does not contribute to endothelium-dependent hyperpolarization in the rabbit ear artery. *Eur. J. Pharmacol.*, **156,** 295 – 297.

TAKAHASHI, K., GHATEI, M.A., LAM, H.-C., O'HALLORAN, D.J. & BLOOM, S.R. (1990). Elevated plasma endothelin in patients with diabetes mellitus. *Diabetologia*, **33,** 306 – 310.

TAKEDA, Y., MIYAMORI, I., YONEDA, T. & TAKEDA, R. (1991). Production of endothelin-1 from the mesenteric arteries of streptozotocin-induced diabetic rats. *Life Sci.*, **48,** 2553 – 2556.

TARE, M., PARKINGTON, H.C., COLEMAN, H.A., NEID, T.O. & DUSTING, G.J. (1990). Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. *Nature*, **346,** 69 – 71.

TESFAMARIAM, B. & COHEN, R.A. (1992). Free radicals mediate endothelial cell dysfunction caused by elevated glucose. *Am. J. Physiol.*, **263,** H321 – H326.

TESFAMARIAM, B., JAKUBOWSKI, J.A. & COHEN, R.A. (1989). Contraction of diabetic rabbit aorta due to endothelium-derived PGH_2/TXA_2. *Am. J. Physiol.*, **257,** H1327 – H1333.

TOMLINSON, K.C., GARDINER, S.M., HEBDEN, R.A. & BENNETT, T. (1992). Functional consequences of streptozotocin-induced diabetic mellitus, with particular reference to the cardiovascular system. *Pharmacol. Rev.*, **44,** 103 – 150.

TSUNODA, K., ABE, K., SATO, T., YOKOSAWA, S. & YOSHINAGA, K. (1991). Decreased conversion of big endothelin-1 to endothelin-1 in patients with diabetes mellitus. *Clin. Exp. Pharmacol. Physiol.*, **18,** 731 – 732.

VAN RENTERGHEM, C. & LAZDUNSKI, M. (1994). Identification of the Ca^{2+} current activated by vasoconstrictors in vascular smooth muscle cells. *Pflügers Archiv.*, **429,** 1 – 6.

VAN DE VOORDE, VANHEEL, J.B. & LEUSEN, I. (1992). Endothelium-dependent relaxation and hyperpolarization in aorta from control and renal hypertensive rats. *Circ. Res.*, **70,** 1 – 8.

VANHOUTTE, P.M. (1989). Endothelium and control of vascular function; state of the art lecture. *Hypertension*, **13,** 658 – 667.

WALDRON, G.J. & GARLAND, C.J. (1994). Contribution of both nitric oxide and a change in membrane potential to acetylcholine-induced relaxation in the rat small mesenteric artery. *Br. J. Pharmacol.*, **112,** 831 – 836.

WHITE, R.E. & CARRIER, G.O. (1990). Vascular contraction induced by activation of membrane calcium ion channels is enhanced in streptozotocin-diabetes. *J. Pharmacol. Exp. Ther.*, **253,** 1057 – 1062.

WOLFF, S.P. & DEAN, R.T. (1987). Glucose autooxidation and protein modification; The role of oxidative glucosylation in diabetes. *Biochem. J.*, **245,** 234 – 250.

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