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Impaired endothelium-dependent relaxation in isolated resistance arteries of spontaneously diabetic rats

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1 Previous studies have shown that endothelium-dependent relaxation in the aorta of spontaneously diabetic bio bred rats (BB) is impaired.

2 We have investigated noradrenaline (NA) contractility, endothelium-dependent acetylcholine (ACh) and bradykinin (BK) relaxation, and endothelium-independent sodium nitroprusside (SNP) relaxation in mesenteric resistance arteries of recent onset BB rats and established insulin treated BB rats, compared to their age-matched non diabetic controls.

3 There was no significant difference in the maximum contractile response or sensitivity to noradrenaline in either of the diabetic groups compared to their age-matched controls.

4 Incubation with the nitric oxide synthetase inhibitor N^G-nitro-L-arginine (L-NOARG) resulted in a significant increase in maximum contractile response to noradrenaline in the recent onset age-matched control group ($P<0.05$). Analysis of the whole dose-response curve (using ANOVA for repeated measures with paired t test) showed a significant left-ward shift following the addition of L-NOARG ($P<0.001$). A similar but less marked shift ($P<0.01$) was evident in vessels from recent onset diabetics. An overall shift in both sensitivity and maximum response was also evident in the age-matched non diabetic controls of the insulin-treated group ($P<0.05$). However, by contrast, there was no significant change in sensitivity in the insulin-treated diabetic rats.

5 ACh-induced endothelium-dependent relaxation was significantly impaired in the recent onset diabetic rats compared to their age-matched controls ($47\pm11\%$ versus $92\pm2\%$, $P<0.05$, $n=6$), and in the insulin treated diabetic rats ($34\pm5\%$ versus $75\pm6\%$, $P<0.05$, $n=6$). The relaxation responses to BK also were significantly impaired in the diabetic rats compared to their age-matched controls (recent onset: $20\pm3\%$ versus $72\pm7\%$, $P<0.05$, $n=6$; insulin treated: $12\pm9\%$ versus $68\pm7\%$, $P<0.05$, $n=7$).

6 Incubation with either the nitric oxide synthetase substrate, L-arginine, or the free radical scavenging enzyme superoxide dismutase (150 μml^{-1}) failed to improve the attenuated response of acetylcholine-induced relaxation in the diabetic vessels.

7 Endothelium-dependent relaxation mediated by ACh and BK was significantly attenuated in both the diabetic and control vessels after incubation with L-NOARG.

8 Pretreatment with a cyclo-oxygenase inhibitor, indomethacin, significantly enhanced the relaxation to ACh in both the recent onset and insulin treated diabetic rats ($42\pm10\%$, $n=7$ versus $64\pm7\%$, $n=7$, $P<0.05$, and $40\pm5\%$, $n=7$ versus $65\pm9\%$, $n=6$, $P<0.05$).

9 Following endothelium removal, there was a marked impairment in endothelium-dependent relaxation responses to ACh and BK in both the diabetic and control vessels.

10 Incubation with the thromboxane A_2 receptor antagonist SQ29548, did not significantly improve the ACh endothelium-dependent relaxation response in the diabetic vessels.

11 Endothelium-independent relaxation to sodium nitroprusside was significantly impaired in the first group of diabetic vessels studied; however, subsequent studies showed no impairment of the sodium nitroprusside response in the diabetic vessels.

12 In conclusion, the ability of the endothelium to regulate vascular contractility is reduced in recent onset diabetic vessels, and significantly impaired in established insulin treated diabetics. Relaxation to the endothelium-dependent vasodilators ACh and BK was impaired in both the recent onset and the established insulin treated diabetics, and the ACh response was significantly improved following pretreatment with indomethacin, suggesting a role for a cyclo-oxygenase-derived vasoconstrictor. Preliminary studies with a thromboxane A_2 receptor antagonist, SQ29548 did not significantly improve the impaired relaxation to ACh, indicating that the vasoconstrictor prostanoid is not thromboxane A_2.

Keywords: Mesenteric resistance arteries; endothelium; nitric oxide; endothelium derived relaxing factor (EDRF); Bio Bred rats; N^G-nitro-L-arginine (L-NOARG); cyclo-oxygenase; indomethacin; vasoconstrictor prostanoids

Introduction

Patients with diabetes are at an increased risk of hypertension, macrovascular disease (such as atheriosclerosis, and thrombosis) and microvascular disease (Garcia *et al.*, 1974; Christlieb *et al.*, 1976). Microangiopathy is in fact a major cause of morbidity leading to renal failure, blindness and peripheral neuropathy. The pathophysiological processes underlying this deterioration of the structure and function of these microvascular beds are poorly understood, but an increase in intracapillary pressure and blood flow has been implicated (Tooke, 1986). These changes have been found in diverse vascular beds, including the kidney (Ditzel & Junker, 1972; Vora *et al.*, 1992), retina (Kohner *et al.*, 1975), skin (Sandeman

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et al., 1992; Houben et al., 1992), and the forearm (Halkin et al., 1991). It has been suggested that this alteration of blood flow depends on precapillary vasodilatation, and this may be the initiating factor in diabetic microangiopathy.

These blood vessel abnormalities in diabetes may depend on an altered smooth muscle response to neurohumoral activity (Wiedmann et al., 1979). However, the endothelium has an important role modulating vascular tone (Furchgott & Zawadzki, 1980), and it has been suggested that abnormal endothelial function may be a contributory factor to large and small vessel disease in diabetes mellitus (Porta et al., 1987). Thus, impaired relaxation responses to an endothelium-dependent vasodilator, acetylcholine, have been demonstrated in the large blood vessels of animals with experimentally induced diabetes (Oyama et al., 1986; Kamata et al., 1989; Tesfamariam et al., 1990; Mayhan et al., 1991). Similarly, defective endothelium-dependent relaxation has been demonstrated in the smooth muscle of the corpora cavernosa in diabetic man (de Tejada et al., 1989), and isolated subcutaneous resistance arteries from juvenile onset insulin-dependent diabetics (McNally et al., 1994). Other studies have demonstrated that acetylcholine relaxation response attenuation of diabetic rabbit aorta and normal rabbit aorta exposed to elevated glucose *in vitro* is restored with a cyclo-oxygenase inhibitor, suggesting a release of a vasoconstricting prostanoid in diabetes (Tesfamariam et al., 1990; Tesfamariam 1994). Recently, studies have demonstrated impaired ACh-induced endothelium-dependent relaxation and increased noradrenaline (NA) sensitivity of mesenteric resistance arteries from streptozotocin (STZ)-induced diabetic rats (Taylor et al., 1992). However, other studies suggest enhanced noradrenaline sensitivity in arteries from chemically induced diabetic animals, independent of impaired relaxation to acetylcholine (Pieper & Gross, 1988; Cohen et al., 1990; Taylor et al., 1994a,b), which contrasts to observations of impaired contractile responses of isolated subcutaneous vessels from human diabetics (McNally et al., 1994).

It is possible that the alterations of NA sensitivity may be a non-specific response to STZ, and an alternative approach lies in studies of the BioBred (BB) diabetic rat. The Bio Breeding rat develops diabetes spontaneously, and therefore has features in common with Type 1 diabetes evident in man, including hyperglycaemia, weight loss, glycosuria, and ketonuria, followed by death if insulin therapy is not initiated (Nakhooda et al., 1977; Marliss et al., 1982; Mordes et al., 1987). The overall incidence of overt diabetes among our colony is 50–60%, with diabetes developing between 60–120 days.

The majority of studies using the BB rat have shown a specific impairment of endothelial-dependent relaxation in conduit arteries such as the aorta (Meraji et al., 1987; Durante et al., 1988). However, no studies have been performed using resistance arteries, and there is little information about the effects of duration of diabetes or insulin treatment. Accordingly, we have studied the contractile and relaxation responses of mesenteric resistance arteries from BB rats at the onset of diabetes, and after long-term insulin treatment (6–8 weeks), compared to age-matched non-diabetic controls.

Methods

BB Wistar rats were obtained from an inbred colony maintained at the Biomedical Services department of Leicester University. The BBs were diagnosed as diabetic when blood glucose levels were >20 mM, and they showed signs of glycosuria and weight loss. Non diabetic BB rats were used as age-matched controls. Rats were fed standard rat chow, and allowed access to water *ad libitum*. All animals were weighed daily, and blood glucose levels measured twice weekly. Once diabetic, insulin therapy was initiated to maintain euglycaemia and daily subcutaneous injections were administered according to weight and blood glucose levels.

Two separate diabetic groups were selected for study along with their age-matched non diabetic controls. The first group of BB rats were recent onset diabetics that had not received insulin therapy, and were killed 24–48 h after diabetes had been diagnosed. These rats were between 11–16 weeks old. The second group of BB rats were insulin treated and had received insulin for up to 6–8 weeks after onset of diabetes. Two groups of age-matched non-diabetic rats acted as controls. Systolic blood pressure was measured by tail cuff plethysmography in conscious, pretreated rats before they were killed (Bunag, 1973).

On the day of death, the treated diabetic rats did not receive their usual insulin injection. Rats were killed by stunning, followed by cervical dislocation. The mesentery and intestine were removed and placed in a small beaker containing cold physiological salt solution (PSS). The hind limbs were removed at the pelvic joint for tibia length measurement, and the heart excised from the thoracic cavity, blotted dry, and weighed. All tissues were stored in cold (4°C) PSS.

Preparation of vessels

Third order resistance arteries (internal diameter <350 μm) were dissected from the mesenteric bed with the aid of a light microscope from a point approximately 5 cm from the posterior end of the colon. Two vessel segments 2 mm in length were mounted in a Mulvany myograph on two 40 μm steel wires. One wire was attached to a force transducer, and the other to a micrometer; this arrangement permitted wall tension measurements at a predetermined internal diameter. Dissection of vessels and mounting was performed in cold (4°C) PSS.

Experimental protocol

Once mounted, the vessels equilibrated for 30 min in PSS at 37°C, gassed with 5% CO_2 and 95% O_2 to maintain a pH of 7.4. Morphological measurements of media thickness and internal diameter were then performed with a pre-calibrated filar micrometer eyepiece with a resolution of 1 μm. As the volume of the wall layers remains constant, the cross-sectional area of each vessel could then be calculated (Patel & Fry, 1969). After this the length tension characteristic for each vessel was determined and then the internal circumference was set to $0.9 \times L_{(100)}$ where $L_{(100)}$ is the internal circumference the artery would have *in vivo* when relaxed under a transmural pressure of 100 mgHg (Mulvany & Halpern, 1976). Following this normalisation procedure, the vessels were incubated in PSS for a further 60 min before the experimental protocol was begun. During this time the PSS was replaced at 20 min intervals.

Vessels were then stimulated three times with high potassium solution (KPSS), followed by one stimulation by KPSS containing 10^{-5} M noradrenaline. Contractions were maintained for 2 min before rinsing with PSS to base line. After this activation procedure had been completed, the vessels were rinsed three times with fresh PSS and left to recover at baseline for 15 min. Then a cumulative dose-response curve to noradrenaline (NA 10^{-8}–3×10^{-5} M) was carried out in the presence of 10^{-6} M cocaine, which was added 20 min before the contraction curve to block neuronal noradrenaline re-uptake. The vessels were rinsed three times with PSS, and allowed to recover at baseline for 15 min. Following this initial contraction curve the vessels were arbitrarily selected for one of the following four experimental protocols.

(1) Vessels from both recent onset and insulin-treated diabetics, and their age-matched non diabetic controls were studied. The arteries were maximally contracted with a bolus dose of NA 10^{-5} M and, once a plateau had been reached, relaxed with cumulative doses of acetylcholine (ACh, 10^{-9}–10^{-5} M). After this, the bath was rinsed three times with PSS and the vessels allowed to recover for 15 min. Then maximally contracted arteries (NA 10^{-5} M) were relaxed with cumulative doses of bradykinin (BK, 10^{-9}–10^{-5} M). The vessels were brought to base line again with three rinses of PSS, and the ACh relaxation curve was repeated following a 20 min incubation with the nitric oxide synthetase substrate L-arginine

(10^{-5} M). After a further period of rinsing with PSS, the vessels were incubated with the nitric oxide synthetase inhibitor N^G-nitro-L-arginine (L-NOARG, 10^{-5} M) for 1 h. The NA, ACh, and BK dose-response curves were repeated in the presence of L-NOARG. Finally, the vessels were maximally contracted with NA 10^{-5} M and relaxed with cumulative doses of the endothelium-independent vasodilator sodium nitroprusside (SNP, $10^{-9}-10^{-4}$ M).

(2) Vessels from recent onset diabetic rats and their age-matched non-diabetic controls were contracted with NA 10^{-5} M and acetylcholine relaxation dose-response curve performed (ACh $10^{-9}-10^{-5}$ M). After being rinsed with fresh PSS, the vessels were incubated with PSS containing superoxide dismutase (SOD, 150 units ml^{-1}) and catalase (CAT, 200 units ml^{-1}) for 10 min. Then, the vessels were maximally contracted with NA (10^{-5} M), and the ACh relaxation dose-response curve repeated. The ACh relaxation curve was repeated again after 30 min incubation with indomethacin (10^{-5} M). Finally vessels were incubated in PSS containing both indomethacin and L-NOARG (10^{-5} M) for 60 min, after which ACh, BK, and SNP dose relaxation-response curves were performed again. Vessels obtained from insulin treated rats and non diabetic age-matched controls were subjected to a similar protocol, except dose relaxation-response curves to ACh, BK, and SNP were performed in the presence of indomethacin alone.

(3) Vessels from recent onset diabetic rats and their age-matched non diabetic controls were arbitrarily selected for studies with an intact endothelium, or after the mechanical removal of the endothelium. Endothelium was removed by the technique described by Osol *et al.* (1989). Briefly, a human hair washed in ethanol followed by rinsing in PSS was inserted into the lumen of the vessel mounted under tension in a myograph. The hair was then repeatedly drawn forwards and backwards through the lumen of the vessel. Following denudation, vessels were left for 45 min before the following protocol was performed. A cumulative dose-contraction response curve to NA($10^{-8}-3\times10^{-5}$ M) was performed in the presence of 10^{-6} M cocaine. After being rinsed with PSS with a rest period of 10 min, the vessels were recontracted with 10^{-5} M NA and relaxation responses to ACh and BK were performed ($10^{-9}-10^{-5}$ M). Then a dose-contraction curve to increasing doses of ACh ($10^{-9}-10^{-5}$ M) was performed. Finally, the vessels were contracted with NA 10^{-5} M and a dose relaxation-response curve to SNP ($10^{-9}-10^{-4}$ M) performed.

(4) The contraction response to NA and dose relaxation-response curves to ACh and BK were performed with vessels from recent onset diabetic rats and age-matched non-diabetic controls. Then, vessels were incubated for 20 min in PSS containing the thromboxane A_2 inhibitor SQ 29548 before dose relaxation-response curves to ACh, BK and SNP were repeated. At the end of each study all vessels were rinsed in calcium free PSS before fixing overnight in 10% formalin for histological examination.

Previous published time control studies from our laboratory have shown there was no difference in either the sensitivity or maximum response to noradrenaline and acetylcholine between dose-response curves performed at baseline and after incubation in PSS for 30, 60 and 120 min (Bennett *et al.*, 1992). Moreover, in subsequent time control studies with mesenteric resistance arteries from Wistar rats, we have observed that maximum responses and sensitivity to NA and ACh remained unchanged over a 6 h time period (Heygate *et al.*, 1993).

Solutions and drugs

PSS composition (mM):NaCl 118, $NaHCO_3$ 25, KCl 4.5, KH_2PO_4 1.0, $CaCl_2$ 2.5, $MgSO_4$ 1.0 and glucose 6. The high potassium physiological salt solution (KPSS) for contraction studies was prepared by replacing NaCl with KCl. All drugs were freshly prepared on the day of study and dissolved in distilled water, except indomethacin and [1*S*-[1α,2β(5Z),-3β,4α]]-7-3-[[2-[phenylamino)carbonyl]hydrazino]-methyl]-7-oxabicyclo-[2.2.1]hept-2-yl]-5-heptenoic acid which were dissolved in ethanol. Previous studies performed in this laboratory have shown that adding this amount of ethanol to the myograph has no effect on either contraction or relaxation responses. Noradrenaline, cocaine hydrochloride, acetylcholine, bradykinin, sodium nitroprusside, L-arginine, superoxide dismutase, catalase and L-NOARG were obtained from Sigma Chemical Company (Poole, Dorset, U.K.). The thromboxane A_2 inhibitor SQ 29548 was a generous gift from Bristol-Myers Squibb Pharmaceuticals Ltd.

Data and statistical analaysis

Results are expressed as the mean ± s.e.mean. Contractile responses to noradrenaline are expressed as active tension (mN mm^{-1}), which is calculated from the measured force divided by twice the vessel length. The media stress of each vessel was then calculated from the wall thickness divided by tension in order to take account of differences in wall thickness. Noradrenaline sensitivity is expressed in terms of ED_{50}, which is the concentration of the drug required to produce 50% of the maximum response. Relaxation responses to ACh, BK, and SNP are expressed as a percentage decline of the maximum contractile response. Statistical significance was determined by use of Student's paired *t* test for comparisons within groups, and unpaired *t* test for comparisons between groups. Differences between the cumulative dose-response curves were compared by analysis of variance (ANOVA) for repeated measures.

Results

Morphology

The media thickness, media volume and media/lumen ratio were significantly increased in the diabetic vessels compared to their age-matched controls ($P<0.05$). There was no significant difference in vessel diameter, or heart weight to tibia ratios between the diabetics and their controls (Table 1). Furthermore, there was no significant difference in the systolic blood

Table 1 Morphological measurements of vessels from recent onset and insulin-treated diabetic rats and age-matched non-diabetic controls

	Recent onset diabetics ($n=12$)	*Age-matched controls* ($n=11$)	*Insulin-treated diabetics* ($n=13$)	*Age-matched controls* ($n=12$)
Media thickness	14.82 ± 0.64**	11.87 ± 0.67	17.6 ± 0.84***	13.08 ± 0.42
Media volume	13131 ± 729*	10790 ± 828	16023 ± 607***	12500 ± 657
Media/lumen	5.68 ± 0.34*	4.52 ± 0.27	6.8 ± 0.51*	4.65 ± 0.31
Vessel diameter (μm)	266 ± 11	263 ± 7	286 ± 12	284 ± 14
Heart weight/tibia	1.93 ± 0.1	2.03 ± 0.04	2.43 ± 0.09	2.27 ± 0.08

Data are shown as means ± s.e.mean.
*$P<0.05$, **$P<0.01$, ***$P<0.001$ (unpaired *t* test, diabetic versus control).

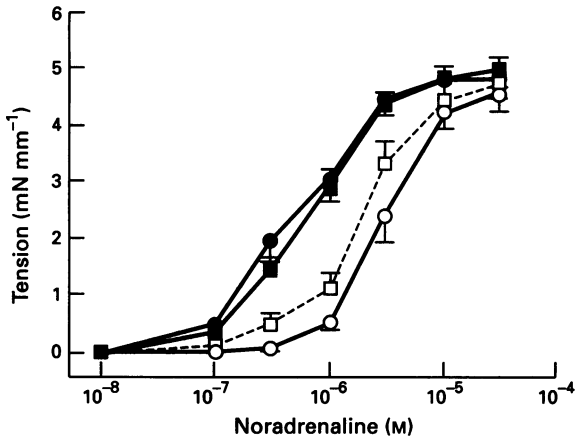

Figure 1 Noradrenaline contractile response of rat mesenteric resistance vessels from recent onset diabetics before (□) and after incubation with N^G-nitro-L-arginine (L-NOARG) (■), and their age-matched controls before (○), and after incubation with L-NOARG (●). Points show means±s.e.mean.

pressures (mmHg) of diabetic BBs and their age-matched controls (recent onset: 136±5 versus 128±5, $n=13$; insulin treated: 119±5 versus 125±7, $n=6$).

Contraction studies

The results showed that there was no significant difference in the maximum contraction or the sensitivity (ED_{50},) of recent onset diabetic rats compared to their age-matched non diabetic controls (4.62±0.27, $n=6$ versus 4.51±0.3 mN mm^{-1}, $n=6$ and 1.52±0.05, $n=6$ versus 1.6±0.03μmoll^{-1}, $n=6$). Similarly, in the insulin-treated diabetic rats there was no significant difference in the maximum contraction or sensitivity compared to the age-matched controls (5.47±0.55, $n=8$ versus 4.8±0.32 mN mm^{-1}, $n=8$ and 1.65±0.07, $n=8$ versus 1.75±0.04 μmol l^{-1}, $n=8$). When the noradrenaline dose-response curves were replotted for media stress, there was no significant difference in contractile response to NA between the diabetic and control vessels.

Incubation with the nitric oxide synthase inhibitor L-NOARG resulted in a significant increase in maximum contractile response to noradrenaline of the recent onset diabetic control group (4.51±0.3 versus 4.81±0.31 mN mm^{-1}, $n=6$, $P<0.05$). Analysis of the whole dose-response curve (using ANOVA for repeated measures and paired t test), showed a significant left-ward shift following the addition of L-NOARG, $P<0.001$. A similar but less marked left-ward shift of the response to NA in the presence of L-NOARG was observed in the recent onset diabetics ($P<0.01$) (Figure 1). An overall shift in both sensitivity and maximum response also was evident in the age-matched controls of the insulin-treated group ($P<0.05$). By contrast, there was no significant shift in the insulin-treated diabetic rats after incubation with L-NOARG (Figure 2).

Mechanical removal of the endothelium resulted in no significant change of the noradrenaline maximum contractile response or sensitivity in the recent onset diabetic vessels, or their controls (4.46±0.54 mN mm^{-1}, $n=8$ versus 3.8±0.27 mN mm^{-1} $n=8$ and 0.98±0.19 μmol l^{-1} versus 1.161±0.17 μmol l^{-1}, respectively). Furthermore, there was no significant difference in maximum contractile response or sensitivity to NA of intact control vessel compared to denuded control vessels, or intact diabetic compared to denuded diabetic vessels. There was no contractile response to cumulative doses of acetylcholine in either the denuded diabetic vessels or their controls.

Acetylcholine relaxation responses

Maximum relaxation to ACh was significantly attenuated in both the recent onset diabetic rats compared to their age-matched controls (47±11% versus 92±2% $P<0.05$, $n=6$), and the insulin-treated diabetic rats compared to their age-matched control group (34±5% versus 75±6% respectively, $P<0.05$, $n=6$) (Figure 3). Incubation with L-arginine failed to improve the impaired acetylcholine response in either of the diabetic groups (recent onset: 47±11% versus 44±11%, $n=6$, and insulin treated: 34±5% versus 43±9%, $n=6$). Inhibition of nitric oxide synthase with L-NOARG virtually abolished acetylcholine-induced relaxation in the recent onset diabetic vessels (47±11% versus 6±4%, $P<0.05$, $n=6$). However, in the age-matched controls ACh relaxation, although significantly impaired, was not abolished (92±2% versus 65±8%, $P<0.05$, $n=6$). There were no changes in acetylcholine sensitivity. Similar results were observed in the in-

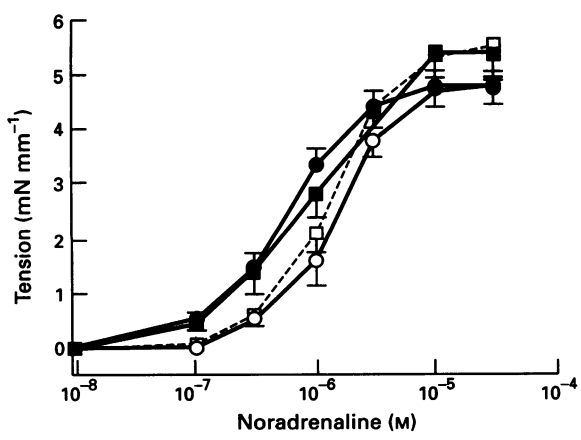

Figure 2 Noradrenaline contractile response of rat mesenteric resistance vessels from insulin-treated diabetics before (□) and after incubation with N^Gnitro-L-arginine (L-NOARG) (■), and their age-matched controls, before (○) and after incubation with L-NOARG (●). Points show means±s.e.mean.

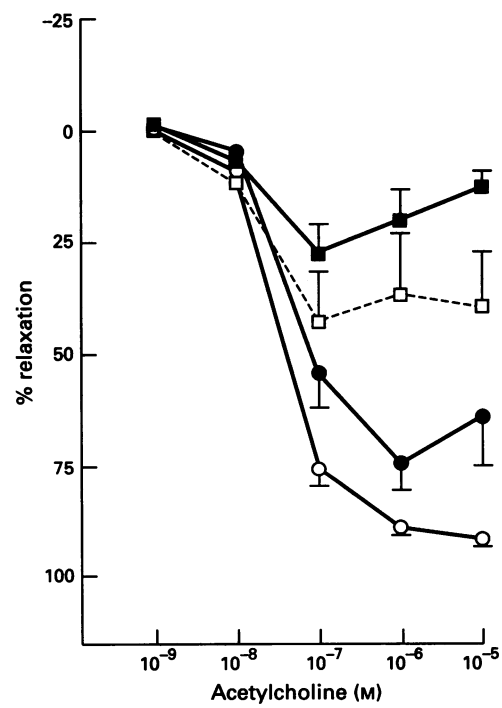

Figure 3 Acetylcholine-induced relaxation response of rat mesenteric resistance vessels from recent onset (□), and insulin-treated diabetics (■), and their age-matched controls (○) and (●) respectively. Points show means ±s.e.mean.

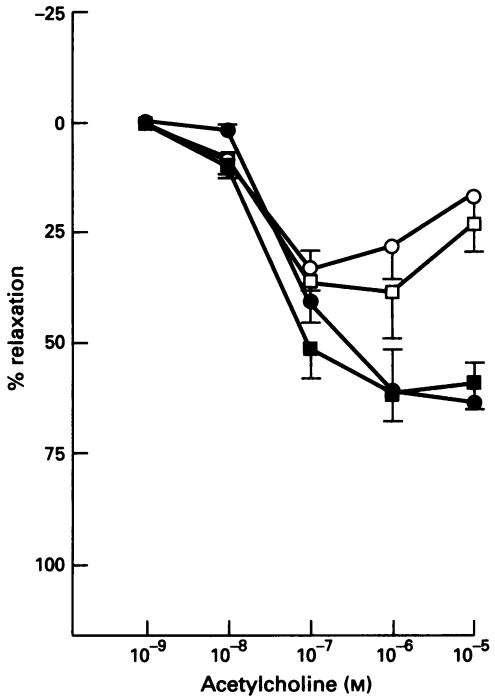

Figure 4 Acetylcholine-induced relaxation response of rat mesenteric resistance vessels from recent onset diabetics before (□), and after incubation with indomethacin (■), and insulin-treated diabetics before (○) and after incubation with indomethacin (●). Points show means ± s.e.mean.

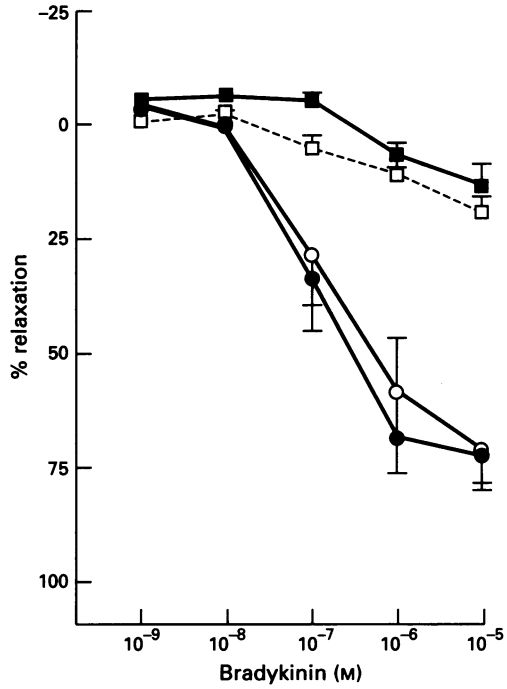

Figure 5 Bradykinin-induced relaxation response of rat mesenteric resistance vessels from recent onset (□), and insulin-treated diabetics (■), and their age-matched controls (○) and (●) respectively. Points show means ± s.e.mean.

sulin-treated diabetic vessels with a reduction in ACh-induced relaxation from 34±5% to 4±6%; but again the relaxation was only partially inhibited in the age-matched control group (ACh 75±6% versus 38±12%, $P<0.05$, $n=6$). This was associated with a decrease in sensitivity to ACh (ED_{50}) (0.063±0.008 μmol l^{-1} versus 0.19±0.035 μmol l^{-1}, $P<0.05$, $n=6$).

Incubation with the free radical scavengers superoxide dismutase and catalase failed to improve the relaxation response to acetylcholine in either the recent onset diabetic rats (42±10%, $n=6$, versus 34±10%, $n=6$) or the insulin treated rats (40±5%, $n=7$ versus 28±7%, $n=7$). Also, there was no significant change in the acetylcholine response after the addition of SOD and CAT in either of the age-matched control groups. By contrast, pretreatment with the cyclo-oxygenase inhibitor indomethacin resulted in a significant improvement in the acetylcholine-induced relaxation response in both the recent onset diabetic rats (42±10%, $n=7$ versus 64±7%, $P<0.05$), and the insulin-treated diabetic group (40±5%, $n=7$ versus 65±9%, $n=6$, $P<0.05$) (Figure 4). However, there was no significant change of acetylcholine relaxation response in either of the control groups after incubation with indomethacin. The addition of the thromboxane A_2 inhibitor SQ 29548 did not cause a significant improvement in the endothelium-dependent relaxation to acetylcholine in the recent onset diabetic rats (46±10%, $n=8$ versus 64±13%, $n=6$).

Incubation with both indomethacin and L-NOARG inhibited relaxation to ACh to a greater extent in the recent onset diabetic vessels (11–16 weeks old) compared to their age-matched controls (7±3%, $n=7$ versus 62±12%, $P<0.05$). A comparison of the relaxation responses of control vessels before and after the addition of indomethacin and L-NOARG showed no significant decrease in the maximum response to ACh (83±4%, $n=7$ versus 62±12%, $n=7$). However a direct comparison of the whole ACh dose-relaxation response curve revealed significant attenuation in the presence of indomethacin and L-NOARG ($P<0.05$).

Mechanical removal of the endothelium dramatically reduced the acetylcholine relaxation response in both the recent onset diabetic vessels and their age-matched non diabetic controls (diabetics denuded: 12±7%, $n=8$ versus intact: 46±10%, $n=8$ and controls denuded: 29±6%, $n=8$ versus intact: 87±2%, $n=8$, respectively, $P<0.05$).

Bradykinin relaxation responses

Compared to their age-matched controls, relaxation responses to bradykinin were reduced in both recent onset and insulin-treated diabetic rats (20±3%, $n=6$ versus 72±7%, $n=6$ and 12±9%, $n=7$ versus 68±7%, $n=8$, respectively, $P<0.05$) (Figure 5). A one hour incubation with L-NOARG significantly inhibited the relaxation to BK in the recent onset diabetic groups (20±3%, $n=6$ versus 2±2%, $n=6$, $P<0.05$). There was no significant difference between the maximum relaxation to bradykinin, before or after the addition of L-NOARG, in the insulin-treated diabetic rats (18–20 weeks old) (11±3%, $n=7$ versus 14±10%, $n=6$). The response of both control groups was attenuated, but not completely abolished in the presence of L-NOARG (11–16 weeks: 72±7%, $n=6$ versus 44±10%, $n=6$ and 18–20 weeks: 68±7%, $n=8$ versus 23±6%, $n=7$, $P<0.05$).

Incubation with indomethacin produced a slight improvement in the relaxation response to bradykinin of the established insulin-treated diabetic group, but the change did not achieve statistical significance (11±8%, $n=7$ versus 24±13%, $n=6$). Similarly, there was no significant improvement in the relaxation response to bradykinin in the age-matched control vessels (63±9%, $n=7$ versus 73±6%, $n=7$). The addition of the thromboxane A_2 inhibitor SQ 29548 did not cause a significant improvement in the endothelium-dependent relaxation to bradykinin in the recent onset diabetic rats (14±10%, $n=8$ versus 18±12%, $n=6$). Incubation with indomethacin and L-NOARG produced a significantly greater inhibition of relaxation responses in the recent onset diabetic vessels compared to their age-matched controls (8±4%, $n=7$ versus

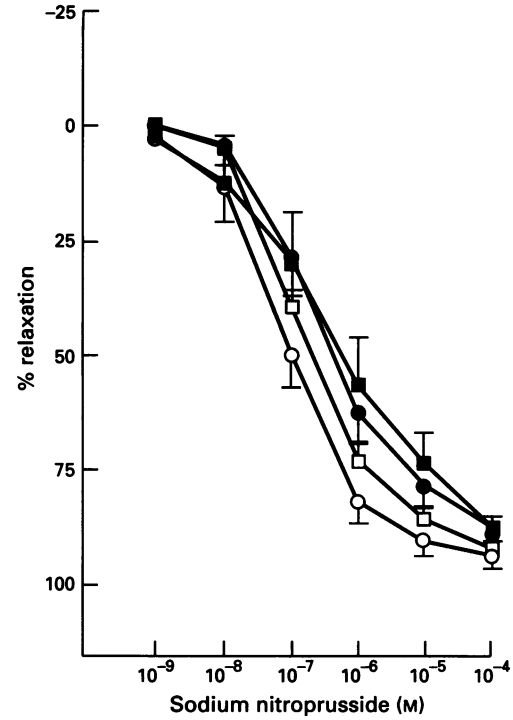

Figure 6 Endothelium-independent relaxation response to sodium nitroprusside of rat mesenteric resistance vessels from recent onset (□), and insulin-treated diabetics (■), and their age-matched controls (○) and (●) respectively. Points show means ± s.e.mean.

48 ± 12%, $n=7$ respectively, $P<0.05$). Removal of the endothelium attenuated the relaxation responses to bradykinin in both the recent onset diabetic vessels and their age-matched controls (diabetics denuded: 1 ± 1%, $n=7$ versus intact: 14 ± 10%, $n=8$, $P<0.05$ and controls denuded: 16 ± 5%, $n=8$ versus intact: 75 ± 5%, $n=8$, $P<0.05$).

Relaxation responses to sodium nitroprusside

The response to the endothelium-independent vasodilator sodium nitroprusside was impaired in the recent onset diabetics compared to their age-matched controls (74 ± 5%, $n=6$ versus 94 ± 2%, $n=6$, $P<0.05$). On the other hand, the maximum relaxation to SNP in the establshed insulin-treated diabetic rats was not significantly reduced (71 ± 7%, $n=8$ versus 88 ± 4%, $n=8$), although there was a significant right-ward shift of the dose-response curve. After the addition of indomethacin and L-NOARG, sodium nitroprusside relaxation responses in the recent onset diabetic rats were very similar to their age-matched controls (92 ± 2%, $n=7$ versus 93 ± 3%, $n=7$). This was also the case in the established insulin-treated diabetic rats and their age-matched controls incubated with indomethacin alone (87 ± 3%, $n=6$ versus 87 ± 3%, $n=7$) (Figure 6). The relaxation to sodium nitroprusside in endothelium denuded recent onset diabetic vessels and their age-matched controls (11 – 16 weeks old) was unchanged (79 ± 4%, $n=8$ versus 86 ± 2%, $n=8$, respectively). However, there was a significant difference in sensitivity to sodium nitroprusside, with the denuded diabetic vessels having less sensitivity than their controls (1.14 ± 0.27 μmol l^{-1} versus 0.29 ± 0.06 μmol l^{-1}, $P<0.05$).

Discussion

The present study shows no significant difference in the maximum contractile response or noradrenaline sensitivity between either the recent onset or insulin-treated diabetic rats and their age-matched non-diabetic controls. This is in agreement with other results with the aortae from diabetic BB rats, where contractile responses were normal (Durante *et al.*, 1988), and in chemically-induced diabetic rats where responses were impaired (Pfaffman *et al.*, 1982; Head *et al.*, 1987; Oyama *et al.*, 1986). By contrast, the majority of studies of both large isolated arteries and resistance from rats with chemically-induced diabetes have shown an enhanced senstivity to contractile agents (Harris & MacLeod, 1988; Legan, 1989; Owen & Carrier, 1980; MacLeod & McNeill, 1985; Friedman, 1989; Taylor *et al.*, 1992). Moreover, a study of insulin-treated STZ-induced diabetic rats, showed that insulin did not prevent enhanced contractility to NA, although it did prevent impaired endothelium-dependent relaxation to ACh (Taylor *et al.*, 1994a). These findings contrast with those from human studies, which have shown resistance arteries from insulin dependent diabetis mellitus (IDDM) patients without complications other than background retinopathy, display impaired contractile responses to NA, and impaired endothelium-dependent relaxation to ACh (McNally *et al.*, 1994); furthermore, resistance arteries from healthy volunteers exposed to increasing concentrations of insulin show decreased contractile response to NA in a dose-dependent manner (McNally *et al.*, 1995). Thus, the altered reactivity found with chemically induced diabetes may depend on the vascular effect of streptozotocin *per se*.

Recently, one study has shown evidence of vascular hypertrophy in the chemically-induced diabetic rat (Cooper *et al.*, 1994). This study demonstrated increased media wall:lumen ratio in chemically-induced diabetes, with no associated significant increase in systolic blood pressure. However, these changes were not evident until three weeks duration of diabetes (contrasting with the finding of the current study, of changes in the recent onset BB rats). Another study investigating the cerebral arterioles of the STZ-induced diabetic rat also showed abnormal morphology of the vascular smooth muscle as well as damaged endothelium (Moore *et al.*, 1985). In our study, morphological measurements in both the recent onset and insulin-treated BB rats revealed a significant increase in the media wall: lumen ratio, as well as increased media volume and media thickness compared to the age-matched controls. There was no significant difference in systolic blood pressure in the diabetic groups compared with their age-matched non-diabetic controls.

Incubation with the nitric oxide synthetase inhibitor L-NOARG produced the expected increased noradrenaline sensitivity in both control groups indicating inhibition of basal endothelium derived relaxing factor (EDRF) release. However, compared to their controls, the shift in the NA dose-response curve was less marked in the recent onset diabetics and no change occurred in the insulin-treated diabetics. This suggests a reduction of basal EDRF release in the recent onset diabetics with a significant impairment in the insulin-treated diabetic rats. These findings are in keeping with a report of reduced spontaneous release of nitric oxide in mesenteric arteries from rats with chemically-induced diabetes mellitus (Taylor *et al.*, 1992), although a later study showed enhanced noradrenaline sensitivity in untreated and insulin treated diabetic rats compared to controls after incubation with the nitric oxide synthetase inhibitor L-NAME (Taylor *et al.*, 1994a).

In the first protocol, there was impaired relaxation to the nitric oxide donor SNP in the diabetic vessels, suggesting a diminished vascular smooth muscle sensitivity to nitric oxide, whereas all the subsequent studies showed normal relaxation. The literature reveals a number of conflicting results of SNP relaxation in diabetes; one *in vivo* study of anaesthetized STZ-treated rats showed a decrease in relaxation to SNP (Ha & Dunham, 1987), whereas in other *in vitro* studies similar relaxation responses from both control and diabetic rats were observed (Oyama *et al.*, 1986; Pieper & Gross, 1988; Taylor *et al.*, 1992). Similarly, most studies in human insulin-dependent diabetic studies have demonstrated a normal response to SNP (Elliott *et al.*, 1993; Johnstone *et al.*, 1993; McNally *et al.*, 1994), whilst others have shown the response is impaired (Calver *et al.*, 1992).

There was a significant impairment in endothelium-dependent relaxation to both acetylcholine and bradykinin in both the recent onset and the insulin treated diabetic BB rats, indicating that the attenuated endothelium-dependent relaxation responses do not depend on the duration of diabetes. This conflicts with a study with the chemically induced diabetic rat which found that impaired endothelium-dependent relaxation was dependent on the duration of diabetes (Otter & Chess-Williams, 1994). Other studies of chemically-induced diabetic rats have shown an impaired response to acetylcholine, but bradykinin relaxation was normal (Taylor *et al.*, 1992). Impaired endothelium-dependent relaxation responses to acetylcholine with normal relaxation responses to bradykinin have also been demonstrated in human subcutaneous resistance arteries (McNally *et al.*, 1994), suggesting an abnormality at the G-protein receptor level. We therefore decided to investigate further the mechanisms specifically behind the attenuated endothelium-dependent relaxation to acetylcholine and bradykinin uniquely evident in both the recent onset untreated diabetic rats and the established insulin-treated diabetic rats.

The potent effects of the nitric oxide synthase inhibitor N^G-nitro-L-arginine (L-NOARG) on acetylcholine induced endothelium-dependent relaxation have been confirmed in studies of rat aortae (Moore *et al.*, 1990), and mesenteric resistance arteries (Bennett *et al.*, 1992). In our study, incubation with L-NOARG caused a variable decrease in maximum relaxation produced by acetylcholine and bradykinin in both diabetics and age-matched controls, suggesting that EDRF partly mediates ACh and BK relaxation in these vessels. Mechanical removal of the endothelium markedly attenuated ACh and BK relaxation responses in all vessels, confirming the pivotol role of the endothelium in mediating relaxation to these vasodilators. Although L-NOARG only partially inhibited endothelium-dependent relaxation, it is unlikely that the inhibitor concentration was insufficient to block the L-arginine pathway. Previous studies using similar concentrations have achieved comparable results (Bennett *et al.*, 1992). It is possible that the residual relaxation in the presence of L-NOARG may depend on the release of other vasodilators from the endothelium. One such vasodilator is an endothelium-derived hyperpolarizing factor (EDHF), which relaxes vascular smooth muscle cells by opening potassium channels, and causing hyperpolarization of the cell membrane (Van de Voorde *et al.*, 1992).

Several mechanisms have been postulated to explain the attenuated relaxation response to acetylcholine in diabetic rats: these include a decrease in the production and release of EDRF, accelerated destruction of EDRF by free radicals, or an alteration in smooth muscle to respond to EDRF (Durante *et al.*, 1988). Incubation with the nitric oxide synthetase substrate L-arginine failed to improve the attenuated endothelium-dependent relaxation of the diabetic vessels, and it is therefore reasonable to accept that the impaired response was not due to a lack of substrate *per se*. Other studies indicate a possible role for superoxide anions interfering with EDRF activity (Katusic *et al.*, 1991; Gryglewski *et al.*, 1986; Tesfamariam & Cohen, 1992). The literature concerning the role of free radicals in man is controversial and conflicting; however, increased levels of diene conjugation products have been demonstrated in insulin-dependent patients with microangiopathy (Jennings *et al.*, 1987). Our studies showed no improvement of ACh relaxation after incubation with the free radical scavengers superoxide dismutase (SOD) and catalase (CAT), suggesting that excessive NO destruction by free radicals was not the cause of the impaired relaxation in the diabetic vessels. However, the present study does not exclude a role for endothelial cell membrane damage by lipid peroxidation and *in vivo* studies of the effects of treatment with free radical scavengers are needed to resolve this issue.

It is well established that the endothelial cyclo-oxygenase pathway can produce thromboxane A_2, and prostaglandin H_2, in addition to the vasodilators prostacyclin and EDRF, and these endothelium-derived contracting factors can profoundly affect vascular tone. Hypertension and atheriosclerosis have both been shown to affect the endothelium so that the role of contracting factors becomes more dominant, leading to an imbalance in endothelium-dependent vascular regulation (Luscher *et al.*, 1992; Bennett *et al.*, 1993). Pretreatment with the cyclo-oxygenase inhibitor indomethacin significantly improved the acetylcholine relaxation response in the diabetic vessels, suggesting increased vasoconstrictor prostanoid production may be overriding nitric oxide-mediated relaxation. Similar observations have been obtained in the aorta of the STZ-induced diabetic rat (Shimizu *et al.*, 1993), and rabbit aorta (Tesfamariam *et al.*, 1989), where exposure to high glucose levels caused an attenuated response to ACh-induced relaxation, which was normalized by inhibitors of cyclo-oxygenase and/or by prostaglandin H_2 (PGH_2)/ thromboxane A_2 receptor antagonists. Moreover, direct radioimmunoassay measurements revealed increased release of several prostanoids including thromboxane A_2 and PGF_{2a} (Tesfamariam *et al.*, 1989; 1990). However, in the present study, incubation with indomethacin did not significantly improve the impaired bradykinin response in the diabetic vessels. These differential responses to ACh and BK following pretreatment with indomethacin, could lie at the receptor G-protein level (Taylor *et al.*, 1995). It has been established that in bovine mesenteric arteries (Ljusengren *et al.*, 1990), and rabbit aorta (Cohen & Tesfamariam, 1992), ACh-induced responses are linked to an endothelial petussis toxin-sensitive G_i-protein, whereas BK is linked to a different G-protein, G_q (Taylor *et al.*, 1991). Therefore, it is possible that the G_i-protein may be linked to the release of both a vasodilator such as EDRF as well as the release of a vasoconstricting prostanoid; whereas the G_q-protein responsible for BK-induced relaxation is linked only to the release of vasorelaxing factors.

In our study, the role of thromboxane A_2 (TXA_2) was assessed by observing the effects of the thromboxane A_2 antagonist SQ 29548 on relaxation of vessels from recent onset diabetic rats. No significant increase in ACh-induced relaxation was observed, suggesting that the contracting factor was not TXA_2. Similarly, the impaired relaxation in rabbit aorta which develops with exposure to a high glucose concentration *in vitro* can be ameliorated by indomethacin, but not by the TXA_2 antagonist (Tesfamariam *et al.*, 1990). A role for vasoconstrictor prostanoids is supported by a recent study showing treatment with aspirin restored forearm blood flow in reactive hyperaemia in diabetic patients free from complications (Steel *et al.*, 1993). On the other hand, another group observed that methacholine-induced relaxation remained impaired despite aspirin therapy (Johnstone *et al.*, 1993).

The mechanism underlying the altered endothelial function has not been elucidated, but attention has focussed on glucose-induced increases of protein kinase C (Tesfamariam *et al.*, 1991; Williams & Schrier, 1992; Johnstone *et al.*, 1993; Cohen, 1993). These studies indicate that the abnormality of endothelium-dependent relaxation caused by elevated glucose is the result of an alteration in endothelial cell receptor-mediated signal transduction, probably mediated by protein kinase C activation (Tesfamariam *et al.*, 1991). This view is supported by the observation that the addition of protein kinase C inhibitors prevents abnormal release of prostanoids, restores endothelium-dependent relaxation, and preserves the release of PGI_2 from endothelial cells exposed to high concentrations of glucose (Tesfamariam *et al.*, 1991). It is therefore possible that protein kinase C activation, mediated by elevated glucose, may lead to an alteration in endothelial prostanoid synthesis and play a role in the development of diabetic vascular complications.

In conclusion, the present study demonstrated abnormal endothelium-dependent relaxation in resistance arteries of both recent onset and established insulin treated diabetic BB rats. The impaired relaxation does not appear to be due to a lack of substrate for the nitric oxide synthase enzyme, or to excessive destruction of nitric oxide by free radicals. The significant improvement of endothelial-dependent relaxation fol-

lowing incubation with indomethacin indicates that the abnormality results from over-production of a vasoconstricting prostanoid. However, pretreatment with the thromboxane A_2 inhibitor SQ 29548 failed to produce a similar improvement of endothelium-dependent relaxation, suggesting that the vasoconstrictor prostanoid is not TXA_2. Further studies with protein kinase C inhibitors, and *in vivo* studies of the effects of antioxidants (such as vitamin E) are required to elucidate the nature of the alteration in endothelial function.

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