Br. J. Pharmacol. (1994), **111**, 35–41

Prevention by insulin treatment of endothelial dysfunction but not enhanced noradrenaline-induced contractility in mesenteric resistance arteries from streptozotocin-induced diabetic rats

[1]Paul D. Taylor, *Beryl B. Oon, *Chris R. Thomas & Lucilla Poston

[1]Division of Physiology, *Department of Endocrinology and Diabetes, United Medical and Dental Schools Smooth Muscle Group, St. Thomas' Campus, London SE1 7EH

1 Streptozotocin-induced diabetic rats (Wistar) were implanted with sustained release insulin pellets (release rate = 4 u day^{-1}) or with placebo pellets (palmitic acid) from the onset of glycosuria.

2 Noradrenaline sensitivity, endothelium-dependent relaxation to acetylcholine and endothelium-independent relaxation to sodium nitroprusside were assessed in mesenteric resistance arteries from the insulin-treated (IT) diabetic animals and compared to placebo-implanted (PI) diabetics and age-matched controls.

3 Arteries from PI-diabetic rats (8–10 weeks) demonstrated an enhanced maximal response to noradrenaline compared to controls, which was not prevented by insulin treatment (control 2.65 ± 0.17 mN mm^{-1}, $n = 18$ arteries *versus* PI-diabetic 3.73 ± 0.40 mN mm^{-1}, $n = 5$, $P < 0.05$; control *versus* IT-diabetic 4.02 ± 0.19 mN mm^{-1}, $n = 22$, $P < 0.001$). Sensitivity to noradrenaline was similar between the three groups.

4 In the presence of the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), IT and PI arteries were more sensitive to noradrenaline than control arteries (pEC_{50}: control 5.75 ± 0.08, $n = 17$, *versus* PI-diabetic 6.14 ± 0.09, $n = 8$, $P < 0.05$; control *versus* IT-diabetic 6.38 ± 0.08, $n = 20$, $P < 0.001$).

5 The maximum contractile response to depolarizing 125 mM K^+ was significantly enhanced in IT-diabetic arteries but not PI-diabetic when compared to control arteries (maximum response: control 3.74 ± 0.15 mN mm^{-1}, $n = 18$, *versus* PI-diabetic 3.61 ± 0.19 mN mm^{-1}, $n = 11$, NS; control *versus* IT-diabetic 4.66 ± 0.18 mN mm^{-1}, $n = 22$, $P < 0.001$).

6 Endothelium-dependent relaxation to acetylcholine was profoundly impaired in the PI-diabetic arteries, but in the IT-diabetic arteries was not significantly different from controls (pEC_{50}: control 7.64 ± 0.19, $n = 17$, *versus* PI-diabetic 6.07 ± 0.12, $n = 8$, $P < 0.001$; control *versus* IT-diabetic 7.36 ± 0.09, $n = 22$, NS).

7 Endothelium-independent relaxation to sodium nitroprusside was slightly but significantly impaired in the PI-diabetic arteries, but was not significantly different in the IT-diabetic arteries compared to controls (pEC_{50}: control 7.78 ± 0.10, $n = 13$, *versus* PI-diabetic 7.31 ± 0.13, $n = 13$, $P < 0.05$; control, *versus* IT-diabetic 7.64 ± 0.09, $n = 16$, NS).

Keywords: Mesenteric resistance arteries; vascular endothelium; vascular smooth muscle; chemically induced diabetes in rats; streptozotocin: insulin treatment

Introduction

Abnormal function of the vascular endothelium has been implicated as one of the underlying mechanisms of microvascular disease in diabetes. Recent studies in man have identified impaired endothelium-dependent relaxation to acetylcholine in the smooth muscle of the corpora cavernosa of impotent diabetic men (De Tejada *et al.*, 1989), in isolated resistance arteries from gluteal subcutaneous fat from type I (insulin-dependent) diabetic subjects (McNally *et al.*, 1992), and in the forearm of subjects with type II (non-insulin dependent) diabetes mellitus (McViegh *et al.*, 1992). Elliot *et al.* (1992), have also reported altered basal and stimulated nitric oxide (NO) synthesis to carbachol in the forearm of microalbuminuric type I diabetic patients. There is also some evidence to suggest that the sensitivity of the underlying smooth muscle to exogenously derived nitric oxide may be altered in human diabetes (McViegh *et al.*, 1992; Calver *et al.*, 1992). Endothelium-dependent and -independent defects could, therefore, account for impaired NO mediated relaxation. In a recent study by Smits *et al.* (1993), however, forearm blood flow responses to both endothelium-dependent and -independent relaxation were found to be normal.

Extensive functional studies in conduit arteries from animals with chemically-induced diabetes comprise a largely confusing literature. Several studies, however, describe enhanced reactivity to noradrenaline (MacLeod & McNeill, 1985; Harris & MacLeod, 1988; Pieper & Gross, 1988; White & Carrier, 1988; Cohen *et al.*, 1990) and impaired endothelium-dependent relaxation (Oyama *et al.*, 1986; Pieper & Gross, 1988; Durante *et al.*, 1988; Kamata *et al.*, 1989; Tanz *et al.*, 1989; Mayhan, 1989; Abiru *et al.*, 1990; Tesfamariam *et al.*, 1990; Mayhan *et al.*, 1991; Cameron & Cotter, 1992). We have previously demonstrated in streptozotocin-induced diabetes in rats, that both sensitivity to noradrenaline and endothelium-dependent relaxation to acetylcholine are abnormal in isolated mesenteric resistance arteries (Taylor *et al.*, 1992) and in the whole perfused mesenteric circulation (Taylor *et al.*, 1994 following paper). The first of these studies (Taylor *et al.*, 1992) also suggested the observed abnormalities can be explained, at least in part, by a deficit in the production of NO in the endothelial cells of the resistance vasculature.

In animal models of diabetes, insulin treatment has been shown to reverse the decreased responsiveness to endothelin-1 in aortic rings (Hodgson & King, 1992), to prevent impaired endothelium-dependent relaxation to histamine in aortic rings

[1] Author for correspondence.

(Tanz *et al.*, 1989) and to prevent both contraction and relaxation defects (Takiguchi *et al.*, 1989) in the rat perfused mesentery. No previous study has determined the effect of insulin treatment on abnormalities of isolated resistance arteries. In the present study, therefore, we aimed to investigate the effect of sustained release insulin replacement on resistance vascular function in rats with streptozotocin-induced diabetes.

Methods

Induction of experimental diabetes

Diabetes was induced in female Wistar rats by intraperitoneal injection of streptozotocin (45 mg kg^{-1}) dissolved in citrate buffer. Onset of diabetes was confirmed by the presence of glycosuria 48 h after injection. Blood samples for the measurement of plasma glucose (glucose oxidase method: YSI model 23 AM Glucose Analyser, Yellow Springs, OH, U.S.A.) and rat and bovine plasma insulin concentrations (RIA, Sönksen, 1976) were obtained by cardiac puncture after cervical dislocation. Control rats were housed separately from the diabetic animals and all animals were given free access to food and water.

Insulin treatment

Following the onset of glycosuria, 48 h after streptozotocin administration, diabetic animals were treated with insulin with sustained release insulin pellets, containing bovine insulin (17%) and palmitic acid (83%) as an excipient (Wang, 1991). Slow surface erosion of the implants *in vivo* gradually releases the entrapped insulin, equivalent to a dose of 4 units day^{-1}. The insulin pellets (2 mm × 7 mm) were sterilised in 10% betadine solution before being implanted subcutaneously between the scapulae using a specially designed (12 gauge) trocar and stylet. This procedure was carried out under a short acting anaesthetic (Hypnorm, 0.5 ml kg^{-1} bodyweight, i.m.) requiring no suturing or clips for closure; 48 h after implantation of the insulin pellets, and in all subsequent routine tests, there was no evidence of glycosuria or ketonuria using reagent strips for urinalysis (Labstix). As controls for the implantation of the insulin, diabetic animals were implanted with placebo pellets containing only palmitic acid which were otherwise identical to the insulin pellets.

After 8–10 weeks animals were killed by cervical dislocation and a blood sample was taken by cardiac puncture for the plasma insulin assays and the determination of plasma glucose concentrations.

Assessment of vascular function

The mesentery was removed and placed in physiological salt solution (PSS). The solution consisted of (mM): NaCl 119, $NaHCO_3$ 25, D-glucose 5.5, KH_2PO_4 1.18, $MgSO_47H_2O$ 1.17, KCl 4.7, $CaCl_2$ 2.5, ethylenediamine-tetra-acetic acid disodium salt (EDTA) 0.026. In order to obtain arteries of approximately equal diameter in control and experimental animals, the third branch mesenteric arteries were routinely dissected from control and insulin-treated (IT)-diabetic rats and the fourth order mesenteric arteries dissected from the placebo-implanted (PI)-diabetic animals (mean internal diameter ± s.e.mean: control 286 ± 9 μm, $n = 18$: IT-diabetic 316 ± 12 μm, $n = 22$: PI-diabetic 293 ± 12 μm, $n = 13$, P not significant). The arteries were dissected free of connective tissue under a light microscope and mounted as ring preparations on a small vessel myograph (Mulvany & Halpern, 1977) capable of measuring isometric tension. Arteries were bathed in PSS at 37°C and bubbled with 5% CO_2/95% O_2 and their passive tension and internal circumference were determined. The arteries were then set to an internal circumference equivalent to 90% of that which they would experience when relaxed *in situ* under a transmural pressure of 100 mmHg (the maximum active tension for the minimum resting tension is developed at approximately this circumference; Mulvany & Halpern, 1977). Arteries were then subjected to a routine run-up procedure in which they were contracted for 2 min every 10 min on four occasions according to the following protocol. The first and fourth contractions were produced with 5 μM noradrenaline in 125 mM potassium solution (KPSS, equimolar substitution of KCl for NaCl in PSS). The second was with 5 μM noradrenaline in PSS and the third with KPSS. Arteries failing to produce a maximum active tension equivalent to a pressure of 100 mmHg on the final contraction were rejected.

All experiments were conducted in the presence of the cyclo-oxygenase inhibitor indomethacin; as in a previous study of endothelial function of STZ-diabetic rats (Taylor *et al.*, 1992) we found no evidence for abnormalities of indomethacin-sensitive components of relaxation. Following the routine run up procedure, the vessels were incubated for 10 min in indomethacin (10 μM) and their responses to increasing concentrations of noradrenaline then determined at 3 min intervals (0.1 mM–10.0 mM cumulative addition). The bath was then washed three times with PSS and a further 15 min washout period allowed before the arteries were submaximally contracted for 3 min with the concentration of noradrenaline required to produce approximately 80% of the maximum response. Relaxation to acetylcholine was subsequently assessed by adding increasing concentrations of acetylcholine at 2 min intervals (final bath concentrations 1 nM–10 μM). After a washout period of 5 min, the NO synthase inhibitor, L-NAME (N^G-nitro-L-arginine methyl ester 10^{-4} M) was added to the bath and 10 min later a cumulative concentration-response to noradrenaline repeated. Finally, and in the continued presence of L-NAME, arteries were precontracted with noradrenaline as described, and after 3 min were subjected to increasing concentrations of the endothelium-independent nitrovasodilator sodium nitroprusside (SNP), at 2 min intervals (0.01–10 μM).

Drugs

Chemicals used in this investigation were noradrenaline (Winthrop Laboratories): acetylcholine, indomethacin, sodium nitroprusside, L-NAME, (all from Sigma); streptozotocin (gift from Dr MacLeod, Upjohn Co. Kalamazoo, U.S.A.) and Hypnorm (Jannsen Pharmaceutics). Chemicals were prepared as stock solutions solubilized in PSS, except indomethacin which was prepared as a 1 mM stock solution in phosphate buffer consisting of (mM): KH_2PO_4 20, $NaH_2PO_4.2H_2O$ 120, pH balanced to 7.8. All concentrations are expressed as the final molar concentrations in the organ bath. Sustained release insulin implants and placebo implants (Linplant) from Mollegaard, Denmark.

Statistical analysis

All values are given as the mean ± s.e.mean. Tension is given as active wall tension (mN mm^{-1} artery length) ± s.e.mean and the relaxation responses to acetylcholine as a percentage of the initial precontraction to noradrenaline. The negative log of the concentration of a drug required to affect 50% of the maximum response (pEC_{50}) was calculated as the mean ± s.e.mean of the individual pEC_{50}s using non linear regression, and the sigmoid equation of the curve fitting programme 'GraphPad' (GraphPad Software Inc., San Diego, CA, U.S.A.). Statistical comparisons of the pEC_{50} values and maximum responses between the groups were achieved by one way analysis of variance with the Bonferonni correction of P values for multiple comparisons. Paired two-tailed t tests were employed in the comparisons of sequential concentration-effect curves in the presence and absence of inhibitors. Significance was assumed if $P < 0.05$.

Results

The glucose concentration of the plasma obtained from PI-diabetic animals at the time of study (8–10 weeks after induction of diabetes) was 44.28 ± 4.10 mM ($n = 5$) whereas the plasma glucose of the insulin-treated diabetic animals was not significantly different from that of controls (8.72 ± 1.81 mM, $n = 11$, *versus* 6.31 ± 0.44 mM, $n = 14$). Bovine insulin concentrations in the implanted animals were 46.68 ± 11.11 mu l^{-1} ($n = 11$) and below the detectable limit in the control and placebo-treated animals. Endogenous levels of insulin in the control animals were 19.64 ± 6.25 mu l^{-1} ($n = 14$), lower than that of bovine insulin in the treated animals ($P < 0.05$). At the time of implantation, IT-diabetic animals weighed 222 ± 2 g, and following insulin treatment weighed 302 ± 4 g, a significant weight gain ($P < 0.0001$), and greater than the normal weight for age matched animals (250–270 g). In contrast PI-diabetics did not show any significant weight gain, being 240 ± 8 g before, and 249 ± 4 g after treatment (P not significant).

Contractile responses to noradrenaline

The exposure of arteries to noradrenaline led to a concentration-dependent rise in tension in all experimental groups. There was no significant difference in the sensitivity between the three groups (pEC_{50}: control 5.64 ± 0.07, $n = 18$; PI-diabetic 5.79 ± 0.12, $n = 5$; IT-diabetic 5.78 ± 0.05, $n = 22$, P not significant). However, the PI-diabetic arteries demonstrated an enhanced maximum contractile response to noradrenaline compared to controls (maximum response; PI-diabetic 3.73 ± 0.40 mN mm^{-1}, $n = 5$, *versus* control 2.65 ± 0.15 mN mm^{-1}, $n = 18$, $P < 0.001$). Insulin treatment did not prevent this enhanced contractile response to noradrenaline (maximum response: IT-diabetic 4.02 ± 0.19 mN mm^{-1}, $n = 22$, *versus* control 2.65 ± 0.17 mN mm^{-1}, $n = 18$, $P < 0.001$) Figure 1.

The addition of L-NAME in control arteries led to a highly significant increase in the maximum response to noradrenaline from 2.65 ± 0.17 mN mm^{-1} to 3.56 ± 0.16 mN mm^{-1}, $n = 17$, $P < 0.001$, although there was no significant increase in sensitivity (pEC_{50}: from 5.64 ± 0.07, to 5.75 ± 0.08, $n = 17$, P not significant). In the PI-diabetic arteries the addition of L-NAME did not affect the maximum response to noradrenaline (from 3.73 ± 0.40 mN mm^{-1} to 4.01 ± 0.18 mN mm^{-1}, $n = 5$, P not significant) but led to a significant increase in sensitivity (pEC_{50}: from 5.79 ± 0.12 to 6.12 ± 0.13, $n = 5$, $P < 0.005$). The PI-diabetic arteries in the presence of L-NAME demonstrated enhanced sensitivity to noradrenaline compared to control arteries in the presence of L-NAME (pEC_{50}: control 5.75 ± 0.08, $n = 17$ *versus* PI-diabetic 6.14 ± 0.09, $n = 8$, $P < 0.05$). In the arteries from the IT-diabetic rats the addition of L-NAME caused both an increase in maximum response (from 4.02 ± 0.19 mN mm^{-1} to 4.65 ± 0.20 mN mm^{-1}, $n = 20$, $P < 0.005$) and a substantial increase in sensitivity to noradrenaline (pEC_{50}: from 5.80 ± 0.05, to 6.38 ± 0.08, $n = 20$, $P < 0.001$). In the presence of L-NAME the IT-diabetic arteries demonstrated greater sensitivity to noradrenaline than control arteries (pEC_{50}: 6.38 ± 0.08, $n = 20$, *versus* 5.75 ± 0.08, $n = 17$, $P < 0.001$). No significant difference was observed between the maximum responses of the PI-diabetic arteries and that of the control arteries (maximum response: 4.01 ± 0.18 mN mm^{-1}, $n = 8$ *versus* 3.56 ± 0.16 mN mm^{-1}, $n = 17$, P not significant) or

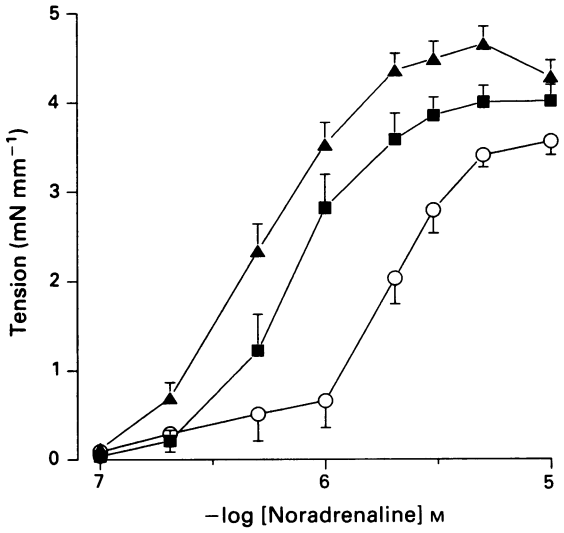

Figure 2 Noradrenaline concentration-effect curves, in the presence of indomethacin (10 μM) and the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (10^{-4} M), in isolated mesenteric resistance arteries from control (○, $n = 17$) placebo-implanted-diabetic (■, $n = 8$) and insulin-treated-diabetic rats (▲, $n = 20$).

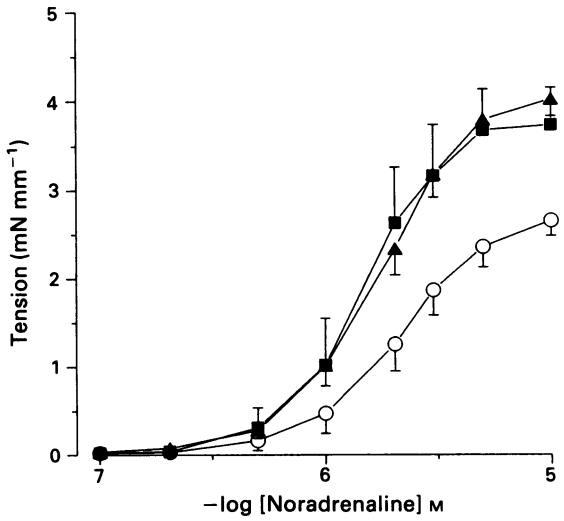

Figure 1 Noradrenaline concentration-effect curves, in the presence of indomethacin (10 μM), in isolated resistance mesenteric arteries from control (○, $n = 18$), placebo-implanted-diabetic (■, $n = 5$) and insulin-treated-diabetic rats (▲, $n = 22$).

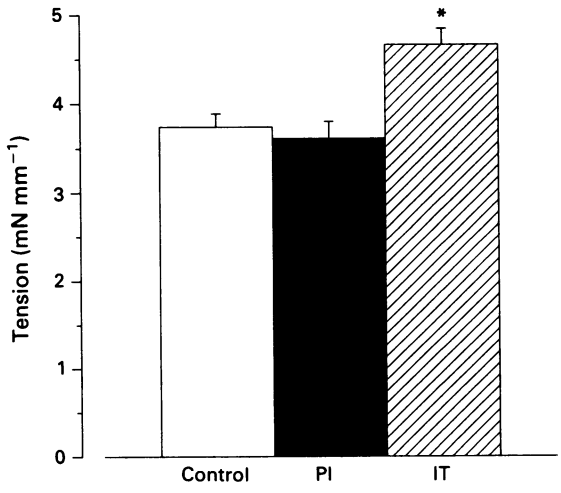

Figure 3 Maximal contraction to 125 mM depolarizing potassium (KPSS) in isolated mesenteric resistance arteries from control ($n = 18$), placebo-implanted (PI)-diabetic ($n = 11$) and insulin-treated (IT)-diabetic rats ($n = 22$).

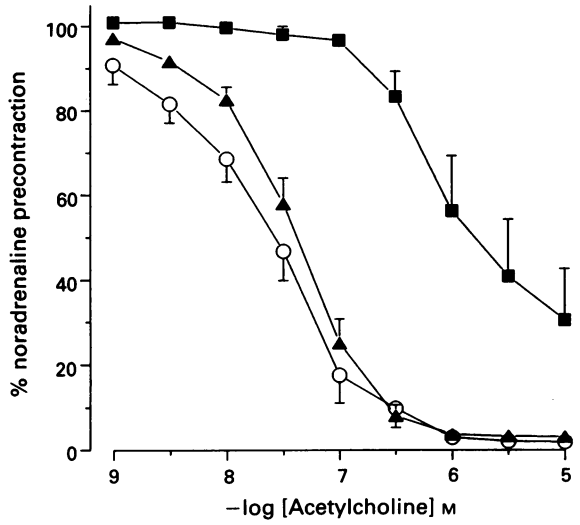

Figure 4 Acetylcholine concentration-effect curves, in the presence of indomethacin, in isolated mesenteric resistance arteries from control (○, $n = 17$), placebo-implanted-diabetic (■, $n = 8$), and insulin-treated-diabetic rats (▲, $n = 22$).

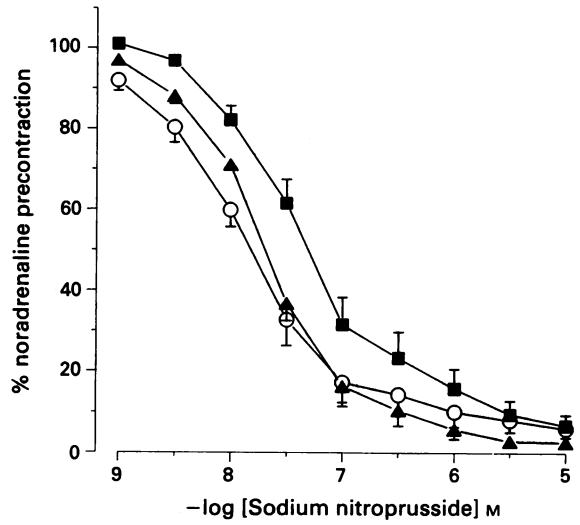

Figure 5 Endothelium-independent relaxation responses to sodium nitroprusside in isolated mesenteric resistance arteries from control (○, $n = 13$) placebo-implanted-diabetic (■, $n = 13$) and insulin-treated-diabetic rats (▲, $n = 16$) in the presence of indomethacin and N^G-nitro-L-arginine methyl ester (10^{-4} M).

between the PI-diabetic arteries and the IT-diabetic arteries (maximum response: 4.01 ± 0.18 mN mm^{-1}. $n = 8$, *versus* 4.65 ± 0.20 mN mm^{-1}, $n = 20$, P not significant). However, in the IT-diabetic arteries the maximum response to noradenaline in the presence of L-NAME was significantly raised above the controls (maximum response: 4.65 ± 0.20 mN mm^{-1}, $n = 20$, *versus* 3.56 ± 0.16 mN m^{-1}, $n = 17$, $P < 0.001$) Figure 2.

Arteries from the IT-diabetic animals demonstrated an enhanced response to 125 mM potassium (KPSS) relative to controls (4.66 ± 0.18 mN mm^{-1}, $n = 22$, *versus* 3.74 ± 0.15 mN mm^{-1}, $n = 18$, $P < 0.001$). Those from PI-diabetic animals were not significantly different from the controls (3.61 ± 0.19 mN mm^{-1}, $n = 11$) Figure 3.

Endothelium-dependent relaxation

The cumulative addition of acetylcholine, in half log molar increments, led to a concentration-dependent relaxation of arteries precontracted submaximally with noradrenaline, in all three experimental groups. Arteries from the PI-diabetic animals demonstrated profoundly reduced relaxation to acetylcholine compared to the control group (pEC_{50}: PI-diabetic 6.07 ± 0.12, $n = 8$, *versus* control 7.64 ± 0.19, $n = 17$, $P < 0.001$). Maximum relaxation was also significantly reduced in the PI-diabetic arteries compared to controls (maximum relaxation: PI-diabetic 69.5 ± 12.1, $n = 8$, *versus* control 98.5 ± 0.4, $n = 17$, $P < 0.001$). In the IT-diabetic arteries there was, however, no obvious impairment of acetylcholine-induced relaxation, compared with controls (pEC_{50}: IT-diabetic 7.36 ± 0.09, $n = 22$, *versus* control 7.64 ± 0.19, $n = 17$, P not significant. Maximum relaxation: IT-diabetic $93.8 \pm 0.6\%$, $n = 22$, *versus* control 98.5 ± 0.4, $n = 17$, P not significant) Figure 4.

Endothelium-independent relaxation

Concentration-dependent relaxation to increasing concentrations of sodium nitroprusside was observed in arteries from all three groups. PI-diabetic arteries did, however, show slight attenuation of the relaxation response compared to the control group (pEC_{50}: PI-diabetic 7.31 ± 0.13, $n = 13$, *versus* control 7.78 ± 0.10, $n = 13$, $P < 0.05$). This attenuation of the response was not observed in the insulin-treated group in which the response was not significantly different from that of the control group (pEC_{50}: IT-diabetic 7.64 ± 0.09, $n = 16$, *versus* control 7.78 ± 0.10, $n = 13$, P not significant) Figure 5.

Discussion

The results of this study, although in a different strain of rat, are in agreement with our previous observations in the STZ-diabetic rat model (Taylor *et al.*, 1992) in which we demonstrated impaired endothelium-dependent relaxation to acetylcholine. In addition we have demonstrated that insulin treatment prevents the development of impaired endothelium-dependent relaxation in isolated resistance arteries from the mesenteric circulation of these animals. This is in agreement with two previous studies in streptozotocin-diabetic rats; Takiguchi *et al.* (1989) have demonstrated that insulin treatment improves impaired acetylcholine relaxation in the isolated perfused mesentery and Tanz *et al.* (1989) have shown that insulin treatment prevents impaired histamine relaxation in 12 week streptozotocin-diabetic rat aorta. Our previous study suggested that the impaired endothelium-dependent relaxation resulted from reduced NO release and insulin therapy may, therefore, prevent this deficit. This is suggested by the experiments using the NO synthase inhibitor L-NAME which indicated greater noradrenaline-stimulated NO release in the arteries from the treated compared to untreated diabetic rats. Reduced NO release in diabetes is consistent with reports of impaired endothelium-dependent relaxation in human isolated resistance arteries in type I diabetes (McNally *et al.*, 1992) and in a study of forearm blood flow in type II diabetes (McVeigh *et al.*, 1992). In addition, other studies in type I diabetes have reported an impaired basal release of NO in the forearm circulation (Calver *et al.*, 1992; Elliot *et al.*, 1992). However, not all have found evidence of impaired NO production (Halkin *et al.*, 1991; Smits *et al.*, 1993). The differences could reflect glycaemic control: better controlled patients may be analogous to the sustained release, insulin-treated rats, whereas, poor control could be associated with poor endothelial function.

Insulin therapy results not only in the normalisation of serum glucose, but also many of the attendant sequelae of diabetes. Reversal of hyperglycaemia is, however, the most likely factor which prevents endothelial dysfunction. Vascular endothelial cells are particularly susceptible targets as glucose transport is independent of insulin (Green & Jaspan, 1990) and the cells are, therefore, at risk of excessive glucose entry when ambient concentrations are high. Activation of the polyol pathway by glucose has been cited as a possible mechanism contributing to reduced nitric oxide production (Cameron & Cotter, 1992). However, in our recent study in which STZ-diabetic rats were treated with the aldose reductase inhibitor,

ponalrestat, no reversal of the endothelial dysfunction in the mesenteric circulation was apparent (Taylor *et al.*, 1994). Other mechanisms by which hyperglycaemia may directly affect endothelial function include the formation of advanced glycosylation end products (AGEs) which can effectively quench nitric oxide, so reducing relaxation (Brownlee *et al.*, 1988; Bucala *et al.*, 1991; Makita *et al.*, 1991; Hogan *et al.*, 1992). Glucose metabolites such as glucose-6-phosphate (G6P) may also lead to intracellular glycosylation and quenching of NO, and may cause extensive damage of intracellular constituents by the generation of free radicals (Molinatti *et al.*, 1990). The breakdown of nitric oxide by free radicals, generated by hyperglycaemia-induced cyclo-oxygenase activity may also contribute to impaired endothelium-dependent relaxation (Tesfamariam & Cohen, 1992). Also a decrease in antioxidant activity, including the reported reductions in glutathione through stimulation of the polyol pathway (Dvornik, 1987), and associated superoxide dismutase activity (Tagami *et al.*, 1992), would lead to a reduced scavenging of free radicals. This has been shown to be prevented in aortic endothelial cells of alloxan diabetic rabbits by treatment with insulin (Tagami *et al.*, 1992).

Insulin also reverses hyperlipidaemia in man and in animal models of diabetes. It is highly relevant, therefore, that hypercholesterolaemia (Creager *et al.*, 1990; Chowienczyk *et al.*, 1992) and atherosclerosis (Zeiher *et al.*, 1991) in man are associated with impaired endothelium-dependent relaxation. Moreover, oxidised low density lipoproteins (LDLs) inhibit relaxations mediated by NO (Jacobs *et al.*, 1990), and LDLs are elevated in diabetes (Sosenko *et al.*, 1980). Reversal of hyperlipidaemia may, therefore, provide an alternative mechanism for insulin reversal of endothelial dysfunction. Some lipids, notably 3-omega fatty acids, stimulate NO production and it is of interest that McVeigh *et al.* (1993) have recently reported dietary fish oil supplementation reverse to abnormal endothelium-dependent relaxation in the forearm of patients with type II diabetes.

Mesenteric resistance arteries from the PI-diabetic rats demonstrated an enhanced maximum response to noradrenaline, but no change in sensitivity, in contrast to our previous study in which we observed both were increased (Taylor *et al.*, 1992). The difference could reflect the strain of rat used or the shorter duration of diabetes (5–6 weeks) in the earlier study. The increased contractility to noradrenaline was unlikely to be the result of enhanced constrictor prostanoid production as suggested by some (Agrawal & McNeill, 1987) as the experiments were carried out in the presence of indomethacin. In our earlier study (Taylor *et al.*, 1992) in rats with shorter duration of diabetes, we attributed enhanced noradrenaline reactivity to reduced release of NO since the addition of L-NAME to the arteries resulted in identical tension development in control and diabetic arteries when contracted with noradrenaline. In the present study in animals with untreated diabetes of longer duration (8–10 weeks), the diabetic arteries continued to display an enhanced response to noradrenaline in the presence of L-NAME when compared to controls. This suggests a second, duration-dependent mechanism and might be associated with autonomic neuropathy. Autonomic neuropathy develops several weeks after STZ injection resulting in reduced endogenous noradrenaline release (Sato *et al.*, 1989), and could lead to upregulation of adrenoceptors and increased sensitivity to exogenous noradrenaline. Cameron & Cotter (1992), Scarborough & Carrier (1983) and Wong & Tzeng (1992), have all implicated an enhanced α_2-adrenoceptor mediated component of noradrenaline contraction in animal models of diabetes. Enhanced pressor responses to exogenous noradrenaline have also been documented in insulin-dependent, diabetic patients (Moorhouse *et al.*, 1966; Christlieb *et al.*, 1976; Eichler *et al.*, 1992).

The increase in reactivity to noradrenaline in the diabetic rats was not prevented by sustained release insulin treatment despite normalisation of plasma glucose. As these treated animals would not have had diabetes for a sufficient duration to develop neuropathy this is unlikely to be a reflection of irreversible autonomic neuropathy. This would suggest that insulin itself may be affecting contractility in the treated group. Indeed serum levels of exogenous bovine insulin in the treated diabetics were moderately raised compared to those of endogenous insulin in the non-diabetic controls. The weight gain following insulin treatment is also indicative of a degree of hyperinsulinaemia. The observation that the contractile response to 125 mM potassium (KPSS) was elevated in the insulin-treated diabetic animals but not in the placebo-implanted group could suggest a chronic effect of raised exogenous insulin on the contractile mechanism. This is consistent with the data of Pfaffman *et al.* (1982) in which there was a tendency for K^+-induced contractions (70 mM) in insulin-treated diabetic aortas to develop greater tension than controls. Insulin could increase contractile responses through the promotion of cell growth and proliferation of vascular smooth muscle (Stolar, 1988; Banskota *et al.*, 1989).

Most studies investigating the effects of insulin on vascular smooth muscle function have been acute studies *in vitro* and are, therefore, likely to be of little relevance to the abnormalities of noradrenaline-induced contraction seen in diabetic and insulin-treated animals in this *in vitro* preparation in the absence of insulin. Indeed acute and chronic responses to insulin may be quite different (Gans & Donker, 1991). Acutely, insulin, usually at supraphysiological concentration, causes vasodilatation in animals (Liang *et al.*, 1982; Yagi *et al.*, 1989; Standley *et al.*, 1991; Wambach & Liu, 1991) and in a man (Scott *et al.*, 1988), but there is also evidence that insulin may act synergistically with noradrenaline to enhance constriction (Gans *et al.*, 1990; Townsend *et al.*, 1992).

Relaxation to SNP was normal in the IT-diabetic rats but significantly reduced in the PI-diabetic rats. This would be expected if AGEs were present in the untreated animals, as quenching of NO would lead to reduced efficacy of SNP since this compound generates free NO in aqueous solution (Ignarro *et al.*, 1980). In our earlier study we reported a normal response to SNP in diabetic animals of 6 weeks duration; the difference between that and the present study may reflect AGE production associated with the longer duration of diabetes. Bucala *et al.* (1991), also found impaired acetylcholine and nitroglycerin vasodilator responses *in vivo* in STZ rats of 2 month duration which were associated with AGE formation in the artery wall. They found that short term insulin treatment did not reverse this defect and suggested that it was due to the irreversible effect of these NO quenching compounds. It would be of interest to determine, in this preparation, therefore, whether insulin treatment reverses rather than prevents the endothelium-dependent and -independent relaxation defects after prolonged diabetes.

In conclusion, sustained release insulin treatment from the onset of streptozotocin-induced diabetes prevents the development of impaired endothelium-dependent and endothelium-independent relaxation in rat mesenteric resistance arteries. The most likely mechanisms, would appear to be the preservation of endothelial NO production, but the prolongation of the half-life of NO through reduced free radical, superoxide and AGE formation may also play a role. In contrast insulin-treatment does not prevent the enhanced contractility to noradrenaline associated with streptozotocin-induced diabetes. This underlying enhancement of contractility to noradrenaline in insulin-treated experimental diabetes could contribute to increased peripheral vascular resistance and to the development of diabetic hypertension.

This work was funded through a collaborative PhD studentship between the Medical Research Council and ZENECA Pharmaceuticals. We are grateful to Dr Richard Jones for thoughtful discussion and suggestions.

References

ABIRU, T., KAMATA, K., MIYATA, N. & KASUYA, Y. (1990). Differences in vascular responses to vasoactive agents of basilar artery and aorta from rabbits with alloxan-induced diabetes. *Can. J. Physiol. Pharmacol.*, **68**, 882–888.

AGRAWAL, D.K. & MCNEILL, J.H. (1987). The effect of diabetes on vascular smooth muscle function in normotensive and spontaneously hypertensive rat mesenteric artery. *Can. J. Physiol. Pharmacol.*, **65**, 2274–2280.

BANSKOTA, N.K., TAUB, R., ZELLNER, K., OLSEN, P. & KING, G.L. (1989). Characterization of induction of protooncogene C-myc and cellular growth in human vascular smooth muscle cells by insulin and IGF-I. *Diabetes*, **38**, 123–129.

BROWNLEE, M., CERAMI, A. & VLASSARA, H. (1988). Advanced glycosylation endproducts in tissue and the biochemical basis of diabetic complications. *N. Engl. J. Med.*, **318**, 1315–1321.

BUCALA, R., TRACEY, K.J. & CERAMI, A. (1991). Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilation in experimental diabetes. *J. Clin. Invest.*, **87**, 432–438.

CALVER, A.L., COLLIER, J.G. & VALLANCE, P.J.T. (1992). Inhibition and stimulation of nitric oxide synthesis in the human forearm arterial bed of patients with insulin-dependent diabetes. *J. Clin. Invest.*, **90**, 2546–2554.

CAMERON, N.E. & COTTER, M.A. (1992). Impaired contraction and relaxation in aorta from streptozotocin-diabetic rats: role of polyol pathway. *Diabetologia*, **35**, 1011–1019.

CHOWIENCZYK, P.J., WATTS, G.F., COCKCROFT, J.R. & RITTER, J.M. (1992). Impaired endothelium-dependent vasodilation of forearm resistance vessels in hypercholesterolaemia. *Lancet*, **340**, 1430–1432.

CHRISTLIEB, A.R., JANKA, H.-U., KRAUS, B., GLEASON, R.E., ICASES-CABRAL, E.A., AIELLO, L.M., CABRAL, B.V. & SOLANO, A. (1976). Vascular reactivity to angiotensin II and to norepinephrine in diabetic subjects. *Diabetes*, **25**, 268–274.

COHEN, R.A., TESFAMARIAM, B., WEISBROD, R.M. & ZITNAY, K.M. (1990). Adrenergic denervation in rabbits with diabetes mellitus. *Am. J. Physiol.*, **259**, H55–H61.

CREAGER, M.A., COOKE, J.P., MENDELSOHN, M.E., CALLAGHER, J.P., COLEMAN, S.M., LOSCALZO, J. & DZAU, V.J. (1990). Impaired vasodilation of forearm resistance vessels in hypercholesterolaemic humans. *J. Clin. Invest.*, **86**, 228–234.

DE TEJADA, I.S., GOLDSTEIN, I., AZADZOI, K., KRANE, R.J. & COHEN, R.A. (1989). Impaired neurogenic and endothelium-mediated relaxation of penile smooth muscle from diabetic men with impotence. *New Engl. J. Med.*, **320**, 1025–1030.

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.

DVORNIK, D. (1987). Hyperglycaemia in the pathogenesis of diabetic complications. In *Aldose Reductase Inhibition. An Approach to the Prevention of Diabetic Complications.* ed. Porte, D. pp. 69–151. New York: Biomedical Information Corporation.

EICHLER, H.G., BLASCHKE, T.F., KRAEMER, F.B., FORD, G.A., BLOCHL-DAUM, B. & HOFFMAN, B.B. (1992). Responsiveness of superficial hand veins to α-adrenoceptor agonists in insulin-dependent diabetic patients. *Clin. Sci.*, **82**, 163–168.

ELLIOT, T.G., COCKROFT, J.R., GROOP, P.-H., EARLE, K., MOROCUTTI, A., VIBERTI, G.C. & RITTER, J.M. (1992). Vascular responses in the forearm of microalbuminuric type I (insulin-dependent) diabetic patients: evidence of altered nitric oxide biosynthesis. *Diabetiologia*, **35**, (suppl. 1) A19.

GANS, R.O.B., BILO, H.J.G., V MAARSCHALKERWEERD, W.W.A. & DONKER, A.J.M. (1990). Divergent effects of insulin on the pressor response to angiotensin-II and noradrenaline in healthy men. *Nephrol. Dial. Transplant.*, **5**, 687–688.

GANS, R.O.B. & DONKER, J.M. (1991). Insulin and blood pressure regulation. *J. Int. Med.*, **229**, 49–64.

GREEN, A.J. & JASPAN, J.B. (1990). *Treatment of Diabetic Neuropathy with Inhibitors of the Aldose Reductase Enzyme. Scholarly Review.* Amsterdam: Elsevier Scientific Publishing Co., Inc.

HALKIN, A., BENJAMIN, N., DOKTOR, H.S., TODD, S.D., VIBERTI, G. & RITTER, J.M. (1991). Vascular responsiveness and cation exchange in insulin-dependent diabetes. *Clin. Sci.*, **81**, 223–232.

HARRIS, K.H. & MACLEOD, M. (1988). Influence of the endothelium on contractile responses of arteries from diabetic rats. *Eur. J. Pharmacol.*, **153**, 55–64.

HODGSON, W.C. & KING, R.G. (1992). Effects of glucose, insulin or aldose reductase inhibition on responses to endothelin-1 of aortic rings from streptozotocin-induced diabetic rats. *Br. J. Pharmacol.*, **106**, 644–649.

HOGAN, M., CERAMI, A. & BUCALA, R. (1992). Advanced glycosylation endproducts block the antiproliferative effect of nitric oxide. *J. Clin. Invest.*, **90**, 1110–1115.

IGNARRO, L.J., EDWARDS, J.C., GRUTEER, B.K., OHLSTEIN, E.H. & GRUETTER, C.A. (1980). Possible involvement of S-nitrosothiols in the activation of guanylate cyclase by nitroso compounds. *FEBS Lett.*, **110**, 275–278.

JACOBS, M., PLANE, F. & BRUCKDORFER, K.R. (1990). Native and oxidised low-density lipoproteins have different inhibitory effects on endothelium-dependent relaxing factor in rabbit aorta. *Br. J. Pharmacol.*, **100**, 21–26.

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

LIANG, C.S., DOCHERTY, J.U. & FAILLACE, R. (1982). Insulin infusion in conscious dogs. Effects on systemic and adrenal medullary responses. *J. Clin. Invest.*, **69**, 1321–1336.

MACLEOD, K.M. & MCNEILL, J.H. (1985). Influence of chronic experimental diabetes on contractile response of rat isolated blood vessels. *Can. J. Physiol. Pharmacol.*, **63**, 52–57.

MCNALLY, P.G., WATT, P.A.C., BURDEN, A.C., HEARNSHAW, J.R. & THURSTON, H. (1992). Impaired vascular contraction and endothelium-dependent relaxation in normotensive Type 1 diabetes. *Diab. Med.*, **9**, 32A.

MCVEIGH, G.E., BRENNAN, G.M., JOHNSTON, G.D., MCDERMOTT, B.J., MCGRATH, L.T., HENRY, W.R., ANDREWS, J.W. & HAYES, J.R. (1992). Impaired endothelium-dependent and independent vasodilation in patients with type 2 (non-insulin-independent) diabetes mellitus. *Diabetologia*, **35**, 771–776.

MCVEIGH, G.E., BRENNAN, G.M., JOHNSTON, G.D., MCDERMOTT, B.J., MCGRATH, L.T., HENRY, W.R., ANDREWS, J.W. & HAYES, J.R. (1993). Dietry fish oil augments nitric oxide production or release in patients with type II (non-insulin-dependent) diabetes mellitus. *Diabetiologia*, **36**, 33–38.

MAKITA, Z., RADOFF, S., RAYFIELD, E.J., YANG, Z., SKOLNIK, E., DELANEY, V., FRIEDMAN, E.A., CERAMI, A. & VLASSARA, H. (1991). Advanced glycosylation end products in patients with diabetic nephropathy. *N. Engl. J. Med.*, **325**, 836–842.

MAYHAN, W.G. (1989). Impairment of endothelium-dependent dilatation of cerebral arterioles during diabetes mellitus. *Am. J. Physiol.*, **256**, H621–H625.

MAYHAN, W.G., SIMMONS, L.K. & SHARPE, G.M. (1991). Mechanism of impaired responses of cerebral arterioles during diabetes mellitus. *Am. J. Physiol.*, **260**, H319–H326.

MOLLINATTI, G.M., BAR, R.S., BELFIORE, F. & PORTA, M. (ed.). (1990). Endothelial cell function in diabetic microangiopathy: problems in methodology and clinical aspects. *Front. Diabetes* vol. 9. Basel: Karger.

MOORHOUSE, J.A., CARTER, S.A. & DOUPE, J. (1966). Vascular responses in diabetic peripheral neuropathy. A clinical and pathological study. *Br. Med. J.*, **78**, 883–888.

MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arterial resistance arteries in spontaneously hypertensive and normotensive rats. *Circ. Res.*, **41**, 19–26.

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.

PFAFFMAN, M.A., BALL, C.R., DARBY, A. & HILMAN, R. (1982). Insulin reversal of diabetes-induced inhibition of vascular reactivity in the rat. *Am. J. Physiol.*, **242**, H490–H495.

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

SATO, N., HASHIMOTO, H., TAKIGUCHI, Y. & NAKASHIMA, N. (1989). Altered responsiveness to sympathetic nerve stimulation and agonists of isolated left atria of diabetic rats: no evidence for involvement of hypothyroidism. *J. Pharmacol. Exp. Ther.*, **248**, 367–371.

SCARBOROUGH, N.L. & CARRIER, G.O. (1983). Increased alpha-2 adrenoceptor mediated vascular contraction in diabetic rats. *J. Auton. Pharmacol.*, **3**, 177–183.

SCOTT, A.R., BENNET, T. & MACDONALD, I.A. (1988). Effects of hyperinsulaemia on the cardiovascular response to graded hypervolaemia in normal and diabetic subjects. *Clin. Sci.*, **75**, 85–92.

SMITS, P., KAPMA, J.A., JACOBS, M.C., LUTTERMAN, J. & THEIN, T. (1993). Endothelium-dependent vascular relaxation in patients with type I diabetes. *Diabetes*, **42**, 148–153.

SÖNKSEN, P.H. (1976). Double antibody technique for the simultaneous assay of insulin and growth hormone. In *Hormones in Human Blood: Detection and Assay*. ed. Antoniades, H.N. Harvard: University Press.

SOSENKO, J.M., BRESLOW, J.L., MIETTINEN, O.S. & GABBAY, K.H. (1980). Hyperglycaemia and plasma lipid levels: a prospective study of young insulin-dependent diabetic patients. *N. Engl. J. Med.*, **302**, 650–654.

STANDLEY, P.R., ZHENG, F., RAM, J.L., ZEMEL, M.B. & SOWERS, J.R. (1991). Insulin attenuates vasopressin-induced calcium transients and a voltage-dependent calcium response in rat vascular smooth muscle cells. *J. Clin. Invest.*, **88**, 1230–1236.

STOLAR, M.W. (1988). Atherosclerosis in diabetes: the role of hyperinsulinaemia. *Metabolism*, **37**, 1–9.

TAGAMI, S., KONDO, T., YOSHIDA, K., HIROKAWA, J., OHTSUKA, Y. & KAWAKAMI, Y. (1992). Effect of insulin on impaired antioxidant activity in aortic endothelial cells from diabetic rabbits. *Metabolism*, **10**, 1053–1058.

TAKIGUCHI, Y., SATOH, N., HASHIMOTO, H. & NAKASHIMA, M. (1989). Reversal effect of thyroxine on altered vascular reactivity in diabetic rats. *J. Cardiovasc. Pharmacol.*, **13**, 520–524.

TANZ, R.D., CHANG, K.S.K. & WELLER, T.S. (1989). Histamine relaxation of aortic rings from diabetic rats. *Agents Actions*, **28**, 2–8.

TAYLOR, P.D., MCCARTHY, A.L., THOMAS, C.R. & POSTON, L. (1992). Endothelium-dependent relaxation and noradrenaline sensitivity in mesenteric resistance arteries of streptozotocin-induced diabetic rats. *Br. J. Pharmacol.*, **107**, 393–399.

TAYLOR, P.D., WICKENDEN, A.D., MIRRLEES, D.J. & POSTON, L. (1994). Endothelial function in the isolated perfused mesentery and aortae of rats with streptozotocin-induced diabetes: effect of treatment with aldose reductase inhibitor, ponalrestat. *Br. J. Pharmacol.*, **111**, 42–48.

TESFAMARIAM, B., BROWN, M.L., DEYKIN, D. & COHEN, R.A. (1990). Elevated glucose promotes generation of endothelium-derived vasoconstrictor prostanoids in rabbit aorta. *J. Clin. Invest.*, **85**, 929–932.

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

TOWNSEND, R.R., YAMAMOTO, R. & NICKOLS, M. (1992). Insulin enhances pressor responses to norepinephrine in rat mesenteric vasculature. *Hypertension*, **19**, II, 105–110.

WAMBACH, G.K. & LIU, D. (1991). Insulin attenuates vascoconstriction induced by noradrenaline, serotonin and potassium chloride in rat mesenteric resistance arteries. *J. Hypertens.*, **9**, S198–S199.

WANG, P.Y. (1991). Palmitic acid as an excipient in implants for sustained release of insulin. *Biomaterials*, **12**, 57–62.

WHITE, R.E. & CARRIER, G.O. (1988). Enhanced vascular α-adrenergic neuroeffector system in diabetes: importance of calcium. *Am. J. Physiol.*, **255**, H1036–H1042.

WONG, K.K. & TZENG, S.F. (1992). Norepinephrine-induced contractile responses in isolated rat aortae from different duration of diabetes. *Artery*, **19**, 1–13.

YAGI, S., TAKATA, S. & KIYOK, H. (1989). Effects of insulin on vasoconstrictor responses to norepinephrine and angiotensin II in rabbit femoral artery and vein. *Diabetes*, **37**, 1064–1067.

ZEIHER, A.M., DREXLER, H., WOLLSCHLAGER, H. & JUST, H. (1991). Modulation of coronary vasomotor tone in humans: progressive endothelial dysfunction with different early stages of coronary atherosclerosis. *Circulation*, **83**, 391–401.

(Received April 23, 1993
Revised August 13, 1993
Accepted September 3, 1993)