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Effects of glucose, insulin or aldose reductase inhibition on responses to endothelin-1 of aortic rings from streptozotocin-induced diabetic rats

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1 This study investigated the constrictor responsiveness to endothelin-1 (ET-1, 0.1 nM–0.1 μM) of aortic rings (under 10 g resting tension in Krebs solution) from 2- and 6-week streptozotocin (STZ, 60 mg kg^{-1}, i.v.)-induced diabetic rats and vehicle-treated control rats.

2 In aortae from 2- and 6-week STZ-treated rats, and their corresponding controls, removal of endothelium caused leftward shifts of ET-1 concentration-response curves without affecting maximum responses.

3 Maximum responses to ET-1 were reduced in aortae from both 2- and 6-week STZ-treated rats compared to those from control rats. Such reductions were still evident after removal of the endothelium.

4 Decreased responsiveness to ET-1 of aortae from 2-week STZ-treated rats was still evident after chronic treatment with the aldose reductase inhibitor epalrestat, but not after chronic insulin treatment or in aortae bathed in high glucose (30 mM) Krebs solution.

5 Decreased responsiveness to ET-1 of aortae from 6-week STZ-treated rats (compared with those from controls) was still evident after chronic epalrestat treatment and in high glucose Krebs solution.

6 These data suggest that the decreased responsiveness to ET-1 observed in aortae from 2- and 6-week STZ-induced diabetic rats is not due to abnormal activity of the polyol pathway. The altered responsiveness in aortae from 2-week diabetic rats (compared with those from control rats) may possibly be a manifestation of changes (adaptive or otherwise) which occur as a result of high glucose concentrations *in vivo*. However, in aortae from rats with diabetes of longer duration, other mechanisms may also play a role in the altered responsiveness, since it was no longer reversible by bathing in high glucose Krebs solution.

Keywords: Diabetes mellitus; rat aortae; endothelial cells; endothelin; aldose reductase inhibitor; insulin

Introduction

Elevated glucose levels can trigger the release of the potent vasoconstrictor peptide endothelin (ET) from endothelial cells (Yamauchi *et al.*, 1990). Changes in endothelial cell function have been found to occur in various cardiovascular disorders (Luscher *et al.*, 1989), some of which are associated with diabetes mellitus. We have previously reported that maximum responsiveness to ET-1 of aortic rings from 2-week streptozotocin (STZ)-induced diabetic rats was significantly reduced compared to that of rings from control rats (Fulton *et al.*, 1990; 1991). Such reduced responsiveness was also evident in the presence of the cyclo-oxygenase inhibitor indomethacin, or in the absence of extracellular calcium. The present study was designed to determine whether aortae from 6-week STZ-treated rats were less responsive to ET-1. The effect of insulin treatment on the altered responsiveness of aortae from 2-week STZ-treated rats was examined. Also, the effects of *in vitro* changes in extracellular glucose concentration, as well as the effect of aldose reductase inhibition, on responsiveness to ET-1 was examined in aortae from both 2- and 6-week STZ-treated rats.

Methods

Male Wistar rats (290–385 g) were treated with STZ (60 mg kg^{-1}, i.v.) or vehicle (50 mM citrate buffer) under 4% halothane anaesthesia (O_2/N_2O 2:1) as previously described (Fulton *et al.*, 1991). The animals were then housed in treatment pairs, being allowed free access to food and water at all times. Rat body weights and blood glucose levels were measured on the day of STZ or citrate buffer administration and again after either 2 or 6 weeks. Only rats displaying elevated blood glucose levels (> 16 mM, Ames Minilab 1) after 2 or 6 weeks were considered to be diabetic. Vehicle control rats had normal blood glucose levels when tested at the same times. After 2 or 6 weeks, a STZ-induced diabetic and a control animal were killed, and two 5 mm rings cut from each descending thoracic aorta (Fulton *et al.*, 1991). Endothelial cells were removed from one ring from each treatment group by rubbing (Fulton *et al.*, 1991). Rings were placed in 15 ml organ baths containing Krebs solution of the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.2, glucose 11.1. Where indicated, high-glucose Krebs consisted of Krebs solution with 30 mM glucose. Physiological solutions were maintained at 37°C, and bubbled with 5% CO_2 in O_2. Rings were placed under 10 g resting tension throughout the experiment, as this approximates more to the physiological wall tension than does the more commonly used *in vitro* tension of 1–2 g (Fulton *et al.*, 1991). Apart from the area of tissue directly in contact with the wire within the lumen, effective transmural pressure is assumed to apply uniformly when a blood vessel ring is stretched between two fixed points (Mulvany & Halpern, 1977). After 1 h equilibration at 10 g tension, a submaximal concentration of phenylephrine (0.3 μM) was added to the bath. At the plateau of contraction, acetylcholine

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(10 μM) was added. The presence of functional endothelial cells was indicated by subsequent relaxation (Fulton *et al.*, 1991). A cumulative concentration-response (CR) curve to ET-1 was then obtained. ET-1 was added at intervals of between 5–20 min depending on the time taken for the previous response to reach a plateau. Contractions were recorded with a Grass FTO3 tension transducer connected to a Grass polygraph (Model 79E).

After each experiment, aortic rings were oven dried (50°C) and then weighed. The order of killing STZ-treated and control rats and the arrangement of tissues in organ baths were randomized.

Epalrestat treatment

Where indicated the aldose reductase inhibitor (ARI) epalrestat (40 mg kg^{-1} daily, p.o. by gavage), in 0.5% carboxymethyl-cellulose, was administered for 2 or 6 weeks, starting on the day of STZ or citrate buffer treatment.

Insulin treatment

Where indicated STZ-treated rats were injected with a single daily dose of Lente MC insulin zinc suspension (5 units day^{-1}). This treatment commenced on the second day after the STZ injection, and was continued until the rats were killed. Insulin treatment was started on the second day after STZ administration, since STZ-induced diabetes is characterized by the following pattern of blood glucose levels. After an initial delay of approximately 45–60 min there is a short period of hyperglycaemia (up to 6 h duration; Dulin *et al.*, 1967) followed by a severe hypoglycaemia lasting up to 48 h, and then chronic hyperglycaemia (Rerup, 1970).

Drugs

The following drugs were used: acetylcholine chloride (Sigma), carboxymethyl-cellulose (Sigma), endothelin-1 (Auspep; Batch no. CP36), epalrestat (ONO Pharmaceuticals), insulin (Lente MC zinc suspension, CSL-Novo), (−)-phenylephrine HCl (ICN Pharmaceutical), streptozotocin (Sigma).

Phenylephrine was dissolved in catecholamine diluent (0.312 g NaH_2PO_4 and 0.08 g ascorbic acid per litre of 0.9% (w/v) saline). Acetylcholine was dissolved in distilled water. Endothelin stock was dissolved in distilled water, divided into aliquots and frozen. On the day of use it was thawed and diluted in 0.9% saline.

Statistics

Single comparisons between responses in aortae from STZ-treated and control rats were made by Student's unpaired *t* test. Paired *t* tests were used for comparison of values from the same animal. Multiple comparisons were analysed by two way analysis of variance (ANOVA) and Tukey tests on the CLR ANOVA package (Apple Macintosh). Values shown are means ± s.e.mean. In all cases, statistical significance is indicated by $P<0.05$. EC_{50} values were determined from the E_{max} of each individual curve and the geometric mean (i.e. mean of the log values) determined.

Results

As shown in Table 1, dry weights of aortic rings from 2-week STZ-treated rats were not significantly different from those of 2-week controls. However weights of rings from 6-week STZ-treated rats were significantly reduced. Rats with diabetes of both 2 and 6 weeks duration displayed significantly reduced body weights compared to their pre-injection weights. However, 2- and 6-week control rats displayed significantly increased body weights compared to their corresponding pre-injection weights. The reduction in body weight observed in 2-week STZ-treated rats was not observed in 2-week diabetic rats treated with insulin. Increased blood glucose levels in 2-week STZ-treated rats were evident in rats chronically-treated with epalrestat, but were normalized (i.e. were between 4–8 mM) by chronic insulin treatment (Table 1).

Cumulative CR curves were obtained to ET-1 (0.1 nM–0.1 μM) in aortic rings from 2-week (Figure 1a,b) and 6-week (Figure 2a,b) STZ-treated rats and their corresponding controls, with and without endothelial cells. As shown in these Figures and Table 2, maximum responses of aortae from 2- and 6-week STZ-treated rats, with or without endothelial cells, were significantly reduced compared with those of aortae from controls. This decrease in responsiveness observed in aortae from 2-week STZ-treated rats was no longer significant in aortae from insulin-treated diabetic rats (Figure 1a,b). As shown in Figure 3a and b and Table 2, the reduction in maximum responsiveness to ET-1 in 2-week STZ-treated rats (compared to those from control rats) was not prevented by chronic treatment of diabetic rats with the aldose reductase inhibitor (ARI) epalrestat.

When experiments were performed in high glucose (30 mM) Krebs solution, there was no significant difference in responses to ET-1 between rings obtained from 2-week STZ-

Table 1 Body weights, blood glucose levels and aortic ring dry weights of control and streptozotocin (STZ)-treated rats

		Body weights (g)		*Blood glucose levels* (mM)		*Aortic ring dry weights* (mg)	
	(n)	*Initial*	*Final*	*Initial*	*Final*	*With E*	*Without E*
Control (2-week)	11	326 ± 8	379 ± 11†	6.3 ± 0.2	6.3 ± 0.2	1.67 ± 0.03	1.64 ± 0.03
Control (6-week)	12	330 ± 7	431 ± 10†	5.7 ± 0.3	6.0 ± 0.1	1.90 ± 0.08	1.83 ± 0.05
STZ (2-week)	11	332 ± 4	310 ± 9†*	5.8 ± 0.3	20.4 ± 0.6*†	1.60 ± 0.04	1.66 ± 0.04
STZ (6-week)	12	334 ± 5	281 ± 12*†	6.1 ± 0.2	21.1 ± 1.3*†	1.61 ± 0.04*	1.57 ± 0.04*
Control (2-week) /epalrestat	5	345 ± 11	390 ± 15†	6.8 ± 0.4	6.4 ± 0.3	1.69 ± 0.03	1.68 ± 0.04
STZ (2-week) /epalrestat	5	336 ± 13	321 ± 13*	5.7 ± 0.4	19.4 ± 0.9*†	1.56 ± 0.02	1.54 ± 0.06
Control (6-week) /epalrestat	6	335 ± 8	416 ± 12†	6.0 ± 0.5	5.6 ± 0.2	1.64 ± 0.06	1.62 ± 0.04
STZ (6-week) /epalrestat	6	349 ± 4	283 ± 13*†	6.8 ± 0.5	21.2 ± 1.1*†	1.40 ± 0.03*	1.42 ± 0.02*
STZ (2-week) /insulin	5	309 ± 7	359 ± 8†	5.5 ± 0.3	6.1 ± 0.8	1.47 ± 0.05	1.49 ± 0.07

Initial measurements were made at the time of STZ or vehicle injection, and final measurements made 2 or 6 weeks later.
E = endothelial cells.
*Significantly different from corresponding control group, $P<0.05$, unpaired *t* test.
†Significantly different from initial value in same treatment group, $P<0.05$, paired *t* test.

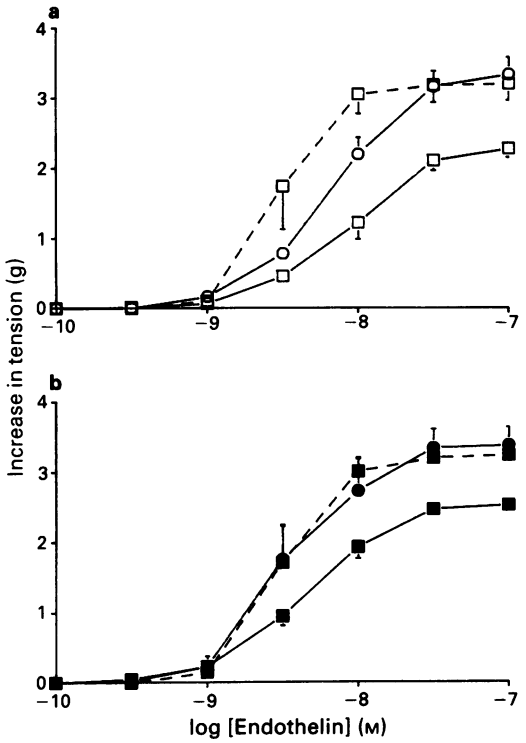

Figure 1 Responses to endothelin-1 of aortic rings from 2-week streptozotocin (STZ)-treated rats. (a) With endothelium: STZ-treated (□—□, $n = 6$), control (○—○, $n = 6$) and STZ-treated with insulin (□--□, $n = 5$). (b) Without endothelium: STZ-treated (■—■, $n = 6$), control (●—●, $n = 6$) and STZ-treated with insulin (■--■, $n = 5$).

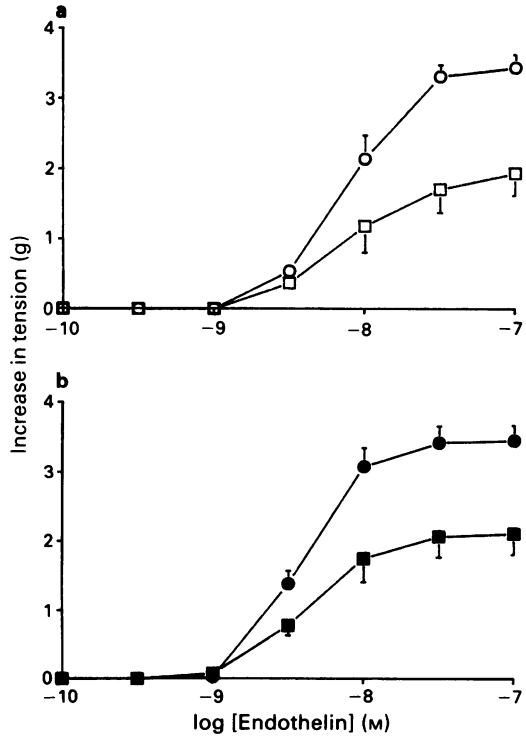

Figure 2 Responses to endothelin-1 of aortic rings from 6-week streptozotocin (STZ)-treated rats ($n = 6$). (a) With endothelium: STZ-treated (□) and control (○). (b) Without endothelium: STZ-treated (■) and control (●).

treated versus those from control rats (Figure 3a,b). There was also no significant difference when responses of aortic rings from 2-week control rats bathed in normal Krebs solution were compared to those from 2-week diabetic rats bathed in 30 mM glucose Krebs solution (Table 2, ANOVA). However, in high glucose Krebs solution there was a significant difference between responses of aortae from 6-week diabetic versus those from control rats (Figure 4a,b). There was also a significant difference in responsiveness to ET-1 of aortae bathed in normal Krebs solution from control rats, compared to that of aortae bathed in 30 mM glucose Krebs solution from diabetic rats (Table 2, ANOVA, $P < 0.05$). As shown in Figure 4a and b and Table 2, the reduction in maximum responsiveness to ET-1 in 6-week STZ-treated rats (compared to those from control rats) was not prevented by chronic treatment of diabetic rats with the ARI epalrestat. In all cases (except for endothelium-intact rings from 6-week diabetic rats bathed in 30 mM glucose Krebs solution) there was no significant effect of diabetes on EC_{50}s for ET-1 (Table 2).

Discussion

Results of the present study indicate that the previously reported reduced maximum responsiveness to ET-1 of aortic rings from 2-week STZ-treated rats (compared with 2-week controls; Fulton *et al.*, 1991) also occurs in aortae of 6-week diabetic rats. In the case of endothelium-intact or -denuded aortic rings from 2-week diabetic rats, the difference in maximum responses in the presence of normal glucose versus 30 mM glucose was not significant. However, the difference in responsiveness of aortae from 2-week diabetic versus control rats was not observed in physiological bathing solution containing 30 mM glucose. Thus it is possible that the high glucose concentrations which occur *in vivo* during diabetes may affect responsiveness to ET-1. Indeed, it is possible that the reduced responsiveness to ET-1 of aortae from 2-week diabetic rats (compared with controls), observed in normal Krebs solution, may be a manifestation of an adaption of the vasculature to high levels of circulating blood glucose. However, normalization of ET-1 responses in high glucose Krebs solution was not observed in aortae from 6-week rats. One conceivable explanation for this observation could be that with a longer duration of diabetes (6 weeks), changes and/or damage occurs to the vasculature which hinder the adaptive responses to high glucose levels postulated above. In addition to causing a change in responsiveness to ET-1, high glucose levels may also affect ET-1 release. Yamauchi *et al.* (1990) found that increasing the glucose concentration from 5.5 to 11.1 mM or 22.2 mM significantly stimulated ET-1 release from cultured bovine aortic endothelial cells. However, Hattori *et al.* (1991) found that hyperglycaemia caused inhibition of ET-1 release from cultured porcine aortic endothelial cells: under hyperglycaemic conditions (27.5 and 55 mM glucose) ET-1 release was inhibited by greater than 50%.

Results of the present study indicate that streptozotocin *per se* was not responsible for the changed responsiveness to ET-1 of aortae from 2-week diabetic rats, since insulin treatment prevented this changed responsiveness.

Some cells, including endothelial cells of blood vessels (Dvornik, 1978), do not require insulin to enable glucose entry, and are consequently susceptible to the fluctuating plasma levels of glucose associated with diabetes. During periods of hyperglycaemia the glycolytic pathway in these cells may become saturated and the polyol pathway activated. It has been postulated that this increased activity leads to many of the complications of diabetes (Beyer & Hutson, 1986). Inhibition of the rate-limiting enzyme of the polyol pathway, aldose reductase, has been shown to restore normal biochemical and functional parameters in some of these affected tissues (Beyer & Hutson, 1986). Indeed, Williamson *et al.* (1990) have reported that 30 mM glucose caused micro-

Table 2 Maximum responses and EC_{50} values for endothelin-1 (ET-1) of aortae from streptozotocin (STZ)-treated and control (C) rats with (+) and without (−) endothelial cells (E)

	(n)		*C + E*	*STZ + E*	*C − E*	*STZ − E*
2-week	6	Maximum (g)	3.33 ± 0.25	2.28 ± 0.13*	3.38 ± 0.26	2.53 ± 0.12*
		EC_{50} (-log M)	8.17 ± 0.08	8.06 ± 0.10	8.41 ± 0.13	8.34 ± 0.05‡
		Slope	2.38 ± 0.61	1.65 ± 0.45*	2.06 ± 0.81	1.54 ± 0.29
6-week	6	Maximum (g)	3.44 ± 0.19	1.94 ± 0.32*	3.45 ± 0.22	2.11 ± 0.31*
		EC_{50} (-log M)	8.11 ± 0.07	8.03 ± 0.10	8.42 ± 0.04‡	8.37 ± 0.05‡
		Slope	2.77 ± 0.68	1.34 ± 0.87*	3.04 ± 0.57	1.68 ± 0.65*
2-week/Epalrestat	5	Maximum (g)	3.51 ± 0.16	2.29 ± 0.19*	3.81 ± 0.25	2.45 ± 0.23*
		EC_{50} (-log M)	8.22 ± 0.06	8.28 ± 0.05	8.69 ± 0.01‡	8.53 ± 0.09‡
		Slope	2.62 ± 0.63	1.59 ± 0.49*	3.40 ± 0.81	1.94 ± 0.53*
6-week/Epalrestat	6	Maximum (g)	3.91 ± 0.14	2.49 ± 0.18*	3.94 ± 0.11	2.36 ± 0.17*
		EC_{50} (-log M)	8.03 ± 0.09	7.87 ± 0.06	8.38 ± 0.03‡	8.30 ± 0.08‡
		Slope	3.17 ± 0.72	1.73 ± 0.52*	3.64 ± 0.49	1.77 ± 0.53*
2-week/Insulin	5	Maximum (g)	NA	3.20 ± 0.24	NA	3.24 ± 0.19
		EC_{50} (-log M)		8.51 ± 0.10		8.51 ± 0.11
		Slope		2.94 ± 1.19		2.86 ± 0.98
2-week/30 mM Glucose	5	Maximum (g)	2.84 ± 0.30	2.76 ± 0.15	3.04 ± 0.24	2.80 ± 0.07
		EC_{50} (-log M)	8.33 ± 0.02	8.13 ± 0.10	8.45 ± 0.11	8.37 ± 0.06
		Slope	1.94 ± 0.54	1.68 ± 0.40†	2.43 ± 0.83	2.14 ± 0.51
6-week/30 mM Glucose	6	Maximum (g)	3.23 ± 0.12	2.11 ± 0.20*†	3.46 ± 0.18	2.26 ± 0.32*†
		EC_{50} (-log M)	8.25 ± 0.01	7.95 ± 0.07*	8.13 ± 0.18	8.13 ± 0.20
		Slope	2.58 ± 0.58	1.43 ± 0.47*†	2.79 ± 0.63	1.30 ± 0.92*†

Values are given ± s.e.mean (except for slopes, which are ± 95% confidence limits).
*Significantly different from corresponding control group, $P<0.05$ unpaired t test.
‡Significantly different from same treatment group with endothelial cells, $P<0.05$ unpaired t test.
†Significantly different from corresponding control group in normal Krebs solution, $P<0.05$ ANOVA and Tukey test.
NA = not applicable.

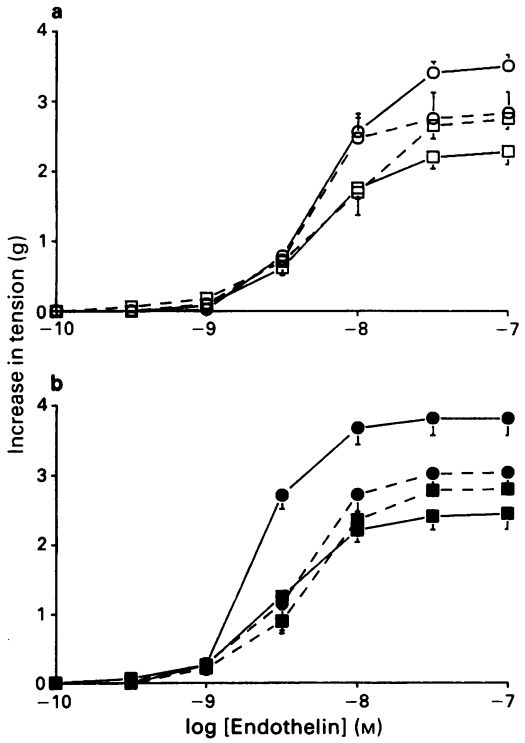

Figure 3 Responses to endothelin-1 of aortic rings from 2-week streptozotocin (STZ)-treated rats. (a) With endothelium: STZ/epalrestat-treated (□—□, $n = 5$), control/epalrestat-treated (○—○, $n = 5$), STZ-treated in 30 mM glucose Krebs solution (□- -□, $n = 5$) and control in 30 mM glucose Krebs solution (○- -○, $n = 5$). (b) Without endothelium: STZ/epalrestat-treated (■—■, $n = 5$), control/epalrestat-treated (●—●, $n = 5$), STZ-treated in 30 mM glucose Krebs solution (■- -■, $n = 5$) and control in 30 mM glucose Krebs solution (●- -●, $n = 5$).

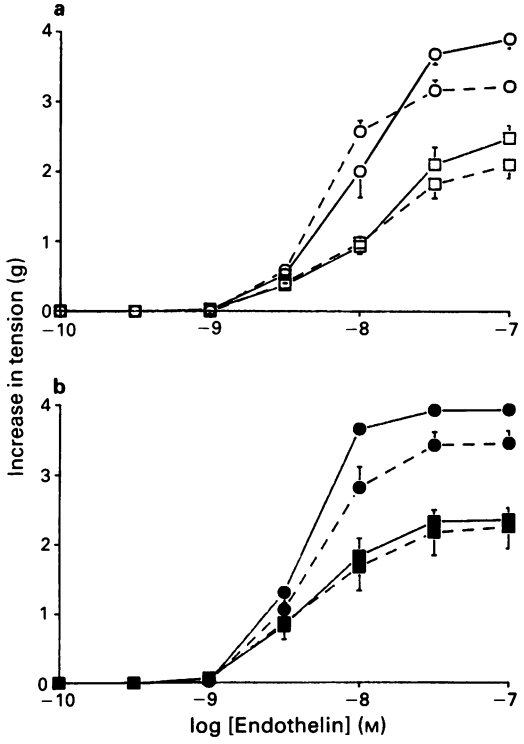

Figure 4 Responses to endothelin-1 of aortic rings from 6-week streptozotocin (STZ)-treated rats. (a) With endothelium: STZ/epalrestat-treated (□—□, $n = 6$), control/epalrestat-treated (○—○, $n = 6$), STZ-treated in 30 mM glucose Krebs solution (□- -□, $n = 6$) and control in 30 mM glucose Krebs solution (○- -○, $n = 6$). (b) Without endothelium: STZ/epalrestat-treated (■—■, $n = 6$), control/epalrestat-treated (●—●, $n = 6$), STZ-treated in 30 mM glucose Krebs solution (■- -■, $n = 6$) and control in 30 mM glucose Krebs solution (●- -●, $n = 6$).

vascular functional changes in non-diabetic rats (indicating that such changes were the consequence of hyperglycaemia *per se*) and that these changes were prevented by the aldose reductase inhibitor (ARI) tolrestat.

Aldose reductase has been detected in human aortae (Srivastava *et al.*, 1986). It was therefore of interest to determine whether the reduced maximum responsiveness to ET-1 of aortae from diabetic rats was prevented by chronic treatment with an ARI. Yorek & Dunlap (1989) showed that incubation of cultured bovine aortic endothelial cells in high ambient glucose (30 mM) led to a reduction in intracellular myo-inositol and an accumulation of sorbitol. This occurred after a minimum incubation period of one week. Therefore these changes could possibly occur in the time-frame of the present study. However, in the present study, reduced responsiveness to ET-1 was still observed in aortae from diabetic rats chronically treated with the ARI, epalrestat. The dose of epalrestat used was greater than that which had been previously shown to prevent peripheral nerve dysfunction in STZ-treated rats (Kikkawa *et al.*, 1984). The same dose as used in the present study has also been shown to improve responsiveness to nerve stimulation in isolated atria of STZ-treated rats, but to have no significant effect on postsynaptic stimulation (by exogenous noradrenaline) (Hashimoto *et al.*, 1990). It is therefore possible that the inability of epalrestat to normalize responses to ET-1 in the present study may be because altered polyol pathway activity is not the chief cause of the abnormality occurring in 2- or 6-week STZ-induced diabetic rats.

Previous workers have shown that in isolated aortic rings from non-diabetic rats, removal of endothelial cells shifted the CR curve to ET-1 to the right with no significant change in maximum responses (Topouzis *et al.*, 1991). This has been confirmed in the present study in aortae from both control and STZ-treated rats. However, this is in contrast to the observations of Rodman *et al.* (1989) who reported that removal of endothelial cells from non-diabetic rat aortic rings enhanced maximum responses to ET-1 but did not significantly affect EC_{50} values.

As has been reported previously (Fulton *et al.*, 1991), in the present study the dry weights of aortic rings were not significantly affected by 2 weeks of diabetes. However, dry weights of aortae from 6-week STZ-treated rats were significantly reduced. A reduction in wet weight of aortic rings from 8-week STZ-treated rats has been previously reported by Head *et al.* (1987), indicating that these changes may be dependent on the duration of diabetes.

A comparison of the potency of ET-1, in aortae from 2-week diabetic and control rats (both with and without endothelium), between the present study and a previous study (Fulton *et al.*, 1991) shows a mean difference in EC_{50} values of 0.52 log (M) units. This discrepancy may be due to the different batches of ET-1 used in each study, as an examination of the amino acid analysis from each batch indicates a slight variation.

In summary, results of the present study suggest that the decreased responsiveness to ET-1 of aortae from 2-week diabetic rats may not be due to abnormal activity of the polyol pathway. Similarly, it was probably not due to streptozotocin *per se* as responses were normalized by insulin treatment. The results of our previous study (Fulton *et al.*, 1991) did not indicate a role for cyclo-oxygenase products or extracellular Ca^{2+} in the change in ET-1 responsiveness observed during diabetes. In both the earlier study and in the present study, decreased responsiveness to ET-1 has still been observed in denuded aortae, indicating that changes in endothelium-derived relaxing factor/NO are probably not responsible for this change. However, Nayler *et al.* (1989) have reported a reduction in the density of [^{125}I]-ET binding sites in cardiac membrane fragments from STZ-treated rats. If a similar change occurs in rat aortae then this may explain the reduced responsiveness to ET-1 observed in the present study.

It is conceivable that the altered responsiveness following 2 weeks of diabetes (compared with controls) was a manifestation of an adaptive change to hyperglycaemia occurring *in vivo*, since it was not observed when aortae were bathed in high glucose Krebs solution. The finding that responsiveness to ET-1 of aortae from 6-week diabetic rats was still reduced in the presence of high glucose Krebs solution suggests that in longer term diabetes, other factors may play a role in the altered responsiveness to ET-1.

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