Attenuated responses to endothelin-1, KCl and CaCl₂, but not noradrenaline, of aortae from rats with streptozotocin-induced diabetes mellitus

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1 This study investigated the responsiveness to vasoconstrictor agents (including endothelin-1, ET-1) of aortic rings from rats with two-week streptozotocin (STZ, 60 mg kg⁻¹, i.v.)-induced diabetes and vehicle-treated control rats. The basal tension was 10 g, which was estimated to be more physiological than the tension of 1–2 g that has been previously used for most studies of aortic rings from diabetic rats.

2 Maximum responses to ET-1 (0.13–18 nM), KCl (2–20 mM) or CaCl₂ (10 μM–10 mM) were reduced in aortae from STZ-treated rats compared to those from control rats. Such reductions were still evident after removal of the endothelium.

3 Responses to noradrenaline (NA, 0.1 nM–26 μM) of aortae from STZ-treated rats were not significantly different from responses of aortae of control rats.

4 Removal of endothelium resulted in a significant reduction in the EC₅₀ values for NA of rings from both STZ-treated rats (6.90 ± 0.13 and 8.17 ± 0.35 (–log M) with and without endothelium, respectively, n = 5) and control rats (6.90 ± 0.15 and 8.37 ± 0.44 (–log M) with and without endothelium, respectively, n = 5).

5 In calcium-free medium (with 1 mM EGTA), responses to NA and ET-1 were reduced compared with those in normal Krebs solution and maximum responses were less in rings from STZ-treated compared with control rats.

6 Indomethacin (5 μM) did not prevent the reduced maximum responsiveness to ET-1 in rings from STZ-treated rats compared with those from controls.

7 This study indicates that changes in vascular responsiveness to ET-1, KCl and CaCl₂ (but not NA) occur in aortae of two-week STZ-treated rats. The endothelium does not appear to play a major role in mediating changes in responsiveness to ET-1.

Keywords: Diabetes mellitus; rat aortae; endothelial cells; endothelin; noradrenaline; calcium; indomethacin

Introduction

The release of the potent vasoconstrictor peptide endothelin (Yanagisawa *et al.*, 1988), from endothelial cells, can be triggered by elevated glucose levels (Yamauchi *et al.*, 1990). Changes in endothelial cell function have been linked to various pathological states such as hypertension, atherosclerosis and vascular occlusion (Vanhoutte, 1986; Lüscher *et al.*, 1989; Vane *et al.*, 1990), all of which occur more frequently in diabetes mellitus (Colwell *et al.*, 1979). This study examined the role of the endothelial cell in modulating responsiveness to various vasoconstrictors in aortic rings from rats with streptozotocin (STZ)-induced diabetes mellitus of two weeks duration. Most previous studies of vascular responsiveness of aortic rings from diabetic rats have used basal tensions of 1 g (e.g. Mulhern & Docherty, 1989) or 2 g (e.g. Owen & Carrier, 1979; Scarborough & Carrier, 1983; Head *et al.*, 1987; Harris & MacLeod, 1988). Such tensions probably do not approximate to those found *in vivo* (see below, and Mulvany & Halpern, 1977; Angus *et al.*, 1986), and therefore the present study was designed to examine the responsiveness of aortic rings under a more physiological tension.

Methods

Male Wistar rats (300–425 g) were lightly anaesthetized (4% halothane, O₂/N₂O 2 : 1) and made diabetic by a single injection into the tail vein of STZ (60 mg kg⁻¹) in citrate buffer (50 mM, pH 4.5), as previously described (Law & King, 1990). Control rats received an equivalent volume of citrate buffer. The animals were then housed in treatment pairs (i.e. one

STZ-treated and one control). Rats were allowed free access to food and water at all times. Animals were weighed and their blood glucose levels measured (Ames Glucometer II), from a tail vein sample, immediately prior to injection, and 1 and 2 weeks after injection. Only rats displaying elevated blood glucose levels (> 18 mM) after 2 weeks were considered to be diabetic. Control rats had normal blood glucose levels over the two week period (4–8 mM).

After 2 weeks, a STZ-treated and a control animal were decapitated and a section of each descending thoracic aorta removed, flushed through with Krebs solution, and placed in a Petri dish containing oxygenated Krebs solution. Fat and connective tissue were removed under magnification. Two rings (5 mm in length) were cut from each aorta with a twin-bladed scalpel. Endothelial cells were removed from 2 of the 4 aortic rings (i.e. 1 from each treatment group but randomly caudal or cephalad) by gentle rubbing with stainless steel wire within the lumen. Each ring was then placed with 2 hooks through its lumen in a 15 ml organ bath containing Krebs solution. To avoid absorption of peptides onto glass, baths were siliconized. One hook from each aorta was connected to a tissue holder, the other to a force displacement transducer (Grass FTO3). Contractions were recorded on a Grass polygraph (Model 7D). Tissues were equilibrated for 60 min under a resting tension of 1 g. After this period tissues were placed under a resting tension of 10 g for the duration of the experiment. This tension of 10 g results in a more close approximation to the wall tension of the aorta *in vivo*, than the more commonly used tension of 1–2 g.

The effective transmural pressure 'p' is an estimate of the pressure which would have been required to maintain the *in situ* vessel at the same internal wall circumference and wall tension. 'p' can be estimated, assuming the vessel wall is sufficiently thin for Laplace's equation to apply (Mulvany & Halpern, 1977).

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$p = 2\pi T/L$, where 'T' is the wall tension, and 'L' is the internal circumference (even if non circular). At a tension of 10 g between hooks, $T = 10 \text{ g}/2 \times 5 \text{ mm}$ (i.e. $10 \text{ g} \div$ twice the length of the ring); thus $T = 1 \text{ g/mm}$. $L = \pi d + 2x$, where 'x' is the distance between the two hooks, and 'd' is the diameter of the hooks (0.6 mm). 'x' was found to be relatively constant between aortae (2 mm). Thus, at a tension of 10 g between hooks, $L = 5.88 \text{ mm}$, and $p = 2\pi T/L = 1.07 \text{ g mm}^{-2} = 0.107 \text{ kg cm}^{-2} = 10.5 \text{ kPa} \approx 80 \text{ mmHg}$. i.e. the effective transmural pressure approximates to that *in vivo*.

Krebs solution was of the following composition (mM): NaCl 118.4, KCl 4.7, CaCl_2 2.5, NaHCO_3 25.0, KH_2PO_4 1.2, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2 and glucose 11.1. Where indicated, calcium-free Krebs consisted of Krebs solution without Ca^{2+} but with 1 mM EGTA added. In high K^+ Krebs solution, the osmotic pressure was maintained by a corresponding reduction of Na^+ . Physiological solutions were maintained at 37°C , and bubbled with carbogen (5% CO_2 in O_2). After equilibration, a submaximal concentration of phenylephrine ($0.3 \mu\text{M}$) was added to the bath. At the plateau of contraction, acetylcholine ($10 \mu\text{M}$) was added. The presence of functional endothelial cells was indicated by subsequent relaxation. Cumulative concentration-response curves to agonists were then obtained, with only 1 agonist being examined in each experiment. Noradrenaline, KCl and CaCl_2 were added at intervals of between 1–5 min depending on the time taken for the response to reach a plateau. ET-1 was added at intervals of between 5–15 min due to the longer time taken for the responses to reach a plateau. Where stated, indomethacin was added to the Krebs solution reservoir for the duration of the experiment. After each experiment, aortic rings were dried and then weighed. The order of killing STZ-treated and control animals was alternated between days and the arrangement of tissues in organ baths randomised.

Drugs

The following drugs were used: acetylcholine chloride (Sigma), ethyleneglycol-bis-(β -amino-ethyl ether)*N,N'*-tetra-acetic acid (EGTA; Sigma), endothelin-1 (Auspep), indomethacin (Sigma), noradrenaline bitartrate (Sigma), phenylephrine HCl (ICN Pharmaceutical), streptozotocin (Sigma). Noradrenaline and phenylephrine were made up 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. Indomethacin was dissolved in 1% Na_2CO_3 . Endothelin was initially dissolved in distilled water and frozen. On the day of use it was thawed and diluted in 0.9% saline. All other drugs were prepared daily.

Statistics

Single comparisons between responses in aortae from STZ-treated and control rats were made by Student's unpaired *t* test. Values shown are means $\pm$ s.e.mean. Parallel shifts and EC_{50} s were calculated by regression analysis of the linear portions of the log concentration-response curves. Multiple comparisons between aortae from STZ-treated and control rats were analyzed for interactions using two way analysis of

variance (ANOVA) on the CLR ANOVA package (Apple Macintosh), and a series of 4 one-way ANOVAs for individual comparisons. In all cases, statistical significance is indicated by $P < 0.05$.

Results

Table 1 shows rat body weights and dry weights of aortic rings. There was no significant difference in pre-injection weights of STZ-treated and control rats. However, 1 and 2 weeks post-injection, STZ-treated rats showed a significant decrease in body weight relative to control rats, and also relative to their pre-injection weight (ANOVA, $P < 0.05$). There was no significant difference between aortic ring weights of STZ-treated and control rats with or without endothelial cells.

Vasoconstrictor responses to endothelin-1 (ET-1)

Cumulative concentration-response curves were obtained to ET-1 (0.13 nM–18.00 nM) in aortic rings from STZ-treated and control rats, with and without endothelial cells (Figure 1a, b). Maximum responses of aortae from STZ-treated rats, with or without endothelial cells, were reduced relative to those of aortae from controls (ANOVA, $P < 0.05$, Table 2). There was

Figure 1 Responses of rat aortic rings to endothelin-1 ($n = 7$). (a) Streptozotocin (STZ)-treated ($\square$ — $\square$ normal calcium; $\square$ --- $\square$ calcium-free) and control ($\circ$ — $\circ$ normal calcium; $\circ$ --- $\circ$ calcium-free) with endothelium. (b) STZ-treated ($\blacksquare$ — $\blacksquare$ normal calcium; $\blacksquare$ --- $\blacksquare$ calcium-free) and control ($\bullet$ — $\bullet$ normal calcium; $\bullet$ --- $\bullet$ calcium-free) without endothelium.

Table 1 Body weights and aortic ring dry weights of control and 14-day streptozotocin (STZ)-treated rats

	Animal weights (g)			Aortic ring dry weights (mg)	
	Initial	1 week	2 weeks	With EC	Without EC
Control ($n = 43$)	338 ± 3	$369 \pm 4^*$	$378 \pm 3^*$	1.82 ± 0.03	1.84 ± 0.03
STZ-treated ($n = 43$)	343 ± 4	$315 \pm 3^{**}$	$307 \pm 4^{**}$	1.83 ± 0.03	1.83 ± 0.02

EC = endothelial cells.

Significantly different from control at corresponding time, $*P < 0.05$.

Significantly different from initial weight in same treatment group, $**P < 0.05$.

Table 2 Maximum responses and EC₅₀ values for control (C) and 14-day streptozotocin (STZ)-treated rats, with (+) and without (−) endothelial cells (EC)

	n		C + EC	STZ + EC	C − EC	STZ − EC
ET-1	7	Max response (g)	2.88 ± 0.17	1.93 ± 0.25*	2.81 ± 0.26	1.87 ± 0.36*
		EC ₅₀ (−log M)	8.80 ± 0.20	8.71 ± 0.22	8.95 ± 0.19	8.58 ± 0.06
ET-1 + Indo	5–7	Max response (g)	2.74 ± 0.34	1.90 ± 0.16*	3.66 ± 0.31	2.26 ± 0.12*
		EC ₅₀ (−log M)	8.61 ± 0.22	8.40 ± 0.07	8.71 ± 0.33	8.43 ± 0.12
ET-1 Ca ²⁺ -free	5	Max response (g)	1.00 ± 0.16	0.56 ± 0.04*	1.06 ± 0.17	0.57 ± 0.07*
		EC ₅₀ (−log M)	8.10 ± 0.10	8.15 ± 0.10	8.14 ± 0.09	8.10 ± 0.03
NA	5	Max response (g)	3.12 ± 0.33	2.70 ± 0.36	2.96 ± 0.40	2.86 ± 0.34
		EC ₅₀ (−log M)	6.90 ± 0.15	6.90 ± 0.13	8.37 ± 0.44*	8.17 ± 0.35*
NA Ca ²⁺ -free	5	Max response (g)	0.99 ± 0.15	0.57 ± 0.11*	0.88 ± 0.06	0.51 ± 0.14*
		EC ₅₀ (−log M)	7.20 ± 0.25	7.03 ± 0.24	6.65 ± 0.16	6.86 ± 0.21
KCl	9	Max response (g)	2.03 ± 0.16	1.34 ± 0.12*	2.18 ± 0.17	1.73 ± 0.12**
		EC ₅₀ (−log M)	2.21 ± 0.04	2.27 ± 0.05	2.26 ± 0.06	2.35 ± 0.07
CaCl ₂	5	Max response (g)	3.22 ± 0.15	2.02 ± 0.25*	3.54 ± 0.14	2.32 ± 0.28*
		EC ₅₀ (−log M)	3.30 ± 0.19	2.98 ± 0.14	3.53 ± 0.16	3.17 ± 0.29

Indo = indomethacin; NA = noradrenaline.

Significantly different from corresponding control value, **P* < 0.05.Significantly different from same treatment group with endothelial cells, #*P* < 0.05.

no significant difference between EC₅₀ values for STZ-treated versus controls, with or without endothelial cells (Table 2). As shown in Figure 2a and b, in the presence of the cyclooxygenase inhibitor indomethacin (5 μM), maximum responses to ET-1 of aortic rings from STZ-treated rats, with or without endothelial cells, were significantly reduced compared to control (ANOVA, *P* < 0.05). There were no significant differences in EC₅₀ values between aortae from STZ-treated versus control rats, either with or without endothelial cells (Table 2, with indomethacin). Removal of endothelial cells did not significantly affect maxima or EC₅₀ values of rings from either STZ-treated or control rats.

In calcium-free Krebs solution with 1 mM EGTA, there were significant reductions in maximum responses to ET-1 of aortae from STZ-treated rats, with or without endothelial cells, compared to those of controls (Figure 1a and b, Table 2, ANOVA, *P* < 0.05). Also, in calcium-free Krebs solution with

EGTA, responses to ET-1 in all groups were markedly reduced compared with those obtained in normal Krebs solution.

Vasoconstrictor responses to noradrenaline

Cumulative concentration-response curves were obtained to noradrenaline (NA, 0.1 nM–26.0 μM) in aortic rings, with or without endothelial cells. As shown in Figure 3a and b and Table 2, there was no significant difference between maximum responses of rings from STZ-treated and control rats, with or without endothelium. Removal of endothelium resulted in a parallel shift to the left in concentration-response curves of aortae from STZ-treated and control rats with a corresponding significant change in EC₅₀ values (ANOVA, *P* < 0.05;

Figure 2 Responses of rat aortic rings to endothelin-1, in the presence of 5 μM indomethacin (*n* = 5–7). (a) Streptozotocin (STZ)-treated (□) and control (○) with endothelium. (b) STZ-treated (■) and control (●) without endothelium. **Figure 3** Responses of rat aortic rings to noradrenaline (*n* = 5). (a) Streptozotocin (STZ)-treated (□) normal calcium; (□---□) calcium-free and control (○) normal calcium; (○---○) calcium-free with endothelium. (b) STZ-treated (■) normal calcium; (■---■) calcium-free and control (●) normal calcium; (●---●) calcium-free without endothelium.

Figure 4 Responses of rat aortic rings to KCl ($n = 9$). (a) Streptozotocin (STZ)-treated (□) and control (○) with endothelium. (b) STZ-treated (■) and control (●) without endothelium.

Table 2). Maximum responses to NA in calcium-free Krebs solution with EGTA were significantly reduced in aortic rings from STZ-treated rats compared to those from controls, both with or without endothelial cells (ANOVA, $P < 0.05$; Figure 3a and b, Table 2). Also, in calcium-free Krebs solution with

Figure 5 Responses of rat aortic rings to CaCl_2 ($n = 5$). (a) Streptozotocin (STZ)-treated (□) and control (○) with endothelium. (b) STZ-treated (■) and control (●) without endothelium.

EGTA, responses to NA in all groups were markedly reduced compared to those obtained in normal Krebs solution.

Vasoconstrictor responses to KCl

Cumulative concentration-response curves to KCl (2 mM–20 mM) were obtained. As shown in Figure 4 and Table 2, maximum responses to KCl in aortic rings from STZ-treated animals, with or without endothelial cells, were significantly reduced relative to control (ANOVA, $P < 0.05$). There was no significant difference in EC_{50} values between aortae from STZ-treated and control rats, with or without endothelium (Table 2).

Vasoconstrictor responses to CaCl_2

Cumulative concentration-response curves to CaCl_2 (10 μM –10 mM), in high K^+ (60 mM) Krebs solution, were obtained in aortic rings from STZ-treated and control rats, with or without endothelial cells. Maximum responses of rings from STZ-treated rats were significantly reduced compared to those from control rats, either with or without endothelium (Figure 5 and Table 2, ANOVA, $P < 0.05$). However, there were no differences in EC_{50} values between responses of rings from STZ-treated and control rats, with or without endothelial cells (Table 2).

Discussion

In the present study, maximum responses to ET-1 were reduced in aortic rings from STZ-treated rats when compared to those from control rats. This reduction did not appear to be due solely to alterations in the endothelium, or to altered output of cyclo-oxygenase products and still occurred in the absence of extracellular calcium. Elevated plasma glucose levels in diabetes may cause an increase in secretion of ET. Takahashi *et al.* (1990) found elevated plasma ET levels in patients with diabetes, and elevated glucose levels can release ET (Yamauchi *et al.*, 1990). Such elevation of ET levels may result in homologous down-regulation of receptors (Miasiro & Paiva, 1990). This may account for at least part of the decreased responsiveness to ET-1 found in the present study but further experiments are required to substantiate this hypothesis. Furthermore, it is not known what the effect would be on ET-1 responses of bathing solutions appropriately adjusted for the differences in the composition of the extracellular fluid (including osmolality) of control and STZ-treated rats. Indeed, no such comparative *in vitro* studies with adjusted bathing solutions have been performed (Kiff *et al.*, 1991a). Also, changes in aortic endothelial cell function may not reflect changes in other vascular beds. Indeed Kiff *et al.* (1991b) found regionally selective and agonist-specific vasodilator abnormalities in diabetes, and emphasised the problems associated with *in vitro* studies of aortic ring preparations.

In the present study, the dry weights of aortic rings were not significantly affected by 2 weeks of diabetes. Although other workers have found a small but significant, reduction of aortic weights in diabetes, the diabetes has been of longer duration, and wet rather than dry weights were quoted (Scarborough & Carrier, 1983; 1984; Head *et al.*, 1987).

Results of the present study indicate that the reduced maximum responsiveness to ET-1 of aortic rings from STZ-treated rats was not totally non-specific. Although significantly reduced maximum responsiveness was observed to KCl and CaCl_2 , it did not occur to NA, either in the presence or absence of endothelium. Also, reduced maximum responsiveness to ET-1 in STZ-treated rats was not totally dependent on extracellular calcium. Indeed, in the absence of extracellular calcium, maximum responsiveness to NA was reduced in STZ-treated rats compared to controls, suggesting that in diabetes there may be (at least in some circumstances) an impairment of mobilisation of intracellular calcium stores.

It has been shown that ET-1 stimulates the release of the vasodilator eicosanoid, prostacyclin, from rat mesenteric arteries (Rakugi *et al.*, 1989). However, in the present study indomethacin had no significant effect on EC₅₀s or maximum responses, suggesting that cyclo-oxygenase products do not normally play a major role in modifying constrictor effects of ET-1 in this preparation. The concentration of indomethacin used was greater than that previously shown to inhibit prostaglandin production in aortae from rats (Jeremy *et al.*, 1990).

Increased maximum responsiveness of aortae from STZ-treated rats to NA has been reported previously (Owen & Carrier, 1979 (4–5 week diabetic rats); Scarborough & Carrier, 1983 (8–10 week diabetic rats); Harris & MacLeod, 1988 (3 month diabetic rats)). In the latter study, the altered sensitivity to NA was no longer apparent after removal of endothelial cells. Abebe *et al.* (1990) have recently reported that enhanced responses of arteries from 3 month STZ-treated rats to α -adrenoceptor agonists are mediated by α_1 -adrenoceptors and are largely dependent on the presence of extracellular calcium. It is possible that changes in responsiveness to NA of rat aortae are dependent on the duration of diabetes, since Mulhern & Docherty (1989), using aortae from 2–3 week STZ-treated rats found no change in responsiveness to NA. However, many previous studies have examined the responsiveness of isolated aortae to NA in the presence of a β -adrenoceptor antagonist (Harris & MacLeod, 1988; Mulhern & Docherty, 1989; Abebe *et al.*, 1990), since it is known that

β -adrenoceptors mediate relaxation in rat aortae and that diabetes may change the β -adrenoceptor-mediated response (Kamata *et al.*, 1989). Nevertheless, the present study supports the findings of Mulhern & Docherty (1989), since after 2 weeks of diabetes, aortic responses to NA were not significantly different between STZ-treated and control rats. Results of the present study also support previous reports of decreased maximum responsiveness to KCl of aortae from diabetic rats (Pfaffman *et al.*, 1980; Head *et al.*, 1987) which is not altered by endothelial removal (Wakabayashi *et al.*, 1986).

In the present study, removal of endothelium reduced the EC₅₀ value to NA by factors of 29.5 (2.5, 347, 95% confidence limits, controls) and 18.6 (2.6, 135, 95% confidence limits, diabetics). These shifts in potency are somewhat greater but not significantly so, than those reported by Martin *et al.* (1986) (6 fold, control) and Harris & MacLeod (1988) (4.6 fold, control; 5.6 fold, STZ-treated). The latter study was performed in the presence of a β -adrenoceptor antagonist.

In summary, a decreased responsiveness to endothelin, KCl and CaCl₂ (but not NA) of aortic rings from STZ-treated rats, was observed in the present study. The decreased responsiveness to ET-1 does not appear to be dependent on the presence of endothelial cells, or due to altered output of cyclo-oxygenase products or availability of extracellular calcium.

This work was supported in part by an ASCEP small equipment grant.

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(Received March 22, 1991
Revised July 22, 1991
Accepted July 23, 1991)