The effects of aldose reductase inhibition with ponalrestat on changes in vascular function in streptozotocin diabetic rats

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1 The responses of rat isolated aortae to vasoconstrictor and vasodilator agents have been studied in 14-day streptozotocin-diabetic rats. The effects of treatment with the aldose reductase inhibitor, ponalrestat, on these responses have also been investigated.

2 Maximum contractile responses and aortic sensitivity to phenylephrine were significantly enhanced in 14-day diabetic aortae.

3 In contrast, endothelium-dependent relaxations to carbachol were depressed in diabetic rats, whilst endothelium-independent relaxations to forskolin and sodium nitroprusside were unchanged.

4 Pretreatment with ponalrestat (25 mg kg⁻¹, daily) prevented both the enhanced maximum contractile responses to phenylephrine and the depressed endothelium-dependent relaxations to carbachol in aortae from 14-day diabetic rats. Ponalrestat however, had no effect on the reduced phenylephrine EC₅₀ values observed in tissues from diabetic animals.

5 It is concluded that ponalrestat prevents the depression of endothelium-dependent aortic relaxations induced by diabetes of 14 days duration, suggesting that the polyol pathway is involved in these vascular changes. Ponalrestat does not prevent the increase in aortic sensitivity to α_1 -adrenoceptor agonists.

Keywords: Aldose reductase; diabetes mellitus; aorta; α -adrenoceptor; endothelium-dependent relaxation; nitric oxide; ponalrestat; forskolin

Introduction

Diabetes mellitus is associated with several cardiovascular complications, predisposing the patient to diseases such as hypertension, atherosclerosis and thrombosis (Christlieb *et al.*, 1976). This may be due in part, to altered responsiveness of blood vessels to neurotransmitters and hormones (Weidmann *et al.*, 1979). Studies of vascular α -adrenoceptor responsiveness are contradictory with reports of increased (Agrawal *et al.*, 1987; Taylor *et al.*, 1993), decreased (Turlapaty *et al.*, 1980; Cameron & Cotter, 1992) and unchanged (Fulton *et al.*, 1991) vascular α -adrenoceptor responsiveness and receptor density in diabetes.

In addition to changes in vascular smooth muscle function, endothelial alterations have also been documented. However, results are again contradictory. Several studies report impaired endothelium-dependent relaxation (Kamata *et al.*, 1989; Cameron & Cotter, 1992; Taylor *et al.*, 1993), but there have been reports of unchanged (Head *et al.*, 1987; Mulhern & Docherty, 1989) and also enhanced (White & Carrier, 1986) endothelium-dependent relaxation responses induced by diabetes.

In tissues which are insulin-independent for glucose transport, e.g. endothelium (Dvornik, 1978), hyperglycaemia is known to activate the polyol pathway resulting in sorbitol accumulation (Gabbay, 1973). Aldose reductase, the first enzyme in this pathway, has been identified in many tissues susceptible to damage in diabetes, and inhibitors of this enzyme have been shown to be effective in the prevention of diabetic neuropathy (Carrington *et al.*, 1991), reduction of diabetic albuminuria and retinal complications (McCaleb *et al.*, 1991) and reversal of diabetic cataracts (Beyer-Mears & Cruz, 1985).

Few studies have examined the effects of aldose reductase inhibition on diabetes-induced alterations of the cardiovascular system. Ponalrestat has been shown to prevent the decrease in muscarinic receptor density in diabetic rat atria (Kofo-Abayomi & Lucas, 1987) and we have also found this compound prevents the diabetes-induced changes in cardiac

β -adrenoceptor sensitivity and receptor density (Austin & Chess-Williams, 1991). In vascular tissue, Cameron & Cotter (1992) have shown prevention of impaired endothelium-dependent relaxation with nitromethane but Taylor *et al.* (1994) have recently reported that ponalrestat treatment does not prevent the depression of endothelium-mediated relaxations of mesenteric arteries from diabetic rats.

In man endothelium-dependent relaxations are depressed (Saenz de Tejada *et al.*, 1989; McVeigh *et al.*, 1992) and we have observed a similar depression in aortae from 14 day streptozotocin diabetic rats. The present study investigates the effects of treatment with ponalrestat on these endothelium-dependent relaxations in diabetic and control rats.

Methods

Pretreatment of animals

Male Wistar rats (230–270 g) were given a single intraperitoneal injection of streptozotocin (STZ, 65 mg kg⁻¹) dissolved in 0.01 M citrate buffer (pH 4.5). Age-matched control rats received citrate buffer alone. Animals were fed normal rat chow and water *ad libitum*. Blood glucose was measured with glucose-sensitive BM glycaemie 1-44 test strips (Boehringer Mannheim) and a Glucochek S.C. (Medistron Limited). STZ-treated animals with blood glucose levels greater than 15 mM were considered diabetic and used in the study. Animals to be pretreated with ponalrestat (Statil, I.C.I.) received the initial intraperitoneal injection of streptozotocin or vehicle and the following day the animals were given an oral dose of ponalrestat (25 mg kg⁻¹). Oral dosing via gavage was continued daily for 2 weeks.

Fourteen days after the induction of diabetes, animals were killed by exsanguination, thoracic aortae rapidly excised, and the adherent fat and connective tissue removed. Segments, approximately 3 mm in length were cut from the aortae and carefully cut longitudinally to form strips. The strips were then mounted in 30 ml organ baths containing Krebs-bicarbonate solution (composition in mM: NaCl 118.4, KCl

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4.7, NaHCO₃ 25, glucose 11.7, MgSO₄ 1.2, KHPO₄ 1.2 and CaCl₂ 1.9) and gassed with 5% CO₂ in O₂ at 37°C. Isometric tension developed by the circular smooth muscle was recorded via Lectromed UF1 force transducers on a Lectromed M19 chart recorder. Tissues were equilibrated for 60 min under a resting tension of 1 g, with several changes of bathing medium. Cumulative concentration-response curves were then obtained to phenylephrine. Separate tissues were precontracted with phenylephrine (1.1 µM) and relaxation responses obtained to carbachol (0.1–30 µM), forskolin (1 µM) and sodium nitroprusside (300 nM).

Drugs

(–)-Phenylephrine hydrochloride, carbamylcholine chloride, forskolin, sodium nitroprusside and streptozotocin were

obtained from Sigma (St. Louis, U.S.A.). Ponalrestat (I.C.I 128436, Stalil; MK538, prodiar) was a gift from Zeneca pharmaceuticals, Macclesfield, Cheshire.

Statistics

Drug-induced changes in tension were plotted as a percentage of the maximum change for each concentration-response curve. Individual EC₅₀ values (molar concentration producing half-maximal response) were determined and geometric mean EC₅₀ values with 95% confidence limits calculated. Differences in mean EC₅₀ values were analysed by Analysis of Variance and Student's modified *t* test (Newman-Keuls) applied to individual logarithmic values. Responses were also plotted as a percentage of the precontraction to phenylephrine. Differences in mean responses were analysed by Analysis of Variance and Student's modified *t* test. Differences with a *P* value of 0.05 or less were considered significant.

Results

The pretreatment of animals with streptozotocin resulted in the development of symptoms characteristic of diabetes i.e. polydipsia, polyphagia and polyuria. In addition, rats suffered a significant weight loss (18.3 ± 3.4 g) over the 14 day period compared with a weight gain of 60.5 ± 7.9 g for control animals. Blood glucose levels were also elevated (> 16 mM) relative to control animals (8.1 ± 1.1 mM). Treatment of diabetic rats with ponalrestat did not alleviate weight loss (23.3 ± 9.3 g) or the rise in blood glucose levels (> 18 mM). Ponalrestat treatment of control animals did not affect weight gain (66.0 ± 3.8 g) or blood glucose levels (9.4 ± 0.4 mM) over 14 days.

Contractile responses of isolated aortae

Diabetes of 14-days duration enhanced α₁-adrenoceptor-mediated responses of aortae to phenylephrine. The maximum developed tensions were significantly (*P* < 0.01) increased in diabetic aortae, whilst concentration-response curves were shifted to the left, resulting in significantly (*P* < 0.001) reduced EC₅₀ values (Table 1, Figure 1).

Ponalrestat treatment of control animals did not affect responses to phenylephrine, but ponalrestat treatment of diabetic animals significantly (*P* < 0.05) reduced the maximum tensions developed to phenylephrine (Table 1, Figure 1). The values for these ponalrestat-treated diabetic animals were then not significantly different from values for control or ponalrestat-treated control rats. However, the diabetes-induced reduction in phenylephrine EC₅₀ values was not prevented by ponalrestat and remained significantly reduced in diabetic animals compared with values for control or ponalrestat-treated control animals.

Figure 1 Mean ($n \geq 8$) cumulative concentration-response curves to phenylephrine of aortae from non-diabetic (○), diabetic (●), ponalrestat-treated non-diabetic (△) and ponalrestat-treated diabetic animals (▲). Responses are plotted as (a) mean responses as a percentage of individual maximum responses and (b) the mean tensions developed ($\pm$ s.e.mean) for each concentration of phenylephrine.

Table 1 Responses of aortae to phenylephrine and carbachol

	Control	Diabetic	Ponalrestat-treated control	Ponalrestat-treated diabetic
Phenylephrine				
Maximum developed tension (g)	0.47 ± 0.06	0.70 ± 0.05**	0.43 ± 0.04	0.52 ± 0.04*
Mean EC ₅₀ (nM)	401 (161–995)	10.0*** (4.0–2.1)	214.9 (60.0–768.9)	45.9** (35.1–60.2)
Carbachol				
Maximum relaxation (g)	0.43 ± 0.03	0.29 ± 0.02***	0.38 ± 0.03	0.38 ± 0.06
Maximum relaxation (%)	78.2 ± 4.4	60.2 ± 4.3**	76.1 ± 7.4	76.3 ± 5.6*
Mean EC ₅₀ (µM)	0.89 (0.61–1.29)	1.31 (1.05–1.64)	1.20 (0.82–1.88)	1.25 (0.80–1.80)

Geometric mean ($n \geq 8$) EC₅₀ values (with 95% confidence limits) and maximum responses ($\pm$ s.e.mean) to phenylephrine and carbachol. Relaxation responses to carbachol were recorded following precontraction with 1.1 µM phenylephrine. Responses are expressed as the maximal relaxation in g and also as a percentage of the precontraction to phenylephrine.

P* < 0.05 relative to diabetics. *P* < 0.01; *** *P* < 0.001 relative to controls.

Relaxation responses of isolated aortae

In tissues precontracted with phenylephrine, carbachol induced concentration-related relaxations which were dependent on the presence of an intact endothelium. In aortae from diabetic animals, the relaxation responses were depressed compared with controls (Figure 2a). Maximum relaxation responses were significantly ($P < 0.001$) reduced in diabetics compared with controls (Table 1) and this reduction was still observed when responses were expressed as a percentage of the phenylephrine precontraction ($P < 0.01$) (Figure 2b). Carbachol EC_{50} values were not significantly different between control and diabetic animals (Table 1).

Maximal relaxations obtained to forskolin ($1 \mu\text{M}$) were significantly ($P < 0.01$) greater in aortae from diabetic animals compared with controls (Table 2). However, this was due to the larger precontraction obtained to phenylephrine in aortae from diabetic animals ($0.65 \pm 0.1 \text{ g}$) compared with controls ($0.43 \pm 0.1 \text{ g}$). When responses were expressed as a

Figure 2 Mean ($n \geq 4$) cumulative concentration-response curves to carbachol of aortae from non-diabetic (○), diabetic (●), ponalrestat-treated non-diabetic (△) and ponalrestat-treated diabetic animals (▲). Responses are plotted as (a) relaxation in g, and (b) a percentage of the phenylephrine precontraction.

percentage of the precontraction, responses to forskolin were similar in control and diabetic tissues (Table 2). Similar results were obtained with nitroprusside (300 nM), maximum responses to this agent being similar in aortae from control and diabetic animals when responses were expressed as a percentage of the precontraction to phenylephrine (Table 2).

Relaxation responses to carbachol, whether expressed in absolute tension or as a percentage of the precontraction to phenylephrine, were similar in tissues from ponalrestat-treated control and ponalrestat-treated diabetic animals (Figure 2, Table 1). The values for ponalrestat-treated animals, both diabetic and non-diabetic, were also similar to those for untreated control animals. However, maximum relaxations to carbachol of tissues from ponalrestat-treated diabetic rats were significantly greater than those for tissues from untreated diabetic animals (Table 1). Ponalrestat treatment did not affect carbachol EC_{50} values in any of the animal groups studied.

Relaxation responses to forskolin and nitroprusside were similar in tissues from ponalrestat-treated control and ponalrestat-treated diabetic rats and these were not significantly different from the values obtained for control non-diabetic and diabetic animals (Figure 2).

Discussion

In the present study aortae from rats with 14-day streptozotocin-induced diabetes demonstrated enhanced α -adrenoceptor-mediated vasoconstrictions and depressed endothelium-dependent relaxations. Treatment with ponalrestat prevented some but not all of these vascular changes.

The enhanced vasoconstrictor responses to phenylephrine were exhibited as a change in both sensitivity (decrease in EC_{50}) and responsiveness (increase in maximum response). Ponalrestat prevented the latter but not the former effect suggesting that two mechanisms were operating to increase aortic responses to phenylephrine in diabetes, only one of which involves the polyol pathway. An increase in the maximum responses to noradrenaline and phenylephrine has been reported by other investigators (Agrawal *et al.*, 1987; White & Carrier, 1988). The reduced endothelial-mediated effects observed in diabetic animals may provide an explanation for the effect of diabetes on the maximum responses to phenylephrine. The contractile responses of vascular tissues with intact endothelium can be inhibited by simultaneous nitric oxide release (Martin *et al.*, 1986). If this is reduced in diabetes, as observed in the present study, responses to contractile agents such as phenylephrine may be enhanced. In support of this hypothesis, ponalrestat was found to prevent both the depressed endothelial-mediated relaxation responses to carbachol and the increased maximal responses to phenylephrine. In contrast, the change in sensitivity (decreased EC_{50} values) to phenylephrine in aortae from diabetic rats was not affected by aldose reductase pretreatment, suggesting that a different mechanism may be operating. This suggestion is supported by the report that maximal responses

Table 2 Relaxation responses of aortae to forskolin and nitroprusside

	Control	Diabetic	Ponalrestat-treated control	Ponalrestat-treated diabetic
Forskolin				
Maximum relaxation (g)	0.46 ± 0.04	$0.69 \pm 0.07^{**}$	0.55 ± 0.05	0.62 ± 0.05
Maximum relaxation (%)	111.4 ± 6.2	107.5 ± 1.9	108.28 ± 3.0	113.1 ± 5.4
Nitroprusside				
Maximum relaxation (g)	0.26 ± 0.03	$0.59 \pm 0.09^{***}$	$0.48 \pm 0.05^*$	$0.51 \pm 0.05^*$
Maximum relaxation (%)	101.8 ± 3.9	101.6 ± 4.6	107.34 ± 5.3	105.9 ± 3.1

Geometric mean ($n \geq 7$) EC_{50} values (with 95% confidence limits) and mean maximum responses ($\pm$ s.e.mean) to forskolin ($1 \mu\text{M}$) and nitroprusside (300 nM). Responses are expressed as the relaxation in g and also as a percentage of the initial precontraction to phenylephrine.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ relative to controls.

and sensitivity of isolated aortae to noradrenaline and methoxamine are enhanced in diabetes when tissues have an intact endothelium but in denuded tissues only sensitivity is increased in diabetes (Harris & MacLeod, 1988).

Taylor *et al.* (1994) have also found ponalrestat to be ineffective in preventing the reduction in EC_{50} values for noradrenaline-induced contractile responses of mesenteric arteries from the diabetic rat. Possible mechanisms which may be operating to increase aortic sensitivity to α_1 -adrenoceptor agonists include an alteration in calcium handling as observed in the hearts of diabetic animals (Heygler *et al.*, 1987). The α_1 -adrenoceptor-mediated responses of the rat aorta are at least partially coupled to the influx of extracellular calcium (MacLeod & McNeill, 1985). Alternatively, a diabetes-induced reduction in sympathetic tone to the vasculature may increase vascular α -adrenoceptor density. Such a change in sympathetic nerve activity has been proposed to explain the increase in β -adrenoceptor sensitivity and receptor density observed in 2-week diabetic rat hearts (Austin & Chess-Williams, 1991). The mechanisms involved in the sensitivity changes were not investigated in the present study, but they would appear to be specific for the α -adrenoceptor since previous studies reporting increased α -adrenoceptor responsiveness have found responses to 5-hydroxytryptamine and calcium to be unaltered in diabetic animals (Agrawal *et al.*, 1987; Harris & MacLeod, 1988).

The depressed endothelium-dependent relaxations to carbachol observed in the present study agree with reports from other laboratories using streptozotocin-diabetic rats (Kamata *et al.*, 1989; Cameron & Cotter, 1992) and spontaneously diabetic rats (Durante *et al.*, 1988). In addition, endothelium-dependent relaxations are impaired in human diabetes (Saenz de Tejada *et al.*, 1989; McVeigh *et al.*, 1992). The cause of this depression is unknown but in the present study the relaxation responses of the smooth muscle to forskolin, a direct activator of adenylate cyclase (Seamon & Daley, 1983), were unchanged suggesting that there was no general depression of relaxation responses. Furthermore, responses to nitroprusside, a nitric oxide donor, were not affected by the diabetic state and suggest that the responsiveness of vascular smooth muscle to guanosine 3':5'-cyclic monophosphate (cyclic GMP) was unaffected and the defect in endothelium-dependent relaxations to carbachol exists at the level of nitric oxide synthesis and/or release. The elevation in responses to forskolin and nitroprusside when the data were expressed in absolute values (i.e. g) was solely due to the increased precontraction induced by phenylephrine in tissues from diabetic animals and the effect was not seen when the data were expressed as a percentage of the precontraction values. Relaxation responses to carbachol were depressed in aortae from diabetic animals whichever method was used to express the results.

There have been suggestions that increased production of vasoconstrictor arachidonic acid metabolites is responsible for impaired endothelium-dependent relaxation in diabetes (Tefsamariam *et al.*, 1990). Although the present study cannot rule this out, a number of reports have shown that blocking the action of these substances in aortae does not prevent impaired endothelium-mediated responses (Pieper & Gross, 1988; Hattori *et al.*, 1991). Furthermore, in our preliminary studies we found that indomethacin (10 μ M) failed to inhibit responses to carbachol, and relaxations were depressed in aorta from diabetic rats even in the presence of

indomethacin (unpublished data). N^G -monomethyl-L-arginine (L-NMMA, 50 μ M) was also found to inhibit responses equally in aortae from control and diabetic animals whilst methylene blue (10 μ M) virtually abolished responses to carbachol in both experimental groups. These data indicate that relaxation responses to carbachol were mediated via nitric oxide.

The most probable explanation for the reduced relaxation responses would appear to be an alteration in the vascular endothelium resulting in reduced synthesis, release and/or breakdown of EDRF (NO). Indeed endothelial damage has been reported in diabetes (Moore *et al.*, 1985), although others have found functional changes without apparent endothelium damage (Hattori *et al.*, 1991). The beneficial effects of ponalrestat on the diabetes-induced changes in relaxation in the present study imply that the polyol pathway is involved in the functional defect, at least in larger capacitance vessels. Recent reports however, suggest that this protective effect may not be exerted on smaller resistance vessels, since ponalrestat treatment did not reverse the diabetes-induced defect in endothelium-dependent relaxations in perfused mesenteric vessels (Taylor *et al.*, 1994). If proved correct, this may limit the usefulness of aldose reductase inhibitors in preventing diabetic neuropathy which, at least in experimental models of diabetes mellitus, has been shown to be preceded by reductions in nerve blood flow (Stevens *et al.*, 1994).

A number of mechanisms may explain the role of the polyol pathway in endothelial dysfunction (see Cameron & Cotter, 1992). When the polyol pathway flux is high, glucose is diverted through the pentose phosphate shunt (Dvornik, 1987), which requires three molecules of ATP for each molecule of glucose (Davidson & Murphy, 1985) and this can produce an ATP deficit. Since agonist-stimulated NO release requires energy (Griffith *et al.*, 1986) nitric oxide production may be limited. Alternatively, free radicals may be involved in the endothelial dysfunction. Reduced glutathione (GSH) and superoxide dismutase (SOD) levels in diabetes have been reported (Loven *et al.*, 1986), possibly caused by reductions in NADPH and NAD^+ due to the increased flux through the polyol pathway. The reduction in GSH and SOD would render cells less able to cope with oxidative stress, as seen in the reports of increased sensitivity to superoxide damage (Pieper & Gross, 1988) and increased accumulation of superoxide anions in diabetes (Hattori *et al.*, 1991). Superoxide ions break down nitric oxide (Gryglewski *et al.*, 1986) and it has been reported that SOD can restore impaired endothelium-dependent relaxation to normal (Hattori *et al.*, 1991). In our laboratory however, SOD potentiates carbachol relaxations in both diabetic and control tissues. Relaxations to carbachol, even when experiments were performed in the presence of SOD (30 units ml^{-1}), were still significantly reduced in aortae from diabetic rats ($92.7 \pm 8.0\%$ of the precontraction) compared to controls ($121.0 \pm 8.8\%$).

In conclusion, the present study demonstrates enhanced α -adrenoceptor contractile responses and depressed endothelium-dependent relaxations in aortae from 14 day streptozotocin diabetic rats. The increase in vascular α_1 -adrenoceptor sensitivity was independent of the change in endothelial function. Ponalrestat proved to be beneficial in the prevention of these functional changes implicating the involvement of the polyol pathway in diabetes-induced alterations in endothelium-dependent relaxations.

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