

Impairment of endothelium-dependent relaxation and changes in levels of cyclic GMP in aorta from streptozotocin-induced diabetic rats

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- 1 Acetylcholine (ACh)-induced relaxation of aortic strips with endothelium and production of cyclic GMP between streptozotocin-induced diabetic and age-matched control rats were compared.
- 2 The concentration-response curve for ACh-induced relaxation was shifted to the right in diabetic rats. IC₅₀ values for ACh were $4.57 \pm 0.67 \times 10^{-8}$ M and $1.00 \pm 0.87 \times 10^{-7}$ M in aortic strips from age-matched control and diabetic rats, respectively ($n = 6$, $P < 0.05$).
- 3 Relaxations produced by atrial natriuretic peptide (ANP) in diabetic aortae were similar to those in age-matched vessels.
- 4 Relaxations produced by sodium nitroprusside (SNP) in diabetic aortae were similar to those in age-matched vessels.
- 5 Basal levels of cyclic GMP and ACh-induced production of cyclic GMP were significantly decreased in diabetic rats.
- 6 These results suggest that functional changes in endothelium but not in guanylate cyclase activity in the aorta may occur in diabetes, and, thus, spontaneous and ACh-induced formation of cyclic GMP may be decreased. This decrease in production of cyclic GMP may be responsible for the decreased response of the aorta to the relaxant effect of ACh.

Introduction

It is now well-established that vascular disease is one of the complicating features of diabetes mellitus in man (Christrieb, 1973). It has been suggested that this is in part a consequence of altered sensitivity or reactivity of vascular smooth muscles to neurotransmitters and hormones (Weidmann *et al.*, 1979).

In this respect, the reactivity of vascular smooth muscles to contractile agents in diabetic rats has been extensively studied (Brody & Dixon, 1964; Pfaffman *et al.*, 1982; Scarborough & Carrier, 1984; Agrawal & McNeill, 1987; Head *et al.*, 1987; Kamata *et al.*, 1988a). However, only a few studies have been undertaken to investigate the responses to relaxant agents in diabetic animals.

The discovery that the vascular endothelium plays a vital role in the relaxation of rabbit aorta to acetylcholine (ACh) *in vitro* (Furchgott & Zawadzki, 1980) has led to the realization that many vasodilator agents act through the release of endothelium-derived relaxing factor (EDRF) (Demey & Van-

houtte, 1981). There is general agreement that endothelium-dependent relaxation is associated with increased levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in smooth muscle cells (Rapoport & Murad, 1983; Furchgott *et al.*, 1984; Ignarro *et al.*, 1984).

It has been shown, in diabetic rats that the relaxation response of blood vessels to acetylcholine (ACh) is inconsistent: both decreases (Oyama *et al.*, 1986; Durante *et al.*, 1988) and increases (White & Carrier, 1986) in endothelium-dependent relaxation have been obtained. Even though ACh-induced relaxant responses are mediated through production of cyclic GMP, there have been, until now, no data on changes in levels of cyclic GMP in blood vessels in response to ACh.

Therefore, to determine whether or not there is a causal relationship between changes in relaxation and in production of cyclic GMP, basal levels of cyclic GMP and the effect of ACh on the levels of cyclic nucleotides were examined in age-matched control and streptozotocin-induced diabetic rats.

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Methods

Male Wistar rats, 8 weeks old and 200–220 g in weight, received a single injection in the tail vein of streptozotocin (Stz) 60 mg kg^{-1} , dissolved in a citrate buffer. Age-matched control rats were injected with the buffer alone. Food and water were given *ad libitum*. The concentration of the glucose in plasma was determined by the *o*-toluidine method (Dubowski, 1962).

Eight weeks after treatment with Stz or buffer, rats were killed by decapitation. A section of the thoracic aorta between the aortic arch and the diaphragm was then removed and placed in oxygenated, modified Krebs-Henseleit solution (KHS). The solution consisted of (mM): NaCl 118.0, KCl 4.7, NaHCO_3 25.0, CaCl_2 1.8, NaH_2PO_4 1.2, MgSO_4 1.2, dextrose 11.0. In some experiments, the endothelium of the aortic strips was removed by gentle rubbing of the endothelial surface with a disposable cotton applicator. The aorta was cleaned of loosely adhering fat and connective tissue and cut into helical strips 3 mm in width and 20 mm in length. The tissue was placed in a well-oxygenated (95% O_2 , 5% CO_2) bath of 10 ml KHS at 37°C with one end connected to a tissue holder and other to a force-displacement transducer (Nihon Kohden TB-611T). The tissue was equilibrated for 60 min under a resting tension of 1.0 g. During this time the KHS in the tissue bath was replaced every 20 min.

After equilibration, each aortic strip was contracted by treatment with 10^{-7} M noradrenaline (NA). The presence of functional endothelial cells was confirmed by demonstrating relaxations to 10^{-5} M ACh, and aortic strips in which 85% relaxation occurred were regarded as tissues with endothelium. The removal of endothelial cells by rubbing was confirmed by loss of ACh-induced relaxation. The relaxation responses to ACh, atrial natriuretic peptide (ANP) and sodium nitroprusside (SNP) were expressed as a percentage of the decreased tension of contractile force induced by 10^{-7} M NA. Since the maximal contraction of aortic strips in response to NA was enhanced in diabetic rats (Kamata *et al.*, 1988a), for the relaxation studies, the aortic strips were precontracted with an equieffective concentration of NA (3×10^{-8} – 10^{-7} M). This concentration produced 75–85% of the maximal response. Aortic strips, which were weighed at the end of each experiment, were precontracted with 3×10^{-8} – 10^{-7} M NA so that the strips developed a tension of approximately 90 mg mg^{-1} tissue in both age-matched control and diabetic rats. When the NA-induced contraction reached a plateau, ACh (10^{-8} – 10^{-5} M), ANP (10^{-9} – 10^{-6} M) or SNP (10^{-9} – 10^{-6} M) was added in a cumulative manner. Each aortic strip was exposed only to one relaxant agent.

Measurement of cyclic nucleotides

Basal concentrations of, or drug-induced changes in levels of cyclic nucleotide were measured in a separate series of experiments. Endothelium-intact aortic strips were allowed to equilibrate in tubes that contained KHS, gassed with 95% O_2 , 5% CO_2 , at 37°C for 60 min. After equilibration, the aortic strips were exposed to 10^{-7} M NA for 10 min. Then a single dose of ACh was added to the tubes. One minute after the addition of the drugs, tissues were frozen in liquid N_2 , and then homogenized in 1 ml of 6% trichloroacetic acid, and centrifuged at $3000g$ for 10 min. The supernatants were extracted three times in three volumes of water-saturated ether, and were then stored at -80°C until assayed for cyclic GMP. Following succinylation, levels of cyclic GMP were determined with radioimmunoassay kits as previously described by Kamata *et al.* (1988b). Protein was determined by the method of Lowry, with bovine serum albumin as standard (Lowry *et al.*, 1951).

Drugs

Streptozotocin, noradrenaline hydrochloride and sodium nitroprusside were purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A.). Acetylcholine chloride was purchased from Daiichi Pharmaceuticals (Tokyo, Japan). Atrial natriuretic peptide was purchased from Peptide Institute (Osaka, Japan). All drugs were dissolved in saline. All concentrations are expressed as final molar concentrations of the base in the organ bath. Radioimmunoassay kits for cyclic AMP and cyclic GMP were purchased from Yamasa Shoyu Co. (Choshi, Japan).

Statistical analysis

The contractile force of aortic strips from age-matched control and diabetic rats is expressed as mg tension mg^{-1} of tissue. Results are expressed as mean \pm s.e. Significance was tested by the unpaired *t* test and considered significant if $P < 0.05$.

Results

Eight to ten weeks after treatment with Stz, the concentration of glucose in plasma was elevated significantly, from $113.6 \pm 2.9 (\text{mg dl}^{-1})$ in age-matched controls to $755.5 \pm 44.0 (\text{mg dl}^{-1})$ in diabetic rats, respectively ($n = 20$, $P < 0.01$).

When aortic strips were precontracted with NA, ACh relaxed the strips in a concentration-dependent manner. However, the concentration-response curve

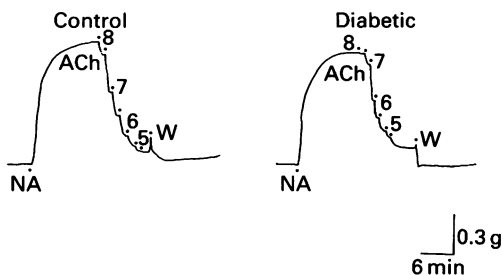


Figure 1 Relaxation responses to acetylcholine (ACh) of aortic strips with endothelium from age-matched control and diabetic rats. The aortic strips were precontracted with 3×10^{-8} – 10^{-7} M noradrenaline (NA).

for ACh was shifted to the right in diabetic rats (Figure 1).

IC_{50} values for ACh were $4.57 \pm 0.67 \times 10^{-8}$ M and $1.00 \pm 0.87 \times 10^{-7}$ M in aortic strips from age-matched control and diabetic rats, respectively ($n = 6$, $P < 0.05$). In contrast to the effects of ACh, the concentration-dependent relaxation produced by ANP and SNP in diabetic rat aorta without endothelium was not different from the controls (Table 1).

Basal concentrations of cyclic GMP in aortic strips with endothelial cells were lower in strips from diabetic rats than in those from age-matched control rats (Table 2).

In both age-matched control and diabetic rats, ACh increased levels of cyclic GMP in isolated aortic strips with endothelium. The increase in levels

of cyclic GMP (measured 1 min after addition of ACh) was greater in strips from age-matched control rats than in those from diabetic rats (Table 2). Basal levels of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in diabetic vessels were similar to those in age-matched control vessels (data not shown).

Discussion

It has been shown previously that the relaxation response of isolated aortic strips to ACh from spontaneously hypertensive rats and New Zealand hypertensive rats is reduced when compared to that in strips from age-matched, normotensive, Wistar Kyoto rats (Cheng & Shibata, 1981; Konishi & Su, 1983; Winquist *et al.* 1984a). In the present study, a similar result was observed in diabetic rats. However, the concentration-response curves for the relaxant effects of ANP and SNP, both endothelium-independent agents (Rapoport & Murad, 1983; Winquist *et al.*, 1984b), in aortic strips from diabetic rats, showed no shift and no depression of the maximal response. It is likely that the decrease in the relaxant response of the aortic strips with endothelium to ACh may be due to an impairment of endothelial cells but not smooth muscle guanylate cyclase activity in diabetic rats. Our observations are consistent with the findings of Oyama *et al.* (1986). The action of EDRF on blood vessels has been demonstrated to be mediated by increasing rates of formation of cyclic GMP in vascular smooth muscles (Rapoport & Murad, 1983).

Table 1 IC_{50} values for acetylcholine (ACh)-, atrial natriuretic peptide (ANP)- and sodium nitroprusside (SNP)-induced relaxation of aortic strips from age-matched control and diabetic rats

Drugs	Control	Diabetic
ACh	$4.57 \pm 0.67 \times 10^{-8}$ M	$1.00 \pm 0.87 \times 10^{-7}$ M*
ANP	$3.51 \pm 0.83 \times 10^{-9}$ M	$4.53 \pm 0.69 \times 10^{-9}$ M
SNP	$3.46 \pm 0.66 \times 10^{-9}$ M	$4.30 \pm 0.60 \times 10^{-9}$ M

Values are mean \pm s.e.; $n = 6$ animals.

* Statistically different from age-matched control ($P < 0.05$).

Table 2 Basal and acetylcholine (ACh)-induced production of cyclic GMP in aortic strips from age-matched control and diabetic rats

Agents	Control cyclic GMP (pmol mg ⁻¹ protein)	Diabetic cyclic GMP (pmol mg ⁻¹ protein)
None	7.46 ± 2.02	$1.28 \pm 0.72^*$
NA (10^{-7} M)	7.30 ± 0.58	$3.56 \pm 1.13^*$
NA (10^{-7} M) + ACh (10^{-5} M)	43.88 ± 4.50	$21.10 \pm 1.32^{**}$

Values are mean \pm s.e.; $n = 4$ animals. Significantly different from age-matched control, * $P < 0.05$, ** $P < 0.01$. NA = noradrenaline.

In the present study, basal concentrations of cyclic GMP in aortic strips with endothelium were lower in strips from diabetic rats than in those from age-matched control rats. Furthermore, the production of cyclic GMP induced by ACh was also lower in strips from diabetic rats than in those from age-matched control rats. It is most likely, therefore, that decreases in basal and ACh-induced levels of cyclic GMP may be due to an impairment of the endothelium in diabetic rats. Changes in the responsiveness of vascular smooth muscle *in vitro* to ACh have been observed in pathological states, such as hypertension and atherosclerosis. In such cases, release of EDRF might be reduced (Konishi & Su, 1983; Winquist *et al.*, 1984a; Jayakody *et al.*, 1985).

In conclusion, ACh-induced endothelium-dependent relaxation of aortic strips precontracted with NA was significantly attenuated in strips from diabetic rats. Furthermore, basal levels of cyclic GMP and ACh-induced production of cyclic GMP were significantly decreased in strips of aorta from diabetic rats. However, relaxations produced by sodium nitroprusside in aortic strips from diabetic rats were similar to those in age-matched control vessels. This decreased production of cyclic GMP may be responsible for the decreased relaxation of the aorta induced by ACh.

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References

- AGRAWAL, D.K. & McNEILL, J.H. (1987). Vascular responses to agonists in rat mesenteric artery from diabetic rats. *Can. J. Physiol. Pharmacol.*, **65**, 1484–1490.
- BRODY, M.J. & DIXON, R.L. (1964). Vascular reactivity in experimental diabetes mellitus. *Circ. Res.*, **14**, 494–501.
- CHENG, J.B. & SHIBATA, S. (1981). Vascular relaxation in the spontaneously hypertensive rat. *J. Cardiovasc. Pharmacol.*, **3**, 1126–1140.
- CHRISTRIEB, A.R. (1973). Diabetes and hypertensive vascular disease. Mechanism and treatment. *Am. J. Cardiol.*, **32**, 592–606.
- DEMEY, J.G. & VANHOUTTE, P.M. (1981). Role of the intima in cholinergic and purinergic relaxation of isolated canine femoral arteries. *J. Physiol. (Lond.)*, **316**, 347–355.
- DUBOWSKI, K.M. (1962). An *o*-toluidine method for body-fluid glucose determination. *Clin. Chem.*, **8**, 215–235.
- 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.
- FURCHGOTT, R.F., CHERRY, P.D., ZAWADZKI, J.V. & JON-ARHIANANDAN, D. (1984). Endothelial cells as mediators of vasodilation of arteries. *J. Cardiovasc. Pharmacol.*, **6**, suppl. 2, s336–343.
- FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature (Lond.)*, **288**, 373–376.
- HEAD, R.J., LONGHURST, P.A., PANEK, R.L. & STIZZEL, R.E. (1987). A contrasting effect of the diabetic state upon the contractile responses of aortic preparations from rat and rabbit. *Br. J. Pharmacol.*, **91**, 275–286.
- IGNARRO, L.J., BURKE, T.M., WOOD, K.S., WALIN, M.S. & KADOWITZ, P.J. (1984). Association between cyclic GMP accumulation and acetylcholine-elicited relaxation of bovine intrapulmonary artery. *J. Pharmacol. Exp. Ther.*, **228**, 682–690.
- JAYAKODY, L., SENARATNE, M.P.J., THOMSON, A.B.R. & KAPPAGODA, T. (1985). Cholesterol feeding impairs endothelium-dependent relaxation of rabbit aorta. *Can. J. Physiol. Pharmacol.*, **63**, 1206–1209.
- KAMATA, K., MIYATA, N. & KASUYA, Y. (1988a). Mechanisms of increased responses of the aorta to α -adrenoceptor agonists in streptozotocin-induced diabetic rats. *J. Pharmacobiodyn.*, **11**, 707–713.
- KAMATA, K., SAKAMOTO, A. & KASUYA, Y. (1988b). Similarities between the relaxations induced by vasoactive intestinal peptide and stimulation of the non-adrenergic non-cholinergic neurons in the rat stomach. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **338**, 401–406.
- KONISHI, M. & SU, C. (1983). Role of endothelium in dilator responses of spontaneously hypertensive rat arteries. *Hypertension*, **5**, 881–886.
- LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
- OYAMA, Y., KAWASAKI, H., HATTORI, Y. & KANNO, M. (1986). Attenuation of endothelium-dependent relaxation in aorta from diabetic rats. *Eur. J. Pharmacol.*, **132**, 75–78.
- PFAFFMAN, M.A., BALL, C.R., DARBY, A. & HILMAN, R. (1982). Insulin reversal of diabetes-induced inhibition of vascular contractility in the rat. *Am. J. Physiol.*, **242**, H490–H495.
- RAPOPORT, R.M. & MURAD, F. (1983). Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through c GMP. *Circ. Res.*, **52**, 352–357.
- SCARBOROUGH, N.L. & CARRIER, G.O. (1984). Nifedipine and α_2 -adrenoceptors in rat aorta. II Role of extracellular calcium in enhanced α_2 -adrenoceptor-mediated contraction in diabetes. *J. Pharmacol. Exp. Ther.*, **236**, 603–609.
- WEIDMANN, P., BERRETTA, P., COLLI, C., KEUSCH, G., GLUCK, Z., MUJAGIC, M., GRIMM, M., MEIYER, A. & ZEIGLER, W.H. (1979). Sodium-volume factor, cardiovascular reactivity and hypotensive mechanism of diuretic therapy in mild hypertension associated with diabetes mellitus. *Am. J. Med.*, **67**, 779–784.
- WHITE, R.E. & CARRIER, G.O. (1986). Supersensitivity and endothelium dependency of histamine-induced relaxation in mesenteric arteries isolated from diabetic rats. *Pharmacology*, **33**, 34–38.

WINQUIST, R.J., BUNTING, P.B., BANSKIN, E.P. & WALLACE, A.A. (1984a). Decreased endothelium-dependent relaxation in New Zealand genetic hypertensive rats. *J. Hypertension*, **2**, 541-545.

WINQUIST, R.J., FAISON, E.P., WALDMAN, S.A., SCHWARTZ, K., MURAD, F. & RAPOPORT, R.M. (1984b).

Atrial natriuretic factor elicits an endothelium-independent relaxation and activates particulate guanylate cyclase in vascular smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 7661-7664.

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