Effect of diabetes and elevated glucose on nitric oxide-mediated neurotransmission in rat anococcygeus muscle

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1 Nitric oxide (NO)-mediated neurotransmission is impaired in anococcygeus muscle from 8-week streptozotocin-induced diabetic rats. This study investigated the effects of insulin treatment, and the duration of diabetes on this impairment. In addition, the effect of *in vitro* exposure to elevated glucose has been investigated on NO-mediated relaxations, in muscles from untreated rats.

2 Relaxant responses to field stimulation (0.5–5 Hz, 10 s train), sodium nitroprusside (SNP; 5 and 10 nM) and NO (1 and 3 μ M) were significantly impaired in anococcygeus muscles from 8-week diabetic rats, compared to responses from control rats. Insulin treatment (5 u Lente day⁻¹, s.c.) of diabetic rats prevented the development of this impairment.

3 Consistent with findings in 8-week diabetic rats, relaxations induced by field stimulation, SNP and NO were attenuated in tissues from 2-week and 4-week diabetic rats compared to corresponding control responses, whereas relaxations to papaverine (3 and 10 μ M) were not reduced. In contrast, diabetes of 3-days duration did not affect relaxations to field stimulation, SNP or NO.

4 Incubation of anococcygeus muscles from untreated rats in medium containing elevated glucose (44.1 mM) for 6 h, significantly impaired relaxations to field stimulation compared to responses obtained after normal glucose (11.1 mM) incubation. Relaxations to SNP and to NO were not affected by 6 h exposure to elevated glucose. Similarly, incubation in hyperosmolar solutions containing mannose or sucrose for 6 h, impaired relaxations to field stimulation, but not to SNP or NO.

5 The results indicate that the diabetes-induced impairment of NO-mediated neurotransmission in the rat anococcygeus muscle develops between 3 days and 2 weeks after the induction of diabetes with streptozotocin. Prevention of the impairment by insulin treatment suggests that it is specific for the diabetic state. In addition, the impairment may be related to hyperglycaemia and the consequent rise in osmolarity, since *in vitro* exposure to high glucose as well as to other hyperosmolar media impaired NO-mediated relaxations to field stimulation.

Keywords: Anococcygeus muscle (rat); diabetes; elevated glucose medium; insulin; nitrergic transmission; nitric oxide (NO); nonvascular smooth muscle; sodium nitroprusside; streptozotocin

Introduction

Dysfunction of the autonomic nervous system is an important complication of diabetes mellitus contributing to alterations in cardiovascular, gastrointestinal and genitourinary function (Hosking *et al.*, 1978; Clarke *et al.*, 1979). Morphological, histological and functional alterations in autonomic neurotransmission have been reported for noradrenergic, cholinergic, 5-hydroxytryptaminergic, peptidergic and purinergic nerves (Lincoln *et al.*, 1984; MacLeod & McNeill, 1985; Longhurst & Belis, 1986; Nowak *et al.*, 1986; Belai *et al.*, 1985; 1988; Mathison & Davison, 1988), using experimental animal models of diabetes mellitus, in particular the streptozotocin (STZ) diabetic rat. However, few studies have examined the effects of the diabetic state on nonadrenergic noncholinergic (NANC) inhibitory neurotransmission mediated by nitric oxide (NO), or nitrergic neurotransmission. We have recently shown that nitrergic neurotransmission is attenuated in the anococcygeus muscle from diabetic rats 8 weeks after treatment with STZ (Way & Reid, 1994a,b). Relaxant responses to nitrergic nerve stimulation, sodium nitroprusside (SNP) and NO were impaired, whereas responses to papaverine, which does not act through NO, were not (Way & Reid, 1994a). In addition, relaxations to the cell permeable analogue of guanosine 3':5'-cyclic

monophosphate (cyclic GMP), 8-bromo-cyclic GMP, were attenuated in tissues from diabetic rats (Way & Reid, 1994b). Together these results suggest that diabetes impairs reactivity to NO in anococcygeus smooth muscle, at least partly through alterations in the cyclic GMP-relaxation pathway.

In the present study we investigate further the specificity and the development of this impaired nitrergic neurotransmission in diabetes. Firstly, the effects of insulin treatment on NO-mediated relaxations have been examined in muscles from 8-week STZ-treated rats, to assess whether the impairment of responses is specific for the production of a diabetic state and not due to any non-specific effect of STZ. Secondly, the effects of diabetes durations shorter than 8 weeks on smooth muscle responsiveness have been determined to assess the development of changes in nitrergic neurotransmission; relaxant responses were examined in muscles from rats at 3 days, 2 weeks and 4 weeks after treatment. Finally, in a separate series of experiments, anococcygeus muscles isolated from untreated rats were exposed to medium containing elevated glucose, to determine the direct effects of an elevated-glucose environment *per se* on NO-mediated relaxations in rat anococcygeus muscle.

Methods

Induction of diabetes

Diabetes was induced in male Sprague-Dawley rats (200–250 g) by a single injection of STZ (65 mg kg⁻¹, i.v.).

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Age-matched control rats were treated with citrate-buffer vehicle alone (20 mM, pH 4.5, i.v.). All rats were placed under light anaesthesia before injection (pentobarbitone 40 mg kg⁻¹, i.p.). Food and water were available *ad libitum*, however for 48 h after treatment the drinking water for STZ-treated rats contained 2% sucrose to help reduce the severity of the hypoglycaemia following STZ administration. Thereafter, rats were monitored for glucosuria (Tes-Tape urine sugar-analysis paper; Eli Lilly, Australia) and for changes in body weight. Blood samples were collected from all animals after decapitation for measurement of blood glucose levels (Ames Glucometer II; Miles Laboratories Inc., Australia).

Insulin treatment

Insulin (5 u day⁻¹ Lente MC, s.c.) was administered to a separate group of STZ-treated rats, between 09 h 00 min–12 h 00 min for 8 weeks, except on the day of the experiment. Treatment commenced 48 h after STZ injection when animals displayed glucosuria and hyperglycaemia. Blood samples were obtained from the tail vein every 2–4 days for measurement of blood glucose levels; samples were collected approximately 8 h after insulin injection.

Tissue preparation

At specific time periods after treatment, rats were killed by stunning with a blow to the head followed by decapitation. Anococcygeus muscles were then isolated according to the method described by Gillespie (1972). After exposure, the colon was cut at the pelvic brim and the pelvic portion pulled forward to reveal two underlying muscles surrounded by connective tissue. A maximal length of muscle was obtained and mounted under 1 g tension in a 5 ml organ bath for isometric recording, using a Grass FT03 C force displacement transducer and a Rikadenki potentiometric recorder. Muscles were bathed in physiological salt solution (PSS) of the following composition (mM; pH 7.4): NaCl 118, KCl 4.7, CaCl₂ 2.5, NaHCO₃ 25, KH₂PO₄ 1.03, MgSO₄ 0.45, D-(+)-glucose 11.1, disodium edetate 0.067, and ascorbic acid 0.14. The PSS was maintained at 37°C and gassed continuously with 95% O₂/5% CO₂. Field stimulation was delivered from a Grass S88 stimulator through two platinum wire electrodes, one on either side of the tissue with square wave pulses of 0.8 ms duration and supramaximal voltage (17 V cm⁻¹).

Relaxant responses

After a 20 min equilibration period, guanethidine (10–30 µM) and clonidine (0.01–0.05 µM) were used to block noradrenergically-mediated contractions and to raise tissue tone. Relaxant responses were obtained to increasing frequencies of field stimulation (0.5–5 Hz, 10 s train) at 3 min intervals, or to SNP (5 and 10 nM) and NO (1 and 3 µM), or to papaverine (3 and 10 µM). In some experiments relaxant responses were obtained in PSS containing 30 mM D-(+)-glucose.

Elevated glucose incubation of tissues from untreated rats

In a separate series of experiments, anococcygeus muscles isolated from untreated rats (450–500 g) were mounted under 1 g tension for isometric recording, and incubated for 6 h in PSS containing normal (11.1 mM) or elevated glucose (44.1 mM); mannose or sucrose (33 mM plus 11.1 mM glucose) were used as hyperosmolar controls. After incubation, relaxations to field stimulation (0.5–5 Hz, 10 s train), SNP (5 and 10 nM) and NO (1 and 3 µM) were obtained in the continued presence of normal or elevated glucose.

Drugs and drug solutions

Guanethidine sulphate, papaverine hydrochloride, sodium nitroprusside and streptozotocin were all purchased from Sigma Chemical Co. Ltd. (U.S.A.). Clonidine hydrochloride and pentobarbitone sodium were obtained from Boehringer Ingelheim (Australia), and insulin zinc suspension (Lente MC) from Commonwealth Serum Laboratories-Novo (Australia). A saturated solution of NO (2 mM) was prepared daily by a modification of the method described by Feelisch (1991). In brief, distilled water initially deoxygenated by gassing with argon (1 h), was bubbled with NO gas (Commonwealth Industrial Gases, Australia) for 30 min.

Statistical analysis

Results are expressed as mean ± s.e. mean and *n* indicates the number of animals tested. Differences between means were assessed with Student's *t* test or by one- or two-way analysis of variance (ANOVA). Analyses were performed using the computer package, Complete Statistical System (Statsoft, U.S.A.). Probability levels less than 0.05 were considered significant.

Results

Assessment of diabetes

At 3 days and 2, 4 and 8 weeks after injection, blood glucose levels of all STZ-treated rats were significantly greater than levels for the corresponding vehicle-treated groups (Table 1; *P* < 0.05, Student's unpaired *t* test or one-way ANOVA). Furthermore, increases in body weight of all STZ-treated rats were significantly less than those of vehicle-treated rats of the same duration (Table 1; *P* < 0.05, Student's unpaired *t* test or one-way ANOVA). Successful induction of diabetes was also confirmed in the STZ-treated groups by the presence of glucosuria, polyuria and polydipsia.

Insulin treatment of 8-week diabetic rats prevented the reduction in growth rate, and the increase in body weight was not different from that for vehicle-treated rats of the same duration, but was significantly greater than that for the corresponding STZ-treated group (Table 1; *P* < 0.05, one-way ANOVA). Blood glucose levels of insulin-treated rats were significantly less than those of STZ-treated animals (Table 1; *P* < 0.05, one-way ANOVA); however, as rats were not administered insulin on the morning of the experiment and as final blood samples were therefore obtained approximately 24 h after insulin injection, blood glucose levels of

Table 1 Blood glucose levels and body weights of vehicle- and streptozotocin (STZ)-treated rats at different time periods after injection

Duration and treatment	Blood glucose (mM)	Body weight (% initial weight)
3 day – Vehicle	8.2 ± 0.2 (6)	106.9 ± 1.1 (6)
– STZ	22.1 ± 0.9 (6)*	99.2 ± 1.4 (6)*
2 week – Vehicle	7.8 ± 0.1 (33)	138.3 ± 1.8 (40)
– STZ	29.4 ± 0.7 (31)*	115.5 ± 1.9 (33)*
4 week – Vehicle	6.7 ± 0.2 (19)	166.3 ± 4.1 (21)
– STZ	29.2 ± 0.7 (20)*	124.7 ± 4.3 (21)*
8 week – Vehicle	7.8 ± 0.1 (8)	249.7 ± 8.7 (8)
– STZ	26.3 ± 1.7 (8)*	129.9 ± 5.1 (8)*
– STZ plus insulin	14.5 ± 0.6 (6)*	240.2 ± 9.8 (6)

Values are mean ± s.e. mean for the number of animals tested indicated in parentheses. *Significant difference from the corresponding vehicle-treated group (*P* < 0.05, Student's unpaired *t* test, and one-way ANOVA for 8-week treatment group).

insulin-treated rats remained significantly greater than control values (Table 1; $P < 0.05$, one-way ANOVA). Regular blood glucose measurements of insulin-treated rats demonstrated that levels remained consistently below 10 mM approximately 8 h after insulin injection (e.g. 8.2 ± 0.9 mM, $n = 6$, taken 42 days after induction of diabetes).

Effect of insulin treatment on relaxant responses

Consistent with our previous findings (Way & Reid, 1994a,b), relaxations to field stimulation (0.5–5 Hz, 10 s train), SNP (5 and 10 nM) and NO (1 and 3 μ M) in muscles from diabetic rats were significantly reduced, compared to responses obtained from control tissues (Figures 1–3; $P < 0.05$, two-way ANOVA). These reductions were completely prevented by insulin treatment; relaxant responses in tissues from diabetic rats which received insulin, were not significantly different from the corresponding control values (Figures 1–3; $P > 0.05$, two-way ANOVA).

In a separate series of experiments, relaxant responses from both control and diabetic tissues were obtained in the

presence of PSS containing high glucose (30 mM) in order to confirm that the alteration in functioning of diabetic tissue was not due to the acute change in glucose concentration from that present *in vivo* (25–30 mM) to that in PSS *in vitro* (11.1 mM). Relaxant responses to field stimulation (0.5–5 Hz, 10 s train), SNP (5 and 10 nM) and NO (1 and 3 μ M) in muscles from 8-week diabetic rats were still significantly smaller than responses from 8-week control tissues, in PSS containing 30 mM glucose (Table 2; $P < 0.05$, two-way ANOVA). Furthermore, relaxant responses from 8-week control and diabetic tissues obtained in PSS containing 30 mM glucose (Table 2) were not significantly different ($P > 0.05$, two-way ANOVA) from those obtained in PSS containing 11.1 mM glucose ($n = 4–7$; data not shown).

Effect of duration of diabetes on relaxant responses

Relaxant responses to field stimulation (0.5–5 Hz, 10 s train) were significantly smaller in tissues from 2- and 4-week diabetic rats than in tissues from corresponding control rats (Figure 4; $P < 0.05$, two-way ANOVA). Relaxations to SNP (5 and 10 nM) and NO (1 and 3 μ M) were also reduced in muscles from 2- and 4-week diabetic rats compared to control responses (Figure 5). However, at 3-days duration, responses to field stimulation, SNP and NO were not

Figure 1 Representative traces showing relaxant responses to field stimulation (Stim; 1 Hz, 10 s train), to sodium nitroprusside (SNP; 5 nM) and to nitric oxide (NO; 1 μ M) in anococcygeus muscles from 8-week control, diabetic and insulin-treated diabetic rats. Tissues were precontracted with guanethidine (10–30 μ M) and clonidine (0.01–0.05 μ M) and *w* indicates tissue washes.

Figure 2 Relaxant responses to field stimulation (0.5–5 Hz, 10 s train) in anococcygeus muscles from 8-week control (O), diabetic (Δ) and insulin-treated diabetic (▲) rats. Results are mean $\pm$ s.e. mean from 6–8 experiments, expressed as reductions in tension produced in tissues precontracted with guanethidine (10–30 μ M) and clonidine (0.01–0.05 μ M).

Figure 3 Relaxant responses to sodium nitroprusside and nitric oxide in anococcygeus muscles from 8-week control (open columns), diabetic (solid columns), and insulin-treated diabetic (cross-hatched columns) rats. Results are mean $\pm$ s.e. mean from 6–8 experiments expressed as reductions in tension produced in tissues precontracted with guanethidine (10–30 μ M) and clonidine (0.01–0.05 μ M). *Significant difference from the corresponding control value ($P < 0.05$, two-way ANOVA).

Table 2 Relaxant responses to field stimulation (0.5–5 Hz, 10 s train), sodium nitroprusside (5 and 10 nM) and nitric oxide (1 and 3 μ M) in anococcygeus muscles from 8-week control and diabetic rats, incubated in physiological salt solution containing 30 mM glucose

		Relaxant response (g)	
		Control	Diabetic
Field stimulation (Hz)	0.5	6.24 $\pm$ 1.29 (5)	0.14 $\pm$ 0.11 (4)*
	1	7.98 $\pm$ 0.75 (5)	0.79 $\pm$ 0.44 (4)*
	2	9.13 $\pm$ 0.78 (5)	2.49 $\pm$ 0.91 (4)*
	5	9.69 $\pm$ 0.65 (5)	4.28 $\pm$ 1.05 (4)*
	10	9.42 $\pm$ 0.79 (5)	1.08 $\pm$ 0.22 (4)*
Sodium nitroprusside (nM)	5	7.05 $\pm$ 0.90 (5)	0.40 $\pm$ 0.12 (4)*
	10	9.42 $\pm$ 0.79 (5)	1.08 $\pm$ 0.22 (4)*
Nitric oxide (μ M)	1	9.07 $\pm$ 1.03 (5)	3.28 $\pm$ 0.83 (4)*
	3	9.56 $\pm$ 0.65 (5)	4.53 $\pm$ 0.91 (4)*

Values are mean $\pm$ s.e. mean for the number of animals tested indicated in parentheses. *Significant difference from the corresponding control value ($P < 0.05$, two-way ANOVA).

Figure 4 Relaxant responses to field stimulation (0.5–5 Hz, 10 s train) in anococcygeus muscles from rats at 3 days (a), 2 weeks (b) and 4 weeks (c) after induction of diabetes. Responses from control (O) and diabetic (Δ) rats are shown as reductions in tension produced in tissues precontracted with guanethidine (10–30 μ M) and clonidine (0.01–0.05 μ M). Values are mean $\pm$ s.e. mean from 6–13 experiments.

significantly different between control and diabetic animals (Figures 4 and 5; $P > 0.05$, two-way ANOVA and Student's unpaired t test).

Relaxant responses to papaverine (3 and 10 μ M) in tissues from diabetic rats of both 2-weeks and 4-weeks duration were not reduced compared to the corresponding control values (Table 3); the response to 3 μ M papaverine in muscles from 4-week diabetic rats was in fact slightly but significantly greater than the response from 4-week control rats (Table 3; $P < 0.05$, Student's unpaired t test).

Effect of exposure to elevated glucose on relaxant responses in tissues from untreated rats

Incubation of anococcygeus muscles from untreated rats in PSS containing high glucose (44.1 mM) for 6 h, significantly impaired relaxant responses to field stimulation (0.5–5 Hz, 10 s train), compared to relaxations obtained in tissues exposed to normal glucose (11.1 mM) for 6 h (Figure 6; $P < 0.05$, two-way ANOVA). Responses to SNP (5 and 10 nM) or to NO (1 and 3 μ M) in the presence of elevated glucose were not significantly different from responses obtained after normal glucose incubation (Figure 7; $P > 0.05$, one-way ANOVA). Similarly, incubation of muscles in hyperosmolar solutions containing mannose or sucrose (33 mM plus 11.1 mM glucose) for 6 h significantly reduced relaxations to field stimulation compared to relaxations in

Figure 5 Relaxant responses to sodium nitroprusside and nitric oxide in anococcygeus muscles obtained at 3 days (a), 2 weeks (b) and 4 weeks (c) after induction of diabetes. Responses from control (open columns) and diabetic (solid columns) rats are shown as reductions in tension produced in tissues precontracted with guanethidine (10–30 μ M) and clonidine (0.01–0.05 μ M). Values are mean $\pm$ s.e. mean from 6–15 experiments. *Significant difference from the corresponding control value ($P < 0.05$, Student's t test).

Table 3 Relaxant responses obtained to papaverine (3 and 10 μ M) in anococcygeus muscles from control and diabetic rats at 2 and 4 weeks after the induction of diabetes

Duration and group	Relaxant response (g)	
	Papaverine (μ M)	
	3	10
2 week – Control	1.08 $\pm$ 0.11 (12)	6.71 $\pm$ 0.33 (11)
– Diabetic	0.82 $\pm$ 0.12 (9)	6.38 $\pm$ 0.27 (9)
4 week – Control	0.55 $\pm$ 0.12 (8)	4.10 $\pm$ 0.41 (11)
– Diabetic	0.88 $\pm$ 0.09 (8)*	4.94 $\pm$ 0.37 (8)

Values are mean $\pm$ s.e. mean for the number of animals tested indicated in parentheses. *Significant difference from the corresponding control value ($P < 0.05$, Student's unpaired t test).

muscles incubated in normal glucose (Figure 6; $P < 0.05$, two-way ANOVA), but did not alter responses to SNP or NO (Figure 7; $P > 0.05$, one-way ANOVA).

Discussion

The present study confirms our previous findings (Way & Reid, 1994a,b) that smooth muscle reactivity to NO is

Figure 6 Relaxant responses to field stimulation (0.5–5 Hz, 10 s train) in anococcygeus muscles after 6 h incubation in medium containing normal glucose (11.1 mM; ○), or elevated glucose (44.1 mM; ●) or an equiosmolar medium containing mannose (▼) or sucrose (■; 33 mM plus 11.1 mM glucose). Values are mean $\pm$ s.e. mean from 5–8 experiments, expressed as reductions in tension produced in tissues precontracted with guanethidine (10–30 μ M) and clonidine (0.01–0.05 μ M).

Figure 7 Relaxant responses to sodium nitroprusside and nitric oxide in anococcygeus muscles after 6 h incubation in medium containing normal glucose (11.1 mM; open columns), or elevated glucose (44.1 mM; cross-hatched columns) or an equiosmolar medium containing mannose (right-hatched columns) or sucrose (left-hatched columns; 33 mM plus 11.1 mM glucose). Values are mean $\pm$ s.e. mean from 4–8 experiments, expressed as reductions in tension produced in tissues precontracted with guanethidine (10–30 μ M) and clonidine (0.01–0.05 μ M).

attenuated in anococcygeus muscle from 8-week diabetic rats. We now report that treatment of 8-week diabetic rats with 5 u Lente insulin day⁻¹ prevents the development of impaired responses to field stimulation, to the NO donor SNP and to NO, in anococcygeus muscle. These results suggest that the observed alteration in nitrergic neurotransmission is due to the production of a diabetic state by STZ and is not due to any non-specific effect of STZ itself. This is in agreement with numerous studies using other smooth muscle preparations where the effects of STZ-induced diabetes have been reversed or prevented by insulin treatment (Nowak *et al.*, 1986; Longhurst *et al.*, 1991; Hodgson & King, 1992; Stevens *et al.*, 1993).

Exposure of tissues from 8-week diabetic rats to a high glucose medium (30 mM), containing a similar concentration of glucose to blood levels in diabetic rats, did not affect the impairment in smooth muscle reactivity to NO. Other studies have also reported no change in functioning of diabetic tissue after incubation in high glucose medium (Cameron & Cotter, 1992; Booth & Hodgson, 1993). However, prevention of diabetes-induced changes in tissue responsiveness has been reported in aortae from 2-week but not 6-week STZ-treated rats after incubation in PSS containing 30 mM glucose (Hodgson & King, 1992). Nevertheless, in the present study, we have demonstrated that the impairment of nitrergic transmission in the anococcygeus muscle from 8-week diabetic rats is not related to the acute change in glucose concentration from that present *in vivo* (approximately 25–30 mM), to the comparatively reduced concentration *in vitro* (11 mM).

Other studies have also shown changes to NANC-inhibitory innervation induced by the diabetic state. These include alterations to both the purinergic (Belai *et al.*, 1991) and peptidergic (Belai *et al.*, 1987; D'Amato & Curró, 1990) components of rat gastrointestinal tissue, and impaired NANC neurotransmission in human corporal smooth muscle from impotent diabetic men (Saenz de Tejada *et al.*, 1989). Furthermore, previous studies have demonstrated that the development of diabetes-induced changes may depend on the duration of the diabetic state (MacLeod & McNeill, 1985; Belai *et al.*, 1988; Morff, 1990; Nowak *et al.*, 1990). For example, in the myenteric plexus of STZ-diabetic rats, biochemical results showed a trend at 8 weeks for an increase in noradrenaline content, followed by a decrease in both tissue content and nerve fibre density at 16 and 24 weeks after diabetes induction (Belai *et al.*, 1988). Another study described defective cholinergic neuromuscular transmission in diabetic rat small intestine after 4 weeks, but this defect was

less evident at 8 weeks, and after 12 weeks the changes had spontaneously resolved (Nowak *et al.*, 1990). Therefore, diabetes-induced changes do not necessarily follow a consistent pattern with an increase in the duration of the diabetic state.

To address the question of changes in the development of diabetes-induced alterations over time, we have examined the relationship between altered NO-mediated relaxations and the time course after the onset of diabetes, up to an 8-week period. At 3 days after the induction of diabetes with STZ, there were no apparent changes in relaxant responses to field stimulation, SNP or NO, when compared with appropriate controls. However, consistent with our findings at the 8-week period, relaxations to field stimulation, SNP and NO were attenuated at 2 weeks and 4 weeks after the induction of diabetes, whereas relaxant responses to papaverine, which does not act through NO, were not impaired at either duration. Our results therefore indicate that the specific impairment of NO-mediated relaxations in rat anococcygeus muscle develops within the first 2 weeks of diabetes and persists for a further 6-week period; the influence of chronic experimental diabetes on the progression of the changes seen at 8 weeks remains to be determined.

The direct effects of an elevated glucose environment *per se* on tissue responsiveness has been previously examined by exposing preparations to elevated glucose media for a sufficient period (Williamson *et al.*, 1990; Tesfamariam *et al.*, 1990; 1991; Bohlen & Lash, 1993; Taylor & Poston, 1994). Tesfamariam and colleagues (1990) demonstrated that the incubation of rabbit aortic rings in high glucose for 6 h impaired cholinergic endothelium-dependent relaxations, similar to the impairment demonstrated in aortae from alloxan-diabetic rabbits. In the present study a similar experimental protocol to that used by Tesfamariam *et al.* (1990, 1991) was employed to investigate whether impaired NO-mediated neurotransmission in diabetic rat anococcygeus muscle could be mimicked by exposure of muscles from untreated rats to acute hyperglycaemic conditions. With a glucose concentration of 44 mM and an incubation period of 6 h (see Tesfamariam *et al.*, 1990; 1991), exposure of muscles *in vitro* to elevated glucose specifically attenuated relaxant responses to field stimulation, but not to SNP or to NO. However, the effects of 44 mM glucose were also produced after a 6 h incubation in equiosmolar solutions containing mannose or sucrose, suggesting that hyperosmolarity *per se* was responsible for the impairment.

The differences in the effects on NO-mediated neurotrans-

mission produced by incubation in high glucose media and by the diabetic state, may reflect acute *versus* chronic exposure to elevated glucose. Acute 6 h exposure to a hyperosmolar environment specifically impaired the response to nitrgic nerve stimulation, possibly affecting NO synthesis, release, or breakdown, without altering the smooth muscle responsiveness to NO. In contrast, diabetes of 2-, 4- and 8-weeks duration, with consequent chronic exposure of muscles to high glucose, impaired the smooth muscle reactivity to NO; this may follow an initial impairment in the release of NO from nitrgic nerves, or a concurrent impairment could exist. The mechanism by which *in vitro* exposure to elevated glucose impairs the response to nitrgic nerve stimulation in anococcygeus muscle is as yet unknown. Free radical generation has been implicated in the impairment of endothelium-dependent relaxations in rabbit aortae exposed to elevated glucose (Teschfariam & Cohen, 1992), whereas the increased metabolism of glucose via the polyol pathway is thought to contribute to functional changes in rat microvessels subjected to high glucose media (Williamson *et al.*, 1990). Such mechanisms could similarly contribute to altera-

tions in NO-mediated neurotransmission produced in anococcygeus muscle by elevated glucose both *in vitro* and by the diabetic state *in vivo*.

In conclusion, impaired NO-mediated neurotransmission in anococcygeus muscle from 8-week diabetic rats, is prevented by insulin treatment and is thus specific for the production of a diabetic state by STZ. Relaxations to nitrgic nerve stimulation and to exogenously added NO were similarly impaired at diabetes durations of 2 and 4 weeks but not 3 days, suggesting the observed alterations develop during the first 2 weeks of diabetes. *In vitro* incubation of anococcygeus muscles in high osmolar media specifically impaired NO-mediated relaxation to nerve stimulation. Therefore elevated glucose levels *in vivo* may contribute towards the development of impaired NO-mediated neurotransmission in anococcygeus muscles from diabetic rats.

This work was supported by a Programme Grant from the National Health and Medical Research Council of Australia.

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(Received January 23, 1995
Accepted February 13, 1995)