Nitric oxide and penile erection in streptozotocin-diabetic rats

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ABSTRACT

The purpose of this investigation was to study the time course, response to insulin and characteristics of erectile dysfunction in streptozotocin (STZ)-diabetic Sprague–Dawley rats, and the function of the NO-generating system in these animals. Copulation-induced and reflex erection were quantified in conscious Sprague–Dawley rats at different times after injection of STZ. The corporal vasodilatation response to nerve stimulation was studied by measuring the rise in corporal pressure in pithed rats following electrical stimulation of sacral spinal nerve roots. The activity of NO synthase was determined in corporal tissue by measuring the generation of [3H]citrulline from [3H]arginine. Copulation-induced erection was inhibited at 1 and 2 months after STZ treatment, but this could be prevented by a short (2-week) pretreatment with insulin. Reflex erection was inhibited at 1, 4, 6 and 9 months after STZ; at 6 months, this inhibition was also reversible by insulin pretreatment. Following pithing, the basal corporal pressure was elevated in diabetic rats. At 4 months after STZ, this increase was normalized by a 2-week, but not by a 1-week, pretreatment with insulin; however, at 9 months after STZ, insulin pretreatment did not normalize corporal pressure. The increase in corporal pressure caused by stimulation of sacral nerve roots in pithed rats was enhanced in diabetic animals. This enhancement was also normalized at 4 months, but not at 9 months, by 2 weeks of insulin treatment. The inhibition of the stimulation-induced increase in corporal pressure by N\textsuperscript{G}-nitro-L-arginine methyl ester (5 mg/kg) was less following 9 months of diabetes, although NO synthase activity was normal in cavernosal tissue following 6–8 months of diabetes. In conclusion, STZ-induced diabetes caused changes in the erectile system that were initially reversible by a short insulin treatment, but which with time (more than 6 months) became irreversible. NO synthase activity in cavernosal tissue was normal, but the response to N\textsuperscript{G}-nitro-L-arginine methyl ester was inhibited in long-term diabetes (9 months).

INTRODUCTION

The streptozotocin (STZ)-diabetic rat has been used as a model system in the study of penile erectile impotence in human diabetes. Most [1–3], but not all [4], workers have described erectile dysfunction in the STZ rat, including reduced erection during copulatory behaviour [1–3] and alterations in the characteristics of the erectile reflex arc [5,6]. A number of possible explanations exist for the diabetes-induced reduction in erectile function of the STZ rat, including autonomic nerve dysfunction, a reduction in nerve conduction properties, an alteration in...
the release or post-synaptic action of neurotransmitters, including nitric oxide (NO), altered smooth muscle and vascular function, and derangements of central nervous system control of behavioural drive or autonomic outflow [7].

Only a few studies have been published on the function of the cavernosal tissue itself in STZ-diabetic rats, mainly because of the technical difficulties associated with the small size of the organ. We have previously described a preparation of the pithed rat which allows study of nerve-stimulation-induced corporal vasodilatation under reproducible conditions, and permits study of the erectile system in the absence of anaesthetic [8]. This is of particular importance when comparing responses between normal and diabetic rats, which may respond differently to anaesthetic agents. We have also described the inhibitory effect of the NO synthase (NOS) inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME), on stimulation-induced erectile responses in this preparation [9]. We have now employed the pithed rat preparation to study erectile response [increase in corporal pressure (CP)] to pelvic nerve stimulation in diabetic rats. The activity of the NO-generating system of the corpora has been studied by determining the response to the blockade of NOS activity by L-NAME administration, and by biochemical measurement of NOS activity in corporal tissue.

In order to establish the appropriate conditions for studying copulatory dysfunction, we initially determined erectile performance in our Sprague–Dawley STZ rats at different time intervals after STZ administration, by measuring behavioural and reflexogenic erection. Most studies on erectile dysfunction in STZ rats have been carried out using hyperglycaemic animals not treated with insulin. Some studies, however, have shown that insulin may correct some aspects of sexual dysfunction in the diabetic rat [10]. The effects of short-term insulin therapy were therefore determined on erectile responses in conscious STZ-diabetic rats, as well as on the corporal response to pelvic nerve stimulation in the pithed rat.

MATERIALS AND METHODS

Animals

Sprague–Dawley-derived rats, raised in the Rappaport Family Medical Faculty animal house, were kept under a 12 h light/dark regimen, and fed on rat pellets ad libitum. At the time of injection with STZ, the animals weighed 180–220 g. Animals were divided randomly into groups, and received either STZ (60 mg/kg, intravenously) or the same volume of vehicle (0.1 M citrate buffer, pH 4.5). Blood glucose levels were checked in all animals 48 h after the injection, and STZ animals with glucose levels below 350 mg/100 ml of plasma were discarded. In some experiments, insulin was administered to diabetic animals, as a single daily subcutaneous injection of 5 i.u. NPH (neutral protamine Hagedorn).

Tests in conscious animals

These observations were carried out as described by Dewbury [11] in a darkened room between 19:00 h and midnight. Individual male rats were placed in a Perspex cage with a mirror to enable observation. A single female in oestrus (injected with oestrogen and progesterone before the test) was introduced into the cage, and the times were determined for latency of mounting and intromission; the numbers of mounts and intromissions, the ejaculation latency and the post-ejaculatory interval were also measured. Animals were studied 1 and 2 months after STZ treatment, and an additional group of STZ rats was treated with insulin for 14 days before the 2-month observations.

Reflex erection was determined as described by Sachs et al. [4], with the animals lightly restrained in a Perspex cylinder. Numbers of clusters, flips and erections were determined over a 20 min period. Rats were studied 1, 4, 6 and 9 months after STZ treatment. The effect of insulin treatment was studied in an additional group of rats, at 6 months after STZ, with insulin administered daily for 14 days before the reflex test.

Pithed rat preparation

The basal level of CP, and the effect of stimulation of pelvic nerve roots on CP, were measured in pithed rats that had been diabetic for 1, 4, 6 and 9 months; modification of the CP response to stimulation by L-NAME was studied after 1, 4 and 9 months of diabetes. Rats that had been diabetic for longer than 1 month did not maintain a stable cardiovascular system after pithing for a long enough time to permit completion of the L-NAME administration and stimulation protocol, and therefore the 4-, 6- and 9-month diabetic animals were treated with insulin for 2 weeks before pithing. An additional group of 4-month diabetic rats were studied for the effects of stimulation alone (without administration of L-NAME) after 1 week of insulin treatment.

The pithed rat preparation was as previously described [8,9]. The rats were anaesthetized with halothane, pithed, and the pithing rod replaced by an electrode insulated except for the terminal 1 mm. Mean arterial pressure was measured from a carotid artery catheter. Cavernosal pressure was measured from a 26-gauge needle inserted into one corpus cavernosum and connected to a pressure transducer via a saline-filled polyethylene catheter. Square-wave stimuli were applied to the electrode at 1, 2, 5, 10 and 20 Hz, for 1 min at each frequency, with 3 min intervals between stimulation periods. The maximal CP attained during stimulation was expressed as a fraction of
the systemic blood pressure (BP) measured simultaneously. A second set of stimuli was administered 30 min after injection of L-NAME (5 mg/kg, intravenous) or saline (control).

**Determination of NOS activity**

The activity of NOS was determined by measuring the production of \(^{3}H\)citrulline from \(^{3}H\)arginine [12]. Whole corpora cavernosa (including tunica albuginea) were dissected from the penises of control and diabetic rats, 6–8 months after STZ injection. Urethra were dissected separately. The tissues were frozen in liquid nitrogen, and pulverized while frozen in a stainless steel pestle and mortar. The powdered tissue was extracted with incubation buffer containing 2 μM leupeptin, 1 μM pepstatin A and 1 mM PMSF. The cytosolic fraction was incubated in buffer containing 50 μM L-arginine, 2 μM NADPH, 3 μM tetrahydrobiopterin and 30 units/ml calmodulin, and arginine was separated from citrulline by filtration over a mini-column of Dowex AG50W-X8.

**Statistical analysis**

The significance of differences between group mean data was evaluated using the Student t-test for comparisons between pairs of data, or by one-way ANOVA with Student–Newman–Keuls post hoc test for comparisons between several groups.

**RESULTS**

Diabetic rats were considerably lighter in body weight than age-matched controls (296 ± 10 and 310 ± 7 g at 6 and 13 weeks respectively after STZ, compared with 390 ± 8 and 540 ± 10 g respectively in control animals), and showed the other usual signs of diabetes, such as cataract, polyuria and polydipsia. Diabetic rats showed significantly reduced erectile activity in the copulation test, at both 1 and 2 months following STZ. Data at 2 months are presented in Figure 1, and essentially similar results, with the same levels of statistical significance of differences between groups, were obtained at 1 month. This change was manifest in significantly reduced numbers of mounts and intromissions, with a significantly increased mount latency. Ejaculation latency and post-ejaculatory interval were not affected by diabetes. The effect of insulin on copulatory activity was studied 2 months after STZ. Insulin treatment largely prevented the effects of diabetes on mount and intromission number and latency (Figure 1).

The diabetic state also reduced reflex erectile activity. The number of reflex erections (visible increase in penile width and length) was significantly reduced at 4, 6 and 9 months after the onset of STZ-induced diabetes (Figure 2), but not at 1 month. Essentially similar results were obtained when the numbers of clusters of erectile responses (flips, flares and erections) were recorded (results not shown). The effect of insulin on reflex...
erectile function was studied 6 months after the onset of STZ diabetes. As in the copulatory test, insulin treatment for 2 weeks reversed the suppressive effects of the diabetic state on reflex erections (Figure 2).

After pithing, the basal CP in control rats remained at a consistent low level (5–10 mmHg) over the whole 3–4 h period of the experiment, but in pithed diabetic rats a significant increase in basal CP was seen (Figure 3). This effect was detected at 1 month after STZ in rats not treated with insulin, and also at 4 months after STZ when the animals had received only 1 week of insulin treatment before pithing, but was not seen when the rats had received 2 weeks of insulin treatment at 4 and 6 months after STZ. At 9 months after STZ, the basal CP was elevated even after 2 weeks of insulin pretreatment. The penises of the animals with elevated CP were also visibly tumescent. These alterations in resting CP were independent of systemic arterial pressure measured after pithing, which had not changed significantly at 4, 6 or 9 months after STZ, although it was decreased (P < 0.01) at 1 month after STZ treatment (resting BP averaged 66.7 ± 6.2, 51.5 ± 2.2 and 60.8 ± 3.7 mmHg for 1-month control, 1-month STZ and 4-month STZ rats respectively).

Following pelvic nerve stimulation, pithed STZ rats attained similar or higher values for the CP/BP ratio compared with control rats (Figure 4). At 1 month after STZ administration, the CP/BP ratio in STZ rats was significantly higher than in controls at a stimulation frequency of 1 Hz. At 4 months after STZ and after 1 week of insulin treatment, the CP/BP ratio had reached significantly higher values than in controls at all stimulation frequencies, but following 2 weeks of insulin there was no difference between STZ rats and controls. Similarly, at 6 months after STZ and after 2 weeks of insulin treatment, identical CP/BP values were seen in control and treated rats (results not shown), but at 9 months after STZ and following 2 weeks of insulin, significantly higher values were seen at stimulation frequencies of 5, 10 and 20 Hz. These increases in the CP/BP ratio in STZ rats were the result of an increase in CP, since BP values during stimulation were not lower in STZ compared with control rats. In this connection, it should be noted that application of pelvic nerve stimulation produced an insignificant change in BP.

Administration of l-NAME (5 mg/kg intravenous) caused an initial pressor response. BP then returned to control levels, so that at the time of stimulation after l-NAME, BP was not significantly different between the various groups of animals. Peak BP following l-NAME was 146 ± 8.8, 86.9 ± 12.6, 153.5 ± 5.1 and 150 ± 4.7 mmHg in control and 1-, 4- and 9-month diabetic animals respectively (P < 0.01 for difference between control and 1-month-diabetic values). The value of CP/BP attained during stimulation was reduced by l-NAME.
Erectile dysfunction in streptozotocin-diabetic rats

Figure 5 CP response to stimulation of sacral nerve roots in pithed rats treated with L-NAME (5 mg/kg).

NAME in all control and STZ animals (Figure 5). The response to L-NAME (decrease in CP/BP during stimulation) was similar in control and STZ rats at 1 and 4 months, but was significantly less marked in STZ rats after 9 months of diabetes, as shown by significantly higher CP/BP values after L-NAME at stimulation frequencies of 5, 10 and 20 Hz (Figure 5), and by a significantly greater (P < 0.01) ratio of CP/BP after L-NAME to CP/BP before L-NAME at each frequency of stimulation (results not shown).

The biochemical activity of NOS in the tissues of STZ rats was studied at 6–8 months after STZ administration.

Table 1 NOS activity in tissues from control and STZ-diabetic rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>n</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol · min$^{-1}$ · mg protein$^{-1}$)</th>
<th>n</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol · min$^{-1}$ · mg protein$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebellum</td>
<td>11</td>
<td>32.6 ± 4.3</td>
<td>475 ± 23</td>
<td>15</td>
<td>32.3 ± 4.1</td>
<td>660 ± 50**</td>
</tr>
<tr>
<td>Urethra</td>
<td>10</td>
<td>37.0 ± 3.7</td>
<td>121 ± 11</td>
<td>7</td>
<td>3.8 ± 4.4</td>
<td>127 ± 15</td>
</tr>
<tr>
<td>Corpus cavernosum</td>
<td>9</td>
<td>40.2 ± 3.9</td>
<td>11 ± 1</td>
<td>9</td>
<td>38.9 ± 8.5</td>
<td>11 ± 3</td>
</tr>
</tbody>
</table>

No significant differences were seen in $K_m$ or $V_{max}$ between control and STZ rats in either corporeal or urethral tissue, whereas diabetic rats had a significantly increased $V_{max}$ for NOS in the cerebellum (Table 1).

DISCUSSION

Our study has confirmed other reports [1–3] of impaired behaviourally induced erection in STZ-diabetic rats. Many of these studies have also used Sprague–Dawley strain animals, although one group of workers [4] failed to observe altered male sexual function in STZ-treated Sprague–Dawley rats. Our study also shows the ability of insulin to reverse this erectile dysfunction at a relatively early stage (2 months).

We also found a reduced reflex erectile response in diabetic animals. Previous reports of reduced reflex erection in STZ rats [5,6] were carried out using electrical stimulation of the dorsal nerve in the anaesthetized animal, and found decreased afferent nerve conduction [5]. Our study confirms the finding of reduced reflex erection in the conscious animal in vivo. Again, the decrease in reflex erections was reversed by insulin, even 6 months after STZ treatment. Other workers have reported on the ability of short-term or continuous insulin treatment to reverse erectile, vascular and nerve conduction defects associated with STZ-induced diabetes [10,13,14]. At present, we cannot state whether the reversal of behavioural and reflex erectile dysfunction by insulin is the result of hypoglycaemic or other effects of the hormone. Short-term insulin treatment (15 days) reversed many of the neuroendocrine disorders of STZ rats seen at 1.5 months after STZ, and normalized the depressed plasma testosterone levels [10].

Altered behavioural activity in untreated diabetes could well be the result of an altered metabolic state of the animal. However, the suppression of reflex erection shows that the erectile defect in STZ-induced diabetes cannot be entirely explained by a lack of sexual drive resulting from poor general subjective feeling. On the other hand, erection induced by behavioural sexual stimulation was suppressed at 1 month after STZ treatment, whereas reflex erection was not. This may indicate that the erectile deficit at 1 month may be central and not...
Peripheral in origin, but the possibility must also be considered that the hyperglycaemic state affects vascular or even skeletal muscle function. Evidence has been produced for alterations in central nervous system neurotransmitter release in diabetic rats, including reduced noradrenaline and dopamine turnover in the hypothalamus and limbic system, in addition to decreases in plasma gonadotropin, prolactin and testosterone levels [1,10,15]. The sexual dysfunction of the hyperglycaemic STZ rat may therefore result from multiple deficits in nervous and endocrine function, at both central and peripheral sites, many of which can be reversed by insulin.

Nerve conduction defects probably play an important part in erectile dysfunction both in STZ rats and in human diabetes subjects [16]. A decrease in endoneurial blood flow is claimed to be an important factor in the reduced conduction rate of nerve fibres in STZ-diabetic rats [17]. However, our additional findings in the STZ rat [18] indicate that changes also occur in the mechanical properties of corporal vasculature.

The finding of corporal vasodilatation in STZ-diabetic rats is interesting in view of other reports of vasodilatation of renal and mesenteric vascular beds in this model [7]. An increase in resting blood flow in retina, kidney, skeletal muscle and skin of humans occurs in the early stages of insulin-dependent (Type I) diabetes, although thermal and reactive hyperaemic responses are impaired [19]. Isolated resistance vessels from diabetic subjects show attenuated responses to noradrenaline and other vasoconstrictors, and a decreased relaxant response to acetylcholine, mainly during poor metabolic control [20]. In studies of isolated tissues from diabetic animals, variable changes in endothelial function have been reported. In one study [13], the endothelium-dependent vasodilator response to acetylcholine in isolated mesenteric resistance vessels of STZ rats was enhanced at 6 weeks, but not at 12 weeks, after STZ. The increased response to acetylcholine was explained by a possible increase in the production of NO, endothelium-derived hyperpolarizing factor or vasodilator eicosanoids. On the other hand, other studies have reported an attenuation [21], or no change [22], in the relaxant response to acetylcholine in isolated mesenteric arteries from STZ rats. The relaxant action of acetylcholine was impaired in aortic strips from diabetic rabbits, and this was related to the increased production of a constrictor eicosanoid, such as thromboxane [23]. This variability in response may well be the result of removal of the tissues from the in vivo environment.

In the pithed rat, there is no basal autonomic tone to the blood vessels. The fact that, in control pithed rats, basal CP is maintained at a low level indicates either that a local vasoconstrictor tone exists or that the structure of the helicine artery maintains a low flow rate in the absence of active vasodilatation. The vasodilatation (increased resting CP) in the corpus cavernosum from diabetic rats could therefore be the result of either a reduction in local vasoconstrictor release or the release of an active vasodilator.

The high level of resting corporal vasodilatation in the STZ rat was reflected in the CP response to pelvic nerve stimulation. Diabetic rats attained a higher value of CP/BP following stimulation. This observation correlates well with other data showing an enhanced vasodilator response to acetylcholine in the mesenteric vasculature [13]. As also seen in the erectile function tests in conscious animals, a short course of insulin therapy was successful in reversing this change at 4 months after STZ treatment. At 9 months after STZ, however, the same 2-week treatment with insulin was not able to completely reverse the change. This indicates that diabetes-induced vascular dysfunction progresses with time in the STZ-diabetic rat model.

In the pithed diabetic rat model, therefore, we could not find evidence for a reduced vasodilatory response to nerve stimulation in the corpora cavernosa. It should be noted that insulin-treated animals were used for the pithed rat experiments involving stimulation when diabetes had existed for longer than 1 month. This was necessary because rats not treated with insulin did not survive the pithing procedure. Conceivably, the administration of insulin permitted a normal vasodilator response to stimulation in the face of the adverse effects of diabetes; however, a normal or enhanced vasodilator response to stimulation was also seen at 1 month (in the absence of insulin). An endothelium-dependent vasodilator response to insulin has been demonstrated in rat vessels [24], and insulin blunts the effect of vasoconstrictors, including noradrenaline, in resistance vessels from rat and humans [25].

The present study did not provide any indication of a decrease in corporal NOS activity in diabetic rats up to 6 months after STZ injection, since: (a) the CP response to pelvic nerve stimulation was normal or enhanced, and NO is thought to play a major role in this response; (b) l-NAME produced a similar degree of corporal vasoconstriction in control and STZ-treated rats; and (c) NOS activity determined by biochemical techniques in corporal tissue was similar in control and STZ rats. On the other hand, at 9 months after STZ, we found a reduced response to l-NAME, indicating either a decrease in NOS-dependent vasodilatation or altered postsynaptic responsiveness of the vascular tissue to NO.

In the present study, we were careful to determine NOS activity in corporal tissue. Other studies have reported increased NOS activity in whole penile tissue of STZ-treated rats [5], whereas reduced activity was found in penile tissue of BB rats [26].

In conclusion, this study shows that defects were induced in the erectile system of rats following STZ treatment. Up to 6 months after STZ, these changes could
be partially reversed by 2 weeks of treatment with insulin, but by 9 months after STZ this short insulin treatment was not capable of reversing the resting corporal vasodilatation and normalizing the response to L-NAME. Although the NO-generating system showed normal function biochemically, evidence was produced for reduced participation of NO in the corporal vasodilatation following erectile stimulation at 9 months after STZ. Thus a prolonged period (6 months) of diabetes did not decrease the function of the neurally induced corporal vasodilatory system, provided that a short insulin treatment was given before the experiment. The fact that the response to L-NAME was diminished at 9 months indicates that neurotransmitter systems other than NOS may become important in long-term diabetes, but further studies are required to elucidate this point.

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