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Impaired endothelium-dependent and neurogenic relaxation of corpus cavernosum from diabetic rats: improvement with L-arginine

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Abstract This study describes the relaxant response to acetylcholine, electrical field stimulation and sodium nitroprusside after contraction by phenylephrine (10^{-5} M) in corpus cavernosum from control and diabetic rats. The response to acetylcholine (10^{-9}–10^{-3} M) and electrical field stimulation (0.5–64 Hz) is decreased and can be restored by the addition of nitric oxide synthatase substrate, L-arginine(10^{-5} M). The response to sodium nitroprusside is not changed in diabetic rats compared to control rats. NADPH-diaphorase staining was enhanced in a diabetic preparation compared to control preparations. The findings suggest a role for the depletion of L-arginine in diabetes mellitus. The enhanced NADPH-diaphorase staining may be due to a deficiency of NOS substrate L-arginine in the endothelium and nerves of diabetic tissues.

Key words Acetylcholine · Diabetes mellitus · L-arginine · Corpora cavernosa

Introduction

Erection follows the relaxation of penile corpus cavernosum smooth muscle that is initiated by sacral parasympathetic stimulation. Acetycholine is generally regarded as the classic postganglionic parasympathetic neurotransmitter for penile erection. It stimulates endothelial nitric oxide synthase pathway to generate nitric oxide mediated smooth muscle relaxation of human and rabbit corpus cavernosum [19]. Locally, relaxation is mediated by non-adrenergic-noncholinergic (NANC) neurotransmitters [16]. In vitro, electrical stimulation of isolated corpus cavernosum strips elicits a neurogenic, frequency-dependent relaxation that is resistant to adrenergic and cholinergic blockers [16]. Thus, it is characterized as a NANC mediated response. Nitric oxide (NO) is strongly associated with the physiology of the penis, based on mounting basic science and clinical evidence [5]. NO is synthesized during the conversion of L-arginine into L-citrulline in a reaction catalyzed by the enzyme nitric oxide synthetase (NOS) [8, 13]. This chemical is clearly understood to have major importance in mediating penile erections as it mediates signal transduction mechanisms in which guanylate cyclase is activated to produce cyclic guanosine monophosphate (cGMP), an effector of corporal smooth muscle relaxation [5].

Diabetes mellitus is associated with an increased incidence of erectile dysfunction in men and laboratory animals [12, 17]. Previous studies showed that diabetes mellitus could directly disrupt NO-mediated erectile mechanism(s) [17, 21]. The STZ diabetic rat model has been used largely to study diabetes-induced changes. There is nevertheless little knowledge about how diabetes-induced changes affect the local control of penile erection. The object of this study was to determine the effect of diabetes mellitus on the neural and endothelial cell-mediated physiological mechanisms that control corporal smooth muscle tone in the penis.

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Materials and methods

Induction of diabetes mellitus

Male Wistar rats weighing 200–250 g were fasted for 18–24 h and were made diabetic under ether anesthesia by intravenous injection

of streptozotocin (45 mg/kg) into the tail vein. Streptozotocin was dissolved in 0.1 M citrate buffer (pH 4.5) and injected within 5 min of preparation. Control animals ($n = 8$) received citrate buffer only. The streptozotocin treated rats ($n = 8$) developed glycosuria and polyuria within 48 h of injection. Rats were considered diabetic and included in the study when random urine samples tested at least 3+ on clinistix (corresponding to a glucose level of 1 g/100 ml or more) using Keto-Diastix test strips (Ames Co., Division of Miles Laboratories, Elkhart, Ind.), and subsequent analysis of serum obtained at time of death showed glucose levels of at least 400 mg/100 ml. Each group of animals was caged separately and kept under precisely the same conditions as other groups. The experimental animals were randomly divided into control and diabetic groups. The animals were fed with standard Purina chow and provided with water ad libitum and sacrificed 6 weeks later.

Corpus cavernosum tissue

Rats were sacrificed by decapitation. Blood was collected into ice-chilled tubes and separated and analyzed for serum glucose concentration using an Ames Glucometer 3 (Bayer Diagnostics, France). Sacrifice was followed by exsanguination and en bloc removal of the penis. The tunica was gently dissected and the corpus cavernosum tissue was exposed. After dissection, the corporal tissue was immediately placed in organ chambers. Each rat provided one strip of corporal smooth muscle.

Preparation of tissues for organ bath studies

Strips of corpus cavernosum tissue measuring approximately $1 \times 1 \times 7$ mm were mounted in a 25-ml organ bath for measurement of isometric tension. The solution was gassed with 95% O_2 and 5% CO_2 during the study. The pH of the solution was 7.4 and the temperature was maintained at 37 °C. The strips were tied at one end with silk to a wire connected to a force displacement transducer (Ugo Basile, Milan, Italy) and at the other secured with silk to a metallic support. The preparations were incubated in the bath at a resting tension of 1.0 g for 2 h before beginning experiments. The organ baths contained Krebs-bicarbonate buffer (mM; NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 1.5; $NaHCO_3$, 25.0; glucose, 11.1 and disodium EDTA, 0.023).

Organ bath studies

Tissues were contracted by phenylephrine (10^5 M). All acetylcholine experiments (10^{-9}–10^{-3} M) were performed in the presence of indomethacin (10 μM) and guanethidine (5 μM). The acetylcholine-induced relaxation responses were repeated in the presence of the nitric oxide synthetase inhibitor, N^G-nitro-L-arginine methylester (L-NAME) (0.1 mM) or 20 min incubation with the nitric oxide synthetase substrate, L-arginine (10^{-5} M).

Electrical stimulation after phenylephrine contraction was accomplished by means of two platinum plate electrodes, positioned on either side of the tissue (Grass S44, Grass Instruments, Quincy, Mass.). Each stimulation lasted 20 s at the indicated frequencies (0.5–64 Hz), at constant parameters: 10 V, 0.5 ms pulse duration. After precontraction with phenylephrine (10^{-5} M), the electrical stimulation-induced response curves were also performed in the presence of L-NAME (0.1 mM). Then, electrical stimulation-induced relaxation was also performed after a 20-min incubation with L-arginine (10^{-5} M). Acetycholine and electrical field stimulation were not applied in the same tissues.

Finally, the corporal tissues were maximally contracted with phenylephrine (10^{-5} M) and relaxed with cumulative doses of the endothelium independent vasodilator sodium nitroprusside (10^{-9}–10^{-3} M).

During the control studies, we showed that there were no differences in either sensitivity or maximum response curves performed at baselines after incubation in Krebs solution for 30, 60, 120 min. Moreover, in subsequent time control studies with corporal tissue from Wistar rats, we have observed that maximum responses and sensitivity to phenylephrine and acetycholine remained the same for a total of 6 h.

Tissues receiving electrical stimulation and sodium nitroprusside were also treated atropine (1 μM), as well as indomethacin and guanethidine.

Histochemical staining for NADPH-diaphorase

Samples that were taken from the corpus cavernosum of control and diabetic rats were stored in Krebs-bicarbonate solution until the cryostat sections were prepared. The storage time did not exceed 30 min. Serial cryostat sections of 8–12 μm thickness were prepared.

For the NADPH-diaphorase histochemical reaction the slides were put in 0.1 M triton-X-buffer solution containing 0.25 mg/ml nitro blue tetrazolium and 1 mg/ml β-NADPH (β-nicotineamide adenine dinucleotide phosphate). The reaction occured in a humid chamber and slides were kept at room temperature and in the dark for 2 hours. To control the intensity of the staining, the slides were observed under the microscope until a dark blue color occurred. After sufficient staining was obtained the preparations were washed with tris buffer to stop reaction. The preparations were then covered with glycerin in Phosphate buffered saline solution (3:1). By using a Zeiss Photomicroscope the images were taken on 25 ASA, black and white film.

Analysis of data and statistics

All values are expressed as means ± SEM. To evaluate the relaxant effect of agents, such as acetylcholine on corporal preparations of control and diabetic rats, pD_2 values (apparent affinity constant) were calculated according to the method described earlier [1, 2]. The pD_2 values were calculated as the negative logarithm of the concentration of agonists that produced 50% of the maximal effect of agents in rat corporal tissue [i.e., $pD_2 = -\log (EC_{50})$]. EC_{50} values were calculated by the linear regression analysis of concentration–response curves. Maximal effects of relaxant agonists were expressed as percentage inhibition of phenylephrine stimulated contractions. Statistical analysis of the data was performed by Student's unpaired *t*-test for comparison of two groups and one-way analysis of variance for comparison of three or more groups. Differences were considered to be statistically significant when $P < 0.05$, $P < 0.01$.

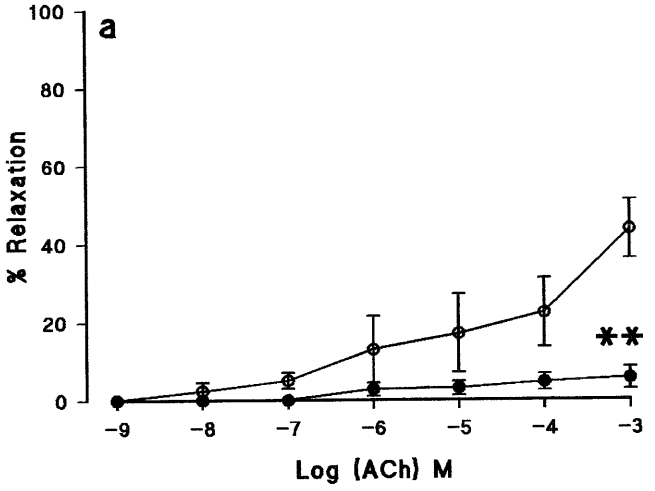
Chemicals and solutions

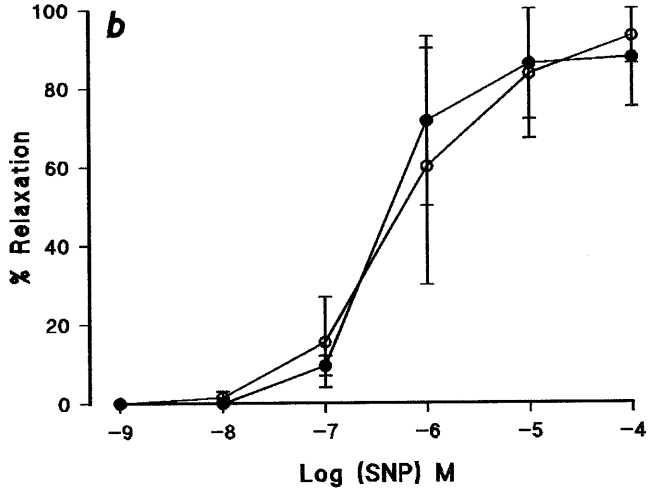
Phenylephrine hydrochloride, guanethidine sulfate, L-arginine hydrochloride, D-arginine hydrochloride, atropine sulfate, L-NAME, acetylcholine chloride, sodium nitroprusside, indomethacine, nicotineamide adenine dinucleotide phosphate (β-NADPH) and nitro blue tetrazolium were purchased from Sigma Chemical (St. Louis, Mo.). Indomethacin was dissolved with 0.1 M $NaCO_3$ and 0.1 M Na_2PO_4 (pH: 6.9). Other drugs were dissolved in deionized distilled water. All drugs were kept at 4 °C while in solution.

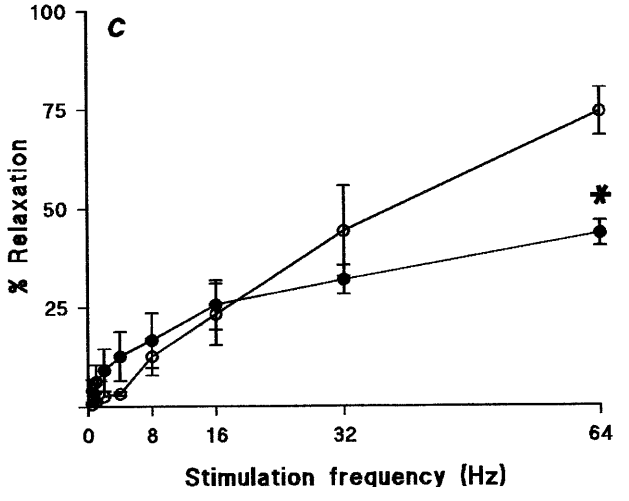
Results

Body, corpus cavernosum weight and blood glucose levels

Six weeks after injection, diabetic rats (201 ± 955 g, $P < 0.05$) had body weights significantly less than those of control rats (295 ± 17 g). At 6 weeks the average blood glucose level was significantly greater in the diabetic group (442 ± 18 mg/100 ml, $P < 0.01$) compared







to the control group (112 ± 19 mg/100 ml). There was no significant difference in corpus weights of the diabetics and their controls (control; 0.028 ± 0.003 g; diabetic 0.017 ± 0.003 g).

◀

Fig. 1a–c Relaxation of smooth muscle from the corpora cavernosa from diabetic rats ($n = 8$, filled circle) and control rats ($n = 8$, open circle) in the presence of acetylcholine (**a**), L-arginine (**b**), and sodium nitroprusside (**c**). A comparison of concentration–response curves in tissues from diabetic and nondiabetic rats revealed statistically significant differences (*$P < 0.05$; **$P < 0.01$)

Contraction with phenylephrine

There was no significant difference between contractions induced by phenylephrine (10^{-5}) in corpus cavernosum strips from diabetic rats and those from age-matched nondiabetic control rats (control; 1.21 ± 0.13 g, diabetic; 1.26 ± 0.15 g tension, $n = 6$).

Responses to acetylcholine

Rat corpus cavernosum strips were contracted with phenylephrine and treated with increasing concentrations of acetylcholine (10^{-9}–10^{-3} M). Only the maximal response to acetylcholine was significantly decreased in diabetic animals (Table 1; Fig. 1a). With the other concentrations of acetylcholine (10^{-9}–10^{-4} M), there were no significant differences between control and diabetic tissues.

In the presence of 0.1 mM L-NAME, relaxant responses to acetylcholine were significantly diminished in control tissues (Table 1). In preparations from diabetic rats, diminished relaxant responses to acetylcholine were not altered in the presence of L-NAME (Table 1).

L-arginine incubation did not alter phenylephrine-induced contractile response one between control and diabetic strips. Although addition of L-arginine did not

Table 1 The pD_2 and maximum values of the inhibitory response to acetylcholine and electrical stimulation (64 Hz) in phenylephrine-contracted corpus cavernosum tissue from both control rats ($n = 8$) and diabetic rats at 6 weeks ($n = 8$)

	pD_2	Maximum response (% relaxation)
ACH		
Control	4.88 ± 0.69	43.6 ± 7.50
+L-NAME	4.60 ± 0.35	16.0 ± 2.08*
+L-arg	4.90 ± 0.31	41.2 ± 5.60
Diabetic	5.11 ± 0.52	5.5 ± 2.90**
+L-NAME	5.90 ± 0.3	5.1 ± 0.76**
+L-arg	5.23 ± 0.56	36 ± 9.16
EFS		
Control	–	74.3 ± 6.06
+L-NAME	–	18.3 ± 5.07**
+L-arg	–	65.1 ± 5.23
Diabetic	–	43.6 ± 3.17
+L-NAME	–	20.2 ± 4.09**
+L-arg	–	57.0 ± 6.65

*$P < 0.05$, **$P < 0.01$, paired t-test against contol
L-NAME, N^G-nitro-L-arginine methyl-ester; L-arg, L-arginine; ACh, acetylcholine; EFS, electrical field stimulation

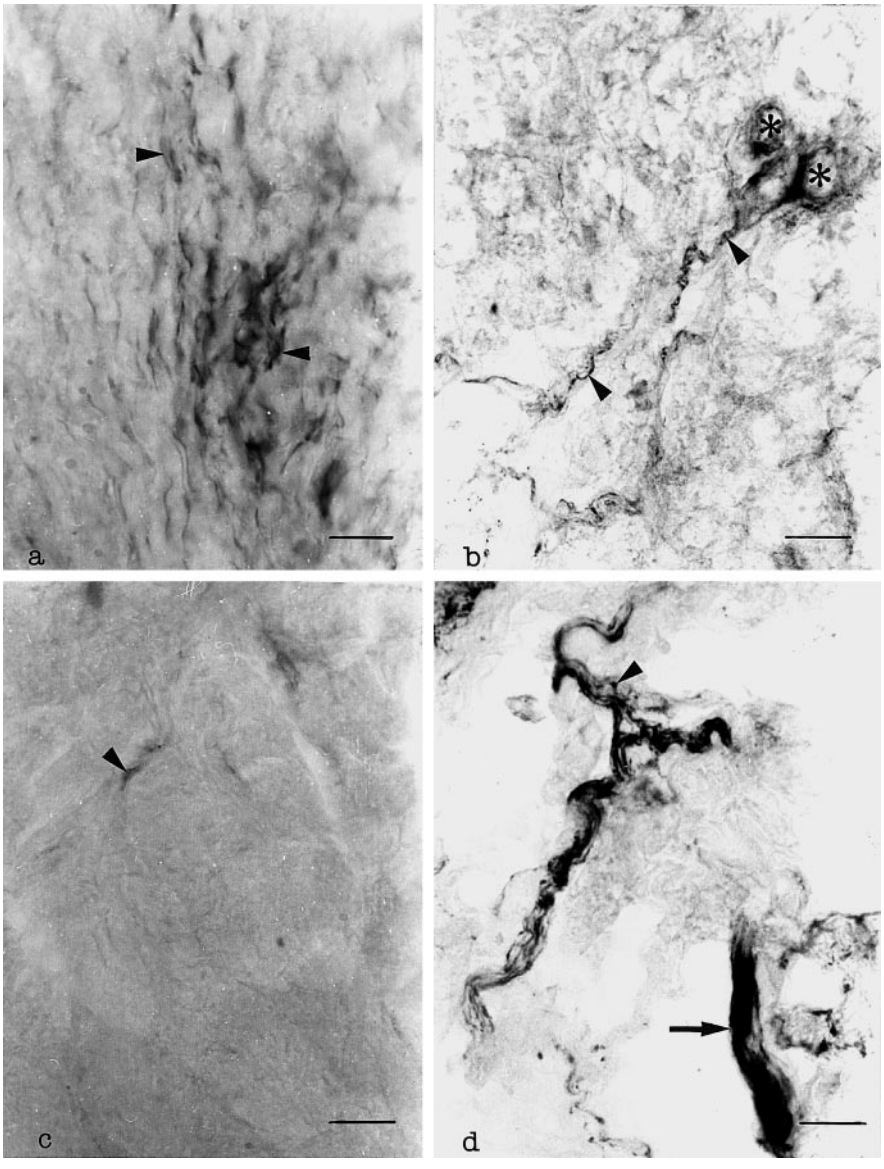


Fig. 2a–d NADPH-diaphorase stained nerves in the corpus cavernosum from the penis of both control and diabetic rats. **a** In control rats, fine nerve fibers lay between the caverns (*arrowhead*; scale bar 25 μ). **b** In diabetic rats, the nerves (*arrowhead*) entered the corpus cavernosum of the penis around the vessels (*asterisks*) (scale bar 20 μ). **c** In transverse section from the control preparation, stained nerve fibers were not seen in any areas except thicker bundles (*arrowhead*; scale bar 20 μ). **d** In diabetic rats, the thickness of the nerve bundles was increased, and varicose enlargements (*arrowhead*) were observed (scale bar 17 μ)

alter the relaxant responses to acetycholine in tissues from control animals, tissues from diabetic rats exhibited an improvement in the diminished relaxant responses to acetylcholine (Table 1).

Responses to sodium nitroprusside

There was no significant difference in the relaxation of corporal muscle induced by sodium nitroprusside (10^{-9}–10^{-3} M) between the tissue from diabetic and control rats (Fig. 1b). The pD_2 values were not altered in these diabetic preparations (data not shown).

Responses to electrical stimulation

The neurogenically mediated relaxation of corpus cavernosum strips induced with electrical stimulation at 0.5–32 Hz was not significantly different between control and diabetic strips (Fig. 1c). Remarkably, low frequency stimulation (0.5–8 Hz) produced greater relaxation effects in the diabetic tissues compared with the diabetic tissues, although these observations were not significant. However, when the stimulation was 64 Hz, relaxation was significantly less in the tissues from diabetic rats (Table 1).

No differences in neurogenic relaxation at 64 Hz stimulation was observed between corporal tissues obtained from diabetic and those of control rats after L-arginine incubation (Table 1). In the presence of 0.1 mM L-NAME, relaxant responses to electrical stimulation were significantly diminished in tissues from both control and the diabetic rats (Table 1).

Histochemical findings

NADPH-diaphorase stained nerves were observed in corpora cavernosa of both control and diabetic rats. The

nerve cell fibers that were stained with NADPH-diaphorase entered the corpus cavernosum penis around the vessels. These nerves disseminated between the caverns after entering the corpus cavernosum penis. In the control rats, the nerve bundles especially were organized as small bundles (Fig. 2a, c). In some microscopic areas, none of these nerves were stained with NADPH-diaphorase, except thicker ones. On the stained nerves, some varicose enlargements were seen in controls. In the diabetic rats, the NADPH-diaphorase stained nerve cell fibers formed thick bundles in both the connective tissue around the corpus cavernosum penis and cavern walls (Fig. 2b, d). These nerve bundles also contained varicose enlargements that were seen as well in the control preparations. NADPH-diaphorase stained nerve cell bodies were not observed in preparations from control and diabetic rats. Preparations from the corpus cavernosum penis of diabetic rats showed thickening of NADPH-diaphorase stained nerve fibers in the central and peripheric areas of the tissues.

Discussion

The process of cavernous smooth muscle relaxation is thought to be locally controlled by neurotransmitters and by vascular endothelium that lines the sinusoidal spaces [16]. The present findings demonstrated that corporal tissues obtained from diabetic rats exhibited similar diminished relaxant responses to acetylcholine, and electrical stimulation, but not sodium nitroprusside.

In our data, acetylcholine induced endothelium-dependent relaxation in corpora cavernosa in control preparations. Inhibition of these effects of acetylcholine in the presence of L-NAME is strong evidence that cholinergic relaxation of control cavernous smooth muscle in rats is predominantly mediated by nitric oxide release. Our results are consistent with previous findings in diabetic corporal tissues [3, 17]. In these studies, diminished endothelium dependent mechanisms that mediate the relaxation of the smooth muscle of corpora cavernosa has been found in diabetic men and New Zealand white rabbit with alloxan induced diabetes. In the present study, L-arginine supplementation in vitro prevented a reduction in the response to acetylcholine in diabetic tissues compared to control tissues. The mechanism(s) of the beneficial effects of L-arginine on impaired acetylcholine relaxation (endothelium dependent) in isolated diabetic corporal tissues is unknown. In a recent study using aortic preparations, Pieper and Peltier [15] found that pretreatment with 3 mM L-arginine potentiated the relaxation to acetylcholine in diabetic rings suggesting a defect in utilization of L-arginine by nitric oxide synthase for production of EDRF/NO. Furthermore, reduced plasma levels of arginine have been reported in diabetic patients [9, 11] and experimental diabetic animals [14]. In another study, it has been proposed that a decrease in the level of L-arginine available for nitric oxide synthesis could be the result of the catabolic state in diabetes [4]. There is a generalized reduction in receptor responsiveness in corporal tissue due to diabetes-induced L-arginine deficiency. Previous results and our findings suggest that in diabetic tissues, the attenuated responses to acetylcholine may be due to depletion of L-arginine as a substrate for nitric oxide synthase.

Penile corporal smooth muscle from diabetic rats was found to relax normally with the endothelium independent vasodilatator sodium nitroprusside. This finding suggests that the corporal smooth muscle is functioning normally, which supports previous findings [3, 17]. Our findings further suggest that the lack of changes in the smooth muscle itself may be due to artificially induced diabetes.

In the present study, it has been shown that the response to electrical stimulation between 0.5–32 Hz was the same in both groups and it was only with stimulation at 64 Hz that a reduced relaxation was found in the diabetic samples. This study showed that there is an impairment of NO synthesis or at high frequencies of electrical stimulation, a release of NO from the corporal nerve. In contrast, other studies have shown that the neurogenically mediated relaxation of corpus cavernosum was significantly less pronounced at all levels of frequencies in the tissues of diabetic preparations [3, 17]. The difference between these data and ours may be due to a difference between the species and diabetogenic agents used.

In this study, the NOS substrate L-arginine prevented the reduced responsiveness at 64 Hz in diabetic preparations. There is evidence indicating that electrical stimulation induced inhibition was reversed by the addition of an excess of L-arginine, but not of D-arginine in the presence L-NAME, similar to the previous control study in vitro. On the other hand, our results provide the first evidence for restoration of defective relaxations to acetylcholine and electrical stimulation by L-arginine in diabetic rat corporal tissues. The action was sterospecific, in that relaxation was not normalized by the D-arginine isomer. Furthermore, these effects were specific for diseased corporal tissues, as L-arginine had no effect on normal corporal tissues. These results suggest that endothelial cells and neurone concentrations of L-arginine are sufficient to saturate enzymatic production of NO in normal corporal tissues. Our data may suggest that a deficient supply of arginine for NO synthesis in diabetic strips might have an impact on acetylcholine and electrical stimulation-induced relaxation.

Morphologically, the activity of NOS can be assessed by using NADPH-diaphorase staining while NADPH-diaphorase and neurone specific nitric oxide synthase (nNOS) seem to be identical in peripheral tissues and could be used to demonstrate nNOS [6, 22]. Similar to our NADPH-diaphorase staining results, Elabbady et al. [15] observed that NOS activity was significantly higher in the penile tissues of diabetic rats after 9 and 14 weeks. In a recent study, Haas et al. [10] showed up-regulation of NOS in aging. Our data suggested that in diabetics, NOS

activity was significantly higher in diabetes. By contrast, diabetic rats were shown to be impotent suggesting impaired neurogenic relaxations. With the technique of NADPH-diaphorase, Schirar [18] and Vanhatalo [20] demonstrated stained nerves in various areas of normal cavernosal tissue samples of Sprague-Dawley and Wistar rat. The results from our histochemical studies confirmed those from our other parallel studies. In our results, the increase of NOS in cavernosal tissue may explain the corresponding decrease of the L-arginine.

In conclusion, this study found an impairment of relaxation of corpus cavernosum from diabetic rats. In this model, physiological alteration of the corporal tissue that occurs in a relatively short period of time is comparable to those reported in corporal tissue of diabetic men with impotence. These changes contribute to the diabetic data of impotent men. These findings may be of further significance for new therapeutic approaches to the treatment of erectile dysfunction associated with diabetes.

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