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Changes in Mating Behavior, Erectile Function, and Nitric Oxide Levels in Penile Corpora Cavernosa in Streptozotocin-Diabetic Rats[1]

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ABSTRACT

This study assessed whether the in vivo production of nitric oxide (NO) in the penis is impaired in experimental diabetes and whether this phenomenon can be explained by abnormal levels of NO synthase isoenzymes and/or plasma androgens. Adult male Sprague-Dawley rats were injected with streptozotocin (STZ) (40 mg/kg, i.p.) or vehicle. One half of the STZ-treated animals received daily insulin replacement. Twelve weeks later, the animals were tested for mating behavior and erectile reflexes. They were then anesthetized with urethane (1 g/kg), and the NO levels in their corpora cavernosa were monitored electrochemically with porphyrin microsensors before and after electrostimulation of the cavernous nerve. The intracavernous pressure (ICP) was measured simultaneously. The diabetic animals had substantial impairment in the mating and erectile reflexes tests, decreased basal and stimulated NO levels in the corpora, and a reduced ICP response to cavernous nerve stimulation. Insulin replacement fully reversed the effects of diabetes on the mating reflexes, the basal NO signals, and the ICP responses to electrical field stimulation and partially restored the stimulated NO release. Neither diabetes nor diabetes with insulin treatment had significant effects on serum testosterone levels or NOS isoform (nNOS, eNOS, and iNOS) protein content in penile homogenates, indicating that the changes found in erectile function were independent of such variables. These results also suggest that the diabetes-induced reduction in corporeal NO levels could be mainly due to the lack of some essential cofactors for NOS activity rather than to changes in the amount of enzyme proteins.

insulin, male sexual function, nitric oxide, penis, testosterone

INTRODUCTION

Diabetes has long been recognized as a major risk factor for erectile dysfunction (ED) in man. The prevalence of ED is higher in diabetic men than in age-matched nondiabetic men, and this difference increases with age (reviewed in [1, 2]). A variety of mechanisms have been suggested for the erectile disorders associated with diabetes. Autonomic neuropathy can account for a decreased function of erectogenic nerves or an altered balance of the proerectile and antierectile transmitters reaching the cavernosal smooth muscle. The vascular supply of the penis is highly sensitive to atherosclerotic changes, which are accelerated in diabetic individuals. In the human, psychological factors often add to these organic disturbances.

Research in animals with either spontaneous or experimentally induced diabetes has also shown deficits in several indices of sexual function. In the male rat, such effects have been documented for mating behavior [3–7], ex copula penile reflexes [4, 6–10], and erectile responses to electrostimulation of the cavernous nerves as evidenced by the changes in intracavernous pressure (ICP) [9, 11–13]. Only a few studies have assessed the effects of insulin replacement [7, 9, 11].

The mechanisms responsible for diabetes-induced ED in these animal models are controversial. A main suspect is the degeneration of the erectogenic innervation to the penis, which uses nitric oxide (NO) as a main neuromediator. Some studies have reported reductions in NO synthase (NOS) activity as measured in vitro and in the neuronal (nNOS) isoenzyme content in the corpus cavernosum of diabetic animals [10, 12, 13]. However, there are also reports describing no changes [7] or even increases [14] in NOS activity in the penis of diabetic rats. Likewise, there is no general agreement about the effects of experimentally induced diabetes on sexual behavior and erectile function in rats since some studies have found no changes in copulatory activity or erectile reflexes [15], and other studies have found increased ICP responses to nerve stimulation [16]. A variety of additional mechanisms including central neuropathy [6] and decreased levels of circulating androgens [3–5, 10] have also been proposed to explain the sexual deficits found in diabetic rats.

Recently, we have developed an electrochemical approach for monitoring NO levels in the corpora cavernosa in a rat model of erection. The changes in the NO voltammetric signal can be recorded at nearly 1-min intervals concomitantly with those in ICP [17]. Furthermore, in some experimental situations such as orchiectomy and testosterone replacement [18] or repeated intracavernous prostaglandin (PG) E_1 injections [19], the NO and ICP responses to cavernous nerve stimulation correlate well with the levels of constitutive NOS isoenzymes (nNOS and the endothelial isoform eNOS) in penile homogenates. In the present study, these measures were used together with standard tests for mating behavior and erectile reflexes ex copula and with evaluation of androgenic status. Such a comprehensive assessment of the effects of streptozotocin (STZ)-induced diabetes and insulin replacement on sexual behavior and erectile function at behavioral and molecular levels should help to clarify the conflicting reports in the literature.

As pointed out previously, serum testosterone levels are reduced in rats with both STZ-induced [3, 5] and spontaneously occurring [4, 10] diabetes. Previous work from this and other laboratories has documented the androgenic dependence of NOS enzyme levels and activity in erectile tissues ([18] and references therein). Thus, it was considered of interest to carry out the study in animals with a mild degree of diabetes in which, according to preliminary observations, androgen levels seem to remain unaffected.

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This approach should help to elucidate whether the observed sexual effects are due to diabetes itself or are secondary to impaired androgenic function.

MATERIALS AND METHODS

Animals

The experiments were done in adult Sprague-Dawley rats reared at the University of La Laguna (ULL) Animal Facility. The animals were housed under a 12L:12D cycle with free access to standard rat chow and tap water.

All of the experimental procedures complied with national regulations for the Care and Use of Laboratory Animals (similar to the U.S. National Research Council guidelines) and were approved by the local Ethics Committee.

Treatments

The animals were injected with STZ (Sigma Chemical Co., St. Louis, MO) (40 mg/kg i.p.) or vehicle (0.1 M citrate buffer, pH 4.6). Preliminary observations in this laboratory have shown that this STZ dose results in a mild diabetic status with no evidence of decreased androgen levels. Starting 72 h after the injection, one half of the STZ-treated animals were given daily insulin (Humulina lenta, Lilly SA, Madrid, Spain) injections (1–3 U, s.c.) to maintain normal body weight and glucose levels in tail blood, both of which were monitored at weekly intervals. Each group consisted of 12 animals.

Behavioral Testing

Twelve weeks after the STZ or vehicle injection, the animals were tested first for mating behavior and then for ex copula penile reflexes 2 days later.

The mating tests were conducted under a dim red light at the onset of the lights-off period, as previously described [20, 21]. The animal was placed in the test arena; 5 min later, we introduced a female rat that had been injected s.c. with 20 μg estradiol benzoate (Sigma) and 500 μg progesterone (Sigma) at 48 and 4–6 h, respectively, before the test. The female was changed if no intromission occurred after 10 min. A total of 48 females were used for the training (before STZ treatment) and test sessions. The latency to the first mount, intromission, and ejaculation and the length of the subsequent postejaculatory interval as well as the frequency of mounts and intromissions corresponding to the first ejaculatory series were recorded.

Penile reflexes were tested as previously described [15, 22] by holding the awake animal in supine position inside a plastic cylinder and keeping the penile sheath retracted to extrude the glans. The latency to the first erection and number of penile responses including erections, erections with a flared glans (cups), and dorsal extensions of the penis (flips) were recorded for 30 min.

Surgical Procedures

The day after the penile reflexes test, the animals were anesthetized with urethane (1 g/kg i.p.) and placed on a homeothermic blanket to keep body temperature at 37°C. A polyethylene cannula was inserted into the trachea. The penile skin was removed, and the abdomen was opened by a midline suprapubic incision to allow access to the corpora and the cavernous nerves, respectively.

Pressure Recordings

A 23-gauge needle was inserted into the corpora pointing toward the base of the penis to monitor the ICP. The arterial pressure was measured through a carotid line. The arterial and cavernosal catheters were connected via PE50 tubing filled with heparinized saline (200 U/ml) to Statham P23Db pressure transducers (Statham, Hato Rey, Puerto Rico). The amplified signals were digitized with an FPC-011 interface (Flight-Tech, Southampton, U.K.) at a rate of 4 sec^{-1} and stored in a PC-compatible computer.

Electrostimulation of the Cavernous Nerve

The major pelvic ganglion was identified on one side of the dorsolateral prostate and used as a landmark for placing the stimulating electrode on the emerging cavernous nerve. A bipolar platinum electrode connected to a Grass S48 square wave stimulator (Grass Instrument Co., West Warwick, RI) was used to deliver 1-msec pulses of 6 V at 12 Hz for 1 min as previously described [17–19].

Voltammetric Recordings

Differential normal pulse voltammetry (DNPV) with carbon fiber microelectrodes covered with a polymeric porphyrin film and coated with Nafion was performed as previously described [17–19]. Briefly, the working electrode consisted of a carbon fiber (30 μm in diameter, 500 μm in length) that was coated by electrodeposition with a polymeric film of Ni (II) tetrakis (3-methoxy-4-hydroxyphenyl) porphyrin (Interchim, Montlucon, France) and by subsequent dipping into 5% Nafion (Aldrich Chemical Co., Milwaukee, WI). These electrode coatings are applied, respectively, to enhance the NO signal and to exclude interfering anions such as nitrite.

The working electrode was mounted into a telescopic carrier assembly (Unimécanique, Épinay-sur-Seine, France) so that the electrode could be inserted through the tunica albuginea. The microelectrode could thus be placed undamaged into the cavernosal space. The auxiliary (platinum) and reference (Ag/AgCl) electrodes were inserted into nearby abdominal muscles.

Voltammetric recordings were made at 100-sec intervals with a microprocessor-controlled potentiostat system (Bioelectrochemical Analyzer; ULL, Spain). The following DNPV parameters were used: potential range, −100 to 1000 mV; scan rate, 20 mV/sec; pulse amplitude, 40 mV; pulse duration, 40 msec; and prepulse duration, 50–120 msec. Under these conditions, NO shows a measurable oxidation peak at approximately 650 mV. Details of the electrochemical procedures, electrode calibration, signal specificity, and pharmacologic characterization of the NO signals have been given elsewhere [17].

Biochemical Analyses Ex Vivo

After the ICP and electrochemical recordings were obtained, the animals were given a lethal injection of the anesthetic. Blood was collected from the abdominal aorta to measure serum testosterone levels by ELISA [18]. The corpora cavernosa were dissected out, homogenized, and assayed for both the constitutive (nNOS, eNOS) and inducible (iNOS) NOS proteins by Western blot analysis [18, 19].

Data Processing

Data from the three groups studied were compared by one-way ANOVA and post hoc Newman-Keuls tests with Graphpad Prism software (San Diego, CA). To this aim, the data describing the NO responses to cavernous nerve stimulation were summarized as area under the curve (AUC) as determined by the trapezoid rule. The AUC was defined as change over baseline values (as percentage of basal NO level) × time (20 min from onset of stimulation, including 12 NO recordings). The baseline value was defined as the average value of the three recordings preceding the nerve stimulation. The ICP responses were expressed as maximal values recorded during the electrical field stimulation.

RESULTS

As shown in Table 1, serum glucose levels of the STZ-treated animals rose to approximately three times the control group values and were restored apparently by insulin replacement. The final body weight decreased in the diabetic animals and was preserved in the diabetic animals given insulin. No differences in serum testosterone levels were observed between the three groups. Likewise, the weights of the ventral prostate and seminal vesicles, expressed as a percentage of body weight, of both the diabetic and the insulin-treated diabetic animals were similar to those of the control animals.

The number of animals displaying copulatory activity in the mating test was considerably lower in the diabetic group than in the control group (42% vs. 100%, $P < 0.05$); this value increased up to 75% in the insulin-treated group (P not significant vs. control). Furthermore, those diabetic animals still copulating showed substantial deficits in all of the standard measures of the mating test, and these deficits were reversed by insulin treatment (Fig. 1). In the penile

TABLE 1. Effects of STZ diabetes and insulin replacement on body and sex accesory organ weights and serum glucose and testosterone levels.*

	Final body weight (g)	Ventral prostate (mg/100 g BW)	Seminal vesicles (mg/100 g BW)	Serum glucose (mmol/L)	Serum testosterone (nmol/L)
Intact control	410 ± 15	129 ± 12	132 ± 11	4.72 ± 0.55	11.79 ± 2.08
Diabetic	320 ± 20†	131 ± 10	114 ± 10	15.54 ± 0.83†	10.75 ± 2.43
Diabetic + insulin	398 ± 22	138 ± 16	125 ± 12	6.66 ± 0.66	11.44 ± 1.73

* Values are expressed as mean ± SEM; n = 12 animals per group.
† $P < 0.001$ vs. intact control and diabetic + insulin groups.

reflexes test, the diabetic group also displayed fewer erections, flips and cups and a longer latency to the first erection, but insulin replacement had no significant effect (Fig. 2).

The basal NO signals recorded in the corpora of diabetic animals were considerably reduced, and this reduction was fully reversed by insulin treatment (Fig. 3A). The diabetic group also had a much attenuated NO response to cavernous nerve stimulation, which was restored by insulin replacement to approximately 50% of the control values (Fig. 3, B and C). The ICP response was decreased in the diabetic group relative to the control group; in contrast, the ICP response in the insulin-treated diabetic group was similar to that in the control group (Fig. 3D). The arterial pressure recordings (not shown) were similar in the three groups of animals.

No significant changes in the protein content of the three NOS isoenzymes measured (nNOS, eNOS, iNOS) were observed in the corpora of diabetic animals with or without insulin replacement (data not shown).

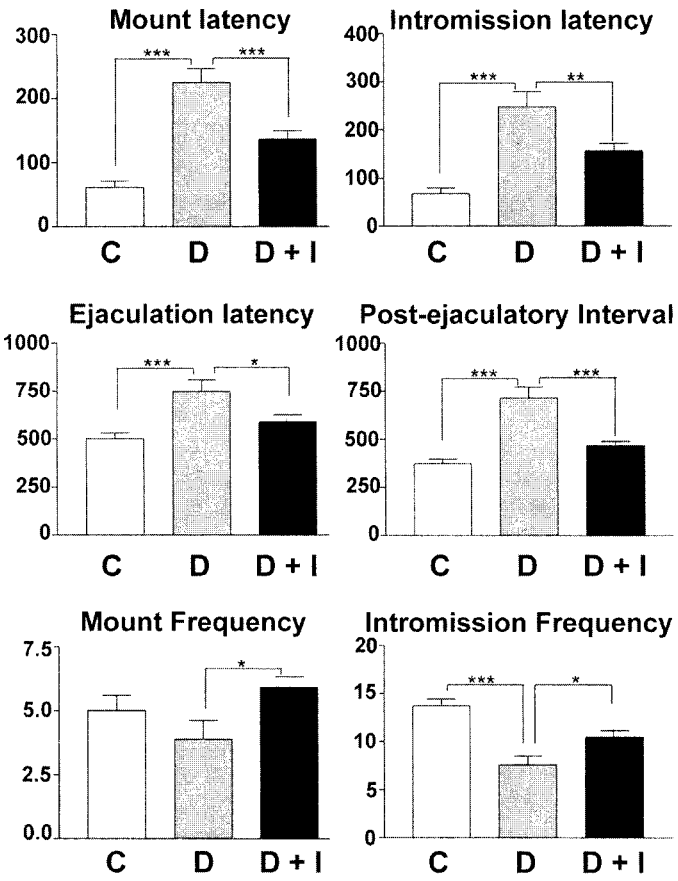

FIG. 1. Effects of STZ-induced diabetes and insulin replacement on measures of copulatory behavior. C, Intact control (n = 12); D, diabetic (n = 5); and D + I, diabetic with insulin (n = 9). Latencies are expressed in seconds, and frequencies are expressed as number of events. Values are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

DISCUSSION

This is the first description of changes in the actual levels of NO in the corpora cavernosa of diabetic animals with or without insulin replacement. The present data show a greatly decreased corporeal NO concentration in STZ-induced diabetes as observed both in basal recordings and in recordings obtained after the stimulation of the cavernous nerve. This finding confirms previous suggestions based on more indirect data. Thus, it is consistent with previous reports describing decreased NOS activity as measured ex vivo by the citrulline assay [10, 12] or NADPH diaphorase staining [13] in the penises of diabetic rats. All of these findings support the notion that impaired NO production is a main cause of the erectile failure associated with diabetes.

The present study also shows that insulin replacement fully reverses the basal NO signals recorded in the corpora and partially restores the NO response to electrical field stimulation to about one half of the control levels. Yet the ICP responses to nerve stimulation in the insulin-treated animals were similar to those in the control animals. A possible explanation for this apparent discrepancy is that the partial restoration of the stimulated NO release found in the insulin-treated diabetic rats was sufficient to yield a normal ICP response. Thus, it is likely that with the standard parameters commonly used for electrical field stimulation (the parameters used in the present experiment) the nitrergic nerves are supramaximally stimulated. In other words, the amount of NO released in normal animals exceeds what is needed for eliciting the ICP response. This

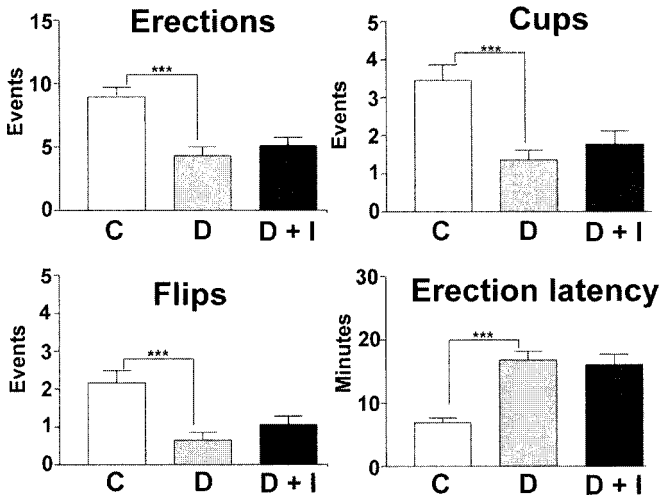

FIG. 2. Effects of STZ-induced diabetes and insulin replacement on erectile reflexes ex copula. C, Intact control (n = 12); D, diabetic (n = 9); D + I, diabetic with insulin (n = 10). Values are expressed as mean ± SEM. ***$P < 0.001$.

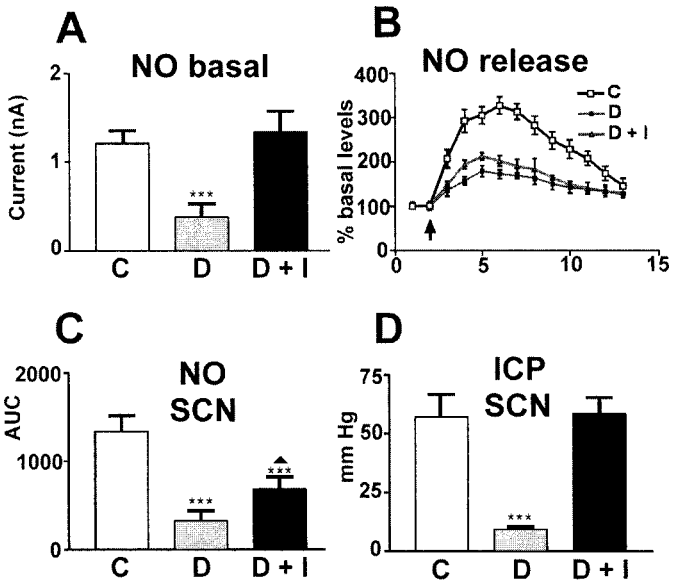

FIG. 3. Effects of STZ-induced diabetes (D) and insulin replacement (D + I) on **A**) basal levels of NO oxidation current recorded in the corpus cavernosum penis; **B**) changes in NO levels after electrostimulation (arrow) of the cavernous nerve (SCN); **C**) the overall NO response to SCN expressed as area under the curve (AUC); **D**) maximal values of intracavernous pressure (ICP) recorded during SCN. Values are expressed as mean ± SEM; n = 12 animals per group. ***$P < 0.001$ vs. control group, ▲$P < 0.001$ vs. diabetic group.

postulate is supported by our previous studies showing that the increases in NO levels after cavernous nerve stimulation found in normal rats [17], in testosterone-treated castrates [18], and in animals given intracavernous injections of PGE_1 or its vehicle [19] long outlive the changes in ICP. In all of these studies, NO levels were still rising to maximal values after electrical field stimulation stopped, when the ICP values had returned to baseline levels. This issue should be clarified in future studies by using nerve stimulation parameters closely mimicking the physiologic firing pattern of the cavernous nerves, which is unknown at present.

In the present study, no changes were found in the protein levels of any of the three NOS isoenzymes in the corpora of diabetic animals. This is at variance with the results of other reports describing diabetes-related decreases in nNOS protein content [10–13] and nNOS and iNOS mRNA expression [13]. A likely reason for this discrepancy is that the smaller dose of STZ used in our experiment resulted in diabetes that was milder than that in the aforementioned studies. Thus, in the preceding reports showing decreased NOS protein content in diabetic rats, the rats' blood glucose levels were considerably higher than those in the present work.

It is noteworthy that in the one previous report that described low nNOS protein levels in the penis of diabetic rats and assessed androgen levels, the serum testosterone concentration was found to be markedly reduced [10]. As shown by our previous work, the protein content of the main NOS isoforms (nNOS and eNOS) and NO levels in the rat penis are greatly dependent on serum androgen levels [18]. It is thus likely that an impaired androgenic function in severe diabetes could account for the low NOS isoenzyme levels found in the previously cited reports. In the present study, however, the normal steady state levels of NOS isoforms and plasma androgens found in mild diabetes were associated with reduced corporeal NO levels and impaired ICP responses to cavernous nerve stimulation. These data suggest that in diabetes, the loss of NOS enzyme activity in the corpora precedes the loss of NOS proteins. Thus, a relatively mild diabetes would only affect the enzyme activity, whereas a more severe diabetes would also decrease the amount of NOS proteins, possibly as a consequence of low androgen levels. This interpretation is also consistent with the previous observation that in type 1 and type 2 spontaneously diabetic rats, the reduction in nNOS protein content is considerably less marked than the reduction in NOS activity [10].

Regarding the possible mechanisms for the observed decrease in NO levels in the corpora of diabetic animals, it should be noted that NOS enzymatic activity requires a substrate (L-arginine) and several redox cofactors such as NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH4), the availability of which can be decreased in diabetes. For example, the impaired endothelial function present in diabetic rats has been found to improve after treatments with either L-arginine [23, 24] or BH4 analogues [25]. An increased production of oxygen free radicals, as found in the aorta of diabetic rats, could also account for the reduction in NO levels since superoxide anions have been reported to inactivate the NO produced by the endothelium [26]. Thus, our finding of decreased NO levels, both at baseline and after stimulation of the cavernous nerve, in spite of normal steady state levels of NOS isoforms in the corpora cavernosa of diabetic animals could reflect a deficit of substrate or some of the previously mentioned essential cofactors for NOS activity and/or increased oxidative stress.

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