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The Effect of Aging and Exogenous Testosterone Replacement on Nitric Oxide Concentration and Activity of Nitric Oxide Synthase in the Rat Corpus Cavernosum

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The effects of testosterone replacement on the nitric oxide (NO) concentration and activity of NO synthase (NOS) in the penis were investigated. Male Wistar rats (*n* = 39) were divided into 5 groups: 14-week-old males, 13-month-old males, 15-month-old males, 15-month-old males treated with low-dose testosterone replacement and 15-month-old males treated with high-dose testosterone replacement. The testosterone concentration in serum and the NO concentration in penile tissue were measured, and the endothelial NOS (eNOS) and neuronal NOS (nNOS) expressions were examined immunohistochemically. The testosterone concentration in serum tended to decrease with aging, but the 15-month-old testosterone-replaced rats maintained almost the same level as the 14-week-old rats. Nitrite and nitrite/nitrate concentrations in penile tissue tended to decrease with aging. Nitrite concentrations in the 15-month-old rats were significantly higher in the testosterone-replaced groups than in the non-replaced group, but no significant difference in nitrite/nitrate concentration was recognized between the 15-month-old rats not treated and treated with testosterone replacement. Immunohistochemical staining for eNOS and nNOS demonstrated a decreasing expression of the 2 NOSs with aging and recovering of the NOSs by testosterone replacement. The results of this study suggest that NO plays a major role in the mediation of penile erection, and testosterone replacement may favorably alter age-related erectile dysfunction.

Key words: aging; immunohistochemical staining; nitric oxide; testosterone replacement

Recently, several studies have revealed that nitric oxide (NO) is an important neural messenger which mediates penile erection (Ignarro et al., 1990; Holmquist et al., 1991; Kim et al., 1991; Burnett et al., 1992; Rajifer et al., 1992). Erection is mediated by the release of NO from non-adrenergic non-cholinergic nerve terminals, the endothelium of penile blood vessels, and corporal smooth muscle, producing smooth muscle relaxation and vasodilation (Burnett, 1997). NO stimulates the formation of guanylate cyclase in smooth muscle cells, converting GTP to 3'5'-cyclic GMP (cGMP) (Burnett, 1997). A cascade of cGMP-dependent intracellular events then leads to a decrease in intracellular calcium, ultimately causing smooth muscle relaxation, in part through changes in potassium conductance (Seftel et al., 1996; Burnett, 1997).

The production of NO is mediated by a family of NO synthase (NOS) that all represent distinct gene products. These enzymes produce NO through a complex set of redox reactions that result in the conversion of L-arginine to L-citrulline. The isoforms of NOS have been categorized as being either inducible or constitutive (Forstermann et al., 1995). The inducible isoform of NOS (iNOS) is associated primarily with macrophages and is activated by specific cytokines as part of the immune response. The endothelial (eNOS) and neuronal (nNOS) isoforms of NOS are constitutive and are activated,

Abbreviations: eNOS, endothelial NOS; iNOS, inducible NOS; mRNA, messenger RNA; nNOS, neuronal NOS; NO, nitric oxide; NOS, NO synthase; PBS, phosphate-buffered saline

in part, by an increased concentration of intracellular calcium and calmodulin binding to the enzyme. Experimental evidence suggests that the constitutive isoforms of NOS may be responsible for NO production in penile erection (Bush et al., 1992; Burnett et al., 1993). Recent evidence suggests that eNOS and its subsequent production of NO may be a significant route by which NO-mediated cavernosal relaxation is brought about because transgenic mice lacking nNOS are still capable of erectile activity with pelvic nerve stimulation (Burnett et al., 1996).

A decrease in the serum testosterone level with aging may contribute to a reduction of sexual potency (Meisel and Sachs, 1994; Garban et al., 1995), although this reduction with aging is a multi-factorial phenomenon.

A recent study also hypothesized that androgens maintained and facilitated male sexual potency through enhancement or maintenance of NOS activity in the corpus cavernous tissue in the penis (Mills et al., 1996). In addition, several studies have shown that androgen replacement facilitated neural activities in some areas of the brain which mediate sexual function (Okamura et al., 1994a, 1994b; Pu et al., 1996).

In this article, the changes in the serum tes tosterone concentration, NO concentration and NOS protein expression in penile tissue were examined by using the rat as our model of aging, and the changes brought about by testosterone replacement, as well. The goal in this study was to evaluate whether testosterone replacement helps to resolve age-related erectile dysfunction.

Materials and Methods

Subjects used were 14-week-old (G1, n = 10), 13-month-old (G2, n = 5) and 15-month-old male Wistar rats (n = 24) (SLC, Shizuoka, Japan). All rats were housed in a room with controlled lighting and allowed access to food and water ad libitum. The 15-month-old rats were divided into 3 groups as follows: no testosterone replacement group (G3, n = 8), low-dose testosterone replacement group (G4, n = 8) and high-dose testosterone replacement group (G5, n = 8). In the replacement groups, Silastic tubes (Dow Corning, Midland, MI; outer diameter, 3.17 mm; inner diameter, 1.57 mm) which were 3 cm in length, and contained 40 mg of testosterone powder (Sigma, St. Louis, MO) were subcutaneously implanted in the backs of the rats when they were anesthetized with pentobarbital (30 mg/kg) at the age of 13 months, and the replacement was continued for 2 months. In G4, 4 tubes were implanted and in G5, 8 tubes were implanted. As each group reached the age desired for the experiment as mentioned above, blood (7–8 mL) was collected through the inferior vena cava of each rat under pentobarbital (30 mg/kg) anesthesia, and the serum was separated by centrifugation and stored at –80˚C until it was assayed. Total testosterone and free testosterone concentrations in serum were determined by radioimmunoassay (Total Testosterone Kit and Free Testosterone Kit, DPC Corp., Tokyo, Japan). Rats were killed in succession by means of additional pentobarbital (30 mg/kg), and the penis was removed and weighed. The penis was divided into 2 pieces, and one was used for measurement of NO concentration and the other was used for immunohistochemistry.

Measurement of NO concentration in penile tissue

The final products of NO in vivo are nitrite and nitrate. The relative proportion of nitrite and nitrate is variable and cannot be predicted with certainty. Thus, the best index of total NO production is the sum of both nitrite and nitrate.

NO was measured by means of the Griess method. The penile tissue was weighed and homogenized in phosphate-buffered saline (PBS) (pH 7.4) and centrifuged at 10,000 × g for 20 min. The supernatant was ultracentrifuged at 100,000 × g for 15 min, and then the supernatant was ultrafiltered using a 30 kDa molecular weight cut-off filter. The sample was assayed by means of a nitrate/nitrite colorimetric assay kit (Cayman Chemical Co., Ann Arbor, MI). The values were estimated per tissue weight and

Table 1. Body and penile weight, and testosterone concentration in serum

	Young adult rats	Middle-aged rats			
	14-week-old	13-month-old	15-month-old		
	G1	G2	G3	Testosterone-replaced	
	[10]	[5]	[8]	Low-dose G4 [8]	High-dose G5 [8]
Body weight (g)	367 ± 14	596 ± 59	562 ± 39*	554 ± 19	587 ± 47
Penis weight (mg)	394 ± 40	659 ± 73	596 ± 42*	621 ± 45	621 ± 38
Total testosterone (ng/mL)	2.0 ± 0.4	1.0 ± 0.2	0.8 ± 0.1*	3.7 ± 0.4**	5.0 ± 1.1**
Free testosterone (pg/mL)	8.3 ± 1.9	5.2 ± 0.8	4.2 ± 1.2*	14.5 ± 3.6**	20.0 ± 6.7**

Values represent mean ± SEM.
[ ], number of animals.
Significant difference ($P < 0.05$): *G3 versus G1; **G4 or G5 versus G3.

per amount of protein in the tissue. Protein was determined using a commercial kit (BCA Protein Assay Reagent Kit, Pierce, Rockford, IL).

eNOS and nNOS immunohistochemistry in penile tissue

The penis was immediately fixed with neutral buffered 15% formaldehyde-saline. The tissues were embedded in paraffin after the fixation. Sections (3 μm) were subjected to immunohistochemical stains for eNOS and nNOS. The sections were retrieved by micro-wave and treated with 3% methanol/hydrogen peroxide for 15 min at room temperature to reduce background staining. They were then reacted with 200 μg/mL of rabbit anti-eNOS or nNOS polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:5000 dilution in Dako antibody diluent (Dako, Carpenteria, CA) overnight at 4˚C. Sections were washed with PBS and the specifically bound first antibodies were visualized by means of biotinylated anti-rabbit secondary antibody and with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Again, sections were washed with PBS and incubated for 30 min with horseradish peroxidase-labeled streptavidin at room temperature. After the incubation period, tissue sections were washed and a diaminobenzidine peroxidase substrate solution (Vector) was applied for 5 min. The reaction was stopped by washing the sections in water. Mayer's hematoxylin was used for counterstaining.

Statistical analysis

Experimental values were expressed as mean ± SEM for the number of separate determinations indicated in each case. The non-parametric *t*-test was used for calculating probabilities when comparing 2 groups independent from the others, and those with *P* values less than 5% ($P < 0.05$) were considered significant.

Results

Body weight and penile weight

Among the group of 15-month-old rats, body weight and penile weight of rats treated without testosterone replacement (G3) were not significantly different from those treated with testosterone replacement groups (G4 and G5) (Table 1).

Serum testosterone concentration

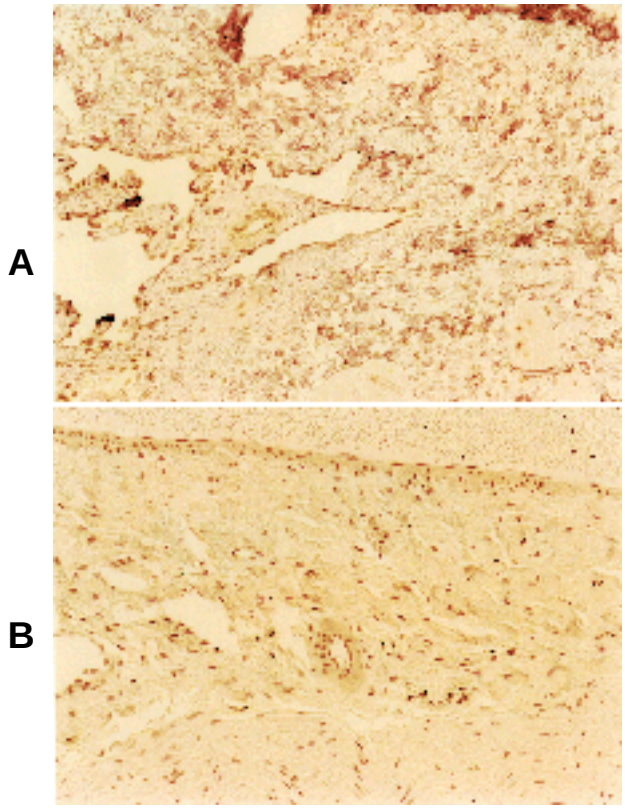
The total testosterone level tended to decrease with aging, but the level in the 15-month-old rats without testosterone replacement (G3) was significantly lower than that in 14-week-old rats (G1). Among 15-month-old rats, the rats with

Table 2. Nitrite and nitrite/nitrate concentration in penile tissue

	Young adult rats	Middle-aged rats			
	14-week-old	13-month-old	15-month-old		
	G1	G2	G3	Testosterone-replaced	
	[10]	[5]	[8]	Low-dose G4 [8]	High-dose G5 [8]
Nitrite† (μmol/g wt)	17.3 ± 3.4	16.2 ± 3.4	11.0 ± 1.9	31.5 ± 3.6*	27.0 ± 6.6*
Nitrite‡ (nmol/mg pro)	1.11 ± 0.24	1.05 ± 0.22	0.54 ± 0.05	2.59 ± 0.73*	1.61 ± 0.50*
Nitrite/nitrate† (μmol/g wt)	48.4 ± 7.1	42.1 ± 5.2	40.6 ± 4.3	43.4 ± 6.7	49.8 ± 10.4
Nitrite/nitrate‡ (nmol/mg pro)	3.28 ± 0.53	2.47 ± 0.33	2.32 ± 0.23	3.27 ± 0.54	3.10 ± 0.66

†Per tissue weight (μmol/g weight).
‡Per amount of protein in the tissue (nmol/mg protein).
Values represent mean ± SEM.
[ ], number of animals.
pro, protein; wt, weight.
Significant difference ($P < 0.05$): *G4 or G5 versus G3.

low- and high-dose testosterone replacement (G4 and G5) showed significantly higher levels of total testosterone than the rats without replacement (G3), but they did not have significantly higher levels than 14-week-old rats (Table 1). The free testosterone level in 15-month-old rats without testosterone replacement was significantly lower than that in 14-week-old rats. Among 15-month-old rats, the free testosterone level in the low- and high-dose testosterone replacement groups was significantly higher than that in the non-replacement group (Table 1).



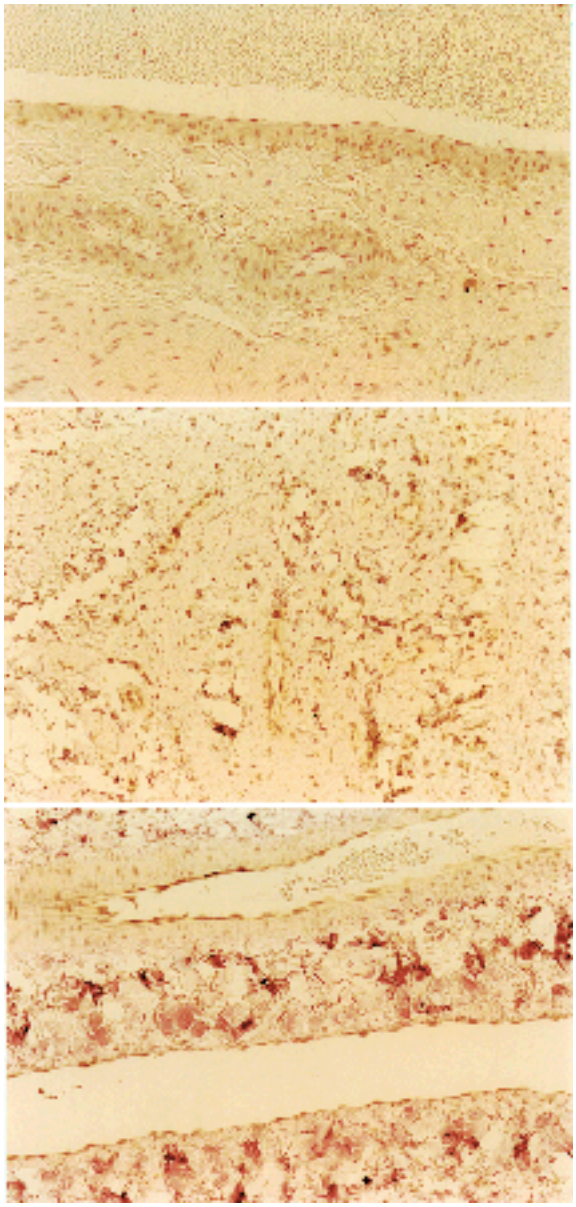
A: G1, young adult rat *(14-week-old)*
B: G2, middle-aged rat *(13-month-old)*

Fig. 1. Sections of rat corpus cavernosum tissue immunostained by ant-endothelial nitric oxide synthase (eNOS) antibodies (original magnification × 200). Sections are counterstained with hematoxylin.
A: Immunohistochemical localization of eNOS shows strong staining of the vascular endothelium.
C: Aged rats without replacement have weak staining.
D and E: Testosterone replacement restored the same level of staining as in the young adult group.
[Figs. 1A and B on p. 48 and Figs. 1C–E on p. 49]

Nitrite and nitrite/nitrate concentration in penile tissue

Nitrite level

This level tended to decrease with aging, but there was no significant difference between 14-week-old rats (G1) and 15-month-old rats (G3). Among 15-month-old rats, the low- and high-dose testosterone replacement rats showed a significantly higher nitrite level per amount of protein in the tissue and per tissue weight than rats without replacement. There was no significant difference in nitrite levels between the high- and the low-dose replacement rats (Table 2).



C: G3, middle-aged rat
D: G4, low-dose testosterone replaced middle-aged rat
E: G5, high-dose testosterone replaced middle-aged rat
(G3–G5: 15-month-old)

Figs. 1C–E. *Continued from the previous page.*

Nitrite/nitrate level

This level tended to decrease with aging, but there was no significant difference between 14-week-old rats and 15-month-old rats without testosterone replacement. The nitrite/nitrate level increased with testosterone replacement; however, the difference was not significant between 15-month-old rats treated with and without testosterone replacement (Table 2).

eNOS and nNOS immunohistochemistry in penile tissue

Figures 1 and 2 show light microscopic photographs of the corpus cavernosum from G1 to G5. Immunohistochemical localization of eNOS showed strong staining of the vascular endothelium in 14-week-old rats (G1) (Fig. 1A).

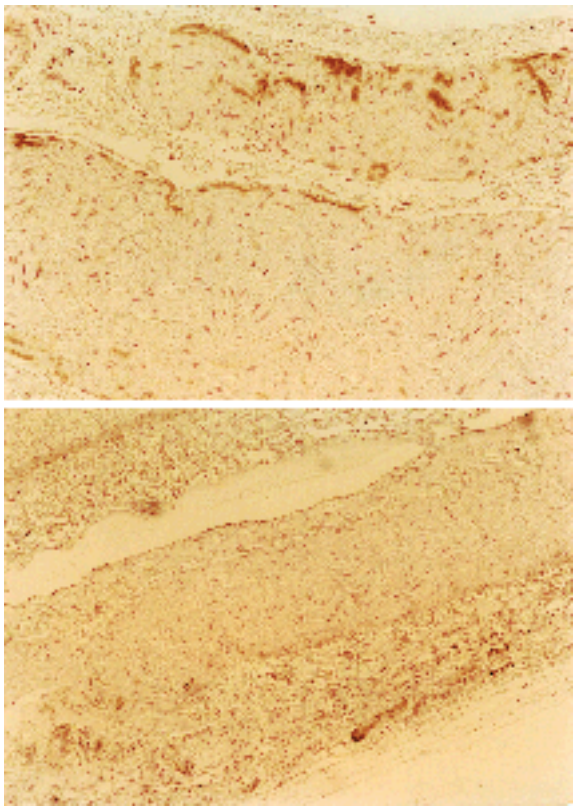
Thirteen-month-old (G2) and 15-month-old rats without testosterone replacement (G3) had weak staining (Figs. 1B and C), and testosterone replacement restored the level of staining (Figs. 1D and E). Immunohistochemical stainings in 14-week-old rats, 15-month-old rats with low- and high-dose testosterone replacement rats were almost at the same level. Immunohistochemical staining with anti-nNOS

polyclonal antibody revealed the presence of nNOS in the nerves. The staining in 14-week-old rats was strong (Fig. 2A), and the staining in 15-month-old rats with testosterone replacement was almost the same in level as in 14-week-old rats (Figs. 2D and E). In 15-month-old rats without testosterone replacement, nNOS was weakly positive (Fig. 2C).

Discussion

The results of this study using young adult and middle-aged rats showed that there was an age-related decrease in serum testosterone concentration, NO level and NOS expression in the corpus cavernosum, and moreover, that in middle-aged rats with 2 months of testosterone replacement which was continued until the rats reached the age of 15 months, those levels were restored to the level in young adult rats. Several reports show that a low testosterone level decreases penile erection and treatment with testosterone restores it (Mills et al., 1992; Heaton and Varrin, 1994; Meisel and Sachs, 1994; Garban et al., 1995). One important question is what is the role of testosterone by itself in the maintenance of penile erection. The author has significantly extended these previous findings by demonstrating that the most likely mechanism of penile dysfunction is an aging-induced reduction in the level of penile NO and NOS, a reduction which is prevented by testosterone.

Recently, several physiological studies (Holmquist et al., 1991; Burnett et al., 1992) of electrically induced erections as well as pharmacological studies (Ignarro et al., 1990; Kim et at, 1991; Rajifer et al., 1992) have indicated that NO plays a major role in the mediation of penile erection. Penile erection results from relaxation of the corporal smooth muscle and penile cavernosal arteries. NO mediates this process as supported by evidence showing that NO has a direct vasodilatory effect on cavernosal tissue and that relaxation of penile smooth muscle is prevented by NOS inhibitors (Krane et al., 1989; Seftel et al., 1994; Burnett, 1997). The production of NO is mediated by a family of NOS that all represent distinct gene products (Forstermann et al., 1995). There are 3 iso-



A: G1, young adult rat *(14-week-old)*
B: G2, middle-aged rat *(13-month-old)*

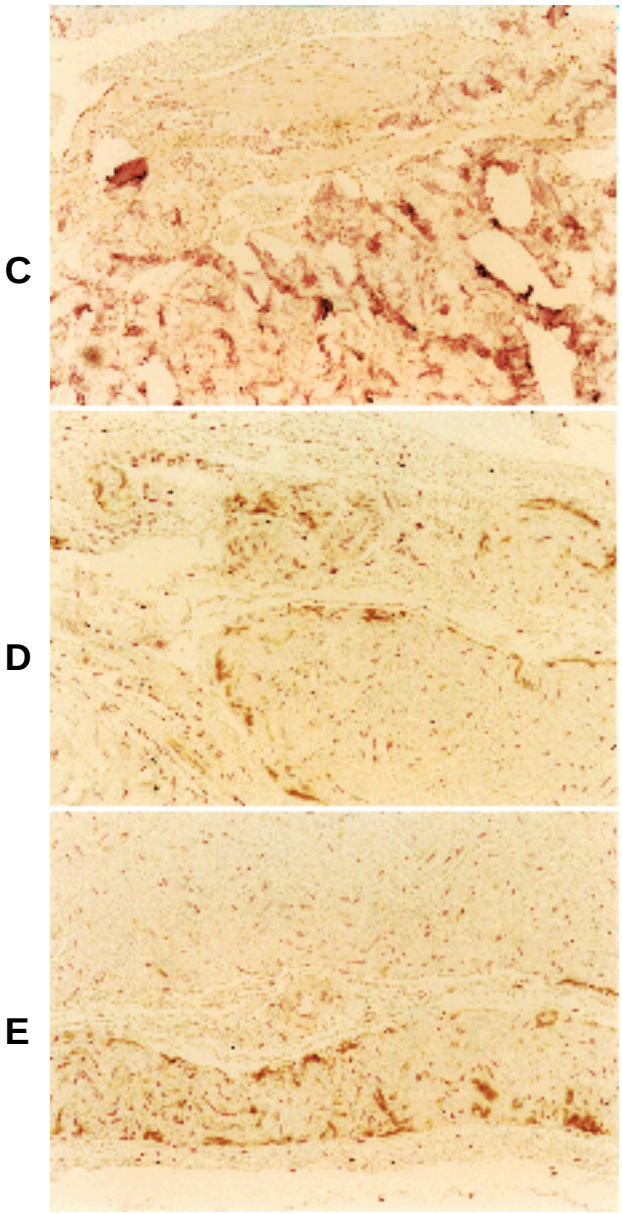
Fig. 2. Sections of rat corpus cavernosum tissue immunostained by ant-neuronal nitric oxide synthase (nNOS) antibodies (original magnification × 200).
A, D and E: Nerve fibers are stained at the same level in G1, G4 and G5.
C: Staining is weaker than that of the young adult group.

[Figs. 2A and B on p. 50 and Figs. 2C–E on p. 51]

forms, 2 constitutive and 1 inducible, which differ in their dependence on intracellular calcium.

Of the constitutive isoforms, eNOS was first identified in bovine aortic endothelial cells and appears to play an important role in the transduction of signals from the bloodstream to the underlying smooth muscle which causes vasorelaxation (Lamas et al., 1992; Sessa et al., 1992). The expression of eNOS protein and mRNA has been examined in several disease processes. Increased expressions of eNOS mRNA and protein has been found in atherosclerotic vessels and in response to shear stress as well as estrogen (Kanazawa et al., 1992; Nishida et al., 1992; Weiner et al., 1994). Increased eNOS activity has been found in causes of renal failure, whereas decreased activity has been found in pulmonary hypertension (Weiner et al., 1994; Conger et al., 1995). These suggest that the eNOS expression is varied and that its expression is a function of the offending disease process. eNOS has been thought to be a putative key enzyme of penile erection in that acetylcholine stimulates the release of endothelium-derived relaxation factors from intact rabbit cavernosal smooth muscle cells (Seftel et al., 1994) and also induces the relaxation of penile smooth muscle, a condition which is reversed by NOS inhibitors (Kim et al., 1991). Furthermore, impaired endothelium-dependent corporal smooth muscle relaxation has been found in diabetic men with erectile dysfunction (Saenz et al., 1989). The recent immunohistochemical localization of eNOS in the endothelial layers of the dorsal penile arteries, veins and corporal sinusoids places the enzyme in a physiologically critical location for mediating penile erection (Burnett et al., 1996).

nNOS has been localized in the rat penile autonomic nerves, the adventitia of rat penile arterioles, and the autonomic innervation of the human penis (Burnett et al., 1993). It has also been reported that there are



C: G3, middle-aged rat
D: G4, low-dose testosterone replaced middle-aged rat
E: G5, high-dose testosterone replaced middle-aged rat
(G3–G5: 15-month-old)

Figs. 2C–E. *Continued from the previous page.*

numerous NOS-containing nerve fibers and terminals innervating corpus cavernosum smooth muscles and vessels in the spaces of the corpus cavernosum.

The presumed role of nNOS in penile erection has been studied in rats by using electrical stimulation of the pelvic nerves, which caused penile erection that was prevented by the NOS inhibitor L-nitroarginine methyl ester (Burnett et al., 1992, 1996; Bush et al., 1992). The NOS activity (presumed to be nNOS) has been indirectly measured in penile tissue homogenates by using a [^{3}H]-L-arginine to [^{3}H]-citrulline conversion assay, which, along with Western blotting analysis, showed a decrease in penile NOS activity and penile nNOS protein content in diabetic rats with erectile dysfunction (Vernet et al., 1995). A recent demonstration that nNOS-deficient transgenic mice maintain a normal penile erection throughout eNOS-dependent production of NO suggests that eNOS may also have an important primary and compensatory role in mediating penile erection (Burnett et al., 1996).

Previous studies have shown a decreased penile NOS activity in models of erectile dysfunction, such as hypogonadism, androgen receptor blockade and diabetes (Burnett et al., 1992). Our model of middle-aged rats had a low level of the penile NOS expression. Lugg et al. (1995) reported that in rats, aging induced a considerable reduction in the erectile response to electric field stimulation which was accompanied by a decrease in NOS activity in very old rats. Several reports have shown that a low testosterone level decreases penile erection and testosterone restores it (Mills et al., 1992; Heaton and Varrin 1994; Meisel and Sachs, 1994; Garban et al., 1995).

The results of this study showed that total and free testosterone levels tended to decrease with aging, simultaneously with the penile NO concentration and NOS expression. Testosterone replacement restored these levels. As mentioned above, testosterone may play a major role in the mediation of penile erection by affecting the pathway relaxing the corpus cavernosum smooth muscle in a manner that is dependent on the NOS activity. It is believed that testosterone replacement might be an effective method of treating erectile dysfunction that occurs with aging.

In summary, the serum testosterone concentration, penile NO concentration and NOS expression decreased with aging and were restored by testosterone replacement. According to the literature, NO plays a major role in the mediation of penile erection, and the present results suggest that testosterone replacement might be an effective method of treating erectile dysfunction that occurs with aging.

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