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Androgenic Regulation of NO Availability in Rat Penile Erection

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ABSTRACT: Prior studies from this laboratory, using untreated castrated (CASTRATE) rats and testosterone-treated castrated (TESTO) rats, have shown that the magnitude of the intracavernosal pressure increase during erection is androgen dependent. Studies from this and other laboratories have also presented evidence suggesting that penile erection is mediated principally by nitric oxide (NO). The present report was designed to confirm that androgens maintain the availability of cavernosal NO and to determine if this androgenic action is exerted at the genomic level modulating the expression of the neuronal form of the nitric oxide synthase gene (nNOS). The results showed that administration of supplemental L-arginine failed to augment the erectile response in either group, suggesting that substrate availability is not a cause of the reduced response in CASTRATE animals. Inhibition of NO synthesis with a nitro-arginine competitive inhibitor of nitric oxide synthase enzyme protein (NOS) resulted in strong inhibition of erection in both TESTO and CASTRATE rats. When given in conjunction with ganglionic stimulation to induce erection, the NO releasing drug, sodium nitroprusside (SNP), increased intracavernosal pressure in CASTRATE rats but not in TESTO rats, suggesting a deficiency of the available NO in CASTRATE animals. Finally, reverse transcription-polymerase chain reaction (RT-PCR) demonstrated that mRNA levels for the enzyme nNOS in the penis were greater in TESTO animals than in CASTRATE rats. These results support the hypothesis that androgens mediate the erectile response in the rat penis by stimulating the expression of the neuronal isoform of nitric oxide synthase, thus maintaining an adequate supply of NO.

Key words: Rats, testosterone, nitric oxide.

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Studies from this and other laboratories, have demonstrated that erection in the rat is androgen dependent and is mediated by nitric oxide (NO) (Andersson and Holmquist, 1994; Burnett, 1995; Lugg et al, 1995a). Result from several groups support the hypothesis that the primary action of androgens is to stimulate the synthesis of NO (Mills et al, 1992, 1994; Chamness et al, 1995; Garban et al, 1995a,b; Lugg et al, 1995b; Zvara et al, 1995); specifically the neuronal isoform of the nitric oxide synthase gene (nNOS), which has been identified in nerve fibers innervating blood vessels and the corpus cavernosum of the penis (Burnett et al, 1992; Keast, 1992; Vizzard et al, 1994). These findings included the observation that the amount of nitric oxide synthase (NOS) enzyme protein was reduced in castrated animals when compared to animals with normal blood levels of testosterone (Chamness et al, 1995). However, none of these prior studies established the mechanism by which androgens stimulate an increase in NO. The present study includes experiments that were designed to confirm that NO is a principal neurotransmitter in the erectile response of the rat penis and to investigate two mechanisms by which androgens could elevate NO levels. These two mechanisms include an androgenic action to increase the availability of L-arginine substrate for the NOS reaction and an action of androgens to increase the expression of the nNOS.

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Materials and Methods

Animals

Male Holtzman rats (Harlan), 100–240 days old, weighing 400–600 g, were used in these studies. Each animal was castrated under ether anesthesia and a pellet of testosterone (50% testosterone: 50% cholesterol—group designation: TESTO) or cholesterol pellet alone (group designation: CASTRATE) was implanted subcutaneously. Animals were castrated and implanted with pellets 6–8 days before measurements were made. At the end of each experiment, blood was collected from the carotid artery for measurement of circulating levels of testosterone by radioimmunoassay (RIA) as previously described (Melner and Abney, 1980). Animals were maintained in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited laboratory facility with animal-use protocols and justifications approved by the Committee for Animal Use in Research and Education at the Medical College of Georgia.

Measurement of Intracavernosal Pressure

The procedure used to induce erection and measure intracavernosal pressure has been previously published (Mills et al, 1992, 1994). Briefly, the animals were anesthetized with ketamine (87 mg/kg) and xylazine (13 mg/kg) by intramuscular injections and supplemented with additional anesthesia as needed. Standard methods were used to cannulate the left carotid artery for continuously monitoring mean arterial blood pressure (MAP). The abdominal cavity was opened and the viscera retracted and wrapped in warmed saline-soaked sponges and cellophane wrap to prevent drying. The right major pelvic ganglion was exposed by clearing the overlying fascia. The shaft of the penis was dissected free of skin and fascia to expose the paired corpora cavernosa distal to the crura. The right corpus cavernosum was cannulated with a 30-gauge needle attached to PE 200 tubing to monitor intracavernosal pressure. A second cannula was inserted into the left corpus cavernosum for intracavernosal drug delivery. Stainless steel bipolar electrodes were then positioned on the major pelvic ganglion for ganglionic stimulation to induce erection. Intracavernosal pressure (CCP), mean arterial blood pressure (MAP) and duration, as well as frequency and amplitude of the ganglionic stimulation, were continuously recorded on a polygraph recorder. Results are expressed as the ratio of CCP/MAP. According to this method of expression, a ratio of 0 would indicate that there was no measurable intracavernosal pressure and the CCP/MAP ratio would be 1 if the CCP and MAP were equal. Stimulatory voltage was varied from 1 to 6 V (5 millisecond duration, 12 Hz frequency), and the CCP/MAP ratio at each voltage was compared to determine the threshold voltage and the minimum voltage required for the maximal response. A voltage of 5 or 6 V was used in subsequent aspects of each experiment. The duration of each ganglionic stimulation was 1 minute, with a 1-minute rest period between subsequent stimulations. Pressure transducers were calibrated with a mercury manometer prior to each use.

Intracavernosal Infusion of L-Arginine

To determine if substrate availability was limiting the erectile response in castrated animals, the response was measured after direct administration of L-arginine into the corpus cavernosum. Following a control measurement of erection, a single injection of L-arginine (25 μg/kg in 10 μl saline) was made into the right cavernous sinus and the erectile response was measured 5 minutes later. Additional L-arginine was given by continuous intraaortic infusion (50 μg/kg/minute infused at 20 μl/minute) with the erectile response measured after 5, 10, and 15 minutes of infusion.

Drug Administration

Intracavernosal injections were made of two drugs. Sodium nitroprusside (SNP): 8 μg/kg body weight in 1 μl saline was injected 3 minutes before ganglionic stimulation to induce erection. This drug acts as a vasodilator via the release of NO (Feelisch, 1991; Martinez-Pineiro et al, 1993). *N*-nitro-L-arginine (L-NNA): 200 μg/kg body weight in 5 μl saline was injected 10 minutes before ganglionic stimulation. L-NNA is a competitive inhibitor of nitric oxide synthase (Ward and Angus, 1993).

Analysis of the Effects of Androgen Treatment on Cavernosal nNOS Gene Expression

Preparation of Cavernosal mRNA—Following death of the animal by decapitation, the entire penis was removed from TESTO and CASTRATE rats and the proximal shaft and the crural regions collected. The distal portion of the penis containing the os penis was discarded and the tissue was immediately placed in RNAzol (2 ml/100 mg tissue wet weight) and RNA isolated according to the protocol supplied with the RNAzol (Biotecx Laboratories, Houston, Texas). The concentration of RNA in each sample was determined by measurement of the absorbance at λ = 260 and 280 nm and diluted with 75% ethanol to a final concentration of 1 μg/μl for storage at −70°C.

Reverse Transcription Reaction—The RNA in each sample was precipitated by addition of one-tenth volume of 2 M sodium acetate (pH 4.7) followed by a 15 minute centrifugation at 12,000 × *g*. The RNA was re-suspended in diethyl pyrocarbonate (DEPC) treated water and subjected to the reverse transcriptase reaction to synthesize cDNA using M-MLV reverse transcriptase (Promega, Madison, Wisconsin). In this procedure 0.8 μg of total RNA was primed with 0.5 μg of the oligo $(dT)_{12-18}$ and incubated for 10 minutes at 70°C in a 10-μl volume and then snap frozen on ice. Next, for each sample 1× M-MLV buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol [DTT]), 0.5 mM dNTP mix, and 200 units of M-MLV reverse-transcriptase in a volume of 20 μl were combined and incubated for 40 minutes at 40°C. After incubation, the samples were stored at −20°C.

Polymerase Chain Reaction—The oligonucleotide primers for nNOS had the following sequence: upper primer (sense strand), 5′-ACC TGA AGA GCA CAC TGG AAA C-3′ and lower primer (antisense strand), 5′-GAT GGC CGA CCT GAG ATT C-3′ that amplified a 428-bp fragment for the nNOS sequence. For an internal standard, cyclophilin mRNA was also amplified. The primers for the cyclophilin gene had the following sequence: upper primer, 5′-TGT TCT TCG ACA TCA CGG C-3′ and lower primer, 5′-TTA TGG CGT GTG AAG TCA CC-3′ amplifying a transcript of 216 bp. In preliminary cycling experiments the expression of cyclophilin was found to reach the plateau phase that was not proportional to that of the nNOS gene. To correct for the difference in the original mRNA levels the "primer dropping" method (Wong et al, 1994) was used. In this method, 1.0 μl of the cDNA product was added to a final volume of 49 μl containing 1.5 mM $MgCl_2$, 1× PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH 8.4, Triton X-100), 2 units of *Taq* DNA polymerase (Promega) and 0.2 mM dNTPs. Each tube contained 100 pM of nNOS upper and lower primer and the reaction mixture was covered with 100 μl of mineral oil. The thermal cycler was allowed to complete eight cycles consisting of heat denaturation at 94°C for 1 minute, primer annealing at 58°C for 1 minute and polymerization at 72°C for 1 minute. After eight cycles the primer for cyclophilin (100 pM concentration of upper and lower) were added and the thermal cycler allowed to complete 24 additional cycles at 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. A 10-μl aliquot of each reverse transcriptase polymerase chain reaction (RT-PCR) product was subjected to electrophoresis using a 2.0% agarose gel in Tris acetate/ethylenediaminetetraacetic acid (TAE) buffer for 50 minutes at 75 V

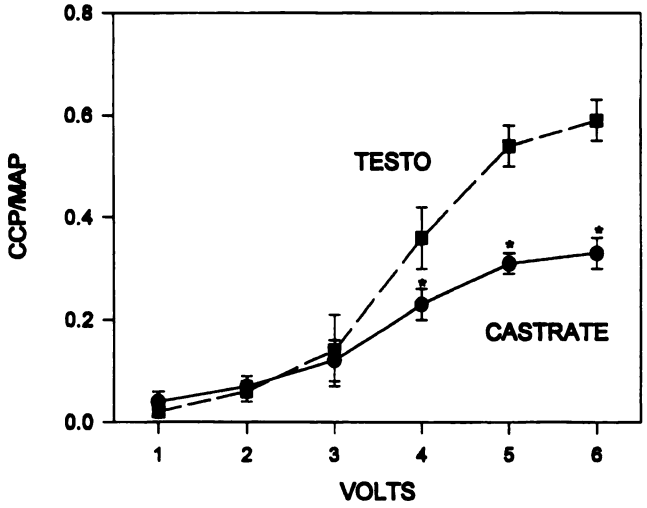

FIG. 1. The erectile response, ratio of intracavernosal pressure to the mean arterial pressure (CCP/MAP), in rats that have been castrated (CASTRATE) or castrated with testosterone replacement (TESTO). The response in each rat was generated by electrical stimulation of the major pelvic ganglion using increasing voltage. Each point is the mean ± 1 standard error of the mean (SEM) of measurements made in 10 rats. Asterisks indicate values significantly different from TESTO at the same voltage ($P < 0.05$).

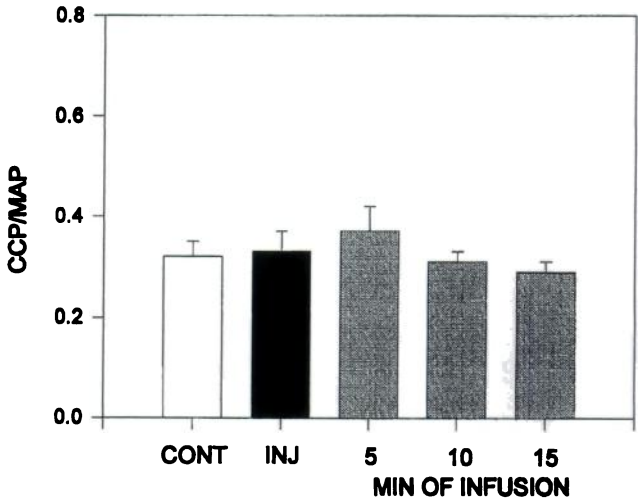

FIG. 2. The erectile response in castrated (CASTRATE) rats treated with intracavernosal L-arginine by injection (INJ; 25 μg/kg in 10 μl of saline) followed by intra-aortic infusion at 50 μg/kg/min for 5, 10, and 15 minutes. CONT indicates the magnitude of the intracavernosal pressure response in the same animals prior to L-arginine treatment. Each bar represents the mean ± 1 standard error of the mean (SEM) of 4 rats. Means are not significantly different from one another.

followed by staining with ethidium bromide (0.5 pg/ml). The intensities of the ethidium bromide fluorescence were determined using an IS-1000 digital imaging system (Alpha Immotech, San Leandro, California). The area under the curve for each peak was normalized with the area of the cyclophilin peak and the values expressed as arbitrary units (AU).

Statistical Analysis

All results are expressed as means ± the standard error of the mean of the ratio of CCP/MAP measured at the same time. Results were analyzed using one and two way analysis of variance (ANOVA) with and without repeated measures. Following ANOVA, means were compared by Newman-Keuls *post hoc* analysis. Data analysis for the RT-PCR experiment was compared by Student's *t*-test. Statistical significance was set at $P < 0.05$.

Results

Testosterone-treated, castrated animals had blood levels of androgen of 1090 ± 105 pg/ml, while in castrated animals implanted with a single cholesterol pellet, androgen levels were 21 ± 3 pg/ml.

The results in Figure 1 show that in the rats used in these studies, as the intensity of ganglionic stimulation is increased from 1 to 6 V, there is a stepwise increase in the magnitude of the erectile response (CCP/MAP). As previously reported, the maximum response was measured at 5 or 6 V, and the magnitude of the response in TESTO animals is greater than that measured in CASTRATE animals at equal voltage.

The experiment depicted in Figure 2 was designed to determine if the decreased magnitude of the erectile response in CASTRATE animals was due to a reduction in the availability of the amino acid, L-arginine, the substrate used by nNOS in the generation of NO. When high concentrations of this amino acid were introduced into the cavernous sinuses by injection followed by 15 minutes of infusion into the aorta, there was no increase in the CCP of the CASTRATE rats during ganglionic stimulation. This finding suggests that substrate availability is not a cause of the reduced response in the CASTRATE animals.

It has been previously reported that the intra-jugular infusion of 50 μg/kg/minute of L-NNA (a competitive inhibitor of NOS activity) leads to a significant decline in the magnitude of the erectile response after 45 minutes (Mills et al, 1992). In those prior studies, the inhibitor also significantly increased mean arterial blood pressure in both CASTRATE and TESTO rats. The experiments shown in Figure 3 demonstrate that a single intracavernosal injection of 200 μg L-NNA/kg resulted in a marked decline in the intracavernosal pressure response in both TESTO and CASTRATE animals. This dose of the inhibitor did not, however, raise blood pressure (not shown). Figure 3 also shows that the marked difference in the magnitude of the stimulated erectile response between TESTO and CASTRATE animals is no longer apparent after L-NNA injection; both are sharply suppressed, although intracavernosal pressure remains slightly elevated. To confirm that L-NNA is a competitive inhibitor of NOS, administration of a 100-fold excess of L-arginine partially reversed the effect of L-NNA and increased cavernosal

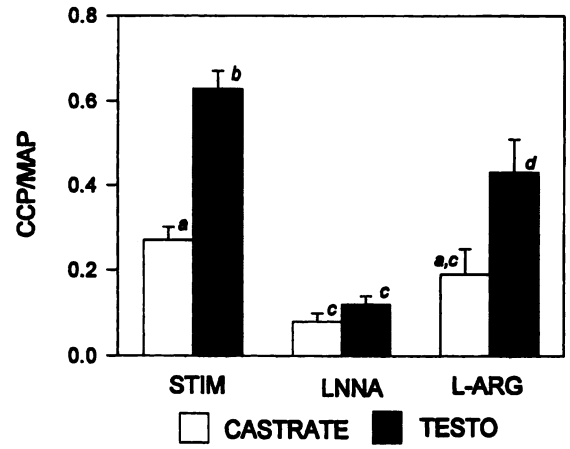

FIG. 3. The electrically stimulated erectile response, ratio of intracavernosal pressure to the mean arterial pressure (CCP/MAP), in castrated with testosterone replacement (TESTO) and castrated (CASTRATE) rats before, 10 minutes after an intracavernosal injection of 200 μg L-NNA/kg body weight, and after injection of a 100 fold excess of L-arginine. Each bar represents the mean ± 1 standard error of the mean (SEM) of five rats. Means with different superscripts are significantly different from one another ($P < 0.05$).

pressures in both CASTRATE and TESTO animals. The recover was about 80% in both TESTO and CASTRATE animals.

In the present studies, we are testing the hypothesis that androgens act primarily to regulate the quantity of NO available during erection. It follows from this hypothesis that in castrated rats with little or no androgen secretion, there would be a deficiency of NO and that exogenous NO should enhance the response in the CASTRATE rats more than in the TESTO rats. To test this hypothesis, CASTRATE and TESTO rats were treated with an intracavernous injection of SNP, a drug which releases NO, and measurements were made both before and after ganglionic stimulation. Figure 4 shows representative tracings of the CCP and MAP responses to ganglionic stimulation only (STIM), to SNP injection only (SNP), and to a combination of ganglionic stimulation and SNP (STIM + SNP). This experiment was repeated several times and the results are shown in Figure 5. During STIM, the expected difference in the response in CASTRATE and TESTO rats is apparent. Following SNP injection into the cavernous sinuses, there is a similar rise in the CCP/MAP ratio in both treatment groups. Although stimulation following SNP administration (STIM + SNP) fails to increase significantly the response in TESTO rats, in the CASTRATE group the supplemental NO yields an additive effect with the magnitude of the response significantly greater than in either STIM or SNP.

We next sought to determine if androgens affect the NO production by a regulation of nNOS mRNA levels in the penile tissue. In this experiment, we measured the availability of nNOS mRNA using the RT-PCR method.

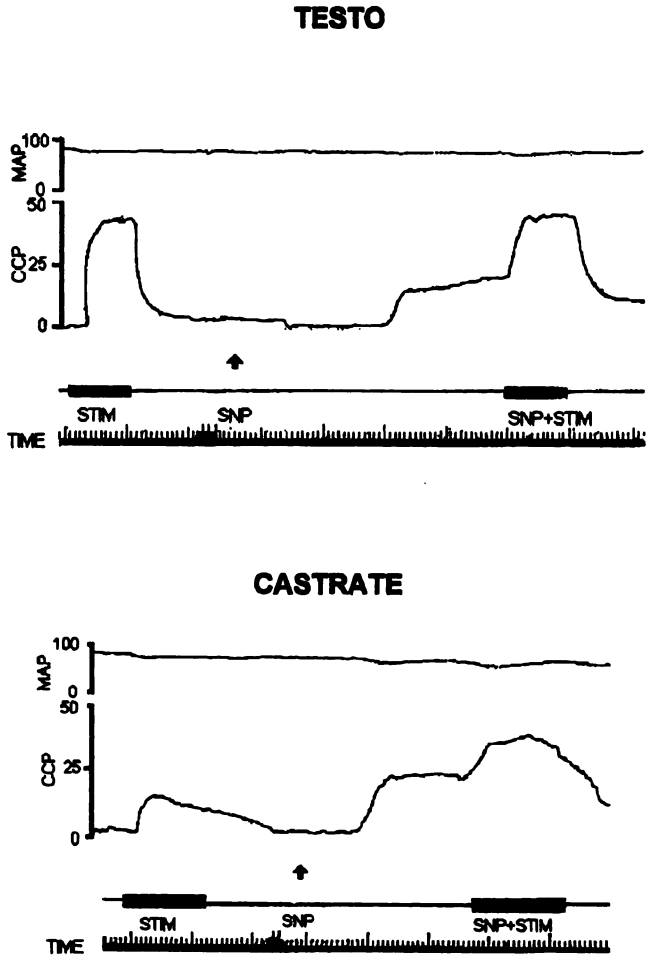

FIG. 4. The effect of ganglionic stimulation and sodium nitroprusside (SNP) on the intracavernosal pressure (CCP) response, showing typical erectile responses in castrated with testosterone replacement (TESTO) and castrated (CASTRATE) animals to ganglionic stimulation alone, to SNP alone, and to SNP in combination with stimulation. Major tick marks represent 1-minute duration. Note the combination of stimulation and SNP leads to an enhanced CCP in the CASTRATE animals but not in the TESTO rats. This experiment was repeated 6 times and the results are depicted in Figure 5.

Figure 6a shows the electrophoretic analysis of the RT-PCR products in TESTO and CASTRATE animals. The expected size for the PCR products for both nNOS (428 bp) and the reporter gene cyclophilin (216 bP) were obtained. The results are further analyzed in Figure 6b by computation of the ratio of nNOS peak area to cyclophilin peak area for each sample; results show that in TESTO animals the amount of nNOS mRNA in cavernosal tissue is significantly greater than the amount in CASTRATE animals.

Discussion

The aims of the present report are twofold: to confirm that NO is the principal mediator of the erectile response,

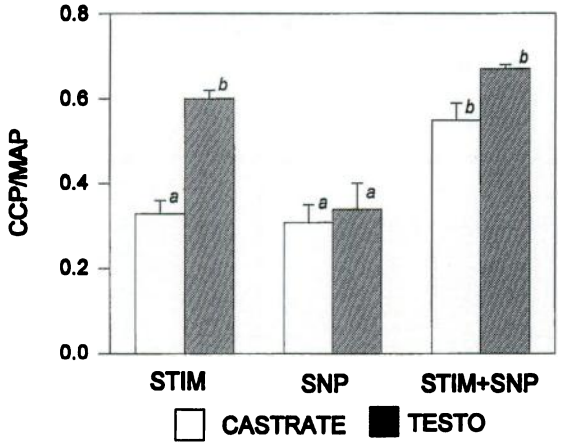

FIG. 5. The erectile response, ratio of intracavernosal pressure to the mean arterial pressure (CCP/MAP) in castrated with testosterone replacement (TESTO) and castrated (CASTRATE) rats resulting from electrical stimulation (STIM), intracavernosal injection of 8 μg/kg body weight sodium nitroprusside (SNP), or stimulation following SNP injection (STIM + SNP). Each bar is the mean ± 1 standard error of the mean (SEM) of observations in 10 rats. Means with different superscripts are significantly different from one another ($P < 0.05$).

and to investigate two potential mechanisms by which androgens could regulate NO synthesis during erection. Many investigators have presented convincing evidence that NO is critical to erection, and although our studies use a somewhat dissimilar approach, the results serve to confirm the general importance of NO in erection. The mechanistic studies, on the other hand, extend our understanding of how androgens act by showing that substrate availability is not regulated by androgens while expression of the nNOS gene appears to be under direct androgenic control.

As reports from this and other laboratories have shown, the magnitude of the intracavernosal pressure rise during erection increases as the voltage applied to the autonomic innervation of the cavernosal vasculature is increased. Furthermore, the pressure rise in castrated animals falls well below the increase in intact rats or animals treated with exogenous androgen. These studies have led to the conclusion that there is an androgen-dependent and androgen-independent portion of the erectile response in rats. The experiments using L-NNA show that both the androgen-dependent and the androgen-independent portions are mediated by NO, because both are diminished by more than 80% with only 10 minutes of treatment with the inhibitor. In prior studies from this laboratory, systemic infusion through the jugular vein of L-NNA (50 μg/kg/minute for 45 minutes) leads to a significant reduction in the erectile response, but, at this rate of drug delivery, systemic blood pressure was significantly increased. In the present study, mean arterial blood pressure was not affected by the intracavernosal delivery of L-NNA even though there was a marked reduction in the CCP. Furthermore, the inhibitor reduced the CCP to the same extent in both CASTRATE and TESTO rats indicating that both portions of the response (androgen dependent and androgen independent) are mediated by NO. However, the small increase in CCP that remained after L-NNA treatment indicates that the dose of L-NNA was suboptimal or that a small portion of the erectile response involves a pathway independent of NO.

The experiment depicted in Figure 2 was designed to determine if the decreased magnitude of the erectile response in CASTRATE animals was due to a reduction in the availability of the amino acid, L-arginine, the substrate used by nNOS in the generation of NO. When high concentrations of this amino acid were introduced into the cavernous sinuses by injection or by 15 minute infusion

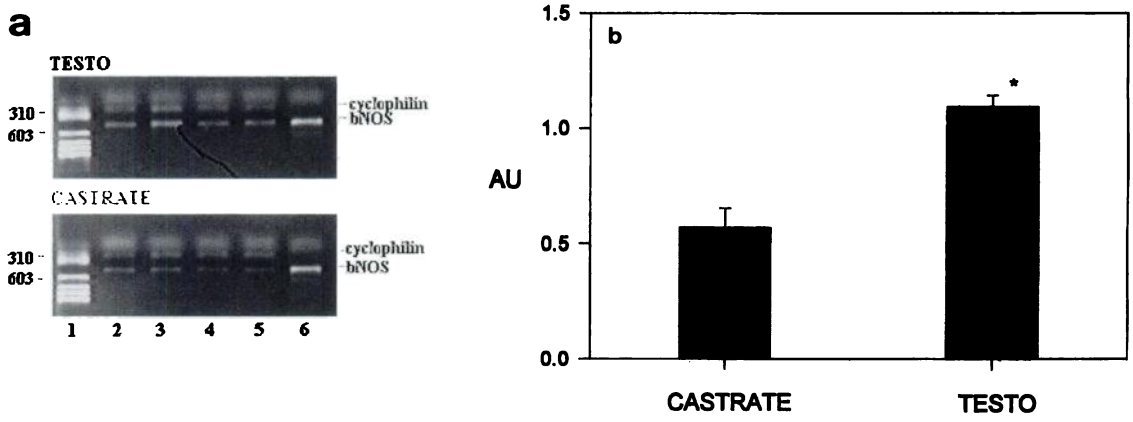

FIG. 6a. Reverse transcription polymerase chain reaction (RT-PCR) amplification of cyclophilin (216 bp) and the neuronal form of the nitric oxide synthase gene (nNOS) (428 bp) cDNA in castrated with testosterone replacement (TESTO; above) and castrated (CASTRATE; below) animals. Lane 1—*ϕX174/Hae III* DNA molecular weight standards. Lanes 2–5—co-amplified cDNA from cavernosal tissue RNA of four TESTO and four CASTRATE rats. Lane 6—amplified nNOS cDNA obtained from rat brain mRNA. Peak areas of the nNOS bands were normalized to the areas of the cyclophilin bands and are expressed in **6b** as arbitrary units (AU) for CASTRATE and TESTO animals. Each bar is the mean ± 1 standard error of the mean (SEM) of observations in six rats; the asterisk indicates a significantly greater AU in cavernosal tissue from TESTO than from CASTRATE rats ($P < 0.05$).

into the aorta, there was no increase in the CCP of the CASTRATE rats during ganglionic stimulation. Although circulating levels of L-arginine were not measured, this finding suggests that substrate availability is not a cause of the reduced response in the CASTRATE animals and that testosterone is not acting to increase substrate availability to the NOS reaction.

SNP acts as an NO donor (Martinez-Pineiro et al, 1993) and the sustained action of this drug or the relatively low rate of clearance from the corpus cavernosum results in a prolonged increase in intracavernosal pressure. Injection of SNP into the cavernosum without ganglionic stimulation resulted in increases in intracavernosal pressure that were similar in both TESTO and CASTRATE animals. That the magnitude of the response to SNP was nearly identical in the two treatment groups suggests that there is no difference in the responsiveness of the cavernosal mechanisms to NO. Rather, it suggests that the difference in the erectile response to ganglionic stimulation in TESTO and CASTRATE rats may depend solely on differences in the availability of NO, possibly due to differences in the capacity to synthesize NO. When the levels of NO were elevated by the administration of SNP and the ganglion stimulated, TESTO treated animals exhibited no significant increase in cavernosal pressure over that seen with stimulation alone, whereas in CASTRATE animals the pressure increased to that observed in TESTO rats. This finding can be interpreted to mean that the erectile response can be augmented with additional NO in the CASTRATE animals where NO is deficient, but not in TESTO rats where NO levels are not limited. Some CASTRATE animals received an additional intracavernosal injection of 16 μg SNP/kg body weight, but the additional NO failed to increase further the response, suggesting that the dose of SNP used was optimal (data not shown).

The final series of experiments was performed to determine if androgens alter the availability of NO by regulation of nNOS mRNA levels. In this experiment expression of the nNOS gene in cavernosal tissue was measured using the reverse transcription polymerase chain reaction method. Our studies showed that in cavernosal RNA preparations, there was significantly less nNOS mRNA in CASTRATE animals than in TESTO animals. This finding extends the work of Chamness et al (1995), who reported that nNOS protein levels in the rat penis decrease 57% following castration, and with the studies of Zvara et al (1995), who demonstrated that castration decreases by 60% NADPH-diaphorase staining in the NANC nerve fibers innervating the corpus cavernosum. The fact that castration does not lead to a total disappearance of nNOS mRNA may serve to explain the androgen-dependent and androgen-independent portions of the erectile response. From these results, we propose that in CASTRATE animals, a basal level of nitric oxide is released by nerve fibers in response to ganglionic stimulation that results in vasodilation and a partial erectile response. Androgens increase the amount of nNOS mRNA by enhancing nNOS gene expression or by decreasing mRNA degradation, resulting in a greater amount of enzyme available for the production of NO in the rat penis.

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