Improved erectile function after Rho-kinase inhibition in a rat castrate model of erectile dysfunction

Christopher J. Wingard, John A. Johnson, Andre Holmes, and Anita Prikosh

1Department of Physiology and 2Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, Georgia 30912

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Wingard, Christopher J., John A. Johnson, Andre Holmes, and Anita Prikosh. Improved erectile function after Rho-kinase inhibition in a rat castrate model of erectile dysfunction. Am J Physiol Regul Integr Comp Physiol 284: R1572–R1579, 2003. First published February 6, 2003; 10.1152/ajpregu.00041.2003.—Androgens are reported to act as strong modulators of erectile function influencing both nitric oxide and vasoconstrictor signaling. Castration results in a depressed erectile response that is associated with a loss of nitric oxide production and increased responsiveness to constrictive agents. The increased vasoconstrictor response may be a result of an active RhoA/Rho-kinase signaling pathway. We report here results of studies designed to test the hypothesis that inhibition of the Rho-kinase pathway restores erectile function in a castrate model by relaxing the smooth muscle. Mean arterial (MAP) and corpus cavernosal (CCP) pressures were monitored during intracavernosal injection of the Rho-kinase inhibitor Y-27632. Castration reduced the maximal erectile response (CCP/MAP) by 33%, and testosterone replacement restored the response (intact, 0.736 ± 0.040; castrate, 0.492 ± 0.022; testosterone, 0.681 ± 0.073). Injection of Y-27632 increased CCP in all experimental groups; it also left shifted the voltage response curve and increased the maximal CCP/MAP response (intact, 0.753 ± 0.091; castrate, 0.782 ± 0.081; testosterone treated, 0.894 ± 0.033). Y-27632 dose dependently relaxed phenylephrine-stimulated cavernosal tissues. Cavernosal tissues showed increased RhoA and Rho-kinase protein levels after castration. Our data support the hypothesis that an active RhoA/Rho-kinase pathway contributes to the reduced erectile response after castration due to an upregulation of RhoA/Rho-kinase protein levels and that inhibition of this pathway may serve as an effective treatment for erectile dysfunction.

ANDROGENS are recognized as strong modulators of male sexual behavior although the exact role of androgens in the maintenance of erectile responsiveness remains controversial (20, 22). Castration has been found to decrease the erectile responses to a variety of stimuli while androgen replacement reversed these effects (1, 26, 38). In animal models of erectile function the intracavernosal pressure response to ganglionic stimulation is suppressed when administered antiandrogenic therapy or after surgical castration and restored with the androgen replacement therapies (4, 5, 16, 34).

An essential requirement for normal erectile function is the relaxation of the cavernosal sinuses and arterial smooth muscle. This relaxation is a complex interplay of messenger molecules that are capable of shifting the balance of smooth muscle tone from constricted to relaxed. Castration has been associated with alteration in the expression and action of the major relaxation pathway element nitric oxide (NO) and its generating enzyme NO synthase (NOS) (4, 14–16, 28). Such alterations are assumed to be major contributors to the loss of erectile function. Additionally, there are reported changes in the sensitivity of the sympathetic pathway response and adrenergic vasoconstrictor effects increasing smooth muscle tone contributing to the depressed erectile response (27).

While most studies examining the role of androgens on erectile function have focused on the impact of androgens on the NO signaling system, few studies have examined other regulators of erectile function. We were particularly interested in the impact of the loss of androgens on the vasoconstrictor action of the RhoA/Rho-kinase pathway. The constricted state of penile vasculature is considered to be mediated by release of norepinephrine, endothelin-1, and a host of other vasoconstrictors (2). These agents bring about vasoconstriction by elevating intracellular calcium and activating myosin light-chain kinase, resulting in myosin phosphorylation and crossbridge activation. Additionally, a calcium sensitization process is activated through agonist activation of heterotrimeric G protein-coupled receptors, activation of RhoA through exchange of GTP for GDP, and dissociation from a guanine nucleotide dissociation inhibitor (GDI). The activated RhoA then activates Rho-kinase, which inhibits myosin light-chain phosphatase, resulting in a net increase in myosin phosphorylation and force at constant calcium (31, 32).

We have recently demonstrated that the vasomotor activity of the penile circulation was under the influence the RhoA/Rho-kinase pathway, which has a very strong vasoconstrictor effect, and its inhibition results in substantial relaxation and an augmented erectile

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response (8, 18, 19). Reported here are results of experiments testing the hypothesis that an active Rho/Rho-kinase pathway contributes to the reduced erectile response after castration due to an upregulation of RhoA/Rho-kinase protein levels, and inhibition of the Rho-kinase restores erectile function by relaxing cavernosal smooth muscle.

**MATERIALS AND METHODS**

**Animals.** Male Holtzman rats (90–120 days of age, 300–325 g, Harlan Laboratories, Indianapolis, IN) were used in all experiments. Animals were divided into three experimental groups: 1) intact: no surgery and served as control; 2) castrate: surgically castrated, implanted with a cholesterol pellet, and allowed to recover for 7–10 days; and 3) testosterone: surgically castrated, with testosterone replacement therapy via a subcutaneous pellet implantation for 7–10 days.

Testosterone pellets were made in the laboratory using a pellet press. Each animal in the castrate and testosterone groups was surgically castrated under anesthesia and implanted with a 3-mm pellet (~ 5 mg) composed of 100% cholesterol or 50% cholesterol-50% testosterone. This concentration and duration of treatment have previously been determined to severely reduce or maintain normal serum testosterone levels (23, 26). Animals were maintained in an American Association for Accreditation of Laboratory Animal Care facility with animal use protocols and justification approved by the Institutional Committee on Animal Use and Care in Research and Education in accordance with National Institutes of Health guidelines.

**Erectile response measurements.** Rats were anesthetized with an intramuscular injection of ketamine (87 mg/kg body wt) plus xylazine (13 mg/kg), and anesthesia was maintained on supplemental ketamine as needed. The left carotid artery was cannulated for continuous monitoring of mean arterial pressure (MAP). The shaft of the penis was freed of skin and fascia, and the right corpus cavernosum was cannulated by insertion of a 30-gauge needle connected to a pressure transducer, permitting continuous monitoring of corpus cavernosal pressure (CCP). The left corpus cavernosus was cannulated with 30-gauge needles attached to 10–μl syringes via short lengths of PE-10 tubing and used for administration (intracavernosal injection) of vasoactive drugs. The abdominal cavity was opened, exposing the right major pelvic ganglion (MPG), containing autonomic nerve fibers that innervate the cavernosal vascular tissue). Platinum bipolar electrodes were positioned on the MPG, and their position was adjusted during stimulation until a maximal voltage-induced response was achieved. During the experiment, stimulatory voltages applied to the MPG ranged from 1 to 5 V delivered in 5-ms pulses at a frequency of 12 Hz. The duration of stimulation was for 1–2 min with rest periods of 2–3 min between subsequent stimulations. All pressure data were collected for analysis using Polyview data-acquisition software (AstroMed, Grass Instrument Division).

**Isolated cavernosal tissue force measurements.** Cavernosal strips were prepared by amputating the distal portion of the penis and then removal of the corpus spongiosum and dorsal vein. Strips were bathed in a physiological salt solution (PSS) at pH 7.4, 37°C bubbled with breathing air. The PSS was composed of (in mM) 140.0 NaCl, 5.0 KCl, 1.6 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 1.2 Na<sub>2</sub>HPO<sub>4</sub>, and 5.6 D-glucose. Tissue resting force was set to 500 mg by a series of stretches and length releases, followed by a period of stress relaxation. Setting the tissue to a preset resting force of 500 mg was found to correlate to an optimal length-force generation in response to maximal K<sup>+</sup> depolarization (data not shown). All tissues were contracted by the addition of 109 mM K<sup>+</sup> physiological saline solution (KPSS). KPSS was prepared by stoichiometric substitution of KCl for NaCl in PSS. Tissues were depolarized for 10 min and then relaxed with repeated washes of PSS at 10-min intervals before the start of any experimental protocol. The collected results were used to construct cumulative dose-response curves for the α-adrenergic agonist phenylephrine (PE) (0.1–100 μM) or the Rho-kinase inhibitor Y-27632 (0.3–30 μM). The relaxation curves with Y-27632 required the strips to be precontracted with 10 μM PE. All dose-response curves were constructed in the presence of inhibitors of the NO pathway (10 μM N<sup>ω</sup>-nitro-L-arginine methyl ester).

**Immunoblot analysis of cavernosal RhoA and Rho-kinase protein expression.** Cavernosal strips (cleaned of the corpus spongiosum and dorsal vein) were frozen in dry ice-acetone slurry and then pulverized in liquid nitrogen. Tissues were homogenized in cold radioimmunodetection buffer that contained 50 mM Tris-HCl (pH 7.6), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSE, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 mM Na<sub>3</sub>VO<sub>4</sub>, and 1 mM Na<sub>2</sub>F. Samples were centrifuged (1,000 g, 4°C, 10 min), and the supernatant was collected for protein quantification and immunoblot analysis. In control experiments, no RhoA or Rho-kinase immunoreactivity was found in the 1,000-g pellet. Equal amounts of protein (100 μg total protein/lane) were loaded and resolved by 10% (Rho-kinase) or 15% (RhoA) SDS-PAGE (at room temperature overnight). Rat brain extract served as a positive control. Proteins were transferred to a nitrocellulose membrane (Immobilon-P, Millipore) using a Bio-Rad Mini-Protran III apparatus (2 h at 4°C) in the presence of 25 mM Trizma base, 191.8 mM glycine, and 20% methanol. The nitrocellulose membrane was then incubated with 5% skimmed milk in phosphate-buffered saline (30 min, room temperature). After blocking, the membrane was incubated overnight (4°C) with primary goat polyclonal antibody to the carboxy terminus of Rho-kinase (1:500 dilution, ROCK-2, Transduction Labs) or primary monoclonal antibody to RhoA, (1:100 dilution, Transduction Labs) and subsequently incubated (2 h at 4°C) with a rabbit anti-mouse antibody (1:500 dilution, Transduction Labs). Antibody-bound protein was visualized using an [125I]-protein A incubation (2 h at room temperature) (13). Rho-kinase protein expression corresponded to a band in the 170-kDa range while RhoA protein expression corresponded to a band in the 25-kDa range. Total protein determinations were accomplished using a Micro bicinechonic acid protein assay (Pierce Biotechnology, Rockford, IL).

**Drugs.** The present study utilized a specific inhibitor of Rho kinase, Y-27632, generously supplied by the Mitsubishi Pharma Group (Osaka, Japan). Testosterone was purchased from Sigma Chemical (St. Louis, MO).

**Data analysis and statistics.** Raw force responses were recorded digitally with POLYview data acquisition (AstroMed, West Warwick, RI). Postacquisition analysis included conversion of force (in g) to stress (mN/mm<sup>2</sup>). Force was converted to stress values (force/cross-sectional area) by the following equation: [(force (mg) × 0.00987]/area/1.055 (density conversion), where area is calculated from wet weight (mg)/length (mm). Dose-response profiles were constructed for the individual traces for each experimental condition. Normalized stress was calculated by dividing the measured stress level by the maximal stress level measured during the construction of the dose-response profile. The profiles were fit by Sigma Plot (SPSS Science, Chicago, IL), using a Hill fit.
protocol, allowing for the report of the estimated EC_{50}. Data were presented as means ± SE. Statistical differences were determined by ANOVA followed by Bonferroni’s complementary analysis, where relevant, and Student’s t-test using the SigmaStat Analysis Program (SPSS Science, Chicago, IL). A P value of <0.05 was considered to be significant.

RESULTS

Effect of castration and testosterone-treatment on the voltage-dependent erectile response. To examine the voltage-dependent erectile response, the MPG was stimulated over a range of 1–5 V while MAP and CCP were recorded. The average value of CCP was divided by the average MAP for each stimulus level to provide a quantitative assessment of the erectile response. Ganglionic stimulation resulted in a voltage-dependent increase in CCP/MAP, in accordance with previously published findings (9, 21, 37). Surgical castration of male rats (10 days postsurgery) resulted in a depressed erectile response to voltage stimulation of the major pelvic ganglion compared with intact age-matched animals (Fig. 1). Statistically significant depression in the erectile responses was seen in the castrate group at voltages > 3 V (Fig. 1B, left). Testosterone treatment (surgical castration with implantation of a 50% testosterone-50% cholesterol subcutaneous pellet) restored the voltage-dependent erectile response to that of intact animals (Fig. 1, A and B, left). Castration and testosterone treatment had no effect on MAP or CCP before MPG stimulation (Table 1).

Effect of Y-27632 on the voltage-dependent erectile response. We have previously established the importance of Rho-kinase in the maintenance of penile vasoconstriction in the normal adult rat. We sought to examine the effects of Rho-kinase inhibition on the erectile response in hypogonadal rats, hypothesizing that a loss in androgen-dependent erectile function was due to upregulation of RhoA/Rho-kinase signaling and could therefore be reversed by inhibition of Rho-kinase activity. Injection of the selective Rho-kinase antagonist Y-27632 (5 μl, 20 nM) into the left corpus...
Table 1. MAP and CCP in response to corpus cavernosal injection of 5 μl of 20 nM Y-27632 before MPG stimulation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MAP</th>
<th>MAP</th>
<th>CCP</th>
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<tr>
<td></td>
<td>Before Y-27632</td>
<td>After Y-27632</td>
<td>Before Y-27632</td>
<td>After Y-27632</td>
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<tr>
<td>Intact</td>
<td>108.1 ± 8.2</td>
<td>92.0 ± 5.7</td>
<td>7.7 ± 2.3</td>
<td>18.4 ± 4.0†</td>
</tr>
<tr>
<td>Castrate</td>
<td>98.5 ± 5.9</td>
<td>87.0 ± 1.1</td>
<td>8.7 ± 1.6</td>
<td>35.1 ± 7.1‡</td>
</tr>
<tr>
<td>Testosterone treated</td>
<td>115.7 ± 7.6</td>
<td>97.0 ± 4.3</td>
<td>10.9 ± 1.9</td>
<td>33.3 ± 7.0*</td>
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Values are means ± SE of mean arterial (MAP) and corpus cavernosal pressures (CCP) in mmHg; n = 4/group. MPG, major pelvic ganglion. *Statistical significance (P < 0.05) vs. before Y-27632. †Near-statistical significance at P = 0.06 vs. before Y-27632.

cavernosum sinuses resulted in an increase in CCP/MAP in all experimental groups without MPG stimulation (Fig. 1, A and B, right). We found that the intracavernosal injection of Y-27632 had a small but not significant depression in MAP in all experimental groups, while producing a significant threefold elevation in CCP in both castrate and testosterone-treated groups without MPG stimulation (Table 1). The doubling in CCP with Y-27632 injection in intact animals was close to significance with a P value of 0.06 and likely reflects a small sample size. However, each animal demonstrated an elevation in CCP upon injection of Y-27632.

We next examined the effect of Y-27632 on the voltage-dependent increase in CCP/MAP. Y-27632 (5 μl of 20 nM) was injected into the left corpus cavernosum, and the increase in CCP pressure was allowed to plateau (5 min after injection). The subsequent CCP/MAP response to MPG stimulation was assessed over the voltage range of 0–5 V. The increase in CCP/MAP with MPG stimulation after administration of Y-27632 was elevated in all treatment groups (Fig. 1B, right). Saline or vehicle injections produced no significant effect on MAP or CCP (data not shown). Administration of Y-27632 resulted in an improvement of the voltage-dependent erection of castrate group to levels that were not significantly different from the intact or testosterone-treated group responses (Fig. 1B, right).

α-Adrenergic-mediated contractile response of isolated cavernosal tissue. We examined the concentration-dependent contractile response of isolated corpus cavernosum to the cumulative addition of the α-adrenergic agonist PE. Cavernosum from intact and testosterone-treated animals displayed a biphasic response to increasing concentration of PE, rising from 0.1 to 10 μM, and then declining at concentrations >10 μM. Cavernosal strips from castrate animals displayed a monotonic response with a plateau in stress >10 μM and a maximal stress response that was significantly larger than the intact and testosterone-treated responses (Fig. 2A). The increased stress response with castration was also associated with a significant rightward shift in the PE dose-response profile as reflected by changes in the average EC50 values (intact 0.299 ± 0.057 μM; testosterone treated 0.209 ± 0.075 μM; castrate, 0.729 ± 0.092 μM; P < 0.05, castrate vs. intact) (Fig. 2B).

Effect of Y-27632 on α-adrenergic-mediated contraction of isolated cavernosal tissue. To examine the effect of Rho-kinase inhibition on the maintenance of adrenergic-mediated stress, isolated corporal strips were first contracted with 10 μM PE and subsequently relaxed with increasing concentrations of Y-27632. The increasing concentrations of Y-27632 resulted in a graded relaxation of corporal strips from all experimental groups. There was a significant higher stress level in response to 10 μM PE pretreatment in corpora from castrate animals (Fig. 3A). However, the sensitivity to Rho-kinase inhibition was not different between the experimental groups with average EC50...
Effect of castration and testosterone treatment on expression of RhoA and Rho-kinase levels in isolated cavernosal tissues.

We have demonstrated that castration results in a depressed erectile response and increased constriction of corporal cavernosal tissues to the \(-\alpha\)-adrenergic agonist PE. We sought to examine the impact of castration and testosterone treatment on the expression pattern of the enzyme Rho-kinase and its upstream regulator RhoA in the corporal tissues to test the hypothesis that increases in the of RhoA and/or Rho-kinase protein levels contribute to increased constrictor activity and decreased erectile response. Castration was associated with a significant increase in the amounts of both RhoA and Rho-kinase in the cavernosal tissues (Figs. 4A and 5A). Testosterone treatment of castrated rats lowered the levels of RhoA/Rho-kinase protein detected but did not return them to intact levels. Castration resulted in a >15-fold increase in the amount of RhoA detected by Western blotting (Fig. 4B). The mean optical density units for RhoA, normalized for the total protein levels, were 6.3 ± 1.0 (intact), 74.3 ± 8.1 (castrate; \(P < 0.05\) vs. intact), and 57.8 ± 10.5 (testosterone treated; \(P < 0.05\) vs. intact; \(n = 4–9\)). A similar pattern of protein detection was seen with Rho-kinase. Castration resulted in a greater than twofold increase in the amount of Rho-kinase detected by Western blotting (Fig. 5B). The mean optical density units for Rho-kinase normalized for the total protein levels were 16.0 ± 3.6 (intact), 27.5 ± 6.6 (castrate; \(P < 0.05\) vs. intact), and 24.9 ± 12.5 (testosterone treated; \(P < 0.05\) vs. intact; \(n = 3–6\)). The 70-kDa band seen in the RhoA and Rho-kinase blots was determined to be nonspecific staining.

DISCUSSION

The findings reported from these studies demonstrate that castration decreased erectile response to ganglionic stimulation and increased constrictor responsiveness to PE stimulation, which was reversed by inhibition of the calcium-sensitizing pathway involving Rho-kinase. Furthermore, the decreased erectile response and increased constrictor response was associ-
Previous studies using the rat model have found a significant suppression of ganglion-induced erectile responses (CCP/MAP) with castration, suggesting a depressed NO release (26–28). We found in these studies that voltage stimulation of the major pelvic ganglion induced an increase in CCP/MAP, which was significantly suppressed in the 10-day castrate male rat, compared with intact age-matched controls (Fig. 1). The in vitro constrictor response to the α-adrenergic agonist PE was elevated in corpus cavernosal strips from the castrate animals (Fig. 2). This response differs from that reported by Alcorn and coworkers (1), who examined the contractile response to 100 μM noradrenaline and found no difference between intact, castrate, and testosterone-treated animals. This discrepancy may reflect a difference in the tissue responsiveness to the agonist of choice, its concentration, or to the duration of castration protocol employed. Our studies show a significant reduction in contractile force to 100 μM levels of PE, which may reflect the receptor desensitization to this high agonist concentration. Using a rat castrate model similar to the one employed in these studies, Reilly and coworkers (27) found that a portion of the depressed erectile response associated with castration was attributed to an increased α-adrenergic responsiveness. These later results are confirmed by our observation of an increased constrictor response in vitro in cavernosal tissue from castrated animals.

In addition to the increased maximal stress generation, we found the response profile to the α1-adrenergic agonist PE was shifted to the right in castrated animals (Fig. 2B) and was restored to the intact profile with testosterone treatment. Such a response could reflect a change in receptor population or subtype. A recent review by Andersson (2) cites studies that demonstrated changes in receptor populations with erectile disorders and that some of the most recently identified α1-receptor subtypes have different affinities for selective antagonists. Traish and coworkers (34) reported that castration reduced the expression of α1-adrenergic receptor in cavernosal tissue, and whose reduction was prevented or reversed by testosterone replacement. Our data are consistent with a possible change in receptor population or subtype as we observed a change in maximal response and a shift in the calculated EC50 values (effective concentration to invoke 50% of the maximal response). In addition, our results suggest that with the loss of androgen through castration, there is an increased calcium sensitization effect, resulting in the augmented force generation seen in isolated strips.

It is tempting to speculate that the increased responsiveness to α-adrenergic stimulation in this model may be associated with an increased activity of Rho-kinase through lowered NO production. There is evidence for an inhibitory effect of NO on the RhoA/Rho-kinase signaling pathway such that NO activation of protein kinase G (PKG) results in phosphorylation of RhoA and its inhibition (17, 29).
activity may be depressed and unable to curtail Rho-kinase activity. Some evidence is reported to suggest that exogenously applied NO can suppress the RhoA/Rho-kinase pathway action and aid in normal erection (17, 19). If such a mechanism of action of NO occurs, then in conditions of low NO, the resulting maintained Rho-kinase activity levels would indirectly inhibit myosin phosphatase and maintain cavernosal smooth muscle tone as seen in this model of erectile dysfunction. Alternatively, the balance between activities or expression levels of elements of calcium-dependent and calcium-sensitizing pathways regulating contracture may be altered by castration.

Interestingly, testosterone treatment of castrated animals restores the erectile responsiveness. Several groups report that testosterone acts to maintain the nNOS expression or NO levels (1, 6, 15, 16, 28, 30, 38). This may result in not only restoration of an NO-mediated relaxation via increased Ca$^{2+}$ sequestration and decreased myosin light-chain kinase activity but also through the inhibition of Rho-kinase and maintained myosin phosphatase activity.

The calculated EC$_{50}$ values of isolated corpora cavernosal strips to Rho-kinase inhibition in all experimental groups from our studies were similar with reported values near 1 nM (16, 28) for rabbit permeabilized corpora cavernosa. Whereas our values were slightly lower those reported by Rees et al. (24) in intact human tissues, our results may suggest a difference in the ROA/Rhokinase pathways regulating contracture. This has particular significance as most previous studies have examined altered NO relaxation mechanisms and not mechanisms associated with maintenance of smooth muscle tone.

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