Effect of Rho-kinase inhibition on vasoconstriction in the penile circulation

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Mills, Thomas M., Kanchan Chitaley, Christopher J. Wingard, Ronald W. Lewis, and R. Clinton Webb. Effect of Rho-kinase inhibition on vasoconstriction in the penile circulation. J Appl Physiol 91: 1269–1273, 2001.—A recent report from this laboratory (Chitaley K, Wingard C, Webb R, Branam H, Stopper V, Lewis R, and Mills T. Nature Medicine 7: 119–122, 2001) showed that inhibition of Rho-kinase increased the erectile response (intracavernosal pressure and mean arterial pressure) by a process that does not require nitric oxide or cGMP. The present study investigated whether vasoconstrictor agents, which are active in the penis, act via the Rho-kinase pathway. Western analysis revealed RhoA and Rho-kinase protein in the penis. Treatment with the selective Rho-kinase inhibitor Y-27632 significantly increased the magnitude of the erectile response. Intracavernous administration of endothelin-1 (ET-1, 50 pmol) or methoxamine (10 μg/kg) reduced the erectile response to autonomic stimulation. If Y-27632 was given before ET-1 or methoxamine, the vasoconstrictor effect was reduced, and intracavernosal pressure and mean arterial pressure remained elevated. However, when given after methoxamine, Y-27632 had a reduced vasodilatory effect, and Y-27632 had no vasodilatory effect when given after ET-1. These findings suggest that ET-1 and methoxamine increase Rho-kinase activity in the cavernous circulation and support the hypothesis that the vasoconstriction that maintains the penis in the nonerect state is mediated, in part, by the Rho-kinase pathway.

penile erection; vasodilation; penis; methoxamine; endothelin-1

penile erection occurs when smooth muscle relaxes in the walls of the cavernous arterioles and sinuses. This relaxation results in increased blood flow into the sinuses under the driving force of the mean arterial pressure (MAP). As the sinuses fill, the pressure-dependent-venoocclusive mechanism is activated, and blood outflow is limited. The combination of increased inflow and decreased outflow results in the rigidity of the corpora cavernosa necessary for erection (1, 2). Smooth-muscle relaxation leading to erection is thought to be mediated by the nitric oxide (NO)/cGMP pathway (4), although other control pathways may be involved as well (17). However, in the absence of an active NO/cGMP pathway, the arteriolar and sinusoidal smooth muscles remain in the contracted state, possibly mediated by the actions of norepinephrine (NE), endothelin-1 (ET-1), or other vasoconstrictors, including neuropeptide Y and angiotensin II (3, 7, 9, 10, 23). The vasoconstriction resulting from ET-1 and α-adrenergic agonists (NE, methoxamine (Methox)) is thought to activate phospholipase C and, via increased inositol trisphosphate and diacylglycerol production, increase myosin light-chain (MLC) kinase activity. These processes lead to increased MLC phosphorylation and smooth muscle contraction (21). In order for erection to occur, the balance must shift away from α-adrenergic- and ET-1-dependent vasoconstriction in favor of NO- and cGMP-dependent vasorelaxation (5).

Recent studies from this laboratory suggest that vasoconstriction in the penile circulation may be regulated, in part, by the RhoA/Rho-kinase calcium sensitization pathway (6). This pathway has been shown to be regulated by NE and ET-1 in other tissues (22). Our results showed that injection of a selective inhibitor of Rho kinase leads to a prompt and significant increase in intracavernosal pressure (ICP). This increase in ICP was also found to occur in the presence of inhibitors of NO synthase or guanylate cyclase, demonstrating that the increase in ICP in the presence of the Rho-kinase inhibitor does not rely on the relaxation actions of the NO/cGMP pathway. Our results suggest that inhibition of Rho-kinase stimulates erection by blocking endogenous vasoconstriction rather than by directly stimulating vasorelaxation mechanisms.

Whereas our studies indicated the presence of considerable Rho-kinase-mediated vasoconstrictor activity in the penis, the regulation of this pathway in the cavernosal vasculature remains to be established. The present studies were undertaken to test the hypothesis that the constrictor actions of α-adrenergic agonists and ET-1 in the cavernosal vasculature are mediated, in part, by the Rho-kinase pathway.

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METHODS

Animals. Intact male Holtzman rats (90–120 days of age; Harlan Laboratories) were used in these studies. All procedures were performed in accordance with the “Guiding Principles in the Care and Use of Animals” endorsed by the American Physiological Society and approved by the Medical College of Georgia Committee on the Use of Animals in Research and Education.

Erectile response measurements. Our laboratory has previously described methods for the measurement of corpus cavernosum pressure (ICP) and MAP (14, 15, 18, 19). In this protocol, rats were anesthetized with intramuscular ketamine (87 mg/kg body wt) plus xylazine (13 mg/kg) and maintained on supplemental ketamine as needed. The left carotid artery was cannulated for continuous monitoring of ICP. 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 ICP. The left corpus cavernosum was cannulated by insertion of a 30-gauge needle 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; contains autonomic nerve fibers that innervate the cavernosal vascular tissue). Stainless steel bipolar electrodes were positioned on the MPG, and their position was adjusted during stimulation until a maximal voltage-induced erectile 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 30 s, after which the voltage was increased by 1 V for another 30 s, and this was continued over the range of 0–5 V. All pressure data were collected for analysis using Polyview data-acquisition software (AstroMed, Grass Instrument Division).

Immunoblot analysis of cavernosal RhoA and Rho-kinase protein expression. Cavernosal strips (cleaned of the corpus spongiosum and dorsal vein) were snap frozen in liquid nitrogen and homogenized in cold radioimmunoprecipitation buffer [50 mM Tris-HCl (pH 7.4), 1.0% Nonidet P-40, 0.25% sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na3VO4, and 1 mM NaF]. Samples were centrifuged (10,000 rpm, 4°C, 10 min), and the supernatant was collected for protein quantification (11) and immunoblot analysis. Equal amounts of protein (50 μg per lane, rat brain extract as a positive control) were loaded and resolved by 6 or 15% SDS-PAGE. Protein was transferred to a nitrocellulose membrane (Immobilon-P, Millipore) using a Bio-Rad Mini-Protein III apparatus (100 V, 1 h, 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, 22°C). After blocking, the membrane was incubated overnight (4°C) with primary goat polyclonal antibody to the carboxy terminus of Rho-kinase (1:200 dilution, ROCK-2 or primary monoclonal antibody to RhoA; Santa Cruz) and subsequently incubated for 1.5 h with an anti-goat horseradish peroxidase-linked secondary antibody as appropriate (1:15,000 dilution, 22°C; Jackson). Antibody bound protein was visualized by using an enhanced chemiluminescence kit (Amersham). Rho-kinase protein expression corresponded to a band in the 170-kDa range. Another band at ~55 kDa was found in the cavernosal samples and was presumed to be nonspecific. RhoA protein expression corresponded to a band in the 23-kDa range.

Drugs used. The present study utilized a selective inhibitor of Rho-kinase (Y-27632) generously supplied by Welfide (Osaka, Japan) that was dissolved in saline such that 2 μl contained 50 nmol. ET-1 was obtained from American Peptide (Sunnyvale, CA) and was dissolved in saline containing 0.1% bovine serum albumin and administered at a rate of 50 pmol/rat in 5 μl. Methox was purchased from Sigma Chemical (St. Louis, MO) and dissolved in 2 μl saline to yield a concentration of 10 μg/kg.

Statistical analysis. Data were analyzed using ANOVA with post hoc comparisons made by Student-Newman-Keuls test (25). Statistical significance was set at P < 0.05.

RESULTS

Western blot analysis demonstrates the endogenous expression of both RhoA and Rho-kinase (ROCK-2) proteins in homogenates from isolated rat cavernosal tissue (Fig. 1). Protein (50 and 100 μg) was loaded for RhoA and Rho-kinase immunoblot analyses, respectively. RhoA protein corresponded to a band in the 23-kDa range, whereas Rho-kinase protein corresponded to a band of ~170 kDa. Rat brain homogenate was loaded as a positive control.

The results in Fig. 2 demonstrate the relationship between electrical stimulation of the MPG (0–5 V) and the ICP and the MAP, with measurements made before and after injection of Y-27632. Figure 2 shows that, with increases in the level of stimulation, ICP increases, whereas the MAP remains unchanged at all levels of stimulation. When Y-27632 (50 nmol) is injected into the cavernous sinuses and the voltage stimulation is repeated (0–3 V), the ICP is significantly enhanced over preinjection levels, whereas at 4 and 5 V, the response is not different from the measurements made before injection of the Rho-kinase inhibitor. Figure 2 also shows that injection of Y-27632 causes a small, but statistically insignificant, reduction in MAP at all levels of ganglionic stimulation.

Figure 3 presents the results of studies to determine whether the vasoconstrictor effects of Methox and ET-1 are mediated by the Rho-kinase pathway in the caver-
nosal vasculature. After an initial set of ganglionic stimulations (0–5 V), Y-27632 (50 nmol) was injected into the cavernous sinuses, and ganglionic stimulation was repeated. Next, Methox (10 mg/kg) was injected into the cavernous sinuses during a period when there was still a significant effect of the Rho-kinase inhibitor. Figure 3A shows that Methox appears to reduce the ICP and MAP when it is injected after Y-27632, but this reduction proved to be statistically insignificant from the response to Y-27632 only. Inhibition of Rho-kinase also altered the vasoconstriction that followed injection of Methox. The results in Fig. 3B show that Methox acted as a potent vasoconstrictive agent in the cavernosal circulation and blocked the expected increase in ICP and MAP in response to stimulation of the ganglion. When Y-27632 was given after Methox, the erectile response to ganglionic stimulation was restored at all voltages.

A similar experiment was performed to investigate the interaction between ET-1 and Y-27632. When the Rho-kinase inhibitor was injected first, ET-1 (50 pmol) failed to alter the response to ganglionic stimulation (Fig. 4A). However, when ET-1 was administered first, it caused vasoconstriction and prevented the Y-27632-induced erection (Fig. 4B).

**DISCUSSION**

The results of the present study confirm that the Rho-kinase pathway plays an important role in the

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**Fig. 2.** Effects of electrical stimulation of the major pelvic ganglion on the intracavernosal pressure (ICP) and mean arterial pressure (MAP) with measurements made before (●) and after (□) injection of Y-27632 (50 nmol). Each point is the mean ± SE of 19 individual measurements. *Statistically significant difference before vs. after Y-27632 treatment at that voltage (P < 0.05).

**Fig. 3.** Effects of methoxamine (Methox) and Y-27632 on the erectile response to ganglionic stimulation (0–5 V). A: after an initial ganglionic stimulation (Cont), Y-27632 (50 nmol) was injected into the cavernous sinuses, and ganglionic stimulation was repeated (Y only). After an additional 5 min, Methox (10 μg/kg) was injected, and ganglionic stimulation was repeated (Y-Methox); n = 5. B: after an initial ganglionic stimulation at 0–5 V (Cont), Methox was injected, and the ganglionic stimulation was repeated (Methox). Y-27632 was then administered, and ganglionic stimulation was repeated (Methox-Y); n = 4. Values are means ± SE. *Significant difference from Cont (P < 0.05).

**Fig. 4.** Effects of endothelin-1 (ET-1) and Y-27632 on the erectile response to ganglionic stimulation (0–5 V). A: after an initial ganglionic stimulation (Cont), Y-27632 (50 nmol) was injected into the cavernous sinuses, and ganglionic stimulation was repeated (Y only). After an additional 5 min, ET-1 (50 pmol) was injected, and ganglionic stimulation was repeated (ET-1 only). Y-27632 was then administered, and ganglionic stimulation was repeated (ET-1-Y); n = 4. Values are means ± SE. *Significant difference from Cont (P < 0.05).
maintenance of the vasoconstrictive state of the cavernosal vasculature. Endogenous expression of RhoA and Rho-kinase protein was found in the corpus cavernosum tissue through Western blot analysis. Additionally, as previously published (6), our results demonstrate that blockade of Rho-kinase activity with Y-27632 results in an increase in ICP. Our results examined the effects of Y-27632 on Methox- and ET-1-mediated cavernosal vasoconstriction and support the hypothesis that a portion of the vasoconstrictor effects of α-adrenergic stimulation and ET-1 is mediated by the Rho-kinase pathway.

A portion of α-adrenergic stimulation, as well as ET-1-induced contraction, has been reported to be mediated by RhoA and Rho-kinase activity in other vascular beds (24). Y-27632 was found to relax dose dependently strips that were prepared from rabbit aorta or pig coronary artery and contracted with phenylephrine or ET-1. However, Rho-kinase inhibition was significantly less effective at relaxing tissue precontracted with KCl (24). Y-27632 was also reported to relax premeabilized tissue in which the calcium concentration was maintained constant, indicative of the calcium-independent effects of RhoA and Rho-kinase on contraction. It is likely that Methox and ET-1 activate both phospholipase C and inositol trisphosphate-mediated, calcium-dependent mechanisms, as well as the calcium sensitizing RhoA/Rho-kinase pathway.

Comparison of the results in Figs. 3 and 4 show that, when administered first, Y-27632 prevents the vasoconstrictor activities of ET-1 and Methox. However, if ET-1 is injected before Y-27632, the Rho-kinase inhibitor fails to prevent the vasoconstriction, even at 8 min after administration (data not shown). The erectile response when Methox is injected before Y-27632 is reduced at 3 min but shows some recovery of the vasoconstrictor effect by 8 min after injection of the Rho-kinase inhibitor (data not shown). These differences in the action of the Rho-kinase inhibitor at preventing the vasoconstrictive actions of Methox or ET-1 were surprising in light of its action when given after the vasoconstrictors. A possible explanation for the difference is that the vasoconstrictive agents elevate Rho-kinase to such an extent that administration of 50 nmol Y-27632 fails to or only partially blocks the vasoconstriction, and higher doses of Y-27632 may then be needed to block the vasoconstrictor activity. In addition, the location of the Y-27632-sensitive Rho-kinase step between ligand binding of ET-1 and the α-adrenergic agonist and inhibition of MLC phosphatase could account for our observation that the inhibitor is more active at preventing vasoconstriction than reversing it.

The results of the present study extend our laboratory’s prior findings that the RhoA/Rho-kinase pathway exists in the penile circulation and that its inhibition results in erection. Our laboratory demonstrated that the selective Rho-kinase inhibitor Y-27632 induced cavernosal smooth-muscle relaxation and increased ICP/MAP via a NO/cGMP-independent pathway (5, 6). It has been previously assumed that erection is due primarily to a direct effect of NO to induce vasorelaxation. However, it has also been reported that NO, via increased protein kinase G activity, may inhibit the membrane association and thus the activity of RhoA by phosphorylation (8, 20) or ADP ribosylation (16). The demonstration that Y-27632 induces a large increase in ICP suggests that inhibition of vasoconstriction may be critical to the erectile response. Our results suggest the possibility that the principle action of NO is not to directly stimulate cavernosal smooth-muscle relaxation but rather to inhibit cavernosal smooth-muscle contraction by inhibiting the activity of the Rho-kinase pathway.

An inhibitory action of NO on ligand-induced vasoconstriction has been demonstrated in other studies from this laboratory. When injected directly into the vasculature of the penis, ET-1 acted as a potent vasoconstrictor via the ET \(_{\alpha}\) receptor and reduced the erectile response to ganglionic stimulation (7). However, if the same dose of ET-1 was given into the cavernous sinuses during erection from ganglionic stimulation or from administration of a NO donor drug, the vasoconstrictor action of ET-1 was sharply reduced (13). Similar observations have been made with the α-adrenergic agonist Methox, for which the vasoconstrictor action was also inhibited during erection (26). The results of the present study extend these findings by showing that ET-1- or Methox-induced vasoconstriction is prevented if either of these constrictors is given immediately after administration of Y-27632. This finding strongly supports our hypothesis that the RhoA/Rho-kinase pathway is involved in ET-1- and α-adrenergic-induced vasoconstriction of the cavernosal vasculature.

In the present study, we assumed that Rho-kinase-regulated vasoconstriction occurs in the smooth muscle of the cavernosal arterioles and the walls of the cavernosal sinuses. However, based on recent findings examining the regulation of the cavernosal vasculature (12), we cannot rule out the possibility that Methox, ET-1, and Y-27632 act on the prepenile arteries and not directly on the cavernosal sinuses and arterioles. However, our laboratory’s recent demonstration that strips of cavernosal tissue in a muscle bath relax in response to Y-27632 demonstrates the presence of Rho-kinase activity in the cavernosal tissue (6).

The RhoA/Rho-kinase calcium-sensitizing pathway has not been extensively studied in the cavernosal circulation, although its importance has been reported in other vascular beds (22). In these other vascular systems, heterotrimeric G protein activation activates the monomeric small protein RhoA by promoting GTP binding. The resulting activated RhoA GTP, in turn, activates Rho-kinase. Rho kinase has a variety of cellular functions, including the phosphorylation and resulting inactivation of MLC phosphatase. In the active form, MLC phosphatase removes the phosphate group from MLC. Because the phosphorylated state of MLC is required for myosin binding to α-actin and contraction, inhibition of MLC phosphatase would favor a net phosphorylation of myosin and smooth-muscle contraction.
Conversely, inhibition of Rho-kinase would permit greater activity of the MLC phosphatase, resulting in reduced levels of MLC phosphorylation and smooth-muscle relaxation. The results of the study show that selective inhibition of Rho-kinase activity elevates ICP, resulting in penile erection, and that the known vasoconstrictor effects of an α-adrenergic agonist and ET-1 are prevented by Y-27632. These findings may have implications in the development of new methods for the treatment of erectile dysfunction.

REFERENCES