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Inhibition of Tonic Contraction—A Novel Way to Approach Erectile Dysfunction?

THOMAS M. MILLS,*† RONALD W. LEWIS,† CHRISTOPHER J. WINGARD,* KANCHAN CHITALEY,* AND R. CLINTON WEBB*

*From the *Departments of Physiology and †Surgery (Urology Section), Medical College of Georgia, Augusta, Georgia.*

Experiments from this laboratory have demonstrated that vasoconstriction in the cavernosal circulation is mediated, in part, by the RhoA/Rho-kinase calcium-sensitization pathway. When RhoA/Rho-kinase signaling is inhibited with Y-27632 in cavernosal smooth muscle, the intracavernosal pressure (ICP) is markedly elevated without a significant change in mean arterial pressure (MAP). To explain how erection can occur in the presence of this strong vasoconstrictive signal, we have suggested that nitric oxide (NO) induces vasodilation, leading to erection by directly inhibiting activity of the RhoA/Rho-kinase pathway. The following discussion summarizes the importance of Rho-kinase signaling in the erectile response and introduces its possible clinical value in 2 animal models of erectile dysfunction (ED) (severe hypogonadism and hypertension). In addition, we suggest that topical application of Y-27632 could be an effective mode of treatment for ED.

Physiology of Penile Erection

Smooth muscle cells that line the arterioles and sinuses of the erectile tissue are the target for the actions of dilators and constrictors in the erectile vasculature of the penis. When this smooth muscle is contracted, blood inflow through the arterioles is limited, and the penis remains in the flaccid (nonerect) state. Relaxation of this smooth muscle permits increased rates of inflow through the arterioles and, as the cavernosal sinuses fill with blood, the veno-occlusive mechanism is activated to reduce the rate of outflow. Thus, the initial increased rate of inflow and the resulting reduction in outflow lead to the high ICP characteristic of the erect penis (Andersson and Wagner, 1995).

Vasoconstriction in the Cavernosal Circulation: Keeping the Penis in the Nonerect State

Constriction in the penile vasculature is mediated by α-adrenergic agonists (norepinephrine [NE]), along with endothelin-1 (ET-1) and possibly other agents (Andersson, 2001). These agents are thought to bring about vasoconstriction by activating phospholipase C, which catalyzes the cleavage of phosphatidylinositol to inositol triphosphate and diacyl glycerol, increasing intracellular levels of calcium (Ca^{2+}). Ca^{2+} binds to calmodulin, which activates myosin light chain (MLC) kinase, leading to increased levels of phosphorylation of MLC. Phosphorylated MLC interacts with α-actin and smooth muscle contraction results. While this is the widely accepted mechanism, the studies of DeFeo and Morgan (1985) have shown that, in addition to changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), smooth muscle contraction is also regulated by a Ca^{2+}-sensitization mechanism. These investigators demonstrated that the α-adrenergic agonist phenylephrine increased the force generation in strips of ferret aorta (ie, contraction). However, this force generation was associated with only a transitory increase in $[Ca^{2+}]_i$, as $[Ca^{2+}]_i$ declined rapidly to near basal levels despite continued force generation. This disconnection between contraction and continued elevation of $[Ca^{2+}]_i$ indicated that a Ca^{2+}-sensitization mechanism was also activated in the contraction process. Unpublished studies from our laboratories have likewise shown that ET-1 causes only a transitory increase in $[Ca^{2+}]_i$ in smooth muscle cells isolated from rat penis.

The RhoA/Rho-Kinase Calcium-Sensitization Pathway

While there is evidence supporting the primary role of $[Ca^{2+}]_i$ in cavernosal smooth muscle contraction and relaxation, the Ca^{2+}-sensitization process in the vascular tissue of the penis has not been investigated. In most vascular tissues, Ca^{2+} sensitization is, at least in part, under the control of the RhoA and Rho-kinase systems (Somlyo et al, 1999; Sauzeau et al, 2000; Somlyo and Somlyo,

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Correspondence to: Dr Thomas M. Mills, Department of Physiology, Medical College of Georgia, Augusta, GA 30912-3000 (e-mail: tmills@mail.mcg.edu).

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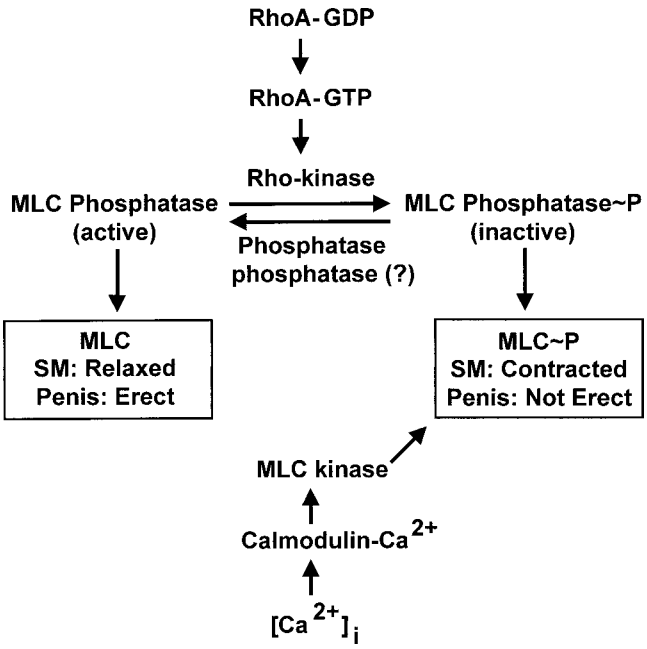

The state of myosin light chain (MLC) phosphorylation in cavernosal smooth muscle (SM) is regulated by MLC kinase and MLC phosphatase. MLC kinase is activated by increased intracellular levels of calcium $[Ca^{2+}]_i$; Ca^{2+} binds to calmodulin to activate the kinase. In the active form, MLC phosphatase promotes SM relaxation, leading to penile erection, but when MLC phosphatase is phosphorylated (and hence inactive), cavernosal SM remains contracted, and the penis is not erect. Thus, by regulating the phosphorylation of MLC phosphatase, the RhoA/Rho-kinase pathway exerts regulatory control over the erectile process.

2000). The following discussion will review RhoA and Rho-kinase signaling and summarize our studies in the role of RhoA and Rho-kinase in the erectile response (see Figure).

RhoA is a small, guanosine triphosphatase (GTP)-binding protein that is involved in many processes, including morphology, cytoskeletal function, secretion, and smooth muscle contraction (Somlyo et al, 1999). When in the inactive state, RhoA binds to guanosine diphosphate (GDP), but upon stimulation (ie, vasoconstrictor ligand binding), GDP is exchanged for GTP. Activation of RhoA also involves posttranscriptional modification (ie, geranylgeranylation) and migration to the cell membrane. The GDP/GTP-binding process is regulated by several factors, including guanine nucleotide dissociation inhibitors (GDI), which inhibit RhoA activation, and guanine nucleotide exchange factors (GEF), which promote the exchange of GTP for GDP, leading to RhoA activation. Since RhoA activation involves GTP binding and migration to the cell membrane, any factor that blocks GTP binding of RhoA, prevents the secondary modification of RhoA, or inhibits its migration will prevent RhoA activation and block smooth muscle contraction.

Activated RhoA has several downstream targets, but in the process of smooth muscle contraction, the downstream target is Rho-kinase (Uehata et al, 1997). Rho-kinase promotes vasoconstriction by utilizing adenosine triphosphate (ATP) to phosphorylate MLC phosphatase, leading to inhibition of MLC phosphatase activity. With reduced MLC phosphatase activity, the phosphorylated form of MLC becomes predominant, and smooth muscle contraction is the result. Inhibition of RhoA-induced activation of Rho-kinase therefore inhibits vasoconstriction and permits vasodilation.

RhoA/Rho-kinase Activity in the Cavernosal Circulation

We have reported the results of a series of experiments dealing with the activity of the RhoA/Rho-kinase pathway in the circulation of the rat penis (Mills et al, 2001a,b, 2002; Chitaley et al, 2001b,d, in press). Western analysis of rat penile protein extracts showed the presence of both RhoA and Rho-kinase proteins (Rees et al, 2001, 2002). Our studies have also utilized the drug Y-27632 (Mitsubishi Pharma Corp, Osaka, Japan), which occupies the ATP-binding site on Rho-kinase to prevent the phosphorylation and inactivation of MLC phosphatase (Ishizaki et al, 2000; Narumiya et al, 2000). When Y-27632 is injected directly into the cavernous sinuses of the rat penis, vasodilation, as measured by increased ICP, occurs in a dose-dependent fashion (Chitaley et al, 2001d). We also used inhibitors of NO synthase and guanylate cyclase to show that Y-27632 does not cause erection by activating NO-dependent vasodilation. Our studies also showed that treatment with Y-27632 blocked the vasoconstriction of cavernosal smooth muscle resulting from intracavernous injection of methoxamine and ET-1 (Mills et al, 2001a). Taken together, our findings that inhibition of Rho-kinase leads to vasodilation and penile erection strongly support our contention that the RhoA/Rho-kinase pathway regulates constriction in the penile circulation (Mills et al, 2001b; Chitaley et al, in press).

Erection of the Penis: Vasodilation of Cavernosal Smooth Muscle

From the preceding discussion, it is clear that penile erection can occur with the reversal of Ca^{2+}-mediated vasoconstriction and suppression of RhoA/Rho-kinase-controlled Ca^{2+} sensitization. This process is initiated by the release of NO from both the nonadrenergic, noncholinergic innervation of the cavernosal circulation and cavernosal endothelial cells (Burnett, 1995). NO diffuses into the cavernosal smooth muscle cells to activate soluble guanylate cyclase and increase intracellular levels of cyclic guanosine monophosphate (cGMP), resulting in increased activity of cGMP-dependent protein kinase (PKG). The NO/cGMP/PKG signaling lowers $[Ca^{2+}]_i$, resulting in cavernosal smooth muscle relaxation and erection. On the basis of this proposed scheme, we suggest

that NO also acts to inhibit RhoA/Rho-kinase-mediated Ca^{2+} sensitization as part of the erectile process.

There is support for an inhibitory effect of NO signaling on the RhoA/Rho-kinase pathway in other vascular beds. Sauzeau et al (2000) showed that NO-regulated vasodilation in rat aorta was due, in part, to the PKG-regulated phosphorylation (and inactivation) of RhoA. Studies from our laboratories also showed that rat aorta rings that were contracted with phenylephrine relaxed in response to Rho-kinase inhibition with Y-27632. However, these studies reported that if the endothelial cells (the primary source of NO in isolated aorta) were removed or in the presence of inhibitors of NO production (L-NNA) or guanylate cyclase activity (ODQ), Y-27632 was less effective at causing relaxation. When the NO donor drug sodium nitroprusside was added back to the aortic rings incubation, the inhibitory effect of Y-27632 was restored (Chitaley et al, 2001c; Chitaley and Webb, 2001). These experiments suggest a role for NO-mediated inhibition of RhoA/Rho-kinase as an integral part of vascular smooth muscle relaxation.

Other studies from our laboratories have investigated the interaction between NO and RhoA/Rho-kinase signaling in penile circulation. In these in vivo experiments, the erectile response to the NO donor drug NOR-1 was measured before and after administration of a threshold dose of Y-27632 to block Rho-kinase activity (Mills et al, 2002). Y-27632 potentiated the NO effect; the response to the combination of the 2 drugs (ie, NOR-1 plus Y-27632) was significantly greater than the response to either drug alone or to the sum of the individual responses. The concept that the NO/cGMP/PKG pathway inhibits RhoA/Rho-kinase signaling is also supported by our observations that Y-27632 enhances the effect of endogenous NO release resulting from electrical stimulation of the autonomic innervation of the penile circulatory system (Chitaley et al, 2001b; Mills et al, 2001b).

Working Hypothesis for the Relationship Between NO and RhoA/Rho-kinase in the Erectile Response

We propose that the cavernosal and arteriolar smooth muscles remain in the contracted state to limit blood inflow and that this, along with veno-occlusion, maintains the flaccid state of the penis (Chitaley et al, 2001b, in press; Mills et al, 2001b). Vasoconstriction is regulated by NE and ET-1 stimulation of the RhoA/Rho-kinase Ca^{2+}-sensitization pathway. In addition, there may be significant RhoA/Rho-kinase activity that is not regulated directly (ie, constitutively expressed). In either case, the high level of Rho-kinase activity maintains high levels of phosphorylated (and therefore inactive) MLC phosphatase, so that even though $[Ca^{2+}]_i$ has declined, the cells remain contracted. With the initiation of erection, NO, released from autonomic nerve fibers and endothelial cells, stimulates guanylate cyclase, resulting in increased cGMP production and PKG activity. PKG could have any one of several actions, including closure of L-type Ca^{2+} channels to lower $[Ca^{2+}]_i$ levels or inhibition of RhoA/Rho-kinase signaling. We propose that the significant action of NO in the erectile response is to inhibit RhoA/Rho-kinase-mediated Ca^{2+} sensitization. This action of NO reverses the phosphorylation state of MLC phosphatase, permitting increased phosphatase activity. Dephosphorylation of MLC leads to cavernosal smooth muscle relaxation, vasodilation, and penile erection.

Potential for Use of Rho-kinase Inhibitors in the Treatment of ED

Topical Application—Although intracavernous injection therapy has been widely used in the clinical treatment of ED, transurethral, topical, and oral modes of drug administration have been well accepted by men suffering from ED. To determine the efficacy of topical application of the Rho-kinase inhibitor, Y-27632 was dissolved in dimethylsulfoxide to achieve 0 (control) or 100-, 200-, or 400-nmol/2 μL doses. Y-27632 was spread over the dorsal proximal regions of the rat penis, and ICP and MAP were continuously recorded. Our unpublished results show that at all doses, Y-27632 caused a statistically significant increase in ICP. However, further analysis also revealed that MAP was significantly reduced at high concentrations of the drug. Y-27632 was concluded to be effective when applied topically to enhance the erectile response. The unique structure of the rat penis likely contributed to the Y-27632-induced decline in MAP in some of the rats; the large central vein running between the paired corpora cavernosa is very close to the dorsal surface of the penis, and rapid diffusion of Y-27632 into the systemic circulation is possible.

Effect of Rho-kinase Inhibition on the Erectile Response in Animal Models of ED

Because inhibition of Rho-kinase leads to penile erection in normal animals, we have investigated the potential value of Y-27632 in 2 animal models of ED: rats made severely hypogonadal by castration and rats that spontaneously develop hypertension.

Hypogonadism-Induced ED

Although the exact role of androgens in the maintenance of penile responsiveness remains controversial, it is known that they regulate erectile function (Arver et al, 1996) and that the frequencies of nocturnal penile tumescence episodes are suppressed in severely hypogonadal men (Horita and Kumamoto, 1994; Granata et al, 1997). Our prior studies have demonstrated that with castration, erectile function is significantly diminished in rats (Mills et al, 1999; Mills and Lewis, 1999). Recently, we have

found by Western analysis that the protein content of both RhoA and Rho-kinase is up-regulated in castrated animals compared to normal, intact rats (unpublished). Furthermore, injection of Y-27632 restores the erectile response in castrated animals.

ED in Hypertension

The extent to which it is the antihypertensive medication or the hypertension itself that is responsible for the ED in patients is not entirely clear (Jensen et al, 1999; Kochar et al, 1999; Burchardt et al, 2000). To investigate this question, we utilized spontaneously hypertensive rats that are also stroke prone (SHRSP). All SHRSP had elevated systemic blood pressures (tail cuff) of at least 180 mm Hg prior to use. Measurement of ICP and MAP in these animals demonstrated a sharply reduced erectile response to ganglionic stimulation. When the animals were given a single intracavernous injection of Y-27632, their erectile response was significantly improved (Chitaley et al, 2001a). Further analysis showed that Y-27632 caused an increase in ICP despite a significant reduction in MAP. Similar studies have been reported using the mineralocorticoid-salt model of hypertension, in which the erectile response was also strongly compromised (Chitaley et al, 2001a). Again, treatment with Y-27632 enhanced erectile response and lowered MAP in these animals.

Development of RhoA/Rho-kinase Inhibition as a Strategy for the Treatment of ED

Our studies have demonstrated that Y-27632 will cause increased erection in normal rats. In addition, we have shown that inhibitors of the RhoA/Rho-kinase pathway may have potential clinical application by showing a beneficial effect of Y-27632 in 2 animal models of ED including severe hypogonadism and hypertension. Both intracavernous injection and topical application of Y-27632 are effective at elevating intracavernous pressure. Except at the highest doses, injection of Y-27632 does not significantly lower systemic blood pressure. Topical application of Y-27632 did lead to suppression of MAP, but the unique structure of the rat penis may contribute to this potentially adverse finding. Oral administration of the Rho-kinase inhibitor remains an option that we have not yet investigated. However, in studies from other laboratories, oral Y-27632 did not lower systemic blood pressure in normotensive rats but did reduce systolic pressure in hypertensive animals (Uehata et al, 1997).

Our studies show that Y-27632 initiates vasodilation by directly inhibiting Rho-kinase and not by stimulating the release of NO. For this reason, Rho-kinase inhibition may be an option for use in the treatment of ED in patients also taking nitrate-containing antihypertensive drugs; the use of these drugs is contraindicated in patients taking sildenafil and other phosphodiesterase inhibitor drugs. Our results show that the prior treatment of rats with the NO donor drug NOR-1 potentiates the effect of Y-27632 to increase Y-27632-induced vasodilation. However, this NOR-1/Y-27632 combination did not measurably affect systemic blood pressure in these animals. Despite this report, we cannot conclude that NO donor drugs and Rho-kinase inhibitors do not interact synergistically to lower systemic blood pressure until a wider range of doses for each of the drugs has been tested. Furthermore, there is the possibility that additional research studies may reveal distinct isoforms of RhoA or Rho-kinase proteins in the cavernosal circulation, and drugs developed to target these isoforms may be useful to reduce penile-specific effects. In addition, other approaches to the inhibition of RhoA/Rho-kinase signaling could include altering the activity of associated molecules (GDI and GEF), which are essential for activity of the pathway.

Thus, the further development of ED treatment strategies will include determination of the best mode of delivery of drugs that target the inhibitors to the cavernosal circulation and minimize systemic effects.

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Appendix

Question 1—In the pathway that controls Rho-kinase-mediated vasoconstriction, you show that norepinephrine and endothelin are primarily initiating the process. Is there anything else that may be initiating this response, or is there some regulation originating at a higher level?

Answer—There is a strong possibility that this is a constitutively expressed pathway. I think there is a real likelihood that the vessels in the penis default to the state of penile flaccidity. These vessels are tonically constricted because of the constitutive expression of Rho-kinase. Only with the advent of NO-mediated vasorelaxation can erection occur. It is likely that even without these vasoconstrictors, this system is vasoconstricted all the time.

Question 2—I am fascinated by the data on Rho-kinase inhibitors in castrated animals. Do you think the testosterone effect on the regulation of the erectile response at the penile level may be actually mediated through the Rho-kinase system?

Answer—What I can tell you is that Western blot analysis performed in our lab has been done with both RhoA and Rho-kinase proteins in castrate and noncastrate rats. Actually, both of these proteins are up-regulated. But there is more protein in the penises of castrated animals, suggesting that they may have a higher level of tonic contraction and therefore less response to the limited amount of NO that may be available. There is a real possibility that testosterone is somehow regulating the amount of these enzymes that are present.

Question 3—There are about 7 Rhos involved in cytoskeletal reorganization. Some agents (eg, botulism C toxin) have been used in in vitro models to inhibit their action. Has this kind of experimental approach been used to compare Rho-kinase inhibitors?

Answer—No, we have not done this. We have exclusively used Y-27632 to inhibit Rho-kinase. I am aware that there is a variety of other inhibitors, but this one is convenient and very soluble in aqueous solution, and one can inject very tiny amounts of it into the erectile tissue without activating the veno-occlusive mechanism.

Question 4—Does Rho-kinase mediate the action of all isoforms of Rho, or is it specific only for RhoA?

Answer—The specificity of this Rho-kinase inhibitor is much higher for RhoA than for the other isoforms.

Question 5—You imply that Rho-kinase somehow overcomes cavernosal smooth muscle relaxation by the NO system. What about other smooth muscle relaxing systems like prostaglandin E, vasoactive intestinal polypeptide, calcitonin gene-related peptide, etc? Are they also involved in this Rho-kinase system?

Answer—That is a difficult question to answer because protein kinase A (PKA) and PKG cross-talk. We have investigated the cyclic adenosine monophosphate (cAMP)-dependent PK pathway using forskolin to activate the erectile response. Forskolin gives a similar erectile response to NO-dependent relaxation. The Y compound enhances the effect of forskolin in the same way as NO-dependent relaxation. Whether there is a real synergism, as we see with the cGMP-dependent pathway, is unknown. We have not done enough animal experiments to answer this question yet, but this is an active area of investigation in our laboratory.