RhoA/Rho-kinase in erectile tissue: mechanisms of disease and therapeutic insights

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ABSTRACT
Penile erection is a complicated event involving the regulation of corpus cavernosal smooth muscle tone. Recently, the small monomeric G-protein RhoA and its downstream effector Rho-kinase have been proposed to be important players for mediating vasoconstriction in the penis. RhoA/Rho-kinase increases MLC (myosin light chain) phosphorylation through inhibition of MLCP (MLC phosphatase) thereby increasing Ca\(^{2+}\) sensitivity. This review will outline the RhoA/Rho-kinase signalling pathway, including the upstream regulators, guanine nucleotide exchange factors, GDP dissociation inhibitors and GTPase-activating proteins. We also summarize the current knowledge about the physiological roles of RhoA/Rho-kinase in both male and female erectile tissues and its aberrations contributing to erectile dysfunction in several disease states. Understanding the RhoA/Rho-kinase signalling pathway in the regulation of erection is important for the development of therapeutic interventions for erectile dysfunction.

INTRODUCTION
ED (erectile dysfunction) is defined as the inability to achieve or maintain an erection of sufficient rigidity for vaginal penetration. Epidemiological studies suggest that ED is often associated with aging and chronic illnesses such as diabetes mellitus, hypogonadism and cardiovascular diseases. It is estimated that ED currently affects 30 million men in the United States and the number will increase to 322 million by 2025 [1,2]. In the past decade, knowledge of penile erection and the pathogenesis of ED has advanced dramatically. Penile erection ultimately depends on the states of contraction and relaxation of SMCs (smooth muscle cells) of the cavernosal arterioles and trabeculae of the corpora cavernosa. However, most studies have focused on vasodilatory mechanisms involving NO (nitric oxide), which is released from non-adrenergic and non-cholinergic nerve endings and endothelial cells in the penis. Impaired NO production or bioavailability leads to increased cavernosal smooth muscle contraction, resulting in ED. In contrast, understanding of contractile signalling pathways involved in erectile function has just emerged recently. In this review, we highlight the contractile mechanisms of erection through activation of the RhoA/Rho-kinase-mediated Ca\(^{2+}\) sensitization pathway and provide an update on the

Key words: corpus cavernosum, erectile dysfunction, penis, RhoA, Rho-kinase, smooth muscle.

Abbreviations: CNF, cytotoxic necrotizing factor; DAG, diacylglycerol; DH, Dbl homology; ED, erectile dysfunction; ET-1, endothelin-1; GAP, GTPase-activating protein; GDI, GDP dissociation inhibitor; GEF, guanine nucleotide exchange factor; GPCR, G-protein-coupled receptor; GTP[S], guanosine 5′-\(\gamma\)-thio]triphosphate; ICP, intracavernosal pressure; IP\(_3\), inositol trisphosphate; LARG, leukaemia-associated RhoGEF; MLC, myosin light chain; MLCK, MLC kinase; MLCP, MLC phosphatase; MMAS, Massachusetts Male Aging Study; NO, nitric oxide; NOS, NO synthase; eNOS, endothelial NOS; PH, pleckstrin homology; PIP\(_2\), phosphatidylinositol 4,5-bisphosphate; PKA, cAMP-dependent protein kinase A; PKB, protein kinase B; PKC, protein kinase C; CPI-17, PKC-potentiated inhibitor protein of 17 kDa; PLC, phospholipase C; PP1, protein phosphatase 1; RGS, regulators of the G-protein signalling; SHR, spontaneously hypertensive rat; siRNA, small interference RNA; SMC, smooth muscle cell; SNP, sodium nitroprusside; WKY, Wistar–Kyoto.

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rapidly accumulated information about this pathway as it pertains to normal erection physiology and to vascular-genic ED.

**PHYSIOLOGY OF PENILE ERECTION**

Penile erection is a complex neurovascular event which is initiated by stimulation of central and peripheral neuronal systems [3,4]. In the flaccid state, sympathetic neuronal input is dominant, releasing neurotransmitters, such as noradrenaline (norepinephrine), that maintain cavernosal smooth muscle contraction. Following sexual stimulation, parasympathetic activity is increased, leading to increased release of NO synthesized by nNOS (neuronal NOS (NO synthase)) from nerve endings in the penis. NO activates the cGMP/PKG (protein kinase G) signalling cascade, inducing corporal smooth muscle relaxation. Reduced peripheral resistance of cavernosal arterioles allows blood flow into the penis under the driving force of systemic blood pressure. The increased blood flow causes shear stress which subsequently activates eNOS (endothelial NOS) to produce NO [5,6]. As a result, sinusoidal spaces are filled with blood, creating a pressure to compress the venules against the tunica albuginea and thus limiting venous outflow. Because of this veno-occlusion mechanism, ICP (intracavernosal pressure) increases dramatically, resulting in penile erection.

**Ca²⁺ SENSITIZATION PATHWAYS IN SMOOTH MUSCLE CONTRACTION**

Vascular smooth muscle contraction involves Ca²⁺-dependent mechanisms and Ca²⁺ sensitization mechanisms, which are initiated by activation of receptors or depolarization of the plasma membrane [7,8]. In the classic Ca²⁺-dependent pathway, agonist-induced GPCR (G-protein-coupled receptor) activation increases PLC (phospholipase C) activity, resulting in increased formation of IP₃ (inositol trisphosphate) and DAG (diacylglycerol). IP₃ subsequently induces Ca²⁺ release from the sarcoplasmic reticulum, leading to a rise in intracellular Ca²⁺ levels [9]. The binding of Ca²⁺ to calmodulin causes a conformational change in the protein and enables Ca²⁺/calmodulin to activate MLCK [MLC (myosin light chain) kinase]. Phosphorylation of the regulatory MLC by MLCK leads to the force generation. Meanwhile, DAG together with Ca²⁺ activates PKC (protein kinase C), promoting contraction by phosphorylation of L-type Ca²⁺ channels or other proteins that regulate crossbridge cycling. Finally, voltage-operated Ca²⁺ channels in the membrane open in response to membrane depolarization brought on by vascular smooth muscle stretch.

Vascular smooth muscle is able to maintain contractile force for an extended period of time at a low cost of energy. The maintenance of force may not be tightly correlated with an elevation in intracellular Ca²⁺ levels. Early studies have demonstrated agonist-enhanced Ca²⁺ sensitivity by simultaneous measurements of force and cytoplasmic Ca²⁺ levels. Intracellular levels of Ca²⁺ are raised by the addition of an α-adrenergic agonist only during the initial phase of force generation, while falling to near basal levels during the maintenance phase of force [10]. Furthermore, adrenergic agonists and GTP[S] (guanosine 5′-y-thiotriphosphate), a non-hydrolysable analogue of GTP, have been shown to induce contractions without alterations in intracellular Ca²⁺ levels in smooth muscle fibres permeabilized by α-toxin [11]. The phenomenon of increased force at a low intracellular Ca²⁺ level is termed Ca²⁺ sensitization [12]. Regulation of MLCP (MLC phosphatase) has been suggested as one of the mechanisms to increase Ca²⁺ sensitivity in various vascular tissues [13].

This Ca²⁺-independent regulation involves the monomeric GTP-biding protein RhoA [14]. The Rho family belongs to the Ras superfamily of GTPases, including five subfamilies: Rho-like (RhoA, B and C), Rac-like (Rac1, 2 and 3 and RhoG), Cdc42-like, RhoBTB-like and Rho-like (Rnd 1, 2 and 3) [15]. Although RhoA, RhoB and RhoC are highly related proteins that share more than 80% amino acid homology, their cellular functions can be distinct [16,17]. Compared with that of RhoB and RhoC, the functions of RhoA have been well characterized. In addition to its important role in mediating smooth muscle contraction, RhoA is also involved in the regulation of various cellular processes, such as stress fibre formation, cell proliferation and migration and apoptosis [1,18,19]. The best characterized effector of RhoA is Rho-kinase, which is directly involved in smooth muscle contraction (discussed below). In addition, RhoA regulates the activities of other kinases, including citron, mammalian diaphanous protein and protein kinase N, whose functions are involved in cytokinesis, cell cycle, actin polymerization and endosomal trafficking [20,21].

**REGULATION OF RhoA GTPase**

RhoA functions as a molecular switch, cycling between the inactive GDP-bound state and the active GTP-bound state (Figure 1). The activity of RhoA is tightly regulated by three proteins: RhoGEF (guanine nucleotide exchange factor), RhoGAP (GTPase-activating protein) and RhoGDI (GDP dissociation inhibitor) [19,22-24]. In the inactive state, RhoGDI binds to RhoA-GDP to form a complex, stabilizing RhoA-GDP and trapping the complex in the cytosol. The activation of RhoGEF facilitates the exchange of GDP for GTP on RhoA and dissociation from RhoGDI. The active RhoA-GTP then translocates from the cytosol to the plasma membrane and binds to the membrane through geranylgeranylation, initiating signalling transduction. RhoGAP accelerates the intrinsic GTPase activity of RhoA and promotes hydrolysis of GTP on RhoA-GTP thereby inactivating.
Figure 1  \(Ca^{2+}\) sensitization and \(Ca^{2+}\)-dependent pathways in penile smooth muscle

Stimulation of GPCRs leads to the activation of both \(Ca^{2+}\) sensitization and \(Ca^{2+}\)-dependent pathways. PLC catalyses PIP\(_2\) into IP\(_3\) and DAG. IP\(_3\) increases intracellular \(Ca^{2+}\) levels and, together with CaM (calmodulin), \(Ca^{2+}\) activates MLCK. MLCK then phosphorylates MLC, resulting in smooth muscle contraction. After activation by DAG, PKC phosphorylates CPI-17. Phosphorylated CPI-17 has a high affinity for the catalytic subunit of MLCP and decreases MLCP activity through phosphorylation. Activation of GPCRs also stimulates RhoGEF activity, which facilitates the exchange of GTP for GDP on RhoA and dissociates RhoA from RhoGDI. The active RhoA-GTP translocates from the cytosol to the plasma membrane and activates Rho-kinase. Subsequently, Rho-kinase phosphorylates the targeting subunit of MLCP, leading to increased MLC phosphorylation. In addition, Rho-kinase has been shown to phosphorylate CPI-17. Meanwhile, RhoGAP accelerates the intrinsic GTPase activity of RhoA and promotes hydrolysis of GTP; thus, inactive RhoA-GDP re-associates with RhoGDI and relocates to the cytosol.


**RhoGDI**

Three isoforms of RhoGDI have been identified so far. RhoGDI\(_\alpha\) appears to be universally expressed, whereas RhoGDI\(_\beta\) and RhoGDI\(_\gamma\) are restricted to haematopoietic tissues and the brain respectively [26]. RhoGDI acts as an endogenous inhibitor for RhoA through two mechanisms: stabilizing RhoA in its inactive GDP-bound form and translocating RhoA-GDP to the cytosol after extracting it from the membrane. Although RhoGDI is able to interact with both the GTP- and GDP-bound Rho, the binding of GDP to Rho increases the affinity for RhoGDI more than 10 times [27]. Overexpression or exogenous administration of high concentrations of RhoGDI in tissues inhibits Rho-mediated biological activities [28,29]. In a cell-free system derived from rat kidney, RhoA is extracted from the brush-border membrane after addition of a GST (glutathione S-transferase)–GDI fusion protein [30]. However, the effect of GST–GDI on membrane-bound RhoA is abolished at physiological ionic strength of salt (150 mmol/l KCl) and normal body temperature, suggesting that the shuttling activity of RhoGDI may normally be down-regulated under physiological conditions.

The ability of RhoGDI to extract RhoA from the plasma membrane is not solely dependent on the exchange of nucleotide on RhoA. Phosphorylation of RhoA on Ser\(^{188}\) by PKA (cAMP-dependent protein kinase A) has been shown to increase the RhoA interaction with RhoGDI even when it is in the GTP-bound state [31]. Forskolin, a cAMP activator, markedly reduces the amount of active RhoA in renal CD8 cells as determined by RhoA-GTP pull down assay and Western blot analysis. Forskolin treatment leads to increased phosphorylation of RhoA as well as decreased membrane fraction of GDI, which results in increased RhoGDI–RhoA complexes in the cytosol [32]. Transfection of human microvascular endothelial cells with adenovirus encoding the PKA inhibitor gene blocks the inhibitory effect of forskolin on RhoA [33]. These studies suggest that phosphorylation of RhoA is an alternative mechanism to inhibit RhoA activity, independently from its nucleotide state.
RhoGEF

Various forms of RhoGEFs have been identified and among them p115RhoGEF, PDZRhoGEF and LARG (leukaemia-associated RhoGEF) are most studied. These RhoGEFs, as with other GEFs, contain a DH (Dbl homology) domain and a PH (pleckstrin homology) domain linked in tandem. Crystallographic studies provide the physical evidence that RhoGEFs directly interact with RhoA at both DH and PH domains [34]. The DH domain is thought to be responsible for catalysing the nucleotide exchange reaction, whereas the PH domain may stabilize the binding of DH domain to RhoA and direct the subcellular localization of RhoA to the membrane, allowing RhoA to interact with actin [35,36]. In some cases, the PH domain can also modulate the nucleotide exchange activity of the DH domain [37]. Kristelly et al. [38] have demonstrated that the DH/PH domains of LARG catalyse nucleotide exchange more efficiently than the DH domain only.

More importantly, p115RhoGEF, PDZRhoGEF and LARG have a unique motif known as the RGS (regulators of the G-protein signalling) domain at the N-terminal that serves as a binding site for the α-subunits of the heterotrimeric G-proteins. Additionally, the RGS domain of these RhoGEFs functions as a GAP, accelerating Gα intrinsic GTPase activity [39,40]. It has been reported that Gα12 and Gα13, members of the G12 family, can physically associate with p115RhoGEF, PDZRhoGEF and LARG [41–43]. Mutation of Gα12 on residues Glu229, Lys234 or Arg232 attenuates the activation of p115RhoGEF and LARG and the subsequent RhoA activation [44,45]. In addition, the variant Gαq reportedly interacts with RhoGEFs, leading to the activation of RhoA [46,47]. In the inactive state of RhoGEFs, the RGS domain and the DH domain have reciprocal inhibitory effects. When Gα is activated, the RGS domain of RhoGEF binds to Gα and exposes the DH domain, which is able to interact with RhoA and initiate nucleotide exchange [48].

Activation of RhoGEFs may not only be dependent on α-subunits of the G-protein, but also on the PKC pathway. In a study of p115RhoGEF-mediated thrombin-induced cytoskeletal rearrangement, direct phosphorylation of p115RhoGEF by PKCα is observed within 1 min of thrombin stimulation [49]. Inhibition of PKCα results in attenuation of thrombin-induced RhoA activation, along with decreased p115RhoGEF phosphorylation. The study suggests that G12 and PKCα pathways may run in parallel to modulate RhoGEF activity.

RhoGAP

RhoGAP inhibits RhoA through enhancing the intrinsic rate of GTP hydrolysis; thus active RhoA-GTP is converted into inactive RhoA-GDP. So far, the knowledge about RhoGAP and how RhoGAP regulates RhoA is incomplete. The mechanism of RhoGAP-facilitated GTP hydrolysis is predicted based on crystallographic studies demonstrating that Arg55 on RhoGAP engages the GTPase active site and stabilizes the transition state of GTP hydrolysis [50]. Several GAPs have been identified to selectively interact with Rho such as Graf, p122RhoGAP and p190RhoGAP [51–54].

Effectors of RhoA GTPase

Active RhoA binds to its downstream targets to initiate numerous cellular responses. Rho-kinase (ROCK or ROK), a serine/threonine kinase, is one of the most studied RhoA effectors and is involved in regulation of the actin cytoskeleton, cell proliferation, local adhesion/stress fibre formation and smooth muscle contraction. Rho-kinase consists of two isoforms: Rho-kinase-α (ROCK-II/ROKα) and Rho-kinase-β (ROCK-I/ROKβ). Rho-kinase contains a RhoA-binding site at the C-terminus and a catalytic site at the N-terminus [55]. Binding of RhoA to Rho-kinase causes a conformational change in Rho-kinase and its subsequent autophosphorylation, increasing Rho-kinase activity [55,56].

In smooth muscle, Rho-kinase induces vasoconstriction through phosphorylation of MLC. MLC is composed of three subunits: MYPT1 (a 110–130 kDa MLC-targeting subunit), PP1c (a 38 kDa catalytic subunit of protein phosphatase type 1) and a small 20 kDa subunit whose function is not clear [57]. MYPT1 is ubiquitously expressed with a relatively higher concentration in SMCs. With the PP1c-binding domain on the N-terminal region and the myosin-binding domain on the C-terminal region, MYPT1 targets MLC to the myosin filaments and increases the catalytic activity of PP1c [58].

The state of MLC phosphorylation involving a balance between MLCK and MLCP activities determines the contractile activity of smooth muscle [59,60]. MLCP inhibition results in the maintenance of phosphorylated MLC at low intracellular Ca2+ concentrations, thus promoting the binding of actin and myosin for force generation. Phosphorylation of MYPT1 at Thr696 is thought to decrease PP1c catalytic activity, which inhibits MLCP and maintains MLC phosphorylation. Agonists such as prostaglandin F2α, lysophosphatidic acid and angiotensin II have been shown to increase phosphorylation of MYPT1 at Thr696 and enhance Ca2+ sensitivity through activation of Rho-kinase [61–63]. In chicken amnion smooth muscle and rat caudal arterial smooth muscle, Rho-kinase inhibits MLCP through phosphorylation of MYPT1 at Thr585 or Thr552, reducing the binding of MLCP to MLC [64–66].

It is noteworthy that Rho-kinase may be directly activated by arachidonic acid, independent of RhoA [67,68]. However, the detailed mechanism through which arachidonic acid activates Rho-kinase is not clear. In
addition, recent studies suggest that Rho-kinase can be inhibited by Rnd3 (RhoE), a member of the Rho family which does not have an intrinsic GTPase activity. Rnd3 is predominantly in a GTP-bound active form and it binds to the N-terminal of Rho-kinase. Studies have shown that Rnd3 competes with RhoA for binding Rho-kinase, although the binding sites for Rnd3 and RhoA on Rho-kinase are distinctively different [69,70]. Rho-kinase inactivation by Rnd3 may result from Rnd3 causing a conformational change of Rho-kinase or RhoA binding to part of the Rnd3-binding site of the enzyme [71]. In addition, Rnd3 stimulates RhoGAP activity, increasing the GDP-bound form of RhoA [72]. Functional studies demonstrated that overexpression of Rnd3 prevents phosphorylation of MLCP and stress fibre formation induced by Rho-kinase [71].

It has been reported that Rho-kinase may also increase Ca$^{2+}$ sensitization by phosphorylating CPI-17 (a 17 kDa PKC-potentiated phosphatase inhibitor). CPI-17 is a downstream target of PKC, which serves an important role in smooth muscle contraction [73]. Molecular and structural studies demonstrate that the phosphorylation of Thr$^{38}$ on CPI-17 by PKC enhances the affinity of CPI-17 and its inhibitory activity on MLCP more than 1000 times [74]. CPI-17 isolated from porcine aorta smooth muscle specifically inhibits PP1c as well as the MLCP holoenzyme [75,76]. It is suggested that phosphorylated CPI-17 selectively suppresses MLCP among various PP1 holoenzymes [77].

The complexities of the PKC/CPI-17 and the RhoA/Rho-kinase pathways have recently been demonstrated. The Rho-kinase inhibitor Y27632 ([(+)-(R)]-trans-4-[(1-aminoethyl)N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride) blocks CPI-17-mediated Ca$^{2+}$ sensitization in response to agonists of this pathway. The specificity of Y27632 for Rho-kinase may be questioned, since Y27632 inhibits phosphorylation of CPI-17 by purified PKC$\beta$ at a concentration similar to that required to inhibit Rho-kinase [78]. Nevertheless, there is evidence suggesting that Rho-kinase can directly phosphorylate native and recombinant CPI-17 at Thr$^{38}$ in vitro. Substitution of Thr$^{38}$ to alanine on CPI-17 significantly decreases its phosphorylation levels by Rho-kinase [79]. Overexpression of an active mutant RhoA or stimulation with thrombin in vascular SMCs increases CPI-17 protein expression as well as its phosphorylation. This phenomenon is attenuated by Y27632 or the RhoA inhibitor exoenzyme C3, but not a PKC inhibitor [80].

**REGULATION OF THE RhoA/Rho-KINASE PATHWAY BY NO**

NO is the primary vasodilator in many vascular beds. NO diffuses into SMCs and activates guanylate cyclase, leading to an increase in cGMP levels. cGMP then activates PKG, thereby decreasing intracellular Ca$^{2+}$ concentration and inducing membrane hyperpolarization. Moreover, NO reduces Ca$^{2+}$ sensitization mediated by the RhoA/Rho-kinase pathway. Evidence has shown that Y27632 is less effective in causing relaxation if the endothelial cells (the primary source of NO) are removed in rat aorta strips [81]. If exogenous NO is added back to the aortic strip incubation, the inhibitory effect of Y27632 is restored. Furthermore, acute or chronic inhibition of NOS increases $\alpha_2$-adrenergic receptor agonist-induced RhoA/Rho-kinase activity [82]. The direct inhibitory effect of NO on RhoA/Rho-kinase activity is supported by molecular studies [83,84]. These studies demonstrated that PKG phosphorylates RhoA at Ser$^{188}$, destabilizing membrane binding of RhoA. Interestingly, administration of SNP (sodium nitroprusside) or a cGMP analogue enhances RhoA mRNA and protein expression, whereas long-term inhibition of NOS significantly reduces RhoA mRNA and protein expression [85]. This result suggests that the basal release of NO is required to maintain RhoA expression and RhoA-dependent functions.

The relationship between NO and RhoA/Rho-kinase becomes more complicated with the evidence that RhoA/Rho-kinase negatively regulates NO production by inhibition of NOS. Transfection of HUVECs (human umbilical vein endothelial cells) with constitutively active RhoA decreases eNOS phosphorylation at Ser$^{1177}$ through inhibition of PKB (protein kinase B) [86]. Additionally, eNOS gene expression is decreased by RhoA overexpression, but this is independent of PKB regulation [86]. Inhibition of Rho-kinase by Y27632 prevents up-regulation of the RhoA/Rho-kinase pathway and down-regulation of eNOS protein expression in the left ventricle from hypertensive rats [87]. Taken together, these studies suggest there is a homodynamic balance between the NO signalling pathway and the RhoA/Rho-kinase pathway to maintain smooth muscle function.

**TOOLS TO STUDY THE RhoA/Rho-KINASE PATHWAY**

The C3 exoenzyme isolated from Clostridium botulinum is the most commonly used toxin for the examination of the functional role of RhoA, because it specifically inhibits RhoA through ADP-ribosylation at Asn$^{41}$ and has little effect on other proteins in the Rho family. The inhibition of RhoA by C3 exoenzyme is thought to block the dissociation of RhoA and RhoGDI complex and prevent the activation of RhoA by RhoGEF, as RhoA agonists GTP[S] and PIP$_2$ (phosphatidylinositol 4,5-bisphosphate) are unable to release RhoA from RhoGDI in the presence of C3 [88]. ADP-ribosylation of RhoA does not appear to interfere with its nucleotide binding, RhoGTPase activity or interaction with downstream effectors such as Rho-kinase [89–91]. On the other hand, CNF (cytotoxic necrotizing factor)-1 and -2, first isolated...
from *Escherichia coli*, have been shown to turn RhoA into a constitutively active form by deamidating Gln to glutamine, which blocks the RhoGAP-increased GTP hydrolysis [92,93]. CNF-1 and -2 also affect other RhoGTPases such as Rac and CDC42 [94]. Recently, CNF₁ identified in *Yersinia pseudotuberculosis* strains has been demonstrated to selectively activate RhoA without activation of Rac or CDC42 [95].

Y27632, a highly selective inhibitor of Rho-kinase, has been a useful pharmacological tool for examining the role of Rho-kinase in various cellular functions [96]. It competes for the ATP-binding site on Rho-kinase, preventing the Rho-kinase-mediated phosphorylation of MLCK, resulting in relaxation of smooth muscle. Y27632 is transported into cells or tissue by a carrier facilitated diffusion mechanism to inhibit Rho-kinase activity; therefore, the extracellular concentration of Y27632 is similar to the intracellular concentration. The *Kᵢ* value of Y27632 for Rho-kinase is about 200-fold lower than that for other protein kinases involved in smooth muscle contractility, such as PKC, PKA and MLCK [96,97]. Although Y27632 is relatively less selective for the Ca²⁺-dependent PKC isoforms, it is a potent inhibitor of the Ca²⁺-independent PKCζ and PKCδ isoforms, which have been shown to mediate Ca²⁺ sensitization [78,97]. The effects of Y27632 on Ca²⁺ mobilization are concentration dependent. Y27632 has minimal effects on intracellular Ca²⁺ at 10 µmol/l, whereas it significantly inhibits the rise of intracellular Ca²⁺ levels induced by agonists at 100 µmol/l [98,99].

Another family of compounds derived from isoquinolinesulphonamide shows selectivity for Rho-kinase. HA1077 (or fasudil hydrochloride), like Y27632, competes for the ATP-binding site on Rho-kinase with a *Kᵢ* = 0.3 µmol/l [100]. However, HA1077 is also a potent inhibitor of PKA and PKC with a *Kᵢ* = 1 and 9.3 µmol/l respectively, which makes it difficult to separate activity of the Rho-kinase-mediated pathway from PKC-mediated Ca²⁺ sensitization using this reagent [101]. H1152P is similar in structure to HA1077, except that it has two extra methyl groups, which significantly enhances its inhibitory effect and selectivity for Rho-kinase. Its *Kᵢ* value for Rho-kinase is approx. 1.6 nmol/l, 400 times more potent than that for PKA, PKC and MLCK [101].

The biological activities of RhoA and Rho-kinase can also be manipulated by overexpression of constitutively active and dominant-negative mutants of RhoA or Rho-kinase as well as the Rho GTPase regulators [102]. The dominant-negative RhoA (T19NRhoA) is generated by the substitution of Asp¹⁹ for threonine, which leads to increased affinity for RhoGEF and decreased GTP binding, competing with endogenous RhoA for RhoGEF. The substitution of Leu⁶⁴ for glutamic acid results in a constitutively active RhoA (V14RhoA). Rho-kinase dominant-negative and active mutants are also used to investigate RhoA/Rho-kinase-mediated Ca²⁺ sensitization [103,104]. Recently, siRNA (small interference RNA) has emerged as a new powerful tool to selectively silence expression of the target gene. This post-transcriptional gene silencing by these 21–25 nucleotides leads to degradation of target mRNAs. Anti-RhoA siRNA has been reported to successfully knock down RhoA expression in cells [105,106].

**RhoA/Rho-kinase signalling in penile erection**

Although the contribution of RhoA/Rho-kinase signalling to vasoconstriction and vascular abnormalities in peripheral vasculature has been extensively studied, it was not until recently that RhoA/Rho-kinase was identified to operate in the penis. The functional role of RhoA/Rho-kinase was first demonstrated in rat penile corpus cavernosum [107]. ICP is significantly increased after administration of Y27632 to the rat penis in vivo without nerve stimulation, suggesting RhoA/Rho-kinase is constantly active and plays an important role in maintaining the penis in the flaccid state. Inhibition of Rho-kinase also potentiates electrical stimulation-induced erections; however, this effect is independent of NO because pre-injection of a NOS inhibitor, N-nitro-L-arginine, and guanylate cyclase inhibitors Methylene Blue or ODQ (1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1), does not block Y27632-induced penile relaxation.

Subsequently, RhoA/Rho-kinase was identified in rat and rabbit penile tissue by immunohistochemistry and Western blot analysis [108,109]. Furthermore, RhoA protein concentration is 17 times greater in rabbit corpus cavernosum than that in ileum, suggesting that RhoA/Rho-kinase Ca²⁺ sensitization may play a significant role in corpus cavernosum smooth muscle contraction [109]. In permeabilized rabbit corpus cavernosal strips, the addition of GTP[S] increases RhoA-GTP as an active form in plasma, suggesting that RhoA is functionally available for Ca²⁺ sensitization in the penis. In addition, RhoGDI, MYPT1, PPIc and CPI-17 protein expression were detected in human and rabbit corpus cavernosum [109]. The localization of CPI-17 supports the possibility of PKC/CPI-17-mediated Ca²⁺ sensitization in the penis. Finally, the significance of the RhoA/Rho-kinase pathway in penile erection was confirmed by adenoviral gene transfer of a dominant-negative RhoA mutant (T19NRhoA) into the rat corpus cavernosum. T19NRhoA markedly increases basal ICP as well as cavernous nerve stimulation-induced ICP in vivo [110].

ET-1 (*endothelin-1*), α-adrenergic agonists and angiotensin II are the primary vasoconstrictors responsible for keeping the penis in the flaccid state [111,112]. Evidence has shown that Y27632 abolishes corporal smooth muscle contractions induced by these vasoconstrictors, suggesting their actions involve the activation of the
RhoA/Rho-kinase pathway in the penis [107,113–115]. In rabbit cavernosum smooth muscle strips, Y27632 has no effect on intracellular $\text{Ca}^{2+}$ concentrations, but attenuates the contraction caused by phenylephrine [116]. The RhoA/Rho-kinase pathway was also shown to mediate the synergistic vasoconstrictive effects of ET-1 and phenylephrine [114]. These results underscore that the RhoA/Rho-kinase signalling pathway plays an important role in tonic contraction of the penis.

Limited information currently exists with regard to the functional relevance of PKC- or PKC/CPI-17-mediated $\text{Ca}^{2+}$ sensitization in penile erection. One study suggests that PKC isoforms may be involved in $\alpha$-adrenergic agonist-induced contraction in rat corpus cavernosum smooth muscle strips [117]. Another study has shown that a non-selective PKC inhibitor decreases contractions in response to an $\alpha$-adrenergic agonist while it does not affect intracellular $\text{Ca}^{2+}$ concentrations [116]. The authors suggested that PKC may mediate $\text{Ca}^{2+}$ sensitization in the penis. However, further studies are required in order to determine whether PKC is important in regulation of $\text{Ca}^{2+}$ sensitivity and whether the PKC/CPI-17 pathway interacts with the RhoA/Rho-kinase pathway in the penis.

Since RhoA/Rho-kinase is continuously active to contract the corporal smooth muscle during the flaccid state, it is plausible that NO may induce penile erection through inhibition of the RhoA/Rho-kinase pathway. Recent studies have demonstrated that NO suppresses RhoA/Rho-kinase-mediated vasoconstriction in the penis both in vivo and in vitro. Injection of Y27632 potentiates the rise in ICP resulting from intracorporeal injection of a NO donor, NOR-1 [118]. In isolated rat corpus cavernosal smooth muscle strips, H1152P or Y27632 has a marked synergistic inhibition with SNP, an NO donor, on both $\alpha$-adrenergic agonist and electrical field stimulation-induced contractions [119]. On the other hand, RhoA/Rho-kinase may exert regulatory control of NO signalling in the penis. In streptozotocin-induced diabetic rat penes, RhoA/Rho-kinase is up-regulated in association with suppressed eNOS activity and protein expression [120]. Inhibition of RhoA using gene transfer of T19NRhoA restores eNOS expression and activity, demonstrating the regulatory basis of RhoA/Rho-kinase signalling.

**ROLE OF RhoA/Rho-KINASE IN ED AETIOLOGICAL CONDITIONS**

Given the important role of RhoA/Rho-kinase-mediated $\text{Ca}^{2+}$ sensitization in smooth muscle contraction, irregular RhoA/Rho-kinase activity may lead to abnormal contractility of smooth muscle. Abundant evidence has shown that elevated RhoA/Rho-kinase activity contributes to the pathogenesis of diseases such as diabetes and hypertension. Studies have suggested that ED is strongly associated with these chronic diseases. Below, we examine the role of the RhoA/Rho-kinase pathway in different diseases associated with ED.

**Hypertension**

ED is associated with cardiovascular diseases such as hypertension [121,122]. The MMAS (Massachusetts Male Aging Study) showed that approx. 30% of patients with ED have a history of hypertension [2,123]. The high prevalence of ED in hypertensive patients was also suggested in a study where 27% of the hypertensive patients had ED compared with 4% with ED in the general population living in the same area [124]. Among those hypertensive patients with ED, 89% was caused by penile arterial dysfunction.

Since Uehata et al. [97] first demonstrated enhanced RhoA/Rho-kinase activity contributing to the increased peripheral vascular resistance in several models of hypertension, over the years substantial evidence has suggested the importance of RhoA/Rho-kinase signalling in the pathogenesis of hypertension. Structurally based studies of penile vascular beds in SHRs (spontaneously hypertensive rats) reveal that penile vasculature undergoes similar structural changes to other vascular beds [125,126]. Therefore increased activity of the RhoA/Rho-kinase pathway observed in hypertension may also be the cause of ED in hypertensive subjects. In stroke-prone SHRs, a well-recognized genetic model of hypertension, and DOCA (deoxycorticosterone)-salt-induced hypertensive rats, ICP/MAP (mean arterial pressure) was significantly lower than that of control rats in response to electrical stimulation of the major pelvic ganglion [127]. Administration of Y27632 into the corpus cavernosum improves erectile function in these hypertensive rats, but to a lesser extent when compared with normotensive rats [127]. The authors suggested that elevated RhoA/Rho-kinase activity contributed to hypertension-associated ED. In addition, impaired erectile function was reported in SHRs in which RhoA protein expression was found to be increased in SHR penes [128]. Intracavernosal injection of Y27632 restores the erectile function in SHR; however, the effect of Y27632 was not determined in normotensive WKY (Wistar–Kyoto) rats.

**Diabetes mellitus**

Diabetes mellitus is a well-recognized risk factor for ED. The risk of ED is 3-fold greater for diabetic men and occurs at an earlier age than for non-diabetic men. In the MMAS, the age-adjusted prevalence of complete ED was 28% in diabetic patients but only 9.6% in the general population [2]. Consistently, the MALES (Men’s Attitudes to Life Events and Sexuality) study also showed the prevalence of ED was 39% in diabetic men compared with 16% in the overall population [123]. The underlying
mechanisms causing the increased risk of ED in diabetes are complicated and remain unclear.

The RhoA/Rho-kinase pathway may be a major molecular mechanism involved in the pathogenesis of diabetes-related ED. Increased RhoA/Rho-kinase activity is observed in penile tissue in diabetic rodents. Chang et al. [129] reported enhanced sensitivity to ET-1 but not phenylephrine in diabetic rabbit corpus cavernosum smooth muscle. The molecular studies demonstrate that the increased sensitivity to ET-1 is due to increased ET1 receptor expression as well as increased Rho-kinase β protein expression. However, there are no significant differences between ET-1-induced contractions of diabetic and non-diabetic human corpus cavernosum [130]. The authors explained the discrepancy as relating to other pre-existing aetiologies in diabetic men. Furthermore, in vivo injection of Y27632 to corpus cavernosum increases ICP to a larger extent in streptozotocin-induced diabetic rats than in control rats, suggesting increased Rho-kinase activity in diabetic rat penes [120]. This increased Y27632 sensitivity is accompanied by increased membrane-fraction RhoA protein and MYPT1 phosphorylation levels in the penile tissue of diabetic rats [120]. However, in diabetic mice, although acetylcholine-induced corpus cavernosal tissue relaxation and erectile function are significantly decreased when compared with that of non-diabetic mice, Rho-kinase inhibitors cause a similar dose–response relaxation curve, indicating that RhoA/Rho-kinase signalling may not be altered in diabetic mouse penes [131].

In diabetes, insulin-mediated smooth muscle relaxation is significantly impaired. It has been reported that insulin inactivates Rho-kinase and decreases MYPT1 phosphorylation in aortic SMCs isolated from normal WKY rats, leading to activation of MLCP [132]. The inhibition of Rho-kinase by insulin is mediated by the PI3K (phosphoinositide 3-kinase)/NO/cGMP pathway. In aortic SMCs derived from Goto–Kakizaki rats, a Type II diabetic rat model, insulin loses its ability to inhibit Rho-kinase because of severely impaired IRS-1 (insulin receptor substrate-1) tyrosine phosphorylation induced by insulin [133]. The relationship between RhoA/Rho-kinase and insulin has not been investigated in penile tissue.

Hypogonadism

Hypogonadism refers to primary testicular failure or insufficient testicular stimulation due to the lack of pituitary gonadotropins. Androgens are important sex hormones in the regulation of male sexual function, including sexual desire and erectile function [3]. It has been reported that hypogonadism is frequently related to ED and testosterone replacement has shown beneficial effects on erectile function [134–136].

Studies in the rat model of castration have suggested that up-regulation of the RhoA/Rho-kinase pathway in the penis could be an underlying mechanism in hypogonadism-associated ED. Contractile responses to phenylephrine are increased in corpus cavernosum muscle strips isolated from castrated rats in parallel with increased sensitivity to Y27632 and increased protein expression of RhoA and Rho-kinase in penes [137]. Testosterone replacement restores erectile function and decreases RhoA and Rho-kinase protein expressions in castrated rats, although these are still significantly higher than those in intact rats. This may be explained by the multifactorial effects of testosterone, including its stimulation of the production of the pro-erectile factor NO [137]. Topical application of Y27632 to the tunica albuginea of intact rat penis or the glans and surrounding skin of castrated rat penis also causes a significant increase in ICP [138].

Aging

ED is strongly associated with aging. In the MMAS, it was estimated that the prevalence of ED is 52 % among men aged 40–70 years [2]. Previous studies have shown that age-associated ED results from decreased sex hormone levels, decreased NOS-containing nerve endings and eNOS expression, decreased VEGF (vascular endothelial growth factor) mRNA levels and increased superoxide production, which ultimately leads to diminished NO production or bioavailability [139–142]. In contrast, vasoconstrictors such as ET-1 in aged penile tissue are increased. Gene transfer of ecSOD (extracellular superoxide dismutase) or eNOS restores erectile function in aged rats [142,143].

The role of RhoA/Rho-kinase in age-related ED has just begun to be investigated. A recent study demonstrates that Y27632 significantly improves erectile responses to electrical stimulation of the major pelvic ganglion in aged Brown–Norway rats, suggesting the beneficial effects of Rho-kinase inhibition for correcting ED in the aged rat [144]. To evaluate whether increased RhoA/Rho-kinase activity is uniquely responsible for age-associated ED, we compared the effects of inhibition of RhoA/Rho-kinase by Y27632 and gene transfer of the dominant-negative RhoA on erectile responses in young and aged Fischer 344 rats [145]. Inhibition of RhoA/Rho-kinase signalling improves erectile responses to a much larger extent in aged rats, suggesting increased RhoA/Rho-kinase activity in aged rat penile tissue. These data are supported by molecular studies showing that MYPT1 phosphorylation is markedly increased in aged rat penes when compared with young rat penes, although the protein expressions of RhoA, Rho-kinase and MYPT1 are not altered.

RhoA/Rho-KINASE IN FEMALE SEXUAL FUNCTION

In the past few decades, the majority of basic science studies in sexual medicine have focused on male ED
with little attention given to female sexual dysfunction. Studies have shown that decreased clitoral engorgement and lack of vaginal wall relaxation contributes to sexual dysfunction in women. The Rho-kinase inhibitor Y27632 reduced phenylephrine- and angiotensin II-induced contraction of vaginal and clitoral smooth muscle isolated from rabbits in a concentration-dependent manner [146,147]. The potency of Y27632 seems greater in female genital tissue than that of males; however, it is not clear whether the difference is due to increased protein expression levels of components of the RhoA/Rho-kinase signalling pathway or a high sensitivity of RhoA/Rho-kinase in response to agonists. Inhibition of NOS does not affect the EC₅₀ of Y27632, indicating the relaxation effect of Rho-kinase inhibition is independent of NO in rabbit vaginal wall and clitoral corpus cavernosum [147]. These studies provide evidence that the RhoA/Rho-kinase pathway is involved in the regulation of vaginal wall and clitoris smooth muscle tone. Although no study has been done to determine the role of RhoA/Rho-kinase signalling in female sexual dysfunction, one can speculate that increased activity of RhoA/Rho-kinase may contribute to this disorder.

**THE RhoA/Rho-KINASE PATHWAY AS A THERAPEUTIC TARGET FOR ED**

Although there are a variety of pharmacological treatments available for ED, it is important to discover new therapies because current treatments are not universally suitable or efficacious in many patients. Much attention has focused on the development of therapies that increase corporal smooth muscle relaxation as a means to improve erectile function. A potential new approach for the treatment of ED may be based on directly inhibiting biochemical pathways that control smooth muscle contraction. Of particular interest is the activity of the RhoA/Rho-kinase-mediated Ca²⁺ sensitization pathway which is relevant to the pathogenesis of ED. Studies have demonstrated that inhibition of tonic contraction of corporal smooth muscle by intracavernosal injection or topical application of Y27632 to the penis results in increased blood flow into erectile tissue causing an erection [107,115,127,137,138,144]. However, Rho-kinase inhibitors usually have profound systemic effects, lowering mean arterial blood pressure, when they circulate into the vascular system. Thus such pharmacological therapy would be restricted to local delivery only. The primary question about the potential clinical value of Rho-kinase inhibitors is whether the compounds can be developed to target the RhoA/Rho-kinase pathway in the vasculature of the penis while minimizing unwanted effects on other vascular beds.

An alternative way to inhibit the RhoA/Rho-kinase pathway in penile tissue would be to identify and manipulate tissue specific isoforms of RhoA regulatory proteins. Increasing genital-tissue-specific RhoGDI and RhoGAP activity or suppressing specific RhoGEF activity could be effective therapies for the treatment of ED.

Specifically targeting RhoA or Rho-kinase by gene transfer offers an additional possible treatment option for ED. Success in improving erection in animal models of ED using gene transfer of T19NRhoA strongly supports this possibility clinically [110,120]. Because the penis is easily accessible by its external location, it allows local application of therapeutic vectors with reduced risk of systemic effects. Studies have shown that the placement of a tourniquet around the base of the penis when introducing virus vectors encoding dominant-negative RhoA directly into the corpora cavernosa limits the virus entering the systemic circulation, having little effect on mean arterial blood pressure. Recently, siRNA has been employed to knock down RhoA protein expression in vitro [148]. Although some studies have demonstrated successful delivery of siRNA in vivo, the stability of siRNA expression still needs to be improved. Whether this technology can be employed specifically targeting the RhoA/Rho-kinase pathway in the penis remains to be investigated. Further studies in animal models may also clarify the beneficial effects and side effects of long-term inhibition of RhoA/Rho-kinase in the penis.

**CONCLUSIONS**

A growing body of evidence has demonstrated that RhoA/Rho-kinase plays a pivotal role in the modulation of corpus cavernosum smooth muscle contraction and penile erection. Overactive RhoA/Rho-kinase signalling results in ED. Although the importance of RhoA/Rho-kinase in erectile function is well documented, the majority of studies are limited in using pharmacological inhibitors to reduce RhoA/Rho-kinase activity. The upstream regulation of RhoA/Rho-kinase in the penis is largely unknown. Recent studies have shown that RhoGEFs, including p115RhoGEF, LARG and PDZ-RhoGEF, are expressed in the rat penile tissue [119,149]; however, the functional roles of these RhoGEFs in the regulation of RhoA activity have not been investigated. Although physiological studies have suggested that NO inhibits RhoA/Rho-kinase activity, the detailed mechanisms by which this regulation occurs are yet to be determined. More studies are needed to determine whether NO regulates RhoA/Rho-kinase activity at transcriptional, translational or post-translational levels. In addition, improved understanding of the upstream regulation of the RhoA/Rho-kinase pathway and the discovery of regulators of RhoA/Rho-kinase uniquely expressed in penile tissue will probably foster the development of new therapeutic avenues for the treatment of ED.
ACKNOWLEDGMENTS

We acknowledge the support from the National Institute of Health (DK 02568 and DK 67223) and American Heart Association National Center (0530007N).

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Received 12 August 2005/2 September 2005: accepted 12 September 2005
Published on the Internet 17 January 2006. doi:10.1042/CS20050255
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