Increased corpus cavernosum smooth muscle tone associated with partial bladder outlet obstruction is mediated via Rho-kinase

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SIGNIFICANT CLINICAL EVIDENCE has been accumulating over the last decade suggesting an association between lower urinary tract symptoms (LUTS) and erectile dysfunction (ED) in men (2, 7, 23, 26, 35, 37, 43, 46). Although these studies are too numerous to describe in this paper, one of the largest and more recent studies was a 12,000 participant multinational study Multinational Survey of the Aging Male-7 (MSAM-7) (40). The results of this study revealed a strong correlation between LUTS and sexual function. However, a finding just as important was that this correlation existed regardless of age and other comorbidities, contradicting earlier arguments by others that LUTS was not an independent risk factor for ED.

The findings of the MSAM-7 study support previous studies examining the relationship between LUTS and ED that also ruled out age and comorbidities as contributing factors. For example, Baniel et al. (4), studying 131 patients with benign prostatic hyperplasia (BPH) before prostatectomy, found that men with relatively mild BPH were more than two-fold more likely to perform normal coitus than men with severe BPH. In this study, the two groups were of the same age and general health, ruling these issues out as confounding factors. Also, Puente et al. (38) found that when controlling for age, International Prostate Symptom Score (IPSS) was significantly correlated with three of five ED domains. Later, in 2000, Richard et al. (39) examined a cohort of 3,500 French men between 50 and 80 yr of age and found sexual function (i.e., erection, penetration, ejaculation, and sexual intercourse) was altered by the severity of LUTS as measured by IPSS, regardless of age.

One-third of men over the age of 50 will develop LUTS (18), and a recent study diagnosed ED in 62% of patients presenting for LUTS (25). In addition, in a separate study, a 72.2% prevalence of LUTS was found in patients with ED compared with only a 37.7% prevalence of LUTS in non-ED patients (9). Thus the importance of establishing the exact relationship between these two pathologies and identifying the molecular mechanisms linking them is clear. A recent editorial has even highlighted the need for basic research in this area (32). However, surprisingly, to date, there have been limited studies performed toward this goal.

Our laboratory has previously demonstrated a change in the isoforms of smooth muscle myosin (SMM) at the mRNA and protein levels in the corpus cavernosum smooth muscle (CCSM) in the rabbit model for partial bladder outlet obstruction (PBOO; 15). Furthermore, we found that CCSM obtained from PBOO rabbits with documented bladder dysfunction produced greater force in response to stimulation with KCl or phenylephrine and did not relax as well to electrical field stimulation compared with sham-operated control rabbits (sham). Results from in vitro kinase and phosphatase assays using purified smooth muscle myosin showed increased kinase and decreased phosphatase activities in cellular extracts from corpora cavernosa isolated from PBOO vs. sham rabbits. Increased Rho-kinase expression in the CCSM of PBOO compared with sham rabbits. Increased Rho-kinase expression in the CCSM of PBOO rabbits was suggested by the observations that Rho-kinase inhibitors attenuated the increased kinase activity and were less effective in relaxing CCSM strips from PBOO vs. sham rabbits. This hypothesis was then confirmed by RT-PCR and Western blotting, which demonstrated increased expression of both isoforms of Rho-kinase (ROKa and ROKβ). Increased SMM basal phosphorylation (necessary for SM contraction) in the CCSM of PBOO rabbits, mediated via an increase in Rho-kinase expression/activity, would be expected to make the CCSM more difficult to relax (necessary for erection), which suggests that the RhoA/Rho-kinase pathway as being involved in the mechanism for LUTS-associated ED.

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The production of force in all smooth muscle (SM) requires the phosphorylation of the 20-kDa regulatory light chain (LC20) of SMM (1). Although it was originally thought that only myosin light chain kinase (MLCK) could increase the level of LC20 phosphorylation, more recently, it has been shown that small GTPase RhoA-activated Rho-kinase could also increase the phosphorylation of LC20 either by directly phosphorylating LC20 (3) or indirectly by phosphorylating and thus inactivating the SMM phosphatase (the major phosphatase in SM responsible for dephosphorylating SM myosin) (29). It has been demonstrated that Rho-kinase is crucial for maintenance of the CCSM-contracted state (flaccid penis) as injection of Y-27632 (a selective Rho-kinase inhibitor) into male mice resulted in penile erection (16).

The results presented in this study show that 1) the basal level of SMM phosphorylation is increased in the CCSM isolated from rabbits with PBOO compared with sham-operated animals, 2) the increased SMM phosphorylation level is associated with an increase in the kinase and a decrease in the phosphatase activities regulating the phosphorylation state of SMM, and 3) the increased kinase and decreased phosphatase activities responsible for maintaining the phosphorylated state of SMM appear to be the result of an upregulation of Rho-kinase activity via overexpression of Rho-kinase enzyme (both the ROKα and ROKβ isoforms).

MATERIALS AND METHODS

Partial bladder outlet obstruction. The use of rabbits, including the surgical procedure, was approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania and the Children’s Hospital of Philadelphia. PBOO is created in 12-wk-old adult male New Zealand White rabbits (weight 2.5–3.0 kg) by a surgical procedure, was approved by the Institutional Animal Care and Use Committee of the Children’s Hospital of Philadelphia. PBOO is created in 12-wk-old adult male New Zealand White rabbits (weight 2.5–3.0 kg) by a surgical procedure, was approved by the Institutional Animal Care and Use Committee of the Children’s Hospital of Philadelphia. PBOO is created in 12-wk-old adult male New Zealand White rabbits (weight 2.5–3.0 kg) by a surgical procedure, was approved by the Institutional Animal Care and Use Committee of the Children’s Hospital of Philadelphia.

Isolation of rabbit corpus cavernosum and physiology. The two corpora cavernosa (CC) were removed from both sham and PBOO rabbits, cleaned and either prepared for physiological measurements or frozen in liquid nitrogen and stored at −70°C, as previously described (15). The CCMS was stimulated to maximal contraction by increasing concentrations (0–200 μM) of phenylephrine (Sigma, St. Louis, MO) and then relaxed from the maximally precontracted state by the addition of increasing concentrations (0–5 μM) of Y-27632 (Calbiochem, La Jolla, CA).

Two-dimensional gel electrophoresis. Two-dimensional (2D) gel electrophoresis was performed as previously described (21). Briefly, extracts were prepared from the frozen CC of sham and PBOO rabbits by extraction directly into isoelectric focusing buffer (50 mg of tissue/ml extraction buffer). Endogenous kinase and phosphatase activity was inactivated by adding the frozen powder to a mixture of dry ice and acetone and allowing it to thaw before extraction, as described (10, 21). The supernatant was obtained, and 50 μl of it was applied to mini-isoelectric focusing (IEF) cylindrical gels (1 × 65 mm) and electrophoresed using an IEF apparatus from Bio-Rad (Hercules, CA). IEF gels were then subjected to 14% SDS-PAGE (7 cm × 10 cm × 1 mm gels) and stained with Coomassie blue (Bio-Rad); the spots corresponding to unphosphorylated and mono-phosphorylated LC20 were quantitated by scanning densitometry and a program (Bio-Rad) for analysis of the 2D gels. The identities of these spots were confirmed by Western blotting of the 2D slab gels and staining with monoclonal antibody (Sigma) to LC20, as described in the Western blot analysis section of MATERIALS AND METHODS.

Kinase assay. Kinase activity was determined as previously described (11, 48). Briefly, kinase was extracted from the frozen CC of sham and PBOO rabbits, and an in vitro kinase assay was performed in which the incorporation of [γ-32P]ATP into exogenous purified chicken gizzard SMM was determined over a period of 4 min. The protein concentrations of the extracts used for the kinase assay were determined by the Bradford microassay (8). The same assay was conducted in the absence of Ca2+ with 2 mM EGTA so as to determine the Ca2+-independent kinase activity capable of phosphorylating SMM. The effect of the Rho-kinase selective inhibitor HA-1077 (Calbiochem) on both calcium-dependent and calcium-independent kinase activity was also determined.

Phosphatase assay. The cell extracts prepared as described under the kinase assay were assayed for phosphatase activity as previously described (11, 48). Briefly, using exogenous purified chicken gizzard SMM phosphorylated with [γ-32P]ATP as the substrate, the ability of the extracts to release free [γ-32P]phosphate was determined over a period of 3 min.

RNA extraction and semi-quantitative RT-PCR. Total RNA was extracted from frozen CC of sham and PBOO rabbits, and reverse transcription and PCR were carried out as previously described (19). In all reactions, α-actin was amplified as an internal control. The sequence of the primers used for RT-PCR and the predicted product sizes were ROKα, upstream 5’GTGATGTTACTATGGGCCGAGAAAT’3’, downstream 5’GTAAAGAAGGCACAGTGAATG3’-(202 bp), ROKβ, upstream 5’AGATTGTTCTGCTGTTG3’, downstream 5’TATCATCAGGGAGGTAAGTG3’-(366 bp), α-actin, upstream 5’GTCACTTCTCGGTCGTCTG’3’, downstream 5’GTCCTGGATAGGGATGACT3’-(91 bp), MLCK, upstream 5’GTGAGGCGCCTCCCTCGTCA3’, downstream 5’CATGCCCCGTTTCTGCGATTTC3’-(289 bp). The sequences of all primers were based on the published rabbit sequences with the exception of ROKα, which has not been cloned and was designed based on the known human, mouse, and rat sequences. The GenBank accession numbers of the rabbit sequences on which the other primers were based are U42424 for ROKβ, M76233 for MLCK, and X60732 for α-actin. Quantification of the resulting PCR products was performed by scanning densitometry using a Bio-Rad GS-700 densitometer, as described previously (21). All PCR products were sequenced to confirm their identities (21). Standard curves for our individual target mRNAs were constructed and ensured all amplifications to be in the linear range. Western blot analysis. Total extractable protein was isolated from frozen CC of sham and PBOO rabbits (15), and Western blotting was performed using a 1:2000 dilution of anti-ROKα antibody (cat. #R54520, Clone 21, Transduction Laboratories, Lexington, KY) or anti-ROKβ antibody (cat. #R81520, Clone C-19, Transduction Laboratories), as previously described (14). Western blot analysis was also performed for MLCK expression using a 1:2000 dilution of anti-MLCK antibody (Sigma, Clone K36). Also, for confirmation of the identity of the 20-kDa smooth muscle myosin light chain (LC20) spots on the 2D Coomassie blue-stained gel, a 2D gel was transferred to Immobilon-P membrane before staining, as described above, and then probed with monoclonal antibody against LC20 (cat. #M4401, Clone MY-21, Sigma) at a dilution of 1:10,000 and visualized by enhanced chemiluminescence.

Statistical analysis. All data are expressed as means ± SE with P < 0.05 considered statistically significant. The nonpaired Student’s t-test was applied using SigmaStat Version 2.03 (SPSS, Chicago, IL).

RESULTS

Myosin light chain phosphorylation. As can be seen in Fig. 1, using 2D electrophoresis, we were able to separate the unphosphorylated SMM 20-kDa regulatory light chain (LC20) from phosphorylated LC20. With the use of this method, it was determined that the SMM extracted from the CC of the sham-operated rabbits was phosphorylated at a level of 10 ± 1.5%
Fig. 1. Two-dimensional gel electrophoresis of corpus cavernosum (CC) total protein extracts. The CC was isolated from four different pairs of sham-operated (sham groups; A and C) and partial bladder outlet obstruction (PBOO) (B and D) rabbits, aerated with 95% O2-5% CO2 and maintained at 37°C for 30 min. The CC was then rapidly frozen at −70°C; extracts were prepared in a manner to preserve the phosphorylation state of the smooth muscle myosin (SMM), and then two-dimensional electrophoresis was carried out as described in MATERIALS AND METHODS. The phosphorylated form (P) of SMM LC20 migrates to the more acidic side of the gel than the unphosphorylated form (U). Note the increased relative amount of phosphorylated compared with unphosphorylated myosin in the CC from PBOO rabbits (B) compared with CC from sham rabbit groups (A). For reference, the positions of the actin and tropomyosin isoforms, as well as the position of the essential 17 kDa (LC17) SMM essential light chains are also indicated. A closeup of the 20 U and 20 P spots from a second pair of gels from a sham (C) and PBOO (D) rabbit are also shown, again showing an increased level of SMM phosphorylation in the CC of PBOO rabbits. E: Western blot performed on two-dimensional gel of corporal extract from a sham-operated rabbit using LC20 specific antibody to confirm identity of LC20 spots identified and analyzed (A–D).

(Fig. 1, A and C), similar to the level of LC20 phosphorylation that we have previously reported for the CC of normal 12-wk adult New Zealand White rabbits (21). In contrast, the SMM LC20 of the PBOO rabbits was phosphorylated to a significantly (P < 0.05) higher level (15 ± 1.2%) compared with the sham rabbits (Fig. 1, B and D). The identity of these spots as LC20 was confirmed by Western blotting (Fig. 1E).

Kinase and phosphatase activities. To determine whether the increased level of LC20 phosphorylation in the CC of PBOO rabbits could be the result of an alteration in the kinase and/or phosphatase activities capable of modulating the SMM LC20 phosphorylation level, kinase and phosphatase activities were determined. The results from these studies revealed that the phosphatase activity necessary to hydrolyze the 32P covalently bound to purified gizzard myosin was significantly lower (50%, as reflected by more phosphorylated SMM remaining in the assay) at the 20-s time point in the extract of CC from PBOO rabbits compared with CC from sham rabbits, although by 60 s, the activities were not significantly different (Fig. 2). It was also determined that the kinase activity was significantly higher (~43% at 1 min) in the CC isolated from PBOO rabbits compared with the sham groups (Fig. 3A). To determine whether the increase in kinase activity in the CC of PBOO rabbits resulted from an increase in calcium-independent kinase activity (since Rho-kinase can modulate the phosphorylation of SMM in a calcium-independent manner), the same assay was run in the presence of 2 mM of EGTA. The results from this assay also revealed a significantly higher level of kinase activity at both the 1 min (~53%) and 2 min (~90%) time points in the CC from the PBOO rabbits compared with CC from sham rabbits (Fig. 3B), suggesting that the CC from the PBOO rabbits has an increased calcium-independent kinase activity. Running this same kinase assay in the presence of the Rho-kinase-selective inhibitor HA-1077 (0.3 μM) confirmed that the majority of the increased calcium-dependent kinase activity appears to be due to an increase in Rho-kinase activity since preincubation with HA-1077 normalized the activities of the CC from PBOO and sham animals (Fig. 3C). The data obtained for the kinase assay under these different conditions for the 1 min time point are summarized in Fig. 3D.

Effect of Y-27632 on CCSM relaxation. In an attempt to further confirm that the CC from PBOO rabbits possessed significantly more Rho-kinase activity than the CC from sham animals, CCSM from both types of rabbits was maximally precontracted with phenylephrine, and then a dose-response relaxation curve to the Rho-kinase-selective inhibitor Y-27632 was prepared. As can be seen in Fig. 4, the CCSM from PBOO rabbits was not as efficiently relaxed by Y-27632 compared with the CCSM from the sham groups. Although the relaxation induced by Y-27632 was on average less for all concentrations of the inhibitor tested, only the 5-μM dose was statistically different. However, this contrast is still within the selectivity range of the Y-27632 inhibitor for Rho-kinase.

Expression of Rho-kinase and MLCK. Semiquantitative RT-PCR revealed an increase in the expression of both of the two...
known isoforms of Rho-kinase (ROK\textsubscript{A} and ROK\textsubscript{B}). As can be seen in Fig. 5, the mRNA expression of ROK\textsubscript{A} and ROK\textsubscript{B} was increased by 40\% and 65\%, respectively, while the expression of \(\alpha\)-actin did not vary significantly. This increased mRNA expression correlated with a similar increase in the expression of ROK\textsubscript{A} (35\% \pm 4\%) and ROK\textsubscript{B} (70\% \pm 5\%) at the protein level as determined by Western blot analyses (Fig. 6), as described in the MATERIALS AND METHODS. In contrast, the expression of MLCK or \(\alpha\)-actin (used as a control) did not appear altered at either the mRNA or protein levels (Figs. 5 and 6).

**DISCUSSION**

In the last couple of years, numerous studies performed in a number of different countries, including Brazil (35), Turkey (2), Singapore (46), The Netherlands (7), Japan (26), and England (37), as well as multinational studies (23) have reported an association between LUTS and ED. Although some have questioned whether this relationship is merely due to an increased age, poorer general health and/or lack of sexual desire, a number of studies (as outlined in the introduction) have addressed this concern and found that a relationship between LUTS and ED still exists regardless of age or other comorbidities. However, to date, little investigation into the...
response to maximal contractile stimulation with the α-agonist phenylephrine (200 μM), the SMM phosphorylation level of the CCSM myosin only reached about 25% (21). This is in contrast to some smooth muscles (e.g., rat femoral artery and rat uterus that have been shown to reach up to 80–90% phosphorylation levels from a basal level of 20% in response to contractile stimulation) (5). Thus we feel that the increase from 10 to 15% that we show (representing about 33% of maximum) would be physiologically significant.

To examine the mechanistic basis for this increased SMM LC20 phosphorylation level, we quantitated the kinase and phosphatase activities in the CC as to their ability to modulate the level of SMM phosphorylation. Our results showed that the CC from PBOO rabbits possessed less phosphatase activity capable of releasing [γ-32P] from the LC20 of exogenous purified chicken gizzard SMM (used as an in vitro substrate) compared with CC from sham animals (Fig. 2). In contrast, kinase activity (in the presence of calcium) was significantly higher in the CC from PBOO rabbits compared with CC from sham animals (Fig. 3A). This increased kinase activity capable of phosphorylating SMM, coupled with a decreased phosphatase activity, would be expected to increase the level of SMM phosphorylation.

As described in the introduction, the phosphorylation of SMM is accomplished primarily by MLCK, an enzyme that requires calcium, as well as calmodulin, for activation (24). However, more recently, it was shown that Rho-kinase could increase the level of SMM phosphorylation in a calcium-independent manner (30) by phosphorylating and inactivating SMM phosphatase (29) and thereby allowing the MLCK to work more efficiently. The observation that the Rho-kinase-selective inhibitor HA-1077 was able to normalize the kinase activity in the presence of calcium (Fig. 3, C and D) suggests that the increase in total kinase activity is due to an increase in Rho-kinase activity.

The fact that the calcium-independent kinase activity was higher in CC extracts from PBOO compared with sham rabbits (Fig. 3B) and that this increased activity could be attenuated by

molecular mechanism(s) linking these two pathologies has been undertaken.

We have chosen to use the rabbit model of PBOO to examine the mechanistic link between LUTS and ED. An advantage of an animal model such as this one is that, unlike human clinical data, by its nature an animal model rules out other confounding factors, including age and cardiovascular complications that affect the male genital system. Also, animals can be placed in metabolic cages to determine (based upon voiding parameters) whether indeed the animals develop bladder dysfunction and the degree to which this dysfunction exists. Additionally, it appears that the obstructive symptoms of LUTS are most closely associated with ED (22). Thus the rabbit model of PBOO (which induces outlet obstruction) may indeed be a very clinically relevant model to study the mechanistic link between LUTS and ED.

The results from our current study show that CC obtained from rabbits with PBOO exhibits a higher level of basal SMM LC20 phosphorylation compared with the CC from sham-operated animals (Fig. 1). Because the contraction of all SM is operated animals (Fig. 1). Because the contraction of all SM is dependent upon phosphorylation of LC20 (12, 27) and it has been shown that there is a direct relationship between LC20 phosphorylation and SMM ATPase activity (necessary for contraction) (13, 42), this increased level of LC20 phosphorylation suggests that the CCSM of PBOO rabbits exists at a higher resting tone than the CCSM of shams as predicted by our prior in vitro physiological studies (15). Although the difference in phosphorylation level that we found between the CCSM myosin of the sham and PBOO rabbits was indeed small (10% vs. 15%), we have previously shown that even in

Fig. 5. RT-PCR analysis of Rho-kinase and myosin light chain kinase (MLCK) mRNA expression. Ethidium bromide-stained agarose gels from RT-PCR performed on total RNA isolated from the CC of four pairs of sham-operated and PBOO rabbits for ROKα (A), ROKβ (B), or MLCK (C) are shown. As an internal control, α-actin was amplified in the same reactions. The PCR products migrated to the predicted positions for the predicted product lengths. PCR products were all sequenced to confirm their identity. Note that the expression of ROKα and ROKβ is increased in the CC from PBOO compared with the CC from sham groups, while the expression of MLCK and the α-actin internal control is not significantly altered.

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Fig. 6. Western blot analysis or Rho-kinase and MLCK expression. Protein was extracted from the CC of four pairs of sham-operated and PBOO rabbits, and equal amounts of total extractable protein were then loaded onto a mini 12% SDS-polyacrylamide gel, separated by electrophoresis, and then transferred to Immobilon-P membrane and probed with antibody to ROKα (A), ROKβ (B), or MLCK (C), as described in MATERIALS AND METHODS. Note that the expression of both ROKα and ROKβ is increased in the CC from PBOO compared with the CC from sham groups, while the expression of MLCK protein is not significantly altered. Also, note that the expression of α-actin was not significantly different between the sham and PBOO groups (D).
the HA-1077 Rho-kinase-selective inhibitor (Figs. 3C), but not by removing the calcium (Fig. 3B), further suggests that this increase in kinase activity in CC of PBOO rabbits may be due to an upregulation of Rho-kinase activity. Although HA-1077 also inhibits PKC and MLCK (both enzymes are capable of phosphorylating SMM), the affinity of HA-1077 for these enzymes is 10- and 100-fold lower, respectively (36), suggesting that the effect of this inhibitor is predominantly Rho-kinase-mediated. Furthermore, the finding that HA-1077 appeared also to normalize the kinase activity in the presence of calcium between the CC from sham and PBOO rabbits (Fig. 3D) suggests that there is no difference in the activity of the calcium-dependent MLCK between these two CCs.

To determine whether the apparent increase in Rho-kinase activity observed in the CC of PBOO rabbits compared with the sham rabbits was also observed in the intact CC, CCSM from PBOO and sham rabbits was maximally precontracted with phenylephrine and then the ability of Y-27632—an even more potent and selective Rho-kinase inhibitor than HA-1077 (17, 45)—to relax the CCSM was determined. Correlating with the results of our in vitro biochemical kinase assay, the CCSM from the PBOO rabbits did not relax as efficiently as the CCSM from the sham-operated rabbits (Fig. 4), implying that Rho-kinase activity is increased at the cellular level as well.

Finally, semiquantitative RT-PCR (Fig. 5) and Western blotting (Fig. 6) revealed that the expression of both Rho-kinase isofoms ROKα and ROKβ is upregulated at both the mRNA and protein level in the CCSM from PBOO, respectively, compared with sham rabbits. Interestingly, the expression of the ROKβ isoform was increased more than the expression of the ROKα isoform. These findings imply that the increased Rho-kinase activity in the CC in response to PBOO is at least partly due to a direct alteration in the expression of one or both of the Rho-kinase enzymes. Although the exact functional differences between the ROKα and ROKβ isoforms are not currently known, we have found that the ROKβ isoform is also selectively upregulated in the bladder in response to PBOO (6) and also in the CCSM in response to diabetes (14). Correlating with the ability of the Rho-kinase-selective inhibitor HA-1077 to normalize the kinase activity in the presence of calcium, the expression of MLCK was not significantly altered at either the mRNA or protein levels (Figs. 5 and 6).

In summary, we report for the first time, an increased basal level of SMM phosphorylation in the CCSM from PBOO compared with sham rabbits, providing direct molecular evidence for an association between LUTS and ED. This increased level of phosphorylation would be expected to increase the basal tone of the CCSM, making it more difficult for CCSM relaxation (necessary for erection) to be achieved. This dysfunction is associated with an increased kinase and decreased phosphatase activity to phosphorylate and dephosphorylate the LC20 of SMM, respectively. Furthermore, using a combination of biochemical, physiological and molecular biological techniques, we identify an increase in Rho-kinase activity, resulting from an overexpression of one or both isoforms of Rho-kinase, as being involved in the mechanism of increased basal CCSM tone in PBOO rabbits.

At this time, the exact mechanism by which PBOO can lead to molecular changes in the erectile tissue remains unknown. One possible explanation would be that the large increase in bladder mass alters signal transduction pathways throughout the lower urogenital system. These alterations could be the result of either the enlarged bladder directly effecting nerve or blood supply to other organs and/or the enlarged bladder needing to increase its own blood supply or innervation and hence indirectly impinging upon the supply to other tissues or organs in the lower urogenital system. Indeed, a central coordination of bladder and corpus cavernosum may exist as has been shown for the bladder and colon (41). Because the expression of Rho-kinase has been shown to be upregulated in response to hyoxia (47) and Rho GTPases such as Rho-kinase are involved in the development, maintenance, and function of the nervous system (34), future studies will examine the potential role of hyoxia and altered innervation in the mechanism of PBOO-induced overexpression of Rho-kinase in the CC.

In closing, we must point out that another possible explanation for the effect of PBOO on the erectile tissue is that the surgical ligation of the urethra directly affects the nerve or blood supply to the penis. The fact that sham-operated animals did not show the same molecular changes in the CCSM as the PBOO animals would appear to rule out an effect from the surgical procedure itself; however, it does not rule out an effect resulting from the continuous compression of a nerve or blood vessel. Interestingly, both in vivo ischemia (44), induced by occlusion of abdominal aorta, and in vitro ischemia (28, 31) actually caused a decrease in CCSM contractility in contrast to the increased contractility that we have observed (15). Studies are currently under way to specifically address the issue of possible nerve/blood vessel compression.

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