Increased Corpus Cavernosum Smooth Muscle Tone Associated with Partial Bladder Outlet Obstruction is Mediated via Rho-kinase

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Running Title: Increased PBOO-induced CCSM Tone is Mediated via ROK

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Numerous studies have now demonstrated that LUTS (lower urinary tract symptoms) is associated with ED (erectile dysfunction) in men independent of age or general health. Since one third of men over the age of 50 will develop LUTS and a recent study showed ED in 62% of patients presenting for LUTS, the importance of determining the mechanistic link between these two pathologies is clear. Using a rabbit model of partial bladder outlet obstruction (PBOO), a primary cause of LUTS, we have identified an increased basal corpus cavernosum smooth muscle (CCSM) tone associated with an elevated level of smooth muscle myosin (SMM) phosphorylation in PBOO compared to sham-operated control rabbits. 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 compared to 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 (ROKα & 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) and identifies the RhoA/Rho-kinase pathway as being involved in the mechanism for LUTS associated ED.
INTRODUCTION

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 multi-national study (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 co-morbidities, 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 co-morbidities as contributing factors. For example, Baniel et al., studying 131 patients with benign prostatic hyperplasia (BPH) prior to prostatectomy, found that men with relatively mild BPH were more than 2-fold more likely to perform normal coitus than men with severe BPH(4). 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. found that when controlling for age, International Prostate Symptom Score (IPSS) was significantly correlated with 3 of 5 ED domains(38). Later, in the year 2000, Richard et al. examined a cohort of 3,500 French men between 50-80 years 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(39).

A 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 as compared to 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 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 to sham-operated (sham) animals(15), suggesting an enhanced CCSM tone. Thus, the goal of this study was to determine whether the basal tone of the CCSM form PBOO rabbits is enhanced as suggested by our prior physiological studies described above and to attempt to identify the molecular mechanism responsible for this increase tone.

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 the 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 to 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 appears to be the result of an upregulation of Rho-kinase activity via overexpression of Rho-kinase enzyme (both the ROKα and ROKβ isoforms).
MATERIAL AND METHODS

Partial Bladder Outlet Obstruction and Reversal: The use of rabbits, including the surgical procedure, was approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania and Children’s Hospital of Philadelphia. PBOO is created in 12 week old adult male New Zealand White rabbits (weight 2.5-3.0 kg) by a modification of the procedure by Malkowicz et al.(33) and as previously described(20). Twelve days after surgical obstruction, sham-operated and normal rabbits were kept in metabolic cages (Kent Scientific Corp., Torrington, CT) for two days to monitor the voiding pattern non-invasively as previously described(20).

Isolation of Rabbit Corpus Cavernosum and Physiology: The two corpus cavernosum (CC) were removed from both sham-operated 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 CCSM was stimulated to maximal contraction by increasing concentrations (0-200 µM) of phenylephrine (Sigma; St. Louis, MO) and then relaxed from the maximally pre-contracted state by the addition of increasing concentrations (0-5 µM) of Y-27632 (Calbiochem; La Jolla, CA).

Two-Dimensional Gel Electrophoresis: Two-dimensional 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 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 prior to extraction as
The supernatant was obtained and 50 µl applied to mini isoelectric focusing (IEF) cylindrical gels (1 X 65 mm) and electrophoresed using an IEF apparatus from Bio-Rad (Hercules, CA). IEF gels were then subjected to 14% SDS-PAGE (7 cm X 10 cm X 1 mm gels), stained with Coomassie blue (Bio-Rad) and the spots corresponding to unphosphorylated and monophosphorylated LC20 were quantitated by scanning densitometry and a program (Bio-Rad) for analysis of 2-D gels. The identities of these spots were confirmed by Western blotting of the two-dimensional slab gels and staining with monoclonal antibody (Sigma) to LC20 as described in the “Western Blot Analysis” section of the 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 performed in which the incorporation of [γ-32P]ATP into exogenous purified chicken gizzard SMM was determined over a period of 4 minutes. 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 [\(\gamma\)-\(^{32}\)P]–ATP as the substrate, the ability of the extracts to release free [\(\gamma\)-\(^{32}\)P] phosphate was determined over a period of 3 minutes.

**RNA Extraction and Semi-quantitative RT-PCR:** Total RNA was extracted from frozen CC of sham-operated and PBOO rabbits and reverse transcription and PCR carried out as previously described\(^{(19)}\). In all reactions, \(\alpha\)-actin was amplified as an internal control. The sequence of the primers used for RT-PCR and the predicted product sizes were:  
- ROK\(\alpha\), Upstream 5’GTGATGGTTACTATGGGCAGAAT3’, Downstream 5’GTTAAGAAGGCACAGATGAGAT3’ - (202 bp),  
- ROK\(\beta\), Upstream 5’AAGTAGTTCTTGCATTGG3’, Downstream 5’TATCATCAGGGAAAGGTAAGGTG3’ - (366 bp),  
- \(\alpha\)-actin, Upstream 5’GCTTTGGATAGGCATGACT3’, Downstream 5’GTCACTTCCTGCTCTGT3’,  
- MLCK, Upstream 5’GGTCAGCGGCCTCTCCCCCTTCA3’, Downstream 5’CATTGCCCGTCTTCTGCCATTTTC3’ – (289 bp). The sequences of all primers were based upon the published rabbit sequences with the exception of ROK\(\alpha\), which has not been cloned, and was designed based upon the known human, mouse and rat sequences. The GenBank Accession #s of the rabbit sequences upon which the other primers were based are (U42424 for ROK\(\beta\)), (M76233 for MLCK) and (X60732 for \(\alpha\)-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-operated and PBOO rabbits(15) and Western blotting 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 two-dimensional Coomassie Blue stained gel, a 2D gel was transferred to Immobilon-P membrane prior to 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 ECL.

**Statistical Analysis:** All data are expressed as the mean plus or minus standard error of the mean (SEM) with p <0.05 considered statistically significant. The nonpaired Student 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 two-dimensional electrophoresis we were able to separate the unphosphorylated SMM 20 kDa regulatory light chain (LC20) from phosphorylated LC20. Using 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. 1A & C), similar to the level of LC20 phosphorylation we have previously reported for the CC of normal 12 week 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 to the sham (Fig. 1B & D). The identity of these spots as LC20 was confirmed by Western blotting (Fig. 1E).

Kinase and Phosphatase Activities: In order to determine if 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 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 second time point in the extract of CC from PBOO rabbits compared to CC from sham rabbits, although by 60 seconds the activities were not significantly different (Fig. 2). It was also determined that the kinase activity was significantly higher (~ 43% at 1 minute) in the CC isolated from PBOO rabbits compared to shams (Fig. 3A). In order to determine if 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 EGTA. The results from this assay also revealed a significantly higher level of kinase activity at both the 1 minute (~53%) and 2 minute (~90%) time points in the CC from the PBOO rabbits compared to CC from shams (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-independent kinase activity appears to be due to an increase in Rho-kinase activity since pre-incubation 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 minute 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 pre-contracted with phenylephrine and then a dose response relaxation curve to the Rho-kinase-selective inhibitor Y-27632 prepared. As can be seen in Fig. 4, the CCSM from PBOO rabbits was not as efficiently relaxed by Y-27632 compared to the CCSM from sham. 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 concentration is still within the selectivity range of the Y-27632 inhibitor for Rho-kinase.

**Expression of Rho-kinase and MLCK:** Semi-quantitative RT-PCR revealed an increase in the expression of both of the two known isoforms of Rho-kinase (ROKα & ROKβ). As can be seen
in Fig. 5, the mRNA expression of ROKα and ROKβ were increased by 40 ± 5% and 65 ±6%, respectively, while the expression of α-actin did not vary significantly. This increased mRNA expression correlated with a similar increase in the expression of ROKα (35 ±4%) and ROKβ (70 ±5%) at the protein level as determined by Western blot analyses (Fig. 6) as described in the Methods. In contrast, the expression of MLCK or α-actin (used as a control) did not appear altered at either the mRNA or protein levels (Figs. 5 & 6).
DISCUSSION

In the last couple of years, numerous studies performed in a number of different countries including Brazil(35), Turkey(2), Singapore(46), Netherlands(7), Japan(26) and England(37) as well as multi-country 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 Section) have addressed this concern and found that a relationship between LUTS and ED still exists regardless of age or other co-morbidities. However, to date, little investigation into the 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 *in vitro* animal model such as this one, is that unlike human clinical data, by its nature it 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) if 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 to the CC from sham-operated animals (Fig. 1). Since 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 LC\textsubscript{20} phosphorylation suggests that the CCSM of PBOO rabbits exists at a higher resting tone as predicted by our prior in vitro physiological studies\cite{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 response to maximal contractile stimulation with the \(\alpha\)-agonist phenylephrine (200 \(\mu\)M), the smooth muscle myosin phosphorylation level of the CCSM myosin only reached about 25\%(\textit{21}). This is in contrast to some smooth muscles (e.g. rat femoral artery and rat uterus that have been show to reach up to 80-90\% phosphorylation levels from a basal level of 20\% in response to contractile stimulation)\cite{5}. Thus we feel that the increase from 10-15\% that we show (representing about 33\% of maximum) would be physiologically significant.

In order to examine the mechanistic basis for this increased SMM LC\textsubscript{20} 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 \([\gamma^{\text{32}}P]\) from the LC\textsubscript{20} of exogenous purified chicken gizzard SM (used as an in vitro substrate) compared to 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 to 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\cite{24}. However, more recently it was shown that Rho-kinase could increase the level of SMM phosphorylation in a calcium-independent manner\cite{30} by phosphorylating and inactivating SMM phosphatase\cite{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. 3D) 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 to sham rabbits (Fig. 3B) and that this increased activity could be attenuated by the HA-1077 Rho-kinase-selective inhibitor (Figs. 3C), but not by removing the calcium (Fig. 3D), 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 protein kinase C (PKC) and MLCK (both enzymes which 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 to also 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 CC’s.

In order to determine if the apparent increase in Rho-kinase activity observed in the CC of PBOO rabbits compared to shams in the CC extracts was also observed in the intact CC, CCSM from PBOO and sham rabbits was maximally pre-contracted 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 was not relaxed 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, semi-quantitative RT-PCR (Fig. 5) and Western Blotting (Fig. 6) revealed, respectively, that the expression of both Rho-kinase isoforms (ROKα and ROKβ) is upregulated at both the mRNA and protein level in the CCSM from PBOO compared to 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 is 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 & 6).

In summary, we report for the first time, an increased basal level of SMM phosphorylation in the CCSM from PBOO compared to 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). Since the expression of Rho-kinase has been shown to be upregulated in response to hypoxia(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 hypoxia 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 we have observed(15). Studies are currently underway to specifically address the issue of possible nerve/blood vessel compression.
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REFERENCE


FIGURE LEGENDS

Fig. 1: Two-dimensional gel electrophoresis of CC total protein extracts. The CC was isolated from 4 different pairs of sham-operated (Panel A & C) and PBOO (Panel B & D) rabbits, aerated with 95% O2, 5% CO2 and maintained at 37°C for 30 minutes. The CC was then rapidly frozen at -70°C, extracts prepared in a manner to preserve the phosphorylation state of the SMM and then two-dimensional electrophoresis carried out as described in the 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 myosin in the CC from PBOO rabbits (Panel A) compared to CC from sham-operated rabbits (Panel B). 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 in the figure. A close up of the 20U and 20P spots from a second pair of gels from a sham (Panel C) and PBOO (Panel D) rabbit are also shown. Panel E: Western blot performed on 2D gel of corporal extract from a sham-operated rabbit using LC20 specific antibody to confirm identity of LC20 spots identified and analyzed in Panels A-D.

Fig. 2: Phosphatase Activity. The phosphatase activity capable of decreasing the phosphorylation of the LC20 of SMM in the extracts from the CC of sham-operated and PBOO rabbits was determined as described in the Methods section. The amount of free $[^\gamma-^{32}\text{P}]$ released from exogenous SMM phosphorylated with $[^\gamma-^{32}\text{P}]-\text{ATP}$ in cpms (counts per minute) was plotted as a function of time. The * = p < 0.05 and all assays were performed on 4 pairs of rabbits.
**Fig. 3: Kinase Activity.** The kinase activities capable of phosphorylating the LC20 of SMM in the extracts from the CC of sham-operated and PBOO rabbits were determined as described in the Methods section. The amount of free [γ-32P]-ATP incorporated into SMM in cpms was plotted as a function of time. **Panel A:** Kinase activity in the presence of calcium, **Panel B:** Kinase activity in the presence of EGTA to bind free calcium, **Panel C:** Kinase activity in the presence of calcium but with the 0.3 µM of the Rho-kinase inhibitor HA-1077. **Panel D:** Graphical summary of kinase activity at 1 minute under different conditions. The * = p < 0.05 and the assays were performed on 4 pairs of rabbit CC.

**Fig. 4: Relaxation of CCSM by Y-27632.** CC strips were obtained from sham-operated and PBOO rabbits, suspended between platinum electrodes, equilibrated with 95% O2-5% CO2 in Tyrode’s buffer at 37°C for 30 minutes and then Lo determined. After equilibration at Lo for an additional 15 minutes, the CCSMs were maximally contracted with phenylephrine as described in the Methods section and then relaxation curves to the Rho-kinase-selective inhibitor Y-27632 were prepared. The data points represent the average of 4 pairs of rabbits with the * representing p < 0.05. Note that the CCSM from PBOO rabbits was not relaxed as efficiently as the CCSM from the sham-operated rabbits.

**Fig. 5: RT-PCR Analysis of Rho-kinase and MLCK mRNA Expression.** Ethidium bromide-stained agarose gels from RT-PCR performed on total RNA isolated from the CC of 4 pairs of sham-operated and PBOO rabbits for ROKα (Panel A), ROKβ (Panel B) and MLCK (Panel C) are shown. As an internal control, α-actin was amplified in the same reactions. The PCR
fragments 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 to the CC from sham rabbits while the expression of MLCK and the α-actin internal control is not significantly altered.

Fig. 6: Western Blot Analysis of Rho-kinase and MLCK Expression. Protein was extracted from the CC of 4 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 (Panel A), ROKβ (Panel B) and MLCK (Panel C) as described in the Methods. Note that the expression of both ROKα and ROKβ is increased in the CC from PBOO compared to the CC from sham rabbits 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 rabbits (Panel D).
Figure 1

A

Sham

Actin

Tropomyosin

LC_{20}

LC_{17}

b

a

Obstructed

B

Western

C

UP

LC_{20}

Sham

D

UP

LC_{20}

Obstructed

E

Western
Figure 2

![Graph showing the release of 32P from LC20 (CPM) over time for Sham and PBOO conditions.](image)

- **Sham**
- **PBOO**

32P Liberated from LC_{20} (CPM)

Time (seconds)
Figure 3A

Obs

PBOO

Sham

3^2P Incorporated into LC_{20} (CPM)

Time (minutes)
Figure 3B

![Graph showing PBOO and Sham treatments over time]

- **PBOO**
- **Sham**

**32P Incorporated into LC_{20} (CPM)**

Time (minutes)
Figure 3C

**Graph:**
- **Y-axis:** $^{32}$P Incorporated into LC$_{20}$ (CPM)
- **X-axis:** Time (minutes)
- **Legend:**
  - ○ PBOO
  - ▲ Sham
Figure 3D

- **32P Incorporation into LC\textsubscript{20} (CPM)**
- **Legend:**
  - PBOO
  - Sham

**Total Kinase**
- PBOO: ~1400 CPM
- Sham: ~1000 CPM

**With HA1077**
- PBOO: ~800 CPM
- Sham: ~600 CPM

**With EGTA**
- PBOO: ~400 CPM
- Sham: ~200 CPM

* indicates a significant difference compared to Sham.
Figure 4

![Graph showing percent maximal contraction vs Y-27632 concentration for PBOO and Sham groups. The graph indicates a decrease in percent maximal contraction with increasing Y-27632 concentration. The PBOO group shows a more pronounced decrease compared to the Sham group, with a significant difference indicated by an asterisk.]
Figure 5

Sham | PBOO
-----|-----
1     | 1   
2     | 2   
3     | 3   
4     | 4   

A

ROKα
α-actin

B

ROKβ
α-actin

C

MLCK
α-actin
Figure 6

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- **A**: ROKα
- **B**: ROKβ
- **C**: MLCK
- **D**: α-actin