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Involvement of the Rho-kinase/LIM kinase/Cofilin signaling pathway in corporal fibrosis after cavernous nerve injury in male rats

Rho-kinase/LIM kinase/cofilin 경로가 해면체신경 손상 백서의 발기능과 해면체 평활근 섬유화에 미치는 영향

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서울대학교 대학원
의학과 비뇨기과학
송 상 훈

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송상훈의 의학박사 학위논문을 인준함

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Involvement of the Rho-kinase/LIM kinase/Cofilin signaling pathway in corporal fibrosis after cavernous nerve injury in male rats

by
Sang Hoon Song

A thesis submitted to the Department of Urology in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Urology at Seoul National University College of Medicine

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Abstract

Introduction: The molecular mechanism of corporal fibrosis leading to erectile dysfunction (ED) following cavernous nerve (CN) injury is poorly understood. A recent study demonstrated that the sphingosine-1-phosphate/RhoA/Rho-kinase 1 (ROCK1) signaling pathway could be involved in corporal fibrosis with the loss of smooth muscle (SM) through coordination with TGF-ß1 following CN injury. Activation of ROCK1 is followed by phosphorylation of the downstream effectors LIM-kinase 2 (LIMK2) and cofilin, resulting in fibroblast-to-myofibroblast differentiation, a pathophysiologic feature of fibrosis. Therefore, the purpose of our study is to determine whether the LIMK2/cofilin pathway, the downstream effectors of ROCK1, was involved in ED and corporal fibrosis following bilateral CN injury in male rats.

Methods: Forty-eight, 10-week-old, male Sprague-Dawley rats were equally divided into three groups: sham surgery (S); bilateral CN crush injury (I); and bilateral CN resection (R). Within each of 3 groups, two subgroups were analyzed as a function of time (1 and 4 weeks postoperatively). At each time point, electrostimulation was performed to assess erectile function by the maximal intracavernous pressure (ICP)/mean arterial pressure (MAP) ratio and areas under the curve (AUC). Penile tissue was processed for Masson's trichrome staining, western blot (ROCK1, total LIMK2, phospho-LIMK2, total cofilin, phospho-cofilin), immunohistochemistry (α-smooth muscle actin), and double immunoflouroscent staining (ROCK1, phospho-LIMK2, vimentin).

Results: At each time point, both the I and the R groups showed a significantly lower percent of ICP/MAP and AUC than S group. The mean ICP/MAP and AUC showed a tendency toward a higher level in the I group than in the R group, although it did not differ significantly. There was no significant difference in the mean ICP/MAP and AUC between the two time points for either group. At all time points, the I and R groups had a significantly decreased

SM cell/collagen ratio and expression of α-SMA compared to the S group and a tendency to decrease during the study period. Densitometry revealed a significantly higher expression of ROCK1 in the I and R groups compared to the S group at all time points. The LIMK2 phosphorylation in I and R groups significantly increased at 1 week, but not at 4 weeks postoperatively. The cofilin phosphorylation in the R group significantly increased to that in the S group starting at 1 week, while that in the I group was increased significantly at 4 weeks after surgery. The double immunofluorescent staining noted that co-expression of vimentin with ROCK1 and phospho-LIMK2 in the I and R groups were significantly increased at 1 week but not at 4 weeks postoperatively. Also, it was found mainly in the subtunical area.

Conclusions: Our data suggest that the ROCK1/LIMK2/cofilin pathway can be involved in ED and corporal fibrosis associated with the loss of smooth muscle after CN injury, and appears to be functional particularly in the early phase after CN injury.

Keywords: Erectile dysfunction, Fibrosis, Prostatectomy, cavernosal nerve

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목 차

1. Introduction

Erectile dysfunction (ED) is one of the important functional complications after radical prostatectomy (RP) even in the era of robot-assisted laparoscopic surgery which enhances the optical power of the surgeon that one can maximally spare the neurovascular bundle of prostate (1). To understand the precise pathophysiologic mechanisms of post-RP ED, experimental in vivo models of cavernosal nerve (CN) injury in small rodents have been widely used in short- and long-term functional and morphologic studies (2). It was known that the CNs may have been unintentionally damaged by manipulation during nerve-sparing prostatectomy, resulting in substantial damage to the axons of the CN (3). Pathophysiologic studies have shown that neural injury during RP results in structural changes such as cavernosal fibrosis or apoptosis of smooth muscle and endothelium of the cavernosum (4, 5).

Transforming growth factor-ß (TGF-ß) is a well-known fibrogenic cytokine in the corpus cavernosum (6), which has ability to enhance processes such as epithelial to mesenchymal transition (EMT), and extracellular matrix production (7, 8). The role of TGF-ß in cavernosal fibrosis has been actively investigated until recently. However, molecular events leading to corporal fibrosis and, thereby, ED after CN injury is poorly understood. We have previously demonstrated in the rat model of CN injury, that sphingosine-1-phosphate/RhoA/Rho-kinase1 (ROCK1) signaling pathway could be involved in corporal fibrosis with loss of smooth muscle (SM) through coordination with TGF-ß1 after CN injury (9). Recent literature suggested that activation of ROCK1 was followed by phosphorylation of the downstream effectors LIM-kinase 2 (LIMK2) and cofilin, resulting in fibroblast-to-myofibroblast differentiation, a pathophysiologic feature of fibrosis (8). However, the role of ROCK1/LIMK/cofilin pathway in corporal fibrosis has not been investigated until recently.

Therefore, we hypothesized that the RhoA/ROCK1 pathway activation would turn on LIMK2/Cofilin pathway, which might be involved in corporal fibrosis and ED in a rat model of CN injury. We investigated whether ROCK1/LIMK2/Cofilin signaling pathway could be involved in corporal fibrosis after bilateral CN injury in male rats to supplement the pathophysiologic knowledge on the role of those pathways in the process of ED after RP.

2. Materials and Methods

2-1. CN Injury Rat Model and Treatment

Forty eight 10-week-old male Sprague-Dawley rats, each weighing 300 to 350 g, were randomly divided into three groups (n= 16 in each group): sham surgery (S); bilateral CN crush injury (I); and bilateral CN resection group (R). Within each of the three groups, two subgroups were analyzed as a function of time, i.e. 1 and 4 weeks postoperatively.

The animals were anesthetized with an intraperitoneal injection of zoletil (10 mg/kg) and inhalation of isoflorane, and were placed on the surgical table. The surgical area was iodinated for sterilization. After vertically incising the low abdominal wall, the dorsolateral lobe of prostate, major pelvic ganglion (MPG), and the main branch of the CN were identified. For the S group, both CNs were exposed, but were not manipulated and after which the abdominal wound was closed. For the I group, two 30-second compressions, without physical disruption, were applied to the bilateral CN 5 mm distal to the MPG using a microsurgical vascular clamp. For the R group, we completely resected the bilateral CN 5 mm distal to the MPG using microsurgical scissors. The skin was closed by sutures using black silk at the end of the procedure.

At the end of the treatment, their erectile function was measured and the animals were sacrificed. The animal experiments were approved by the Institutional Animal Care and Use Committee of the Clinical Research Institute at Seoul National University Hospital, an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. In addition, the National Research Council guidelines for the care and use of laboratory animals were followed.

2-2. Assessment of Erectile Response and Sample Procurement

At each time point of 1 and 4 weeks postoperatively, electrostimulation of the cavernous nerve was performed to assess erectile function by using the maximal intracavernous pressure (ICP)/mean arterial pressure (MAP) ratio and areas under the curve (AUC). The animals were anesthetized with an intraperitoneal injection of zoletil (10mg/kg) and inhalation of isoflorane,

and were placed on the surgical table. A 24-gauge angiocatheter was introduced into the carotid artery and connected to a pressure channel to continuously monitor MAP of the rat. The shaft of the penis was freed of skin and fascia, and the corpus cavernosum was exposed and cannulated using a 23-gauge needle in order to permit continuous monitoring of the intracavernous pressure (ICP). Pressure data were collected and electronically analyzed (Powerlab, ADInstruments, Colorado Spring, CO, USA). The CN was stimulated below the injured segment using a bipolar stimulator at a frequency of 15Hz, width of 0.2ms, and duration of 30 seconds. The application of 1, 2.5 and 4 V were used in order to achieve a consistent erectile response. The ratio of the ICP to the MAP, frequency of response curves, and the area under the ICP curves (AUC) were evaluated and compared among the experimental groups. The AUC was also corrected by the MAP (10). The whole penis was removed after assessment of the erectile function. Middle parts of the skin-denuded penile shaft were maintained overnight in 10% formaldehyde solution and paraffin was embedded for histological studies. The remaining penile tissue was rapidly frozen in liquid nitrogen and was stored at -80℃ until processing.

2-3. Histomorphometry and Immunohistochemistry

In order to calculate the smooth muscle cell (SMC)/collagen ratio, paraffin embedded slides of sectioned penile tissue were stained using Masson's trichrome as described in a previous investigation (9). The SMC/collagen ratio was calculated under ×40 magnification images of the penis comprising one-half of the corpora cavernosa by analyzing the area of SMC (stained in red) and collagen (stained in blue) using ImagePro Plus 4.5 software (Medica Cybernetics, SilverSpring, MD, USA).

Immunohistochemical staining for alpha-smooth muscle actin (α-SMA) was performed to evaluate the percentages of the smooth muscle cell component (% α-SMA) as described in a previous investigation (9, 11). Overnight incubation with primary anti-α-SMA antibody (1:100; Dako, Glostrup, Denmark) was performed. At a ×100 magnification, two fields were randomly selected on each slide, and the percentage of smooth muscle fibers in a given area was measured using Image Pro Plus 4.5software.

2-4. Immunofluorescence for Confocal Laser Microscopy

To assess co-localization of fibroblasts and ROCK1 or LIMK2-activated sites in penile tissue,

double immunofluorescence for ROCK1 and vimentin or phospho-LIMK2 and vimentin were performed. For immunofluorescence staining of paraffin embedded sections (2.5µm) were treated and incubated with primary antibody; monoclonal mouse anti-vimentin (1:100, Dako, Glostrup, Denmark) during two overnight periods, monoclonal rabbit anti-ROCK1 (1:10, AbCam, Cambridge, MA, USA) or rabbit polyclonal anti-LIMK2 (phospho T505) (1:100, AbCam, Cambridge, MA, USA) for two overnight periods at room temperature. After three washes in PBS, the sections were incubated with the fluorescence secondary antibody; goat anti-mouse IgG antibody 488 (1:400, Invitrogen, Camarillo, CA, USA) 10min for vimentin, and goat anti-rabbit IgG 594 (1:200, AbCam, Cambridge, MA, USA) 10min for ROCK1 or phospho-LIMK2. Sections were analyzed using a confocal laser fluorescencemicroscope (Leica Microsystems, Heidleberg, Germany) with single and double filter settings at 488 and 594 nm. Images were acquired using LAS AF (Leica Microsystems, Heidleberg, Germany).

2-5. Western Blot Analysis

Penile tissue was processed for Western blot (ROCK1, total LIMK2, phospho-LIMK2, total cofilin, and phospho-cofilin). Western blot analysis was performed as previously described (9, 12). Anti-ROCK1 (1:1,000,Cell SignalingTechnology), anti-LIMK2 (1:500, AbCam, Cambridge, MA, USA), anti-phopho-LIMK2 (1:1000, AbCam, Cambridge, MA, USA), anti-cofilin (1:1000, Cell SignalingTechnology), and anti-phopho-cofilin (1:1000, Cell SignalingTechnology) antibodies were used as primary antibodies. Results were quantified by densitometry and normalized by β-actin expression. Data were represented as the fold-change over that of the S group.

2-6. Statistical Analysis

Results were reported as the mean ± standard errors of the mean. As indicate, variables were analyzed statistically with the Mann-Whitney U, Kruskal-Wallis test. All statistical tests were two-sided, with the level of significance set at 0.05. The SPSS version 18.0 (IBM Corp., Amonk, NY, USA) was used for the analysis.

3. Results

Assessment of the Erectile Response In Vivo

Electrical stimulation of the cavernous nerve induced a voltage-dependent increase in both the percent of ICP/MAP and of AUC/MAP in all experimental groups (Figure 1). The ratios of ICP to MAP and AUC to MAP for groups I and R were significantly lower than those for group S ($P < 0.001$) in all voltage settings at both time points of 1 week and 4 weeks after surgery. However, there was no difference between the I and R groups, except for the 4 V stimulation at 4 weeks for ICP/MAP (%) and at 1 week for AUC/MAP (%). Analysis between the two time points, i.e. 1 week and 4 weeks, revealed that only the AUC/MAP (%) of the I group at 4 V stimulation showed a significant decrease.

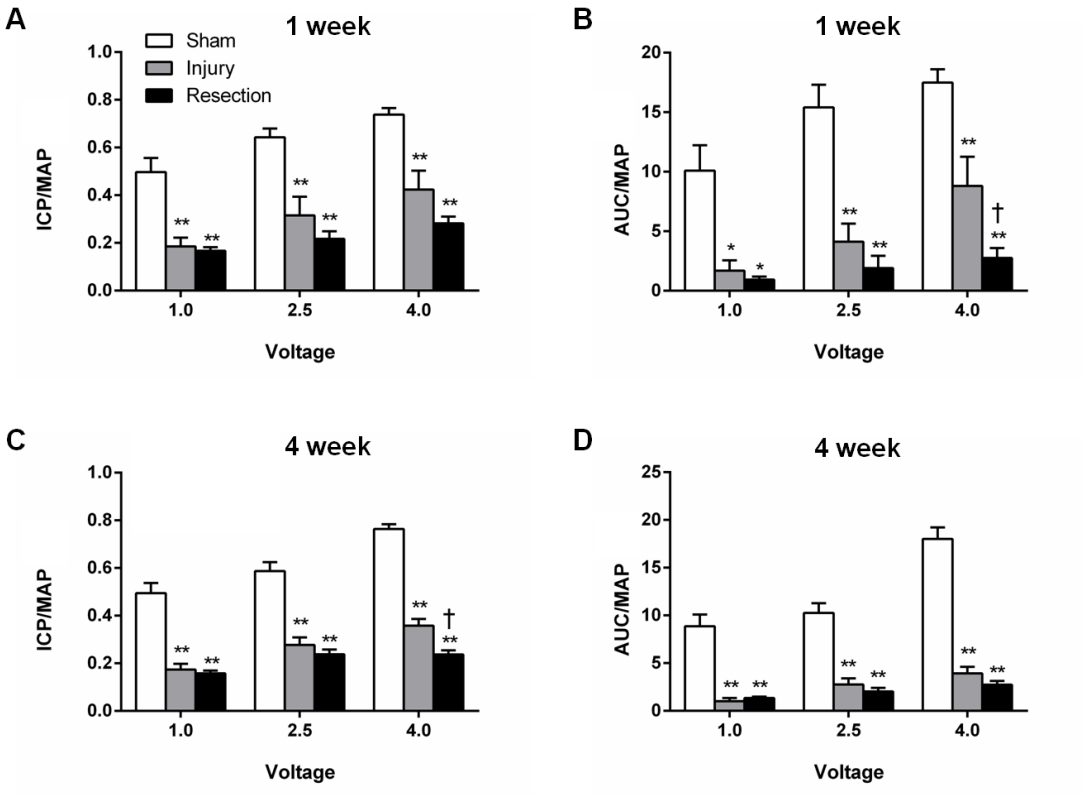

Figure 1 Comparison of the erectile parameters of each group at voltages of 1.0, 2.5, and 4.0 V at week 1 (A, B) or at week 4 (C, D). Bar graphs represent the voltage dependent means and standard errors expressed as the ratio of ICP/MAP (A, C) and the percent of AUC/MAP (B, D). An asterisk or double asterisks indicate a significant difference versus group S ($p < 0.01$ or $p < 0.001$, respectively). A dagger indicates a significant difference versus group I ($p < 0.001$). ICP = intracavernosal pressure; AUC = area under the curve; MAP = mean arterial pressure.

Masson's Trichrome Staining for SMC/Collagen Ratio in the Corpus Cavernosum and the Immunohistochemistry of α-SMA

The SMC/collagen ratio had significantly decreased in the I and R groups compared to that in the S group at 1 week postoperatively, and it was also differed between the I and R groups. At 4 weeks postoperatively, the SMC/collagen ratio had also significantly decreased in the I and R groups compared to that in the S group. In the I and R groups, the SMC/collagen ratio at 4 weeks had significantly decreased compared to those at 1 week postoperatively (Figure 2). The α-SMA immunohistochemical staining results were almost identical to the corresponding results obtained using Masson's trichrome staining. The % α-SMA was decreased in the I and R groups compared to that in the S group at 1 and 4 weeks postoperatively. However, the % α-SMA were not significantly different between the two time points in each groups (Figure 3).

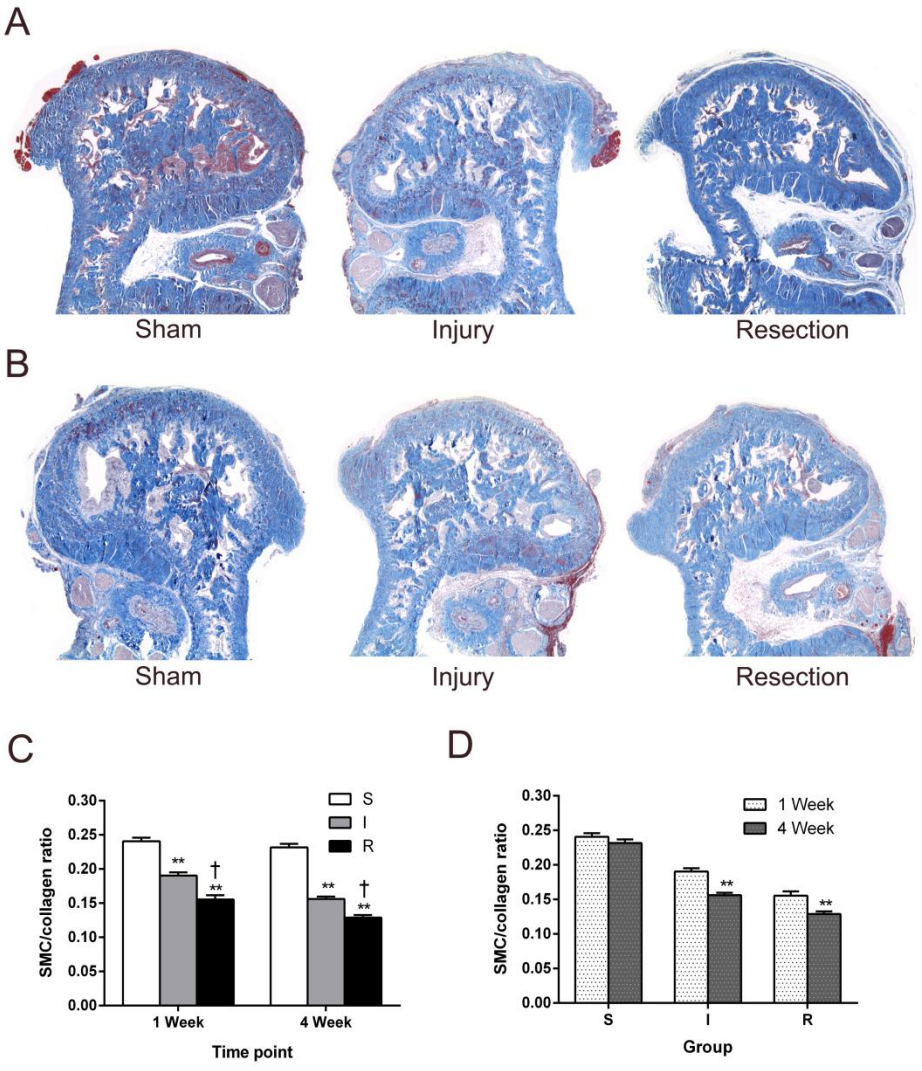

Figure 2 Masson's trichrome staining of rat penile sections. Smooth muscle and collagen fibers were stained in red and blue, respectively (magnification ×40). (A) Representative images for Masson's trichrome staining 1 week postoperatively. (B) Representative images for Masson's trichrome staining 4 weeks postoperatively. (C), (D) Comparison of the Masson's trichrome staining results among the three experimental groups at each time point. The results are presented as the smooth muscle cell (SMC)/collagen ratio (mean, SEM). ***P* < 0.01 vs. S group, †*P* < 0.05 vs. I group. S = sham operation group, I = CN crush injury group, R= CN resection group.

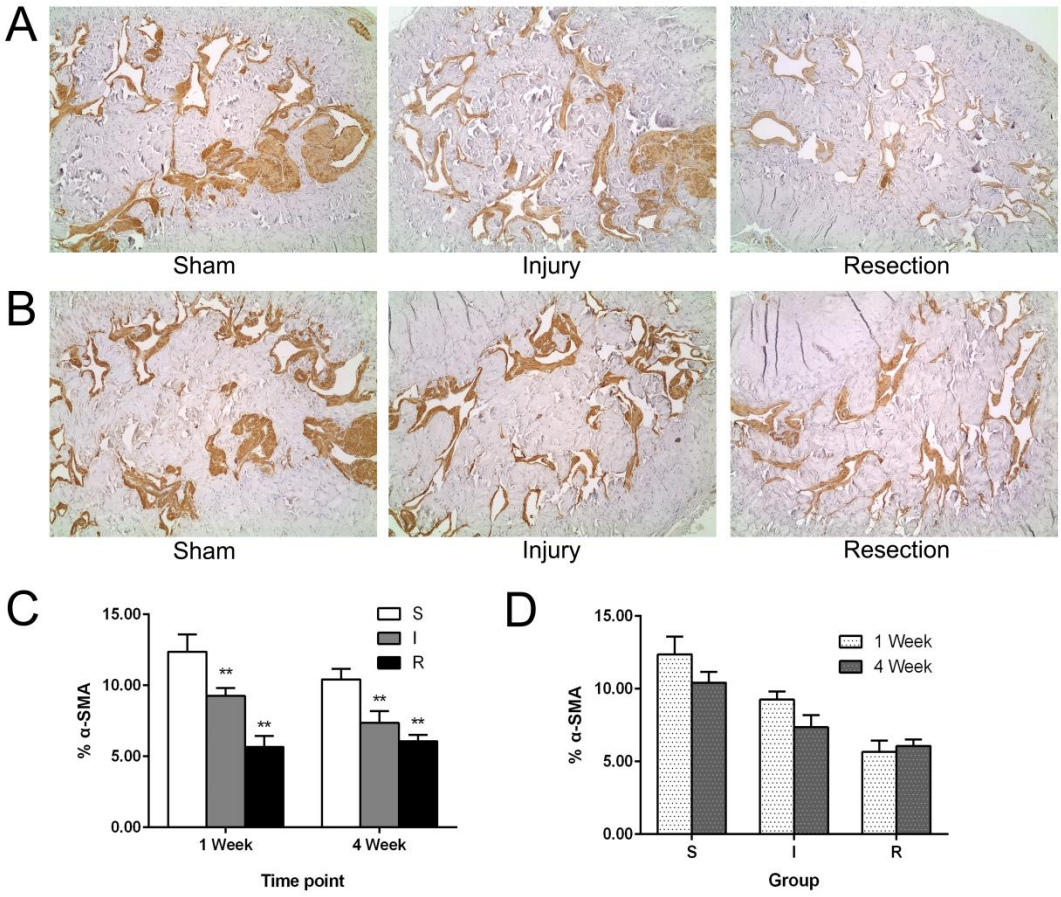

Figure 3 Alpha-smooth muscle actin (α-SMA) expression obtained by immunohistochemical staining of the penile sections. The smooth muscle component is shown as brown areas (magnification x100). (A) Representative images for immune-stained alpha smooth muscle actin (α-SMA) 1 week postoperatively. (B) Representative images for immune-stained α-SMA 4 weeks postoperatively. (C), (D) Comparison of the expression of α-SMA among the three experimental groups and according to the study week. The results are presented as the percentage of smooth muscle fiber in a given area. **P < 0.01 vs. S group. S = sham operation group, I = CN crush injury group, R = CN resection group.

Immunofluorescent Staining of Fibroblasts Positive for phospho-LIMK2

To investigate the phenotypic localization and different expression of fibroblasts positive for

ROCK1 or phospho-LIMK2 in the corpus cavernosum in each experimental group, double immunofluorescent staining of vimentin with ROCK1 or phospho-LIMK2 were performed. In each group, vimentin-positive cells (fibroblasts) were mostly observed in the subtunical areas, but few around the sinusoids of the cavernosum (Figure 4 and 5). In I and R group, fibroblasts positive for ROCK1 or phospho-LIMK2 were significantly increased in the subtunical areas at 1 week, but not at 4 weeks.

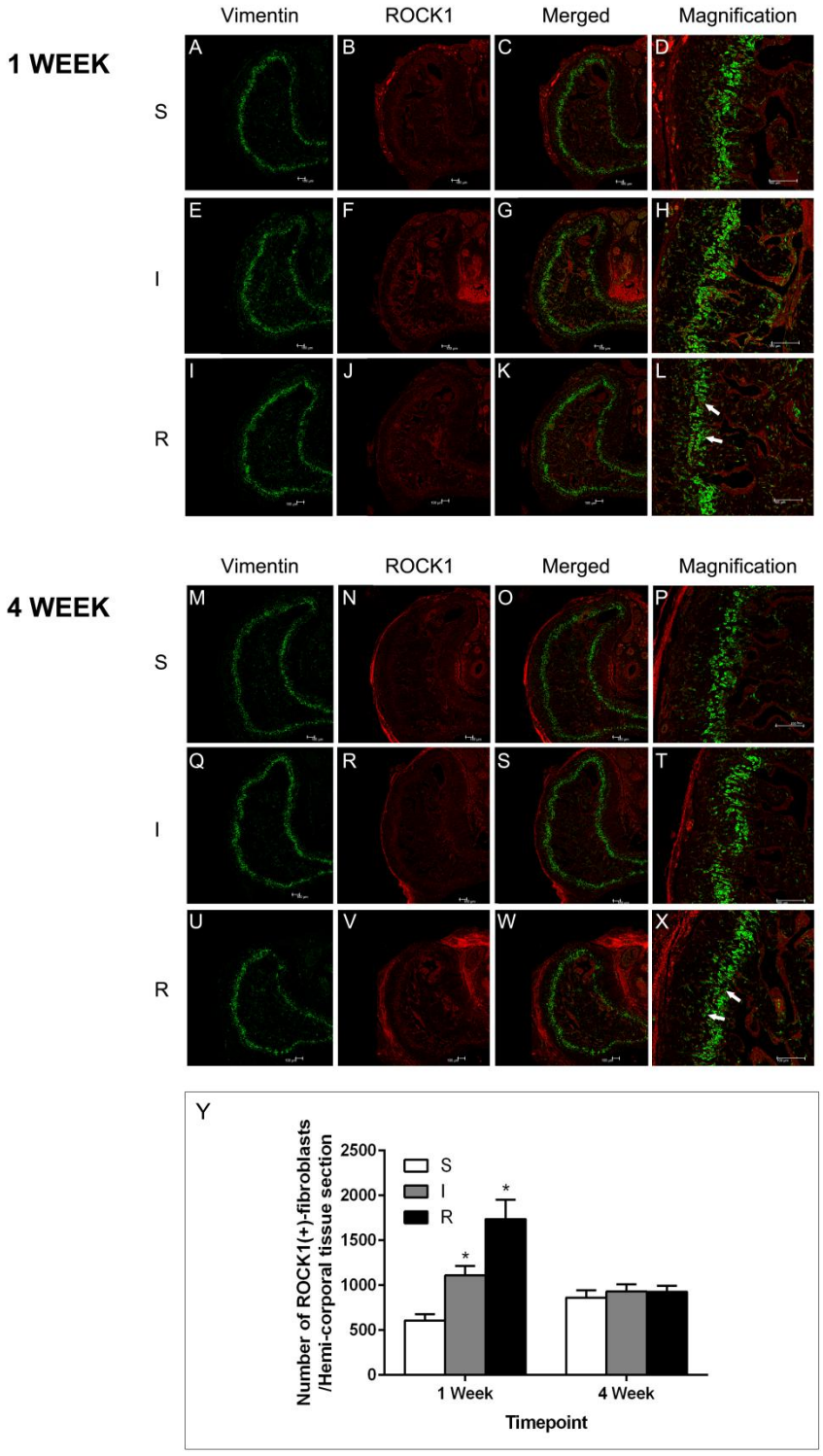

Figure 4. Double immunofluorecent staining of vimentin (green, A, E, I, M, Q, and U) with ROCK1 (red, B, F, J, N, R, and V) in corporal tissue of the each group. White arrows indicate cells double stained with vimentin with ROCK1 in the subtunical areas. In I and R group, fibroblasts positive for ROCK1 were significantly increased in the subtunical areas at 1 week, but not at 4 weeks (Y). S = sham operation group (N = 8 in each subgroup), I = CN crush injury group (N = 8 in each subgroup), and R = CN resection group (N = 8 in each subgroup). *P < 0.05 vs. S group.

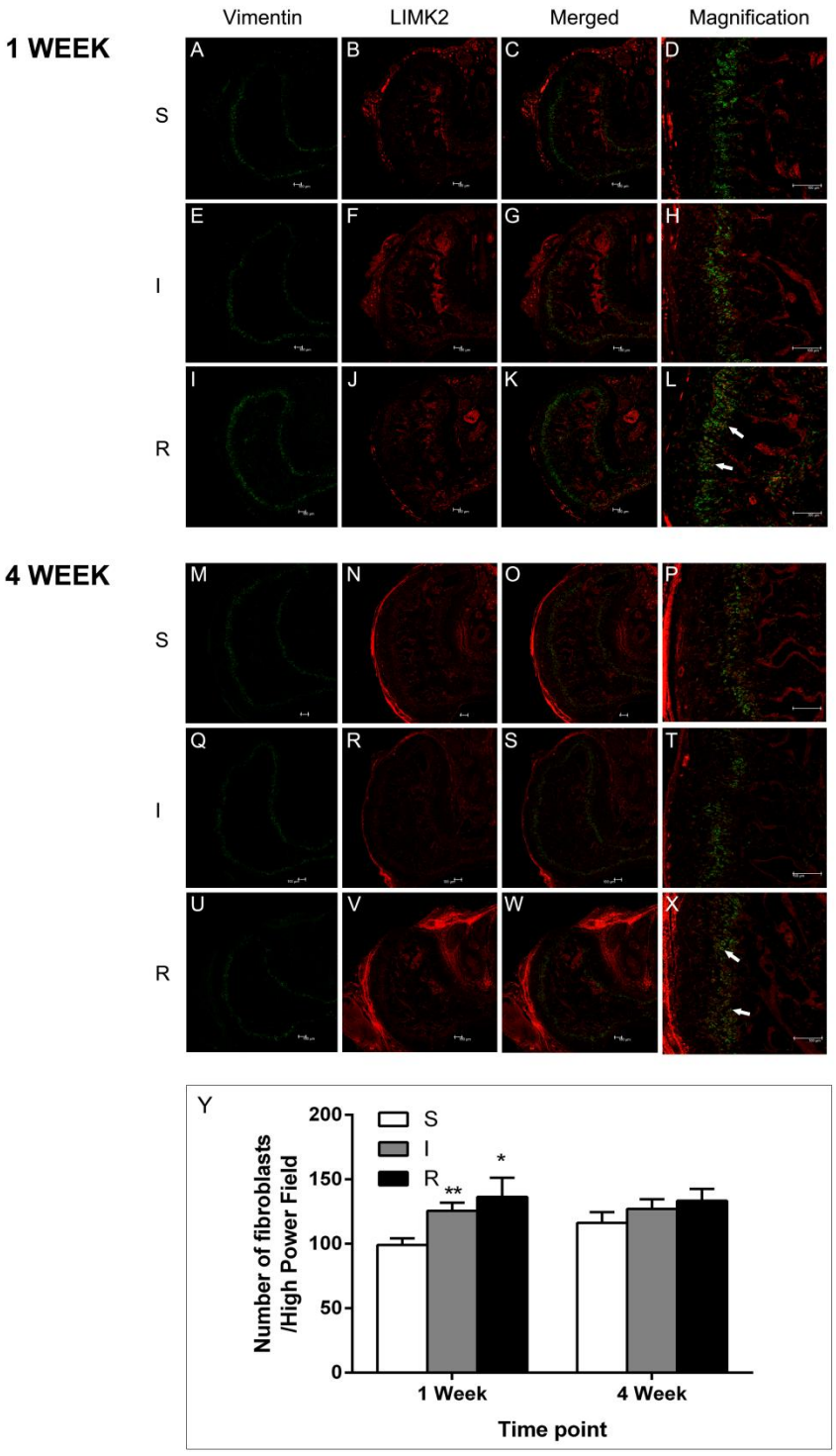

Figure 5. Double immunofluorecent staining of vimentin (green, A, E, I, M, Q, and U) with phospho-LIMK2 (red, B,

F, J, N, R, and V) in corporal tissue of the each group. White arrows indicate cells double stained with vimentin with phospho-LIMK2 in the subtunical areas. In I and R group, fibroblasts positive for phospho-LIMK2 were significantly increased in the subtunical areas at 1 week, but not at 4 weeks (Y). S = sham operation group (N = 8 in each subgroup), I = CN crush injury group (N = 8 in each subgroup), and R = CN resection group (N = 8 in each subgroup). *P < 0.05 vs. S group. **P < 0.01 vs. S group.

Western Blot Analysis

We measured ROCK1, total LIMK2, phospho-LIMK2, total cofilin, and phospho-cofilin protein expression in the penile tissue at 1 and 4 weeks following surgery. The densitometry results showed that the ROCK1 protein level was significantly higher in the I and R groups than in the S group at 1 and 4 weeks. LIMK2 phosphorylation was significantly higher in the I and R groups than in the S group at 1 week, however the ratio did not differ significantly between either group at 4 weeks. Cofilin phosphorylation in the R group significantly increased compared to that in the S group starting after 1 week, while that in the I group significantly increased as late as 4 weeks. These results are shown in Figure 6.

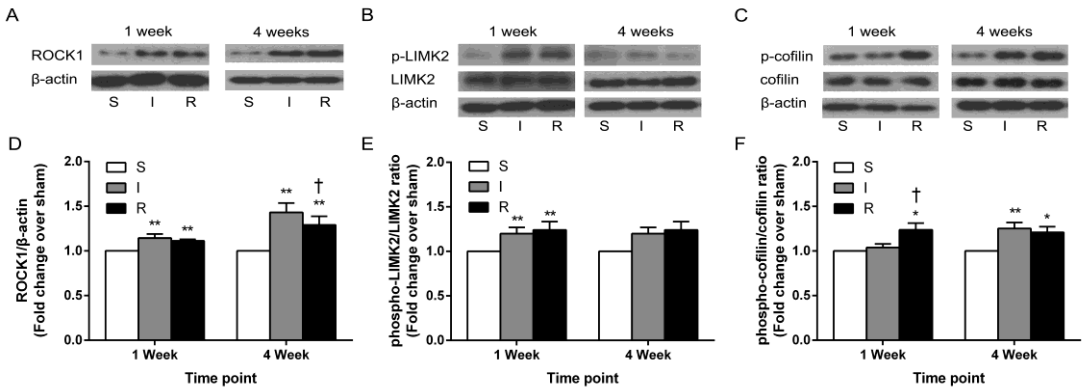

Figure 6. Western blot analysis demonstrating corporal expression of ROCK1, phospho- and total-LIMK, and phospho-and total-cofilin.(A) Representative immunoblots of ROCK1 and β-actin from the corporal tissue of each group. (B) Representative immunoblots of phopho- and the total-LIMK2 and β-actin from the corporal tissue of each group. (C) Representative immunoblots of phospho-, total-cofilin and β-actin from the corporal tissue of each group. (D, E, F) Bar graphs showing the compared protein expression among the three experimental groups using densitometry. The results were normalized by β-actin expression and were presented as fold-changes over the S group. *P < 0.05 vs. sham group, **P < 0.01 vs. sham group, †P < 0.05 vs. crush injury operation group. S = sham operation group (N = 8 in each subgroup), I = CN crush injury group (N = 8 in each subgroup), and R = CN resection group (N = 8 in each subgroup).

4. Discussion

ED is common after RP. The pathophysiology of ED need to be understood properly to maximize the chances of a man to recover functional erections after RP (13). Neural injury, vascular injury, and smooth muscle damage are three key factors of pathophysiology of ED after RP(3). It was reported that CN injury resulted in smooth muscle and endothelium undergo structural changes (4). User et al. have demonstrated early smooth muscle apoptosis after bilateral and unilateral cavernous neurectomy which was maximal at 2–7 days after surgery. They showed that the smooth muscle apoptosis appeared to be clustered in the subtunical area of the corpus cavernosum. The authors suggested that this may be a contributor to the development of venous leak after RP. Another consequence of neural injury is change in smooth muscle-to-collagen ratios. Neural injury has been reported to be associated with upregulation of collagen types I and III as well as upregulation of fibrogenic cascades through cytokines such as TGF-ß (14).

TGF-ß stimulate mesenchymal cells to proliferate and produce extracellular matrix and induce a fibrotic response in vivo (15). Tissue fibrosis is considered to develop due to a failure of the normal wound healing response to finish (16). Myofibroblasts are a specialized form of fibroblasts which express elevated levels of α-SMA and have enhanced ability to contract extracellular matrix (16). It was suggested that the use of TGF-ß as a target for antifibrotic intervention may be limited to acute conditions, such as immediately after surgery(17).

The ROCK pathway is important in the progression of TGF-β-induced vascular fibrosis in cardiovascular disease (18). Gratzke et al. showed that erectile function decreased after CN injury and was accompanied by RhoA/ROCK pathway activation in the rat penis (19). Also, a recent study demonstrated that the RhoA/ROCK1 was involved in corporal fibrosis through coordination with TGF-β/sphingosine-1-phosphate signaling after CN injury (9). In accordance with the studies, the current study showed that ROCK1 up-regulation was accompanied by corporal fibrosis starting from the acute phase following CN injury. Other studies reported the involvement of the ROCK1 in development of fibrosis using murine model. Haudek et al. demonstrated the important role of ROCK1 in the differentiation of monocytes that mature into cardiac fibroblasts (20). Zhang et al. showed that ROCK1 deficiency reduced induction of

fibrogenic cytokines using the ROCK1 knockout mice, and supported the role of ROCK1 in the development of cardiac fibrosis and the induction of fibrogenic cytokines in cardiomyocytes resulting from pathological stimuli (21). Therefore, as shown in the aforementioned studies and in the current study, ROCK1 up-regulation may be involved in the pro-fibrotic cascade in the corporal tissue after CN injury.

This is the first study to evaluate the LIMK2/cofilin pathway, the downstream effectors of ROCK1, which was involved in ED and corporal fibrosis after bilateral CN injury in male rats. The present study shows that up-regulation of ROCK1/LIMK2/cofilin pathway is accompanied by impaired erectile response and corporal fibrosis beginning at 1 week following CN injury. Also, the fibroblasts positive for phospho-LIMK2 were significantly increased after CN injury and mainly distributed in the subtunical areas. Therefore, our study indicates that the ROCK1/LIMK2/cofilin pathway may be involved in corporal fibrosis and erectile dysfunction following CN injury, and that the increase of fibroblasts with LIMK2 up-regulation in the subtunical areas might contribute to a failure of the corporal tissue to expand because of compression of subtunical venules against the tunica. The role of LIMK2 in pro-fibrotic pathway has been previously demonstrated in vitro and in a few in vivo studies. In those studies, LIMK2 as a mediator of actin cytoskeleton reorganization was shown in fibroblasts (22) or in human retinal pigment epithelium (23). However, little is known regarding the role of LIMK2 in corporal fibrosis. Thus, the current study provides the first evidence that the ROCK1/LIMK2/cofilin pathway might be involved in the TGF-ß mediated cascades of fibrosis in the rat model of CN injury.

Previous studies have been evaluated and focused on the role of ROCK in fibrosis cascade after CN injury in rat model (19, 24). The current study elaborated the role of LIMK, downstream effector of ROCK. The epithelial–mesenchymal transition resulting from the Rho/ROCK pathway activation, are mediated by regulators of the actin-cytoskeleton. The LIMK2 is activated downstream of the RhoA/ROCK pathway (25). When active, LIMK is known to phosphorylate and inactivate the functions of cofilin proteins, the principal LIMK substrate, which sever filamentous-actin, resulting in filamentous-actin stabilisation (26). Although ROCK and LIMK lie in a linear pathway downstream of Rho, their roles in epithelial–mesenchymal transition regulation are not identical. Morin et al. studied the differing contributions of LIMK and ROCK and showed that selective inhibition of LIMK, but not ROCK, effectively blocked TGF-ß driven invasion through a layer of matrigel extracellular

matrix protein and suggested that LIMK represents an attractive therapeutic target in TGF-ß driven organ fibrosis and metastatic cancer spread (27). The current study evaluated LIMK activation and its downstream regulation of cofilin in vivo, therefore our data support that LIMK could be another therapeutic target rather than ROCK in corporal fibrosis after CN injury.

Up-regulation of ROCK1/LIMK2/cofilin pathway might be involved in the corporal fibrosis and erectile dysfunction after CN injury. And, up-regulation of the pathway was induced beginning 1 week (acute phase) after CN injury. Furthermore, the present study showed that the protein expression of LIMK2 in the CN crush injury or resection group was significantly increased compared with that in the sham-operated group at 1 week postoperatively. However, the observed increase in the expression of LIMK2 in the CN crush injury or resection group lost significant difference compared to that in the sham-operated group by 4 weeks after surgery. These results suggest that LIMK2 up-regulation may be an acute phase reaction associated with corporal fibrosis following CN injury. In line with the current study, Ferrini et al. showed that corporal veno-occlusive dysfunction(CVOD) developed as a result of the loss of corporal SMC by apoptosis followed by fibrosis(28). They emphasized that early therapeutical intervention would be necessary, because the early histopathological impairment in the cavernosum leads later to the CVOD. Taken together, early therapeutic strategy targeting to the ROCK1/LIMK2/cofilin pathway would be necessary to preserve the integrity of penile structure and, thereby, to prevent CVOD or ED after CN injury.

LIMKs are serine protein kinases that play a central role in the regulation of the actin cytoskeleton by phosphorylating cofilin(25). The LIMK family consists of just two members: LIMK1 and LIMK2 (30). LIMK homologues (LIMK1 and LIMK2) have been identified in various species including; humans, mice, rats and chickens (25). It is known that LIMK1 is required for microtubule disassembly in endothelial cells (29), and it was shown to be involved in cancer metastasis. LIMK2 is known to promote cells cycle progression when activated (30). However, less has been studied about the role of LIMK2 in the various pathologic status regarding fibrosis in vivo. The LIMK2 is a downstream effector of the RhoA/ROCK pathway (25). When active, LIMK2 is known to phosphorylate and inactivate the functions of cofilin proteins, the principal LIMK2 substrate, which sever filamentous-actin, resulting in filamentous-actin stabilization(26). This can lead to cytoskeletal rearrangements and, then fibroblast-to-myofibroblast differentiation, a pathophysiological feature of fibrosis (7). Thus, the current study noted the potential for involvement of LIMK2/cofilin, downstream effectors of

ROCK, in the corporal fibrosis after CN injury. Dai et al. showed that cofilin and LIMK2 were up-regulated in cultured cells in vitro and in an experimental rat pulmonary hypertension model in vivo (31). They demonstrated that LIMK2 was maximally up-regulated at the time of the initial microscopically detectable pulmonary hypertrophy and throughout the progression of vascular hypertrophy. Morin et al. showed that selective inhibition of LIMK effectively blocked TGF-ß driven invasion through a layer of matrigel extracellular matrix protein and suggested that LIMK represents an attractive therapeutic target in TGF-ß driven organ fibrosis and metastatic cancer spread (27). Given the risk for significant adverse effects of Rho-kinase inhibitor, the present study suggests that LIMK2 might be a reasonable therapeutic target for the treatment of corporal fibrosis after CN injury.

A limitation of the current study is that any blocking method such as ROCK or LIMK inhibitor or siRNA knockdown was not performed to further determine the apparent causality between fibrosis and the detailed molecular events of the ROCK1/LIMK2/cofilin pathway in corporal tissue after CN injury. Alternately, we performed a comparative analysis to determine the differences in erectile function, corporal fibrosis and the associated events in the molecular level of the ROCK1/LIMK2/cofilin pathway among rats that underwent a sham surgery or CN crush injury or CN resection. Our results indicated that the higher the expression of ROCK1/LIMK2/cofilin signaling pathways is, the larger the increase in corporal fibrosis is. To a certain degree, this result showed that the dose-dependent relationship between the expression of ROCK1/LIMK2/cofilin signaling molecules and corporal fibrosis. However, further time course studies about therapeutic effect of inhibition of LIMK/cofilin pathway on corporal fibrosis and CVOD after CN injury are needed to get a better understanding of the apparent roles of the pathway in corporal fibrosis and CVOD after CN injury.

5. Conclusions

Our data suggest that the ROCK1/LIMK2/cofilin pathway can be involved in both ED and corporal fibrosis associated with the loss of SM after CN injury, and it appears to be functional particularly in the early phase after CN injury.

6. References

1. Ficarra V, Novara G, Ahlering TE, Costello A, Eastham JA, Graefen M, et al. Systematic review and meta-analysis of studies reporting potency rates after robot-assisted radical prostatectomy. European urology. 2012 Sep;62(3):418-30. PubMed PMID: 22749850.
2. Canguven O, Burnett A. Cavernous nerve injury using rodent animal models. The journal of sexual medicine. 2008 Aug;5(8):1776-85. PubMed PMID: 18774987.
3. Mulhall JP. Penile rehabilitation following radical prostatectomy. Current opinion in urology. 2008 Nov;18(6):613-20. PubMed PMID: 18832948.
4. User HM, Hairston JH, Zelner DJ, McKenna KE, McVary KT. Penile weight and cell subtype specific changes in a post-radical prostatectomy model of erectile dysfunction. The Journal of urology. 2003 Mar;169(3):1175-9. PubMed PMID: 12576876.
5. Paick JS, Goldsmith, P. C., Batra A. K., Nunes, L. L., Padula, C. A., Lue, T. F. Relationshiop between venous incompetence and cavernous nerve injury: ultrastructural alteration of cavernous smooth muscle in the neurotomized dog. Int J Impotence Res. 1991;3:185-95.
6. Moreland RB. Is there a role of hypoxemia in penile fibrosis: a viewpoint presented to the Society for the Study of Impotence. International journal of impotence research. 1998 Jun;10(2):113-20. PubMed PMID: 9647948.
7. Massague J, Gomis RR. The logic of TGFbeta signaling. FEBS letters. 2006 May 22;580(12):2811-20. PubMed PMID: 16678165.
8. Kardassis D, Murphy C, Fotsis T, Moustakas A, Stournaras C. Control of transforming growth factor beta signal transduction by small GTPases. The FEBS journal. 2009 Jun;276(11):2947-65. PubMed PMID: 19490100.
9. Cho MC, Park K, Chai JS, Lee SH, Kim SW, Paick JS. Involvement of sphingosine-1-phosphate/RhoA/Rho-kinase signaling pathway in corporal fibrosis following cavernous nerve injury in male rats. The journal of sexual medicine. 2011 Mar;8(3):712-21. PubMed PMID: 21143420.
10. Allard J, Edmunds NJ. Reflex penile erection in anesthetized mice: an exploratory study. Neuroscience. 2008 Jul 31;155(1):283-90. PubMed PMID: 18599219.
11. Li WJ, Park K, Paick JS, Kim SW. Chronic treatment with an oral rho-kinase inhibitor restores erectile function by suppressing corporal apoptosis in diabetic rats. The journal of sexual medicine. 2011 Feb;8(2):400-10. PubMed PMID: 20233282.
12. Cho SY, Chai JS, Lee SH, Park K, Paick JS, Kim SW. Investigation of the effects of the level of glycemic control on erectile function and pathophysiological mechanisms in diabetic rats. The journal of sexual medicine. 2012 Jun;9(6):1550-8. PubMed PMID: 22489802.
13. Mulhall JP, Morgentaler A. Penile rehabilitation should become the norm for radical prostatectomy patients. The journal of sexual medicine. 2007 May;4(3):538-43. PubMed PMID: 17498093.
14. Leungwattanakij S, Bivalacqua TJ, Usta MF, Yang DY, Hyun JS, Champion HC, et al. Cavernous neurotomy causes hypoxia and fibrosis in rat corpus cavernosum. Journal of andrology. 2003 Mar-Apr;24(2):239-45. PubMed PMID: 12634311.
15. Leask A, Abraham DJ. TGF-beta signaling and the fibrotic response. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2004 May;18(7):816-27. PubMed PMID: 15117886.
16. Gabbiani G. The myofibroblast in wound healing and fibrocontractive diseases.

The Journal of pathology. 2003 Jul;200(4):500-3. PubMed PMID: 12845617.
17. Mead AL, Wong TT, Cordeiro MF, Anderson IK, Khaw PT. Evaluation of anti-TGF-beta2 antibody as a new postoperative anti-scarring agent in glaucoma surgery. Investigative ophthalmology & visual science. 2003 Aug;44(8):3394-401. PubMed PMID: 12882787.
18. Surma M, Wei L, Shi J. Rho kinase as a therapeutic target in cardiovascular disease. Future cardiology. 2011 Sep;7(5):657-71. PubMed PMID: 21929346. Pubmed Central PMCID: 3193795.
19. Gratzke C, Strong TD, Gebska MA, Champion HC, Stief CG, Burnett AL, et al. Activated RhoA/Rho kinase impairs erectile function after cavernous nerve injury in rats. The Journal of urology. 2010 Nov;184(5):2197-204. PubMed PMID: 20851436.
20. Haudek SB, Gupta D, Dewald O, Schwartz RJ, Wei L, Trial J, et al. Rho kinase-1 mediates cardiac fibrosis by regulating fibroblast precursor cell differentiation. Cardiovascular research. 2009 Aug 1;83(3):511-8. PubMed PMID: 19406912. Pubmed Central PMCID: 2709463.
21. Zhang YM, Bo J, Taffet GE, Chang J, Shi J, Reddy AK, et al. Targeted deletion of ROCK1 protects the heart against pressure overload by inhibiting reactive fibrosis. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2006 May;20(7):916-25. PubMed PMID: 16675849.
22. Vardouli L, Moustakas A, Stournaras C. LIM-kinase 2 and cofilin phosphorylation mediate actin cytoskeleton reorganization induced by transforming growth factor-beta. The Journal of biological chemistry. 2005 Mar 25;280(12):11448-57. PubMed PMID: 15647284.
23. Lee J, Ko M, Joo CK. Rho plays a key role in TGF-beta1-induced cytoskeletal rearrangement in human retinal pigment epithelium. Journal of cellular physiology. 2008 Aug;216(2):520-6. PubMed PMID: 18314880.
24. Hannan JL, Albersen M, Kutlu O, Gratzke C, Stief CG, Burnett AL, et al. Inhibition of Rho-kinase improves erectile function, increases nitric oxide signaling and decreases penile apoptosis in a rat model of cavernous nerve injury. The Journal of urology. 2013 Mar;189(3):1155-61. PubMed PMID: 23021998. Pubmed Central PMCID: 4007006.
25. Scott RW, Olson MF. LIM kinases: function, regulation and association with human disease. Journal of molecular medicine. 2007 Jun;85(6):555-68. PubMed PMID: 17294230.
26. Amano T, Tanabe K, Eto T, Narumiya S, Mizuno K. LIM-kinase 2 induces formation of stress fibres, focal adhesions and membrane blebs, dependent on its activation by Rho-associated kinase-catalysed phosphorylation at threonine-505. The Biochemical journal. 2001 Feb 15;354(Pt 1):149-59. PubMed PMID: 11171090. Pubmed Central PMCID: 1221639.
27. Morin P, Wickman G, Munro J, Inman GJ, Olson MF. Differing contributions of LIMK and ROCK to TGFbeta-induced transcription, motility and invasion. European journal of cell biology. 2011 Jan;90(1):13-25. PubMed PMID: 21074289.
28. Ferrini MG, Kovanecz I, Sanchez S, Umeh C, Rajfer J, Gonzalez-Cadavid NF. Fibrosis and loss of smooth muscle in the corpora cavernosa precede corporal veno-occlusive dysfunction (CVOD) induced by experimental cavernosal nerve damage in the rat. The journal of sexual medicine. 2009 Feb;6(2):415-28. PubMed PMID: 19138364. Pubmed Central PMCID: 2756287.
29. Gorovoy M, Niu J, Bernard O, Profirovic J, Minshall R, Neamu R, et al. LIM kinase 1 coordinates microtubule stability and actin polymerization in human endothelial cells. The Journal of biological chemistry. 2005 Jul 15;280(28):26533-42. PubMed PMID: 15897190. Pubmed Central PMCID: 1403832.
30. Croft DR, Olson MF. The Rho GTPase effector ROCK regulates cyclin A, cyclin D1, and p27Kip1 levels by distinct mechanisms. Molecular and cellular biology. 2006 Jun;26(12):4612-27. PubMed PMID: 16738326. Pubmed Central PMCID: 1489131.

31. Dai YP, Bongalon S, Tian H, Parks SD, Mutafova-Yambolieva VN, Yamboliev IA. Upregulation of profilin, cofilin-2 and LIMK2 in cultured pulmonary artery smooth muscle cells and in pulmonary arteries of monocrotaline-treated rats. Vascular pharmacology. 2006 May;44(5):275-82. PubMed PMID: 16524786.

논 문 초 록

서론: 최근 연구에서 해면체신경 손상 후 음경해면체 섬유화에 TGF-β/sphingosine-1-phosphate/RhoA/ROCK1 경로가 관여한다고 보고된 있다. 이에 연구자는 백서모델에서 해면체신경 손상 후 발생하는 음경해면체 섬유화에 ROCK1 경로의 하위 효과기인 LIM kinase2 (LIMK2)/Cofilin 경로의 활성화가 관여하는지 알아보고자 하였다.

방법: 10주령의 수컷 Sprague-Dawley 백서 48마리를 Sham 대조군 (S), 양측 해면체신경 으깸손상군 (I), 양측 해면체신경 절단군 (R)의 세 군 (각 군당 16마리)으로 구분하였다. 술 후 1, 4주 째 각 군당 8마리의 백서에서 발기능을 해면체내압측정 (ICP)으로 평가한 후 음경조직을 적출하였다. 파라핀 포매된 조직절편에 대해 Masson's trichrome 염색과 alpha-smooth muscle actin (α-SMA) 및 ROCK1, phospho-LIMK2 (p-LIMK2), vimentin에 대해 면역조직화학염색 또는 형광염색을 시행하였고, 단백을 추출하여 ROCK1, LIMK2, p-LIMK2, cofilin, phospho-cofilin (p-cofilin)에 대한 Western blot 을 시행하였다.

결과: 술 후 1주와 4주 째 I군과 R군의 평균동맥압에 대한 최대해면체내압의 비 (ICP/MAP)와 해면체내압의 곡선 (Area Under the Curve)은 S군에 비해 유의하게 저하되었다. I군의 ICP/MAP와 AUC은 R군에 비해 높은 경향을 보였으나 유의한 차이는 없었고, 각 군에서 시기별로 유의한 차이를 보이지 않았다. 평활근/콜라겐 비율과 α-SMA 발현은 1주와 4주 째 모두에서 S군에 비해 I와 R군에서 유의하게 감소하였다. 면역형광염색 결과 ROCK1 및 p-LIMK2 발현 양성인 섬유아세포가 1주 째 S군에 비해 I군 및 R군에서 유의하게 증가하였으며, 주로 백막 하 부위에 분포하였다. 하지만, 4주 째에는 S군에 비해 통계적인 차이를 보이지 않았다. Western blot에서 ROCK1 단백 발현은 1주와 4주 째 모두에서 S군에 비해 I군과 R군에서 유의하게 증가하였고, LIMK2의 인산화 정도는 I군과 R군에서 1주 째 S군에 비해 유의하게 증가되었으나, 4주 째는 1주 째에 비해 감소되어 S군과 유의한 차이가 없었다. Cofilin 인산화 정도는 R군에서는 S군에

비해 1주 째 부터 유의하게 증가하였으나, I군에서는 4주 째에 유의하게 증가하였다.

결론: 본 연구결과 해면체신경 손상 후 발생하는 음경해면체 섬유화에 ROCK1/LIMK2/cofilin 경로가 손상후 비교적 초기에 관여함을 알 수 있었다

주요어: 발기부전, 섬유화, 전립선절제술, 해면체 신경

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