The sphingosine-1-phosphate/RhoA/Rho associated kinases/myosin light chain pathway in detrusor of female rats is down-regulated in response to ovariectomy

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Abstract
Background: Dysuria is one of the main symptoms of genitourinary syndrome of menopause, which causes serious disruption to the normal life of peri-menopausal women. Studies have shown that it is related to decreased detrusor contractile function, but the exact mechanism is still poorly understood. Previous results have suggested that the sphingosine-1-phosphate (S1P) pathway can regulate detrusor contraction, and this pathway is affected by estrogen in various tissues. However, how estrogen affects this pathway in the detrusor has not been investigated. In this study, we detected changes of the S1P/RhoA/Rho associated kinases (ROCK)/myosin light chain (MLC) pathway in the detrusor of ovariectomized rats in order to explore the underlying mechanism of dysuria during peri-menopause.

Methods: Thirty-six female Sprague-Dawley rats were randomly divided into SHAM (sham operation), OVX (ovariectomy), and E groups (ovariectomy + estrogen), with 12 rats in each group. We obtained bladder detrusor tissues from each group and examined the mRNA and protein levels of the major components of the S1P/RhoA/ROCK/MLC pathway using quantitative real-time polymerase chain reaction and Western blotting, respectively. We also quantified the content of S1P in the detrusor using an enzyme linked immunosorben assay. Finally, we compared results between the groups with one-way analysis of variance.

Results: The components of the S1P pathway and the RhoA/ROCK/MLC pathway of the OVX group were significantly decreased, as compared with SHAM group. The percent decreases of the components in the S1P pathway were as follows: sphingosine kinase 1 (mRNA: 39%, protein: 45%) (both P < 0.05), S1P (21.73 ± 1.09 nmol/g vs. 18.86 ± 0.69 nmol/g) (P < 0.05), and S1P receptor 2/3 (S1PR2/3) (mRNA: 25%, 27%, respectively) (P < 0.05). However, the protein expression levels of S1PR2/3 and the protein and mRNA levels of SphK2 and S1PR1 did not show significant differences between groups (P > 0.05). The percent decreases of the components in the RhoA/ROCK/MLC pathway were as follows: ROCK2 (protein: 41%, mRNA: 36%) (both P < 0.05), p-MYPT1 (protein: 54%) (P < 0.05), and p-MLC20 (protein: 47%) (P < 0.05), but there were no significant differences in the mRNA and protein levels of RhoA, ROCK1, MYPT1, and MLC20 (all P > 0.05). In addition, all of the above-mentioned decreases could be reversed after estrogen supplementation (E group vs. SHAM group) (all P < 0.05).

Conclusion: In this study, we confirmed that ovariectomy is closely associated with the down-regulation of the S1P/RhoA/ROCK/MLC pathway in the rat detrusor, which may be one mechanism of dysuria caused by decreased contractile function of the female detrusor during peri-menopause.

Keywords: Myosin light chains; Ovariectomy; Rats; Rho-associated kinases; Sphingosine-1-phosphate; Urinary bladder

Introduction
Menopause is defined as the cessation of the menstrual cycle due to decreased ovarian hormone secretion.[1] It is a normal physiological process but is often accompanied by undesirable side effects. Many perimenopausal women suffer from genitourinary syndrome of menopause (GSM) as a result of decreased estrogen. The main urinary system manifestations are urinary frequency and urgency, nocturia, incontinence and dysuria.[2] Dysuria can cause urinary tract infection, urinary retention, or overflow urinary incontinence, which greatly reduces the quality of life of perimenopausal women. Although dysuria may be related to detrusor hypofunction, the exact underlying mechanism remains unknown.

Sphingosine-1-phosphate (S1P) is a biologically active sphingolipid present in various tissue cells, such as blood, endothelial, smooth muscle (SM), and tumor cells.[3-5] It is
involved in numerous physiological processes such as cell growth, apoptosis, and migration. In addition, S1P can regulate the contraction of skeletal muscle, vascular SM, and tracheal SM. Recent studies have shown enhancement of bladder detrusor contraction by S1P. Although the detailed mechanism has not been determined, researchers believe that regulation of detrusor contraction by S1P is mainly achieved through the downstream S1P receptors 1/2/3 (S1PR1/2/3), which are guanine nucleotide-binding protein-coupled receptors. S1P binds to the receptors and activates RhoA. RhoA then activates Rho-associated kinases (ROCK). Finally, ROCK increases the phosphorylation level of myosin light chain (MLC) in one of two ways: through direct phosphorylation of MLC or through phosphorylation of the MYPT1 sub-unit of myosin light chain phosphatase (MLCP), which in turn inhibits the dephosphorylation of phosphorylated forms of MLC (p-MLC) by MLCP. The phosphorylation level of MLC is positively correlated to contractility of the detrusor. S1P is a key component of the sphingolipid pathway, which is closely associated with estrogen. Moreover, researchers have found that the level of S1P in the plasma is significantly higher in pre-menopausal women than in men, and additionally, is higher in pre-menopausal women than in post-menopausal women, which indicates that S1P is correlated with menopause.

Since S1P is associated with menopause, the S1P/RhoA/ROCK/MLC pathway is involved in bladder detrusor contraction and GSM patients show clear dysuria symptoms, we investigated whether regulation of the expression of S1P/RhoA/ROCK/MLC pathway can affect detrusor contraction during menopause. Therefore, we examined the expression levels of the S1P/RhoA/ROCK/MLC pathway in the detrusor tissue of rats undergoing ovariectomy in order to explore the possible mechanisms of dysuria during peri-menopause.

Methods

Ethical approval

All experimental animals were purchased from the experimental animal center of the Peking University Health Science Center. The breeding and experimental procedures for the animals were in accordance with the guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals and World Medical Association Declaration of Helsinki on Ethical Principles for Medical Research involving experimental animals and was approved by the ethics committee on experimental animals (No. LA2018092).

Establishment of the model

Thirty-six 12-week-old female specific pathogen free Sprague-Dawley rats with a body weight of 210 ± 10 g were randomly divided into three groups: sham operation (SHAM), ovariectomized (OVX), and ovariectomized with estrogen treatment (E). The experimental animals were raised in a standard animal facility. The environmental conditions were controlled as follows: temperature of 20 to 26°C, humidity of 50% to 60%, and light/dark cycle of 12 h/12 h. The animals were allowed to eat a non-soybean feed and drink water freely. After 7 days of adaptive feeding, the surgical operation to remove tissue was carried out. One percent pentobarbital sodium (Beijing Guoyiao Chemical Reagent Company, China; 80 mg/kg) was injected intra-peritoneally for anesthesia. Only exploratory laparotomy was performed on the SHAM group, as a control, whereby fat of similar volume was removed from around the ovary without removal of ovarian tissue. The OVX and E groups underwent sterile bilateral ovariectomy. From the third day after the operation, exfoliated vaginal cells of the rats were examined every day for 7 consecutive days. Fourteen days after surgery, all rats were injected subcutaneously with specific drugs between 9 and 10 AM every day. Group E rats were given 17 β-estradiol (Sigma, St. Louis, Mo, USA; 25 μg·kg⁻¹·D⁻¹). The drug was dissolved in ethanol and diluted with sterile sesame oil (across, Belgium; 10 mg/0.1 mL, 0.25 mL/kg). The other two groups were given the same dose of sterile sesame oil. The injection cycle was 28 days.

Tissue sampling

Rats were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (80 mg/kg). After anesthesia, the chest was opened rapidly and blood was taken from the heart. The sample was placed in a 37°C incubator for 30 min and centrifuged at 4°C for 15 min (3000 r/min), followed by storage of the supernatant −80°C. The bladder tissue was excised and placed into a mixture of ice and water. The mucosa tissue of the bladder was quickly scraped using a surgical blade under a stereomicroscope (Olympus, Japan), and the remaining detrusor tissue of the bladder was stored at −80°C.

Radioimmunoassay

Radioimmunoassay was used to detect serum estrogen levels, with a detection limit of 0.01 pg/mL. The standards and samples with labeled antibody were incubated at 37°C for 2 h, separated for 15 min, centrifuged for 15 min at 3600 r/min, and then examined (Xi’an Nuclear Instrument Factory, China).

Hematoxylin-eosin (HE) staining

A cotton rod soaked in normal saline was inserted into the vagina of each rat, rotated for two turns, and applied evenly to a slide. Vaginal smears were placed in an oven for 20 min at 60°C. The smears were then washed with distilled water, stained with hematoxylin for 3 min, rinsed with running water for 15 min, and stained with eosin for 2 min. The smears were then gradually dehydrated with alcohol, transparentized with xylene, and finally sealed with resin.

Quantitative real-time polymerase chain reaction (Q-PCR)

The detrusor tissue was ground with liquid nitrogen. mRNA was isolated using a TransZol Up Plus RNA Kit (TransGen Biotech, Beijing, China, Code#ER501) according to the manufacturer’s instructions. Complementary
Table 1: Primer sequences used in this study.

| Primer name | Forward primer (5'-3') | Reverse primer (5'-3') |
|-------------|------------------------|------------------------|
| GAPDH       | TCCTTCCACCTTGGATGCTTG  | CTCTTGCTCTCAGATCCTTG   |
| SphK1       | AGCTGTCACCATGATACTCG   | GCTCTGATTTCTGACTTCC    |
| SphK2       | CAGCTAGCTTTCACCACCAC  | CTTGAATGCTGACAGCTTTCC  |
| S1PR1       | AACTCAGGCGTGTTGACTG    | GAAACATAGGAAATAAGGAC   |
| S1PR2       | TCTCTCCATTGCCCTTCGCC   | AGTTTTCTCACCAGGAGGCC   |
| S1PR3       | GGTCACTTGACCTATGCTGTC  | CTCAGCCGCGACTTCTCATTG  |
| RhoA        | TCTCAAGCAGGACAGTTC     | CCGTCCACCTCAATTCGCC    |
| ROCK1       | TCTCACCTCCTACACCTTCC   | CATGACCCTCTACACTCAGT   |
| ROCK2       | TGGAATGCGGCTGAGTAAG    | GAAATCGACCTGAGGAGGAG   |
| MYPT1       | GCGACTAGAAAGAGATGACTC  | AATCAGTTGTTCAGCTGATAG  |
| MLC20       | ACACATATCCACAGCATCC    | CTCGATAGGTGAAGAGACA    |

DNA (cDNA) synthesis was performed using 200 ng mRNA and Transcript II All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (TransGen Biotech). The gene expression levels of SPHK1/2, S1PR1/2/3, RhoA, ROCK1/2, MYPT1, and MLC20 were determined using TransStart Top Green qPCR SuperMix (TransGen Biotech) with the Bio-Rad CFX96 PCR system. Relative gene expressions were measured by the comparative period threshold method. Standardization of relative expression was carried out by comparison to the average expression of the housekeeping gene GAPDH. The primer sequences are listed in Table 1.

**Enzyme-linked immunosorbent assay (ELISA)**

Fifty milligrams of detrusor tissue were added to 450-μL phosphate buffer saline (pH = 7.2–7.4, 0.01 mol/L) and homogenized on ice. The homogenate was centrifuged at 5000 rpm for 10 min at 4°C. The concentration of S1P in the supernatant was detected using a rat S1P ELISA Kit (Jiangsu Meimian Industrial Co., Ltd, China).

**Western blotting (WB)**

Twenty-five milligrams of detrusor tissue were ground on ice and added to RIPA lysate buffer containing phenylmethane sulfonfyl fluoride and a phosphatase inhibitor. The protein concentration was measured using a bicinchoninic acid protein detection kit (Beyotime Biotechnology, China). The protein samples were separated by electrophoresis in 10% twelve alkyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to a polyvinylidene fluoride film. The membrane was sealed in Tris-Buffered Saline Tween-20 with 5% skim milk for 1 h and incubated with the primary antibody at 4°C overnight. The solution was replaced the next day with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5000) (Boster Biological Technology, China) and incubated at room temperature for 1 h. After washing, the band was visualized with a super-sensitive electrogenerated chemiluminescence (ECL) chemiluminescence solution (Haigene Detection Co. Ltd, China) and analyzed with a Chemidoc MP Chemiluminescence imaging system (Bio-Rad, USA). Actin was used as the internal reference and the following proteins were examined: Actin (Abcam, UK), SphK1 (Abcam), SphK2 (Thermo, USA), S1PR1 (Abcam), S1PR2 (Thermo), S1PR3 (Abcam), RhoA (Abcam), ROCK1 (Cst, USA), ROCK2 (Cst), MYPT1 (Cst), p-MYPT1 (Cst), MLC20 (Cst), and p-MLC20 (Cst).

**Statistical analysis**

Statistical analysis was performed using SPSS version 17.0 (IBM, USA). All data were expressed as mean ± standard deviation. One-way analysis of variance with the least significant difference was used to evaluate the differences between groups. P < 0.05 was considered to be statistically significant.

**Results**

**HE staining**

The results of HE staining of exfoliated vaginal cells showed that the estrous cycle of the SHAM group was normal. During the estrous phase, the exfoliated vaginal cells were large, polygonal, and rich in the cytoplasm [Figure 1A]. During the non-estrous phase, the exfoliated vaginal cells remained small, round, and deficient in cytoplasm [Figure 1B]. The estrous cycle of the OVX group and E group disappeared, and the morphology of exfoliated vaginal cells remained as shown in Figure 1B. These results confirmed that the ovariectomy was successful.

**Serum estrogen concentration**

Radioimmunoassay was used to measure the serum estrogen levels of the rats [Figure 2]. The results showed that the serum estrogen level of the OVX group was significantly lower than that of the SHAM group (5.24 ± 1.44 vs. 32.02 ± 3.14 pg/mL, P < 0.05), while the estrogen level of the E group, which was treated similarly with bilateral oophorectomy but supplemented with estrogen, was recovered (32.69 ± 3.12 pg/mL, P < 0.05). There was no significant difference between the OVX group and the SHAM group (P > 0.05).

**ELISA**

ELISA was used to detect the content of S1P in the rat detrusor. The results are shown in Figure 3. The contents
of S1P in the detrusor of the SHAM group, OVX group, and E group were 21.73 ± 1.09, 18.86 ± 0.69, and 21.85 ± 0.58 nmol/g, respectively. Compared with the other two groups, the level of S1P in the detrusor of the OVX group was decreased significantly (both \( P < 0.05 \)), but no significant difference was detected between the SHAM group and the E group (\( P > 0.05 \)).

**Q-PCR**

All key components of the S1P/RhoA/ROCK/MLC pathway were examined by Q-PCR [Figure 4]. The mRNA expressions of SphK1 and SphK2, the key enzymes of S1P synthesis, were not the same. No significant difference in SphK2 was found among the three groups (\( P > 0.05 \)), while the SphK1 level of the OVX group was decreased by 39% (\( P < 0.05 \)), as compared with the SHAM group. Similarly, the SphK1 levels were significantly different between the OVX group and the E group (\( P < 0.05 \)), while the SphK1 levels between the E group and the SHAM group were similar (\( P > 0.05 \)). The mRNA expression of S1P receptor S1PR1/2/3 were also different between the three groups. The expression of S1PR1 mRNA was relatively stable among the three groups (all \( P > 0.05 \)), but...
the expression of S1PR2/3 were different among the three groups. The expression of S1PR2/3 in the OVX group were significantly lower than that of the SHAM group (25% and 27%, both \(P < 0.05\)). In the RhoA/ROCK/MLC pathway, we examined the mRNA expression of RhoA, ROCK1/2, MYPT1, and MLC20. Compared with the SHAM group, the mRNA of the above components in the OVX group were decreased, and the decrease of ROCK2 was statistically significant (36%, \(P < 0.05\)). The ROCK2 level in the E group was similar to the level in the SHAM group (\(P > 0.05\)).

**WB**

In addition to the ten proteins measured by Q-PCR, we also examined the phosphorylated forms of MYPT1 and MLC20 (p-MYPT1 and p-MLC20) using WB [Figure 5]. The results of WB were consistent with Q-PCR, except in the cases of S1PR1/2. There were no significant differences in the protein expressions of SphK2, S1PR1, RhoA, ROCK1, MYPT1, and MLC20 between the three groups (all \(P > 0.05\)). The Q-PCR results showed that the mRNA expression of S1PR2/3 were different between groups, but there was no significant difference between the two groups at the protein level (both \(P > 0.05\)). The protein expression of SphK1 and ROCK2 in the OVX group were significantly lower than that of the SHAM group (45% and 41%, both \(P < 0.05\)), which was able to be reversed by estrogen supplementation (E group vs. SHAM group, both \(P > 0.05\)). Although there were no significant differences in the total protein expression of MYPT1 and MLC20 between the groups, contraction-related p-MYPT1 and p-MLC20 were indeed decreased significantly in the OVX group, compared with the SHAM group (54% and 47%, both \(P < 0.05\)). Moreover, the expression of the two proteins in the E group could also be corrected to those of the SHAM group (E group vs. SHAM group, both \(P > 0.05\)).

**Discussion**

In this study, we investigated changes of the S1P/RhoA/ROCK/MLC pathway in the detrusor of SHAM, OVX, and E group rats, as this pathway was expected to be an important factor affecting detrusor contraction of perimenopausal women. The Q-PCR and WB results showed that, compared with the SHAM group, the expression of SphK1 mRNA and protein in the detrusor of the OVX group were decreased significantly. After estrogen supplementation in the E group, expression returned to a level similar to that of the SHAM group. However, the expression of SphK2 between groups was similar. Likewise, SphK1 but not SphK2 could be increased or activated by estrogen in vascular endothelial and breast cancer cells.\(^{21-23}\) The physiological functions of SphK1 and SphK2 are quite different, although both can catalyze the production of S1P. SphK2 is primarily located in organelle and nuclear membranes of cells, and the S1P it produces is mainly involved in cell proliferation and apoptosis, while SphK1 is primarily located in the cytoplasm, and its S1P can be secreted out of cells for regulation of SM contraction.\(^{24-27}\) In the current study, we found that expression of SphK1 was down-regulated after ovariectomy, and we speculate that S1P produced from SphK1 (which is involved in the contraction of the detrusor) was reduced. Additionally,
the regulatory effect of estrogen on SphK1 is likely to be achieved through the membrane-localized estrogen receptor (mER)/G protein coupled receptor 30 (GPR30), as researchers have found that the specific antagonist of GPR30 can block activation of SphK1 by estrogen,[22,23] for which the exact mechanism awaits further investigation.

Although the level of SphK1, the key enzyme for S1P synthesis, decreased significantly after ovariectomy, the content of S1P was regulated by both the synthesis and decomposition pathway.[3] Thus, we further examined the content of S1P in the rat detrusor and found that consistent with the changes in SphK1, the content of S1P in the OVX group was significantly decreased. After estrogen supplementation, the decrease was completely reversed. The changes in estrogen and S1P levels were consistent with previous results. The results of a clinical study have shown that the level of S1P in the plasma of pre-menopausal women is significantly higher than that of men and post-menopausal women. Researchers have also found that the level of S1P in the plasma of female mice is significantly higher than that of male mice. Moreover, the addition of estrogen to human vascular endothelial cells can directly cause an increase of S1P.[21] Since estrogen can activate SphK1 and increase the S1P level and SphK1 is the key enzyme catalyzing the synthesis of S1P, combined with our findings that both SphK1 and S1P in the detrusor of the OVX group were decreased and can be completely restored after estrogen supplementation, we speculate that estrogen causes the change of S1P levels by regulating SphK1 in the detrusor.

As S1P function through S1PR, we further examined the expression of S1PR1/2/3 in the detrusor. We found that, compared with the SHAM group, the expression of S1PR2/3 mRNA in the OVX group were significantly decreased, and in the E group, expression can be rescued to the level of the SHAM group by estrogen supplementation. However, the expression of S1PR1 remained the same.
between the groups. Previous results have suggested that S1PR1/2/3, as G-protein-coupled receptors, are of different G-protein sub-types. In SM tissue, S1PR1 mainly binds to the Gq sub-type, causing change in the nitric oxide level and muscle relaxation, while S1PR2/3 mainly binds to the G12/13 sub-type, regulating the phosphorylation level of MLC and mediating cell contraction. In addition, S1PR2 and S1PR3 were determined to be the main receptors that mediate S1P-related detrusor contraction in rats and rabbits. Interestingly, the decreases of S1PR2/3 mRNA in the OVX group are consistent with the clinical phenomenon of weakened contraction of the detrusor in post-menopausal women. However, the protein expression of S1PR2/3 were similar between the groups. This may be because we utilized a physiological model with a short experimental period, and the inter-group differences in protein expression were not fully developed. In addition, the biological effect of S1PR2/3 is achieved by binding with its ligand, S1P. Even if the level of S1PR2/3 does not change, the decrease of S1P alone is enough to cause the changes of downstream effector proteins.

The contraction of the detrusor is a complex physiological process. The calcium channels of the cell membrane and sarcoplasmic reticulum are activated under specific conditions, which cause intracellular calcium influx and release of calcium stored in cells. The increased concentration of intracellular free Ca²⁺ combines with calmodulin (CAM) to form a Ca²⁺-CAM complex, which then combines with and activates downstream MLCK to phosphorylate Serine 19 of MLC20. The Mg²⁺ ATPase in the head of p-MLC20 myosin is then activated to combine with actin, forming the cross bridge. The ATPase in the head of myosin decomposes ATP to generate energy, induces the swinging of the cross bridge, and finally triggers myofilament contraction. On the other hand, contrary to MLCK, MLCP can cause the dephosphorylation of the phosphorylated MLC20 and in turn, inhibit the contraction. RhoA and ROCK are important regulatory factors of MLCP. The activated RhoA can activate ROCK, and ROCK can increase the phosphorylation level of MLC in two ways. One is through direct phosphorylation of MLC, and the other is through the phosphor-ylation of the MYPT1 sub-unit of MLCP, which in turn inhibits the dephosphorylation of P-MLC by MLCP. In conclusion, the RhoA/ROCK/MLC pathway is one of the key pathways mediating detrusor contraction, and the level of p-MLC is the deterministic factor for muscle contraction. Recently, studies have shown that RhoA/ROCK could be activated by S1P through its receptors, which has been reported as an active pathway in many rapidly advancing areas. For instance, the signal pathway could inhibit human bone marrow-derived mesenchymal stem cells migration and peripheral neurons growth, induce neurite retraction and vasoconstriction, cause stem cells migration and peripheral neurons growth, could inhibit human bone marrow-derived mesenchymal rapidly advancing areas. For instance, the signal pathway contraction. Recently, studies have shown that RhoA/ROCK/MLC pathway is one of the key pathways mediating detrusor contraction, and the level of p-MLC is the deterministic factor for muscle contraction. Therefore, these changes indicated the down-regulation of the RhoA/ROCK/MLC pathway. In general, the RhoA/ROCK/MLC pathway of the detrusor in the OVX group was down-regulated in accordance with the weakened SphK1-1-S1P-S1PR2/3 pathway, which can be reversed by estrogen supplementation. This phenomenon is specifically helpful in confirming the hypothesis that menopause affects detrusor contraction by regulating the expression of the S1P/RhoA/ROCK/MLC pathway.

In general, our study found that OVX can cause the down-regulation of the S1P/RhoA/ROCK/MLC pathway in the detrusor muscle of rats, which may be one of the mechanisms of perimenopausal bladder contraction dysfunction in women. Moreover, estrogen supplementation can completely correct these changes, suggesting that estrogen may be the key factor underlying the down-regulation of this pathway during menopause. Further work is required to unveil the detailed mechanism of mER in the pathway and the specific functionalities of S1PR2/3.

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Conflicts of interest
None.

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