133

Aydin M[1], Downing K[1], Villegas G[1], Zhang X[1], Chua R[1], Melman A[1], DiSanto M[1]
1. *Albert Einstein College of Medicine*

THE SPHINGOSINE-1-PHOSPHATE PATHWAY IS UPREGULATED IN RESPONSE TO PARTIAL URETHRAL OBSTRUCTION IN MALE RATS AND ACTIVATES RHOA/RHO-KINASE SIGNALING

Hypothesis / aims of study

Partial urethral obstruction (PUO) is a common urologic pathology observed in children with posterior urethral valves and in adults with benign prostatic hyperplasia (BPH) or urethral stricture. Obstructive uropathy is known to cause substantial changes in the phenotype of bladder smooth muscle (SM). In general there is an increase in the connective tissue-to-muscle ratio, an increase in detrusor muscle cell hypertrophy and an increase in SM contractility. PUO is associated with bladder overactivity in men.
Sphingosine-1-phosphate (S1P) is a bioactive lipid shown to modulate SM phenotype via activation of three G protein-coupled receptors (S1P1-S1P3). S1P1 is associated with nitric oxide-mediated SM relaxation, while S1P2 and S1P3 receptors are associated more with SM contraction via the RhoA/Rho-kinase (ROK) pathway. We hypothesized that PUO may up-regulate the S1P signaling pathway and that this up-regulation would activate the RhoA/ROK pathway. Thus, the specific aims of this study were to: 1) perform a novel examination of the effect of PUO on the S1P pathway in the bladders of male rats and 2) determine the effect of PUO on the RhoA/ROK pathway and if there is a molecular cross-talk with the S1P pathway.

Study design, materials and methods

Twenty adult male Sprague Dawley rats were divided into two groups and underwent PUO or sham operation (SHAM). Sample size was determined based upon previous experimental standard error to have 80% power at a two-sided Type I error rate of 0.05. After 2 weeks all rats were sacrificed and bladder specimens used for *in vitro* organ bath physiological contractility studies and also for mRNA and protein analyses of major S1P/ROK pathway constituents via Real-Time PCR and Western blotting, respectively. In addition, early passage SM cells were transfected with recombinant sphingosine kinase [SPHK, the enzyme that converts sphingosine (Sph) to S1P]. All studies were approved by our Animal Institute Committee.

Results

Bladder weight was significantly increased on average by 2.49-fold from 145 ± 10.8 mg in SHAM to 363 ± 23.7 mg in PUO rats while the average body weight was significantly less in the PUO (337 ±10.1 g) compared to the SHAM (384 ± 4.3 g) rats. In addition, the ratio of bladder weight to body weight was also significantly increased on average by 2.86-fold from 0.000379 in SHAM rats to 0.00108 in PUO rats. Real-Time RT-PCR showed increased expression of all three of the major S1P receptors in the bladders of PUO rats compared to the bladders of SHAM rats when normalized to the expression of the ribosomal protein large subunit 19 (RPL19). Compared to two other housekeeping genes (GAPDH & β-actin), RPL19 exhibited the most stable expression. However, the S1P1 receptor (associated more with SM relaxation) was increased only an average of 1.31-fold which was determined not to be statistically significant. In contrast, the S1P2 receptor and the S1P3 receptor (associated more with SM contraction and the RhoA/ROK pathway) were increased to a greater degree at 4.78- and 2.04, respectively, but only the increase in S1P2 mRNA expression was determined to be statistically significant. We also determined using Real-Time PCR that the expression of SPHK1 and SPHK2 (enzymes that convert Sph to S1P) were both increased by 2.72- and 1.47-fold, respectively, but only the increase in SPHK1 was statistically significant. At the same time the expressions of SPP1 and SPP2 (enzymes that convert S1P back to Sph) were not significantly altered which, with the increase in SPHK1, would be expected to result in a net driving of the S1P pathway toward increased S1P production. Since the S1P pathway is linked to the RhoA/Rho-kinase pathway via the S1P2 and S1P3 receptors and we have previously found that the expression of ROKβ was increased in a male rabbit model of PUO, we sought to determine whether the RhoA/ROK pathway was upregulated in our rat model of PUO. We determined that the expression of RhoA, ROKα and ROKβ were all increased by 1.76-, 2.19- and 1.32-fold, respectively, when normalized to the expression of the RPL19 but only the increases in RhoA and ROKα were determined to be statistically significant. In order to confirm the significant increases in mRNA expression for S1P pathway molecules, we extracted total protein from the bladder and performed Western blot analyses. Our results revealed that the expressions of SPHK1 and the S1P2 receptor were also increased at the protein level by 1.52 and 2.23-fold, respectively, in the bladders of PUO compared to SHAM rats when normalized to the averaged combined expression of β-actin and GAPDH. The expressions of RhoA and ROKα were also significantly increased at the protein level with the expression of RhoA increased by 1.51-fold while the expression of ROKα was increased by 2.55-fold.
Based upon our molecular findings we hypothesized that the bladder SM from PUO rats would generate more force *in vitro*. Our results revealed that the bladder strips from PUO rats generated more force and maintained force better in response to KCl and carbachol stimulation than bladder strips from SHAM rats when normalized to bladder weight. In addition, the bladder strips from PUO rats were more sensitive to carbachol stimulation. In response to exogenous S1P, bladder strips from PUO rats produced on average more force than bladder strips from SHAM rats when normalized to tissue weight with maximal force at 20 μM. At all concentrations above 5 μM, the force produced by bladder strips from PUO rats was between 3-8 fold higher than for bladder strips from SHAM rats which also represents a significant increase when normalized to either KCl or carbachol-induced contraction. In addition, PUO bladder strips contracted with a mid dose of S1P (10 μM) were treated with 1 μM Rho-kinase inhibitor (H-1152) and it was observed that the S1P-induced contraction was completely returned to baseline reaching sub-basal levels. In order to confirm a role for the S1P2 and S1P3 receptors in the mechanism of bladder contraction, we added JTE-013 or suramin (selective inhibitors of S1P2 and S1P3 receptors, respectively) to bladder strips pre-contracted with 10 μM carbachol. JTE-013 relaxed carbachol pre-contracted bladder SM on average by ~ 60% while suramin only produced on average an ~ 35% decrease in carbachol-induced contraction.Finally, in order to determine if there is a link (molecular cross-talk) between the S1P and RhoA/ROK pathways, we cloned the human SPHK1 or SPHK2 genes into the pVAX1 vector and forcibly overexpressed the recombinant SPHKs into the rat A7r5 SM cell line. Both SPHK1 and SPHK2 overexpression drive an increase in the expression of ROK1 (ROKβ) while only SPHK1 overexpression increases the expression of ROK2 (ROKα). This novel data provides a molecular link between the S1P and RhoA/ROK pathways.

Interpretation of results

In the current study we employed a rat model of surgically-induced PUO and have examined, for the first time, the effect of PUO on the S1P signaling pathway which is emerging as a master regulator of SM phenotype. Increased expression of SPHK1 would be expected to produce more S1P and this, coupled with the increased S1P2 & S1P3 receptor expressions (both associated with the RhoA/ROK pathway) would significantly enhance ROK activity. Moreover, our novel demonstration that S1P pathway activation can also increase the expression of ROK would further enhance ROK activity. Increased ROK activity is known to inhibit smooth muscle myosin phosphatase (SMMP) which would allow the myosin light chain kinase (MLCK) to work more efficiently and result in a greater phosphorylation of SM myosin leading to enhanced bladder SM contraction (overactivity).

Concluding message

We show for the first time that the S1P signaling pathway is significantly upregulated in response to PUO in male rats at both the molecular and *in vitro* functional levels correlating with an activation of the RhoA/Rho-kinase pathway. Further, we provide novel data that SPHK overexpression increases ROK expression *in vitro*, suggesting a novel hypothesis of S1P-induced bladder overactivity in the mechanism for PUO-induced bladder dysfunction and the S1P signaling pathway as a possible therapeutic target for bladder overactivity.

Specify source of funding or grant	**NIH Grant: 5R01DK077116**
Is this a clinical trial?	**No**
What were the subjects in the study?	**ANIMAL**
Were guidelines for care and use of laboratory animals followed or ethical committee approval obtained?	**Yes**
Name of ethics committee	**Animal Institute Committee of the Albert Einstein College of Medicine**