Transient Receptor Potential Melastatin 8 Contributes to Cystitis-Induced Neuronal Sprouting and Pain Hypersensitivity Through AKT/Mtor Signaling Pathway in Interstitial Cystitis/ Bladder Pain Syndrome

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Research Article

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Abstract

Transient receptor potential melastatin 8 (TRPM8) is associated with the pathophysiology of interstitial cystitis (IC)/bladder pain syndrome (BPS). We investigated the mechanism of TRPM8 in neuroproliferation and pain, as well as the relevance of the Akt/mTOR signaling pathway in mice with IC/BPS. The model of IC/BPS was established in wild and TRPM8+/− mice. The mechanical sensitivity was measured. The number of neurite segments, length of neurites, and density of neurites were all counted. IL-6 and norepinephrine levels were detected by ELISA, Western blot was used to detect protein levels of TRPM8, Akt, p-Akt, mTOR, p-mTOR. Immunofluorescence was used to detect TRPM8 expression and distribution in neurites, neurons, and sensory nerves in mouse bladder tissue. Pain threshold in the IC/BPS group was decreased, and neurite segments, length, and density were all significantly enhanced when compared to the control group. The parameters in the TRPM8 agonists(menthol)+IC/BPS group were more statistically significant. Neurite number and density were lower in TRPM8+/−+IC/BPS mice than in IC/BPS mice. The expression of TRPM8 and the ratios of p-Akt/Akt and p-mTOR/mTOR rose in the IC/BPS group. In TRPM8+/−+IC/BPS mice, the ratios of p-Akt/Akt and p-mTOR/mTOR were not substantially different from those in the control group. TRPM8+/−+IC/BPS mice had considerably lower levels of serum IL-6 and urine norepinephrine than wild-type IC/BPS mice. TRPM8 can induce pain hypersensitivity and sensory nerve proliferation by activating Akt/mTOR pathway and raising the expression of IL-6 and norepinephrine in IC/BPS models. These findings offer new perspectives on IC/BPS treatment.

Introduction

The international continence society defines interstitial cystitis (IC)/bladder pain syndrome (BPS) as recurrent exacerbation of pain in the bladder area with bladder filling and symptom relief after emptying, accompanied by other symptoms like frequency and nocturia, but without obvious evidence of urinary tract infection or other maladies¹. "Suprapubic discomfort associated with bladder fullness" is a common symptom of IC/BPS. This pathological pain could be linked to the emergence or alteration of aberrant afferent signaling pathways linked to inflammation². In both human and animal models, the following occurrences have been documented in IC/BPS: proteome modifications³, ion channel sensitization, and enhanced neurotrophic factor release⁴, all occur as the neurons that innervate the bladder recombine⁵.

Transient receptor potential melastatin 8 (TRPM8) is a type of nonspecific ion channel that has a high calcium permeability. A chilly environment (8°C–28°C) and cool substances can activate it, creating inward currents dominated by calcium ions⁶. TRPM8 may be linked to increased innervation of bladder afferent neurons in patients with IC/BPS, resulting in pain symptoms, according to our findings⁵. Ciobanu AC et al revealed that a higher level of TRPM8 expression in dorsal root ganglion neurons lowers pain thresholds in sensory nerves in patients, despite the fact that many specifics of the mechanism are unknown⁷. Furthermore, Lashinger et al found that TRPM8 blocker suppresses pain in normal rats, implying that TRMP8 blocker may play a role in the relief of IC/BPS pain symptoms⁸. However, the study
did not demonstrate the efficacy of TRPM8 blockers in IC/BPS rat models. The role of TRPM8 in IC/BPS pain symptoms is currently unknown.

As a result, we evaluated the effects of TRPM8 on UPK3A65-84-induced wild and TRPM8 knockout IC/BPS mice models, as well as the role and mechanism of TRPM8 in IC/BPS treatment.

**Results**

The number of CD3-positive cells (T cells) in mouse bladder tissue injected with UPK3A65-84 polypeptide was significantly higher (Fig. 1B) than in the control group (Fig. 1A), indicating that the model was successfully constructed.

In the control group, IC/BPS model group, IC/BPS model+ Menthol group, and TRPM8\(^{-/-}\) group, we examined TRPM8 mRNA (Fig. 2A) and protein expression (Fig. 2B). TRPM8 mRNA and protein expression were substantially greater in the IC/BPS model group than in the control group (P<0.01). TRPM8 expression was significantly increased in the IC/BPS model+ menthol group. TRPM8 was absent in the TRMP8\(^{-/-}\) group. We also examined pain perception in the different groups, as shown in Figure 2C. The results demonstrated that the IC/BPS model group had a lower mechanical response threshold (Control vs IC/BPS model, P<0.01), indicating pain hypersensitivity. Pain hypersensitivity was enhanced after menthol intervention (IC/BPS versus IC/BPS+ menthol, P<0.01), and the TRPM8\(^{-/-}\) group had a higher mechanical response threshold (IC/BPS vs TRPM8\(^{-/-}\)+ IC/BPS, P<0.01) (Fig. 2C), demonstrating that TRPM8 expression is linked to pain hypersensitivity in IC/BPS.

The IC/BPS model group had higher levels of IL-6 (Fig. 3A) in serum and noradrenaline (Fig. 3B) in urine (P<0.01). After TRPM8 agonist treatment, the expression of IL-6 in serum and noradrenaline in urine rose much more (P<0.01). The TRPM8\(^{-/-}\) group had significantly lower levels of IL-6 in serum and noradrenaline in urine (P<0.01).

The number of neurite segments, neurite length, and neurite density in IC/BPS model group were significantly enhanced (P<0.05) according to immunofluorescence data (Fig. 4A). After TRPM8 agonist intervention, the phenomena became more noticeable (P<0.01). The number of neurite segments in the TRPM8\(^{-/-}\)+ IC/BPS group reduced significantly (P<0.05) (Fig. 4B), but neurite length (Fig. 4C) and density (Fig. 4D) were not different between the IC/BPS and TRPM8\(^{-/-}\)+ IC/BPS groups.

Neurites (positive for PGP9.5) and neurons (positive for Tuj1), as well as afferent sensory nerves (positive for CGRP), were identified using immunofluorescence. TRPM8 expression was enhanced in neurites, neurons, and afferent sensory nerves in the IC/BPS model group. In the IC/BPS model +menthol group, the results were clearer (Fig. 5). TRPM8 protein was absent in the TRPM8\(^{-/-}\) mice.

Western blots were used to detect the expression of Akt, p-Akt, mTOR, and p-mTOR in the bladder tissue of all groups (Fig. 6A). The p-Akt/Akt ratio (Fig. 6B) was considerably raised (P<0.01) in the IC/BPS model group and aggravated (P<0.01) in the IC/BPS model+ menthol group. The IC/BPS group had a higher
ratio of p-mTOR/mTOR (P<0.05) (Fig. 6C), but there was no difference between IC/BPS and IC/BPS + menthol group.

**Discussion**

There is evidence that the bladder neck and proximal urethra have the highest density of bladder nerves, with epithelial cells organized on the surface exhibiting neuronal-like features. The pathophysiology of UPK3A65-84-induced visceral pain in mice is strikingly similar to that of human IC/BPS. The addition of a TRPM8 agonist or knocking out the TRPM8 gene dramatically increased or decreased visceral pain, suggesting that TRPM8 may play a role in IC/BPS visceral pain.

The pathogenesis of IC/BPS is complicated by inflammation. Chronic pain is frequently generated and maintained by peripheral or central inflammation, which is marked by high levels of inflammatory cytokines, including the well-known IL-6. When infection and tissue damage occur, IL-6 is normally produced quickly. Changes in IL-6 were substantially linked with the regulation of TRPM8, suggesting that TRPM8 may cause IC/BPS by altering the microenvironment and inflammatory response, similar to our findings.

Bladder inflammation also causes continual peripheral nerve stimulation, nociceptive nerve activation, neuron hypersensitivity, and spontaneous central nervous system neuronal activity. The release of neuropeptides is mediated by neural activity, which causes inflammation and creates a vicious cycle. The pain symptoms of IC/BPS rat model are influenced by an increase in sympathetic activity and a rise in norepinephrine levels in the urine. The activation of the α1A adrenoceptor in peripheral blood can cause chronic visceral discomfort by interacting with TRPV1 and ATP release, according to another study. The shifting trend of IL-6 and epinephrine following TRPM8 regulation was similar to the sensitivity of pain, showing that TRPM8 activates epinephrine through an inflammatory response, resulting in chronic pain, and that reducing TRPM8 can considerably improve the above pathophysiological state.

Excessive peripheral signal input causes visceral pain sensitivity in peripheral neuroinflammatory states (such as IC/BPS). As a result, we looked at how neurite expression changed in the bladders of IC/BPS model animals. The number of neurite segments, neurite length, and neurite density are all highest in the bladder of IC/BPS model mice, indicating that increased neurite expression is proportional to increased visceral pain. The alterations in neurite quantity, length, and density after TRPM8 agonist or TRPM8 gene knockout confirmed that the role of TRPM8 in IC/BPS visceral pain was closely tied to peripheral nerve expression.

Furthermore, we discovered a substantial number of TRPM8 positive neurites, neurons, and sensory nerves, indicating that these nerves are important in visceral sensation. The number of TRPM8 positive nerve fibers in the bladder wall of IC/BPS model mice rose considerably when compared to the control group. This behavior becomes more pronounced after TRPM8 agonist intervention. The findings indicate
that increased TRPM8 positive nerve fibers in the bladder wall produce IC/BPS pathological alterations, which is consistent with our prior findings in IC/BPS patients\textsuperscript{5}. After TRPM8 gene knockout, nerve fiber expression dropped dramatically and visceral pain was relieved compared to the IC/BPS group, confirming TRPM8's pivotal function in IC/BPS visceral pain. TRPM8 blockers can decrease pain in normal rats, according to Lashinger et al\textsuperscript{11}. Mukerji found a link between the relative density of TRPM8 immunoreactive nerve fibers in the bladder and pain score\textsuperscript{12}. Both of these investigations corroborate what we've observed.

In bladder tissue, we also found p-Akt and p-mTOR expression. Cell proliferation is linked to the p-Akt/p-mTOR pathway\textsuperscript{13,14}. Furthermore, nociceptive processes are linked to mTOR\textsuperscript{15,16}. The PI3K/Akt/mTOR signaling pathway is activated by afferent signals triggered by peripheral inflammation, and part of it is engaged in neural circuits that promote pain\textsuperscript{17}. Sciatica caused by endometriosis can be relieved by inhibiting the PI3K/Akt/mTOR signaling pathway\textsuperscript{18}. These findings suggest that the PI3K/Akt/mTOR pathway is involved in pain. The trends of p-AKT/AKT and p-mTOR/mTOR across the groups in this investigation were similar to those of TRPM8. As a result, we believe that TRPM8-induced pain sensitization and neuroproliferation in IC/BPS mice is linked to the AKT/mTOR pathway.

TRPM8 positivity colocalized with TRPV1 immunoreactivity in colonic afferent neurons, according to Harrington et al\textsuperscript{19}. TRPM8 activation, according to Ramachandran et al., suppresses TRPV1-dependent neuropeptide release\textsuperscript{20}. These findings show that TRPM8 modulates other TRP channels via intracellular interactions, although more research is needed to confirm this. Nonetheless, the current findings point to TRPM8 as a possible target of IC/BPS pain hypersensitivity.

This research has certain drawbacks. First and foremost, IC/BPS is a chronic disease. Although the UPK3A65-84 polypeptide-induced mouse cystitis model lasts longer than the CYP-induced model, it still fails to fully imitate human IC/BPS. Second, other potential mechanisms besides TRPM8 should be investigated further.

Finally, we show that via activating the Akt/mTOR pathway and boosting the expression levels of IL-6 and norepinephrine, TRPM8 improves pain transmission and causes sensory nerve proliferation in an animal model. These findings suggest new approaches to treating IC/BPS.

**Methods**

Male wild-type C57BL/6 mice (8-10 weeks) (n=30) were purchased from Huazhong Agricultural University. TRPM8\textsuperscript{−/−} mice (8-10 weeks) (n=20) were purchased from the Jackson Laboratory. C57BL/6 mice were randomly assigned to one of three groups: control, IC/BPS model, or IC/BPS model+TRPM8 agonist (menthol). TRPM8\textsuperscript{−/−} mice were randomly separated into two groups: TRPM8\textsuperscript{−/−} and TRPM8\textsuperscript{−/−}+IC/BPS model.
All of the mice were raised in a pathogen-free environment. All animal operation techniques adhere to the National Research Council's criteria for the care and use of experimental animals. The experimental protocol involved was reviewed by the animal experimental ethics review committee of Beijing Chao-Yang Hospital Affiliated to Capital Medical University 2016-2-19-34. In addition, all studies were carried out in strict compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

A subcutaneous injection of 0.4ml complete Freund's adjuvant emulsified in 0.2ml phosphate-buffered saline (PBS) was given to mice in both groups. Mice in the IC/BPS model and TRPM8⁻/⁻ IC/BPS model groups were given a subcutaneous injection of 200g UPK3A65–84 polypeptide (sequence: AMVDSAMSRNVSVQDSAGVP; Cloud-Clone Corp., Wuhan, China) emulsified in 0.2 ml PBS and 0.4 ml complete Freund's adjuvant. One week before the end of model construction, the IC/BPS model + menthol group received a subcutaneous injection of 200 mg/kg menthol once a day for seven days. During the same period, the control group was given the same volume of saline once a day for 7 days in a row.

The mechanical sensitivity of mice was assessed after 35 days, and the mice's bladder tissues were removed to make paraffin sections for CD3 immunohistochemical labeling to identify T cell counts. The findings were used to determine if the models had been developed correctly.

**Mechanical Sensitivity Testing**

Von Frey filaments were used to test mechanical sensitivity. Mice were examined in an individual cage with a stainless-steel wire grid floor after a 2-hour habituation interval. To avoid desensitization, stimulation was limited to the lower abdomen area in the general location of the bladder, with care given to excite diverse sites within this region. Positive responses to filament stimulation were defined as quick retraction of the abdomen and immediate licking or scratching of the stimulation area. Each filament was used for 1-2 seconds, with a 3-5 second interstimulus interval.

**Immunohistochemistry Staining**

Slides were quenched in 0.3% hydrogen peroxide and rinsed in PBS before being blocked for 1 hour at room temperature in 5% normal goat serum in PBS. Sections were then incubated overnight at 4°C in primary antibody (CD3, Abcam, 1:100) in blocking serum before being washed in PBS, and incubated in HRP labeled secondary antibody for 20min at 37°C. After that, the sections were washed and DAB solution was added. After counterstaining in hematoxylin, dehydrating, and clearing in xylene, the slides were mounted in di-n-butyl phthalate in xylene. A fluorescent microscope was used to collect the images (Olympus, BX53).

**qRT-PCR**
Total RNA was isolated from bladder samples using TRizol® reagent (Invitrogen, Carlsbad, CA, USA), which was then used to make cDNA using a kit (Thermo, #K1622). The 7500 Real-Time PCR system (ROCHE, LC480) was used to evaluate quadruplicates of four different experiments using quantitative real-time polymerase chain reaction (qRT-PCR). Initial denaturation at 95°C for 10 minutes, 40 amplification cycles at 95°C for 15 seconds, and annealing and extension at 60°C for 60 seconds were the conditions. Each experiment was repeated three times, with the average values utilized for analysis.

Primer sequence:

hTRPM8-F: CAGAAGGAATGACACTCTGGAC;

hTRPM8-R: TCACCAAGTCGCTTTTCACTGT;

hGAPDH-F: GGAGCGAGATCCCTCCAAAAT;

hGAPDH-R: GGCTGTTGTCATACTTCTCATGG;

Mu-GAPDH-F: AGGTCGGTGTGAACGGGATTG;

Mu-GAPDH-R: GGGTTCGTTGATGGCAAC;

Mu-TRPM8-F: ACAGACGTGTCCTACAGTGAC;

Mu-TRPM8-R: GCTCTGGGCATAACCACACTT;

**Western Blot**

The DC Protein Assay kit (Bio-Rad) and an ELx800 spectrophotometer were used to assess the protein concentration in cell lysates (Bio-TekTM). An equivalent number of proteins were isolated and blotted onto nitrocellulose sheets using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then probed with a primary antibody (TRPM8, Novus, 1:1000; Akt, Abcam, 1:1000; p-Akt, Abcam, 1:1000; mTOR, Abcam, 1:1000; p-mTOR, Abcam, 1:1000) and a peroxidase-conjugated secondary antibody. The Supersignal™ West Dura system (Pierce Biotechnology, Inc., Rockford, IL, USA) was used to visualize protein bands, and a UVITech imager was used to photograph them (UVITech, Inc., Cambridge, UK).

**Immunofluorescence Staining**

Before embedding in O.C.T. compound, frozen transverse slices (14 mm thick) were prepared from bladder fixed with 4% paraformaldehyde in PBS and cryopreserved in 25% sucrose in PBS. Slides were washed in PBS and treated with goat serum for 30 minutes, primary antibodies (PGP9.5, Novus, 1:200; Tuj1, Abcam, 1:100; CGRP, Abcam, 1:100) overnight at 4°C, and Cy3 labeling sheep anti-mouse IgG for 1 hour at 37°C (1:50). After that, the slides were washed in PBS before being incubated with goat serum. The primary antibody (TRPM8, Novus, 1:1000) was used overnight at 4°C, and FITC labeling sheep anti-
rabbit IgG (1:50) for 1 hour at 37°C. DAPI was added before PBS. A fluorescent microscope (Olympus, BX53) was used to collect the images.

**Neurite Quantification**

Neurite (positive to PGP9.5) was detected visually as longer than 10µm with a thickness between 1 and 3µm. We used ImageJ software to analyze the images and measured fibers on 8-10 images per bladder from three animals per condition. Results were expressed as neurite segments/µm² (the number of individual neurites per surface unit), the neurite length, and the neurite density (neurite length per surface unit).

**Statistical Analysis**

SPSS software, version 22.0, was used for statistical analysis (SPSS Inc, Chicago, IL). All data is presented as Mean±SD. The LSD method of One-way ANOVA was used to compare the means observed in different groups. The significance level was established at P <0.05.

**Statement for ARRIVE guidelines.**

We have read the journal's policy and all the authors read and approve the manuscript. We declared that this study was carried out in compliance with the ARRIVE guidelines. The animal was raised in a stress-free environment and handled with extra care. They were euthanized peacefully. All the protocols were followed according to the ARRIVE guidelines for handling the animal.

**Declarations**

**Acknowledgments**

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**Declaration of Conflicting Interests**

The authors declare that there are no conflicts of interest regarding the publication of this paper.

**Author Contributions**

PZ designed the project. FZ and RC collected data and specimen of patients. LW performed qRT-PCR and Western Blot. FW performed immunofluorescence. ZZ analyzed data. LW and JZ wrote the manuscript.

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**Figures**

*Figure 1*

Identification of the model. The UPK3A65-84 polypeptide was used to make the mouse IC BPS model, and the bladder was stained with CD3 immunohistochemistry. The scale bar represents 50μm.
Figure 2

(A) TRPM8 mRNA expression in different groups. (B) TRPM8 protein expression in various groups. The upper section shows a representative Western blot, while the lower section shows a statistical diagram of protein gray value. (C) Pain threshold detection results in various groups. SD is shown by the error line, and ** represents P<0.01.
Figure 3

ELISA was used to detect the expression of IL-6 in serum and norepinephrine in urine. SD is shown by the error line, and ** represents P<0.01.
Figure 4

Quantification of Neurites. (A) Number of neurite segments (/μm²). (B) Neurite length [μm]. (C) Neurite density (/m2). The scale bar represents 50μm. The error line represents SD and ** represents P<0.01.
Figure 5

Representative images of TRPM8-positive nerve sprouting in the bladder wall in various groups. TRPM8 expression increased in bladder neurites (positive for PGP9.5) and neurons (positive for Tuj1), as well as afferent sensory nerves (positive for CGRP), according to immunofluorescence data. After TRPM8 agonist intervention, these phenomena became more noticeable. After the TRPM8 gene was knockout, the protein TRPM8 was not expressed in the bladder tissue of mice. The scale bar represents 50μm.
Figure 6

Detection of the Akt/mTOR signaling pathway in the mice bladder. (A) Western blot was used to detect the protein levels of p-Akt, Akt, p-mTOR, and mTOR in the mice bladder. (B-C) Protein gray analysis. For data statistics, one way ANOVA was used. The error line represents SD, ** P<0.01, * P<0.05, and NS no significant difference between the two groups.