



P2X7 receptor mediates activation of microglial cells in prostate of chemically irritated rats

Heng Zhang, Limei Liu, Zhong Yang, Jinhong Pan, Zhiwen Chen, Qiang Fang, Weibin Li, Longkun Li, Gengsheng Lu, Zhansong Zhou

Department of Urology (HZ, JP, ZC, QF, WL, LL, GL, ZZ); Department of Pathology (LL) and Department of Neurobiology (ZY), Southwest Hospital, Third Military Medical University, Chongqing 400038, China

ABSTRACT

Purpose: Evidence shows that adenosine triphosphate (ATP) is involved in the transmission of multiple chronic pain via P₂X₇ receptor. This study was to investigate the P₂X₇ and microglial cells in the chronic prostatitis pain.

Materials and Methods: Rats were divided into control group and chronic prostatitis group (n = 24 per group). A chronic prostatitis animal model was established by injecting complete Freund's adjuvant (CFA) to the prostate of rats, and the thermal withdrawal latency (TWL) was detected on days 0, 4, 12 and 24 (n = 6 at each time point in each group). Animals were sacrificed and the pathological examination of the prostate, detection of mRNA expression of P₂X₇ and ionized calcium binding adaptor molecule 1 (IBA-1) and measurement of content of tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) in the dorsal horn of L₅-S₂ spinal cord were performed on days 0, 4, 12 and 24. In addition, the content of TNF- α and IL-1 β in the dorsal horn of L₅-S₂ spinal cord was measured after intrathecal injection of inhibitors of microglial cells and/or P₂X₇ for 5 days.

Results: The chronic prostatitis was confirmed by pathological examination. The expression of P₂X₇ and IBA-1 and the content of TNF- α and IL-1 β in rats with chronic prostatitis were significantly higher than those in the control group. On day 4, the expressions of pro-inflammatory cytokines became to increase, reaching a maximal level on day 12 and started to reduce on day 24, but remained higher than that in the control group. Following suppression of microglial cells and P₂X₇ receptor, the secretion of TNF- α and IL-1 β was markedly reduced.

Conclusion: In chronic prostatitis pain, the microglial cells and P₂X₇ receptor are activated resulting in the increased expression of TNF- α and IL-1 β in the L₅-S₂ spinal cord, which might attribute to the maintenance and intensification of pain in chronic prostatitis.

ARTICLE INFO

Key words:

Prostatitis; Cells; Receptors, Purinergic P2X7; Tumor Necrosis Factor-alpha; Interleukin-1

Int Braz J Urol. 2013; 39: 276-85

Submitted for publication:
June 04, 2012

Accepted after revision:
January 17, 2013

INTRODUCTION

Chronic prostatitis, a common urological condition in young and middle-age men, is caused by multiple etiological factors. Pain is a major presentation of chronic prostatitis (1). Previous studies focused on the pathological changes in the prostate, while the pathways related

to neurotransmission and the regulatory mechanisms of chronic prostatitis pain have not been studied. Recent studies have identified the chronic prostatitis pain as a visceral referred pain, which is usually accompanied by the dysfunction of pelvic floor muscles. The prostate is innervated largely by the pelvic nerves arising from the L₅-S₂ spinal cord (2,3).

It has also been shown that the transmission and regulation of pain are associated with not only the neurons but the microglia and astrocytes (4,5). Studies also demonstrated that astrocytes and microglia may secrete pro-inflammatory cytokines such as tumor necrosis factors (TNF), interleukin-1 (IL-1), nerve growth factor (NGF), and nitric oxide (NO), which may lead to the neuronal injury and chronic pain (6,7). Especially, the microglia are widely distributed in the central nervous system (CNS). The detrimental stimulation of CNS (such as trauma, ischemia and infection) may activate microglia. Under this condition, their morphology, the receptor expression on these cells and their function alter; these cells are amoeboid; the markers for activation increase on these cells (8). There is evidence showing that the microglia in the posterior horn of spinal cord are significantly activated after damage to peripheral nerves (9). This suggests that the activation of microglia in the spinal cord is related to the occurrence and transmission of neuropathic pain. However, the role of microglia in chronic prostatitis pain is still poorly understood, and molecules activated after injury on these cells and the exact mechanisms are unclear.

There are a lot of P_2X_7 receptors of adenosine triphosphate (ATP). ATP is a type of pain-causing neurotransmitter, and its receptors can be classified as P_{2X} receptors and P_2Y receptors. P_2X_7 receptor is a special subtype of purinergic receptor P_{2X} family and an ATP-gated non-selective cation channel. P_2X_7 receptor contains 595 amino acids and three P_2X_7 receptors form homologous polymers generally. P_2X_7 receptor is a dual functional receptor. Under pathological conditions, P_2X_7 receptor is involved in the transmission of pain. It was reported that microglia may be activated by the P_2X_7 receptor, which is up-regulated in various types of chronic pain (10). Chessell et al. found P_2X_7 knockout mice failed to present with hyperalgesia to heat and mechanical stimulation after nerve injury (11). However, whether the microglia and P_2X_7 receptor in the posterior horn of L_5-S_2 spinal cord are activated to regulate the chronic prostatitis pain remains unclear. Hence, the present study was to investigate the role of microglia and P_2X_7 receptor in the chronic pros-

titis pain and the possible therapeutic strategies for chronic pelvic pain syndrome.

MATERIALS AND METHODS

Animals

The specific pathogen free (SPF) rats weighing 200 ± 25 g were purchased from the Experimental Animal Center of the Third Military Medical University and randomized into experiment group and control group. All rats were intraperitoneally anesthetized with 1% pentobarbital and then the prostate was exposed through a ventral midline incision (1 cm). For rats in the experiment group, injection with complete Freund's adjuvant (CFA; Sigma-Aldrich, Sigma) was done once at bilateral ventral lobes (10 μ L for each). For rats in the control group, 20 μ L of normal saline was injected (Nackley et al. (12), Butler et al. (13) and Zhou et al. (14)). Then, the wound was closed. Rats were sacrificed on days 0, 4, 12 and 24 d after injection ($n = 6$ at each time point in each group) and pathological examination and detection of the mRNA expression of P_2X_7 and ionized calcium binding adaptor molecule 1 (IBA-1) and the content of TNF- α and IL-1 β in spinal cord were performed. All procedures were performed in accordance with the guidelines for animal care and use of National Institute of Health, and this study was approved by the Ethics Committee of our Hospital.

Prostatic Inflammation Model Identification

Rats were intraperitoneally anesthetized with 10% chloral hydrate (0.3 mL/100 g) on days 0, 4, 12 and 24 after CFA injection, and transcardially perfused with 200 mL of 0.9% saline and then with 0.01 mol/L phosphate buffer (about 300 mL) containing 4% paraformaldehyde at 48° C. Subsequently, the left and right prostatic tissues were collected, fixed in 4% paraformaldehyde at 4° C overnight, embedded in paraffin, cut into sections, stained, and finally examined under a microscope (15).

Detection of heat pain threshold

The thermal withdrawal latency (TWL) of the rats was detected at 4 d, 12 d and 24 d after injection respectively. The rats were placed in a box (2 cm x 12 cm x 22 cm) with a glass floor and allowed to accommodate to the environment for 30 min. Then,

a light spot (5 mm in diameter; 50 W, 12 V) was produced through a radiant heat stimulator and used to stimulate the paw. The time to paw withdraw was recorded as the TWL. The stimulation was done for no longer than 30 s and measurement was performed 5 times in each rat with an interval of 10 min. between two detections. The maximal or minimal TWL was removed, and the TWL in remaining 3 measurements was employed for the calculation of average (16).

Detection of mRNA expression of P₂X₇ and IBA-1 in L₅-S₂ posterior horn

Animals were sacrificed by decapitation. The posterior horn of L₅-S₂ spinal cord was carefully collected on ice under a microscope, and stored at -70° C for use. Total RNA was extracted from the L₅-S₂ spinal cord using the RNeasy Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions. The concentration and purity of total RNA were determined by spectrophotometric analysis at A₂₆₀ and A₂₈₀ (1.8-2.0). The quality of RNA was determined by methanol agarose gel electrophoresis following ethidium bromide staining. Total RNA (2µg) was subjected to reverse transcription using the Reverse Transcription System (Jikang, Shanghai, China) with random primer oligo(DT)₁₈ (0.5µg). The reaction conditions were as follows: 70° C for 5 min, 37° C for 60 min. and 70° C for 10 min., and products were then stored at -70° C.

The resulting cDNA (20 ng) was used as templates for real-time fluorescence quantitative (FQ) PCR using a SYBR green PCR core reagent kit (Applied Biosystems, Foster City, CA) in DNA Engine OPTIONTm² (MJ RESEARCH, USA). The primers were designed using the Geneworks software package as follows: P₂X₇: 5'-GACAAACAAAGT-CACCCGGAT-3' (forward) and 5'-CGCTCACCAAAGCAAAGCTAAT-3' (reverse); IBA-1: 5'-TTGATCTGA

ATGGCAATGGA-3' (forward) and 5'-CCTCC AAT-TAGGGCAACTCA-3' (reverse). The PCR conditions were as follows: reverse transcription at 50° C for 30 min., Hot Start Taq (1.25 unit/sample) activation for 15 min at 95° C, 40 cycles of denaturation at 94° C for 15 s, annealing at 56° C for 30 s, and extension at 72° C for 30 s. The SYBR Green fluorescence was acquired by a final extension at 79° C. The melting curve analysis was performed after each reaction. GAPDH (5'-TTTAACTCTGGTAAAGTGGATATTG-TTG-3' [forward] and 5'-ATTTCCATTGATGACAA GCTTCC-3') served as an internal control (Table-1). The expression of target genes could be calculated according to the amplification standard curve and regression equation of GAPDH automatically by the DNA Engine OPTIONTm². The expression of target genes was normalized to that of GAPDH as the relative expression. Average was obtained from 6 animals in each group.

Contents of TNF-α and IL-1β in L₅-S₂ posterior horn

L₅-S₂ posterior horn was homogenized in 0.5 mL of ice-cold lysis buffer containing 50 mM Tris, 150 mM NaCl, 1% TritonX 100, 0.5% sodium deoxycholate, 1 mM phenylmethanesulfonyl fluoride (PMSF), 0.1% sodium dodecyl sulfate (SDS), 10 mM NaF and 1 mM vitriolu acid sodium. Homogenates were centrifuged at 1800 rpm for 10 min. and the supernatant was collected and stored at -70° C. The contents of IL-1β and TNF-α were detected using the commercially available ELISA kits according to the manufacturer's instructions. Briefly, 50µg) was subjected to reverse transcription using the L of biotinylated antibody was added to 100µg) was subjected to reverse transcription using the L of samples in an anti-rat TNF-α or IL-1β pre-coated plate (Santa Cruz Biotechnology, Inc., Santa Cruz) followed by incubation for 3 h at room temperature. After wa-

Table 1 - Primers and conditions for RT-PCR.

	Forward	Reverse
P ₂ X ₇	5'-GACAAACAAAGT-CACCCGGAT-3'	5'-CGCTCACCAAAGCAAAGCTAAT-3'
IBA-1	5'-TTGATCTGA ATGGCAATGGA-3'	5'-CCTCC AATTAGGGCAACTCA-3'
GAPDH	5'-TTTAACTCTGGTAAAGTGGATATTGTTG-3'	5'-ATTTCCATTGATGACAAGCTTCC-3'

shing three times, a prepared 100µg) was subjected to reverse transcription using the L of strepta vidin horseradish peroxidase solution and 3, 3', 5, 5'-tetramethylbenzidin substrate solution were added followed by incubation. Absorbance was measured at 492 nm in a microplate reader (Salzburger Labortechnik, Salzburg, Austria). A standard curve was delineated to determine the content of TNF-α and IL-1β. The sensitivity of this assay is > 10 pg/mL. Average was obtained from 6 animals in each group.

Intrathecal injection of agonist or antagonist of P₂X₇ and microglial cells

The second part of the experiment involved intrathecal cannulation according to the procedures described by Yaksh and Rudy (17). In brief, rats were intraperitoneally anesthetized with pentobarbital, a cannula (PE-10 tubing) was inserted through the cisterna magna at 6 cm to the L₁ spinal cord via the spinal subarachnoid space. A recovery period of 7 d was allowed, and rats showing no motor impairment following surgery were used for further experiments. The prostatitis was induced as mentioned above. From day 7, intrathecal injection of drug was done for consecutive 5 days. Rats were divided into 5 groups (n = 6 per group) and treated as follows: 1) P₂X₇ receptor agonist: 2'-3'-O-(4-Benzoylbenzoyl)-adenosine 5'-triphosphate (BzATP; 100µmol/L); 2) P₂X₇ receptor antagonist: oxidized ATP (oATP; 100µmol/L); 3) inhibitor of microglial cells: minocycline (50µg); 4) P₂X₇ receptor agonist + inhibitor of microglial cells; 5) in the control group, injection was done with artificial cerebrospinal fluid (ACSF; pH5.5) of equal volume. Six rats were included in each group. Drugs were injected at a

volume of 15µL and flushing was done with 5µL of ACSF. Injections were finished within 5 min. The content of TNF-α and IL-1β in the spinal cord was determined.

Statistical analysis

All data were expressed as means ± standard error (SEM) and statistical analysis was performed with SPSS version 13.0 for Windows. When F-test showed significance, means were compared with the LSD test of post hoc analysis (Dunnett's t test). Analysis of variance (ANOVA) was conducted for comparisons of data among different groups. A value of P < 0.05 was considered statistically significant.

RESULTS

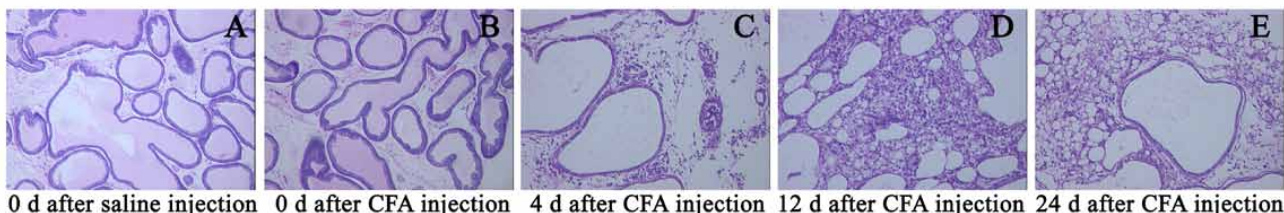
Pathological changes in prostate

Pathological examination showed that CFA treated prostates presented with degeneration, necrosis and exfoliation of mucosal cells in the prostate gland. Infiltration of large amounts of lymphocytes and monocytes was noted in the interstitium, and some lymphocytes aggregated in cluster. In the control group, the mucosal epithelial cells were regularly arranged, and the infiltration of leukocytes was not observed in the interstitium (Figure-1).

Detection of pain sensation

The TWL of CFA treated rats was 14.73 ± 0.93s, 12.15 ± 0.99s and 12.45 ± 1.19 respectively at 4 d, 12 d and 24 d after injection, respectively, which were significantly shorter than that in the

Figure 1 - Pathological changes of the prostate in different groups. In the control group (A) and inflammation 0 day group (B), the mucosal epithelium maintained an orderly organization, without leukocyte infiltration in interstitial tissues. In contrast, in the inflammation 4, 12, 24 days groups (C,D,E), degeneration, necrosis, and exfoliation of mucosal cells in the prostate gland were observed. Interstitial substance was infiltrated with large amounts of lymphocytes and monocytes.



control group ($P < 0.01$). This suggests that hyperalgesia was induced following CFA injection at the prostate and the rat chronic prostatitis model was successfully established (Figure-2).

mRNA expression of P_2X_7 and IBA-1 in posterior horn

The mRNA expression of P_2X_7 and IBA-1 was significantly increased in the posterior horn of L_5-S_2 spinal cord in the experiment group on days 4, 12 and 24 as compared to the control group at the corresponding time points and to the experiment group at baseline ($P < 0.01$). The P_2X_7 expression reached a maximal level on day 12 ($P < 0.05$) (Figures 3A and 3B).

Content of TNF- α and IL-1 β in L_5-S_2 posterior horn

The content of TNF- α and IL-1 β in the posterior horn of L_5-S_2 spinal cord was significantly increased in the experiment group on days 4, 12 and 24, as compared to the control group at corresponding time points ($P < 0.01$) and to the experiment group at baseline ($P < 0.01$). The content of TNF- α and IL-1 β reached a peak on day 12 ($P < 0.05$) (Figures 3C and 3D).

Content of TNF- α and IL-1 β following inhibition of microglial cells and/or P_2X_7

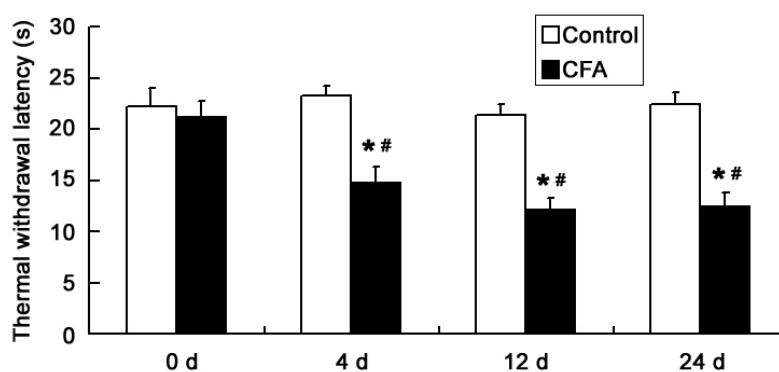
In the experiment group, following injection of minocycline and oATP, the content of TNF- α and IL-1 β was markedly reduced ($P < 0.01$). However, the

P_2X_7 agonist (BzATP) could promote the secretion of TNF- α and IL-1 β ($P < 0.01$ and < 0.05 , respectively), and minocycline inhibited the bioeffects of BzATP ($P < 0.05$). In the control group, intrathecal injection of ASCF had no influence on the contents of TNF- α and IL-1 β in the spinal cord (Figure-4).

DISCUSSION

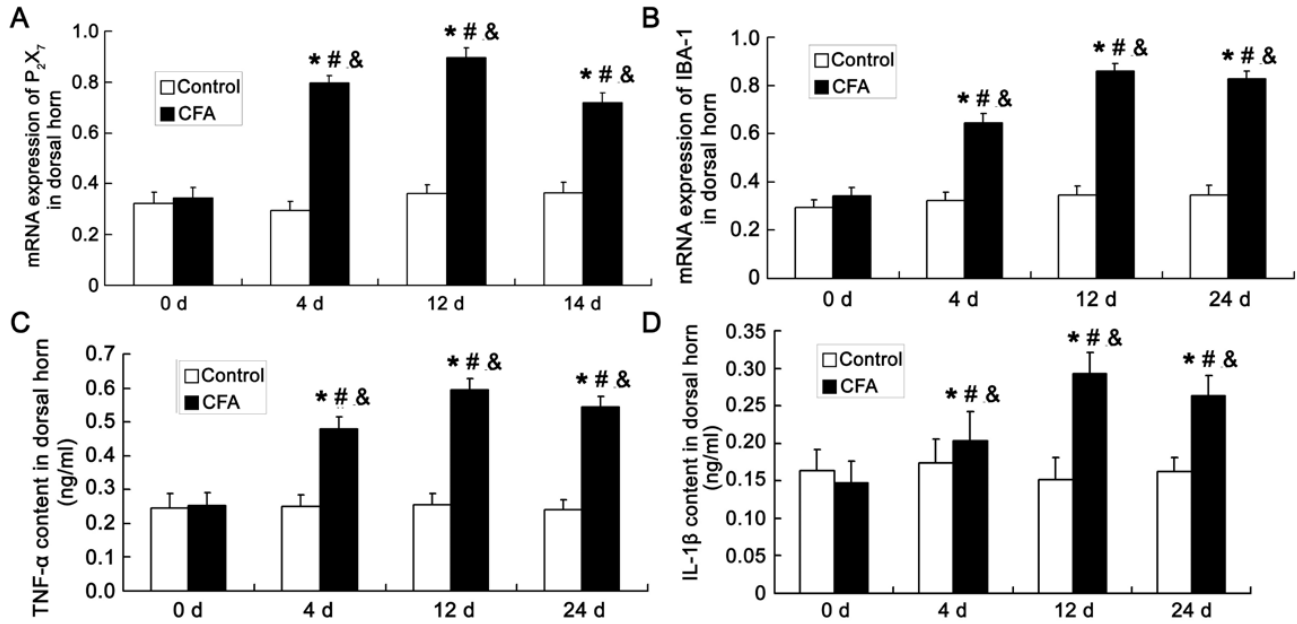
Chronic prostatitis is a common urological disease in young and middle-age men, and patients with chronic prostatitis account for 25–35% of inpatients at the urological clinic. Of all chronic prostatitis of different types, chronic nonbacterial prostatitis (IIIa) or chronic prostatitis/chronic pelvic pain syndrome (IIIb) is the most common and accounts for about 95% of chronic prostatitis (18,19). Generally, chronic prostatitis is characterized by refractory pelvic or perineal pain without evidence of urinary tract infection, which is usually accompanied by bladder and urethra dysfunction. Chronic prostatitis is a major reason for hospital visit. The etiology of chronic prostatitis and the pathogenesis of pain in chronic prostatitis are still poorly understood. In recent years, studies have shown that nanobacteria infection might be a major cause of chronic nonbacterial prostatitis (20). Currently, the antibiotic therapy achieves unfavorable efficacy for patients with chronic prostatitis, and effective strategies have not been developed for these patients to date. The diagnosis

Figure 2 - Thermal withdrawal latency (s) in two groups.



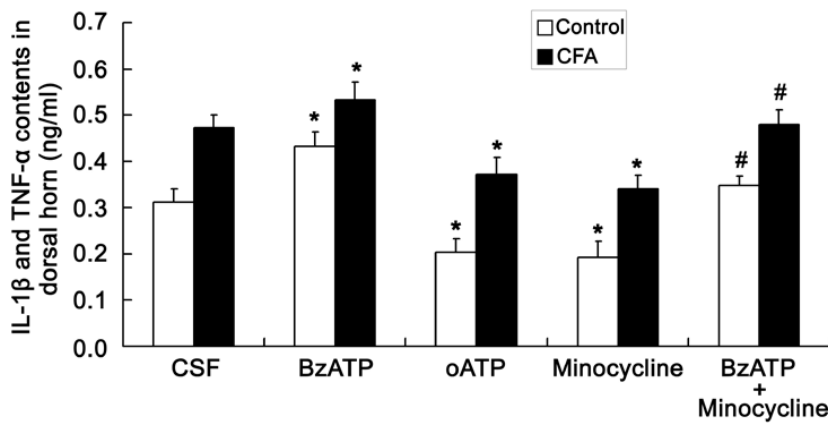
* $P < 0.01$ vs. control group; # $P < 0.01$ vs. 0 d.

Figure 3 - mRNA expression of P₂X₇ (A) and IBA-1 (B) in dorsal horn and contents of TNF- α (C) and IL-1 β (D) (ng/mL) in dorsal horn at different time points in two groups.



*P < 0.01 vs. control group; #P < 0.01 vs. 0 d; &P < 0.05 vs. 4 d and 24 d.

Figure 4 - Contents of IL-1 β and TNF- α in dorsal horn after injection of different agonist or antagonist (ng/mL).



*P < 0.05 vs. CSF; #P < 0.05 vs. BzATP.

and treatment of chronic prostatitis have been a challenge in urology. In addition, the long-lasting pain may result in physical and psychological disorders. Thus, the investigation of the etiology of chronic prostatitis and the pathogenesis of pain in chronic prostatitis are crucial for the accurate diagnosis and development of effective strategies for the treatment of chronic prostatitis.

Pain appears to be a most prominent manifestation of chronic prostatitis. However, the diagnosis and treatment of pain in chronic prostatitis are still challenging because of the complicated pathogenesis of chronic prostatitis pain (21). Patients with chronic prostatitis often experience pains not only at prostate, but at the sites adjacent to or tissues outside the prostate which are found to be also controlled by the L₅-S₂ spinal cord. Moreover, some patients feel pain even after prostatitis disappears. Hence, the pain in the chronic prostatitis is often characteristic of “extra-territorial” and “mirror” image pain. Increasing evidence demonstrates that there are abnormalities in the cell-mediated neurological regulation and the transmitters in the L₅-S₂ spinal cord in chronic prostatitis (22,23).

Accumulating studies have revealed that the pathological pain is due to not only neuronal dysfunction, but the activation of astrocytes and microglia (24), especially in the chronic exaggerated and continuing pain. Microglia and astrocytes are regarded as “immune cells” in the nervous system, and can secrete some pro-inflammatory cytokines such as IL-1 β , TNF- α , NGF, NO, prostaglandin and bradykinin following activation, leading to the exaggeration and persistence of pain by acting on other glial cells and neurons (25,26). Therefore, microglial cells play important roles in the pathogenesis of pathological pain (27,28). In rats with sciatic inflammation, intrathecal injection of minocycline, an inhibitor of microglial cell activation, was found to inhibit the abnormal mechanical pain with low threshold (29).

In the pathogenesis of pathological pain, P₂X₇ plays an important role in the secretion of pro-inflammatory cytokines mediated by microglial cell activation (27). P₂X₇ is an ATP receptor, a transmitter and modulator in the nervous system. P₂X₇ is a special subtype of purinergic

receptor P₂X family. In rats with inflammatory pain, visceral pain and neuropathic pain, focal or intraperitoneal injection of antagonist of P₂X₇ (oATP or A-740003) was found to inhibit the mechanical hyperalgesia, allodynia and hypersensitivity (30,31). In the P₂X₇ receptor deficiency mice, the neuropathic hypersensitivity to mechanical or heat stimulation was absent following nerve injury (11). However, under physiological conditions, P₂X₇ receptor is not activated. Under the pathological conditions, P₂X₇ receptor is activated and involved in the pain transduction. Our findings also revealed that P₂X₇ receptor activation significantly increased the secretion of pro-inflammatory cytokines in animal inflammation model. Following activation, P₂X₇ receptor involves in the pain transduction, which is associated with the calcium related signal transduction (32,33).

In the present study, our results indicated that, in rats with chronic prostatitis pain, the expression of P₂X₇ and IBA-1 was elevated in the posterior horn of L₅-S₂ spinal cord, and the excretion of TNF- α and IL-1 β was also up-regulated. However, after inhibition of P₂X₇ receptor and/or microglial cells, the secretion of TNF- α and IL-1 β was dramatically reduced suggesting that P₂X₇ receptor mediates the microglial cell activation in rat with prostate prostatitis leading to the increased secretion of pro-inflammatory cytokines. It has been well established that TNF- α and IL-1 β are responsive to inflammatory stimuli and cytotoxicity towards neurons, and they can induce chronic inflammation and pain (34). These findings suggest that there is neurogenic inflammation in the L₅-S₂ spinal cord as a result of microglial cell activation via the P₂X₇ receptor in rats with chronic prostatitis pain. These observations demonstrate that P₂X₇ mediated microglial cells activation in the L₅-S₂ spinal cord may take part in the regulation of chronic pain and might lead to the persistence and exaggeration of prostatitis pain. Hence, identification of new neurotransmission pathways and the mechanisms underlying the regulation of chronic prostatitis pain may be helpful to find novel therapeutic targets for chronic prostatitis pain. Currently, oral or intravenous non-steroidal anti-inflammatory drugs have been used to treat spinal cord inflam-

mation. However, the focal drug concentration is at a low level leading to unfavorable efficacy. In Traditional Chinese Medicine, the surface projection of L₅-S₂ spinal cord is also known as Shenshu point and acupuncture of Shenshu point has been used in the treatment of chronic prostatitis pain (35). To date, we have applied “water-needle therapy” for chronic prostatitis pain on the basis of our previous findings, in which the acupuncture of acupoint at L₅-S₂ spinal cord was performed followed by focal injection of B12, B1, hydrocortisone and Chinese herbs. This treatment achieves favorable efficacy, but is still in its infancy stage.

Of note, our findings can not explain the whole molecular mechanisms underlying the activation of microglial cells in chronic prostatitis because there are other receptors (such as P₂X₄, P₂Y₁₂ and Toll like receptor) related to the neuropathic pain (36,37). Studies have shown that the P₂X₇ receptor is related to the P₂X₄ receptor in structure and function, and there is interaction between P₂X₇ and P₂X₄ in the microglial cell mediated pain (38). In addition, the activation of Toll-like receptor 4 in the dorsal horn and the release of IL-1β are dependent on the activation of P₂X₇ receptor, and inhibitors of P₂X₇ receptor (oxidized ATP, A-438079) may suppress the hyperalgesia to heat and mechanical stimulation following intrathecal injection of LPS (a agonist of Toll-like 4 receptor) (39). These findings demonstrate that the P₂X₇ receptor on the microglial cells can interact with the above molecules, which then aggregates the neuropathic pain. However, the specific mechanisms of their interactions require further studies.

CONCLUSIONS

The chronic prostatitis is related to the activation of P₂X₇ and microglial cells and the high expression of TNF-α and IL-1β in the dorsal horn of L₅-S₂ spinal cord. Moreover, TNF-α and IL-1β expression in the L₅-S₂ spinal cord can be inhibited by inhibitors of P₂X₇ receptor and microglial cells. These findings indicate that chronic pelvic pain syndrome may cause secondary inflammation in the L₅-S₂ spinal cord by activating the microglial cells via P₂X₇ receptor, a phenomenon

probably associated with the persistence and intensification of chronic prostatitis pain.

ABBREVIATIONS

ACSF = artificial cerebrospinal fluid
 ANOVA = analysis of variance
 ATP = adenosine triphosphate
 BzATP = 2'-3'-O-(4-Benzoylbenzoyl)-adenosine 5'-triphosphate
 CFA = complete Freund's adjuvant
 CNS = central nervous system
 IBA-1 = ionized calcium binding adaptor molecule 1
 IL-1 = interleukin-1
 NGF = nerve growth factor
 NO = nitric oxide
 oATP = oxidized ATP
 PMSF = phenylmethanesulfonyl fluoride
 SDS = sodium dodecyl sulfate
 SEM = standard error
 SPF = specific pathogen free
 TNF-α = tumor necrosis factor-α
 TWL = thermal withdrawal latency

ACKNOWLEDGMENT

This study was supported by the National Natural Science Foundation (NO.81100537).

CONFLICT OF INTEREST

None declared.

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Correspondence address:

Dr. Zhansong Zhou
Department of Urology
Southwest Hospital, Third Military Medical University
30 Gaotanyanzheng
Chongqing 400038, China
Fax: + 86 23 6546-0268
E-mail: doczhang123@yeah.net