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BLOCKING PAR2 ALLEVIATES BLADDER PAIN AND HYPERACTIVITY VIA TRPA1 SIGNAL

Daihui Chen,
Nian Liu,
Mao Li,
Simin Liang*

*Department of Urology,
The First Affiliated Hospital
of Chongqing Medical University
Chongqing 400012, P.R. China*

Abstract

Bladder disorders associated with interstitial cystitis are frequently characterized by increased contractility and pain. The goals of this study were to examine 1) the effects of blocking proteinase-activated receptor-2 (PAR2) on the exaggerated bladder activity and pain evoked by cystitis and 2) the underlying mechanisms responsible for the role of PAR2 in regulating cystic sensory activity. The protein expression of PAR2 was amplified in rats with cystitis by inducing it with systemic administration of cyclophosphamide (CYP) as compared with control rats. Blocking PAR2 by intrathecal infusion of PAR2 antagonist FSLLRY-NH2 attenuated bladder hyperactivity and pain. In addition, blocking PAR2 attenuated the transient receptor potential A1 (TRPA1) signal pathway, whereas inhibition of the TRPA1 decreased bladder hyperactivity and pain. The data revealed specific signaling pathways leading to CYP-induced bladder hyperactivity and pain, including the activation of PAR2 and TRPA1. Inhibition of these pathways alleviates cystic pain. Targeting one or more of these signaling molecules may present new opportunities for treatment and management of overactive bladder and pain often observed in cystitis.

Keywords

• Bladder activity • Cystic Pain • Cystitis • Proteinase-activated receptor-2 (PAR2) • Transient receptor potential A1 (TRPA1)

Introduction

Interstitial cystitis, also known as bladder pain syndrome (IC/BPS) is a chronic pathological condition of the bladder characterized by symptoms such as pelvic pain, urgency or frequency in urination [1]. IC/BPS impacts normal physical and mental health and presents a remarkable negative effect on the quality of life of patients [1]. Patients with IC/BPS constantly feel pain at normal bladder pressures, suggesting amplified excitability of their micturition reflex pathway [2]. This is likely due to an impairment of the sensory inputs that originated from the bladder to the spinal cord and central nervous system. Nonetheless, treatment options for cystic pain have been limited, partly due to our poor understanding of the underlying mechanisms responsible for pain.

Proteinase-activated receptors (PARs) are a family member of G-protein-coupled receptors and are activated by a proteolytic mechanism [3]. Among the four members of PARs, PAR2 is largely distributed in various tissues, including skin, gastrointestinal, cardiovascular, and respiratory systems. Of note, about 60% of sensory dorsal root ganglion (DRG) neurons at the spinal L4-L6 levels contain PAR2 [4, 5]. Stimulation of PAR2 by peripheral or central administration of non-inflammatory doses of PAR2 agonists evokes mechanical and thermal hyperalgesia in rats [6]. The studies further suggest that the release of substance P and calcitonin gene-related peptide (CGRP) contribute to acute and chronic pain by activation of PAR2 [6]. In experimental animal models, the expression of PAR2 is upregulated in the dorsal horn of the spinal cord after induction of pain and blocking spinal PAR2 eliminates mechanical and thermal hyperalgesia [7]. Nevertheless, it has not been reported that PAR2 pathways specifically contribute to cystitis-induced hyperalgesia. The underlying mechanisms responsible for the role of PAR2 in regulating cystitis-evoked neuropathic pain should also be studied.

The superficial dorsal horn is the first synaptic site from peripheral afferent nerves to the central nervous system [8, 9] and plays an important role in modulating pain [10, 11]. Specifically, the dorsal horn at the lumbar levels (i.e., L5 to L6) is the first synaptic site receiving (sharing) pain inputs from both visceral organs (i.e., bladder) and the hind paw. Thus, in this study we determined the role played by PAR2 at this level of lumbar superficial dorsal horn in regulating bladder hypersensitivity and mechanical hyperalgesia in rats following cystitis induced by systemic administration of cyclophosphamide (CYP). In a cystitis model of CYP, the rats' bladders appear to be hyperactive with an elevated voiding pressure, which leads to mechanical pain [12, 13]. In general, mechanical paw withdrawal threshold (PWT) of a rat's hind paw in response to the stimulation of von Frey filaments was employed to assess mechanical pain under pathophysiological conditions [14].

It has been reported that transient receptor potential A1 (TRPA1) is expressed in sensory nerves and as a main receptor engaged in

pain responses [15, 16]. A recent study has demonstrated that PAR2 appears in the superficial dorsal horn and is involved with neuropathic pain [6]. Prior findings suggest that PAR2 signaling plays a critical role in regulating TRPA1 and thereby leads to mechanical allodynia and thermal hyperalgesia [12].

Therefore, in the present study, we suspected that PAR2 in the superficial dorsal horn of the lumbar spinal cord is likely changed in CYP-rats. We also suspected that PAR2 is likely an important player in the induction and maintenance of cystic pain. We hypothesized that CYP upregulates the protein expression of PAR2 signaling pathways in the superficial dorsal horn, resulting in bladder hyperactivity and pain. Moreover, blocking spinal PAR2 by intrathecal injection of PAR2 antagonist FSLLRY-NH2 should attenuate the amplified bladder activity and pain response evoked by CYP. We also hypothesized that CYP upregulates the expression of TRPA1, a downstream signal of PAR2. We speculated that blocking spinal PAR2 would attenuate the TRPA1 pathway thereby leading to a reduction in bladder hyperactivity and pain.

Materials and methods

Animals

Adult female Wistar rats (250-300 g) were housed in standard care facilities with water and food *ad libitum* on a 12 hour light-dark cycle. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Chongqing Medical University and were carried out in accordance with the guidelines of the International Association for the Study of Pain.

Induction of cystitis

Rats were intraperitoneally (i.p.) injected with CYP (75 mg/kg, in the concentration of 75 mM, Sigma-Aldrich, St. Louis, MO, USA) every 3 days to induce chronic cystitis [12, 13]. On the eighth day experiments were performed. Control rats received saline injections.

Bladder catheterization

Rats were treated perioperatively for 96 hours with trimethoprim-sulfamethoxazole (150 mg/250 mL) in their drinking water. On the day of catheter implantation, rats were anesthetized by sodium pentobarbital (60 mg/kg, i.p.), and 15 μg of buprenorphine in 1 mL of normal saline was given subcutaneously for postoperative analgesia. The dorsal neck and lower abdomen were clipped, prepped with 1% povidone iodine, and sterilely draped. A 4 cm incision was made in the lower abdomen to expose the bladder, and a small incision was made in the dome. A 25 cm polyethylene-50 catheter with a cuff was inserted into the bladder and secured with a 6-0 silk purse-string suture. The catheter was tunneled subcutaneously to the dorsal neck, anchored, and the free end was stoppered with a blunted 22 gauge needle. The abdominal wound was closed with 4-0 silk suture and the skin was closed with three 4-0 silk interrupted sutures. The catheter was covered with a dressing and secured to the rat's back.

Intrathecal catheter for administration of drugs

After completion of the bladder catheter implantation, one end of polyethylene-10 tubing was inserted intrathecally through an incision in the cisternal membrane and advanced 7-9 cm caudally until the tip of the catheter was positioned at the lumbar spinal level (L5 to L6). The other end of the intrathecal tubing was sutured to the musculature and skin at the incision site and externalized to the back of the rat. Animals were allowed to recover and then returned to the animal care facility where they were housed individually. Five days were allowed before the experiments were performed.

Mechanical sensitivity and cystometry

In this study, all experiments were performed in a double-blinded manner. On the day of experimentation, individual rats were placed in a Plexiglas chamber (25 × 8 × 9 cm) with a wire mesh floor. The bladder catheter was connected to a three-way stopcock connected to an infusion pump and pressure transducer. Rats were allowed to acclimate to the chamber for 30-40 min. First, saline was infused into the bladder at 10 mL/h for 1 h and mechanical sensitivity was examined and an urodynamic measurement was performed to collect baseline data.

To quantify the mechanical sensitivity of the hindpaw, rats were placed in individual plastic boxes and allowed to acclimate for > 30 min. Mechanical PWT of rat's hind paw in response to the stimulation of von Frey filaments was determined. A series of calibrated von Frey filaments (ranging from 0.5 to 18.0 g) were applied perpendicularly to the plantar surface of the hind paw with a sufficient force to bend the filaments for 60 s or until paw withdrawal. In the presence of a response, the filament of the next lower force was applied. In the absence of a response, the filament of the next greater force was applied. To avoid injury during tests, the cutoff strength of the von Frey filament was 18 g. The tactile stimulus producing a 50% likelihood of withdrawal was determined using the "up-down" method [14]. Each trial was repeated 2 times at approximately 2 min intervals. The mean value was used as the force producing a withdrawal response.

Following collection of baseline data, a Hamilton microsyringe (250 μL) was connected to the intrathecal tubing to deliver 100 μl of dimethyl sulfoxide (DMSO) as vehicle control, FSLLRY-NH2 (PAR2 antagonist, 10 μg), and HC030031 (TRPA1 antagonist, 10 μg) [17] (obtained from Sigma-Aldrich, St. Louis, MO, USA). The volume of infused FSLLRY-NH2 and HC030031 was 100 μl. An infusion pump was used to deliver those vehicles and drugs constantly over a period of 30 min. Note that a prior study using the same approach was used to intrathecally infuse respective FSLLRY-NH2 and HC030031 and it was shown that 10 μg of FSLLRY-NH2 and HC030031 were effective to attenuate mechanical hyperalgesia [6].

Ten min after intrathecal infusion, mechanical sensitivity of the hindpaws was again determined. Post-treatment cystometry was also performed after completion of mechanical sensitivity examination. Thus, six groups were included in this study as: saline rats + vehicle infusion (n = 9); CYP-rats + vehicle infusion (n = 12); saline rats + FSLLRY-NH2 (n = 8); CYP-rats + FSLLRY-NH2 (n = 15); saline rats + HC030031 (n = 8); and CYP-rats + HC030031 (n = 15).

In a subset of experiments, in order to examine the effects of blocking PAR2 on the levels of TRPA1 (n = 8), FSLLRY-NH2 (10 μg) was intrathecally given using an infusion pump in CYP-rats. The pump was set to constantly deliver vehicle or the drugs over a period of 30 min. At the end of infusion, rats were euthanized by sodium pentobarbital (120 mg/kg, i.p.) and the superficial dorsal horn tissues (L4-L6) were obtained under an anatomical microscope for western blot.

Western blot analysis

Total protein of the superficial dorsal horn tissues was extracted by homogenizing dorsal horn samples in ice-cold immunoprecipitation assay buffer. The lysates were centrifuged and the supernatants were collected for measurements of protein concentrations. After being denatured by heating at 95°C for 5 min in buffer, the supernatant samples containing 20 μg of protein were loaded onto 4-20% Mini-PROTEAN (Bio-Rad Laboratories, Hercules, CA, USA) Tris Glycine eXtended (TGX) gels and electrically transferred to a polyvinylidene fluoride membrane. The membrane was blocked in 5% nonfat milk in 0.1% Tween-TBS buffer and was incubated overnight with respective primary antibody. The primary antibodies included: rabbit anti-PAR2 antibody (1:200, Abcam Co.); rabbit anti-TRPA1 antibody (1:250, Abcam, Cambridge, UK). Next, the membranes were washed and incubated with an alkaline phosphatase conjugated anti-rabbit secondary antibody (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoreactive proteins were detected by enhanced chemiluminescence. The bands recognized by the primary antibody were visualized by exposure of the membrane onto an X-ray film. The membrane was stripped and incubated with mouse anti-β-actin to show equal loading of the protein. Then, the film was scanned and the optical density of all protein bands was first analyzed using the Scion Image public domain software, and values for densities of immunoreactive bands/β-actin band densities from the same lane were determined. Each of the values was then normalized to a control sample.

Statistical analysis

Data for mechanical threshold, voiding pressure and intercontraction interval were analyzed using a two-way repeated-measure ANOVA since multiple interventions were compared among groups. Data for western blot analysis were analyzed by using a two sample t-test. Values were presented as means ± standard deviation. For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed by using SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Expression of PAR2 in spinal cord of control rats and CYP-rats

Figure 1A shows the protein expression of PAR2 in saline control rats (n = 6) and CYP-rats (n = 8). CYP significantly increased the protein levels of PAR2 in the superficial dorsal horn as compared with control rats ($P < 0.05$, CYP-rats vs. control rats).

Effects of PAR2 on mechanical hypersensitivity and bladder activity

After all the data of mechanical sensitivity and cystometry was obtained, control vehicle and FSLLRY-NH2 was intrathecally infused. Figure 1B shows that CYP decreased PWT in rats with vehicle infusion ($P < 0.05$ vs. saline control; n = 12) compared with saline control rats (n = 9). As FSLLRY-NH2 (n = 15) was infused, a decrease in PWT evoked by CYP was blunted ($P < 0.05$ vs. CYP-rats with vehicle infusion). Note that there was no changes of PWT observed in saline control rats after infusion FSLLRY-NH2 (n = 8), suggesting that blocking PAR2 had no significant effects on mechanical sensitivity in rats under normal bladder conditions.

Figure 1C and 1D further demonstrates that CYP increased maximum voiding pressure and
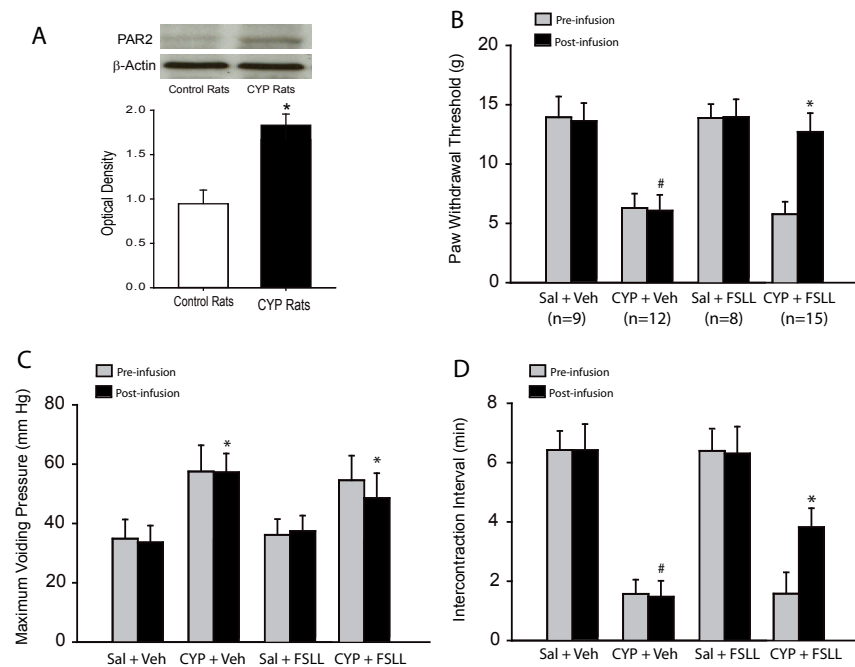
Figure 1. Role of PAR2 in regulating CYP-evoked bladder mechanical hypersensitivity and pain. A. The protein expression levels of PAR2 were increased in in the dorsal horn of the spinal cord of CYP rats (n = 8). Top panel: typical bands and bottom panel: averaged data. *$P < 0.05$ vs. control rats (n = 6). B. CYP decreased PWT and intrathecal administration of FSLL blunted the effects of CYP. *$P < 0.05$ vs. pre-infusion; and #$P < 0.05$ vs. post-infusion of all other groups. Number of animals is shown in the figure and they are the same in C and D. C and D. CYP amplified maximum voiding pressure and decreased intercontraction interval. FSLL significantly recovered the shortened intercontraction interval induced by CYP without significantly altering maximum voiding pressure. In C, *$P < 0.05$ vs. respective saline control rats with post-infusion. In D, *$P < 0.05$ vs. pre-infusion; and #$P < 0.05$ vs. post-infusion of all other groups. Sal, saline; Veh, vehicle; CYP, cyclophosphamide; FSLL: FSLLRY-NH2.

decreased intercontraction interval. As FSLLRY-NH2 was intrathecally infused, the shortened intercontraction interval evoked by CYP was recovered without significantly altering maximum voiding pressure.

TRPA1 signal pathway engaged in effects of PAR2

Figure 2A shows the protein expression of TRPA1 in saline control rats and CYP-rats (n = 6 in each group). CYP significantly increased the protein levels of TRPA1 in the superficial dorsal horn as compared with control rats (P < 0.05 vs. control rats). Furthermore, Figure 2A demonstrates that the amplified expression of TRPA1 evoked by CYP was significantly attenuated (n = 8) when FSLLRY-NH2 was infused into the spinal cord of CYP-rat.

We also examined the effects of blocking TRPA1 on mechanical hypersensitivity and bladder activity. Figure 2B demonstrates that CYP decreased PWT in rats with vehicle infusion (P < 0.05 vs. saline control; n = 12) compared with saline control rats (n = 9). As HC030031 (n = 15) was infused, a decrease in PWT evoked by CYP was blunted (P < 0.05 vs. CYP-rats with vehicle infusion). Likewise, no significant changes of PWT were observed in saline control rats after infusion of HC030031 (n = 8). Figure 2C and 2D also demonstrates that CYP increased maximum voiding pressure and decreased intercontraction interval. Infusion of HC030031 largely recovered the shortened intercontraction interval evoked by CYP, but did not significantly alter maximum voiding pressure.

Discussion

In the current study, we consistently observed the development of mechanical hyperalgesia and hypersensitivity in CYP-rats. We further demonstrated that the expression of PAR2 and TRPA1 pathways were upregulated in CYP-rats as compared with control rats. Blocking PAR2 and TRPA1 by intrathecal infusion of their respective antagonists FSLLRY-NH2 and HC030031 attenuated bladder mechanical hyperalgesia and restored the shortened intercontraction interval evoked by CYP. Moreover, blocking the PAR2 signal attenuated the expression of TRPA1.

In the current study, we identified a greater level of PAR2 expression in the superficial dorsal horn of the spinal cords of rats with mechanical hyperalgesia and hypersensitivity following administration of CYP. This is consistent with findings in a prior study showing upregulation of PAR2 in the superficial dorsal horn of a rat model with neuropathic pain [6]. Transient receptor potential receptors (such as TRPA1 and TRPV1) have been reported to regulate pain responses at spinal cord level [15, 16]. Additional results suggest that PAR2 signaling plays a critical role in regulating TRPA1 and TRPV1, thereby leading to mechanical allodynia and thermal hyperalgesia [6, 7]. Moreover, PLC, PKA and PKC intracellular signaling pathways are involved in the role of PAR2 [6, 7]. In our current study, it was observed that CYP amplifies expression of PAR2 in the superficial dorsal horn and speculatively this is likely to activate PLC, PKA and PKC signaling pathways,

which then lead to increases of TRPA1 and/or TRPV1 and regulate mechanical hyperalgesia in this rat model of cystitis.

TRPA1 also has a functional role in pain and neurogenic inflammation resulting from channel activation to a variety of compounds including pungent agents, irritant chemicals, reactive oxygen and nitrogen species, and products of oxidative stress-induced lipid peroxidation [18-21]. TRPA1 has been shown to co-localize with TRPV1 in subpopulations of sensory neurons [20] and is engaged in development of bradykinin-induced mechanical hypersensitivity [22, 23] and painfully cold temperatures [24]. Our evidence from the current study supports the notion that TRPA1 mediates CYP-induced mechanical hypersensitivity. Results further suggest that PAR2 plays an important role in regulating TRPA1 functions in CYP-evoked pain because blocking PAR2 significantly attenuates the
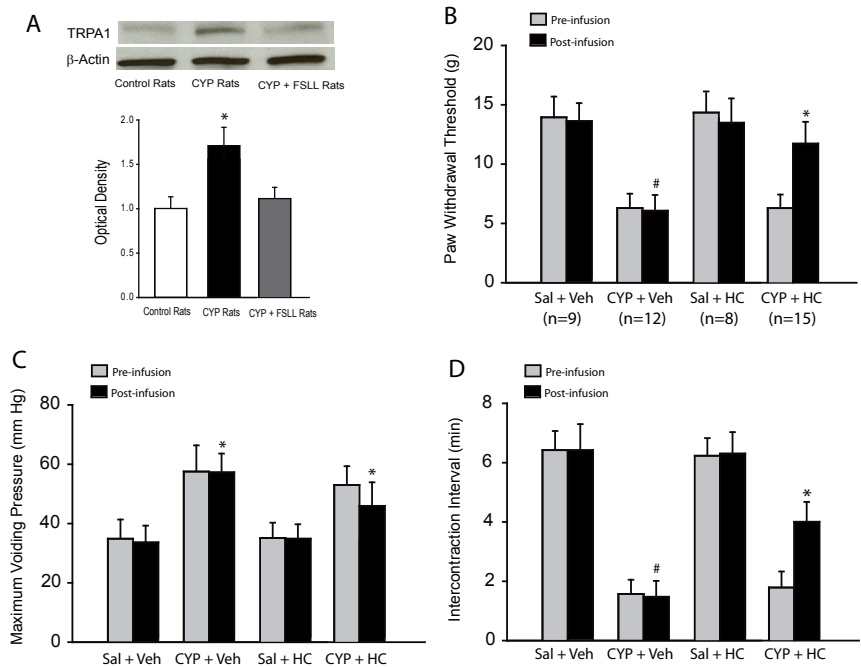
Figure 2. Engagement of TRPA1 in PAR2 regulating bladder mechanical hypersensitivity and pain. A. The expression of TRPA1 was amplified in the dorsal horn of CYP-rats with vehicle infusion (n = 6) as compared with control rats (n = 6). Blocking PAR2 by FSLL attenuated increases in TRPA1 in CYP-rats. Top and bottom panels: typical bands and averaged data. *P < 0.05 vs. control animals and CYP-animals infused with FSLL (n = 8). B. CYP decreased PWT and intrathecal infusion of HC blunted the effects of CYP. *P < 0.05 vs. pre-infusion; and #P < 0.05 vs. post-infusion of all other groups. Number of animals is shown in the figure and they are the same in C and D. C and D. CYP amplified maximum voiding pressure and decreased intercontraction interval. HC recovered the shortened intercontraction interval induced by CYP without significantly altering maximum voiding pressure. In C, *P < 0.05 vs. respective saline control rats with post-infusion. In D, *P < 0.05 vs. pre-infusion; and #P < 0.05 vs. post-infusion of all other groups. Sal, saline; Veh, vehicle; CYP, cyclophosphamide; HC, HC030031.

protein expression of TRPA1 in the dorsal horn engaged in CYP-evoked mechanical hyperalgesia. This study also demonstrated that blocking PAR2 and TRPA1 restored the shortened intercontraction interval observed in CYP-rats. This suggests that the PAR2-TRPA1 signaling pathway is necessary to play a regulatory role in mediating CYP-evoked bladder hyperactivity responses.

It is well known that stimulation of nociceptive receptors in the sensory nerves leads to the releases of substance P and CGRP from the nerve terminal of dorsal root ganglions into the superficial dorsal horn [25]. Numerous receptors present on the presynaptic site of the nerve terminals contribute to the release of substance P and CGRP, thus regulating inflammatory pain [11]. Specifically, our recent study suggests that substance P and CGRP in the dorsal horn are engaged in CYP-induced bladder mechanical hyperalgesia [26].

Inhibition of the PAR2 signal pathway can affect the levels of substance P and CGRP in the dorsal horn and alter the process of pain responses. Thus, it is speculated that blocking PAR2 can decrease the levels of those two neuropeptides in the dorsal horn of the spinal cord of CYP-rats.

Moreover, it needs to be acknowledged that we studied female rats in the present study because the prevalence of IC/PBS among women is estimated to be 2 to 5 times higher than in men [1]. Also, it should be noted that the levels of estrogen generally affect pain sensitivity. However, the estrus cycle was not examined during behavioral experiments in the present study. This could be considered as a limitation of this study.

In CYP-rats, spinal PAR2 and TRPA1 are upregulated, which results in mechanical pain and bladder hyperactivity. Intrathecal administration of PAR2 antagonist FSLLRY-NH2 has a significant analgesic effect in a rat model of bladder pain with cystitis. Moreover, blocking PAR2 blunts the amplified expression of the TRPA1 pathway and thereby alleviates CYP-evoked bladder pain and exaggerated activity. For the first time, our data reveals specific signaling pathways leading to bladder pain and hyperactivity with cystitis, including the activation of PAR2 and downstream TRPA1 pathways. Results of our study provide a base for the mechanisms responsible for CYP-induced cystic pain. Targeting one or more of these signaling molecules involved in activation of PAR2-TRPA1 at the spinal levels may present new opportunities for treatment and management of pain often observed in patients with cystitis.

Acknowledgment

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