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Original Article

Involvement of P2X7 receptors and downstream ERK pathways in chronic inflammatory pain

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Abstract: Analyzing the effects and mechanisms of P2X7 receptor signaling in Complete Freund's Adjuvant (CFA) induced chronic inflammatory pain in mice, the current study used a variety of methods, including behavioral evaluation, Western blotting, Rt-PCR, immunohistochemistry, and pharmacology. Results showed that P2X7 receptors, mainly located at the spinal dorsal horn, were upregulated after the hind paws of mice were injected with CFA. Intrathecal administration of P2X7 receptor antagonist A438079 (A43) alleviated pain behavior and reduced expression of phosphorylated ERK (p-ERK), Iba1, and tumor necrosis factor α (TNF-α) protein following CFA injections. Effects of P2X7 receptor agonist ATP were the opposite. After injections with CFA, pain was statistically significant at 3 hours. On the third day, pain levels were the most severe. The trend of the corresponding substance expression was consistent with the trend of pain. Present results indicate that P2X7 receptors in the spinal dorsal horn may play a role in the onset and development of chronic pain induced by CFA by mediating activation of ERK signaling pathways.

Keywords: P2X7 receptor (P2X7R), chronic pain, microglia, ERK

Introduction

P2X receptors are a group of non-selective ATP-gated cation channels that exist on the cell membrane [1]. When P2X receptors are activated, cells are allowed to pass on monovalent or divalent cations, such as Na^+, K^+, and Ca^{2+} polarization, causing a series of physiological or pathological reactions [2]. At present, seven P2X subunits (P2X1-P2X7) have been reported, including P2X7 receptor, an oligomeric protein consisting of multiple subunits. It is widely distributed in breathing, digestion, cardiovascular, and nervous systems [3]. P2X7 receptor is a unique member, mainly mediated by Ca^{2+} permeability. It can be activated by ATP and BzATP. Continuous activation can form a large membrane pore channel, allowing macromolecular ion organic compounds to pass freely, eventually leading to cell lytic death [4]. When tissues are injured, P2X7 receptor unique natural agonist adenosine triphosphate (ATP) is released as mediator of pain. Accumulating evidence has indicated that P2X7 receptors are involved in the regulation of immune function and inflammatory response [5-7]. P2X7 receptors have been found in the sensory nervous system, both in the central and in the periphery, showing a variety of biological effects in various organizations [8-10].

Many reports have suggested that activated-P2X7R promotes activation of microglia and causes the release of inflammatory factors, modulating inflammatory response and pain transmission [11]. In addition, some evidence has indicated that P2X7 receptors mediate pain through activating microglia and its downstream signaling pathways [2], while P2X7 gene knockout mice have been shown to reduce inflammation and neuropathic pain without changing the nociception of normal stimulation [12]. Some effective and selective P2X7R antagonists have inhibitory effects on pain, suggesting that P2X7 receptors play a significant role in the onset and development of

chronic pain [13, 14]. The spinal cord dorsal horn is an important part of the primary sensory nervous system. Its activity is controlled by the brain. In one study of ERK pathways, it was found that ATP, glutamate, calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY), and other substances in glial cells can activate ERK through their respective receptors or transporters in spinal dorsal horns. Phosphorylated ERK is involved in the regulation of downstream genes, such as COX-2, IL-1β, TNF-α, and IL-6 [15-18]. Although many reports have indicated that P2X7R may mediate pain transmission via activation of microglia, the exact mechanisms of P2X7 receptor involvement in inflammatory pain induced by CFA remain unclear. The current study used a variety of methods, including behavioral sciences, molecular biology, immunohistochemistry, and pharmacological analysis, to analyze the effects and mechanisms of pain signals transmitted and transformed by P2X7 receptors in chronic inflammatory mice model.

Materials and methods

All experimental protocols were approved by the Committee for the Ethical Use of Laboratory Animals, Xuzhou Medical University, Jiangsu, China. They were conducted in accordance with guidelines of the International Association for the Study of Pain. Two hundred and sixteen male Kunming mice (25 ± 5 g), from the Experimental Animal Center, Xuzhou Medical University, were housed at a constant ambient temperature of 24 ± 1°C under a 12-hour light/dark cycle. They had ad libitum access to food and water.

CFA-induced chronic inflammation pain model

Male mice were employed for this study. They were tested for changes in thermal hyperalgesia using heat radiation equipment. They received intraplantar administration of 30 ul of 1 mg/mL CFA (Sigma-Aldrich, USA) or saline after the establishment of paw withdraw latency baseline levels. In saline-operated mice, the same procedure was performed, except CFA was replaced with equivalent amounts of physiological saline as controls. Successful models were determined by thermal radiation.

Intrathecal injections

Under brief sevoflurane anesthesia (2% in oxygen), mice lumbar spines were fixed with the left hand. ATP (10 μM, 4 μl, Sigma, USA) and A43 (10 μM, 4 μl, Sigma, USA) or saline was injected slowly into the subarachnoid space via a 10 μl microinjector. A quick flick was believed to be successful administration. ATP, A43, and saline were injected at an appropriate rate 30 minutes before CFA injections. Saline is used as a solvent for all drugs needing dissolution or dilution.

Thermal and mechanical sensitivity testing

Paw withdrawal thermal latency (PWL) levels were measured using heat radiation equipment (IITC Life Science). The mice were adapted for a time period before behavioral testing. They were measured at -1 day, 3 hours, 1 day, 3 days, 7 days, and 14 days, respectively. On the day of the test, the mice were transferred to the glass surface of the thermal apparatus in the noiseless room. Room temperature was controlled at 23-26°C. After the mice were quiet, paw withdrawal thermal latency was tested. The thermal radiant source was concentrated in the middle of the hind paws. PWL is from the beginning of the irradiation to the mouse lift leg. The automatic cut-off time was set to 25 seconds to prevent tissue damage. The intensity of thermal stimulation was consistent throughout the course of the experiment and each animal was measured 5 times.

Paw withdrawal mechanical threshold (PWT) levels were tested using von Frey hairs (North Coast Medical). They were measured at the same times as above. The mice were adapted in plastic boxes (7 cm by 9 cm by 9 cm) on the barbed wire of the mechanical stimulation device, respectively. After the mice became quiet, von Frey hairs were used to evaluate PWT levels. Von Frey hairs contain different values of force (0.1, 0.16, 0.4, 0.6, 1, 1.4, 2, and 4 g). Each hair was applied to the left hind paw for 5 seconds. Occurrence of paw withdraw was considered a mechanical hyperalgesia response.

Western blotting

The mice were sacrificed immediately after behavioral testing. This was followed by isolation of lumbar enlargement dorsal spinal cords, which were frozen in a refrigerator at -80°C for use. Lumbar enlargement dorsal spinal cords were sonicated in modified RIPA buffer. After centrifuging the lysates, protein concentrations

in the supernatant were confirmed. Protein samples with equal amounts were separated by electrophoresis on a 10% SDS-polyacrylamide gel and electro-transferred to polyvinylidene fluoride membranes. This was followed by blockage with 5% skim milk for 3 hours and incubation overnight at 4°C with rabbit anti-P2X7R (1:500; Alomone) and p-ERK, ERK (1:500; Cell Signaling Technology). Next, the blots were extensively rinsed with washing buffer and incubated with the anti-rabbit IgG antibody (1:1000, Sigma) for 2 hours at room temperature. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control (1:4000, Bioworld). Semi-quantitative analysis of the grayscale images of the Western-blotted P2X7R was conducted using ImageJ software (NIH, USA).

Real time PCR

Spinal dorsal horn tissues were taken out of the -80°C refrigerator and added with 1mL of TRIzol Reagent (Invitrogen) to extract total RNA. Complementary DNA (cDNA) was synthesized with primers (Invitrogen), reverse transcriptase M-MLV, and dNTP Mixture. The gene was extended with SYBR® Premix Ex Taq™ Real-Time PCR kit (TaKaRa) and expression of the gene was examined with the LightCycler®480 System. The reference was Actb, which was used to normalize the data. P2X7 mRNA forward primers were 5'-TTGCTTTGGTGAGCGATAAG-3' and reverse primers were 5'-CATTCTCCGTCACCTCTGCT-3'.

Immunohistochemistry

Under anesthesia with 3% pentobarbital (10 mg/kg, i.p.), the mice were perfused intracardially with 20 mL of saline, followed by 20 mL of ice-cold 4% paraformaldehyde in PBS. The spinal cord was then removed, kept in the same fixative for 4-6 hours, and transferred to 30% sucrose for equilibration. After dehydration, 30 μm transverse series sections were cut on a cryostat (Leica CM1900, Germany) and collected in 3% hydrogen peroxide. After washing with 3% H_2O_2 to PBS, the sections were incubated for 24 hours at 4°C in the rabbit anti-Iba1 antibody (1:100; Abcam). Afterward, the sections were extensively rinsed with PBS and subsequently incubated with donkey anti-rabbit secondary antibody containing A (1:300) and B (1:200) for 30 minutes, respectively, at 37°C. Sections were then visualized with DAB chromogen intensified with nickel for 5 minutes. Images were captured using a microscope (Leica, Germany). Image-Pro Plus (version6.0, Media Cybernetics) was used for image cropping and adjustment.

TNF-α ELISA

Quantifying TNF-α content in lumbar enlargement, ELISA testing was applied. Steps of sample homogenate and protein extraction were same as those with Western blotting. Specific methods of operation were according to manufacturer instructions. Following the biochip sample, incubation, washing, and coloration, absorbance (OD values) of each well was measured at a wavelength of 450 nm, in order, to zero with a blank well. Assays were carried out within 15 minutes after the addition of the stop solution.

Statistical analysis

Graphics and calculations were carried out using Graphpad Prism 5.0 software. Quantitative data are expressed as mean ± SD. Multiple-group comparisons were made using one-way ANOVA. Comparisons between the two groups used Student's t-tests. Behavioral data during the development of inflammatory pain was tested using repeated measures ANOVA tests with post hoc LSD-t pairwise comparisons. The test level was α equal to 0.05. $P < 0.05$ indicates statistical significance in all tests.

Results

Upregulation of P2X7 receptors in the spinal dorsal horn after peripheral inflammatory injury

To determine how thermal allodynia and mechanical hyperalgesia develop in mice undergoing CFA injections, Thermal Plantar testing and von Frey testing were employed, measuring PWL and PWT levels. As shown in **Figure 1A**, **1B**, mice in the control group showed no significant changes in PWL and PWT before and after injections with saline. However, the mice injected with CFA alone showed a significant decrease in PWL and PWT. Hyperalgesia peaked on the third day and lasted until day 14 ($p < 0.001$). As shown in **Figure 1C**, **1D**, Western

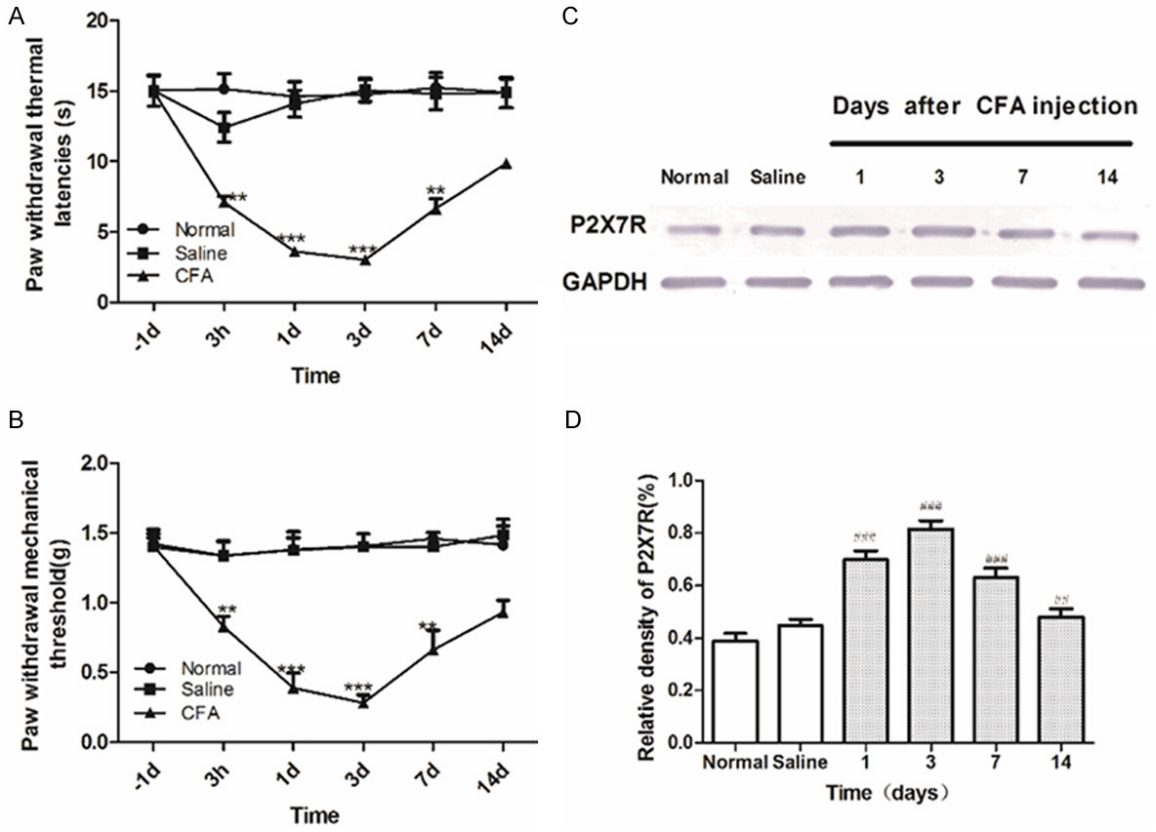

Figure 1. Assessment of behavioral tests (n = 6) and variations of P2X7R protein (69KD) expression (n = 3) in CFA administration mice. (A) Time course of PWLs; (B) Time course of PWTs; (C) Western blotting of P2X7R protein using the total protein from the L4-6 spinal cord; (D) Bar graph showing the relative intensity in the level of P2X7R expression in L4-6 spinal cord, compared with the mean expression level of P2X7R in saline mice (*P < 0.05, **P < 0.01, ***P < 0.001 vs. normal group).

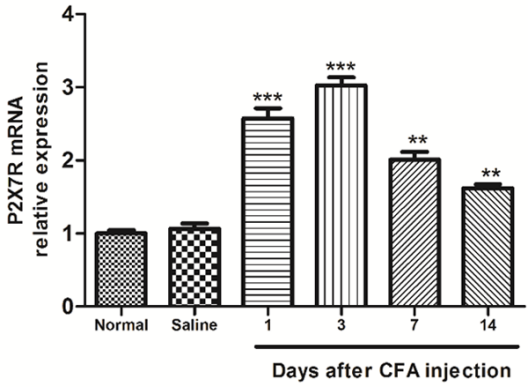

Figure 2. Assessment of expression of P2X7R mRNA (n = 3) in CFA administration mice. Alteration of P2X7 receptor mRNA relative expression in the spinal dorsal horn in CFA mice. (n = 3, *p < 0.05, **p < 0.01, ***p < 0.001, vs. normal group).

blot analysis revealed that levels of P2X7 receptor expression of the spinal dorsal horn were significantly upregulated after CFA injections (p < 0.001). Levels were positively relevant to chronic inflammatory pain behavioral signs. Results suggest that P2X7 receptors exhibit upregulated expression in the spinal dorsal horn after the paws of mice were formalin injected.

Upregulation of P2X7 mRNA in the spinal cord after peripheral inflammatory injury

To further confirm the relationship P2X7 receptor expression with CFA-induced inflammatory pain, quantitative real-time PCR was performed, examining levels of P2X7 gene expression in the spinal dorsal horn of CFA mice. Data analysis revealed that expression of P2X7 in the spinal cord dorsal horn was consistent with the changes in P2X7 receptor protein, markedly upregulated from day 1 to day 14 and peaking on day 3 (**Figure 2**; P < 0.001). Current

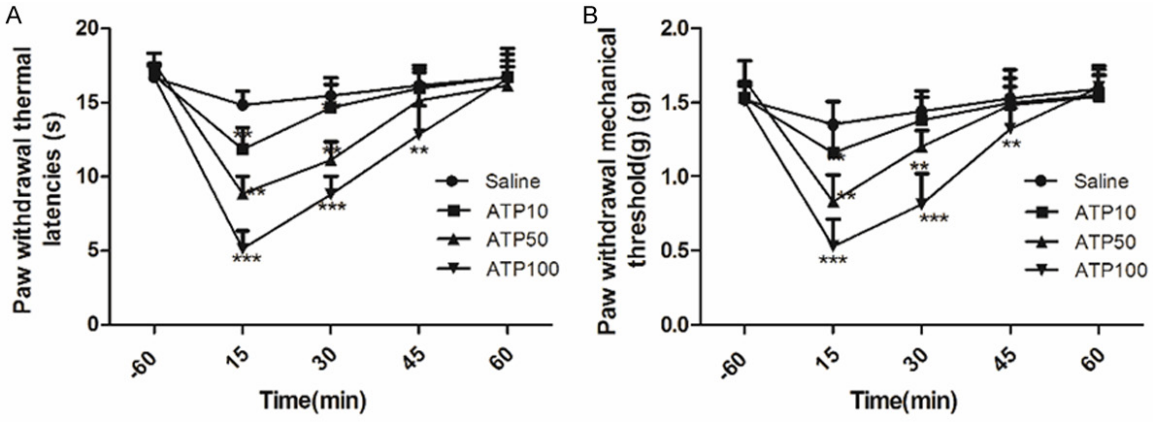

Figure 3. ATP-induced thermal hyperalgesia and mechanical allodynia were dose-dependent (A and B); Effects of intrathecal injections of ATP on PWLs and PWTs of normal mice, respectively. (*p < 0.05, **p < 0.01, ***p < 0.001 vs. saline groups).

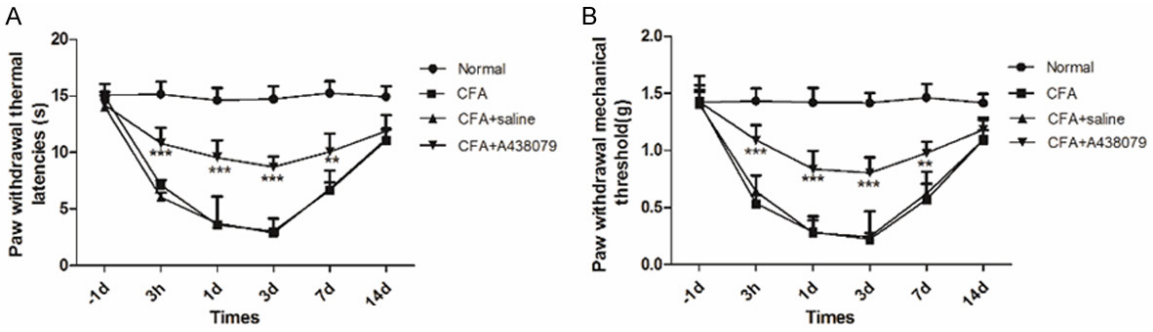

Figure 4. CFA-induced thermal allodynia and mechanical hypersensitivity were partially inhibited by the selective P2X7R antagonist A43; (A and B) Effects of intrathecal injection of A43 on PWLs and PWTs on ipsilateral side of CFA injection mice, respectively. (*p < 0.05, **p < 0.01, ***p < 0.001 vs. saline groups).

results provide further evidence for spinal cord dorsal horn P2X7 receptor involvement in inflammatory pain.

Effects of intrathecal injections with different concentrations of ATP on mice pain behavior

ATP, a P2X7R unique natural agonist, was applied in the current study. Control group values of PWL and PWT remained stable during the 60 minutes of saline administration (0.9% Nacl; 4 μl). However, it was the intrathecal administration of ATP (50 μM and 100 μM; 4 μl), rather than ATP (10 μM; 4 μl), from 15 minutes to 60 minutes, that produced significant thermal allodynia and mechanical hyperalgesia. Pain induced by 100 μM ATP was markedly stronger than 50 μM ATP. Data indicates that changes in thermal allodynia and mechanical hyperalgesia produced by ATP are dose-dependent (**Figure 3A**, **3B**; $p < 0.01$).

P2X7 receptor antagonist A43 alleviates CFA-induced thermal allodynia and mechanical hyperalgesia

To further explore the roles of P2X7 receptors in the spinal dorsal horn in the CFA induced inflammatory pain model, A43 was employed. As shown in **Figure 4A**, **4B**, values of PWL and PWT showed no significant differences after application of NS in every phase, compared with the CFA group ($p > 0.05$). However, intrathecal administration of A43 reduced thermal allodynia and mechanical hyperalgesia in every phase of the response ($p < 0.001$). These antinociceptive effects were apparently rapid and long-lasting. Present results suggest that acti-

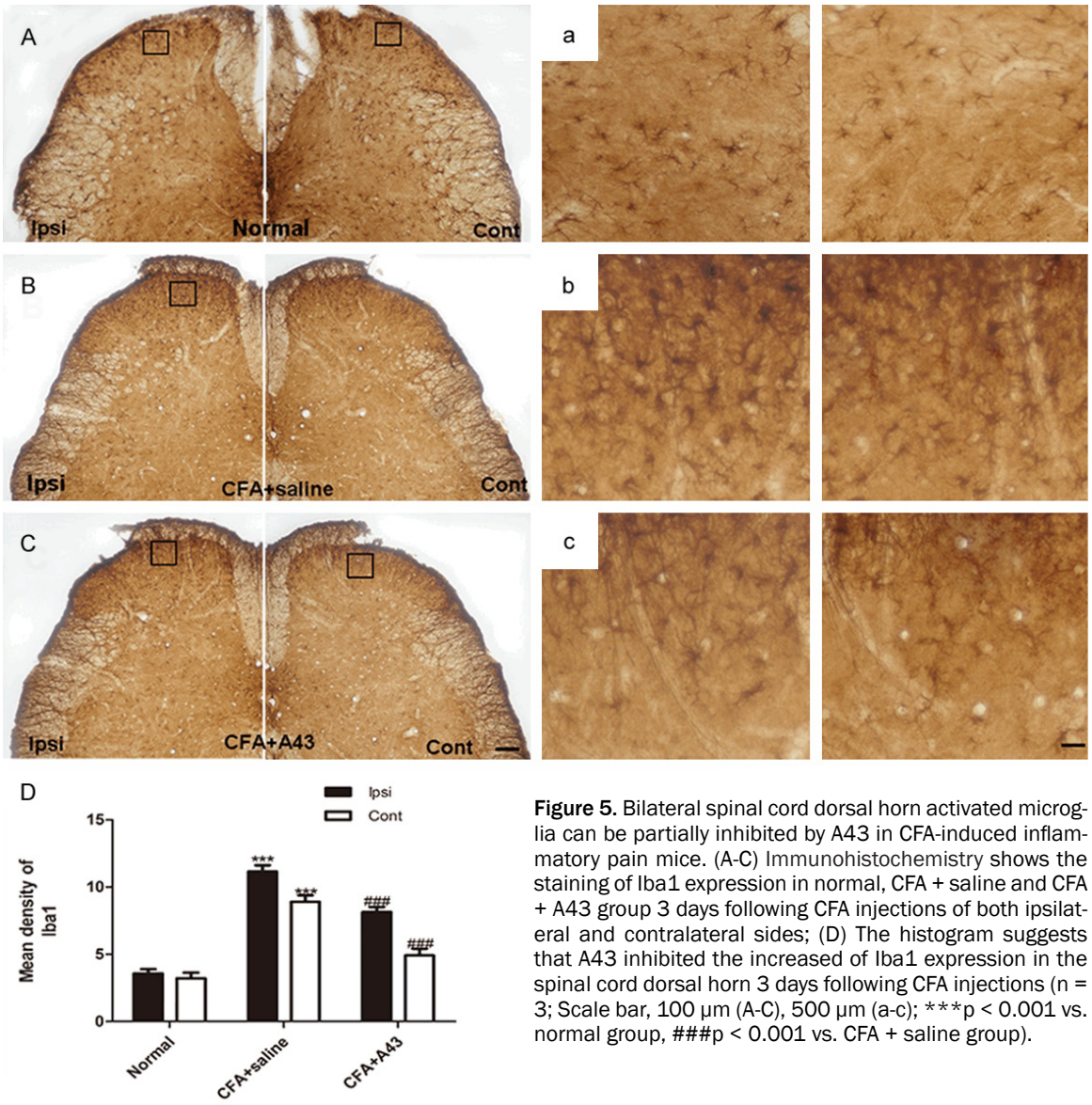

Figure 5. Bilateral spinal cord dorsal horn activated microglia can be partially inhibited by A43 in CFA-induced inflammatory pain mice. (A-C) Immunohistochemistry shows the staining of Iba1 expression in normal, CFA + saline and CFA + A43 group 3 days following CFA injections of both ipsilateral and contralateral sides; (D) The histogram suggests that A43 inhibited the increased of Iba1 expression in the spinal cord dorsal horn 3 days following CFA injections (n = 3; Scale bar, 100 μm (A-C), 500 μm (a-c); ***p < 0.001 vs. normal group, ###p < 0.001 vs. CFA + saline group).

vation P2X7 receptors in the spinal dorsal horn may promote CFA-induced pain behavior.

Bilateral spinal cord dorsal horn activated microglia can be partially inhibited by A43 in CFA-induced inflammatory pain mice

On the third day of mice inflammatory injury, immunohistochemistry was conducted, observing marked upregulation of microglia marker Iba1 in the bilateral spinal cord dorsal horn, compared with normal mice (**Figure 5A**, **5B** and **5D**; $p < 0.001$). Intrathecal administration of A43 significantly suppressed CFA-induced increases of expression of Iba1 in the spinal dorsal horn. In other words, compared with intrathecal administration of saline, intrathecal administration of A43 significantly reduced Iba1 expression (**Figure 5B-D**; $p < 0.01$). Results suggest that P2X7 receptors may participate in chronic inflammatory pain via activation of microglia.

Intrathecal administration of A43 inhibited activation of ERK and inflammatory cytokines in the spinal cord dorsal horn

Western blotting was conducted to explore the effects of A43 on expression of ERK signaling pathways. Following CFA injections, expression of p-ERK was significantly increased, compared to the normal group (**Figure 6A**; $p < 0.001$),

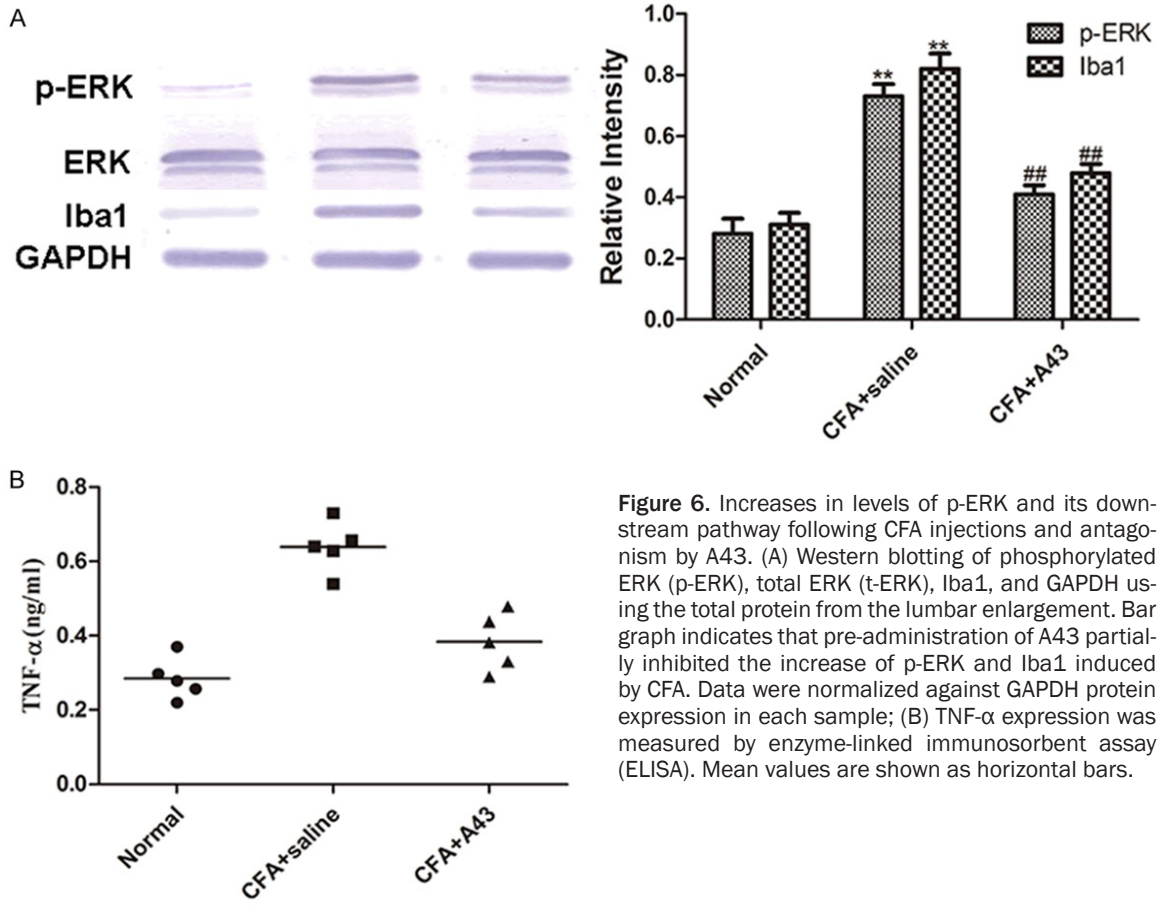

Figure 6. Increases in levels of p-ERK and its downstream pathway following CFA injections and antagonism by A43. (A) Western blotting of phosphorylated ERK (p-ERK), total ERK (t-ERK), Iba1, and GAPDH using the total protein from the lumbar enlargement. Bar graph indicates that pre-administration of A43 partially inhibited the increase of p-ERK and Iba1 induced by CFA. Data were normalized against GAPDH protein expression in each sample; (B) TNF-α expression was measured by enzyme-linked immunosorbent assay (ELISA). Mean values are shown as horizontal bars.

which was partially reversed by pretreatment with A43 (10 μM, 4 μl) ($p < 0.001$). Iba1, a microglia specific marker, has been considered a downstream substance of p-ERK. The trends are consistent with p-ERK (**Figure 6A**; $p < 0.001$). TNF-α is the earliest inflammatory factor mainly released from glial cells. It acts as an ERK downstream material. ELISA analysis showed that TNF-α was dramatically increased, compared to the normal group, on day 3 following CFA injections (**Figure 6B**, $p < 0.001$). Pretreatment with the P2X7 receptor antagonist A43 eased the increase of TNF-α (**Figure 6B**; $p < 0.01$). Results suggest that pain mediated by P2X7R involves ERK and its downstream pathway.

Discussion

Previous studies have shown that the spinal cord is a key part of the modulation of inflammatory pain. Aiming in order to explore the relationship between spinal cord P2X7R and inflammatory pain, a CFA model was applied in the current study. Experiments confirmed that P2X7R was activated and expression increased in inflammatory pain mice. Change trends matched the course of thermal allodynia and mechanical hyperalgesia. It was found that P2X7 gene expression was upregulated in the spinal cord dorsal horn of mice for at least 14 days following CFA administration. Intrathecal administration of ATP activates P2X7R to increase the inflammatory pain response, while intrathecal administration of A43 inhibits P2X7R activation and relieves the inflammatory pain response in mice. In addition, the activity of p-ERK and its downstream pathway substances, Iba1 and TNF-α, were increased. Expression was upregulated in the spinal cords of inflammatory pain mice. This which could be suppressed through intrathecal administration of A43. Results indicate that P2X7 receptors play an important role in CFA-induced inflammatory pain, mediating ERK signaling pathway activation to onset and maintain inflammatory pain. However, the exact mechanisms underlying the appearance have not been fully elucidated.

The current study showed that A43 can inhibit expression of P2X7R. However, the mechanisms by which A43 downregulates P2X7R expression and inhibits microglia activation remain elusive. A high concentration of ATP applied to microglia can trigger the release of inflammatory factors, such as IL-1 and tumor necrosis factor-α (TNF-α) [19, 20]. These induce allodynia and hyperalgesia in a dose-dependent manner. Although present experiments indicate that intrathecal ATP is associated with pain inducement, there remains a lack of continuous dose-related studies. Recent evidence has suggested that activated ERK plays an important role in neuronal plasticity. Long-term activation of ERK has been associated with memory processes and promotes inflammation and nociceptive responses in the spinal dorsal horn of nerve injuries [15, 21]. However, the specific mechanisms remain unclear. Long-term chronic pain stimuli can cause pathological changes in the central nervous system [22]. One study found that inflammatory stimuli can lead to excessive excitability of dorsal horn neurons and dorsal horn tissue remodeling, which is the main cause of chronic pain maintenance [23].

P2X7R, an ion receptor on the cell membrane, is distributed widely in the body. It mainly exists in the immune cells and the central nervous system of microglia and oligodendrocytes [1, 24]. P2X7 receptors have been detected in microglial and astrocytes in the spinal dorsal horn. P2X7 receptors have unique nature agonist ATP. It is an important signal molecule in pain transmission, playing a significant role in the onset and persistence of chronic pain [25, 26]. Aiming to understand the reactivity of P2X7R to ATP, different concentrations of ATP were administered intrathecally. High concentrations of ATP were found to cause persistent thermal allodynia and mechanical hyperalgesia. Some reports have confirmed that administration of P2X7 receptor antagonists, including BBG and A317491, can ease both thermal hyperalgesia and mechanical allodynia in some pain models [27, 28]. A43, a novel, selective, and potent small molecule antagonist of P2X7R [13], was applied in the current study. Pretreatment of A43 was shown to prevent CFA-induced hypersensitivity. Results of the current study strongly suggest that A43 reduces CFA-induced inflammatory pain, likely due to suppression of P2X7R activation.

There is evidence that activated P2X7 receptors lead to the activation of mitogen-activated protein kinase (MAPK) pathway proteins. This, in turn, causes the release of inflammatory factors [2, 29]. Allergic hypersensitivity caused by various pathological conditions also leads ERK to over-activate phosphorylation. ERK phosphorylation has been closely related to the production of inflammatory factors [15, 30], leading to central sensitization and development of pathological pain [31, 32]. In addition, many studies have shown that activated P2X7R activates microglia to release a large number of proinflammatory factors, facilitating the transmission of nociceptive pain [33]. TNF-α is an important inflammatory factor mainly released from microglia. It is thought to be a form of pain sensitization involved in peripheral and central sensory formation. The current study found that CFA-induced upregulation of TNF-α release was inhibited through administration of P2X7R antagonist A43. The same phenomenon occurs in microglia marker Iba1. These suppressive effects may be explained by P2X7R blockade, because peripheral inflammation (or spinal cord injury)-induced activation of P2X7R can result in Ca^{2+} influx and activation of ERK signaling pathways. This readily enhances pain transmission. Previous findings, together with present results, suggest that activation of P2X7R and ERK downstream pathways may be an essential step in CFA-induced inflammatory pain.

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