Role of ERK1/2 activation on itch sensation induced by bradykinin B1 activation in inflamed skin

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Abstract. It has previously been demonstrated that bradykinin receptor B1 (B1R) agonists evoke an itch-related scratching response in inflamed skin via the B1 receptor; however, the mechanisms responsible for this abnormal itch sensation remain unclear. Therefore, the present study utilized a complete Freund's adjuvant (CFA)-induced mouse model of inflammation to elucidate the mechanisms responsible. Over a period of 30 min, scratching behavior was quantified by the number of hind limb scratches of the area surrounding the drug injection site on the neck. Furthermore, western blot analysis was used to investigate the potential role of extracellular signal-regulated kinase (ERK) 1/2 signaling as a mediator of itch in CFA-treated mice. The results demonstrated that CFA-induced inflammation at the back of the neck is associated with sustained enhancement of ERK1/2 activation in the spinal cord. Moreover, B1R agonist treatment resulted in increased expression of phosphorylated ERK1/2 in the spinal cord, which peaked at 45 min. Consistent with these findings, inhibition of either mitogen-activated protein/ERK kinase or ERK1/2, as well as inhibition of ERK1/2 activation following inflammation, attenuated B1 receptor-mediated scratching responses to a greater extent, as compared with control mice. Collectively, the results of the present study indicated that enhanced and persistent ERK1/2 activation in the spinal cord may be required to induce a scratching response to B1R agonists following CFA-induced inflammation.

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Introduction

Although itch, also known as alloknesis, and pain are unpleasant sensations, they are two distinct sensations (1); however, recent studies have identified various interactions between itch and pain transmission and sensitization pathways (2-4). For example, when painful stimuli is applied to lesional skin of patients with atopic dermatitis an itch sensation is evoked rather than pain, which is thought to be the result of central sensitization for itching (5,6). Similarly, intradermal injection of algogenic substances capsaicin or bradykinin has been demonstrated to induce a scratching reflex, rather than pain-related behavior, in inflamed skin of mice (7,8). The authors of the present study have previously reported that activation of bradykinin receptor B1 (B1R) contributes to alloknesis in mouse skin inflamed by treatment with complete Freund's adjuvant (CFA) (9). Moreover, B1R agonists are known to effectively induce scratch in inflamed lesioned skin (7). Although these previous studies have improved understanding of this phenomenon, the underlying mechanisms are yet to elucidated.

It has been well-documented that CFA is capable of activating ERK1/2 in primary sensory and secondary order dorsal horn neurons, and that pERK1/2 is associated with the generation and maintenance of inflammatory pain (10,11). ERK1/2 activation has previously been described in the spinal cord during histamine- and DNFB-induced alloknesis (12), in DRG neurons during ET-1-induced acute alloknesis (13), during chronic alloknesis in dry skin and in allergic contact dermatitis models (14). However, its role in B1R agonist-induced alloknesis in an animal model of CFA inflammation has not been described. Therefore, we hypothesize that prolonged activation of ERK following CFA-induced inflammation may mediate the abnormal scratching response to B1R agonists. ERK1/2 are two closely related members of the mitogen-activated protein kinase family. Upon activation, phosphorylated

ERK1/2 (pERK1/2) transduce extracellular stimuli into intracellular signaling and subsequently trigger the expression of a plethora of nuclear transcription factors to regulate numerous cellular functions (15,16). Following peripheral nerve injury, phosphorylation and activation of ERK1/2 is induced in the dorsal root ganglia (DRG) (17-20) and dorsal horn of the spinal cord (21-23), and inhibition of ERK1/2 activation prevents behavioral pain sensitization (11,24). At the molecular level, previous studies have demonstrated that activation of ERK1/2 signaling in the spinal cord or sensory neurons may be responsible for the relay of distinct types of stimulus-evoked acute itch and spontaneous scratching responses in chronic itch models (12,14,25). It has also been demonstrated that ERK1/2 activation is required at the spinal level for itch responses, which are induced by histamine or dinitrofluorobenzene (DNFB) (12). Furthermore, it has been reported that pruritogenic molecules, including endothelin 1 (ET-1) and cytokine interleukin 31 (IL-31), stimulated alloknesis in cultured primary sensory neurons and induced phosphorylation of ERK1/2, whereas inhibition of ERK1/2 activation blocked ET-1- and cytokine IL-31-induced scratching behavior *in vivo* (13,25). When taken together, these findings demonstrate that ERK1/2 activation in the spinal cord or sensory neurons may have an important role in itch transmission.

To our knowledge, there is a lack of previous studies investigating the mechanisms mediating itch-related scratching in response to algesic chemical stimuli delivered to inflamed tissue in mice. The aim of the present study was to investigate the potential role of ERK1/2 signaling in B1R agonist-induced alloknesis, using a CFA-induced mouse model of inflammation.

Materials and methods

Reagents and antibodies. CFA and MEK1/2 inhibitor (PD0325901) were purchased from Sigma-Aldrich (St. Louis, MO, USA). ERK1/2 inhibitor (328006) and kinin B1 receptor agonist [des-Arg(9)-bradykinin] were purchased from EMD Millipore (Billerica, MA, USA) and Tocris Bioscience (Bristol, UK), respectively. Rabbit anti-pERK1/2 (cat. no. 4370) and anti-ERK1/2 (cat. no. 9102) monoclonal antibodies, goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. 7074) and rabbit anti-tubulin monoclonal antibody (cat. no. 2128) were obtained from Cell Signaling Technology, Inc., (Danvers, MA, USA).

Mice. A total of 98 male C57BL/6J mice, weighing 20-22 g and aged ~6 weeks, were obtained from the Center for Laboratory Animals, Sun Yat-Sen University (Guangzhou, China). Mice were maintained at 22±1˚C on a 12/12 h light/dark cycle with *ad libitum* access to food and water. A mouse model of skin inflammation was established via intradermal injection of 50 μl CFA into the nape of the neck, as previously described (7,8). In the control group, an identical volume of normal saline was administered instead of CFA. The experimental procedures and animal use and care protocols were approved by the Committee on Ethical Use of Animals at the Guangdong Academy of Medical Sciences (Guangzhou, China), and were performed according to the National Institutes of Health guidelines for the care and use of animals.

Behavioral tests. Seven mice from each of the CFA and control groups were used for the behavioral tests. Behavioral studies were conducted at approximately the same time each day between 9:00 a.m. and 4:00 p.m. in order to reduce circadian effects. Four days after injection with CFA, the mice were placed into individual small plastic chambers (22x12x10 cm) for 30 min prior to the experiment for acclimation. From video recordings over a period of 30 min, scratching behavior was quantified by the number of hind limb scratches directed to the area surrounding the drug injection site on the neck. Off-site scratches, such as to the cheek, were excluded from the counts.

Treatment with BIR agonist. As peripheral noxious stimuli is capable of inducing phosphorylation of ERK1/2 (26,27), western blot analysis was used to determine whether increased expression of pERK1/2 could be induced in the spinal cord by stimulation with a B1R agonist. Four days after inflammation was induced with CFA, mice (n=15) were injected with des-Arg(9)-bradykinin B1R agonist (0.4 mmol/l) in the nape of the neck. The control group (n=15) were administered normal saline stead of the B1R agonist. Mice were anesthetized with 1% sevoflurane (Wanshi Company, Osaka, Japan) and sacrificed by cervical dislocation at various time points (5, 15, 30, 45 and 60 min) post-injection. These time points were selected as they correspond with the time-dependent induction of pERK1/2 expression in the spinal cord by histamine (12). Cervical spinal cord samples were collected and preserved at -80˚C for analysis of pERK1/2 levels by western blotting.

Treatment with ERK1/2 and MEK1/2 inhibitors. CFA-treated mice were divided into four groups, as follows: i) 328006 group, in which mice were intraperitoneally injected with 30 mg/kg ERK1/2 inhibitor (100 μl 328006 in 10% DMSO; n=7); ii) PD0325901 group, in which mice were intraperitoneally injected with 10 mg/kg MEK1/2 inhibitor (100 μl PD0325901 in 10% DMSO; n=7); and iii) control group, in which mice were administered an identical volume of 10% DMSO and normal saline into the nape of the neck (n=14). The mice were treated with 328006, PD0325901 or vehicle 30 min prior to intradermal administration of the des-Arg(9)-bradykinin B1R agonist (0.4 mmol/l in 50 μl) into the nape of the neck. Dosages of ERK1/2 and MEK1/2 inhibitors were calculated based the study conducted by Kido-Nakahara *et al* (13).

Western blotting. Mice (n=3/group) were anesthetized with sevoflurane and sacrificed by cervical dislocation. Cervical cord segments and DRG neurons were dissected and preserved at -80˚C. Lysates of DRG neurons or spinal cord sections were prepared by homogenization in lysis buffer containing a protease inhibitor mixture (Roche Diagnostics, Basel, Switzerland) and a phosphatase inhibitor cocktail (Thermo Fisher Scientific Inc., Waltham, MA, USA). Cell debris was removed by centrifugation at

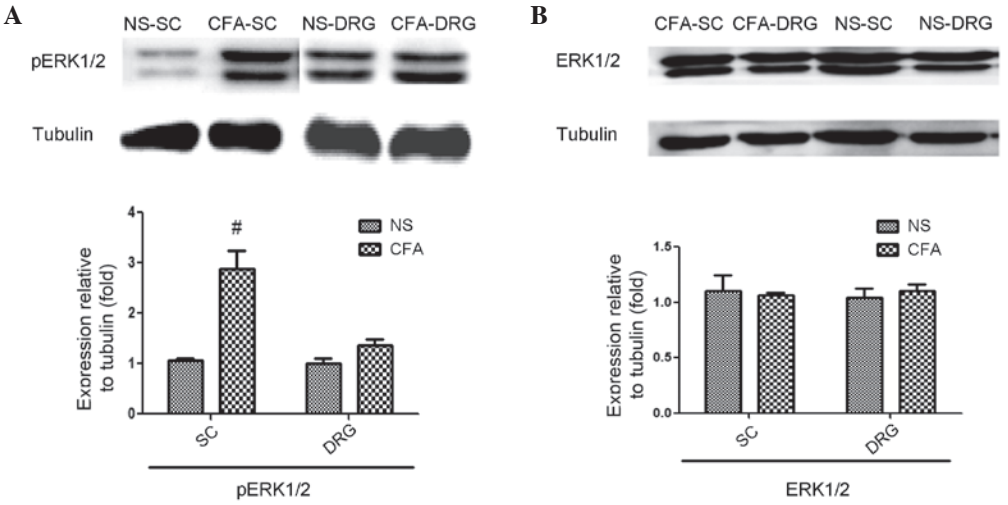

Figure 1. Increased level of pERK1/2 were detected in the spinal cord of CFA-induced mouse model of inflammation, as determined by western blot analysis. (A) Increased expression of pERK1/2 protein was detected in the spinal cord of CFA-inflamed mice, whereas pERK1/2 expression was unchanged in DRG neurons. (B) Invariable total ERK1/2 expression was detected in spinal cord and DRG neurons. Tublin was used as an internal reference. Data are presented as the mean ± standard error of the mean. #P<0.05 vs. the NS group. pERK, phosphorylated extracellular signal-regulated kinase; SC, spinal cord; DRG, dorsal root ganglia; CFA, complete Freund's adjuvant; NS, normal saline.

10,000 x g for 5 min at 4˚C. Samples were boiled for 5 min in sodium dodecyl sulfate (SDS) sample buffer, containing 1.0 mol/l Tris-HCL, 8% SDS, 0.1% bromophenol blue, 10% glycerol and 2.5% 2-mercaptoethanol (pH 6.8). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (GE Healthcare Life Sciences, Chalfont, UK). Membranes were blocked with 5% low-fat milk and incubated overnight at 4˚C with rabbit anti-pERK1/2, anti-ERK1/2 (1:1,000) and anti-tubulin (1:2,000) monoclonal antibodies. Subsequently, the membranes were washed with phosphate-buffered saline and incubated with HRP-conjugated anti-rabbit secondary antibody (1:5,000) for 1 h at 37˚C. Labeled proteins were detected by incubation with enhanced chemiluminescence solution (Thermo Fisher Scientific, Inc.) for 1 min and visualized with an ImageQuant Las 4000 imager (GE Healthcare Biosciences, Pittsburgh, PA, USA). Tubulin was used as an internal control. Band intensities were quantified using ImageJ software, version 1.45 (https://imagej.nih.gov/ij/).

Statistical analysis. All results are expressed as the mean ± standard error of the mean. Between-group comparisons of scratching responses were performed using unpaired t-tests. Two-factorial analysis of variance was used for all other comparisons. Statistical analyses were performed using SPSS software, version 19.0 (IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

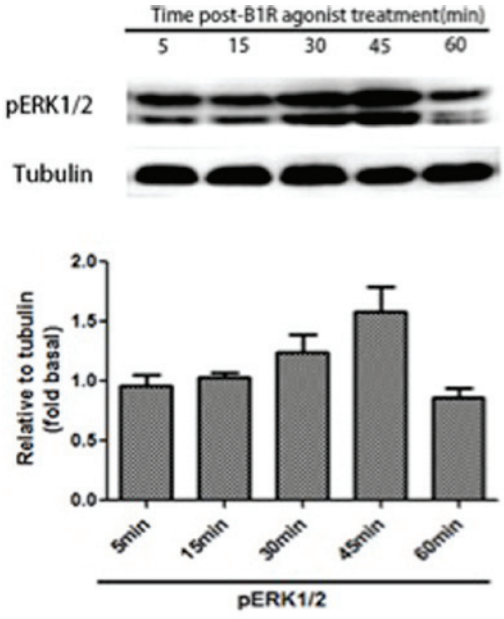

Figure 2. Time-dependent induction of pERK1/2 expression by B1R agonists in the spinal cord of CFA-inflamed mice. Following B1R agonist treatment, cervical spinal cords were harvested from CFA-inflamed mice at the specified time points and western blot analysis indicated that pERK1/2 is gradually upregulated from 30 min post-B1R agonist treatment, peaking at 45 min and subsequently declining to levels comparable with the NS control group after 60 min. Tublin was used as an internal control. pERK, phosphorylated extracellular signal-regulated kinase; B1R, bradykinin 1 receptor; CFA, complete Freund's adjuvant.

Results

pERK1/2 expression levels are significantly increased in the cervical spinal cord of CFA-inflamed mice. To explore the potential role of ERK1/2 signaling as a mediator of alloknesis in CFA-treated mice, the present study investigated whether the expression levels of pERK1/2, which is a well-established indicator of ERK1/2 signaling activation, were increased in the spinal cord or DRG neurons of CFA-induced mice, as compared with control mice. Western blot analysis indicated that pERK1/2 were significantly increased in the cervical spinal cord 4 days after CFA-induced inflammation (P=0.0217), whereas pERK1/2 expression levels in DRG neuron extracts and total ERK1/2 levels were unchanged (Fig. 1). These results indicate that there was sustained ERK1/2 activation in the

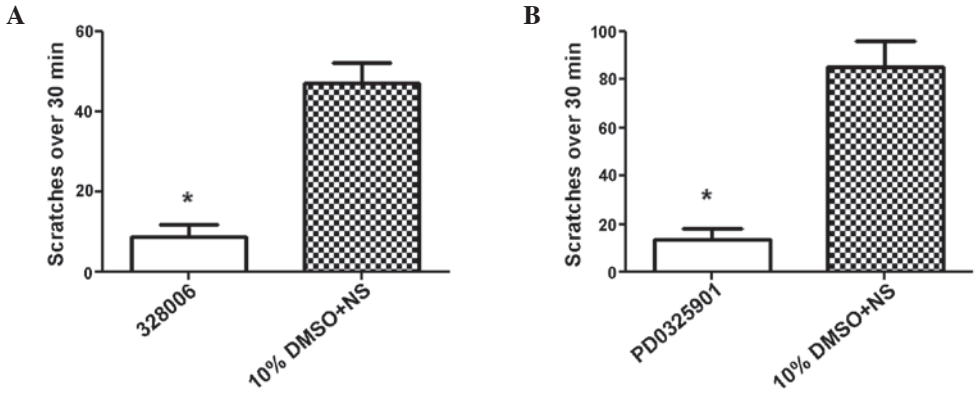

Figure 3. Blocking ERK1/2 signaling decreased B1R agonist-evoked scratching behavior in a mouse model of CFA-induced inflammation. (A and B) The number of scratches induced by B1R agonist treatment over a 30 min period was dramatically reduced by intraperitoneal injection of (A) PD0325901 MEK1/ERK1/2 inhibitor, which targets ERK1/2 phosphorylation and (B) 328006 ERK1/2 inhibitor, which targets ERK1/2, 30 min prior to intradermal bradykinin receptor B1 agonist injection into the nape of the neck. The respective control groups were administered 10% DMSO + NS. Data are presented as the mean ± standard error of the mean (n=7). *P<0.05 vs. the control group. ERK, extracellular signal-regulated kinase; B1R, bradykinin 1 receptor; CFA, complete Freund's adjuvant inflamed mice; NS, normal saline.

spinal cord for at least 4 days following the injection of CFA in the nape region, which was not localized to DRG neurons.

pERK1/2 is upregulated following treatment with B1R agonist. As shown in Fig. 2, pERK1/2 expression levels in the cervical spinal cord increased 30 min post-B1R agonist administration, peaked at 45 min, and then returned to control levels by 60 min post-administration. These results suggest that B1R agonist treatment is able to stimulate greater ERK1/2 activation in CFA-inflamed mice, as compared with the persistent activation induced by CFA treatment alone.

Inhibiting ERK1/2 signaling decreases B1R agonist-evoked scratching behavior. Western blot analysis indicated a potential role for ERK1/2 signaling in B1R agonist-induced pruritus *in vivo*. To further examine this hypothesis, a pharmacological approach was used to determine whether ERK1/2 activation is associated with the scratching response *in vivo*. PD0325901 MEK1/2 inhibitor, which targets ERK1/2 phosphorylation but is inactive against various other MAP kinase pathway kinases (28), was intraperitoneally administered 30 min prior to intradermal B1R agonist injection in the nape of neck. As compared with the control group, behavioral analyses demonstrated that B1R agonist-induced scratching behaviors were significantly reduced (>4-fold) by inhibiting ERK1/2 via systemic application of the PD0325901 MEK1/2 inhibitor (P<0.001; Fig. 3A). Consistent with this result, treatment with the 328006 ERK1/2 inhibitor prior to B1R agonist intradermal injection also significantly decreased scratching (to a similar extent), as compared with the control group (P<0.001; Fig. 3B). These results suggested that the ERK1/2 signaling pathway may be associated with the transmission of itch sensations in response to B1R agonist treatment in inflamed skin and indicated the contribution of ERK1/2 activation in the spinal cord as a mediator of this process.

Discussion

The present study demonstrated two major findings. Firstly, CFA-induced inflammation in the nape of the mouse neck produced a sustained increase in ERK1/2 activation in the cervical spinal cord, which was associated with increased scratching behavior in response to intradermal injection of the des-Arg(9)-bradykinin B1R agonist into the inflamed skin. Secondly, the upregulated levels of pERK1/2 in the CFA-induced mouse model of inflammation were further enhanced by treatment with the B1R agonist. This enhancement of pERK1/2 expression in the spinal cord may account for the abnormally elevated scratching behavior that was observed in response to B1R agonist treatment following CFA-induced inflammation.

Previous studies have demonstrated that ERK1/2 signaling in the spinal cord may contribute to the sensation of pain (11,18,23). However, additional evidence has also suggested that the ERK1/2 signaling pathway is associated with itch transduction (12,13,25). A previous study has demonstrated that pruritogens are capable of activating ERK1/2 signaling in the spinal cord, which is associated with the transduction of acute alloknesis (12). It has also been demonstrated that ERK1/2 phosphorylation in DRG neurons is critical for acute alloknesis, which are induced by cytokines IL-31 and ET-1 (13,25). Furthermore, ERK1/2 signaling in chronic alloknesis was also demonstrated by Zhao *et al* (14), who showed that pERK1/2 is a key mediator of itch sensations in the sensory neurons of mice with chronic alloknesis, since transient pERK1/2 activation in the spinal cord was absent when spontaneous scratching was observed. Therefore, these studies suggested that ERK1/2 activation in the spinal cord or DRG neurons is associated with neural transmission mediation of the itch sensation.

The present study investigated whether pERK1/2 expression increased in the spinal cord or DRG neurons n response to B1R agonist stimulation during CFA-induced inflammation, and whether this increase was capable of mediating scratching behavior. The results of the present study demonstrated a persistent enhancement of pERK1/2 in the spinal cord of CFA-inflamed mice, but not in DRG neurons. These data are consistent with a recent study by Zhang *et al* (12), which showed that intradermal injection of pruritogen histamine into

the nape or cheek of mice induced phosphorylation of ERK1/2 in the spinal cord but not in DRG neurons, and that ERK1/2 activation was crucial for the initiation and maintenance of the itch sensation. Conversely, our findings differ from a study published by Zhao *et al* (14), which indicated that persistent activation of ERK1/2 signaling in DRG neurons, but not in the spinal cord, was required for the maintenance of spontaneous scratching in allergic contact dermatitis and dry skin models of chronic alloknesis. It should be noted that scratching behaviour was detected in the present study, therefore the possibility that increases in pERK1/2 were elicited by the scratching itself and not by the pruritus produced by model treatments cannot be excluded. This may also account for the increased activation of ERK1/2 in DRG neurons that was observed in the chronic itch model (12,29); whereas the present study demonstrated that enhanced levels of pERK1/2 were only required in the spinal cord for the B1R agonist-evoked itch sensation.

To elucidate whether B1R agonist treatment is capable of triggering alterations in the phosphorylation status of ERK1/2, similar to those observed following injection with histamine, the time-dependent induction of pERK1/2 in the cervical spinal cord was assessed in CFA-inflamed mice after B1R agonist injection (9,30). Western blot analysis demonstrated that treatment with a B1R agonist induced further enhancement of ERK1/2 activation, which was already increased due to CFA inflammation. Furthermore, blocking ERK1/2 signal activation via systemic application of MEK1/2 or ERK1/2 inhibitors significantly decreased scratching behavior, indicating that the ERK1/2 signaling pathway may have a role in the itch sensation following B1R agonist application.

Phosphorylation of ERK1/2 in the spinal cord following peripheral inflammation by CFA contributes to persistent inflammatory pain (10,21). The results of the present study suggest that activated ERK1/2 may have a role in the abnormal itch sensation following B1R activation in CFA-inflamed mice. It has previously been reported that the enhancement of nocifensive responses to metabotropic glutamate receptor agonist (RS)-3, 5-dihydroxyphenylglycine was associated with sustained ERK activation in dorsal horn neurons, which persisted for seven days following injection with CFA (31). It is possible that sustained ERK activation regulates plasticity in the spinal cord and underlies a component of central and peripheral sensitization, which leads to scratching in response to B1R agonist stimulation in CFA-inflamed skin (32,33).

The results of the present study suggested that the persistence of ERK1/2 activation in the CFA inflammation model, and further upregulation by B1R agonist treatment in the spinal cord, underlies a change in the behavioral response to B1R activation in inflamed mice. Previous studies have demonstrated the involvement of B1R in pruriceptive processing following CFA-induced inflammation (8,9) and B1R blockade has been shown to ameliorate CFA-induced inflammatory hyperalgesia (34,35). A modulatory function for MAP kinase pathways has been demonstrated to regulate B1R expression during tissue injury (36) and control its function in inflammatory disorders (37). In addition, in previous studies B1R was upregulated in the airways of mice treated with TNF-a and IL-4, which also demonstrated the involvement of the MAPK pathway in this process (38,39). Therefore, in light of these finding and the results of the present study, we hypothesize that there is an association between sustained ERK1/2 activation and B1R in CFA-inflamed mice. The present data suggested that the persistence of ERK1/2 activity itself is important for B1R agonist-mediated itch signaling following inflammation. However, it still remains to be investigated whether there is increased expression of B1R in the CFA-induced inflammation model and if sustained ERK1/2 activity upregulates B1R levels.

The authors of the present study have previously reported that a painful bradykinin stimulus evokes an itch sensation in CFA-inflamed skin, which was substantially blocked by a specific B1R antagonist (8). Therefore, CFA-induced inflammation may induce novel pruriceptive properties in B1R, resulting in B1R becoming responsive to algogenic stimuli. This previous study and the present results demonstrated that ERK1/2 activation in the spinal cord may be a prerequisite for the abnormal itch sensation stimulated by the B1R agonist during chronic inflammation. Therefore, the ERK1/2 signaling pathway may have a pivotal role in itch processing, providing novel insight into the molecular mechanisms that mediate itch responses to algogenic stimuli.

In conclusion, the present study demonstrated that CFA-induced inflammation results in persistent ERK1/2 activation in the spinal cord, which can be further upregulated by B1R agonist treatment, and is associated with itch processing in the spinal cord. As the cellular mechanisms that regulate itch sensations evoked by pain stimuli in inflamed skin remain poorly understood, further examination of the role of ERK1/2 signaling pathways in this process may yield novel mechanistic insights.

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