IP$_3$-Mediated Calcium Signaling Is Involved in the Mechanism of Fractalkine-Induced Hyperalgesia Response

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Background: Fractalkine is widely expressed throughout the brain and spinal cord, where it can exert effects on pain enhancement and hyperalgesia by activating microglia through CX3C chemokine receptor 1 (CX3CR1), which triggers the release of several pro-inflammatory cytokines in the spinal cord. Fractalkine has also been shown to increase cytosolic calcium ([Ca$^{2+}$]$_i$) in microglia.

Material/Methods: Based on the characteristics of CX3CR1, a G protein-coupled receptor, we explored the role of inositol 1,4,5-trisphosphate (IP$_3$) signaling in fractalkine-induced inflammatory response in BV-2 cells in vitro. The effect and the underlying mechanism induced by fractalkine in the brain were observed using a mouse model with intracerebroventricular (i.c.v.) injection of exogenous fractalkine.

Results: [Ca$^{2+}$]$_i$ was significantly increased and IL-1$\beta$ and TNF-$\alpha$ levels were higher in the fractalkine-treated cell groups than in the farctalkine+ 2-APB groups. We found that i.c.v. injection of fractalkine significantly increased p-p38MAPK, IL-1$\beta$, and TNF-$\alpha$ expression in the brain, while i.c.v. injection of a fractalkine-neutralizing antibody (anti-CX3CR1), trisphosphate receptor (IP$_3$R) antagonist (2-APB), or p38MAPK inhibitor (SB203580) prior to fractalkine addition yielded an effective and reliable anti-allodynia effect, following the reduction of p-p38MAPK, IL-1$\beta$, and TNF-$\alpha$ expression.

Conclusions: Our results suggest that fractalkine leads to hyperalgesia, and the underlying mechanism may be associated with IP$_3$/p38MAPK-mediated calcium signaling and its phlogogenic properties.

MeSH Keywords: Chemokine CX3CL1 • Hyperalgesia • Inositol 1,4,5-Trisphosphate • Mitogen-Activated Protein Kinase Kinases

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Fractalkine, which contains an extracellular region, transmembrane region, and intracellular region, is a chemokine found in cell membranes of central nervous system cells [1,2]; its receptor, CX3C chemokine receptor 1 (CX3CR1), is mainly expressed by microglia [3,4]. As a mediator of neuron-to-microglia signaling, fractalkine is closely related to inflammation [5]. Fractalkine sheds off from neurons and its receptor, CX3CR1, to activate microglia, which can promote the release of pro-inflammatory cytokines, including IL-1β and TNF-α, to induce further glial activation, ultimately resulting in an adverse cycle of inflammation [6]. Fractalkine has been shown to lead to increased cytosolic calcium ([Ca^{2+}]_i) in microglia [7]. However, no previous report describes the mechanism by which cytosolic calcium is elevated, nor its relationship to hyperalgesia; indeed, no study reports the brain mechanism induced by fractalkine in a pain state.

In the present study, we assessed the role of inositol 1,4,5-trisphosphate (IP3) signaling in the change of [Ca^{2+}]_i induced by fractalkine in BV-2 cells. First, we used primary cultured microglia, but they did not proliferate substantially, and we could not achieve the quality and quantity necessary for the experiment. It was not successful after 3 months of cultivation, so we changed to the cell line and completed our experiments. Effects and underlying mechanisms were investigated using a mouse model with i.c.v. injection of exogenous fractalkine. The study of this signaling pathway may provide a reference for central mechanisms of pain.

Material and Methods

Cell culture and treatment

BV-2 cells (Cell Resource Centre of the Beijing Union, China), a murine microglial cell line, were cultured in DMEM/F12 containing 10% fetal bovine serum and 1% penicillin and streptomycin, at 37°C with 5% carbon dioxide. When cultures were 70–80% confluent, and 0.25% trypsin was used to digest and passage cells. Cells were seeded in 35-mm-diameter dishes (5 mL/dish), 24-well plates (1 mL/well), or 6-well plates (2 mL/well) at a density of 1×10⁵ cells/ml. After 24 h of growth, culture medium was replaced with serum-free DMEM/F12 medium and synchronized for 12 h.

Cells were randomly divided into 4 groups (n=18 per group): control (C), fractalkine (aa25-105, R&D Systems, Minneapolis, MN) (F), 2-APB (Cayman, Ann Arbor, MI) + fractalkine (AF), and 2-APB (A). Cells were treated with 10 nmol/L fractalkine in groups F and AF, 2-APB (50 µmol/L for 1 h) prior to 10 nmol/L fractalkine addition in group AF, serum-free medium in group C, or 50 µmol/L 2-APB in group A.

Two-photon confocal microscopy of [Ca^{2+}]_i

[Ca^{2+}]_i was determined by measuring the F340/F380 ratio of Fura-2/AM (Molecular Probes, Eugene, OR) fluorescence with a photometer coupled to an Olympus 1×70 inverted microscope and IPLab v3.7 image processing and analysis software (Biovision Technologies, Exton, PA). We determined F340/F380 ratios according to the Ge Liang method [8]. Briefly, microglia were loaded with Fura-2/AM in buffer (in mM: NaCl 126, HEPES 10, KCl 6, CaCl_2 1, MgCl_2 1.2, glucose 10, Fura-2/AM 0.001, pH 7.4) for 30 min at 2°C. Cells were washed twice to remove excess extracellular Fura-2/AM and maintained for 30 min in buffer with or without calcium before testing for [Ca^{2+}]_i. CaCl_2 was chelated by adding 1 mM EGTA in calcium-free buffer. [Ca^{2+}]_i was tested in buffer with or without calcium and containing 0, 0.1, 1, or 10 nM fractalkine in the 4 groups. Fluorescence signals were measured with excitation at 340 and 380 and emission at 510 nm for the last 10 min of each treatment. F340/F380 ratios were averaged from a minimum of 30 cells in 6 separate experiments. [Ca^{2+}]_i was calculated using a previously described ratiometric method [9].

Enzyme-linked immunosorbent assay (ELISA) for IL-1β and TNF-α

Cell culture supernatants were extracted after treatment in the absence or presence of 2-APB or fractalkine (10 nM) for 0, 6, 12, and 24 h. Briefly, samples were centrifuged at 3,000 g at 4°C for 20 min and supernatants were collected. IL-1β and TNF-α were measured by ELISA (Mouse IL-1β Assay Kit and Mouse TNF-α Assay Kit; Boster, Wuhan, China), according to the manufacturer’s instructions. Absorbance values were measured at a 490-nm wavelength using a microplate reader (Bio-Rad, Hercules, CA). Concentrations of samples were calculated using a standard curve drawn according to the absorbance values of standards.

Mice in the 6 groups were rapidly sacrificed before injection (0) or at 6, 12, or 24 h after i.c.v. injection. The hippocampus was collected. IL-1β and TNF-α were measured by ELISA according to the manufacturer’s instructions. Optical density was measured at 450 nm using a microplate reader and values were obtained from standard curves using the recombinant IL-1β and TNF-α provided in the ELISA kits.

Quantitative real-time PCR for IL-1β and TNF-α

Cells were harvested after treatment with or without fractalkine for 6 h, in the absence or presence of 2-APB, and after fractalkine (10 nM) treatment for 0, 6, 12, or 24 h. Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA). Mice in the fractalkine group were treated as described for the ELISA assay. Reverse transcription was performed
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according to standard protocols using a reverse transcriptase assay kit (Invitrogen). Expression levels of target genes were determined from cDNA samples according to standard protocols using a SYBR qPCR mix kit (Toyobo, Osaka, Japan). PCR cycling protocols were as follows: initial denaturation, 95°C for 1 min; cycling, 95°C for 15 s, 58°C for 10 s, and 72°C for 45 s; for 40 cycles. The following primers were used for mRNA detection: IL-1β forward, 5’-TTTGAAGTGGAGGACC-3’; reverse, 5’-TGCTGCTAGGAGATTTGA-3’; TNF-α forward, 5’-GGGAGGCTACATTTTGAG-3’, reverse, 5’-CAGGTC ACTTCCCCACGCTA-3’; actin forward, 5’-CCGTGAAAAG 5’-CGGGCAGGTCTACTTTGGAG-3’, reverse, 5’-CAGGTC ACTTCCCCACGCTA-3’. For relative comparison of each gene, realtime PCR data were evaluated using the equation 2^–ΔΔCt test/2^–ΔΔCt control.

Animal preparation

All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. All procedures were approved by Animal Ethics and Welfare Committee of Inner Mongolia Autonomous Region People’s Hospital (IACUC-20160427). Animals were maintained under controlled conditions (24±2°C, 50–60% humidity, 12/12-h dark/light cycles) with free access to food and water. Anti-mouse CX3CR1 (eBioscience, San Diego, CA) is a neutralizing antibody for CX3CR1, 2-APB (Cayman, Ann Arbor, MI) is an IP3 antagonist, and SB203580 (Sigma-Aldrich, St. Louis, MO) is a p38MAPK inhibitor. Following complete anesthesia (i.v. 30 µg/g), we performed a right lateral ventricle puncture according to the Ge ZJ method to deliver reagents directly into the cerebrospinal fluid. All reagents were diluted in 5 µL of sterile normal saline.

At total of 138 adult male C57BL/6 mice, weighing 25–30 g, were randomized and divided into 6 groups: sham (no i.c.v. injection), n=20; vehicle (i.c.v. injection of 5 µL normal saline), n=23; fractalkine (i.c.v. injection of 100 ng fractalkine), n=35; fractalkine + 1 µg anti-CX3CR1 (i.c.v.), n=20; fractalkine + 3.4 µg 2-APB (i.c.v.), n=20; and fractalkine + 1 µg SB203580, n=20. Pretreatments (i.c.v.) were conducted 1 h before i.c.v. injection of 100 ng fractalkine in the last 3 groups.

Thermal nociceptive thresholds

The time between placement on the heat source and appearance of withdrawal or licking of the hind paw was recorded as paw withdrawal latency (PWL). PWL was assayed according to the Hargreaves method using an Ugo Basile 37370 planar test platform (Ugo Basile, Milan, Italy). Each mouse was tested 30 min before, as control performance (0 min), and at 30, 60, 120, and 240 min after drug administration.

Immunofluorescence assay for the role of fractalkine in microglia

Mice in vehicle and fractalkine groups were rapidly sacrificed 4 h after i.c.v. injection. Coronal sections (10-µm thick) of the hippocampus were made using a cryostat. Sections were analyzed by immunofluorescence as previously described using goat anti-mouse fractalkine antibody (1: 50; SC-7227, Santa Cruz Biotechnology, Dallas, TX), rabbit anti-mouse Iba-1 (1: 500; Wako Chemicals, Richmond, VA), and rabbit anti-mouse GFAP (1: 50; ZSGB-BIO, Beijing, China) with secondary FITC-labeled anti-rabbit IgG Ab (1: 50; Proteintech, San Diego, CA) or TRITClabeled anti-goat IgG Ab (1: 50; Proteintech). Images (400×) were obtained with a fluorescence microscope.

Western blot analysis of p38MAPK and p-p38MAPK

Mice in fractalkine groups were rapidly sacrificed 30 min before injection (0) and at 30, 60, 120, and 240 min after i.c.v. injection; whereas mice in the 6 treatment groups were rapidly sacrificed 30 min after i.c.v. injection. The hippocampus was rapidly isolated on ice and homogenized with a hand-held pestle in SDS sample buffer (10 µL/mg tissue) containing a cocktail of proteinase inhibitors and phosphatase inhibitors (Beyotime, Beijing, China). After complete cleavage, centrifugation at 14 000× g for 5 min at 4°C, supernatants were collected and proteins were quantified using BCA as a standard (Beyotime). A total of 40 µg of protein was analyzed by Western blot using monoclonal anti-mouse phosphor-p38MAPK and anti-mouse p38MAPK (1: 1000, Beyotime). Images were analyzed with Quantity One software (Bio-Rad). Phosphorylation levels of p38MAPK were calculated using the equation: grayscale value of p-p38MAPK/grayscale value of p38MAPK.

Statistical analysis

On the basis of testing the homogeneity of variances, differences among groups were compared by ANOVA. Differences between any 2 groups were compared using the least significant difference (LSD) test, while 2 independent samples were compared by t test. Statistical significance was established at a level of P<0.05. All statistical analyses were performed using SPSS version 13.0 software (IBM, Armonk, NY).

Results

IP3-mediated calcium signaling participates in fractalkine-induced [Ca2+]i release in BV-2 cells

First, we investigated whether IP3-mediated calcium signaling is involved in the elevation of induced by fractalkine. Effects were determined with a [Ca2+]i assay using Fura-2 as a probe.
Fractalkine injection lead to thermal hyperalgesia and activated microglia in vivo

Mice in fractalkine-injected groups exhibited thermal hyperalgesia (i.e., decreased thermal nociceptive threshold) at 60, 120, and 240 min after i.c.v. injection; the lowest thermal nociceptive threshold was observed at 120 min. Pretreatment (i.c.v.) with anti-CX3CR1, 2-APB, or SB203580 decreased fractalkine-induced thermal hyperalgesia at 60, 120, and 240 min (Figure 3A).

Immunofluorescence of Iba-1 and GFAP was used to assess microglia and astrocytes, respectively (Figure 3B). In the vehicle group, a few unactivated and branched Iba-1-positive cells were detected; Fractalkine-positive (Figure 3B-A, red), Iba-1-positive cells (Figure 3B-B, green), and overlap (Figure 3B-C) was not observed in the hippocampus. In the fractalkine group, fractalkine-positive (Figure 3B-D, red), Iba-1-positive cells (Figure 3B-E, green), and overlap (Figure 3B-F) was observed, but many activated and rod-like Iba-1-positive cells were observed in the right hippocampus. Notably, fractalkine (Figure 3B-G, red) and GFAP-positive cells (Figure 3B-H, green) and overlap (Figure 3B-I) were not observed. Yellow fluorescence indicated overlap or double labeling. Most fractalkine was co-localized with the microglial-specific marker, Iba-1, rather than the astrocyte-specific marker, GFAP, indicating extrinsic fractalkine can activate microglia and regulate their function.

Fractalkine upregulated pro-inflammatory cytokines and p-p38MAPK protein in vivo

Quantitative RT-PCR analysis showed a significant increase in mRNA levels of IL-1β and TNF-α in the hippocampus after treatment with fractalkine for 6, 12, or 24 h (Figure 4A, 4B). This increase peaked at 6 h and was maintained until 24 h. ELISA showed a persistent increase in levels of IL-1β and TNF-α in the hippocampus after treatment with fractalkine (Figure 4C, 4D).

We next analyzed the influence of IP$_3$-mediated calcium signaling on IL-1β and TNF-α gene expression. Fractalkine markedly upregulated IL-1β and TNF-α mRNA levels (Figure 2E, 2F), but 2-APB significantly downregulated fractalkine-induced increases of IL-1β and TNF-α mRNA levels (Figure 2G, 2H); however, 2-APB alone did not affect IL-1β and TNF-α mRNA expression. These observations were consistent with expression of IL-1β and TNF-α proteins, suggesting that inhibition of IP$_3$-mediated [Ca$^{2+}$]$_i$ elevation by 2-APB can suppress IL-1β and TNF-α protein expression by directly modulating gene transcription.

We investigated whether IP$_3$-mediated calcium signaling is involved in inflammatory responses stimulated by fractalkine in BV-2 cells. Effects of 2-APB on production of IL-1β and TNF-α are depicted in Figure 2. Expression of IL-1β and TNF-α persistently increased in BV-2 cells exposed to fractalkine for 6, 12, and 24 h (Figure 2A, 2B). In contrast, when treated with 2-APB prior to fractalkine addition, production of these factors was greatly decreased at 24 h, but 2-APB alone did not affect IL-1β or TNF-α protein levels (Figure 2C, 2D). These results demonstrate that fractalkine-stimulated IL-1β and TNF-α production can be inhibited by suppressing IP$_3$-mediated [Ca$^{2+}$]$_i$ elevation in BV-2 cells using 2-APB.

We next analyzed the influence of IP$_3$-mediated calcium signaling on IL-1β and TNF-α gene expression. Fractalkine markedly increased [Ca$^{2+}$]$_i$ in BV-2 microglia cells in calcium-containing and calcium-free media, and this effect was dose-dependent. While fractalkine-induced elevation of [Ca$^{2+}$]$_i$ was inhibited by 2-APB, 2-APB alone did not affect [Ca$^{2+}$]$_i$, indicating that IP$_3$-mediated calcium signaling is involved in fractalkine-induced [Ca$^{2+}$]$_i$ elevation.

IP$_3$-mediated calcium signaling is involved in fractalkine-induced inflammatory responses in vitro

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Figure 2. IP₃-mediated calcium signaling is involved in fractalkine-induced inflammatory responses in vitro. (A, B) The increased of IL-1β and TNF-α by exposed to fractalkine persistently. (C, D) The increased of IL-1β and TNF-α was decreased by 2-APB. (E, F) The mRNA of IL-1β and TNF-α were increased by exposed to fractalkine persistently. (G, H) The mRNA of IL-1β and TNF-α were decreased by 2-APB. * P<0.05, compared with '0 nM'; # P<0.05, compared with the control group; & P<0.05, compared with the fractalkine group.

Figure 3. Fractalkine injection lead to thermal hyperalgesia and activated microglia in vivo. (A) The thermal nociceptive threshold of mice after receiving fractalkine, 2-APB, antiCX3CR1, and SB203580. (B) The immunofluorescence of FKN (fractalkine), Iba-1, and GFAP in mice brain tissues without or with fractalkine. * P<0.05, compared with sham group; # P<0.05, compared with vehicle group; & P<0.05, compared with fractalkine group, n=5.
Immunofluorescence showed that extrinsic fractalkine can pretreatment (i.c.v.) with anti-CX3CR1 decreased this effect. Thermal hyperalgesia in mice, but thermal hyperalgesia in the brain. We showed that i.c.v. injection of fractalkine can cause thermal hyperalgesia in mice, but thermal hyperalgesia in the brain is unknown. The present study showed that the intensity of the p-p38MAPK band was significantly increased in the hippocampus after treatment with fractalkine. These increases have been previously reported in spinal cord inflammation and macrophages. Pretreatment with anti-CX3CR1 downregulated IL-1β and TNF-α secretion. p38MAPK is known to be essential for transcription of pro-inflammatory cytokines. Many studies have confirmed activation of p38MAPK signal transduction proteins in spinal microglial cells during the development of neuropathic and inflammatory pain, and intrathecal injection of p38MAPK inhibitors can ameliorate this pain state [18–20]. However, whether phosphorylation of p38 (p-p38) also mediates thermal hyperalgesia induced by fractalkine in the brain is unknown. The present study showed that the intensity of the p-p38MAPK band was significantly increased in the hippocampus after treatment with fractalkine, while pretreatment with anti-CX3CR1, 2-APB, or SB203580 markedly attenuated fractalkine-induced hyperalgesia and downregulated p-p38MAPK, IL-1β, and TNF-α expression. These results indicate that fractalkine can induce activation of microglia and regulate their function, suggesting that hyperalgesia coincides with microglia activation.

As a chemokine, fractalkine has the characteristics of inducing leukocyte migration and facilitating pro-inflammatory cytokine secretion [15–17]. In previous studies, these cytokines were shown to play crucial roles in glia activation evoked by cytokine release, which exacerbate hyperalgesia. Our findings showed a significant increase in mRNA and protein levels of IL-1β and TNF-α in the hippocampus after treatment with fractalkine. These increases have been previously reported in spinal cord inflammation and macrophages. Pretreatment with anti-CX3CR1 downregulated IL-1β and TNF-α secretion. p38MAPK is known to be essential for transcription of pro-inflammatory cytokines. Many studies have confirmed activation of p38MAPK signal transduction proteins in spinal microglial cells during the development of neuropathic and inflammatory pain, and intrathecal injection of p38MAPK inhibitors can ameliorate this pain state [18–20]. However, whether phosphorylation of p38 (p-p38) also mediates thermal hyperalgesia induced by fractalkine in the brain is unknown. The present study showed that the intensity of the p-p38MAPK band was significantly increased in the hippocampus after treatment with fractalkine, while pretreatment with anti-CX3CR1, 2-APB, or SB203580 markedly attenuated fractalkine-induced hyperalgesia and downregulated p-p38MAPK, IL-1β, and TNF-α expression. These results indicate that fractalkine can induce activation of microglia-derived

**Discussion**

Fractalkine is widely distributed throughout the spinal cord and brain tissue [10]. A pivotal role of activation by fractalkine in inflammation during central nervous system diseases has been well described by previous studies [11,12]. In recent years, fractalkine has been investigated as a new player involved in pain control [13,14]. Previous studies have shown that fractalkine administration can lead to allodynia in the spinal cord [4]. However, little is known about fractalkine-induced thermal hyperalgesia in the brain. We showed that i.c.v. injection of fractalkine can cause thermal hyperalgesia in mice, but pretreatment (i.c.v.) with anti-CX3CR1 decreased this effect. Immunofluorescence showed that extrinsic fractalkine can activate microglia and regulate their function, suggesting that hyperalgesia coincides with microglia activation.

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p38MAPK in the hippocampus, causing the release of cytokines and modulation of thermal hyperalgesia. In the pre-experiment of the study, the threshold of mechanical pain in mice was similar among all groups and no obvious change was observed. Thus, only thermal pain threshold was tested in the experiments.

Fractalkine has been shown to lead to increased cytosolic calcium in microglia [21]. Moreover, calcium antagonists are effective pain treatments [22]. In the present study, our results showed that [Ca^{2+}], significantly increased after incubation with fractalkine in calcium-containing and calcium-free media, and this effect was dose-dependent. This suggests an association between pain and cytosolic calcium signaling. Indeed, our findings show a correlation between fractalkine-induced calcium signaling and thermal hyperalgesia, as pretreatment with 2-APB attenuated fractalkine-induced thermal hyperalgesia and downregulated p-p38MAPK, IL-1β, and TNF-α expression. This supports the involvement of IP_3-mediated calcium signaling in fractalkine-induced hyperalgesia. 2-APB, a functional and membrane-permeable D-myo-IP_3 receptor antagonist [23,24], stimulates calcium release at low concentrations (<10 μM) and inhibits it at higher concentrations (up to 42 μM). 2-APB inhibited IP_3-induced Ca^{2+} release from rat cerebellar microsomal preparations without affecting IP_3 binding to its receptor [25].

Our findings showed that treatment with 2-APB prior to fractalkine addition suppressed fractalkine-induced calcium signaling, but [Ca^{2+}], was still higher than in the control group, suggesting that increased [Ca^{2+}], resulted from both extracellular influx and intracellular release, and IP_3-mediated calcium signaling was involved in the fractalkine-induced elevation of [Ca^{2+}]. To investigate whether IP_3-mediated calcium signaling correlates with the inflammation induced by fractalkine, our studies showed that pretreatment with 2-APB attenuated fractalkine-induced IL-1β and TNF-α expression. This indicates that IP_3-mediated calcium signaling may be involved in fractalkine-induced inflammation, based on the observation that 2-APB, an IP_3 antagonist, ameliorated fractalkine-induced changes of the above indices.

Conclusions

Our results suggest IP_3-mediated calcium signaling is associated with fractalkine-induced thermal hyperalgesia. Underlying mechanisms could involve increased calcium signaling to promote p38MAPK protein activation and upregulated pro-inflammatory cytokine secretion by microglia. Collectively, these data show that fractalkine may be a pivotal factor in the development, expansion, and maintenance of hyperalgesia via IP_3-mediated calcium signaling. Given the important role of fractalkine in pain hypersensitivity and neuronal-glial interactions, targeting fractalkine signaling is emerging as an attractive new strategy for treating hyperalgesia.

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Conflict of interests

None.
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