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Research report

Src/p38 MAPK pathway in spinal microglia is involved in mechanical allodynia induced by peri-sciatic administration of recombinant rat TNF-α

Yu-Ying Li[c,1], Xu-Hong Wei[b,1], Zhen-He Lu[a], Jin-Sheng Chen[a], Qiao-Dong Huang[a], Qing-Juan Gong[a,*]

[a] Department of Pain Medicine, The Second Affiliated Hospital, Guangzhou Medical University, 250 Changgang Road East, Guangzhou 510260, China
[b] Department of Physiology and Pain Research Center, Zhongshan Medical School, Sun Yat-Sen University, 74 Zhongshan Rd. 2, Guangzhou 510080, China
[c] School of Medical Technology & Nursing, Shenzhen Polytechnic, Xili Lake, Nanshan District, Shenzhen 518055, China

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ABSTRACT

Our previous work has shown that peri-sciatic administration of recombinant rat TNF-α (rrTNF) induces mechanical allodynia and up-regulation of TNF-α in the spinal dorsal horn of rats; however, the underlying mechanisms remain unknown. In the current study, we found that the levels of phosphorylated Src-family kinases (p-SFKs) and phosphorylated p38 mitogen-activated protein kinase (p-p38 MAPK) were significantly increased in bilateral lumbar spinal dorsal horn on day 3 after rrTNF administration. Double immunofluorescence staining revealed that p-SFKs and p-p38 MAPK were nearly restricted to the microglia. Intrathecal delivery of SFKs inhibitor PP2 or p38 MAPK inhibitor SB203580, started 30 min before rrTNF administration and given once daily thereafter for 7 days, blocked mechanical allodynia in bilateral hind paws and increase of TNF-α expression in the spinal dorsal horn. Moreover, PP2 inhibited the up-regulation of p-p38 MAPK induced by rrTNF. We also found that intrathecal injection of TNF-α neutralization antibody alleviated mechanical allodynia in bilateral hind paws and suppressed up-regulation of p-SFKs and p-p38 MAPK. These results suggest that activation of the SFKs/p38 MAPK pathway in microglia and subsequent TNF-α expression in the spinal dorsal horn may contribute to the mechanical hyperalgesic state induced by peri-sciatic administered rrTNF.

1. Introduction

It has been shown that tumor necrosis factor-alpha (TNF-α) plays a pivotal role in both peripheral and central sensitization on various animal models of pathological pain (Leung and Cahill, 2010). We have previously demonstrated that recombinant rat TNF-α (rrTNF) injected around the healthy sciatic nerve dose-dependently induces mechanical allodynia in bilateral hind paws of rats, lasting for about 20 days (Wei et al., 2007). In clinical settings, there are some patients undergoing pathological pain without any obvious nerve injury, which may be similar to the pain in this model. However, it remains unclear how peri-sciatic administration of rrTNF causes mechanical allodynia.

Src-family kinases (SFKs), a group of non-receptor tyrosine kinases expressed in the central nervous system (CNS), have been implicated in the regulation of inflammatory responses (Skubitz et al., 1997). It has been demonstrated that SFKs are activated in spinal microglia after peripheral nerve injury and that intrathecal administration of the SFKs inhibitor PP2 reverses nerve injury-induced mechanical hypersensitivity (Katsura et al., 2006). Moreover, recent studies have shown that SFKs are also activated in spinal microglia after high-frequency stimulation (HFS) or brain-derived neurotrophic factor (BDNF)-induced long-term potentiation (LTP) (Zhong et al., 2010; Zhou et al., 2011), which is believed to be an attractive cellular model of central sensitization (Sandkühler, 2007). These suggest that SFKs activation in spinal microglia is critical for nerve injury-induced neuropathic pain. However, whether peri-sciatic administered rrTNF can induce activation of SFKs remains unknown.

Many studies have demonstrated that mitogen-activated protein kinases (MAPKs) play important roles in central sensitization and persistent pain states (Ji and Suter, 2007; Ji et al., 2009). There is emerging evidence that p38 MAPK is activated in spinal microglia after peripheral nerve injury or inflammation (Jin et al., 2003;

* Corresponding author at: Department of Pain Medicine, The Second Affiliated Hospital, Guangzhou Medical University, 250 Changgang Road East, Guangzhou, China. Tel.: +86 20 34152379; fax: +86 20 34153611.
E-mail address: gqj01@sohu.com (Q.-J. Gong).
[1] These authors contributed equally to this work.

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Milligan et al., 2003; Svensson et al., 2003; Tsuda et al., 2004, 2005). Several lines of evidence have indicated that SFKs are a point of convergence for various signaling pathways leading to MAPK activation in pathological or normal physiological events (Callera et al., 2005; Kim et al., 2009; Krady et al., 2002; Watanabe et al., 2009). Some studies have shown that PP2 can reverse activation of the MAPK pathway (ERK or p38 MAPK) in spinal microglia (Katsura et al., 2006; Tan et al., 2012). Nevertheless, whether peri-sciatic injected rrTNF-induced mechanical allodynia is regulated by this Src/MAPK signaling pathway in spinal microglia remains to be explored.

In the current study, we demonstrated that following mechanical allodynia induced by rrTNF administration, SFKs and p38 MAPK were activated only in the spinal microglia but not in neurons or astrocytes. We also showed that inhibition of SFKs activation or p38 MAPK activation induced by rrTNF administration produced a striking alleviation of tactile allodynia and decreased TNF-α expression in the spinal dorsal horn. Meanwhile, inhibition of SFKs activation reversed microglial p-p38 MAPK expression. In addition, mechanical allodynia and up-regulation of p-SFKs and p-p38 MAPK induced by rrTNF application were inhibited by intrathecal injection of TNF-α neutralization antibody. Taken together, these results indicate that the Src/p38 MAPK signaling pathway in spinal microglia plays a crucial role in rrTNF-induced mechanical allodynia.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 150–170 g were used. The rats were housed in separated cages and the room was kept at 24 ± 1 °C temperature and 50–60% humidity, under a 12:12 light–dark cycle and with free access to food and water ad libitum. All experimental procedures were approved by the Local Animal Care Committee and were carried out in accordance with the guidelines of the National Institutes of Health on animal care and the ethical guidelines for investigation of experimental pain in conscious animal (Zimmermann, 1983).

2.2. Drugs

rrTNF (R&D Systems, Inc.) was stored as a stock solution of 10 μg/ml at −80 °C, and diluted to 100 pg/ml in 0.1% bovine serum albumin (BSA) in saline immediately before administration. PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine, Calbiochem), PP3 (4-amino-7-phenylpyrazolo[3,4-d]pyrimidine, Calbiochem), and SB203580 [(4-(4-fluorophenyl)-2-(4-methyl-sulfonylphenyl)-5-(4-pyridyl)-1H-imidazole), Sigma] were first dissolved in dimethyl sulfoxide (DMSO) to make a stock concentration of 50 mM, aliquoted in small volumes and stored at −80 °C and diluted with 0.9% saline for intrathecal administration. TNF-α neutralization antibody (TNF-Ab, R&D Systems) were first dissolved as a concentrated stock solution (10 mg/ml) in 0.1% BSA/PBS (bovine serum albumin/phosphate buffer saline), the stock solution was diluted with 0.9% saline to make final concentrations immediately before administration.

2.3. Administration of drugs

Rats were deeply anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.). Special care was paid to prevent infection and to minimize the influence of inflammation. An indwelling peri-sciatic catheter system was developed to allow 2 days of rrTNF administration. Peri-sciatic catheters were constructed as previously described (Wei et al., 2007). Briefly, sterile gel foam was aseptically cut into 15-mm (*L*) × 5-mm (*W*) × 9-mm (*H*) strips and one end was bisected (3.5 mm *W*) to a depth of 1 cm to allow a 4-cm sterile polyethylene (PE-10) tube to be sutured inside. The assembly was implanted around the left sciatic nerve at mid-thigh level. 200 μl rrTNF or vehicle was injected through the PE-10 tube. PP2, PP3, SB203580, DMSO or TNF-Ab, was injected intrathecally (i.t.) started 30 min before each rrTNF administration, once daily thereafter and lasted for 7 days. For intrathecal delivery of PP2, PP3, SB203580, DMSO or TNF-Ab, rats were implanted with i.t. catheters according to the method described previously (Wei et al., 2007). Briefly, a sterile PE-10 tube filled with saline was inserted through L5/L6 intervertebrae space, and the tip of the tube was placed at the spinal lumbar enlargement level. Any rats with hind limb paralysis or paresis after surgery were excluded. Drugs or vehicle were administered in volumes of 10 μl followed by a flush of 7 μl of saline to ensure drugs delivered into the subarachnoid space.

2.4. Behavioral tests

Animals were habituated and basal pain sensitivity was tested before drug administration or surgery. Mechanical sensitivity was assessed with the up-down method described previously (Chaplan et al., 1994; Gong et al., 2010), using a set of von Frey hairs with logarithmically incremental stiffness from 0.41 to 15.14 g (0.41, 0.70, 1.20, 2.04, 3.63, 5.50, 8.51, 15.14 g). The 2.04 g stimulus, in the middle of the series, was applied firstly. In the event of paw withdrawal absence, the next stronger stimulus was chosen. On the contrary, a weaker stimulus was applied. Each stimulus consisted of a 6–8-s application of the von Frey hair to the sciatic innervation area of the hindpaws with a 5-min interval between stimuli. The quick withdrawal or licking of the paw in response to the stimulus was considered a positive response. Three persons performed the behavioral tests and only one of them knew the design of the study.

2.5. Immunohistochemistry

Rats were perfused through the ascending aorta with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2–7.4, 4 °C. The L5 spinal cord segments were removed and postfixed in the same fixative for 3 h and then replaced with 30% sucrose overnight. Transverse free-floating spinal sections (25 μm) were cut in a cryostat (LEICA CM1900) and processed for immunostaining with immunofluorescence according to the method described previously (Gong et al., 2009). All the sections were blocked with 3% donkey serum in 0.3% Triton X-100 for 1 h at room temperature and incubated over 2 nights at 4 °C with rabbit anti-p-SFKs (1:200; Cell Signaling Technology, Beverly, MA) and rabbit anti-p-p38 MAPK (1:200; Cell Signaling Technology, Beverly, MA). The sections were then incubated for 1 h at room temperature with Cy3-conjugated secondary antibody (1:300; Jackson ImmunoResearch, PA). For double immunofluorescence staining, spinal sections were incubated with a mixture of p-SFKs antibody or p-p38 MAPK antibody and monoclonal neuronal-specific nuclear protein (NeuN) (neuronal marker, 1:200; Chemicon), glial fibrillary acidic protein (GFAP) (astrocyte marker, 1:300; Chemicon), and Iba-1 (microglia marker, 1:800; Abcam) over 2 nights at 4 °C, all the above sections were treated by a mixture of FITC- and Cy3-conjugated secondary antibody for 1 h at room temperature. The stained sections were examined with an Olympus IX71 (Olympus Optical, Tokyo, Japan) fluorescence microscope and images were captured with a CCD spot camera.

2.6. Western blotting

The dorsal quadrants of L4–L5 spinal cord on the ipsilateral side were separated from other parts of the spinal cord and processed for western blot according to the method described previously (Xin et al., 2006). The tissues were put immediately into liquid nitrogen, followed by homogenation in 15 mmol/l Tris buffer, PH 7.6 (250 mmol/l sucrose, 1 mM MgCl, 1 mM DTT, 2.5 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 μg/ml leupeptin, 1.25 μg/ml pepatatin, 2.5 μg/ml aprotin, 2 mM sodium pyrophosphate, 0.1 mM $NaVO_4$, 0.5 mM PMSF, and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN)), and was sonicated on ice. The sample was centrifuged at 13,000 × *g* for 15 min at 4 °C to isolate the supernatant containing protein samples. Protein samples were separated by gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). The blots were placed in block buffer for 1 h at room temperature and then incubated with rabbit polyclonal anti-rat TNF-α antibody (1:1000; Abcam, Cambridge, UK) and rabbit polyclonal anti-rat GADPH antibody (1:1000; Cell Signaling Technology, Beverly, MA) overnight at 4 °C. The blots were washed three times for 10 min each with washing buffer and then incubated with horseradish peroxidase-conjugated IgG (Cell Signaling). The membranes were washed three times for 10 min each with washing buffer and enhanced with a chemiluminescence reagent (ECL kit; Amersham, USA). Then the blots were exposed to autoradiographic film.

2.7. Quantification and statistics

The area of p-SFKs-immunoreactive (IR) and p-p38-IR per section was measured in spinal dorsal horn (laminae I–V) using LEICA Qwin V3 digital-image processing system (Germany). A density threshold was set above background level firstly to identify positively stained structure. The area occupied by these structures was measured as positive area. In each spinal cord, every fifth section was picked from a series of consecutive spinal cord sections; and four to six sections for each condition were selected randomly. An average percentage of area of p-SFKs-IR and p-p38-IR relative to the total area of the spinal dorsal horn of the sections was obtained for each animal across the different tissue sections, and then the means ± SE across animals was determined. Six rats were included for each group for quantification of immunohistochemistry.

Differences in changes of values over time were tested using one-way analysis of variance (ANOVA) followed by individual post hoc comparisons (Tukey post hoc tests). For the data of behavioral tests, nonparametric tests were employed in comparison between various testing days. The data were analyzed with Friedman ANOVA for repeated measurements, followed by Wilcoxon matched pairs test when