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Inhibition of protein tyrosine phosphatases in spinal dorsal horn attenuated inflammatory pain by repressing Src signaling

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ABSTRACT

Tyrosine phosphorylation of *N*-methyl-D-aspartate (NMDA) subtype glutamate receptors by Src-family protein tyrosine kinases (SFKs) plays a critical role in spinal sensitization. Besides SFKs, the tyrosine phosphorylation levels of proteins are also determined by protein tyrosine phosphatases (PTPs). However, whether PTPs are involved in spinal nociceptive processing is largely unknown. The present study found that intrathecal application of broad-spectrum PTPs inhibitors orthovanadate or Bpv (phen) generated little effects on the paw withdrawal thresholds of intact rats to Von Frey filament stimuli. Although the basal nociceptive responses didn't require the involvement of PTPs, the mechanical allodynia evoked by intrathecal injection of NMDA was greatly attenuated by orthovanadate and Bpv (phen), suggesting that PTPs activity, once stimulated by NMDA receptors, became essential for spinal sensitization. Biochemical analysis demonstrated that PTPs functioned to activate SFKs member Src and promote Src interaction with NR2B subunit-containing NMDA receptors (NR2B receptors). As a result, PTPs inhibition largely suppressed Src-mediated NR2B phosphorylation at Tyr1472 and reduced the synaptic concentration of NR2B receptors in spinal dorsal horn of NMDA-treated rats. Importantly, intraplantar injection of Complete Freund's Adjuvant (CFA) naturally activated spinal PTPs to initiate Src signaling, because PTPs inhibition significantly repressed Src activity, reduced Src phosphorylation of NR2B, decreased NR2B synaptic accumulation and eventually ameliorated inflammatory pain. These data indicated an important role played by spinal PTPs in inducing Src-dependent NR2B receptor hyperfunction and suggested that PTPs inhibition might represent an effective strategy for the treatment of inflammatory pain.

1. Introduction

Activation of Src-Family Protein Tyrosine Kinases (SFKs) in spinal dorsal horn is essential for the initiation and development of central sensitization after peripheral tissue and nerve injury (Guo et al., 2002; Liu et al., 2008; Yang et al., 2011). The active SFKs integrate multiple intracellular signaling cascades to modulate the nociceptive conveyance and neuronal excitability (Guo et al., 2004; Slack et al., 2008; Yang et al., 2011). One of the best-characterized SFKs substrates involved in pain processing is NMDA subtype glutamate receptors, especially NR2B subunit-containing NMDA receptors (NR2B receptors) (Guo et al., 2002; Liu et al., 2008). Tyrosine phosphorylation by SFKs increases the synaptic accumulation of NR2B receptors (Li et al., 2011; Prybylowski et al., 2005; Yang et al., 2011), boosts NR2B receptor-triggered signaling transduction (Katano et al., 2011; Matsumura et al., 2010), and causes NR2B receptor-dependent neuronal plasticity and pain sensitization (Katano et al., 2011; Liu et al., 2008; Matsumura et al., 2010).

A growing body of evidence has indicated that SFKs-mediated protein tyrosine phosphorylation is also subjected to the control by Protein Tyrosine Phosphatases (PTPs), which dephosphorylate the tyrosine residues on SFKs *per se* or on SFKs substrates (Alonso et al., 2004; Frank et al., 2004; Roskoski, 2005). A fine balance between tyrosine kinases and phosphatases is critical for many intracellular responses. To date, both receptor-like and non-receptor PTPs have been detected in postsynaptic density (PSD), where they form macromolecular complex with signaling proteins, scaffolding proteins and substrates (Lei et al., 2002; Peng et al., 2012; Yang et al., 2011). A subpopulation of PTPs has been shown to dephosphorylate SFKs at the conserved autophosphorylation site within the catalytic motif (corresponding to Tyr418 at Src), leading to SFKs inactivation (Roskoski, 2005). Nevertheless, more PTPs dephosphorylate SFKs at their carboxyl-terminal inhibitory

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tyrosine residue (corresponding to Tyr529 at Src), leading to SFKs activation (Roskoski, 2005). Of particular importance is that PTPs activities are dynamically regulated by a wide range of extracellular stimuli, which either change PTPs expression (den Hertog et al., 2008), or modulate PTPs binding to substrates (den Hertog et al., 2008; Yang et al., 2011). The altered catalytic efficacy of PTPs produces a profound impact on synaptic transmission and plasticity, which correlates with an array of physiological and pathological processes such as learning and memory and Alzheimer disease (Lei et al., 2002; Snyder et al., 2005).

Although more than 100 members of PTPs superfamily have been identified in mammalian tissues (Alonso et al., 2004), the functional significance of PTPs in pain-related spinal dorsal horn remains largely unknown. The present study found that intrathecal application of broad-spectrum PTPs inhibitors orthovanadate and Bpv (phen) alleviated the mechanical allodynia induced by spinal administration of NMDA or intradermal injection of Complete Freund's Adjuvant (CFA). PTPs inhibition operated to repress Src activity and decrease the tyrosine phosphorylation and synaptic accumulation of NR2B receptors in NMDA- and CFA-injected rats, implicating that PTPs contributed to spinal sensitization by evoking Src-dependent NR2B receptor hyperfunction.

2. Materials and methods

2.1. Animals

Male adult Sprague–Dawley rats weighing 200–250 g were purchased from the Experimental Animal Center of Lanzhou University and housed on a 12 h light/dark cycle with free access to food and water. The animals were acclimatized to the testing environments for at least 3 days before any experiments were conducted. The inflammatory pain was induced by subcutaneous injection of Complete Freund's Adjuvant (CFA; 100 μl; Sigma, St. Louis, MO) into the plantar surface of hind paws under light halothane anesthesia. Control animals received identical volumes of saline. All experimental procedures were carried out with the approval of the Animal Care and Use Committee of Lanzhou University. Every effort was made to minimize the number of animals used and their sufferings.

2.2. Drug preparation, intrathecal injection and behavioral tests

To achieve the maximum inhibition of PTPs activities, sodium orthovanadate (Sigma) was activated according to the following procedure (Gordon, 1991). A stock solution of orthovanadate (100 mM) was prepared in water with pH value adjusted to 10. This yellow solution was boiled until it turned colorless. After cooling down at room temperature, the solution was re-adjusted with NaOH to pH 10 and boiled again to be colorless. After three to four cycles of activation procedure, the solution was stabilized at colorless state and then stored at −20 °C. Just before use, the stocking solution was diluted to the desired working concentration. Another PTPs inhibitor, potassium Bisperoxo (1,10-phenanthroline) oxovanadate (V) [bpv(phen); Calbiochem, MA, U.S.A], and NMDA (TCI, Tokyo, Japan) were dissolved in saline. SFKs inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-D] pyrimidine (PP2; Calbiochem) was dissolved in dimethyl sulfoxide, which was diluted with saline before use. The final concentration of dimethyl sulfoxide was less than 0.5%.

All the chemical reagents in 10-μl volume were intrathecally injected by direct lumbar puncture as previously reported (Mestre et al., 1994). Rats were briefly anesthetized with halothane and a 30-gauge needle attached to a 25 μl microsyringe was inserted between L5–L6 vertebrae. A sudden advancement of the needle accompanied by a slight tail flick was used as the indicator for the proper insertion of the needle tip into the subarachnoid space. The drug vehicles, saline and dimethyl sulfoxide, were used as control, which had no discernable effects on the nociceptive behavioral responses after intrathecal application.

The Von Frey test was performed as previously described (Chaplan et al., 1994). In brief, the rats were placed in a cage with a wire mesh floor for at least 30 min to adapt to the environment. A series of calibrated Von Frey filaments (Stoelting, Wood Dale, IL, USA), which increased in force with approximately equal logarithmic value (δ; 0.22), were applied perpendicularly to the plantar surface for 6 s. The pattern of positive and negative responses was converted to 50% threshold according to the following formula: 50% threshold (g) $= (10^{[xf+K\delta]})/10{,}000$, in which X_f represented the value of the final Von Frey filament used and K was the tabular value for the pattern of positive/negative responses. These behavioral tests were conducted blindly by experimenters without knowledge of the manipulations that had been performed by others on the animals.

2.3. Subcellular fractionation

The rats were deeply anesthetized with sodium pentobarbital (30 mg/kg, *i.p.*) and the spinal cords were quickly removed into ice-cold artificial cerebrospinal fluid (ACSF) (119.0 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 26.0 mM $NaHCO_3$, 11.0 mM D-glucose, bubbled with 95% O_2 + 5% CO_2, pH 7.4). The dorsal quadrant of L4–5 spinal cord was dissected out and homogenized in sucrose-containing Lysis Buffer [10.0 mM Tris–HCl, pH 7.6, 320.0 mM sucrose, 5.0 mM EDTA, plus the inhibitors of proteases and phosphatases (5.0 mM EGTA, 10.0 mM NaF, 1.0 mM Na_3VO_4, 1.0 mM phenylmethylsulfonyl fluoride, 1.0 mg/ml each of aprotinin, chymostatin, leupeptin, antipain and pepstatin)]. The homogenates were centrifuged at 1000 × *g* for 10 min at 4 °C to remove the nuclei and large debris (P1). The supernatant was further centrifuged at 10, 000 × *g* for 15 min to yield the crude synaptosomal fraction (P2). The P2 pellet was resuspended and incubated for 15 min in the Lysis Buffer containing 0.5% Triton X-100, and then centrifuged at 32,000 × *g* for 20 min to obtain the synaptosomal membrane fraction (P3). The final P3 was homogenized in SDS sample buffer and boiled at 95 °C for 5 min before processing. We and others have previously demonstrated that P3 fraction is enriched with postsynaptic density (PSD) marker, PSD-95 (Cao et al., 2011; Goebel-Goody et al., 2009; Smith et al., 2006; Yang et al., 2009). The P3 fraction was used instead of the classic PSD fraction because of the limited amount of the starting materials.

To assay the tyrosine phosphorylation of NR2B (Guo et al., 2002), the spinal dorsal horn was homogenized in Radio-Immunoprecipitation Assay (RIPA) buffer (50.0 mM Tris·HCl, pH 8.0, 150.0 mM NaCl, 1.0 mM EDTA, 1.0% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, and proteases/phosphatases inhibitors). After centrifugation at 14,000 × *g* for 10 min, the supernatant was harvested and the protein concentration was measured by using BCA protein assay kit (Pierce, Rockford, IL, USA).

2.4. Immunoprecipitation and co-immunoprecipitation

To assay Src phosphorylation in spinal dorsal horn, the crude synaptosomal fraction (P2) was lysed in RIPA buffer for 30 min at 4 °C (Yang et al., 2011). After centrifugation at 14,000 × *g* for 10 min, the supernatant was incubated overnight with specific monoclonal antibody against the unique N-terminus of Src (Millipore, Temecula, CA, USA) at 4 °C under gentle rotation. Src was immunoprecipitated by incubation with Protein A/G agarose beads for 4 h at 4 °C. The beads were washed with RIPA buffer three times, and boiled in SDS sample buffer to elute proteins.

For co-immunoprecipitation of proteins associated with NR2B, the P2 fraction was extracted in 50.0 mM Tris–HCl, pH 9.0, 1.0% sodium deoxycholate, 10.0 mM EDTA, and proteases/phosphatases inhibitors at 37 °C for 30 min (Huang et al., 2001). This extract was mixed with equal volume of Tris buffer (50.0 mM Tris–HCl, pH 7.4, 150.0 mM NaCl, 1.0% Triton X-100, 0.1% SDS, proteases/phosphatases inhibitors) and then gently rocked overnight at 4 °C. After centrifugation at 10,000 × *g* for 10 min, the supernatant was harvested and incubated with anti-NR2B antibody overnight at 4 °C. The immune complex was isolated by the addition of Protein A/G agarose beads as described above. Non-specific IgG was utilized for immunoprecipitation as negative control.

2.5. Western blot

The equal amounts of protein samples (20 μg) were resolved on SDS-Polyacrylamide Gel Electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk for 30 min at room temperature before incubation overnight with appropriate primary antibody at 4 °C. After three times washes with PBST for 10 min each, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution, goat anti-rabbit and goat anti-mouse; Jackson ImmunoResearch Laboratories, Baltimore, PA, USA) for 60 min at room temperature. The blots were visualized by Enhanced Chemiluminescence (Beyotime Institute of Biotechnology, Jiangsu, China). The primary antibodies used in the present study included: the rabbit polyclonal anti-NR2B antibody (1:1000), rabbit polyclonal anti-NR2A antibody (1:1000), mouse monoclonal anti-Src antibody (1:500) and rabbit polyclonal antibody against phosphorylated NR2B at Tyr 1472 (1:1000) purchased from Millipore; the rabbit polyclonal antibody against phosphorylated Src at Tyr418 (1:500) from Invitrogen (Camarillo, CA, USA); the mouse monoclonal anti-NR1 antibody (1:1000) from BD Pharmingen (San Diego, CA, USA); the rabbit polyclonal antibody against phosphorylated Src at Tyr529 (1:1000) from BioSource (Camarillo, CA, USA), and the mouse monoclonal anti-β-actin antibody (1:600) from Sigma. In some cases, the PVDF membrane was stripped with the stripping buffer (5.0 mM Tris–HCl, pH 6.8, 2.0% SDS, 0.5% β-mercaptoethanol) for 30 min at 60 °C and re-probed with distinct antibodies (Cao et al., 2011; Yang et al., 2011).

2.6. Motor function tests

The reflexes for surface righting, placing/stepping and grasping/climbing were tested to evaluate the influence of intrathecal drug application on the motor functions (Garraway et al., 2007; Park et al., 2009). In the surface righting test, we had the rats to lie on the back and observed whether they could resume the normal upright