Research Report

Effect of sodium ferulate on the hyperalgesia mediated by P2X3 receptor in the neuropathic pain rats

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Abstract
Neuropathic pain is usually persistent and there is no effective treatment. Activation of P2X3 receptor subtype in primary sensory neurons is involved in neuropathic pain. Sodium ferulate (SF) is an active principle from Chinese herbal medicine and has anti-inflammatory activities. This study observed the effects of SF on the hyperalgesia mediated by P2X3 receptor of rats after chronic constriction injury (CCI). Mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were measured and the expression of P2X3 receptor immunoreactivity and protein in dorsal root ganglion (DRG) neurons was analyzed by immunohistochemistry and western blotting. In CCI rats treated with SF, the MWT and TWL were increased compared with CCI rats treated with normal saline. The expression of P2X3 receptor in DRG neurons was increased after CCI. In CCI rats treated with SF, the up-regulated expression of P2X3 receptor in DRG neurons was reduced. SF may reduce the thermal and mechanical hyperalgesia in CCI rat model by decreasing the pain transmitted by primary afferent neurons mediated by P2X3 receptor during the chronic neuropathic pain injury.

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1. Introduction

Intradermally administered ATP elicits pain in humans under normal conditions and enhances inflammatory-mediated pain (Bleehen and Keele, 1977; Burnstock and Wood, 1996; Burnstock, 1997, 2009; Donnelly-Roberts et al., 2008; Hamilton et al., 2000; Khakh et al., 2001; Liu and Salter, 2005). Adenosine-5'-triphosphate (ATP) activates cation-permeable ion channels (P2X receptors) and G-protein-coupled receptors (P2Y receptors) (Burnstock, 2007; Lecca and Abbacchio, 2008). P2X receptor family (P2X1-7) has been cloned (Burnstock, 2006, 2007, 2009). P2X receptors play a crucial role in facilitating pain transmission at peripheral and spinal sites because both sensory neurons as well as spinal cord dorsal horn neurons could be depolarized by ATP using neurons in culture (Chizh and Illes, 2001; Dunn et al., 2003). P2X3 receptor is most highly expressed in a subpopulation of small diameter primary afferent neurons in the pain pathway. Peripheral injections of P2X receptors agonists enhance nociception in animal models of inflammatory pain (Hamilton et al., 2001) and lead to mechanical allodynia in cutaneous pain model (Dussora et al., 2009; Tsuda et al., 2000). P2X3 receptor contributes to transmission of nociceptive pain signals (Burnstock, 2000; Chizh and Illes, 2001; Cockayne et al., 2000; Jarvis et al., 2002;
Kennedy, 2005; Kennedy et al., 2003; North, 2003a,b; Tsuda et al., 2007). The study indicates that keratinocytes are a potential source of ATP that can be released in response to mechanical and thermal stimulation (Bodin and Burnstock, 2001; Lumpkin and Caterina, 2007), which would in turn activate the DRG neuron of the combined keratinocyte–neuron complex through P2X3 receptor (Dussora et al., 2009).

Neuropathic pain can be experienced after nerve injury or as part of diseases that affect peripheral nerve function (Bennett and Xie, 1988; Christoph et al., 2007, Jarvis et al., 2002). P2X3 expression is up-regulated following chronic constriction injury (CCI) of the sciatic nerve (Novakovic et al., 1999), which also provokes an ectopic sensitivity to ATP (Chen et al., 2005). Under acute and chronic pain conditions, ATP release is enhanced, which may lead to an increase in keratinocyte production to promote increased nociceptor sensitivity and action potential generation through P2X3 in cutaneous nociceptors. P2X3 knockout mice show lack of rapidly desensitizing currents induced by ATP and significant reduction in pain reception in response to ATP (Souslova et al., 2000). P2X3 receptor is important in mediating both acute pain and chronic pain (Burnstock and Wood, 1996; Burnstock, 1997, 2006, 2007, 2009; Kennedy et al., 2003; Wirkner et al., 2007). P2X3 receptor thereby constitutes possible target for analgesic drugs.

Sodium ferulate (SF) is an active principle from Chinese herbal medicines, Angelica sinensis, Cimicifuga heracleifolia, Lignsticum chuangxiong. It has been used for treatment of cardiovascular and cerebrovascular diseases (Ozaki, 1992; Liu, 2005; Wang and Ou-Yang, 2005). SF also expresses antioxidant and anti-inflammatory activities (Fang et al., 2006; Liu, 2005). The previous studies showed tetramethylpyrazine, an active principle from Chinese herbal medicine Lignsticum chuangxiong, reduced both acute pain and chronic pain mediated by P2X3 receptor (Liang et al., 2004, 2005; Gao et al., 2008). Our previous works also showed that sodium ferulate reduced the nociceptive sensory facilitation of neuropathic pain injury mediated by P2X3 receptor (Zhang et al., 2008). The present work further investigates the effect of sodium ferulate on the hyperalgesia mediated by P2X3 receptor of the primary sensory afferent after CCI rat and characterizes P2X3 receptor as the pharmacological target of SF for the therapeutic treatment of neuropathic pain injury.

2. Results

2.1. Effect of SF on mechanical hyperalgesia

14 days after operation, the MWT (mechanical hyperalgesia) in CCI rats treated with normal saline group was lower than those in rats treated with normal saline group, rats treated with SF group, sham rats treated with normal saline group, and CCI rats treated with SF group. There was no difference in MWT in rats treated with normal saline group, rats treated with SF group, sham rats treated with normal saline group. 5 days after operation, there were no significant differences in MWT in CCI rats treated with SF group compared with rats treated with normal saline group (p > 0.05). 7 days after operation, the data in CCI rats treated with SF group were higher than those in CCI rats treated with normal saline group (p < 0.01) (Fig. 1).

2.2. Effect of SF on thermal hyperalgesia

No significant difference in TWL (thermal hyperalgesia) had been observed in rats treated with normal saline group compared with rats treated with SF group and sham rats treated with normal saline group (p > 0.05). At 9 days after operation, the results showed that TWLs in CCI rats treated with normal saline group and CCI rats treated with SF group were lower than that in rats treated with normal saline group [F(4, 26) = 15.234; p < 0.01], and at 11 days after operation, TWL in CCI rats treated with SF group was higher than that in CCI rats treated with normal saline group (p < 0.05) (Fig. 2).

2.3. Effect of SF on the expression of P2X3 immunoreactivity

P2X3 receptor expression in the L4 and L5 DRG neurons was observed by immunohistochemistry. The stain values of P2X3 expression were 103.367 ± 2.606 for rats treated with normal saline group, 105.573 ± 2.881 for sham rats treated with normal saline group, 115.477 ± 7.409 for CCI rats treated with normal saline group, 107.073 ± 5.214 for rats treated with SF group, and 106.777 ± 2.528 for rats treated with SF group, respectively (n = 6 for each group). No difference was found in the intensity of P2X3 receptor expression of DRG neurons among rats treated with normal saline group, sham rats treated with normal saline group, rats treated with normal saline group, rats treated with SF group (p > 0.05). The gray scale of P2X3 receptor expression in CCI rats was higher than those in rats treated with normal saline group (p < 0.01) (Fig. 1).

Fig. 1 – Effect of SF on mechanical withdrawal threshold (MTL). MWT in CCI rats treated with normal saline group (CCI+NS group) was lower than those in rats treated with normal saline group (NS group), rats treated with SF group (SF group), sham rats treated with normal saline group (sham+NS group), and CCI rats treated with SF group (CCI+SF group). There was no difference in MWT in rats treated with normal saline group, rats treated with SF group, sham rats treated with normal saline group. MWT in CCI rats treated with SF group were higher than that in CCI rats treated with normal saline group (n = 6 per group. Data present as mean ± SEM. *p < 0.01 as compared to group sham + NS, #p < 0.01 as compared to group CCI+NS).
treated with normal saline group was significantly higher than those in other groups \( [F(4, 73)=4.447, p=0.01] \). The intensity of P2X3 receptor expression in CCI rats treated with SF group was lower than that in CCI rats treated with normal saline group \( (p<0.01) \) (Fig. 3).

2.4. Effect of SF on the P2X3 protein level

P2X3 expression in protein level was analyzed by western blotting. The gray value of P2X3 expression in CCI rats treated with normal saline group was higher than those in other groups \( [F(4, 26)=12.469, p<0.01] \). In CCI rats treated with SF group, the gray value of P2X3 expression was lower than that in CCI rats treated with normal saline group with statistically significant difference \( (p<0.01) \) (Fig. 4).

3. Discussion

The neuropathic pain is proposed as “pain initiated or caused by a primary lesion or dysfunction of the nervous system” (Backonja 2003). Neuropathic pain due to nerve injury is a prevalent condition, for which currently there is no effective treatment (Przewlocki and Przewlocka, 2005; Saarto and Wiffen, 2007; Tha’n et al., 2007). Neuropathic pain often has spontaneous pain, allodynia (pain response to normally innocuous stimuli), and hyperalgesia (aggravated pain evoked by noxious stimuli) (Bennett and Xie, 1988; Christoph et al., 2007; Fujita et al., 2007). CCI is a rat model of painful peripheral mononeuropathy (Bennett and Xie, 1988; Gao et al., 2008; Novakovic et al., 1999). Our results showed that SF reduced the thermal hyperalgesia and mechanical hyperalgesia of CCI rats. SF may relieve neuropathic pain. Injury to peripheral nerves arising from disease or physical trauma leads to abnormal “neuropathic” pain states. In animal models, this long-lasting syndrome is principally characterized by the emergence of tactile allodynia and thermal hyperalgesia, conditions reminiscent of human neuropathic pain states (Merskrey, 1994; Payne, 1989). SF may decrease the injury of peripheral nerves. What is the mechanism of SF for reducing hyperalgesia of CCI rats?

P2X receptors are localized in the central and peripheral nervous systems. P2X receptors are ion channels that were activated by extracellular ATP (Burnstock, 2007; North, 2002)
and play the important role in excitatory nociceptive processing (Liu and Salter, 2005; Burnstock, 2006). ATP can facilitate the nociceptive sensitivity after tissue injury. The number of P2X3 receptor immunoreactive in DRG neurons was increased after a chronic constriction injury of the sciatic nerve (Burnstock, 2000, 2006, 2007; Chizh and Illes, 2001; Kennedy et al., 2003; Novakovic et al., 1999; Tsuda et al., 2007; Tsuzuki et al., 2003). Activation of P2X3 receptor subtype in primary sensory neurons is involved in neuropathic pain (Tsuda et al., 2000). P2X3 selective antagonist A-317491 can reduce both thermal hyperalgesia and tactile allodynia in the CCI neuropathic pain model (Jarvis et al., 2002). Therefore, blockade of P2X3 receptor can reduce nociception mediated by both small and larger diameter sensory neurons in chronic pain states. An up-regulation of P2X3 receptors in the soma leads to an increase in P2X3 receptor expression at both terminals (North, 2003a,b). An increase in P2X3 receptor expression at peripheral terminals results in the exaggerated pain behavior induced by an injection of P2X3 agonist α,β-meATP. Present studies showed that the thermal hyperalgesia and mechanical hyperalgesia in CCI rats was higher than those in control rats. The effects of SF on the thermal hyperalgesia and mechanical hyperalgesia of CCI rats may be involved in P2X3 receptor in DRG. The present works also observe the up-regulated expression of P2X3 receptor in rat DRG neurons after CCI injury. SF reduced the expression of P2X3 receptor immunoreactive in DRG neurons of CCI rats. SF may decrease the signal transmission of chronic pain mediated by P2X3 receptor in primary afferent nerve. SF administrated by intraperitoneal injection decreased the up-regulated expression of P2X3 protein in CCI rats. It further supports that SF might inhibit the activation of P2X3 receptor during chronic pain. SF may decrease P2X3 receptor expression in DRG neurons to reduce the chronic neuropathic pain injury and alleviate the exaggerated pain behavior of neuropathic pain rats.

4. Conclusion

In summary, SF can decrease P2X3 receptor expression of DRG neurons and the primary afferent transmission of P2X3 receptor activation in CCI rats to increase the threshold of thermal or mechanical hypersensitivity in CCI rats.

5. Experimental procedures

5.1. Animals and drugs

Male Sprague–Dawley rats (200±20 g) were provided by the Center of Laboratory Animal Science of Nanchang University. Use of the animals was reviewed and approved by the Animal Care and Use Committee of Medical College of Nanchang University. The IASP (The International Association for the Study of Pain)’s ethical guidelines for pain research in animals were followed. The animals were housed in plastic boxes in a group of three at 21–25 °C. Rats were divided into five groups randomly. Each group had six rats. The CCI rat model was as the neuropathy pain model (Bennett and Xie, 1988; Gao et al., 2008; Novakovic et al., 1999). Five groups were NS group (rats treated with normal saline group), SF group (rats treated with SF group), sham+NS group (sham rats treated with normal saline group), CCI+SF group (CCI rats treated with SF group), and CCI+NS group (CCI rats treated with normal saline group), respectively. SF (100 mg/kg, Suzhou Lixing Medical Company) was administrated by intraperitoneal injection in rats after
CCI operation. Adenosine-5′-triphosphate disodium (ATP) was obtained from Sigma. All drugs were dissolved and diluted in 0.9% saline.

5.2. CCI model

The CCI model was prepared as a neuropathy pain model (Bennett and Xie, 1988; Gao et al., 2008; Novakovic et al., 1999). Each rat was anesthetized with pentobarbital sodium (Shanghai Xingya Medical Company, Batch No. 050101) during surgical procedures. The sciatic nerve was exposed at the middle level of rat thigh. Proximal to the sciatic trifurcation, four ligatures (4-0 chronic gut) were performed loosely with microsurgical techniques. Intervals among ligatures were about 1 mm. The same investigator created CCI animals to avoid variation. In the sham-operated group the nerve was left untouched. Rats without the operation were used as normal controls. The nociception of rats was assessed on days 0, 1, 3, 5, 7, 9, 11, and 14 after CCI by the observation of spontaneous pain behavior, by measurement of changes in paw withdrawal latency on thermal stimulation, and paw withdrawal threshold using von Frey filaments to assess mechanical hyperalgesia.

5.3. Measurement of MWT

Noxious-pressure stimulation was used to evaluate mechanical hyperalgesia. Unrestrained rats were placed inside a clear plastic chamber (22 cm × 12 cm × 22 cm) on a stainless-steel mesh floor and allowed to acclimate. Withdrawal responses to mechanical stimulation were determined using calibrated von Frey filaments (BME-403, Tianjin) applied through an opening in the stainless-steel mesh floor of the cage (grid 1 cm × 1 cm) to an area adjacent to the paw. Each von Frey filament was applied once starting with 0.0044 g and continuing until a withdrawal response occurred or the force reached 1.4791 g (the cut-off value). The hind paws were tested alternately at 2-min intervals. Three measurements were taken using the up and down method on each side and the lowest value was taken as the threshold value. The filaments were applied in the order of increasing bending force (0.13, 0.20, 0.33, 0.60, 1.30, 3.60, 5.00, 7.30, 9.90, and 20.1 g), with each applied 10 times at intervals of 15 s to different parts of the midplantar glabrous skin. The strength of the filaments in the series that evoked at least five positive response among the ten trials was designated the pain threshold. If the value of the threshold was beyond 20.1 g, the threshold was still recorded as 20.1 g.

5.4. Measurement of TWL

Noxious heat stimulation for assessment of thermal hyperalgesia was applied by the Thermal Paw Stimulation System (BME-410C, Tianjin). Rats were placed in a transparent, square, bottomless acrylic box (22 cm × 12 cm × 22 cm) on a glass plate under which a light was located. Radiant heat stimuli were applied by directing a beam of light at the foot pad of each hind paw through the glass plate. The light beam was turned off automatically when the rat lifted the paw, allowing the measurement of time between the beginning of the light beam and the elevation of the foot. This time was designated as the paw withdrawal latency. The hind paws were tested alternately at 5-min intervals. The cut-off time for the heat stimulation was 25 s.

5.5. Immunohistochemistry for P2X3 receptor immunoreactivity

14 days after operation of CCI, the animals were anesthetized with pentobarbital sodium and perfused transcardially with 200 ml of normal saline, followed by 200–300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The L4 and L5 lumbar DRG were dissected, postfixed for 8 h, and transferred into 30% sucrose in 0.1 M PB overnight, and then were cut with a cryostat. One tissue from six nonadjacent sections from the L4 and L5 segments were selected randomly, and P2X3 receptor expression in L4 and L5 lumbar DRG was observed by immunohistochemistry. P2X3 antibody was bought from Chemicon International Company of America. The gray scale of P2X3 receptor expression was determined with an image analysis system (HMIAS-2000).

5.6. Western blotting for P2X3 receptor protein

14 days after operation of CCI, the animals were anesthetized with pentobarbital sodium and killed. The DRG ganglia were isolated immediately and flushed with ice-cold PB. Ganglia were homogenized by mechanical disruption in lysis buffer (50 mmol/L TrisCl, pH 8.0, 150 mmol/L NaCl, 0.1% dodecyl sodium sulfate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL Aprotinin) and incubated on ice for 40 min. Homogenate was then pelleted at 6000 g for 10 min and supernatant was collected.

Using Lowry method, the quantity of total protein was determined in the supernatant. After diluted with sample buffer (100 mmol/L TrisCl, 200 mmol/L dithiothreitol, 4% sodium deodecylsulfate (SDS), 0.2% bromophenol blue, 20% glycerol) and heated to 95 °C for 10 min, samples containing equal amounts of protein (20 μg) were separated by SDS-polyacrylamide gel electrophoresis by using Bio-Rad system and 12% gel. The electrophoretic transfer onto nitrocellulose (NC) membrane was used in the same system, the membrane was blocked with 5% non-fat dry milk in 25 mmol/L Tris-buffered saline, pH 7.2, plus 0.1% Tween 20 (TBST) for 3 h at room temperature, followed by incubation with primary antisera (rabbit anti-P2X3, 1:1000; Santa Cruz Biotech. Inc.) in the same buffer for overnight at 4 °C. The membrane was then washed in TBST, and incubated with secondary antibody, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (1:3000, Beijing Zhongshan Biotech. CO.) in the same buffer for 1 h at room temperature. After a final wash in TBST, and then using the enhanced chemiluminescence (ECL) kit (Shanghai Pufei Biotech. CO.), chemiluminescent signals were collected on autoradiography film. The quantity of band intensity was carried out using Image Quant software.

5.7. Data analysis

Data for the experiments are expressed as mean±S.E.M. Differences between treatment groups were analyzed by Student’s t-test or, where appropriate, ANOVA followed by
Dunnett’s post hoc test for multiple comparisons. P-value < 0.05 was considered to be statistically significant. Statistical analyses of the data were performed on computer.

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