Vatalanib decrease the positive interaction of VEGF receptor-2 and P2X<sub>2/3</sub> receptor in chronic constriction injury rats

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**A B S T R A C T**

Neuropathic pain can arise from a lesion affecting the peripheral nervous system. Selective P2X<sub>3</sub> and P2X<sub>2/3</sub> receptors’ antagonists effectively reduce neuropathic pain. VEGF inhibitors are effective for pain relief. The present study investigated the effects of Vatalanib (VEGF receptor-2 (VEGFR-2) inhibitor) on the neuropathic pain to address the interaction of VEGFR-2 and P2X<sub>2/3</sub> receptor in dorsal root ganglia of chronic constriction injury (CCI) rats. Neuropathic pain symptoms following CCI are similar to most peripheral lesions as assessed by the Neuropathic Pain Symptom Inventory. Sprague-Dawley rats were randomly divided into sham group, CCI group and CCI rats treated with Vatalanib group. Mechanical withdrawal threshold and thermal withdrawal latency were measured. Co-expression of VEGFR-2 and P2X<sub>2</sub> or P2X<sub>3</sub> in L4-6 dorsal root ganglia (DRG) was detected by double-label immunofluorescence. The modulation effect of VEGF on P2X<sub>2/3</sub> receptor agonist-activated currents in freshly isolated DRG neurons of rats both of sham and CCI rats was recorded by whole-cell patch-clamp technique. The mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) in CCI group were lower than those in sham group (p < 0.05). MWT and TWL in CCI rats treated with Vatalanib group were increased compared with those in CCI group (p < 0.05). VEGFR-2 and P2X<sub>2</sub>, or P2X<sub>3</sub> receptors were co-expressed in the cytoplasm and surface membranes of DRG. The co-expression of VEGFR-2 and P2X<sub>2</sub> or P2X<sub>3</sub> receptor in CCI group exhibited more intense staining than those in sham group and CCI rats treated with Vatalanib group, respectively. VEGF enhanced the amplitude of ATP and αβ-meATP -activated currents of both sham and CCI rats. Increment effects of VEGF on ATP and αβ-meATP -activated currents in CCI rats were higher than those in sham rats. Both ATP (100 μM) and αβ-meATP (10 μM) -activated currents enhanced by VEGF (1 nM) were significantly blocked by Vatalanib (1 μM, an inhibitor of VEGF receptors). The stain values of VEGFR-2, P2X<sub>2</sub> and P2X<sub>3</sub> protein expression in L4/5 DRG of CCI treated with Vatalanib group were significantly decreased compared with those in CCI group (p < 0.01). Vatalanib can alleviate chronic neuropathic pain by decreasing the activation of VEGF on VEGFR-2 and the positive interaction between the up-regulated VEGFR-2 and P2X<sub>2/3</sub> receptors in the neuropathic pain signaling.

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1. Introduction

Neuropathic pain often has a major impact on a patient’s quality of life since it may be severe, chronic, and difficult to treat. Neuropathic pain has been recently redefined by the Assessment Committee of the Neuropathic Pain Special Interest Group (NeuPSIG) as “pain arising as a direct consequence of a lesion or disease affecting the somatosensory system” (Haanpää et al., 2011; Treede et al., 2008). This implies that neuropathic pain can arise from a lesion affecting either the peripheral or the central nervous system. The chronic constriction injury (CCI) rats were made as the neuropathic pain model (Gao et al., 2011; Xu et al., 2011; Zhang et al., 2008b, 2010). Behavior of CCI rats appeared analogous to human neuropathic pain conditions (Wang and Wang, 2003). Despite the location of the lesion within the nerve root, neuropathic pain symptoms following CCI are similar to most peripheral lesions as assessed by the Neuropathic Pain Symptom Inventory. The intradermal administration of ATP elicits pain in healthy humans and enhances inflammation-mediated pain (Burnstock, 2006, 2007). ATP also activates sensory neurons, including those with cell bodies in the dorsal root ganglia (DRG). Nucleotides signal through the P2 family of receptors, which includes both ATP-gated ion channels (the P2X purinergic receptors) and G protein-coupled receptors (P2Y receptors) with more diverse agonist profiles. Extracellular ATP stimulates sensory neurons mainly through interaction with P2X receptors (Gao et al., 2010, 2011; Li et al., 2011; Liang et al., 2012b).
2004; Liang et al., 2010; Shao et al., 2007; Sperlágh et al., 2006; Wan et al., 2010; Wang et al., 2008, 2009; Vizi et al., 2007; Vizi et al., 1992; Vizi and Sperlágh, 1999; Zhang et al., 2007, 2008a). The predominant ATP-gated current in sensory neurons appears to be mediated by channels composed of P2X3 homomers and P2X2/3 heteromers (Li et al., 2011; Liang et al., 2004; Liang et al., 2010; Sperlágh et al., 2006; Vizi et al., 2007; Vizi et al., 1992; Vizi and Sperlágh, 1999; Xu et al., 2009; Xu et al., 2011; Zhang et al., 2010). In experimental pain models, selective P2X3 and P2X2/3 receptor antagonists effectively reduce neuropathic pain (Jarus et al., 2002; McGaraughty et al., 2003).

Vascular endothelial growth factor (VEGF) is a potent regulator of vascular function through its control of multiple endothelial cell functions. Previous studies in our laboratory had observed that the immunoreactivity of VEGF in dorsal root ganglia (DRG) of CCI rats was enhanced (Lin et al., 2010). Treated with Anti-rVEGF antibody (injected to intrathecally the CCI rats), thermal withdrawal latency (TWL) and the mechanical withdrawal threshold (MWT) of the CCI rats were increased, and the expression of VEGF receptor (VEGFR-2), P2X3 and P2X2/3 receptor of DRG in the CCI rats were reduced. There are VEGF-activated cation currents in microvascular endothelial cells (Cheng et al., 2006). This study wants to observe the effects of VEGF on P2X3 and P2X2/3 agonist-activated currents in freshly isolated DRG neurons of both sham and CCI rats and the effects of Vatalanib (an inhibitor of VEGF receptors) on VEGF activating VEGFR-2 by whole-cell patch-clamp technique. The relationship between VEGFR-2 and P2X3, P2X2 receptors in DRG was studied by double-label immunofluorescence. The objective of this study is to investigate the interaction between VEGFR-2 and P2X2/3 heteromer receptors on DRG sensory neurons in neuropathic pain state and explore a new target for preventing and treating neuropathic pain.

2. Materials and methods

2.1. Materials

Vatalanib (PTK787/ZK 222584, a potent inhibitor of the VEGF receptor) was the product of Selleck Chemicals. Adenosine 5'-triphosphate disodium (ATP) and αβ-methylene-ATP (αβ-mATP) were obtained from Sigma. All drugs were dissolved and diluted in 0.9% saline. Rabbit anti-P2X3 and rabbit anti-P2X2 polyclonal antibody were bought from Chemicon International Company of America. VEGFR-2 antibody was bought from Thermo Fisher Scientific (Fremont, CA, USA). Other antibodies and reagents were ascribed as following.

2.2. Experimental animals

Male Sprague-Dawley rats (180–230 g) were provided by the Center of Laboratory Animal Science of Nanchang University. The animals were housed in plastic boxes in a group of three at 21–25 °C. Use of the animals was reviewed and approved by the Animal Care and Use Committee of Medical College of Nanchang University. The IASP's ethical guidelines for pain research in animals were followed. All animals were treated in accordance with ARVO Statement for the use of Animals in Ophthalmic and Vision Research in China.

The chronic constriction injury (CCI) rats were made as the neuropathic pain model. Rats were randomly divided into three groups: sham group, CCI group and CCI rats treated with Vatalanib group (Vatalanib group) (n = 6 for each group). Vatalanib (100 μM) in Vatalanib group and phosphate-buffer saline (PBS) in Sham group and CCI group were intrathecally injected to rats by puncture every 2 days intervals (total six times), respectively. All drugs were diluted in PBS and 15 μl of drugs was injected every time. The injection was initiated 2 h after operation to ensure all rats were awake completely. Then about 30 min later, behavior of all rats were observed.

2.3. Chronic constriction injury (CCI) model

CCI rat model was prepared (Bennett and Xie, 1988; Gao et al., 2008; Novakovic et al., 1999; Zhang et al., 2008b, 2010). Each rat was anesthetized with Nembutal [35 mg/kg intraperitoneally (i.p.)] 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 between every two ligatures were about 1 mm. The same investigator created CCI animals to avoid variation. In the sham-operated group, the sciatic nerve was exposed using the same procedure but not ligatured by chronic gut. We assessed nociception on day 0, 1, 4, 7, 10, 13 after CCI by the observation of spontaneous pain behavior, by measurement of changes of latency in paw withdrawal on thermal stimulation, and of paw withdrawal threshold using von Frey filaments to assess mechanical hyperalgesia.

2.4. Measurement of mechanical withdrawal threshold (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 0.13 g and continuing until a withdrawal response occurred or the force reached 20.1 g (the cut-off value). The right hind paws were tested alternately at 2 min intervals. Measurements of three times were taken by using the method up and down on each side and the mean of the three determinations was taken as the threshold values. 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, 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 responses among the 10 trials was designated the pain threshold.

2.5. Measurement of thermal withdrawal latency (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 30 s.

2.6. Immunofluorescence double labeling

DRG isolated from rats of three groups was washed with phosphate-buffered saline (PBS). DRG was dissected immediately and fixed in 4% paraformaldehyde (PFA) for 24 h at room temperature. Then they were transferred to 20% sucrose for dehydration at 4 °C.
overnight. Tissues were sectioned at 10 μm at a cryostat and put into glass slide covered with poly-d-lysine to be stored in refrigerator at –20°C for pre-emergency. After washed with PBS for three times, the preparations were preincubated with 10% normal donkey serum (NDS; Jackson ImmunoResearch Inc, West Grove PA, USA) for 40 min in a moist chamber at 37°C. The sections were then incubated with goat anti-P2X2 and rabbit anti-P2X3 (1:500 dilutions; CHERMICON International, Inc. USA) diluted in PBS for overnight at 4°C. After three rinses in PBS, the sections were then incubated with the fluorescent donkey anti-goat FITC and donkey anti-rabbit TRITC secondary antibody (1:200 dilutions; Jackson ImmunoResearch, PA, USA) in the dark at 37°C for 40 min. The prepared sections were given further three times washes in PBS before they were mounted in glycerol and then coverslipped. After these steps, the sections were examined with fluorescence microscopy. Image-Pro Plus 6.0 image analysis software (Media Cybernetics Inc.) was used to quantify P2X2 and P2X3.

To specify the immunoreactivity of P2X2 and P2X3 antibodies, as a negative control, normal donkey serum and PBS were substituted for the primary antibody.

2.7. Isolation of DRG neurons

The rats from sham group and CCI group were decapitated after anesthetized with urethane [1.2 g/kg, intraperitoneally (i.p.)]. The thoracic and lumbar segments of the vertebral column were dissected and longitudinally divided into two halves along the median lines on both dorsal and ventral sides. The L4 and L5 lumbar DRGs together with dorsal and ventral roots and attached spinal nerves were taken out from the inner side of each half of the dissected vertebrae with fine dissecting forceps and transferred immediately into Dulbecco’s modified Eagle’s medium (DMEM, Sigma) at pH 7.4 and 340 mOsmol/kg. After the removal of attached nerves and surrounding connective tissues, the DRGs were minced with dissecting spring scissors and incubated with trypsin (0.5 mg/ml; type III, Sigma), collagenase (1.0 mg/ml; type IA, Sigma) and Dnase (0.1 mg/ml; type IV, Sigma) in 5 ml DMEM at 35°C in a shaking bath for 35–40 min, after which soybean trypsin inhibitor (1.25 mg/ml; type II-S, Sigma) was added to stop the enzymatic digestion. The isolated neurons were transferred into a 35 mm culture dish and kept still for 30 min. Experiments were performed at room temperature (20–30°C) (Zhang et al., 2008b).

2.8. Electrophysiological recordings

The whole-cell patch-clamp recording (Li et al., 2010; Liang et al., 2005; Zhang et al., 2008b) was carried out using a patch/whole cell clamp amplifier (CEZ-2400, Nihon Kohden). The micropipette was filled with internal solution containing (in mM): KCl 140, MgCl2 2, HEPES 10, EGTA 11, ATP 5; its osmolarity was adjusted to 340 mOsmol/kg with sucrose and pH adjusted to 7.4 with KOH. The external solution contained (mM): NaCl 150, KCl 5, CaCl2 2.5, MgCl2 1, HEPES 10, D-glucose 10; its osmolarity was adjusted to 340 mOsm with sucrose, pH was adjusted to 7.4 with NaOH. The resistance of recording electrodes were in the range of 1–4 MΩ. A small patch of membrane underneath the tip of the pipette was aspirated to form a seal (1–10 GΩ) and then a more negative pressure was applied to rupture it thus a whole-cell mode was established. Membrane currents were filtered at 1 kHz (−3 dB), data were recorded by a pen recorder (LMS-2B, Chengdu). The holding potential was set at −60 mV. The drugs were dissolved in external solution and delivered by gravity flow from an array of tubules (500 μm O.D., 200 μm I.D.) connected to a series of independent reservoirs. The distance from the tubule mouth to the cell examined was approximately 100 μm. Rapid solution-exchange was achieved by shifting the tubules horizontally with a micromanipulator.

2.9. Western blotting

Animals were anesthetized and tissue collection was performed as described above, except tissues were quick frozen in tubes on dry ice during collection (Gao et al., 2011; Lin et al., 2010; Xu et al., 2012). In brief, on the 14th day after operation, animals were anesthetized with pentobarbital sodium, ipsilateral L4/L5 DRG nerves were dissected. DRG were isolated immediately and rinsed in ice-cold phosphate-buffered saline (PBS). Ganglia were homogenized by mechanical disruption in lysis buffer containing the following; 50 mM TrisCl, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 0.02% sodium deoxycholate, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml Aprotinin, and incubated on ice for 30 min. Homogenate was then centrifuged at 12,000 rpm for 10 min and supernatant was collected. Using Lowry method, the quantity of total protein was determined in the supernatant. After diluted with loading buffer (250 mM Tris–Cl, 200 mM Dithiothreitol, 10% sodium dodecyl sulfate (SDS), 0.5% Bromophenol Blue, 50% Glycerol) and heated to 95°C for 10 min, samples containing equal amounts of protein (20 μg) were separated by SDS–polyacrylamide gel electrophoresis in 10% gel by using Bio-Rad system, protein in gel was transferred onto nitrocellulose (NC) membrane by electrophoretic transfer using the same system, the membrane was blocked with 5% non-fat dry milk in 25 mM Tris buffered saline, pH 7.2, plus 0.05% Tween 20 (TBST) for 3 h at room temperature, and incubated with primary antibodies in blocking buffer overnight at 4°C. The membranes were washed (twice with TBST) and incubated (1 h, room temperature) with horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG (1:3000, Beijing Zhongshan Biotech Co.) in blocking buffer. After another wash cycle, labeled proteins were visualized by enhanced chemiluminescence on high-performance film (Shanghai Pufei Biotech Co.). Chemiluminescent signals were collected on autoradiography film, and the quantity of band intensity was carried out using Image Pro Plus software. The primary antibodies and dilutions used were the following: mouse monoclonal anti-VEGFR-2 (1:100) and mouse monoclonal β-actin (1:1000, Beijing Zhongshan Biotech Co., China).

3. Results

3.1. Effects of Vatalanib on mechanical hyperalgesia of CCI rats

At day 7 after operation, the mechanical withdrawal threshold (MWT) in CCI group was lower than that in sham group (p < 0.05), and constantly exist from day 7 to day 13. MWT in CCI rats treated with Vatalanib group was higher than that in CCI group at days 7–13 (p < 0.05). MWT in CCI rats treated with Vatalanib group was no statistical difference compared with that in sham group from day 1 to day 13 (p > 0.05) (Fig. 1).

3.2. Effects of Vatalanib on thermal hyperalgesia of CCI rats

At day 1 after operation, the thermal withdrawal latency (TWL) in CCI group was lower than that in sham group (p < 0.05), and there is extremely significantly difference from day 3 to day 13 (p < 0.01). TWL in CCI rats treated with Vatalanib group was higher
Effects of Vatalanib on the expression of P2X2 or P2X3 and VEGFR-2 receptors in CCI group exhibited more intense staining than those in sham group and CCI rats treated with Vatalanib group, respectively (Figs. 3 and 4).

3.4. Effects of VEGF on ATP and α,β-meATP-activated currents with or without CCI

The amplitude of ATP and α,β-meATP-activated currents in CCI rats was higher than that in sham rats (Fig. 5A1). VEGF (1 nM) enhanced the amplitude of ATP and α,β-meATP-activated currents of both sham and CCI rats (Fig. 5A2). The amplitude of ATP and α,β-meATP-activated currents enhanced by VEGF in the DRG neurons of sham rats was lower than that in CCI rats (Fig. 5A2). When VEGF (0.1–1000 nM) was applied for 30–60 s prior to application of ATP (100 μM), an augmentation of ATP current (I_{ATP}) in DRG neurons of CCI rats was increased in comparison with that in sham rats (Fig. 5B). Levels of I_{ATP} (100 μM) enhanced by VEGF (0.1, 1, 10, 100 and 1000 nM) were 10.99 ± 5.27% (n = 9), 20.20 ± 3.29% (n = 7), 38.82 ± 8.54% (n = 7), 75.76 ± 6.35% (n = 7) and 80.46 ± 4.89% (n = 7), respectively in CCI rats (Fig. 5B). α,β-MeATP (10 μM) activated currents in DRG neurons of CCI rats were also increased by VEGF (1 nM) to 43.85 ± 6.20% (n = 8).

3.5. Effects of Vatalanib on ATP- and α,β-meATP-activated currents enhanced by VEGF in CCI rats

Current traces demonstrated that both ATP (100 μM)-activated and α,β-meATP (10 μM)-activated currents enhanced by VEGF (1 nM) in DRG neurons of CCI rats were significantly blocked by Vatalanib (1 μM, an inhibitor of VEGF receptors) (Fig. 6A), and almost completely blocked by 10 μM Vatalanib (Fig. 6A). The dose-response curves for ATP-activated current enhanced by VEGF (1 nM) in DRG neurons of CCI rats with or without pretreatment of Vatalanib were showed in Fig. 6B. All responses were normalized to the current induced by 100 μM ATP plus VEGF with pretreatment of Vatalanib. Vatalanib shifted the concentration–response curve of I_{ATP} enhanced by VEGF (1 nM) downward markedly (Fig. 6B). The amplitude of ATP-activated current enhanced by VEGF at maximum concentration was decreased by 74.8%, while the threshold value remained unchanged. The K_{d} values (ATP concentration producing 50% of the maximal response current) for with and without pre-application of Vatalanib were around 64 μM and 75 μM, respectively.

3.6. Effects of Vatalanib on the expression of VEGFR-2, P2X3 and P2X2 protein in L4/5 DRG of CCI rats

The expression of VEGF-2 protein in L4/5 DRG was detected by western blotting. The stain values of VEGFR-2 protein expression in L4/5 DRG of CCI group were significantly enhanced compared with those in sham group (p < 0.01). The expression of P2X3 and P2X2 proteins in L4/5 DRG of CCI are also higher than that in Sham group (p < 0.01). The relative levels of VEGFR-2, P2X3 and P2X2 protein expression in CCI rats treated with Vatalanib group were lower than those in CCI group (p < 0.01) (Fig. 7). There were no significant differences in the staining values of VEGFR-2, P2X3 and P2X2 proteins between sham group and CCI rats treated with Vatalanib group (p > 0.05).

4. Discussion

VEGF is the primary angiogenic molecule in vertebrates in both physiological and pathological states. VEGF acts via two receptors, tyrosine kinases Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2). Our...
previous works showed that the expression of VEGF and VEGFR-2 immunoreactivity in dorsal root ganglia (DRG) was enhanced after neuropathic pain (CCI rats) (Lin et al., 2010). Previous studies in our laboratory showed that VEGFR-1 immunoreactivity of DRG in both of CCI rats and control rats was little, so DRG of rats might mainly express VEGFR-2. It was reported that VEGF expression was associated with inflammatory cells in the lesions of both multiple sclerosis (MS) patients and animals with acute experimental allergic encephalomyelitis (EAE) (Proescholdt et al., 2002). Furthermore, an intracerebral infusion of VEGF in an acute model of EAE induced an inflammatory response in the brain suggesting that neuroinflammatory disease may be exacerbated by the over-expression of VEGF (Proescholdt et al., 2002). Many of the mechanisms in neuropathic pain are the same as those observed with inflammatory pain (Campbell and Meyer, 2006). The up-regulated expression of VEGF and VEGFR-2 immunoreactivities in DRG of CCI rats showed that VEGF and VEGFR-2 are involved in pathological states of neuropathic pain.

Fig. 3. Double immunostaining for VEGFR-2 or/and P2X2 subunits in DRG neurons. VEGFR-2 or/and P2X2 immunoreactivity in sections of DRG was expressed. Double labeling for VEGFR-2 and P2X2 was in a single section of DRG. Red signal represents VEGFR-2 staining with TRITC-conjugated secondary antibody and green signal indicates P2X2 staining with FITC. (A) sham group; (B) CCI group; (C) CCI rats treated with Vatalanib group. The stain intensity of VEGFR-2 and P2X2 immunoreactivity in CCI rats treated with Vatalanib group was lower than that in CCI group, while is still higher than that in sham group. Scale bars denote 50 μm.

Fig. 4. Double immunostaining for VEGFR-2 or/and P2X3 subunits in DRG neurons. VEGFR-2 or/and P2X3 immunoreactivity in sections of DRG was tested. Double labeling for VEGFR-2 and P2X3 was in a single section of DRG. Red signal represents VEGFR-2 staining with TRITC-conjugated secondary antibody and green signal indicates P2X3 staining with FITC. (A) sham group; (B) CCI group; (C) CCI rats treated with Vatalanib group. The expression level of VEGFR-2 and P2X3 receptor in CCI rats treated with Vatalanib group was lower than that in CCI group. Scale bars denote 50 μm.
The results in our laboratory indicated that the thresholds to heat and mechanical stimuli in CCI rats had been lowered (Gao et al., 2008, 2011; Lin et al., 2010; Xu et al., 2011; Zhang et al., 2008b, 2010). Hyperalgesia occurs at the site of tissue injury and is mediated in part by sensitization of primary afferent nociceptors. Intradermally administered ATP enhances inflammatory-mediated pain. ATP can facilitate nociceptive sensitivity after...
tissue injury. Purinergic sensitivity in sensory neurons has been developed after chronic peripheral nerve injury (Burnstock, 2006, 2007; Gao et al., 2008, 2011; Xu et al., 2011; Zhang et al., 2008b, 2010). Mechanical allodynia caused by surgical injury has been considered to involve local release of ATP in the tissue injury area and its action on P2X nociceptive receptors (Gao et al., 2008, 2010, 2011; Tsuda et al., 2001; Vizi et al., 1997; Xu et al., 2011; Zhang et al., 2008b, 2010). VEGF, VEGFR-2 and P2X2/3 receptors’ expressions of L4-6 DRG in CCI group were higher than those in control group (Lin et al., 2010). The expressions of VEGF, VEGFR-2 and P2X2/3 in L4-6 DRG of CCI rats treated with anti-rVEGF antibody group were decreased compared with those in CCI group (Lin et al., 2010). VEGF may activate VEGFR-2 in the neuropathic pain states. Therefore, there is an interaction between VEGF receptor-2 and P2X2/3 Receptor. When CCI rats treated with Vatalanib (an inhibitor of VEGF), mechanical and thermal hyperalgesia was reduced. Vatalanib may inhibit the sensitization of primary afferent P2X2/3 nociceptors induced by VEGF. Double-label immunofluorescence results showed that VEGF-2 and P2X2 or P2X3 receptors were co-expressed in the cytoplasm and surface membranes of DRG. The interaction of VEGF-2 and P2X2/3 receptor may engage in the increase in the neuropathic pain states.

Our results showed that VEGF enhanced the amplitude of ATP and α,β-meATP -activated currents both of sham and CCI rats. Increment effects of VEGF on ATP and α,β-meATP -activated current in CCI rats was markedly higher than those in sham rats. It is possible that the interaction of VEGF-2 and P2X2/3 receptor is increased after CCI treatment. Both ATP and α,β-meATP -activated currents were significantly blocked by Vatalanib (1 μM, an inhibitor of VEGF receptors). VEGF appears to be a factor in pathological situations that involve neuropathic pain. Vatalanib has been shown to be a potent inhibitor of the VEGF receptor tyrosine kinases. It is most potent against KDR (VEGFR-2) and exhibits slightly weaker inhibition of Flt-1(VEGF-R1) (Wood et al., 2000). After induction of painful peripheral neuropathy by sciatic nerve entrapment there is evidence for increased release of ATP from DRG neurons on the side of the injury (Donnelly-Roberts et al., 2008; Matsuka et al., 2004; Surprenant and North, 2009). Involvement of P2X2/3 receptors in neuropathic pain in the chronic constriction injury model has been demonstrated (Gao et al., 2008, 2011; Mcgarvaughy and Jarvis, 2006; Ueno et al., 2003; Xu et al., 2011; Zhang et al., 2008b, 2010). Activation of heteromeric P2X2/3 receptors appears to modulate longer-lasting nociceptive sensitivity associated with nerve injury or chronic inflammation (Burnstock, 2006, 2007; Nakagawa et al., 2007). VEGF may be a pro-inflammatory neuropeptide such as calcitonin gene-related peptide (CGRP) and/or substance P. VEGF activation of its cognate receptor VEGFR-2 on DRG neurons could sensitize nociception through P2X2/3 receptors. This might contribute to the increase in purinergic signaling in chronic pain conditions. Our results showed that the expression levels of P2X2 and P2X3 proteins in L4/5 DRG of CCI rats are higher than those in Sham group. The expression level of VEGFR-2 protein in CCI rats treated with Vatalanib group was decreased in comparison with that in CCI group. The expression levels of P2X2 and P2X3 proteins in L4/5 DRG of CCI rats were concomitantly down-regulated in comparison with those in CCI group. P2X2/3 heteromultimers contain a subunit of P2X2 and two subunits of P2X3. These results suggested that the blocking effects of Vatalanib on the expression of VEGFR-2, and then the concomitant reduction of P2X2/3 expression in CCI rats. The effects of Vatalanib on mechanical and thermal hyperalgesia were also related with inhibiting the up-regulated expression and activation of heteromeric P2X2/3 receptors in L4-6 DRG of CCI rats. Vatalanib treatment in CCI rats can alleviate chronic neuropathic pain by decreasing the positive interaction between the up-regulated VEGF-2 and P2X2/3 receptors in DRG neurons of CCI rats.

In summary, VEGF as a pro-inflammatory neuropeptide could contribute to the increase in purinergic signaling in chronic pain conditions. There is an interaction between VEGF-2 and P2X2/3 receptors in DRG neuron of CCI rats. Inhibitory effects of Vatalanib on mechanical and thermal hyperalgesia were related with decreasing the activation of heteromeric P2X2/3 receptors in L4-6 DRG of CCI rats. VEGF enhanced the amplitude of ATP and α,β-meATP -activated current both of sham and CCI rats. Increment effects of VEGF on ATP and α,β-meATP -activated current in CCI rats was significantly higher than those in sham rats. Both ATP and α,β-meATP -activated currents were markedly blocked by Vatalanib (1 μM, an inhibitor of VEGF receptors). Vatalanib treatment in CCI rats can decrease the up-regulated expression of VEGFR-2 and P2X2/3 receptors in DRG neurons of CCI rats. Vatalanib can alleviate chronic neuropathic pain by decreasing the activation of VEGF on VEGFR-2 and the positive interaction of up-regulated VEGF-2 and P2X2/3 receptors in the neuropathic pain signaling.

5. Conflict of interest

None.

Acknowledgments

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References


