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Research report

Effects of anti-rVEGF on the expression of VEGF receptor-2 and $P2X_{2/3}$ receptors of the spinal dorsal horn in neuropathic pain rats

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ABSTRACT

Neuropathic pain is caused by the peripheral or central nervous system structure damage or dysfunction. VEGF is involved in nociception and inflammation. VEGF may target VEGF receptor-2 (VEGFR-2) on the surface of neurons. $P2X_{2/3}$ receptors play a crucial role in facilitating pain transmission at the spinal sites. Chronic constriction injury (CCI) rats were used as neuropathic pain model. Sprague-Dawley male rats were randomly divided into sham group, anti-recombinant VEGF antibody group with phosphate-buffer saline (anti-rVEGF + PBS group), CCI rats treated with phosphate-buffer saline group (CCI + PBS group) and CCI rats treated with anti-recombinant VEGF antibody group (CCI + anti-rVEGF group). The expressions of VEGFR-2, $P2X_2$ and $P2X_3$ protein in spinal dorsal horn (SDH) were detected by immunohistochemistry, double-label immunofluorescence and western blotting. The protein levels of VEGFR-2, $P2X_2$ and $P2X_3$ in L4/5 SDH of CCI + PBS group were higher than those in sham group. VEGFR-2 and $P2X_2$ or $P2X_3$ receptors were co-expressed in the cytoplasm and surface membranes of SDH. Anti-rVEGF treatment in CCI rats reduced the expression of VEGFR-2 and $P2X_{2/3}$ receptors in L4/5 SDH compared with those in CCI + PBS group. Therefore, VEGF may activate VEGFR-2 to participate the process of neuropathic pain. Anti-rVEGF treatment in CCI rats reduced the expression of VEGFR-2 and inhibited the transmission of neuropathic pain in L4/5 SDH via decreasing the expression of $P2X_{2/3}$. There is a cross-potentiation between VEGFR-2 and $P2X_{2/3}$ receptors in neuropathic pain state.

1. Introduction

Vascular endothelial growth factor (VEGF) is a potent regulator of vascular function through its control of multiple endothelial cell functions. VEGF acts mainly via two receptors, tyrosine kinases Flt-1 (VEGF receptor-1, VEGFR-1) and Flk-1/KDR (VEGF receptor-2, VEGFR-2) [28]. VEGF and its receptors are expressed by neurons [28,29]. VEGF receptors are expressed at low levels in normal conditions, but are overexpressed in tissue injury. VEGF may target VEGFR-2 on the surface of cerebral cortical neurons [14]. VEGF is involved in pain and inflammation [13,24,27]. VEGF and VEGFR-2 are enhanced in the dorsal root ganglia (DRG) following peripheral nerve injury [16,38]. Our previous studies showed that VEGF and its receptor-2 were involved in neuropathic pain transmission mediated by $P2X_{2/3}$ receptor in DRG [20,22].

ATP is an important substrate in the formation of pain [1,2,6–9,19,21,22]. ATP released from the injured nerves or postsynaptic sites can act on P2X receptors [1,2,4,30–34,42,43]. $P2X_3$ and $P2X_{2/3}$ receptors play a crucial role in facilitating pain transmission at the spinal sites [26]. The expression of P2X receptors has been identified in the spinal cord dorsal horn neurons [1,25]. ATP can be released centrally during spinal cord tissue damage and inflammation as a result of numerous disorders in the spinal cord [1,2,11]. P2X receptors on afferent central terminals have important implications in the centrally initiated sensory signals including neuropathic pain associated with disorders in the spinal cord [1–3,12]. Neuropathic pain is caused by the peripheral or central nervous system structure damage or dysfunction. The aim of the study is to observe the effects of anti-rVEGF on the expression of VEGFR-2 and $P2X_{2/3}$ receptors of the spinal dorsal horn (SDH) and the relationship between VEGFR-2 and $P2X_{2/3}$ receptors of the SDH in neuropathic pain rats.

2. Materials and methods

2.1. Animal groups

Male Sprague-Dawley rats (180–250 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.

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2.2. Chronic constriction injury (CCI) model

CCI rats were taken as the neuropathic pain model. Each rat was anesthetized with 1% Nembutal [40 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 chromic gut) were performed loosely with microsurgical techniques. Intervals between every two ligatures were about 1 mm. Tightness of the ligation does not affect the blood supply of epineurium, then sutured and disinfected with 75% alcohol. In the sham-operated group, the sciatic nerve was exposed using the same procedure but not ligatured by chromic gut.

2.3. Animal groups

Rats were divided into four groups randomly: sham group, anti-recombinant VEGF antibody group with phosphate-buffer saline (anti-rVEGF + PBS group), CCI rats treated with phosphate-buffer saline (CCI + PBS group) and CCI rats treated with anti-recombinant VEGF antibody group (CCI + anti-rVEGF group) ($n = 6$ for each group). About 10 μl anti-rVEGF antibody (1 μg/kg) was injected to the rats intrathecally by microinjector (Ningbo Zhenhai Sanai Instrument Factory, China) in anti-rVEGF used group every 2 days (total 7 times), and the same dosage of PBS was injected into sham group and CCI + PBS group at the same time. All drugs were diluted in phosphate buffered saline (PBS).

2.4. Antibodies and reagents

Anti-rVEGF antibody was the product of R&D Systems, Inc. (AF564) [22]. Rabbit anti-$P2X_3$ and rabbit anti-$P2X_2$ polyclonal antibody were bought from Chemicon International Company of America [35]. Mouse anti-VEGFR-2 antibody was bought from Wuhan Boster Co., China [22]. β-Actin was from Beijing Zhongshan Biotech Co., China. Other antibodies and reagents were ascribed as following.

2.5. Immunohistochemistry

Immunohistochemical staining was performed using SP-9001 kit (Beijing Zhongshan Biotech Co., China) according to the manufacturer's instruction. On the 14th day after surgery, Sprague-Dawley rats in three groups were anesthetized with 1% Nembutal (40 mg/kg i.p.). We chest and exposed the heart, cut the pericardium, inputted about 50 ml saline through the left ventricular catheter to wash immediately, meanwhile cut the right atrial appendage, then inputted 4% paraformaldehyde (PFA) to fix. L4/5 SDH was taken from rats. After fixed by 4% PFA for 24 h, and dehydrated by 20% sucrose for overnight at 4 °C. Preparations were fixed and embedded in OCT (tissue freezing medium, Sakura Finetek USA, Inc.), frozen section (the coronal, 10 μm). After washed by 0.1 M PBS for three times, the sections were incubated in 3% H_2O_2 for 10 min to block the endogenous peroxidase activity, then with 10% goat serum for 40 min at 37 °C to block non-specific antigen, and followed by an incubation overnight at 4 °C with primary antibody ($P2X_2$ and $P2X_3$ 1:2500 diluted in PBS; others 1:100 diluted in PBS). On the second day, after three rinses in PBS, the sections were incubated with biotinylated goat anti-rabbit secondary antibody (Beijing Zhongshan Biotech Co., China) for 1 h at 37 °C. The preparations were washed in PBS and then added streptavidin–horseradish peroxidase (Beijing Zhongshan Biotech Co., China) for 30 min. After development of the diaminobenzidine chromogen for 2 min, the slides were washed with distilled water and coverslipped. After immunohistochemistry, the Image-Pro Plus 6.0 was used to analyze the changes in stain values (average optical density) of $P2X_2$, $P2X_3$ and VEGFR-2. Background was determined by averaging the optical density of 10 random areas.

2.6. Immunofluorescence double-labeling

L4/5 SDH, isolated from rats of the three groups, was washed with PBS. The SDH was dissected immediately and fixed in 4% paraformaldehyde (PFA) for 24 h at room temperature, then were transferred to 20% sucrose for dehydration at 4 °C overnight. Tissues were sectioned at 10 μm at a cryostat and put onto 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% goat serum (Beijing Zhongshan Biotech Co., China) for 40 min in a moisture chamber at 37 °C to block non-specific antigen. The sections were then incubated with the mixture of mouse anti-VEGFR-2 (1:100 dilution; Wuhan Boster Co., China) and rabbit anti-$P2X_2$ or rabbit anti-$P2X_3$ diluted in PBS for overnight at 4 °C. After three rinses in PBS, the sections were incubated with fluorescent goat anti-rabbit FITC and goat anti-mouse TRITC secondary antibodies (1:100 dilutions, Beijing Zhongshan Biotech Co., China) for 45 min at 37 °C in dark. The preparations were washed in PBS in dark and mounted with 10% glycerol, examined with fluorescence microscopy (IX-17, Olympus). Image-Pro Plus 6.0 image analysis software (Media Cybernetics Inc.) was used to quantify $P2X_2$, $P2X_3$ and VEGFR-2.

2.7. Western blotting

The rats in three groups were anesthetized by 1% Nembutal (40 mg/kg i.p.). The L4/5 SDH was isolated immediately and flushed with icecold PBS. Tissues 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 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 sample buffer [100 mmol/L TrisCl, 200 mmol/L dithiothreitol, 4% sodium dodecylsulfate (SDS), 0.2% bromophenol blue, 20% glycerol] and heated to 95 °C for 5 min, samples containing equal amounts of protein (20 μl) were separated by SDS-polyacrylamide gel electrophoresis by using Bio-Rad system and 10% gel. In the wake of electrophoretic transfer onto nitrocellulose membrane using 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 1.5 h at room temperature, and incubated with primary antibodies in blocking buffer for 2 h at room temperature or overnight at 4 °C. The membranes were washed three times with TBST and incubated (1 h, room temperature) with horseradish peroxidase-conjugated secondary antibody [goat anti-rabbit IgG (1:2000) or goat anti-mouse IgG (1:5000), Beijing Zhongshan Biotech Co., China] in blocking buffer. After another wash cycle, labeled proteins were visualized using the enhanced chemiluminescence (ECL) kit (Fremont, CA, USA). Chemiluminescent signals were collected on autoradiography film, and the quantity of band intensity was carried out using Image-Pro Plus 6.0. The primary antibodies and dilutions used were the following: rabbit polyclonal anti-$P2X_3$ and anti-$P2X_2$ (1:1000), mouse monoclonal anti-VEGFR-2 (1:100) and mouse monoclonal β-actin (1:1000, Beijing Zhongshan Biotech Co., China).

2.8. Statistical analysis

Statistical analysis of the data was performed on computer (SPSS 11.5). All results were expressed as mean ± SEM. Statistical significance was determined by one factor analysis of variance (ANOVA) followed by the Fisher post hoc test for multiple comparisons. $p < 0.05$ was considered significant.

3. Results

3.1. Effects of anti-rVEGF antibody on the expression of VEGFR-2, immunoreactivity in L4/5 SDH of CCI rats

Immunoreactivity of VEGFR-2 in L4/5 SDH was detected by immunohistochemistry. The staining of VEGFR-2 in L4/5 SDH of CCI + PBS group was higher than that in sham group ($p < 0.01$). After treated with anti-rVEGF in CCI group, the staining of VEGFR-2 in L4/5 SDH was lower than that in CCI + PBS group ($p < 0.05$). There were no significant differences in the staining values of VEGFR-2 protein among sham group, anti-rVEGF + PBS group and CCI + anti-rVEGF group ($p > 0.05$) (Fig. 1).

3.2. Effects of anti-rVEGF antibody on the expression of $P2X_2$ or $P2X_3$ immunoreactivities in L4/5 SDH of CCI rats

The expressions of $P2X_2$ or $P2X_3$ immunoreactivities in L4/5 SDH of each group were examined by immunohistochemistry. The staining values of $P2X_2$ or $P2X_3$ in L4/5 SDH of CCI + PBS group were higher than those in sham group and anti-rVEGF + PBS group, respectively ($p < 0.01$). The expressions of $P2X_2$ or $P2X_3$ in L4/5 SDH of CCI + anti-rVEGF antibody treatment group were lower than those in CCI + PBS group ($p < 0.05$). There were no significant differences in the staining values of $P2X_2$ or $P2X_3$ protein among sham group, anti-rVEGF + PBS group and CCI + anti-rVEGF group ($p > 0.05$) (Figs. 2 and 3).

3.3. Double-label immunofluorescence in L4/5 SDH

VEGFR-2 or/and $P2X_2$ immunoreactivities in sections of L4/5 SDH were tested. Double labeling for VEGFR-2 and $P2X_2$ was in a single section of L4/5 SDH. It was indicated that VEGFR-2 and $P2X_2$ receptor were co-expressed in sections of L4/5 SDH (Fig. 4).

Double labeling for VEGFR-2 and $P2X_3$ was measured in a single section of L4/5 SDH. It was showed that VEGFR-2 and $P2X_3$ receptor were co-expressed in sections of L4/5 SDH (Fig. 5).