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Effect of artemisinin on neuropathic pain mediated by $P2X_4$ receptor in dorsal root ganglia

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ABSTRACT

Neuropathic pain is a type of chronic pain caused by nervous system damage and dysfunction. The pathogenesis of chronic pain is complicated, and there are no effective therapies for neuropathic pain. Studies show that the $P2X_4$ receptor expressed in the satellite glial cells (SGCs) of dorsal root ganglia (DRG) is related to neuropathic pain. Artemisinin is a monomeric component extracted from traditional Chinese medicine and has a variety of important pharmacological effects and potential applications. This study observed the effect of artemisinin on neuropathic pain and delineated its possible mechanism. The chronic constriction injury (CCI) rat model was used in this study. The results demonstrated that artemisinin relieved pain behaviors in the CCI rats, inhibited the expression of $P2X_4$ receptor in the DRG, and decreased the ATP-activated currents in HEK293 cells transfected with $P2X_4$ plasmid. Dual-labeling immunofluorescence showed that the coexpression of $P2X_4$ receptor and glial fibrillary acidic protein (GFAP) in the DRG of CCI rats was increased compared to control rats. After CCI rats were treated with artemisinin, the coexpression of $P2X_4$ receptor and GFAP in the DRG was significantly decreased compared to the CCI group. This finding suggested that artemisinin could inhibit the nociceptive transmission mediated by $P2X_4$ receptor in the DRG SGCs and thus relieve pain behaviors in the CCI rats.

1. Introduction

Pain is a common symptom of most diseases, but it is different in different people. According to the duration and nature of pain, it can be divided into acute pain and chronic pain. Acute pain can be triggered by trauma or surgery and is often alleviated after recovery of the damaged tissue. Chronic pain lasts longer than the normal healing time of an acute injury or disease and recurs after several months or several years. Chronic pain includes inflammatory pain, neuropathic pain and cancer pain (Backonja, 2003; Kidd and Urban, 2001; Moalem and Tracey, 2006). Neuropathic pain is due to nervous system damage and dysfunction, can often induce sudden spontaneous pain, and can produce hyperalgesia especially to mechanical and thermal stimulation (Yan et al., 2015). Due to the long duration of chronic pain, it can be of great harm to a person's physical and mental health. The treatment of chronic pain is not effective in most cases. Because the pathogenesis of chronic pain is

Abbreviations: Art, artemisinin; CCI, Chronic Constriction Injury; SGC, satellite glial cells; GFAP, glial fibrillary acidic protein; DRG, dorsal root ganglia; MWT, Mechanical Withdrawal Threshold; TWL, Thermal Withdrawal Latency; RT-PCR, Reverse Transcription-Polymerase Chain Reaction.

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complicated, it is difficult to develop clinical treatments for it.

Adenosine triphosphate (ATP) is involved in nociceptive signaling transmission by activating purinergic 2 receptors (Burnstock, 2006; Donnelly-Roberts et al., 2008; Inoue, 2007; Jarvis and Khakh, 2009; Kennedy, 2005). The purinergic receptors are divided into P1 and P2 receptors. P2 receptors include $P2X_{1-7}$ (a ligand-gated ion channel receptor) and P2Y (a G protein-coupled receptor) (Burnstock et al., 2011; Coddou et al., 2011). $P2X_4$ receptor is a subtype of P2X receptor. Studies have shown that $P2X_4$ receptor is expressed in the satellite glial cells (SGCs) of dorsal root ganglia (DRG) (Burnstock et al., 2011). After peripheral nerve injury, ATP is released from the nerve endings of primary sensory neurons to increase and activate $P2X_4$ receptor in the spinal cord dorsal horn. Therefore, $P2X_4$ receptor is related to neuropathic pain (Inoue et al., 2005; Tsuda et al., 2003).

DRG receive most of the primary sensory information and transmit the sensory signals to the dorsal horn of spinal cord. SGCs closely surround the neuronal cell bodies in DRG, and studies have shown that neuropathic pain increases the communication between neurons and SGCs in DRG (Hanani et al., 2014). After nerve injury, the expression of glial fibrillary acidic protein (GFAP), which has low expression in an uninjured state, increases significantly (Xie et al., 2009). GFAP is therefore considered a marker of SGC activation. Inflammatory factors released from activated SGCs enhance neuronal sensitivity to ATP by acting on P2X receptors in neurons, cause neuronal hyperexcitability, and reduce the pain threshold of neuropathic pain in rats.

Artemisinin, extracted from artemisia annua leaves, is a type of sesquiterpene lactone. Artemisinin has a variety of important pharmacological effects and potential applications (Lee, 2007), including its use as a drug against malaria, tumors, and inflammation as well as its ability to enhance immune function (Lee, 2007). These applications suggest that it may have a role in chronic neuropathic pain. The data from our laboratory suggest that arteminisin, a monomeric component isolated from traditional Chinese medicine, plays an effective role in acute pain and chronic neuropathic pain mediated by P2X receptors (Li et al., 2011; Lian et al., 2014; Lin et al., 2010; Xin et al., 2012; Xu et al., 2012; Zhang et al., 2010). This study tested whether artemisinin could influence pain behavior in a neuropathic pain rat model, and it assessed the possible mechanism of artemisinin acting on neuropathic pain through the $P2X_4$ receptor in SGCs of DRG.

2. Materials and methods

2.1. Animals and chronic constriction injury model

Male Sprague-Dawley rats weighing 220–250 g were provided by the Center of Laboratory Animal Science of Nanchang University. The rats were fed a standard laboratory diet and housed under controlled temperature (20–22 °C) and 12-h light/dark cycle conditions. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Medical College of Nanchang University. The rats were randomly divided into four groups: control group (Ctrl), sham group (Sham), chronic constriction injury group (CCI), artemisinin-treated CCI group (CCI+Art). Each group consisted of eight animals. The rats in the CCI+Art group were injected intraperitoneally with 5 mg/kg artemisinin daily for 14 days. Artemisinin (purity 99%) was purchased from Chengdu Ruifensi Biological Technology Co. ltd.

The CCI model was established as previously described (Li et al., 2013). Briefly, rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). The sciatic nerve was exposed and loosely ligated with sterile 4-0 catgut thread at four consecutive sites with an interval of approximately 1 mm. Meanwhile, sham surgery was performed with the sciatic nerve exposed but not ligated. Animals were kept warm and allowed to recover from anesthesia.

2.2. Behavior study

Measurement of mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) was performed as previously described (Liu et al., 2016). Briefly, the pain behavior of rats in the four groups were measured before the experiment (day 0) and day 1, 3, 5, 7, 9, 11, 13 after CCI operation. All testing was performed blindly.

2.3. Quantitative real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was performed as previously described (Liu et al., 2016). The total RNA from rat L4-L5 DRGs was extracted using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and the synthesis of complementary DNA (cDNA) was performed according to the ThermoScript RT-PCR System procedure (Invitrogen, USA)(Tsuda et al., 2003). The sequences of the primers are as follows: $P2X_4$ receptor (forward 5′-CCCTTTGCCTGCCCAGATA-3’; reverse 5′-CCGTACGCCTTGGTGAGTGT-3′) and β-actin (forward 5′-AAGATCCTGACCGAGCGTGG-3’; reverse 5′- CAGCACTGTGTTGGCATAGAGG-3′). Reactions (total volume 10 μl) were performed in triplicate by incubating at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Real-time RT-PCR was performed with an ABI Prism 7500 Sequence Detection System (Applied Biosystems) using Maxima SYBR Green qPCR Master Mix ROX. β-actin was used as an internal control. The amount of sample RNA was normalized to β-actin. All samples were analyzed in duplicate. The cycle threshold values were obtained, and the fold changes of gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method.

2.4. Western blotting

L4-L5 DRGs of rats were removed, and the total protein was extracted by homogenizing the DRG samples via mechanical disruption.The protein was separated by electrophoresis and transferred to a polyvinylidene fluoride membrane. The membrane was blocked for 1 h at room temperature in 5% nonfat dried milk and then incubated with primary antibody (rabbit anti-$P2X_4$, Abcam, 1:200; mouse anti-β-actin, 1:800) overnight at 4 °C and then with HRP-conjugated secondary antibody (1:2000 for both $P2X_4$ and β-actin, Beijing Zhongshan Biotech Co.). The quantification of band intensity was carried out using Image-Pro Plus software. Target band densities were normalized to each β-actin internal control.

2.5. Double-labeled immunofluorescence

On the fourteenth day after CCI operation, L4/L5 DRGs were dissected and fixed in 4% paraformaldehyde (PFA) diluted in phosphate-buffered saline (PBS: 145 mM NaCl, 7.3 mM Na_2HPO_4, and 2.7 mM NaH_2PO_4 pH 7.2) for 24 h. The ganglia were cryopreserved in 20% sucrose overnight at 4 °C and then cut into 12 μm-thick sections. The sections were rinsed three times for 5 min in PBS, and nonspecific staining was blocked by incubation with 10% normal goat serum (Jackson ImmunoResearch Inc., West Grove PA, USA) and 0.1% Triton X-100 in PBS for 30 min at room temperature. The sections were then incubated with rabbit anti-$P2X_4$ (1:100, Abcam, USA) and chicken anti-GFAP (1:1000, Abcam, USA) overnight at 4 °C. The secondary antibodies were tetraethyl rhodamine isothiocyanate (TRITC)- (Jackson ImmunoResearch Inc., West Grove, PA, USA) conjugated to goat anti-rabbit IgG and fluorescein (FITC)-conjugated to goat anti-chicken IgG, and the