Dynamic Regulation of Delta-Opioid Receptor in Rat Trigeminal Ganglion Neurons by Lipopolysaccharide-induced Acute Pulpitis

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Abstract

Introduction: Delta-opioid receptor (DOR) and its endogenous ligands distribute in trigeminal system and play a very important role in modulating peripheral inflammatory pain. DOR activation can trigger p44/42 mitogen-activated protein kinase (ERK1/2) and Akt signaling pathways, which participate in anti-inflammatory and neuroprotective effects. In this study, our purpose was to determine the dynamic changes of DOR in trigeminal ganglion (TG) neurons during the process of acute dental pulp inflammation and elucidate its possible mechanism. **Methods:** Forty rats were used to generate lipopolysaccharide-induced acute pulpitis animal models at 6, 12, and 24 hours and sham-operated groups. Acute pulpitis was confirmed by hematoxylin-eosin staining, and TG neuron activation was determined by anti-c-Fos immunohistochemistry. DOR protein and gene expression in TG was investigated by immunohistochemistry, Western blotting, and real-time polymerase chain reaction, and DOR expression in trigeminal nerves and dental pulp was also determined by immunohistochemistry. To further investigate the mechanism of DOR modulating acute inflammation, the change of pErk1/2 and pAkt in TG was examined by immunohistochemistry. **Results:** Lipopolysaccharide could successfully induce acute pulpitis and activated TG neurons. Acute pulpitis could dynamically increase DOR protein and gene expression at 6, 12, and 24 hours in TG, and DOR dimerization was significantly increased at 12 and 24 hours. Acute pulpitis also induced the dynamic change of DOR protein in trigeminal nerve and dental pulp. Furthermore, ERK1/2 and Akt signaling pathways were inhibited in TG after acute pulpitis. **Conclusions:** Increased DOR expression and dimerization may play important roles in peripheral acute inflammatory pain. *(J Endod 2015;41:2014–2020)*

Key Words

Acute pulpitis, δ-opioid receptor, pAkt (Ser473), pERK1/2, rat, trigeminal ganglion

Opioid receptors belong to G protein-couple receptors and play important roles in various physiological and pathologic functions (1–3). Three major types, μ-, δ-, and κ-opioid receptors, have been identified, and they interact with exogenous and endogenous opioid ligands. In recent years, δ-opioid receptor (DOR) has become an attractive drug target because the analgesics working on DOR may produce fewer side effects and have lower abuse potential compared with conventional opioid analgesics that act on μ-opioid receptor (4–6). DOR expression is less abundant in the central nervous system, but it is more prevalent for analgesia in peripheral nervous system (7). In peripheral nervous system, DOR and its endogenous ligands are distributed in the sensory neurons and their processes. In trigeminal ganglion (TG) neurons, DOR is located in cell bodies and their nerve fibers, including nerve fibers in the dental pulp (8, 9). Many studies have demonstrated that inflammation could enhance the analgesic effect of DOR agonist in peripheral tissue (10–12). By using DOR knockout mice, Gaveriaux-Ruff et al (13) found endogenous DOR played a very important role in inflammatory pain, and the activation of DOR could inhibit inflammatory pain. Cvejic and Devi (14) and Wang et al (15) found DOR could form homodimers and heterodimers, and dimerization might be important for regulating their functions. Therefore, the beneficial effects of targeting DOR have been well-documented, especially for chronic inflammatory pain, but the dynamic function of DOR during the process of acute inflammatory pain has not been thoroughly investigated yet.

Activation of DOR can trigger different intracellular signaling pathways, such as mitogen-activated protein kinases (MAPKs), protein kinase C, PI3/AKT, ion homeostasis, and antioxidative signaling pathways (16–19). Both p44/42 MAPK (extracellular signal-regulated kinase [ERK]1/2) and Akt signaling pathways have been suggested to participate in the anti-inflammatory and neuroprotective effects, and DOR agonists acting on DOR provide their neuroprotective effects via MAPK and PI3K/Akt signaling pathways (20, 21). However, it is not fully understood whether DOR could play a role in modulating peripheral inflammation pain through p44/42 MAPK (ERK1/2) and Akt signaling pathways.

Pulpitis is usually characterized by prolonged and severe toothache that is caused by the inflammation of dental pulp tissue. Dental pulp contains abundant sensory nerves that are the afferent fibers of TG neurons (9). The axons of TG neurons are distributed to the craniofacial tissues and play an important role in conducting the sensory information from their tissues, including dental pulp. Dental pulp inflammation can affect the

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primary afferent neurons in TG, so pulpitis is a useful model system to investigate the mechanism of peripheral pain and inflammation. In this study, we aimed to determine the dynamic changes of DOR in TG neurons during the process of acute pulpitis and further elucidate its possible mechanism.

Materials and Methods

Animals

Eight-week-old male Sprague-Dawley rats (250–350 g body weight) were purchased from Vital River Laboratory Animal Technology Company (Beijing, China). All rats were held in a 22°C temperature-controlled room and maintained under a 12-hour light-dark cycle with free access to food and water. All experimental animal procedures were carried out in accordance with the guidelines of the Animal Care and Use Committee of the Liaoning Medical University.

Reagents

All reagents were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise specified.

Lipopolysaccharide-induced Dental Pulp Inflammation Model

Forty rats were randomly assigned to 4 groups: rats without dental pulp exposed (sham-operated), rats with dental pulp exposed and lipopolysaccharide (LPS) (5 mg/mL in saline, *Escherichia coli*; Sigma, Cat#L2880) application for 6, 12, and 24 hours. All animals were anesthetized with 10% chloral hydrates (3 mL/kg, intraperitoneal), and a custom-made mouth gag was used to immobilize their heads and open their mouths widely. The cavity was made in the left maxillary first molar by using a round bur (size 1/4) driven by high-speed dental drill with water cooling. A very small cotton ball soaked with 5 mg/mL LPS was applied to the cavity, and then the cavity was sealed with dental cement. For sham-operated groups, rats were anesthetized only.

Histology and Immunohistochemistry

Rats were transcardially perfused with saline, followed by 4% paraformaldehyde (pH 7.4) in 0.1 mol/L sodium phosphate buffer, and TGs, maxillary nerves, and the maxillae containing maxillary first molars were dissected. After being postfixed, the TG and maxillary nerves were cryoprotected in phosphate-buffered 25% sucrose for 48 hours at 4°C, and the maxillae were decalcified with 10% EDTA solution for 10 days at 4°C, followed by the same cryoprotection. Sections were cut by using a cryostat at 10 μm (CM3050S; Leica, Nussloch, Germany) for the maxillae from their near surface to far surface, the TGs transversely, and maxillary nerves longitudinally. The hematoxylin-eosin staining was performed by using the sections of the maxillae, and immunohistochemistry was conducted with the sections of the left TG maxillary nerves and maxillae.

For immunohistochemistry, sections were blocked with 5% normal goat serum containing 0.1% Triton X-100 for 1 hour at 4°C and then incubated with different primary antibodies overnight at 4°C. The primary antibodies used are rabbit anti-delta-opioid receptor (1:2000, AB1560; Millipore, Billerica, MA), rabbit anti-c-Fos (1:2000, ab190289; Abcam, Hong Kong, China), rabbit anti-phospho-p44/42 MAPK (pERK1/2, 1:200, 4370s; Cell Signaling Technology, Danvers, MA), and rabbit anti-phospho-Akt (1:50, 3787s; Cell Signaling Technology). After being rinsed 3 times with tris buffered saline, the sections were incubated with Cy3-conjugated goat anti-rabbit, fluorescein isothiocyanate–conjugated goat anti-rabbit, or Cy3-conjugated goat anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) for 2 hours at room temperature (RT). Sections were counterstained with DAPI and coverslipped with fluorescent mounting medium (S3023 Dako, Glostrup, Denmark). The images were taken by fluorescence microscopy (DMI4000 B; Leica), and the mean intensity of fluorescence was measured with ImageJ software (National Institutes of Health).

Western Blot Analysis

TGs were dissected and homogenized in RIPA buffer containing 50 mmol/L Tris (pH 7.5), 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate, 150 mmol/L NaCl, 2 mmol/L EDTA, and phenylmethylsulfonyl fluoride (Beyotime, Beijing, China). Equal amounts of protein (40 μg) were loaded onto a 12% polyacrylamide gel and separated by electrophoresis and then transferred to polyvinylidene fluoride membranes (Millipore). After blocking in 1% bovine serum albumin for 2 hours at RT, the membrane was incubated with different primary antibodies overnight at 4°C. The primary antibodies used were rabbit anti-delta-opioid receptor (1:1500, AB1560; Millipore) and rabbit anti-beta-actin antibody (1:2000; Sigma-Aldrich). After being washed 3 times with Tris-buffered saline solution with Tween-20, membrane was incubated with horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse secondary antibodies for 2 hours at RT. The proteins were visualized with enhanced chemiluminescence reagents (Beyotime) under UVP BioSpectrum imaging system (BioSpectrum 510; UVP, Upland, CA), and the density of protein bands was analyzed by VisionWorks LS analysis software (UVP).

Real-time Polymerase Chain Reaction

Total RNA from TGs was isolated by using the TRI reagent, and cDNA synthesis was carried out with ThermoScript RT-PCR System (Invitrogen, Grand Island, NY). Real-time polymerase chain reaction (PCR) was performed with the primers specific for DOR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by using SYBR Green SuperMix-UDG (Invitrogen) with 7500 Fast Real-Time PCR system (Applied Biosystem, Life Technologies, Carlsbad, CA). The primers of DOR and β-actin were as follows: GAPDH forward 5′-TATCGGACGCCTGGTTAC-3′, reverse 5′-TGCTGACAA TCTTGAGGGA-3′; DOR forward 5′-TGGGTCTTGGCTTCAGGTGT-3′, reverse 5′-CGTGCATACCACTGCTCCAT-3′. The PCR amplification conditions were 95°C for 120 seconds, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. The relative gene expression was normalized to the internal control GAPDH.

Statistical Analysis

All experiments were independent and repeated at least 3 times. These data were presented as mean values ± standard deviation. All statistical analyses were performed with SPSS16.0 (SPSS Inc, Chicago, IL) by using one-way analysis of variance followed by a Bonferroni post hoc test. The significance level was set as $P < .05$.

Results

Morphologic Changes of Dental Pulp after LPS-induced Tooth Pulp Inflammation

To examine whether the dental cavities were prepared and dental pulp inflammation was induced successfully, we did hematoxylin-eosin staining by using the sections of left maxillary first molars at 6, 12, and 24 hours of LPS induction and for sham-operated group. Our results indicated that dental cavities were made successfully, and acute inflammation was detectable as early as 6 hours and more severe at 12 and 24 hours after LPS-induced tooth pulp inflammation (Fig. 1).