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Original article

Quercetin alleviates thermal and cold hyperalgesia in a rat neuropathic pain model by inhibiting Toll-like receptor signaling

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ABSTRACT

Neuropathic pain is caused by lesion or disease of the nervous system, which results in abnormal spontaneous and evoked pain. It's common in clinical practice and greatly impairs the life quality of patients, but the effective treatment is still lacking. In this study, we aimed to explore the effect of quercetin (QUE) on neuropathic pain and the underlying mechanisms. Spinal nerve ligation (SNL) was performed in Sprague Dawley rats to establish the neuropathic pain model. Single or continuous oral administration of QUE after the operation or continuous administration before the operation was applied to evaluate the effects of QUE on SNL-induced thermal and cold hyperalgesia. Dorsal root ganglions from these rats were harvested to analyze the expression levels of some inflammatory mediators. Primary cultured astrocytes and HEK293 cells were used to further explore the downstream signaling pathways of QUE. Both single and continuous oral administration of QUE dose-dependently alleviated SNL-induced thermal and cold hyperalgesia. Pre-administration also attenuated neuropathic pain symptoms. Meanwhile, SNL-induced increase in protein or mRNA levels of some inflammatory mediators could be down-regulated by QUE treatment. Furthermore, QUE reduced the phosphorylation of TAK1, IKK and JNK2 in cultured astrocytes. Moreover, luciferase assay in HEK293 cells showed that QUE dose-dependently inhibited NF-κB activity only via TAK1. QUE exerts anti-inflammatory effects and alleviates neuropathic pain through the inhibition of Toll-like receptor signaling pathway. It could shed some light on the potential applications of QUE in chronic pain therapy.

1. Introduction

Neuropathic pain is defined as the pain state initiated by primary lesion or dysfunction in the peripheral or central nervous system. The most prominent symptoms are hyperalgesia (increased pain response to a normally painful stimulus) and allodynia (painful response to a normally innocuous stimulus) [1]. It becomes more prevalent in clinic due to the absence of effective treatments. Commonly used opioids and non-steroidal anti-inflammatory analgesics only have modest alleviating effect on neuropathic pain [2,3]. Therefore, understanding the exact mechanisms and searching for more effective targets and drugs are very important in neuropathic pain therapy.

It is generally accepted that peripheral sensitization and central sensitization are two major mechanisms in the pathogenesis of chronic pain. Inflammatory cytokines and mediators released from immune and glia cells participate in both processes and are considered as promising therapeutic targets [1]. Increasing evidences indicate that Toll-like receptors (TLRs) and their associated components are critical in triggering these proinflammatory immune signaling events and contribute to pain hypersensitivity [4,5]. TLRs are expressed in immune cells, astrocytes and microglia [6]. Upon ligand binding, TLRs associate with myeloid differentiation primary-response protein 88

Abbreviations: QUE, quercetin; SNL, spinal nerve ligation; TLR, Toll-like receptors; MyD88, myeloid differentiation primary-response protein 88; TAK1, transforming growth factor-β-activated kinase; IKK, inhibitor of nuclear factor-κB-kinase; NF-κB, translocation of nuclear factor-κB.

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(MyD88), which in turn recruits many signaling molecules to phosphorylate and activate transforming growth factor-β-activated kinase (TAK1). TAK1 could induce the phosphorylation of inhibitor of nuclear factor-κB-kinase (IKK) complex and allow the translocation of nuclear factor-κB (NF-κB) into the nucleus, which triggers the expression of many cytokines and chemokines, such as tumor necrosis factor (TNF)-α, interleukins (IL) and C-C motif chemokine ligands (CCL). TAK1 also activates mitogen-activated protein kinase (MAPK) signaling pathways, such as extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) [7]. Blockade of TLR signaling has been shown to attenuate pathological pain [8].

Quercetin (QUE), the most abundant dietary flavonoid, processes remarkable antioxidative and anti-inflammatory properties [9]. Increasing studies suggest that QUE could inhibit nociceptive neurotransmission and ameliorate pathological pain in many animal models, including chronic constriction nerve injury model, diabetic model and caner pain model [10–13]. The analgesic effects of QUE were thought to be based on different mechanisms, including the inhibition of oxidative stress and cytokine production [14], reduction of 5-hydroxytryptamine type 3 (5-HT_{3A}) receptor channel activity [15], down-regulation of protein kinase C epsilon isoform (PKCε) and transient receptor potential cation channel subfamily V member 1 (TRPV1) expression [16], and the modulation of γ-aminobutyric acid (GABA)ergic system [17]. The discrepancies result from the experimental design and reflect partial understanding of QUE functions and the underlying mechanisms.

In the present study, we adopted a commonly-used neuropathic pain model termed spinal nerve ligation (SNL) model, which could mimic the symptoms of human patients suffering from causalgia [18]. We systematically evaluated the effects of QUE treatment on SNL-induced heat and cold hyperalgesia. We further investigated the detailed mechanisms by which QUE could exert its anti-inflammatory property to alleviate pathological pain sensation.

2. Material and methods

2.1. Animals

All animal procedures were performed in accordance with International Association for the Study of Pain guidelines. Sprague Dawley (SD) rats were purchased from Nanjing Model Animal Center and housed under a 12-h light/dark cycle at 22 °C–26 °C. For the surgery, the rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally, Sigma, St. Louis, MO). An incision was made at the level of the posterior iliac crest to expose lumbar (L) spinal nerves. L5 nerve was identified and ligated with a cotton thread. For sham rats, the nerves were exposed without ligation. Rats exhibiting severe motor deficiencies were discarded from later behavior tests. For drug treatment, QUE (Sigma) was prepared in 0.5% sodium carboxymethylcellulose solution and orally administrated to the rats at indicated dosages.

2.2. Behavior tests

All behavior tests were carried out blindly. For plantar test, a radiant heat stimulator (BME-410C; CAMS) was used to test the latency of the hindpaw withdrawal response. For hot plate and cold plate tests, the rats were placed on the 55 °C hot plate or 5 °C cold plate (Ugo Basile), respectively. The latency to lift or lick the hindpaw was recorded. The cut-off time was 20 s for plantar test, 30 *s* for cold plate test, and 50 s for hot plate test. For all tests, three measurements, with an interval >5 min between two consecutive tests, were taken and averaged as the final response latency for each rat. The area under the curve (AUC) values was used to evaluate the analgesic effects of the drug treatments.

2.3. Enzyme-linked immunosorbent assay (ELISA)

L5 dorsal root ganglions (DRG) from different groups were dissected out and homogenized in the Neuronal Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA) containing protease inhibitor cocktail (Roche, Penzberg, Upper Bavaria, Germany). The total protein concentration was measured by spectrophotometer (Nanodrop ND-1000; Thermo Fisher) and the protein levels of TNF-α and IL-1β were determined with the commercial ELISA kits (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Each sample was measured three times and the results were standardized as pg of protein to 100 μg of total protein.

2.4. Quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNAs (2 μg) from L5 DRGs were reverse transcribed with SuperScript II reverse transcriptase (Invitrogen, USA). Then, the cDNAs from different groups were used to perform qPCR experiments with SYBR Green PCR kit (Thermo Fisher Scientific). The information of primers that we used was: matrix metalloproteinase (MMP)-9: forward 5′-CAGAGCGTTACTCGCTTGGA-3′, reverse 5′-GGTTGTGGAAACTCACACGC-3′; MMP-2: forward 5′-CACAGAACCCTCACAGGACC-3′, reverse 5′-TGTCTCCAACATGCAGCCAT-3′; CCL2: forward 5′-TAGCATCCACGTGCTGTCTC-3′, reverse 5′-CAGCCGACTCATTGGGATCA-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5′-TGTTCCTACCCCCAATGTG-3′, reverse 5′-GTGTAGCCCAAGATGCCCT-3′. The mRNA levels of MMP-9, MMP-2, and CCL2 were standardized with GAPDH and normalized to the sham group.

2.5. Cell culture

Human embryonic kidney cells 293 (HEK293) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco). For transfection, 2×10^5 cells were seeded onto each well of 24-well plate and transfected with indicated plasmids by calcium phosphate transfection method.

Primary astrocyte cultures were prepared from cerebral cortex of postnatal day 2 mouse pups. Briefly, the cerebral hemispheres were dissected out and digested with papain (Worthington Biochemical, Lakewood, NJ). After digestion, the tissues were gently triturated, filtered with the cell strainer, and collected by centrifugation. The cell pellets were resuspended and cultured in low glucose DMEM (Gibco) containing 10% FBS. Dibutyryl cAMP (Sigma, 0.15 mM) was added to induce differentiation when the cells were grown to 95% confluence. The culture medium was replaced with Opti-MEM (Gibco, USA) when cells were treated with IL-1β (Thermo Fisher Scientific, 10 ng/ml) and QUE (50 μM).

2.6. Western blot

Whole cell extracts from cultured astrocytes were isolated with Neuronal Protein Extraction Reagent containing protease inhibitor cocktail. Protein from each sample was resolved by SDS-PAGE. Primary antibodies that we used were: TAK1, phosphorylated (P)-TAK1, IKKα, P-IKKα/β, JNK2, P-JUK2, β-actin. HRP conjugated secondary antibodies (Pierce, WI, USA) were used at a dilution of 1:2000. LumiGLO chemiluminescent substrate (Cell Signaling Technology, Danvers, MA, USA) was used to detect HRP and light emission was captured by films.