International Immunopharmacology 41 (2016) 66–73

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Gastrodin relieved complete Freund's adjuvant-induced spontaneous pain by inhibiting inflammatory response

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ARTICLE INFO

Article history:
Received 30 April 2016
Received in revised form 8 October 2016
Accepted 27 October 2016
Available online 3 November 2016

Keywords:
Chronic inflammatory pain
CFA
Neuroplasticity
Anxiety
Gastrodin
ACC
IL-6
TNF-α

ABSTRACT

The analgesic effects of gastrodin (GAS), an active component derived from the Chinese herb Tian ma (*Gastrodia elata Blume*), on chronic inflammatory pain of mice and the involved molecular mechanisms were investigated. GAS significantly attenuated mice chronic inflammatory pain induced by hindpaw injection of complete Freund's adjuvant (CFA) and the accompanying anxiety-like behaviors. GAS administration reduced CFA-induced up-regulation of GluR1-containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, GluN2A- and GluN2B-containing *N*-methyl-D-aspartate (NMDA) receptors, and Ca^{2+}/calmodulin-dependent protein kinase II-alpha (CaMKII-α) in the anterior cingulate cortex (ACC). The GluN2A and GluN2B subunits of NMDA receptors, the GluR1 type of AMPA receptor, and CaMKII-α are key molecules responsible for neuroplasticity involved in chronic pain and the accompanying anxiety. Moreover, GAS administration reduced the activation of astrocyte and microglia and the induction of TNF-α and IL-6 in the ACC of the CFA-injected mice. Therefore, GAS administration relieved chronic pain, exerted anxiolytic effects by regulating neuroplasticity molecules, and attenuated the inflammatory response by reducing the induction of TNF-α and IL-6 in the ACC of the CFA-injected mice.

1. Introduction

Noxious or non-noxious stimuli, including inflammation leading to chronic pain, are clinically characterized as hyperalgesia and allodynia, which result in complex maladaptive changes in neural networks [1,2]. A network of brain areas is activated by experimental painful stimulations and is involved in the different dimensions of pain perception [3]. These brain areas include primary sensory, motor, anterior cingulate and insular cortices as well as the amygdala. The anterior cingulate cortex (ACC) is one of the pain-processing regions of the brain and a key component of pain modulation. Evidence shows that electrical stimulation or application of glutamate agonists in ACC results in hyperalgesia by degrading the pain modulation system [4]. Moreover, an ACC lesion in mice exerts an antinociceptive effect [5], suggesting that ACC is implicated in the modulation of nociception. Patients with chronic pain often suffer from affective disorders, such as anxiety [6,7], and anxiety may increase the likelihood of chronic pain development [6,8]. ACC has also been implicated in anxiety in both human and animal studies [9,10]. It plays important roles in sensory perception and emotional responses [6]. Chronic pain can lead to anxiety, and anxiety can enhance the sensation of pain. Anxiety-like behaviors can also be induced by an injection of complete Freund's adjuvant (CFA) into the hindpaws of mice with persistent inflammatory pain [11].

A common mechanism exists between neuroplasticity and chronic pain [12]. Inflammation-induced changes in neural plasticity in the central nervous system (CNS) are important mechanisms involved in chronic pain; these mechanisms are strikingly similar to learning and memory [13]. Long-term potentiation (LTP), a neural substrate of learning and memory, is an underlying mechanism of synaptic plasticity, including nociceptive plasticity [14,15]. LTP in ACC provides a synaptic mechanism for the interactions between anxiety and chronic pain [12]. Glutamate, a major excitatory neurotransmitter in CNS, plays a

Abbreviations: CNS, central nervous system; CFA, complete Freund's adjuvant; NMDA, *N*-methyl-D-aspartate; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ACC, anterior cingulate cortex; CaMKII-α, Ca^{2+}/calmodulin-dependent protein kinase II-alpha; GFAP, glial fibrillary acidic protein; PVDF, polyvinylidene difluoride; ECL, enhanced chemiluminescent solution; ANOVA, analysis of variance.

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http://dx.doi.org/10.1016/j.intimp.2016.10.020
1567-5769/

vital role in learning and memory. Several key molecules are responsible for neuroplasticity formation and are required for the maintenance of LTP. These key molecules include glutamate receptors, such as GluR1-containing α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [16], GluN2A- and GluN2B-containing *N*-methyl-D-aspartate (NMDA) receptors [17,18], and Ca^{2+}/calmodulin-dependent protein kinase II-alpha (CaMKII-α) [14,19].

Gastrodia elata Blume, also known as Tian ma, has been used for thousands of years in China to treat headaches, dizziness, tetanus, epilepsy, and limb numbness because of its analgesic, nootropic, and anti-inflammatory effects [20,21]. Phenolic glucoside gastrodin (GAS) is an active component derived from *Gastrodia elata Blume* root [22]. GAS inhibits the expression of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines in cultured microglia upon LPS stimulation via MAPK pathways [23], suggesting that GAS may suppress specific signaling pathways associated with the inflammatory response. In a previous study, GAS reduced the expression levels of IL-6, IL-1β, and iNOS and inhibited the phosphorylation of P38 MAPK in the hippocampus of a post-traumatic stress disorder model [24]; GAS also reversed animal anxiety-like behaviors. In addition, GAS exhibits anxiolytic-like effects via the GABAergic nervous system [25]. However, the protective effects of GAS on inflammation-induced chronic pain and the involved mechanisms remain unclear.

The present study investigated the possible roles of GAS in CFA-induced chronic pain and the involved mechanisms. GAS attenuated chronic pain induced by CFA injection into the hindpaws of mice and exerted anxiolytic-like effects. Neuroplasticity proteins, including GluR1, GluN2A, GluN2B, and CaMKII-α, as well as inflammatory signaling proteins, including phospho-JNK, phospho-P38, IL-6, and TNF-α, increased in the CFA-injected mice. Administration of GAS relieved hyperalgesia and anxiety by recovering the enhanced expression levels of the related signal proteins.

2. Materials and methods

2.1. Materials

Complete Freund's adjuvant (CFA), Hoechst 33,258 and *anti*-β-actin antibody were purchased from Sigma (St. Louis, MO), anti-GFAP was obtained from Millipore (Billerica, MA), anti-Iba-1 was purchased from Abcam (Cambridge, UK), anti-GluN2A, GluN2B, GluR1, phospho-JNK, phospho-P38, TNF-α, IL-6 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). All secondary antibodies conjugated with horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluro 594 goat anti-rabbit IgG and donkey anti-goat IgG were purchased from Invitrogen (Carlsbad, CA, USA). BCA Kit, M-PER Protein Extraction Buffer and enhanced chemiluminescent solution (ECL) were obtained from Pierce (Pierce, Rockford, IL). PVDF membrane was purchased from Roche (Mannheim, Germany). Gastrodin (purity >98%) was purchased from Shanghai Pure One Biotechnology (Shanghai, China) and reconstituted in sterile 0.9% saline to desire concentrations before use. All chemicals were obtained from Sigma unless otherwise stated.

2.2. Animals

Adult male C57BL/6 mice (aged 6–8 weeks) from the Fourth Military Medical University Experimental Animal Center were used. The animals were housed in groups of six mice with a temperature-controlled colony room (24 ± 2 °C), humidity (50%–60%), and a 12/12 h light/dark cycle with light on 7:00 a.m. Food and water were available ad libitum. All behavioral tests were performed during the light period on the designated day of experiment. All experimental procedures were approved by the Fourth Military Medical University Animal Care and Use Committee. Every effort was made to minimize the number of animals used and their suffering. Mice must adapt to laboratory conditions for at least 2 days prior to testing.

2.3. Experimental designs and gastrodin treatment

The model of chronic peripheral inflammatory pain was established according to previous study [26] after mice acclimatization. 10 μl CFA (50%) was injected intraplantar subcutaneously into the left hindpaws of mice. The same volume of 0.9% saline was injected into the hindpaw as control animals. GAS was dissolved in saline and administrated intraperitoneally (*i.p*) to all GAS groups. The doses of GAS were used in this study according to previous studies [24,27] and our preliminary tests. The mice were injected *i.p* with either GAS (100, 200 mg/kg) or sterile saline (Vehicle, 10 ml/kg) at once after CFA insult. Mice (n = 6 per group) were administrated repeatedly with GAS or saline once a day for 2 weeks for analgesic analysis, and 3 weeks for anxiolytic determination. All the behaviors of animals were tested at a fixed time during testing days and animals were always habituated in the testing room for 15 min before behavioral tests. Animals were sacrificed and the ACC was collected after behavior tests on day 14 to examine molecular events during phase of hyperalgesia and effects of GAS treatment.

2.4. Mechanical allodynia

Prior to experiment, mice were placed in individual plastic boxes and were acclimatized to the environment for 15 min. The mechanical sensitivity was assessed with a set of von Frey filaments using the up-down paradigm. Based on preliminary experiments that characterized the threshold stimulus in untreated animals, the innocuous 0.4 mN (#2.44) filament, representing 50% of the threshold force, was used to detect mechanical allodynia. The filament was applied to the point of bending six times each to the dorsal surface of the hindpaw. Positive responses included prolonged hindpaw withdrawal followed by licking or scratching. For each time point, the percentage response frequency of hindpaw withdrawal was expressed as follows: (number of positive responses) / 6 × 100 per hindpaw.

2.5. Thermal hyperalgesia

The paw withdrawal latency was measured according to previously described methods [26]. The intensity of the thermal stimulus was adjusted to result in an average paw withdrawal latency of approximately 8–12 s in non-inflamed animals. To assess thermal nociceptive responses, a commercially available plantar analgesia instrument (BME410A, Institute of Biological Medicine, Academy of Medical Science, China) was used. Prior to testing, the mice were placed on an elevated glass surface under an inverted transparent plastic cage and allowed to habituate for at least 25 min. Thermal hyperalgesia was assessed by measuring the paw withdrawal latency (PWL) in response to the radiant heat source. The heat source was turned off when the mice lifted the foot, allowing the measurement of time from onset of radiant heat application to withdrawal of the mice's hindpaws. This time was defined as the PWL. Left paws were tested at 5 min intervals for a total of five trials. A 20 s cut off was used to prevent tissue damage.

2.6. Measurement of paw edema

In order to evaluate the effect of GAS on inflammation, paw edema of mice was measured after CFA injection just before (day 0) and after the CFA injection at day 1, 3, 7, 14 and 21 using the method described previously [26]. We used a dial thickness gauge to measure the paw thickness before edema induction at 4 h, day 1, 3, 7, 14 and 21 after CFA injection.