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Full-length Article

Cynandione A attenuates neuropathic pain through p38β MAPK-mediated spinal microglial expression of β-endorphin

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ABSTRACT

Cynanchi Wilfordii Radix (baishouwu), a medicinal herb, has been widely used in Asia to treat a variety of diseases or illnesses. Cynandione A isolated from *C. Wilfordii* is the principle acetophenone and exhibits neuroprotective and anti-inflammatory activities. This study aims to evaluate the antihypersensitivity activities of cynandione A in neuropathy and explored its mechanisms of action. Intrathecal injection of cynandione A dose-dependently attenuated spinal nerve ligation-induced mechanical allodynia and thermal hyperalgesia, with maximal possible effects of 57% and 59%, ED_{50}s of 14.9 μg and 6.5 μg, respectively. Intrathecal injection of cynandione A significantly increased β-endorphin levels in spinal cords of neuropathic rats and its treatment concentration-dependently induced β-endorphin expression in cultured primary microglia (but not in neurons or astrocytes), with EC_{50}s of 38.8 and 20.0 μM, respectively. Cynandione A also non-selectively upregulated phosphorylation of mitogen-activated protein kinases (MAPKs), including p38, extracellular signal regulated kinase (ERK1/2), and extracellular signal regulated kinase (JNK) in primary microglial culture; however, cynandione A-stimulated β-endorphin expression was completely inhibited by the specific p38 activation inhibitor SB203580, but not by the ERK1/2 or JNK activation inhibitors. Knockdown of spinal p38β but not p38α using siRNA also completely blocked cynandione A-induced β-endorphin expression in cultured microglial cells. Furthermore, cynandione A-induced antiallodynia in neuropathy was totally inhibited by the microglial inhibitor minocycline, SB203580, anti-β-endorphin antibody, and μ-opioid receptor antagonist CTAP (but not the κ- or δ-opioid receptor antagonist). These results suggest that cynandione A attenuates neuropathic pain through upregulation of spinal microglial expression β-endorphin via p38β MAPK activation.

1. Introduction

Cynanchi Wilfordii Radix (baishouwu) is an appellative name for the root tubers of *Cynanchum wilfordii* and *Cynanchum auriculatum* (The Health Department and National Chinese Medicine Office, 1999). *C. Wilfordii* has been widely used as a traditional herbal medicine in Asia countries to treat a variety of medical conditions, such as insomnia, anxiety, anemia, senescence, and various geriatric diseases (Koo et al., 2015). In animal experiments, the *C. Wilfordii* extracts and fractions exhibited various biological functions, including blocking free radicals production (Song and Ding, 1997), enhancing immunity (Gu et al., 1987), reducing serum cholesterols (Niu et al., 1988), and exhibiting anti-tumor (Shan et al., 2005) and gastroprotection activities (Shan et al., 2006). Cynandione A, the principle acetophenone isolated from *C. Wilfordii*, has shown to attenuate glutamate-induced cytotoxicity and markedly improve neurological deficit scores in the rat model of cerebral ischemia (Yue et al., 2012). Cynandione A also significantly improved survival of mice with lethal endotoxemia (Kim et al., 2015). However, to date, there are no scientific reports published on its antinociceptive property particularly in neuropathy.

Abbreviations: POMC, proopiomelanocortin; GLP-1, glucagon-like peptide-1; GPR40, G-protein receptor 40; CRF, corticotropin-releasing factor; MAPKs, mitogen-activated protein kinases; ERK1/2, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; IL-1β, interlukin-1β; IL-6, interlukin-6; TNF-α, tumor necrosis factor-α; BDNF, brain-derived neurotrophic factor; LPS, lipopolysaccharides; GsPCR, Gs-protein coupled receptor; DMSO, dimethyl sulfoxide; PEG400, polyethylene glycol; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane chloride salt; % MPE, % maximal possible effect; ANOVA, analysis of variance; E_{max}, maximum effect; ED_{50} or EC_{50}, half-effective dose or half-effective concentration.

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Mitogen-activated protein kinases (MAPKs) are a family of evolutionally conserved proteins and include three major members, i.e., p38, extracellular signal regulated kinase (ERK1/2), and c-Jun N-terminal kinase (JNK) for three different signaling cascades (Ji and Suter, 2007; Pyo et al., 1999, 1998). After being activated via phosphorylation, MAPKs play a critical role in cell signaling and gene expression, particularly in relation to microglial activation (Ledeboer et al., 2005; Sorge et al., 2015; Taves et al., 2013; Tsuda et al., 2004). Lipopolysaccharides (LPS), the main contributing factor to inflammation, can activate several signaling cascades, including MAPKs and the transcription factor NF-κB (Lu et al., 2008; Weinstein et al., 1992; Yang et al., 2000). Accumulated evidence indicates that spinal microglia play a crucial role in initiation and development of neuropathic pain (Jin et al., 2003; Raghavendra et al., 2004; Taves et al., 2013; Wang et al., 2014, 2012) and activated microglia in chronic pain states induce production of different proinflammatory factors (tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β, and brain-derived neurotrophic factor (BDNF)) (Chauvet et al., 2001; Taves et al., 2013). These cytokines released from activated microglia consequently sensitize the neurons in the spinal dorsal horn by altering the excitatory or inhibitory synaptic transmission, contributing to pain facilitation (Kawasaki et al., 2008). Cynandione A and the extracts of *C. Wilfordii* were recently reported to reduce LPS-increased plasma levels of proinflammatory cytokines (TNF-α, IL-6 and IL-1β) in septic mice, and nitric oxide and prostaglandin E2 in microglia and macrophages (Kim et al., 2015; Yang et al., 2014). Cynandione A can also significantly inhibit ERK1/2 and p38 phosphorylation (but barely changing JNK activation) and NF-κB activation (Kim et al., 2015). It is thus possible that cynandione A produced antinociception by blockade of proinflammatory cytokine expression via inhibition of MAPK and NF-κB activation.

Microglia are the key cells in overall brain maintenance and act as the main form of active immune defense in the central nervous system. They also exhibit a "protective state" that involves the anti-inflammatory cascades or tissue repair, in addition to their well-known "destructive state" that induces inflammation (Taves et al., 2013). We have recently illustrated that microglia-released opioid peptide transmitters were actively involved in the protective state of microglia. Specifically, glucagon-like peptide-1 (GLP-1) receptors expressed in microglia were a potential molecular target for treatment of chronic pain (Gong et al., 2014c) and ischemia (Jia et al., 2015). Their agonists, including the peptidic agonists GLP-1(7-36) and exenatide, non-peptidic agonist WB4-24, and herbal agonists shanzhiside methylester, geniposide, loganin, morroniside and catalpol, stimulated microglia in the spinal dorsal horn and hippocampus to express and release β-endorphin, which subsequently activated post-synaptic neuronal μ-opioid receptors, leading to antinociception and neuroprotection (Fan et al., 2015, 2016; Gong et al., 2014a, 2014c; Jia et al., 2015; Xu et al., 2017; Zhu et al., 2014). Furthermore, the herb *aconitum*-derived bulleyaconitine A and bullatine A exhibited antihypersensitivity activity through a direct stimulation of dynorphin A expression in spinal microglia (Huang et al., 2016; Li et al., 2016). Our preliminary data also showed that cynandione A antinociception in neuropathy was blocked by intrathecal injection of the specific opioid receptor antagonist naloxone, suggesting that cynandione A produces antinociception possibly through expression and secretion of opioid peptides from spinal microglia.

In this study, we aimed to investigate whether cynandione A possesses antinociceptive effects in the rat neuropathic pain model induced by tight ligation of L5/L6 spinal nerves. We further explored the mechanisms underlying cynandione A antinociception, particularly the involvement of expression of spinal microglial opioid peptides and proinflammatory cytokines. Our data revealed that cynandione A produced antinociception in neuropathy via β-endorphin overexpression in spinal microglia and subsequent activation of post-synaptic neuronal μ-opioid receptors.

2. Materials and methods

2.1. Drugs and reagents

Cynandione A was extracted and purified by Shandong Academy of Sciences and the purity was more than 95% determined using the high performance liquid chromatography. Its molecular weight was verified by a high resolution mass spectrum (Waters Corporation, Milford, MA, USA). Lidocaine and minocycline were purchased from the First Chengdu Pharmaceuticals Group (Chengdu, China) and Yuanye Biotech (Shanghai, China). 5′-Guanidinonaltrindole (GNTI) and SP600125 were from Sigma Aldrich (St. Louis, MO, USA). SB203580 and U0126 were from Selleck Chemicals (Houston, TX, USA), and CTAP, naltrindole and GW1100 were obtained from Abcam (Cambridge, UK), Tocris (Bristol, UK), and Caymen Chemical (Ann Anbor, MI, USA), respectively. Exendin(9-39) and α-helical CRF(9-41) with peptide contents of 98% were synthesized in Shanghai TASH Biotechnology Co. (Shanghai, China) and GL Biochem (Shanghai, China), respectively. A rabbit polyclonal neutralizing antibody against β-endorphin was purchased from Abcam (Cambridge, UK), which is reported by the manufacturer to be specific to β-endorphin without cross-reactions to methionine-enkephalin, leucine-enkaphalin, γ-endorphin, α-endorphin, ACTH, or α-melanocyte stimulating hormone. Cynandione A was dissolved in 10% dimethyl sulfoxide (DMSO) and 20% polyethylene glycol (PEG400) in 0.9% normal saline for intrathecal injection and dissolve in 0.1% DMSO for cell culture, SB203580, U0126 and SP600125 were dissolved in 20% DMSO in saline, GW1100 and α-helical CRF(9-41) were dissolved in 30% DMSO and 70% PEG400. All other drugs or reagents were dissolved or diluted in normal saline.

The small interference of double-stranded RNA (siRNA) targeting the cDNA sequence of the rat p38α (siRNA/p38α), p38β (siRNA/p38β), and the nonspecific oligonucleotide were designed and synthesized by Shanghai GenePharma Co. (Shanghai, China) and their sequences are listed in Table 1. These oligonucleotides were dissolved in 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) (Avanti Polar Lipids Inc., Alabaster, AL, USA) in a DOTAP: siRNA ratio of 7:1 at room temperature for 15 min.

2.2. Experimental animals

Male adult (160–180 g body weight) and 1-day-old neonatal Wistar rats were obtained from the Shanghai Experimental Animal Institute for Biological Sciences (Shanghai, China). One-day-old neonatal Wistar rats were used for primary glial cell and neuron culture (see below). The adult animals were housed (3–4 per cage) in the Shanghai Jiao Tong University Experimental Animal Center (Shanghai, China) in room temperature (22 ± 2 °C) under conditions of a 12/12-h reversed light-dark cycle (7:00 a.m.-7:00 p.m.), and received food and water *ad libitum*. The adult rats were accustomed to the laboratory environment for 3–4 days before surgery. Experimental study groups (n = 5–6 in each group) were randomly assigned, and the investigator was blinded to the behavior tests. The research protocols were approved by the Animal Care and Welfare Committee of Shanghai Jiao Tong University and carried out in accordance with the animal care guidelines of the US National Institutes of Health.