European Journal of Pharmacology 629 (2010) 40–46

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Neuropharmacology and Analgesia

Attenuation of experimental autoimmune encephalomyelitis in C57 BL/6 mice by osthole, a natural coumarin

Xiaohong Chen [a], Rongbiao Pi [b], Yan Zou [c], Mei Liu [a], Xiaomeng Ma [a], Ying Jiang [a], Xuexuan Mao [b], Xueqiang Hu [a,*]

[a] Department of Neurology, The Third Affiliated Hospital, Sun Yat-sen University, 600 Tianhe Road, Guangzhou, Guangdong 510630, China
[b] Department of Pharmacology and Toxicology, School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, China
[c] Department of Radiology, The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

ARTICLE INFO

Article history:
Received 12 August 2009
Received in revised form 2 December 2009
Accepted 7 December 2009
Available online 16 December 2009

Keywords:
Osthole
Experimental autoimmune encephalomyelitis
Multiple sclerosis
Nerve growth factor
Interferon gamma

ABSTRACT

Osthole, a natural coumarin, is known to have a variety of pharmacological and biochemical uses and is considered to have potential therapeutic applications. Here we examined the effects of osthole on the central nervous system demyelination in experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis and its mechanism(s). C57 BL/6 mice immunized with myelin oligodendrocyte glycoprotein 35–55 amino acid peptide were treated with osthole at day 7 post immunization (7 p.i., subclinical periods, early osthole treatment) and day 13 p.i. (clinical periods, late osthole treatment) respectively and both therapies continued throughout the study. The content of nerve growth factor (NGF) and interferon gamma (IFN-γ) in the sera and brain of mice *in vivo* as well as the splenocytes culture supernatants *in vitro* were detected. The results showed that osthole retarded the disease process when the therapy was initiated at subclinical periods, attenuated the clinical severity of EAE mice when the therapy was initiated at both subclinical and clinical periods, ameliorated inflammation and demyelination and improved the outcomes of magnetic resonance imaging. In addition, osthole blocked the reduction of NGF and suppressed IFN-γ increase in EAE mice. These results suggested that osthole might be a new pharmacological approach to treat multiple sclerosis.

1. Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that affects more than 2.5 million people worldwide (Noseworthy et al. 2000; Frohman et al., 2006). However the etiology of multiple sclerosis is not completely understood and the treatment is not satisfactory. Current treatments for multiple sclerosis have several shortages, such as expensive, subcutaneous injection, limited efficacy, and occasionally causing post injection site irritation/infection (Kieseier et al., 2007). Developing an effective drug that is well tolerated and can slow disease progression would be of great clinical benefits in the treatment of multiple sclerosis (Kieseier et al., 2007).

Osthole (7-methoxy-8-isopentenoxycoumarin, $C_{15}H_{16}O_3$, 244.39 Da, Fig. 1), a natural coumarin, is the main effective constituents of *Cnidium monnieri* (L.) Cusson, which possesses a variety of pharmacological and biochemical properties of central nervous, cardiovascular, endocrine and immune systems, is considered to have potential therapeutic applications (Hoult and Paydt, 1996; Guh et al., 1996; Basnet et al., 2001; Okamoto et al., 2003; Chou et al., 2007; Nakamura et al., 2009). It has been observed to ameliorate several inflammatory diseases, and its antimicrobial 50% effective eoncentration is 21.15–61.62 μg/ml (Tsai et al., 1996; Zhou et al., 2008; You et al., 2009). Osthole was also found to inhibit immune inflammatory disease such as arthritis and hepatitis through modulating inflammatory cytokines, including tumor necrosis factor-α, interferon gamma (IFN-γ) and interleukins (Okamoto et al., 2001;Yang et al., 2003; Chiu et al., 2008; You et al., 2009). However, there is no report about the effects of osthole against CNS immune inflammatory diseases, such as multiple sclerosis.

Experimental autoimmune encephalomyelitis (EAE) is a well-established animal model for the study of the underlying pathogenesis of multiple sclerosis and also widely used to develop new therapies for multiple sclerosis (Stromnes and Goverman, 2006). In this study, we evaluated whether osthole was sufficient to delay or ameliorate the clinical symptoms in C57 BL/6 EAE mice induced by myelin oligodendrocyte glycoprotein 35–55 amino acid peptide (MOG35–55). We further detected the alteration of nerve growth factor (NGF) and IFN-γ in the sera and brain of mice in vivo as well as the splenocytes culture supernatants in vitro, and both of these two factors' expression were implicated in the pathogenesis of experimental autoimmune encephalomyelitis (Kerschensteiner et al., 2009). Our collective results demonstrated that osthole attenuated clinical

* Corresponding author. Tel./fax: +86 20 8751 6822.
E-mail address: huxueqiangzssy@yahoo.com.cn (X. Hu).

0014-2999/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2009.12.008

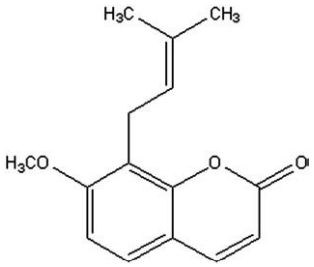

Fig. 1. Chemical structural formula of osthole.

and pathological parameters of experimental autoimmune encephalomyelitis, improved the outcomes of magnetic resonance imaging (MRI), blocked the reduction of NGF and suppressed IFN-γ production in the EAE mice. These results suggested that osthole could be a novel and promising drug for treatment of multiple sclerosis.

2. Materials and methods

2.1. Materials

Osthole (purity>98%) was purchased from Xi'an Green Fount Natural Product Co Ltd (Xi'an, China) and the MOG35–55 peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by CL. Bio-Scientific CO., LTD. (Xi'an, China). Amino acid sequences were confirmed by amino acid analysis and mass spectroscopy. The purity of the peptide was greater than 95%. TRIzol™ and complete Freund's adjuvant (CFA) was purchased from Sigma-Aldrich (St. Louis, MO). Mycobacterium tuberculosis H37RA was purchased from Difco (Detroit, MI). Pertussis toxin (PTX) was purchased from Alexis Corp (San Diego, CA). RPMI 1640 and fetal calf serum was bought from GIBCO/Life Technologies Inc. (Gaithersburg, MD). Gadolinium-DTPA (Gd-DTPA Magnevist™, Schering AG, Berlin, Germany). The cytokine assay by enzyme-linked immunosorbent assay (ELISA) kits for NGF and IFN-γ were purchased from Adlitteram Diagnostic Laboratories (Inc. USA).

2.2. Induction and assessment of EAE

Six- to eight-week-old female C57 BL/6 mice weighing 16–18 g were obtained from the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). Experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Bioethics Committee of Sun Yat-sen University. The procedure used for the induction of EAE has been described previously (Chen et al., 2009). Briefly, mice received a subcutaneous injection in the flanks with 200 μg of MOG35–55 peptide per animal emulsified in CFA containing 500 μg of Mycobacterium tuberculosis H37RA. Immediately thereafter, and again 48 h later, the mice received an intraperitoneal (i.p.) injection of 300 ng of PTX in 100 μl of phosphate buffered saline (PBS). An additional injection of MOG35–55 peptide in CFA was delivered 7 days later. The animals were examined daily for disability. Clinical scores were defined as follows, no signs; 1, loss of tail tonicity; 2, flaccid tail; 3, ataxia and/or paresis of hindlimbs; 4, complete paralysis of hindlimbs; 5, moribund or death.

2.3. Treatment of mice

The dose of osthole was chosen on the basis of previous *in vivo* data and our preliminary dose-finding experiment (Okamoto et al., 2003; Chou et al., 2007). We found osthole used at 30 mg/kg body weight daily could suppress the severity of experimental autoimmune encephalomyelitis. Osthole was dissolved in N,N-dimethylformamide, tween and 0.9% sodium chloride with the ratio 1:1:8, and was administered by intraperitoneal injections at a dosage of 30 mg/kg body weight, twice daily for the first 2 days, then 15 mg/kg body weight twice daily thereafter. Because the apoptosis of neurons starts around 1 week before clinical manifestation of experimental autoimmune encephalomyelitis (Hobom et al., 2004), we initiated the therapies of osthole at day 7 post immunization (7 p.i., subclinical periods, early osthole treatment) and day 13 p.i. (clinical periods, late osthole treatment) respectively and both therapies continued throughout the study. Mice were randomly divided into the following groups ($n=7$): normal control mice, vehicle-treated EAE mice were administered vehicle described above only, early osthole-treated EAE mice received osthole therapy at day 7 p.i. in order to evaluate the therapeutical effects when the therapy was initiated at subclinical periods; late osthole-treated EAE mice received osthole therapy at day 13 p.i. to evaluate the therapeutical effect when the therapy started at clinical periods, and the experiment repeated three times. To evaluate the treatment effects, mice were subjected to MRI examination and histopathology assay at day 20 p.i.. Blood sera and splenocytes of different treated mice were prepared for cytokine assay by ELISA at day 35 p.i.. Brains from different treated mice were harvested for reverse transcriptional-polymerase chain reaction (RT-PCR) at day 35 p.i.

2.4. MRI protocol

All MRI studies were conducted on a GE (Signal Twin Speed Excite II) 1.5-T scanner employing a 77 mT/m (150 mT/m ms) gradient system. Mice were anesthetized by intraperitoneal injection of 300 mg/kg body weight chloral hydrate at day 20 p.i.. For all MRI scans, animals were lying in prone position with their heads fixed in a small dual coil specially designed for investigations of the mice brain. The MRI protocol included T1-weighted (TR 475 ms, TE 13 ms) sequences before and after administration of 0.5 mmol/kg body weight Gd-DTPA and a T2-weighted (TR 2500 ms, TE 80 ms) sequence in the coronal plane with a slice thickness of 1 mm.

2.5. Histopathology assay

To assess the degree of CNS inflammation and demyelination, mice in the osthole-treated and vehicle-treated groups were anesthetized and perfused with ice-cold PBS, followed by 4% paraformaldehyde from the left ventricle at day 20 p.i.. Spinal cords were removed. Tissues were then embedded in paraffin, sectioned and stained with hematoxylin and eosin (H and E) for revealing inflammatory infiltration. And Solochrome cyanin technique was used for myelin staining. Histopathological examination was performed in a blinded fashion. The scale evaluated for inflammation was as follows (O'Neill et al., 2006): 0, no inflammatory cells; 1, a few scattered inflammatory cells; 2, organisation of inflammatory infiltrates around blood vessels; and 3, extensive perivascular cuffing with extension into adjacent parenchyma, or parenchymal infiltration without obvious cuffing. Demyelination in the spinal cords was scored as previously described (Kuerten et al., 2007): 1, traces of subpial demyelination; 2, marked subpial and perivascular demyelination; 3, confluent perivascular or subpial demyelination; 4, massive perivascular and subpial demyelination involving one half of the spinal cord with presence of cellular infiltrates in the CNS parenchyma; and 5, extensive perivascular and subpial demyelination involving the whole cord section with presence of cellular infiltrates in the CNS parenchyma.

2.6. ELISA assay

Spleens and sera of different treated mice were aseptically harvested. Single-cell suspensions of splenocytes were prepared by pushing spleens through a sterile 70 μm pore size nylon mesh. Red blood cells (RBCs) were lysed by RBC lysis buffer. Total splenocytes (1×106 cells/well) were incubated in 96-well flat-bottom plates in RPMI 1640 supplemented with 10% fetal calf serum, with the specific encephalitogenic peptide