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CHRYSIN ATTENUATES EXPERIMENTAL AUTOIMMUNE NEURITIS BY SUPPRESSING IMMUNO-INFLAMMATORY RESPONSES

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Abstract—Guillain–Barré syndrome (GBS) is an acute, post-infectious, immune-mediated, demyelinating disease of peripheral nerves and nerve roots. Experimental autoimmune neuritis (EAN) is an animal model of GBS. Chrysin, which is a naturally occurring flavonoid, exhibits various biological activities. This study was designed to investigate the anti-inflammatory and neuroprotective properties of preventative and therapeutic chrysin treatment in EAN rats. For preventative treatment, chrysin was administered orally from day 1 to day 16 (50 mg/kg once daily) while, for therapeutic treatment, rats received chrysin from day 7 to day 16 at the same dose once daily. Control animals received the same volume of the vehicle (phosphate-buffered saline/2% dimethylsulfoxide). Regardless of the treatment regimen, chrysin attenuated the severity and duration of the clinical course of EAN and reduced inflammatory cell infiltration and demyelination of sciatic nerves. In the sciatic nerves, the expression of inducible nitric oxide synthase, cyclooxygenase-2 and nuclear factor kappa B was reduced. Furthermore, chrysin inhibited the splenic mononuclear cell secretion of interleukin-1β, interleukin-2, interleukin-6, inteleukin-12, interferon γ and tumor necrosis factor α, and elevated the level of inteleukin-4. In summary, our data demonstrate that chrysin is a potentially useful agent for the treatment of EAN with its anti-inflammatory and neuroprotective effects.
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Abbreviations: AIDP, acute inflammatory demyelinating polyneuropathy; ANOVA, analysis of variance; CFA, complete Freund's adjuvant; COX-2, cyclooxygenase-2; DMSO, dimethylsulfoxide; EAN, experimental autoimmune neuritis; ELISA, enzyme-linked immunosorbent assay; GBS, Guillain–Barré syndrome; GM-CSF, granulocyte–macrophage colony-stimulating factor; H&E, hematoxylin–eosin; IFN-γ, interferon γ; IL, interleukin; iNOS, inducible nitric oxide synthase; LFB, luxol fast blue; MNC, mononuclear cell; NF-κB, nuclear factor kappa B; $NO^{\cdot}$, nitric oxide; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; PGs, prostaglandins; PNS, peripheral nervous system; RANTES, regulated upon activation normal T cell expressed and secreted factor; ROS, reactive oxygen species; RT-PCR, real time polymerase chain reaction; TNF-α, tumor necrosis factor α.

Key words: experimental autoimmune neuritis, chrysin, inducible nitric oxide synthase, cyclooxygenase-2, nuclear factor kappa B.

INTRODUCTION

Guillain–Barré syndrome (GBS) is an acute, post-infectious, immune-mediated, demyelinating disease of peripheral nerves and nerve roots. It contains various patterns such as acute motor axonal neuropathy (AMAN), acute motor-sensory axonal neuropathy (AMSAN) and acute inflammatory demyelinating polyneuropathy (AIDP) which is the most common type. Experimental autoimmune neuritis (EAN) is an animal model of the AIDP subtype of GBS which mirrors its many clinical, electrophysiological and histological features. EAN is induced by subcutaneous injection of autoantigen emulsified with complete Freund's adjuvant. Furthermore, the pathology of EAN is characterized by breaching of the blood–nerve barrier, peripheral nervous system (PNS) demyelination and accumulation of inflammatory cells such as T cells and macrophages. There is no doubt that macrophages are the main contributors to the pathogenesis and course of EAN as a result of antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis, and possibly, myelin sheath damage due to the production of reactive oxygen intermediates (Hartung et al., 1988b). Recent studies indicate that nuclear factor kappa B (NF-κB), especially its p65 subunit plays an important role in both in the induction of EAN and during periods of remission (Laura et al., 2006). Furthermore, activation of NF-κB is critical to the regulation of pro-inflammatory responses which involve the recruitment of T cells and the production of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Lee et al., 2011; Liu et al., 2011). The iNOS and COX-2 genes are controlled mainly at the transcriptional level by signaling pathways including NF-κB which is activated by cytokines (Kim et al., 2013). In response to inflammatory stimuli, iNOS and COX-2 can produce copious amounts of prostaglandins (PGs) and nitric oxide (NO) (Kim et al., 2005). This has been considered as a key process in demyelination and inflammation of the PNS (Conti et al., 2004; De La Hoz et al., 2010). Therefore, both iNOS and COX-2 are crucial enzymes in the pathopoiesis of EAN (Lee and Shin, 2002; Shin et al., 2003).

Flavonoids are natural polyphenolic phytochemicals which are present at high levels in honey, propolis and many plant extracts. Among these is chrysin a naturally

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occurring flavonoid known for its various biological activities such as immunomodulation (Lv et al., 2011), anti-cancer (Yu et al., 2013), anti-inflammatory (Rehman et al., 2013), anti-oxidation (Khan et al., 2012a) and anti-allergy (Bae et al., 2011). Several authors have previously reported that chrysin or other flavonoids have an anti-inflammatory and neuroprotective effect on inflammatory neuropathies including diabetic neuropathy, hypoxic-ischemic brain injury and experimental autoimmune encephalomyelitis (Kandhare et al., 2012; Keddy et al., 2012; Yin et al., 2012). The anti-inflammatory mechanism of chrysin is predominantly based on its function as an agonist of peroxisome proliferator-activated receptors gamma (PPARγ) and an antagonist of NF-κB, which ultimately downregulated the production of iNOS and COX-2 (Bae et al., 2011; Wang et al., 2011) and might also be linked with its selective elimination of macrophages (Hougee et al., 2005). The pharmacokinetic clearance and plasma binding of chrysin have been studied relatively clearly that the plasma binding of chrysin is estimated to be >99% and chrysin has a low oral bioavailability and is quickly eliminated ($t1/2$ 6.5 h) (Walle et al., 2001; Velescu et al., 2012). In addition, a new study showed that flavonoids could induce neurotrophic factor synthesis and secretion (Xu et al., 2013). Above all, chrysin has been shown to be a potential immunopreventative and neuroprotective agent (Gresa-Arribas et al., 2010; Lv et al., 2011).

Various drugs including anti-inflammatory agents, immunomodulators and monoclonal antibodies have been tested recently as treatment options for EAN (Xiao et al., 2013). However, the therapeutic potential of flavonoids for EAN is unknown. We hypothesized that chrysin may exert anti-inflammatory and neuroprotective effects in autoimmune disorders of the PNS. Thus, this study was designed to explore the prophylactic and therapeutic effects of chrysin in EAN rats by assessment of clinical, histological and electrophysiological measures.

EXPERIMENTAL PROCEDURES

Experimental animals and grouping

Eighteen Lewis rats, male, 6–8 weeks old, with body weights of 170–190 g, were purchased from the Vital River Corporation (Beijing, China). All animals were acclimated to the environment under temperature-controlled conditions and a 12-h light/dark cycle for 1 week with food and water provided *ad libitum*. Animals were then randomly allocated into three groups (preventative, therapeutic and control). All efforts were made to minimize the number of animals and their suffering. The experimental procedures were approved by the Animal Ethics Committee of the Tianjin Medical University.

Induction and clinical evaluation of EAN

EAN was induced in Lewis rats by the injection of both hind footpads with 300 μL of P0 peptide 180–199 (1 mg/mL; Bio-Synthesis Corporation, Lewisville, TX, USA) emulsified with an equal volume of complete Freund's adjuvant (CFA; Difco, Detroit, MI, USA) containing *Mycobacterium* (*M*) *tuberculosis* (strain H37RA) at a final concentration of 5 mg/mL. Following immunization, animals were weighed and clinical signs of EAN were evaluated as follows: 0, normal; 1, reduced tonus of the tail; 2, limp tail, impaired righting reflex; 3, absent righting reflex; 4, gait ataxia; 5, mild paresis of the hind limbs; 6, moderate paraparesis; 7, severe paraparesis or paraplegia of the hind limbs; 8, tetraparesis; 9, moribund; and 10, death.

Chrysin treatment

Chrysin (50 mg/mL; Sigma–Aldrich, St. Louis, MO, USA), at a purity of 98%, was freshly prepared in phosphate-buffered saline (PBS) containing 2% (v/v) dimethylsulfoxide (DMSO). For preventative treatment, chrysin solution was administered by oral gavage (50 mg/kg body weight/day) from day 1 to day 16 post-immunization (peak phase). For therapeutic treatment, chrysin was administered by oral gavage at the same dose daily from the day on which the first clinical signs were observed, namely, from day 7 to day 16 post-immunization. Control animals received the same volume of the vehicle (PBS/2% DMSO).

Histopathological assessment

Following nerve conduction studies, the right sciatic nerves were harvested at the peak of disease (day 16 post-immunization). Six rats from each group were generally anesthetized and perfused intracardially with 4 °C PBS for 2 min followed by 4% paraformaldehyde in PBS for 5 min. The right sciatic nerve and lumbar spinal cords were rapidly removed and fixed in 4% paraformaldehyde overnight at 4 °C and embedded in paraffin. To evaluate the extent of mononuclear cell (MNC) infiltration and demyelination, serial transverse sections (6-μm slices) were stained with hematoxylin–eosin (H&E) (Solarbio Science & Technology, Beijing, China) and luxol fast blue (LFB) which contains 0.1% LFB solution, 0.1% Cresyl Echt Violet solution and 0.05% lithium carbonate solution. Infiltrating inflammatory cells in H&E stained tissues were enumerated by image analysis using a Nikon Coolscope (×10 and ×100 magnification; Nikon, Dusseldorf, Germany). The average results were expressed as cells per mm^2 tissue section. To evaluate the severity of demyelination, sections containing all perivascular areas were assessed by two independent observers (who were blinded to the treatment) according to the semi-quantitative pathological scale: 0, normal perivascular area; 1, mild cellular infiltrate adjacent to the vessel; 2, cellular infiltration plus demyelination in immediate proximity to the vessel; 3, cellular infiltration and demyelination throughout the section.

Immunohistochemistry

Paraffin tissue sections (6 μm) were deparaffinized with xylene and hydrated with ethanol. Sections were then boiled (in a 600-W microwave oven) for 20 min in citrate

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