International Immunopharmacology 17 (2013) 415–426

Contents lists available at ScienceDirect

International Immunopharmacology

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

Schizandrin C exerts anti-neuroinflammatory effects by upregulating phase II detoxifying/antioxidant enzymes in microglia

Sun Young Park [a], Se Jin Park [b], Tae Gyeong Park [b], Seetharaman Rajasekar [b], Sang-Joon Lee [c], Young-Whan Choi [b,*]

[a] Bio-IT Fusion Technology Research Institute, Pusan National University, Busan 609-735, Republic of Korea
[b] Department of Horticultural Bioscience, Pusan National University, Miryang 627-706, Republic of Korea
[c] Department of Microbiology, Pusan National University, Busan 609-735, Republic of Korea

ARTICLE INFO

Article history:
Received 3 May 2013
Received in revised form 24 June 2013
Accepted 26 June 2013
Available online 13 July 2013

Keywords:
Microglia
Schizandrin C
Anti-neuroinflammation
Lipoteichoic acid
Phase II detoxifying/antioxidant enzymes

ABSTRACT

We investigated the anti-neuroinflammatory properties of schizandrin C by focusing on its roles in the induction of phase II detoxifying/antioxidant enzymes and in the modulation of upstream signaling pathways. Schizandrin C induced expression of phase II detoxifying/antioxidant enzymes including heme oxygenase-1 (HO-1) and NADPH dehydrogenase quinone-1 (NQO-1). Activation of upstream signaling pathways, such as the cAMP/protein kinase A/cAMP response element-binding protein (cAMP/PKA/CREB) and erythroid-specific nuclear factor-regulated factor 2 (Nrf-2) pathways, significantly increased following treatment with schizandrin C. In addition, expressions of schizandrin C-mediated phase II detoxifying/antioxidant enzymes were completely attenuated by adenylyl cyclase inhibitor (ddAdo) and protein kinase A (PKA) inhibitor (H-89). In microglia, schizandrin C significantly inhibited lipoteichoic acid (LTA)-stimulated pro-inflammatory cytokines and chemokines, prostaglandin E2 (PGE2), nitric oxide (NO), and reactive oxygen species (ROS) production, and inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and matrix metallopeptidase-9 (MMP-9) protein expressions. Moreover, schizandrin C suppressed LTA-induced nuclear factor-kappa B (NF-κB), activator protein-1 (AP-1), janus-kinase/signal transducer and activator of transcription (JAK-STATs), and mitogen-activated protein kinase (MAPK) activation. Schizandrin C also effectively suppressed ROS generation and NO production, as well as iNOS promoter activity in LTA-stimulated microglia. This suppressive effect was reversed by transfection with Nrf-2 and HO-1 siRNA and co-treatment with inhibitors ddAdo and H-89. Our results indicate that schizandrin C isolated from *Schisandra chinensis* could be used as a natural anti-neuroinflammatory agent, inducing phase II detoxifying/antioxidant enzymes *via* cAMP/PKA/CREB and Nrf-2 signaling.

1. Introduction

Expressions of phase II detoxifying/antioxidant enzymes are regulated through the activation of transcription factors, including the erythroid-specific nuclear factor-regulated factor 2 (Nrf-2), nuclear factor-kappa B (NF-κB), cAMP response element (CRE)-binding protein (CREB), and activator protein-1 (AP-1). The promoter regions of phase II detoxifying/antioxidant enzyme genes contain antioxidant response elements (AREs) that directly bind Nrf-2 and regulate the expression of many genes involved in adaptive responses and inflammation [1,2]. Several kinase signaling pathways, including protein kinase A (PKA), phosphatidylinositol-3 kinase (PI3K)/AKT, and mitogen-activated protein kinase (MAPK), have been suggested to be involved in regulating the Nrf-2 activation that facilitates its accumulation in the nucleus to promote ARE-related gene expressions [3]. Elevated cAMP levels induce the expression of phase II detoxifying/antioxidant enzymes through PKA-mediated phosphorylation of CREB, a component of the transcription complex at the CRE site on the ARE promoter [4].

Additionally, heme oxygenase-1 (HO-1), and NADPH dehydrogenase quinone-1 (NQO-1) have been intensively studied in the brain for their neuroprotective and anti-inflammatory effects, and results have suggested that they are potential therapeutic targets for many inflammatory diseases [5]. The HO-1 and NQO-1 genes contain an ARE consensus sequence, which enables responses to Nrf-2, as well as to oxidative and nitrosative stresses such as hypoxia, cytokines, nitric oxide (NO), heat shock, and hydrogen peroxide [2]. The major anti-inflammatory function of HO-1 is its rate-limiting catabolic activity during heme degradation. In this reaction, the oxidation of free heme engenders ferrous iron and biliverdin, which are subsequently converted into ferritin and bilirubin, respectively, as well as carbon monoxide, all of which are known to have anti-inflammatory and anti-oxidant properties [6].

* Corresponding author. Tel.: +82 55 350 5522; fax: +82 55 350 5529.
E-mail address: ywchoi@pusan.ac.kr (Y.-W. Choi).

1567-5769/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.intimp.2013.06.032

Microglia are macrophages of the central nervous system (CNS) that play a crucial role in immunological defense against virulent factors in the brain [7]. Under normal conditions, microglia display a ramified morphology in the resting state while continually monitoring the surrounding environment for any changes in the homeostasis of the CNS that may be harmful to neurons or induce damage in the brain [8]. However, in the presence of certain stimuli, microglia become activated and enable proper brain development or repair injured sites *via* secretion of various inflammatory cytokines and phagocytosis, which protects neuronal tissue from subsidiary damages in healthy brains [9]. Upon microglia stimulation, intracellular phosphorylation cascades, including NF-κB, AP-1, janus-kinase/signal transducer and activator of transcription (JAK-STATs), and mitogen-activated protein kinases (MAPKs) become activated. Excessive levels of microglia activation induce chronic inflammatory circumstances *via* constituent activation of pro-inflammatory signal cascades, such as the NF-κB, AP-1, and STAT pathways, ultimately leading to neuronal death and brain injury rather than neuronal survival [10]. In addition, recent studies have emphasized the neuro-inflammation triggered by microglia activation, which has potent effects on the pathogenesis of several neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease [11].

Bacterial lipoteichoic acid (LTA) is known as the major antigenic agent against innate immune cells such as microglia, monocytes, and macrophages. LTA is the smallest bioactive fragment of peptidoglycan (PGN) of gram-positive bacteria membranes. The surfaces of immune cells contain Toll-like receptors (TLRs), which are involved in recognition of various bacterial products such as LTA, PGN, and lipopolysaccharides (LPS) [12]. Recognition by Toll-like receptors leads to activation of cellular signaling pathways involved in the defense against external stimulations. Little information is available pertaining to LTA, but they are both known to trigger activation of immune cells and to promote a diverse array of inflammatory responses through the release of various pro-inflammatory cytokines and mediators, including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, and NO. Moreover, there is accumulating evidence that TLR2 is specifically associated with LTA [13].

In several Asian countries, dried fruit of *Schisandra chinensis* has long been used in traditional medicine as sedatives, analgesics, and antipyretics, and for treatment of hyperlipidemia and hypertension [14]. Researchers have recently found that *S. chinensis* is also useful for treatment of neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease. Moreover, schizandrin C isolated from *S. chinensis* has been shown to have neuroprotective effects on amyloid beta-stimulated PC12 cells [15]. In addition, schizandrin C has been shown to have inhibitory effects on LPS-induced inducible nitric oxide synthase (iNOS) expression *via* MAPKs in RAW264.7 cells [16]. However, to the best of our knowledge, no studies have been conducted to date focusing on the inhibitory effects of schizandrin C on neuroinflammatory responses *via* up-regulation of phase II detoxifying/antioxidant enzymes. Therefore, in order to determine its potential for further development as an anti-neuroinflammatory agent, we investigated the mechanism by which schizandrin C exerts its neuroinflammatory responses. Specifically, experiments were carried out to investigate the effects of schizandrin C on neuroinflammatory responses of murine primary microglia and BV2 microglia cell lines. Here, we provide the first evidence that schizandrin C induces the expression of phase II detoxifying/antioxidant enzymes *via* cAMP/PKA/CREB and nuclear factor-regulated factor 2 (Nrf-2) activation in microglia. The molecular mechanism underlying the observed anti-neuroinflammatory properties of schizandrin C was determined by studying its effects on activation of cAMP/PKA/CREB, Nrf-2, NF-κB, AP-1, STATs, and MAPKs. Overall, results presented herein reveal novel mechanisms by which schizandrin C exerts anti-neuroinflammatory effects, and will therefore be helpful for the development of therapeutic strategies for LTA-mediated neurodegeneration in CNS diseases.

2. Materials and methods

2.1. Materials

The cell culture medium, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were all purchased from Gibco BRL (now Invitrogen Corporation, Carlsbad, CA). LTA and other reagents were obtained from Sigma (St. Louis, MO). Protoporphyrin IX (SnPP), siRNAs against Nrf-2 and HO-1, and antibodies for iNOS, cyclooxygenase-2 (COX-2), HO-1, Nrf-2, c-Jun, c-Fos, NF-κB, IκBα, TATA-binding protein (TBP), and α-tubulin were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated p-p38, p-JNK, p-ERK, p-IκBα, p-STAT-1, STAT-1, P-STAT-3, STAT-3, p-CREB, CREB, and matrix metallopeptidase-9 (MMP-9) were purchased from Cell Signaling Technology (Beverly, MA). The FuGENE 6 transfection reagent and the X-treme GENE siRNA Transfection Reagent were obtained from Roche (Indianapolis, IN).

2.2. Plant material

Fruits of *S. chinensis* (Turcz.) Baill were collected in September 2005 from Moonkyong, Korea. A voucher specimen (accession number SC-PDRL-1) was deposited in the herbarium of the Pusan National University (Miryang, Korea). The plant was identified by one of the authors (Y.W. Choi).

2.3. Purification of schizandrin C

The isolation and purification of schizandrin C from dried fruit of *Schisandra chinensis*, and subsequent evaluation of its structure, were conducted as described previously by Choi et al. Briefly, dried fruits of *S. chinensis* (2.5 kg) were ground to a fine powder and successively extracted at room temperature with n-hexane, EtOAc, and MeOH. The hexane extract (308 g) was then evaporated *in vacuo*, and the remaining sample was chromatographed on a 40 μm silica gel (J.T. Baker, NJ) column (70 × 8.0 cm) with a step gradient of 0, 5, 10, 20, and 30% EtOAc in hexane and 5% MeOH in $CHCl_3$ to obtain 38 fractions. Fraction 8 (1.579 mg) was separated on a silica gel column (100 × 3.0 cm) with CH_2Cl_2 to obtain schizandrin C (501 mg).

2.4. Isolation of mouse primary microglia and cell culture

Isolated primary microglia cultures were prepared as previously described [17]. Briefly, primary mixed glia cell cultures from whole brains of imprinting control region (ICR) mice at postnatal days 2–5 were prepared in culture flasks and maintained in DMEM/F12 containing 10% FBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 mg/ml penicillin/streptomycin at 37 °C under 5% CO_2. After 2 weeks, the culture flasks were shaken in an orbital shaker at 180 rpm at 37 °C for 5 h and the medium was harvested. The attached cells were then removed by trypsinization and seeded onto new plates for subsequent experiments. To monitor purity, cells were immunostained with CD11b antibody, which resulted in more than 90% of cells being stained positively. Mouse BV2 microglial cells were cultured in DMEM supplemented with 5% heat-inactivated FBS and 0.1% penicillin–streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.5. Determination of intracellular cAMP levels

The cAMP concentration was measured using a cAMP EIA kit (Cayman Chemical Company, Ann Arbor, MI). Briefly, cells were lysed in 0.1 M HCl to inhibit phosphodiesterase activity. After neutralization and dilution, a fixed amount of cAMP conjugate was added to compete with cAMP in the cell lysate for sites on rabbit polyclonal antibody immobilized on a 96-well plate. The protein content in the cell lysate was determined