Ginsenoside Rg1 Attenuates Neuroinflammation Following Systemic Lipopolysaccharide Treatment in Mice

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ABSTRACT

Objectives : Neuroinflammation is characterized by microglial activation and the expression of major inflammatory mediators. The present study investigated the inhibitory effect of ginsenoside Rg1 (GRg1), a principle active ingredient in Panax ginseng, on pro-inflammatory cytokines and microglial activation induced by systemic lipopolysaccharide (LPS) treatment in the mouse brain tissue.

Methods : Varying doses of GRg1 was orally administered (10, 20, and 30 mg/kg) 1 h before the LPS injection (3 mg/kg, intraperitoneally). The mRNA expression of pro-inflammatory cytokines in the brain tissue was measured using the quantitative real-time PCR method at 4 h after the LPS injection. Microglial activation was evaluated using western blotting and immunohistochemistry against ionized calcium binding adaptor molecule 1 (Iba1) in the brain tissue, Cyclooxygenase-2 (COX-2) expressions also observed using western blotting and immunohistochemistry at 4 h after the LPS injection. In addition, double-immunofluorescent labeling of tumor necrosis factor-α (TNF-α) and COX-2 with microglia and neurons was processed in the brain tissue.

Results : GRg1 (30 mg/kg) significantly attenuated the upregulation of TNF-α, interleukin (IL)-1β and IL-6 mRNA in the brain tissue at 4 h after LPS injection. Morphological activation and Iba1 protein expression of microglia induced by systemic LPS injection were reduced by the GRg1 (30 mg/kg) treatment. Upregulation of COX-2 protein expression in the brain tissue was also attenuated by the GRg1 (30 mg/kg) treatment.

Conclusion : The results suggest that GRg1 is effective in the early stage of neuroinflammation which causes neurodegenerative diseases.

Key words : Ginsenoside Rg1, Neuroinflammation, Pro-inflammatory Cytokines, Microglia activation, Cyclooxygenase-2

Introduction

Neuroinflammation is the inflammation of the central nervous system (CNS), and it is considered a mediator of secondary damage. It is characterized by microglia activation and expression of major inflammatory mediators, including pro-inflammatory cytokines and cyclooxygenase (COX)1,2. Neurodegenerative diseases, including Alzheimer’s disease (AD) and Parkinson’s disease (PD), are characterized by chronic inflammatory responses in the CNS3. Microglia, the resident immune cells in the CNS, are key cellular elements of the acute neuroinflammatory response and are the primary sources of pro-inflammatory cytokines detected in the brain4. COX-2 is responsible for propagating the inflammatory response and is thus considered the best target for anti-inflammatory drugs5. Therefore, determining the modulatory effects of various drugs...
on neuroinflammation is critical to developing therapeutic approaches for acute and chronic CNS disorders.

Ginsenoside Rg1 (GRg1) is an active ingredient in Ginseng, the root of *Panax ginseng* C.A. Meyer, Araliaceae[^4]. Ginseng is one of the most widely investigated medicinal herb and exerts ameliorating effects on the central nervous system disorders such as stroke, AD, PD, and amyotrophic lateral sclerosis[^5]. Ginseng also alleviates neuroinflammatory processes through regulation of neurotransmitter release and pro-inflammatory mediators[^5]. Ginsenosides can be classified into 3 categories: the panaxadiol group (e.g., Rb1, Rb2, and Rg1), the panaxatriol group (e.g., Rg1, Rg2, and Rh1), and the oleanolic acid group (e.g., Ro[^6]). The characteristic actions of each group are different from other groups[^6]. In particular, the protective effects of panaxatriol GRg1 against neurodegeneration are well studied[^6-13]. With regard to neuroinflammation, panaxadiol ginsenoside Rg1 demonstrated anti-neuroinflammatory effects[^6]. Studies of GRg1 have demonstrated anti-diabetic activity[^15], hematopoietic function[^10], androgenic effects[^7], and anti-inflammatory effects[^10]. Recently, *in vitro* evidence has emerged that GRg1 may play a role in protection against neurodegenerative conditions[^6-9]. In these studies, GRg1 has been shown to attenuate beta-amyloid (Aβ) generation as a peroxisome proliferator-activated receptor-gamma agonist[^9], cause tau phosphorylation in Aβ-stimulated THP-1 cells (human acute monocytic leukemia cell line)[^7], induce neuronal damage in hypoxic ischemic injury[^9], and protect the dopaminergic cell line against 6-hydroxydopamine-induced toxicity[^9]. Moreover, in animal studies, GRg1 reduced Aβ accumulation and improved cognitive impairments in an ovariecotomized rat model of AD[^10], in transgenic AD mice[^11], and in senescence-accelerated mouse prone-1/8 mice[^12]. Furthermore, GRg1 protected dopaminergic neurons against injury to the nigrostriatal dopaminergic pathway and ameliorated dysfunctional behaviors[^13]. Overall, these reports indicate that GRg1 can play a modulatory role in neuroinflammation that causes neurodegenerative diseases.

Microglial activation and pro-inflammatory mediators play a major role in the neuroinflammatory mechanisms of the CNS[^10,20]. There are few *in vivo* studies investigating the effect of GRg1 on microglial activation in the brain tissue except a report from Hu et al.[^21]. Therefore, to better understand the anti-neuroinflammatory effects of GRg1, the present study investigated the effect of GRg1 on microglial activation, which a major character in neuroinflammation with pro-inflammatory cytokine and COX-2 expression, in the brain tissue of LPS-treated mice.

### Materials and Methods

1. Materials

**1) Animals**

Male C57BL/6 mice (25-28 g; Nara Biotechnology, Korea) were used for this study. All animal protocols were approved by the Ethics Committee for the Care and Use of Laboratory Animals at Kyung Hee University. The animals were housed in plastic cages at a constant temperature (22±2 °C) and humidity (55±10%) under 12-h light-dark conditions. The animals were allowed free access to food and water before the experiment.

**2) Reagents**

GRg1 (C_{42}H_{72}O_{14}; molar weight, 801.01; Fig. 1) was purchased from LKT Laboratories (Saint Paul, MN, USA), LPS (from *Escherichia coli* O55:B5) was purchased from Sigma–Aldrich (St. Louis, MO, USA), Rabbit anti-ionicized calcium binding adaptor molecule 1 (Iba1) antibodies (#016-20001, #019-19741) were purchased from Wako Pure Chemical Industries (Osaka, Japan), Goat anti–tumor necrosis factor–α (TNF–α) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Rabbit anti-COX–2 antibody for immunohistochemistry was purchased from Cayman Chemical (Ann Arbor, MI, USA), Goat anti-COX–2 antibody for western blotting was purchased from Abcam (Cambridge, UK), Mouse anti-actin antibody was purchased from Chemicon International (Temecula, CA, USA), Mouse anti-neuronal nuclei (NeuN) antibody and secondary antibodies were purchased from Millipore (Temecula, CA, USA), Cy2–conjugated donkey anti–mouse or donkey anti–rabbit immunoglobulin G (IgG), and Cy3–conjugated donkey anti–mouse IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The other chemicals and reagents used were of high quality and obtained from various commercial sources.

Fig. 1. Chemical structure of ginsenoside Rg1.
2. Methods

1) Experimental groups

Mice were randomly divided into 5 groups. The normal group (Normal) was allowed free access to food and water without any treatment. The control group (LPS) was intraperitoneally (i.p.) injected with a single dose of LPS (3 mg/kg) and received the vehicle (normal saline) orally 1 h before the LPS injection. The GRg1 treatment groups (LPS+GRg110, LPS+GRg120, and LPS+GRg130) were administered an oral dose of GRg1 (10, 20, or 30 mg/kg, respectively, dissolved in normal saline) 1 h prior to LPS injection. Immunohistochemistry studies were processed separately from the polymerase chain reaction (PCR) and western blotting studies in different animals. A total of 48 mice (6 mice per group) were used in this study.

2) Real-time PCR measurement

The mRNA expression of TNF-α, interleukin (IL)-1β, and IL-6 in the brain tissue was measured using the quantitative real-time PCR method. At 4 h after the LPS injection, mice were sacrificed by decapitation and the brain tissue was rapidly dissected. Total RNA was extracted from the samples with Trizol (Qiagen, Germany) according to the manufacturer’s protocol. One microgram of total RNA was transcribed into DNA by using an iScript cDNA synthesis Kit (Bio-Rad, USA). After reverse transcription, quantitative real-time PCR was performed using preoptimized primer/probe mixture with the iQ SYBR Green Supermix kit (Bio-Rad, USA) and the CFX 96 Real-Time PCR Detection System (Bio-Rad, USA). Primer sequences for the analyzed genes are as follows: (1) TNF-α: forward, 5’-TGA GAA GTT CCC AAA TGG C-3’; reverse, 5’-GCC GTA TGG TCA CTC-3’; (2) IL-1β: forward, 5’-TGA GCA CCT TCT TTT CCT TCA-3’; reverse, 5’-TTG TCT AAT GGG AAC GTG ACA C-3’; (3) IL-6: forward, 5’-AGA CTT CAC AGA GGA TAC CA-3’; reverse, 5’-GCA TCA TCG TTC ATGCAAATGGGAC TGC ACA C-3’; (4) β-actin: forward, 5’-TTT CCA GCC TTC CTT GGG TAT G-3’; reverse, 5’-CAC TGT GTG GGC ATA GAG GTC TTT AC-3’. The relative difference in expression between samples was represented using cycle time values normalized in reference to the measurement of the housekeeping gene β-actin. The sample values represent x-fold differences from a sample from the Normal group (given as a designated value of 1) within the same experiment.

3) Western blotting

The brain tissue was homogenized and sonicated on ice in lysis buffer (50 mM Tris–HCl, pH 8.0; 150 mM NaCl; 1% Triton X–100; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate (SDS); 1 mM EDTA; and 1% protease inhibitor cocktail; Sigma, USA). After centrifugation, the supernatant was collected and assayed for protein concentration by using the Bradford method. The lysates containing 50 μg proteins were fractionated by SDS–10% polyacrylamide gel electrophoresis, and then subjected to western blot analysis. The primary antibodies used in this study were rabbit anti–Iba1 antibody (#016–20001; Wako, Japan), goat anti–COX–2 antibody (ab23672; Abcam, UK), and mouse anti–β-actin antibody (MAB1501; Chemicon, USA).

4) Immunohistochemistry

Immunohistochemistry was used to observe microglia, which were immuno–labeled with Iba1 as a microglia marker, and COX–2 in the brain tissue. At 4 h after the LPS injection, the mice were deeply anesthetized and perfused transcardially with 0.05 M phosphate–buffered saline (PBS) containing 4% paraformaldehyde. The brain was removed and postfixed in the same perfusion solution overnight at 4 ℃. Coronal sections of 30 μm thickness were made using a freezing microtome (Leica, 2800N, Germany). The brain sections were stained by a free-floating 3,3’–diaminobenzidine (DAB) reaction. The sections were rinsed with 0.05 M PBS and incubated for 15 min in 1% hydrogen peroxide PBS at room temperature. The sections were incubated overnight at 4 ℃ with primary antibodies against COX–2 (1:200, 160106; Cayman, USA) and Iba1 (1:500, #019–19741; Wako, Japan). The sections were then incubated with biotinylated anti–mouse secondary antibody (1:200, Millipore, USA) for 1 h at room temperature, after which the avidin–biotin complex (Vector Laboratories, USA) method was carried out with peroxidase coupling in a mixture containing 0.05% DAB (Sigma–Aldrich, USA) and 0.03% H2O2 for 2–5 min. Primary antibodies against COX–2 (1:200, 160106; Cayman, USA), NeuN (1:200, MAB377; Millipore, USA), and Iba1 (1:500, #019–19741; Wako, Japan) were used for double–immunofluorescent labeling of COX–2, neuron, and microglia, respectively. Moreover, a primary antibody against TNF–α (1:200, sc–1349; Santa Cruz, USA) was used for the double–immunofluorescent labeling of microglia. The anti–rabbit or anti–mouse Cy2 and Cy3 (Jackson ImmunoResearch, USA) was used as a secondary antibody. Images of the DAB–colorized brain sections were captured using a light microscope (BX51; Olympus, Japan) equipped with a CCD camera (DP70; Olympus, Japan) and the
double-fluorescent-labeled images were captured using confocal laser-scanning microscopy (LSM 510 META; Carl Zeiss, Germany).

5) Analysis of Iba1 and COX–2 immuno-positive cells

Immunohistochemistry stained sections were used to analyze the number of immuno-positive cells. The Iba1 immuno-positive microglia and COX–2 expressed cells counts were analyzed using ImageJ software (Ver. 1.44p, NIH, USA). Four sections and 4 fields per section were chosen for the analysis of each mouse. Data were normalized to the same area ($10^5 \mu m^2$), and the mean values for the 4 sections in each mouse were used for the statistical analysis.

6) Statistical analysis

All data in this study are presented as means ± SEM. Differences between groups were evaluated using Student’s t-test. A probability value less than 0.05 was used to indicate a significant difference.

Results

1. Pro-inflammatory cytokine mRNA expression in the brain tissue

Quantitative real-time PCR measurements of brain TNF-α, IL-1β, and IL-6 mRNA were conducted at 4 h after the LPS injection. The LPS injection increased brain levels of TNF-α mRNA by ~80-fold (80.3±6.2 fold), while the GRg1 treatment significantly attenuated TNF-α mRNA levels in the brain tissue at doses of 10 mg/kg (55.4±8.3 fold, p<0.05), 20 mg/kg (51.3±8.7 fold, p<0.05), and 30 mg/kg (39.0±9.1 fold, p<0.01) compared to the LPS group. The inhibitory effect of GRg1 on brain TNF-α mRNA expression followed a dose-dependent pattern (Fig. 2–A). LPS also increased brain IL-1β mRNA expression by ~110-fold (109.4±14.1 fold), while GRg1 treatment significantly attenuated the percentage increase of Iba1 expression in the brain tissue at a dose of 30 mg/kg (153.2±10.5% compared to the LPS group (†, p<0.05; †, p<0.01) using Student’s t-test.

Fig. 2. (A) The effects of GRg1 on TNF-α mRNA expression in the brain tissue at doses of 10, 20, and 30 mg/kg. (B) GRg1 treatment attenuates brain IL-1β mRNA expression in the brain tissue at doses of 20 and 30 mg/kg. (C) GRg1 treatment attenuates brain IL-6 mRNA expression in the brain tissue at a dose of 30 mg/kg.

2. Microglial activation in the brain tissue

The effects of GRg1 on microglial activation following systemic LPS injection were tested by quantifying Iba1 protein in the brain tissue at 4 h after the LPS injection by using western blot analysis and immunohistochemistry with an Iba1 antibody. LPS increased the percentage of Iba1 expression in the brain tissue (153.2±10.5 %), while GRg1 treatment significantly attenuated the percentage increase of Iba1 expression in the brain tissue at a dose of 30 mg/kg (Fig. 2–C). Overall, the increases in the levels of TNF-α, IL-1β, and IL-6 mRNA in the brain tissue following systemic LPS injection were consistently reduced by the 30 mg/kg GRg1 treatment.
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(112.9 ± 10.6 %, p < 0.05) compared to the LPS group. The brain levels of Iba1 expression in the 10 and 20 mg/kg GRg1 treatment groups were not significantly different from that of the LPS group (Fig. 3).

Immunohistochemistry with the Iba1 antibody was performed on tissue from mice treated with a 30 mg/kg dose of GRg1 because 30 mg/kg of GRg1 was the most effective dose for the inhibition of cytokine and Iba1 expression. The injection of LPS injection activated microglia in the cerebral cortex of the brain, as evidenced by their increased cell size, irregular shape, thickened and shortened processes, and intense Iba1 immunostaining (Fig. 3, LPS vs. Normal). However, GRg1 treatment attenuated the morphological changes associated with the activated form of microglia in all the brain regions from mice treated with LPS (Fig. 3, LPS+GRg130 vs. LPS). To determine the effect of GRg1 on microglial activation, the number of Iba1–immunostained microglia in a standard area (10^5 µm^2) was counted. The LPS group had 60.2 ± 3.0 cells/10^5 µm^2, whereas the LPS+GRg130 group had 51.3 ± 2.4 cells/10^5 µm^2 (p < 0.05) (Fig. 4). These results indicate that GRg1 attenuated microglial activation in the brain induced by systemic LPS injection. In addition, double-immunofluorescent labeling of Iba1 and TNF-α showed that TNF-α-expressing cells in the brain were co-localized mostly with the microglia (Fig. 5, Iba1+TNF-α). This result indicates that TNF-α is mainly expressed on microglia in the brain tissue following systemic LPS injection.

3. COX-2 expression in the brain tissue

The effects of GRg1 on COX-2 expression following systemic LPS injection were tested by quantification of COX-2 protein in the brain tissue at 4 h after the LPS injection using western blot analysis. LPS increased the percentage of COX-2 expression in the brain tissue (171.0 ± 9.8 %), while the GRg1 treatment significantly attenuated the percentage increase of Iba1 expression in the brain tissue at a dose of 30 mg/kg (121.2 ± 9.4 %, p < 0.01) compared to the LPS group. The brain levels of COX-2 expression in the 10 and 20 mg/kg GRg1 treatment groups were not significantly different from that of the LPS group.
For a better understanding of the suppressive effect of GRg1 on COX-2 expression, immunohistochemistry against the COX-2 antibody and double-immunofluorescent labeling of COX-2 with the neurons and microglia were performed on the brain tissue. Brain tissue in the normal group exhibited few COX-2–positive cells in the cortex. The LPS group demonstrated a robust increase in COX-2–positive cells in the cortex (Fig. 7, LPS). In contrast, GRg1 treatment reduced the number of COX-2–positive cells in the cortex (Fig. 7, LPS+GRg130). Based on the COX-2–positive cell count in the corresponding area, GRg1 treatment significantly reduced the number of COX-2–positive cells in the cortex (23.0±3.5 vs. 35.2±3.2 cells/10^5 µm^2, p<0.05) compared with the LPS group (Fig. 7).

In addition, double-immunofluorescent labeling of COX-2 with NeuN or Iba1 identified the properties of the COX-2–positive cells. COX-2–positive cells in the cortex were mostly co-localized with the neurons (Fig. 8, COX-2+NeuN). Interestingly, COX-2 expression in the cortex did not co-localize with the microglia (Fig. 8, COX-2+Iba1). This result indicates that COX-2 is mainly expressed on neurons, not on microglia, in the brain tissue following systemic LPS injection.

Discussion
Systemic LPS treatment (intraperitoneal injection) activates the inflammatory response in the brain and promotes inflammation through the activation of microglia and the upregulation of pro-inflammatory cytokines and COX-2. In an acute systemic LPS treatment study, inflammatory responses in the brain, including morphological activation of microglia and protein/mRNA expression of inflammatory mediators, appeared within 4–8 hours and subsided within 1–3
Microglia, the resident immune cells in the CNS, are activated by an extensive list of pro-inflammatory stimuli, including LPS. Systemic LPS injection activates the inflammatory response in the brain through the toll-like receptors located on glia\(^{26}\). This activates microglia and upregulates pro-inflammatory cytokines, consequently propagating inflammation\(^{27}\). There is abundant evidence suggesting that pro-inflammatory cytokine production and signaling results in neuronal cell death and is closely related to neurodegeneration\(^{28,29}\).

In a previous in vitro study of BV–2 microglial cells, GRg\(_1\) effectively inhibited TNF–α, IL–1β, inducible nitric oxide synthase, and COX–2 via activation of the phospholipase C signaling pathway\(^{18}\). This study showed that GRg\(_1\) significantly reduced the over-expression of TNF–α, IL–1β, and IL–6 mRNA in the brain tissue following systemic LPS treatment. This result may expand our understanding of the anti-inflammatory effect of GRg\(_1\) on neuroinflammation from in vitro to in vivo. In the present study, GRg\(_1\) significantly attenuated microglia activation in the brain tissue of LPS-treated mice. Microglia are key cellular elements of the acute neuroinflammatory response and are the primary sources of pro-inflammatory cytokines detected in the brain\(^{30}\). A previous report showed that GRg\(_1\) attenuated Iba1 expression, which is highly and specifically expressed in microglia and macrophages\(^{30}\), in the cerebral cortex and hippocampus\(^{31}\). The present study demonstrated that GRg\(_1\) reduced the morphological change of microglia into the activated form. The activated form shows increased cell size, irregular shape, and thickened and shortened processes in the cerebral cortex. Moreover, GRg\(_1\) significantly reduced the number of Iba1-expressing microglia in the cerebral cortex when compared to the LPS group. In addition, TNF–α-expressing cells were identified as microglia by double-immunofluorescent labeling of Iba1 and TNF–α. Overall, the results suggest that GRg\(_1\) plays a modulatory role in microglia activation.

COX plays a central role in the inflammatory cascade by converting arachidonic acid into bioactive prostanoids\(^{31}\). COX exists in 2 isoforms: COX–1 and COX–2. COX–1 is a constitutive isoform, which is widely distributed in virtually all cell types and is thought to mediate physiological responses. COX–2 is a second and inducible isoform, which is rapidly expressed in several cell types in response to cytokines and pro-inflammatory molecules and has emerged as the isoform primarily responsible for prostanoid production in acute and chronic inflammatory conditions\(^{31,32}\). Interestingly, excluding a Chinese report by Wang et al.\(^{33}\), there are no in vivo reports about the effect of GRg\(_1\) on COX–2 despite its significant role in the modulation of inflammatory responses. As a result of the present study, a dose of 30 mg/kg of GRg\(_1\) significantly attenuated the upregulation of COX–2 mRNA and protein expressions in the brain tissue induced by intraperitoneal LPS injection. Although there are conflicting views about the role of COX–2 in neurodegenerative diseases caused by neuroinflammation\(^{33}\), the results of this study suggest that GRg\(_1\) might play a role in modulating COX–2 expression induced by LPS. The primary purpose of this study was to show the effect of GRg\(_1\) on COX–2 mRNA and protein expressions in the brain tissue. As the results of COX–2 immunohistochemistry in this study show, COX–2 expression was detected in the cerebral cortex, COX–2 expression in the cerebral cortex was attenuated by GRg\(_1\) treatment. The COX–2-expressing cells were identified as neurons by double-immunofluorescent labeling of COX–2 and NeuN. Additionally, it was revealed that COX–2 expression did not coincide with Iba1-expressing microglia in the brain tissue of LPS-treated mice. This result confirms that microglia are not the main source of COX–2 production\(^{31}\), even COX–2 expression has been detected in activated microglia preceding neuronal cell death\(^{33,32}\).

The findings from this study indicate that GRg\(_1\) can effectively attenuate COX–2 mRNA and protein expression and microglia activation induced in the brain by systemic LPS treatment. Thus, it is thought that GRg\(_1\) and GRg\(_1\)-containing Panax ginseng may potentially protect against neurodegenerative diseases caused by neuroinflammation.

Acknowledgement

This work was supported by a grant (KHU–20110208) from the Kyung Hee University in 2011.

References

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