Glycyrrhiza glabra L. Extract Inhibits LPS-Induced Inflammation in RAW Macrophages

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Summary Glycyrrhiza glabra has been used in medicine for thousands of years. Our previous study revealed that the methanolic extract of Glycyrrhiza glabra L. (EGGR) exhibits significant nitric oxide (NO) inhibitory effect on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages among 100 other extracts. Accordingly, the aim of the present study was to investigate the potential anti-inflammatory effect of EGGR. The anti-inflammatory effect of EGGR on LPS-stimulated RAW 264.7 macrophages was measured by MTT assay, NO content analysis, reactive oxygen species (ROS) level analysis, RT-PCR, Western blot analysis, and ELISA assay. Low doses of EGGR were non-toxic to macrophages and imparted protective effect against LPS induced cell death. Incubation of LPS-treated macrophages with 100 μg/mL EGGR led to an increase in cell viability from 66.6 to 99%. Moreover, EGGR led to down regulation of NO (NO2+NO3) and ROS productions in a dose-dependent manner. In particular, 100 μg/mL EGGR led to a reduction in NO2+NO3 level from 336.2 to 24.1 μM/mL, and ROS level from 483.5 to 128.4%. Consistent with the result related to NO production, EGGR suppressed the ability of LPS to induce mRNA and protein expressions of nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) cytokines, tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), and IL-6 productions which were analyzed by an ELISA assay. These results provide a comprehensive approach into the anti-inflammatory effect of EGGR on LPS-stimulated macrophages; however, efforts are underway on gaining detailed insight into anti-inflammatory signaling pathways.

Key Words Glycyrrhiza glabra L., LPS, inflammation, macrophage

Glycyrrhiza glabra (GG) is an important medicinal herb native to central and south-western Asia and the Mediterranean region (1). For popular use, it has been cultivated in many countries, such as Italy, Russia, France, the UK, the USA, Germany, Spain, China and India (2). The root, which is the most commonly used part, contains many triterpenoid saponins, flavonoids, coumarins, and other compounds (3-5). These compounds are considered to possess therapeutic properties including anti-inflammatory (6, 7), antioxidant (8, 9), anti-ulcer (10), anti-viral (11), anti-microbial (12), immunostimulatory (13), anti-atherosogenic (14), hepatoprotective (15), anticarcinogenic (16), antimitagenic (17), anticarcinogenic (18), and expectorant effects (19). The water and ethanol extracts of this herb have been demonstrated to exhibit strong anti-inflammatory activity in terms of reducing the production of inflammation in lipopolysaccharide (LPS)-stimulated macrophages (20).

Macrophages, the common target of anti-inflammatory studies, play a critical role in the initiation, maintenance, and resolution of inflammation (21). The inflammatory response has been extensively studied in a model of LPS-stimulated RAW 264.7 macrophages. Activation and over production of proinflammatory cytokines including interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) in the activated macrophages is well documented (22, 23). Nitric oxide (NO), as well as reactive oxygen species (ROS), are key inflammatory mediators in inflammation and important signaling molecules in anti-inflammatory investigation (24, 25). Scavenging or deactivation of such mediators can effectively inhibit inflammation, help in repairing damaged tissues with subsequent prevention or treatment of inflammation-related diseases.

Our previous report (26) has demonstrated considerable anti-inflammatory effect of methanolic extracts from different medicinal plants on LPS-stimulated RAW 264.7 macrophages. In particular, the extract from Glycyrrhiza glabra L. root (EGGR), significantly inhibited nitric oxide production. In this study, we investigated the effect of EGGR on the inflammatory cytokines and related products employing a LPS-stimulated RAW 264.7 macrophage model.

MATERIALS AND METHODS

Sample preparation. The methanol extract from the root of Glycyrrhiza glabra L. root (EGGR) was prepared
by the Plant Extract Bank (Korea). The root of the plant was dried in shade and dipped in HPLC grade methanol at 50°C. Subsequently, the solvent was evaporated under reduced pressure by a vacuum rotary evaporator.

**Cell line and cell culture.** Murine macrophage RAW 264.7 cells were purchased from the Korean Cell Line Bank (Seoul, Korea) and grown in Roswell Park Memorial Institute medium 1640 (RPMI 1640), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37°C in 95% humidified air containing 5% CO2.

**Methylthiazolyltetrazolium bromide (MTT) assay.** The RAW 264.7 macrophages were plated at a density of 1×10^5 cells/well in a 96-well plate for 16 h, followed by treatment with various concentrations (12.5, 25, 50, 100, and 200 μg/mL) of EGGR in the presence/absence of LPS (2 μg/mL). After 24 h incubation, a 20 μL aliquot of 2 mg/mL MTT solution was added to each well and allowed to stand for 4 h. Then, supernatant was removed, and 200 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Absorbance was measured at 550 nm by an enzyme-linked immunosorbent assay plate reader (BioTek, Winooski, VT). The percentage of cytotoxicity was determined with respect to the control group.

**Determination of nitric oxide (NO).** NO production (NO2^−+NO3^- level) in the supernatants of the cultured RAW 264.7 macrophages was determined by Griess reagent. Briefly, the macrophages were plated in a 96-well plate (1×10^5 cells/well) for 16 h. After treatment, 100 μL of supernatants were mixed with equal volumes of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride) and incubated at room temperature for 10 min. Next, the absorbance was measured at 550 nm by an enzyme-linked immunosorbent assay plate reader (BioTek).

**Determination of reactive oxygen species (ROS).** The intracellular ROS were measured by a fluorometric assay using dichlorofluorescein-diacetate (DCFH-DA) as the probe. The prepared RAW 264.7 macrophages (1×10^5 cells/well in 96-well plate and incubated for 16 h) were treated with 25, 50, and 100 μg/mL EGGR with/without LPS and incubated for 24 h: subsequently the samples were again incubated with 10 μM of DCFH-DA for 30 min in the dark. After washing twice with PBS, fluorescence was measured in a SpectraMax® M2 Microplate Reader (Molecular Devices, Sunnyvale, CA) with excitation and emission wavelengths of 485 nm and 535 nm, respectively.

**RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis.** The prepared RAW 264.7 macrophages were incubated in 6-well plates with 1×10^5 cells/well. After treatment and incubation for 24 h, the total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA), and stored at −80°C for subsequent analysis. Isolated RNA was reverse transcribed, and 2 μL cDNA was subsequently used as the template for RT-PCR amplification. The conditions for iNos (sense, 5′-GGCTGTCAGAGGCTGTTTGG-3′) and Gapdh (sense, 5′-CCTACTGGAATTTCAAGGCAC-3′; antisense, 5′-GACTTCCAGGATCACTGAC-3′) were initial denaturation at 94°C for 5 min and amplification was followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and primer extension at 72°C for 45 s with a final extension at 72°C for 5 min. The conditions for Cox-2 (sense, 5′-CCTACTACCT-GACCCATTGTTT-3′; antisense, 5′-ATGCTCTGTTGAGTATGT-3′) were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s with a final extension at 72°C for 5 min. The PCR product was analyzed on 1% agarose gel, and DNA bands were visualized by ethidium bromide staining and a Mini BIS image analysis system (DNR Bio-Imaging Systems Ltd., Kiryat Anavim, Israel).

**Western blot analysis.** RAW 264.7 macrophages were plated onto 6-well plates at approximately 1×10^6 cells/well and media were replaced with 5% FBS/RPMI. After 16 h incubation, the cells were treated with/without LPS in the presence of different concentrations of EGGR for 24 h. The harvested cells were washed with PBS twice and then lysed with cell lysis buffer (150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 50 mM Tris-HCl, pH 7.5, 2 mM storage) for 30 min at 4°C. The homogenates were centrifuged at 13,000 ×g for 10 min to isolate the supernatants. Protein concentrations were determined using Pierce bicinchoninic acid (BCA) protein assay. Typically, 30 μg of protein from the supernatants was separated on 10% sodium dodecylsulfate-polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF; Bio-Rad) membranes. After transfer, the membrane was blocked with 5% BSA in Tris-buffered saline-Tween (TBST; 20 mM Tris, 500 mM NaCl, pH 7.5, and 0.1% Tween 20) at room temperature for 1 h. Subsequently, the membranes were incubated with primary antibodies for 1 h, after three washes in TBST for 5 min; the membranes were incubated with secondary antibody for 1 h at room temperature. The immunoreactive protein bands were detected using an enhanced chemiluminescence detection system. Blots were developed with a Mini BIS image analysis system.

**Enzyme-linked immunosorbent assay (ELISA).** The levels of TNF-α, IL-1β, and IL-6 in the medium of macrophages were determined using a mouse ELISA kit (BD Biosciences, San Diego, CA). Typically, 100 μL of pre-incubated media from 96-well plate was used and assayed according to the manufacturer’s protocol for the relevant ELISA kit.

**Statistical analysis.** All tests were carried out independently in triplicate. Data are expressed as mean±standard derivation. All statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test using SPSS 19.0 software (IBM, Armonk, NY). Values of p<0.05 were considered statistically significant. Means with the same letter in each figure are not significantly different.
RESULTS

Effect of EGGR on LPS-induced cytotoxicity in RAW 264.7 macrophages

To evaluate possible toxicity of EGGR on RAW 264.7 macrophages, MTT assay was performed. Cytotoxicity of EGGR on RAW 264.7 macrophages was observed to be dose-dependent. When the concentration of EGGR was ≤100 μg/mL, cell viability was 100% (Fig. 1); however, when the concentration was 200 μg/mL, cell viability was reduced to 90.2%. Furthermore, LPS inhibited proliferation of RAW 264.7 macrophages; a reduction in cell viability from 100 to 66.6% was observed after incubation with 2 μg/mL of LPS for 24 h. When compared to the LPS-stimulated group, an increase in cell viability was observed in the EGGR-stimulated group: EGGR increased cell viability to 78.6, 83.0, 92.6, and 99% after incubation with 12.5, 25, 50, and 100 μg/mL EGGR for 24 h, respectively.

Effect of EGGR on LPS-induced NO production in RAW 264.7 macrophages

For NO content (NO₂⁺NO₃⁻ level) analysis, the medium from the LPS-incubated group became purple after being mixed with Griess reagent. A marked increase in NO₂⁺NO₃⁻ level (336.2 μM/mL) was noted in RAW 264.7 macrophages after 24 h of incubation with LPS. However, EGGR exhibited inhibitory effect on NO production in LPS-stimulated macrophages in a dose-dependent manner. As compared to LPS-incubated group, the NO₂⁺NO₃⁻ level was down-regulated to 289.1, 272.0, 164.5, and 24.1 μM/mL after treatment with 12.5, 25, 50, and 100 μg/mL EGGR, respectively (Fig. 2).

Effect of EGGR on LPS-induced ROS level in RAW 264.7 macrophages

The DCFH-DA assay was employed to investigate the
intracellular ROS level in LPS-stimulated macrophages. LPS stimulated an increase in ROS level from 100 to 483.5% (Fig. 3). Treatment of LPS-stimulated macrophages with 25, 50, and 100 μg/mL EGGR downregulated the ROS level to 311.3, 187.7, and 128.4%, respectively.

**Effect of EGGR on LPS-induced iNOS and COX-2 expression in RAW 264.7 macrophages**

To determine that EGGR can reduce LPS-induced NO production, we studied the effect of EGGR on iNOS mRNA and protein expressions in RAW 264.7 macrophages using RT-PCR and Western blot analysis, respectively. Consistent with the findings related to NO level, reduction in both mRNA and protein expression of iNOS induced by LPS in macrophages was observed subsequent to EGGR treatment (Fig. 4). RT-PCR and Western blot analysis revealed EGGR induced significant reduction in COX-2 (another important cytokine) expression. Figure 4B and C represent complete repression in iNOS and COX-2 protein expression in macrophages treated...
Anti-Inflammatory Effect of *Glycyrrhiza glabra* L.

with 100 μg/mL EGGR. The band relative intensity (RI) of iNOS and COX-2 mRNA/protein expressions are exhibited in Fig. 4D and E.

**Effect of EGGR on LPS-induced TNF-α, IL-1β, and IL-6 production in RAW 264.7 macrophages**

To investigate the effect of EGGR on the inflammatory cytokines, TNF-α, IL-1β, and IL-6, the production of cytokines in the medium of macrophages treated with LPS (2 μg/mL) alone or in combination with EGGR was measured by ELISA assays. Data are expressed as mean±standard deviation (n=3).

**DISCUSSION**

*Glycyrrhiza glabra* L. as a member of the Fabaceae family has been used as a source of novel food ingredients and a medicinal plant for thousands of years. In recent years, many researchers have focused on studying biologically active compounds isolated from *Glycyrrhiza glabra* L. and their clinical safety (19, 24, 27). In the current study, the MTT assay was employed to test the potential toxicity of EGGR. A low dose (100 μg/mL) of EGGR had no effect on the cell viability of RAW 264.7 macrophages. Thus, the low dosage of EGGR was considered to be clinically safe. On the other hand, LPS inhibited cell viability of RAW 264.7 macrophages in a significant manner. EGGR exerted a protective effect to macrophages against LPS-induced toxicity. When *Glycyrrhiza glabra* is used as a food ingredient, the ingested amount is limited. As per the European Food Safety Authority (EFSA) report up to 120 mg/d of Glavonoid, an extract from *Glycyrrhiza glabra*, is safe for consumption amongst the general adult population (28). In our study, the employed dosages of EGGR were also limited, and in future studies 25, 50, and 100 μg/mL EGGR will be employed.

LPS-induced toxicity in macrophages may be related to the high NO$_2$+NO$_3$ level, which can induce toxic reactions against the host tissues (29). NO, as a marker of inflammation, is a common research target in anti-inflammatory studies. Measurement of the NO$_2$+NO$_3$ level can be used for the diagnosis and monitoring of responses to anti-inflammatory therapy (30). In this study, the NO$_2$+NO$_3$ level was tested employing Griess reagent. When compared to the LPS-stimulated macrophages, a decline in the reaction in the EGGR treated groups was observed. Moreover, EGGR suppressed the ROS level in a dose-dependent manner, which was increased sharply in macrophages in the presence of LPS. ROS are known to be crucial inflammatory mediators contributing to damaging effects of inflammatory reactions (31). ROS are considered to be involved in the down-regulation of inflammatory response. In the inflammatory model of LPS-stimulated RAW macrophages, many studies have demonstrated that samples, such as procyanidins and red ginseng marc oil, possessed anti-inflammatory effects with respect to a decrease in ROS production (32, 33). Hence, based on the significant inhibitory effect of EGGR on NO and ROS, the anti-inflammatory effect of EGGR is apparent.

In macrophages, NO is usually produced and controlled by iNOS. The iNOS is highly expressed during the inflammatory process, thus producing large amounts of nitric oxide, which subsequently leads to inflammatory and autoimmune diseases (34, 35). COX-2 is another important enzyme catalyzing the conversion of arachidonic acid to prostaglandins, the precursor of inflammatory mediators (36). Therefore, inhibition of iNOS and COX-2 has been considered as an effective means for preventing or treating inflammation-related diseases. The RT-PCR and Western blot analyses demonstrated induction of iNOS and COX-2 expressions in RAW 264.7 macrophages after treatment with LPS. In unstimulated RAW 264.7 macrophages, the iNOS and COX-2 mRNA expressions were not detected; however, LPS remarkably up-regulated the mRNA level. Nevertheless, pre-treatment with EGGR antagonized the up-regulatory effect. In addition, LPS-induced iNOS and COX-2 protein expressions were attenuated in macrophages co-treated with different concentrations of EGGR. Therefore, the inhibitory effect of EGGR on iNOS and COX-2 expressions may be one of the operations
accountable for the anti-inflammatory activity of EGGR.

The pro-inflammatory enzymes iNOS and COX-2 are known to be modulated by nuclear factor-kappa B (NF-κB), which is one of the most ubiquitous transcription factors in cellular proliferation, inflammatory responses, and cell adhesion (37). The activated NF-κB can regulate gene expression of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (38). In LPS-stimulated macrophages, TNF-α plays a key role in the induction and perpetuation of inflammation. TNF-α has also been reported to induce apoptotic cell death (39). The EGGR down-regulated LPS induced TNF-α production, which further demonstrates the protective effect of EGGR on LPS-induced cell death. Furthermore, IL-1β is one of the most important inflammatory cytokines secreted by macrophages (40). By contrast, EGGR inhibited LPS-induced production of IL-1β in a dose-dependent manner. During inflammation, the increased level of IL-1β in LPS-incubated macrophages can lead to damage (41), and it is assumed that EGGR might retard inflammatory responses. Additionally, IL-6 is a pleiotropic cytokine, which can be induced by several factors and produced by T cells, macrophages, and synovial fibroblasts in inflamed joint tissues (42, 43). The inducing factor, LPS, significantly increased IL-6 production, while EGGR significantly reduced the induction at a high dosage. Cytokine balance contributes to the onset, progression, and resolution of inflammation. The ELISA analysis indicated decreases in TNF-α, IL-1β, and IL-6 by EGGR, which suggested EGGR might be a good candidate for treatment of inflammation and related diseases.

In summary, our results demonstrate that EGGR exerts anti-inflammatory effects, resulting in the inhibition of LPS-induced NO and ROS productions and expressions of pro-inflammatory cytokines, iNOS, COX-2, TNF-α, IL-1β, and IL-6, in RAW 264.7 macrophages. Moreover, EGGR was found to protect the macrophages against cell death, which may be induced by overproduction of NO and activation of TNF-α. Additional detailed studies on the mechanism of anti-inflammatory signaling pathways are currently underway, which are proposed to contribute to the elucidation of the marked anti-inflammatory effect of EGGR.

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