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Glycyrrhizic Acid and 18β-Glycyrrhetinic Acid Modulate Lipopolysaccharide-Induced Inflammatory Response by Suppression of NF-κB through PI3K p110δ and p110γ Inhibitions

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ABSTRACT: The roots and rhizomes of licorice (*Glycyrrhia*) species have been used extensively as natural sweeteners and herbal medicines. The aim of this work was to determine the in vitro anti-inflammatory effects of glycyrrhizic acid (GA) and 18β-glycyrrhetinic acid (18βGA) from licorice in a lipopolysaccharide (LPS)-stimulated macrophage model. The results showed that treatment with 25−75 μM GA or 18βGA did not reduce RAW 264.7 cell viability but did significantly inhibit the production of LPS-induced nitric oxide (NO), prostaglandin E_2 (PGE_2), and intracellular reactive oxygen species (ROS). Western blotting and reverse transcriptase polymerase chain reaction (RT-PCR) analyses revealed that GA and 18βGA significantly reduced the protein and mRNA levels of iNOS and COX-2 in LPS-induced macrophages. Both GA and 18βGA inhibited the activation of NF-κB and the activities of phosphoinositide-3-kinase (PI3K) p110δ and p110γ isoforms and then reduced the production of LPS-induced tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β in a dose-dependent manner. In conclusion, these results indicate that GA and 18βGA may provide an anti-inflammatory effect by attenuating the generation of excessive NO, PGE_2, and ROS and by suppressing the expression of pro-inflammatory genes through the inhibition of NF-κB and PI3K activity. Thus, the results suggest that GA and 18βGA might serve as potential agents for the treatment of inflammatory-mediated diseases.

KEYWORDS: glycyrrhizic acid, 18β-glycyrrhetinic acid, anti-inflammatory, lipopolysaccharide, macrophage

INTRODUCTION

Inflammation, the first line of physiological defense in biological systems, can protect against injuries caused by harmful stimuli, such as pathogens and poisons. During mobilization of the host defense against pathogenic infections, macrophages exposed to lipopolysaccharides (LPS) on the outer membrane of bacterial toxins play crucial roles in inflammation.[1] In the initiation and maintenance of inflammation, activation of macrophages by pathogens causes phosphoinositide-3-kinase (PI3K) and nuclear factor-κB (NF-κB) activation, resulting in the production of reactive oxygen species (ROS), nitric oxide (NO), prostaglandins (PG), cytokines, tumor necrosis factor-α (TNF-α), and other eicosanoid mediators to promote inflammatory responses. These inflammation-inducible factors are mediators of cellular injury and are involved in the onset of cellular damage during endotoxemia.[2] During an inflammatory response, pro-inflammatory genes, such as nitric oxide synthase (iNOS) and cyclooxygenase (COX), are induced via signal transduction pathways that lead to NO and PG production, respectively. However, excessive amounts of iNOS and COX have been implicated in the pathogenesis of inflammatory tissue injury and in several additional disease states.[3] Thus, inhibiting the expression of inducible pro-inflammatory genes may be useful in the development of therapeutic drugs to control inflammation associated with human disease in a clinical environment.[4]

An increasing public preference for plant food components over conventional drug therapies in the prevention and treatment of chronic inflammatory disease has prompted the discovery and development of new bioactive natural products with anti-inflammatory properties. Various plant extracts and isolated compounds have been scientifically demonstrated to exhibit anti-inflammatory activities by modulating the levels of inflammation-associated gene expression.[5] Licorice, derived from the root of *Glycyrrhiza uralensis*, is an annual crop that has long been considered a traditional folk herbal medicine in many Asian and European countries. Licorice and its principal component, glycyrrhizic acid (GA), have been widely used as a flavoring additive in the food industry, and licorice has been the subject of medical research for the treatment of allergies, gastric ulcers, liver disease, and adrenal insufficiency.[6,7] Numerous studies have reported that licorice extracts have anti-inflammatory and anticarcinogenic effects. For example, the ethanol extract from roasted licorice has been demonstrated to suppress the LPS-induced inflammatory response in murine macrophages and to increase the survival rate in LPS-induced mice macrophages.[8] Cho et al.[9] have also reported that a hexane/ethanol extract from licorice displayed anti-inflammatory activities toward murine macrophages and in mouse skin. In addition, GA and 18βGA have marked anti-inflammatory activities, for example, inhibiting LPS/D-galactosamine-induced liver injury and preventing free fatty acid-induced hepatic lipotoxicity.[10,11]

Our previous studies have demonstrated that GA acts via phosphoinositide-3-kinase/Akt/glycogen synthase kinase-3β (PI3K/Akt/GSK3β) to reduce cytokine production, whereas 18βGA leads to the dissociation of a glucocorticoid receptor−heat shock protein 90 (GR-HSP90) complex to block inflammation.[12]

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Hence, the objective of this study was to investigate the anti-inflammatory effects of GA and its metabolite, 18β-glycyrrhetinic (18βGA), on protein and mRNA levels of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. Moreover, the inhibitory effects of GA and 18βGA on the production of intracellular ROS and pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) and on the activation of transcription factors NF-κB and PI3K p110δ and p110γ were also examined.

MATERIALS AND METHODS

Chemicals. GA, 18βGA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and LPS from *Escherichia coli* 0111:B4 were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM), Trizol, and SYBR Safe DNA gel stain were obtained from Invitrogen (Carlsbad, CA). Antibodies against iNOS (2977) and COX-2 (4842) were purchased from Cell Signaling (Boston, MA). All fine chemicals were obtained from Showa Chemical (Tokyo, Japan) and Sigma-Aldrich.

Cell Culture. The murine RAW 264.7 cell line (BCRC 60001) was obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan). The cells were maintained in DMEM, 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin−streptomycin. All cell cultures were incubated in a humidified chamber at 37 °C with 5% CO_2.

Cell Viability Assay. The RAW 264.7 cells were cultured in triplicate wells at a density of 1×10^5 cells/well in 96-well flat-bottomed plates for 24 h. The cells were treated with various concentrations of GA and 18βGA and further incubated for 4 h at 37 °C in 5% CO_2. The cytotoxic effect was evaluated by MTT assay.[13] MTT solution (10 μL, 5 mg/mL) was added to each well and further incubated for 2 h at 37 °C. After the media had been discarded, dimethyl sulfoxide (DMSO) was added to dissolve the formazan dye. The optical density was measured at 570 nm with a FLUOstar galaxy spectrophotometer (BMG Lab Ltd., Offenburg, Germany). The relative cell viability (%) related to the control wells was calculated as $A_{570\ nm}$ (sample)/$A_{570\ nm}$ (control)] × 100.

Nitrite Assay. The amount of nitrite, the end-product of NO generation by activated macrophages, was determined according to the Griess reaction.[14] Briefly, the RAW 264.7 cells were incubated with 10−75 μg/mL GA or 18βGA in the presence or absence of LPS (1 μg/mL) for 24 h. One hundred microliters of Griess reagent (1:1 mixture of 1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid solution) was added to 100 μL samples of cellular supernatants in 96-well plates and agitated for 10 min at room temperature. Absorbance at 550 nm was read using a FLUOstar galaxy spectrophotometer (BMG Lab Ltd.). The reduction or increase in nitrite levels was estimated as the percentage of absorbance of the sample relative to the respective control. Standard calibration curves were prepared using sodium nitrite dilutions.

Measurement of Prostaglandin E_2 (PGE_2). The RAW 264.7 cells were cultured with 10−75 μM GA or 18βGA in the presence or absence of LPS (1 μg/mL) for 24 h. The cell culture media supernatants (100 μL) were collected to determine the prostaglandin E_2 concentration using prostaglandin E_2 ELISA kits (Cayman Chemical Co., Ann Arbor, MI). The concentration of PGE_2 was photometrically determined using a microplate reader (Awareness Technology, Palm City, FL) at 405 nm. Standard calibration curves were prepared using PGE_2 dilutions.

Evaluation of mRNA Expression of COX-2 and iNOS. The RAW 264.7 cells were plated in 6-well tissue culture plates overnight and pretreated with 25−75 μM GA or 18βGA in the presence or absence of LPS (1 μg/mL) for 24 h. After stimulation, total RNA was isolated using the TRIzol RNA isolation kit (Life Technologies, Rockville, MD) according to the manufacturer's instructions and treated with DNase. cDNA was synthesized from 240 ng of total RNA with a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) using a thermal cycler (Thermo Electron Corp.). Specific oligonucleotides used to amplify the desired cDNA were as follows: iNOS forward and reverse primers (5′-AATGGCAACATCAGGTCGGCCATCACT-3′ and 5′-GCTGTGTGTCACAGAAGTCTCGAACTC-3′); COX-2 forward and reverse primers (5′-GGAGAGACTATCAAGATAGT-3′ and 5′-ATGGTCAGTAGACTTTTACA-3′); and β-actin forward and reverse primers (5′-TCATGAAGTGTGACGTTGACATCCGT-3′ and 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′). The expected PCR products for iNOS, COX-2, and β-actin were 454, 861, and 285 bp, respectively.[15] The PCR products were analyzed by electrophoresis on a 1% agarose gel and visualized by SYBR Safe DNA gel stain (Invitrogen). Quantitative analysis was performed using photographs and the BioSpectrum AC Imaging System (UVP, Cambridge, U.K.). The relative levels of iNOS and COX-2 mRNA expression were compared using the ratio to a common reference, β-actin, from the same template.

Western Blot Analysis. The RAW 264.7 cells were incubated with 25−75 μM GA or 18βGA in the presence or absence of LPS (1 μg/mL) for 24 h. After stimulation, total protein was extracted from the cells and prepared according to our previous study.[16] The cells were collected and lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 500 μM sodium orthovanadate, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 10 μg/mL leupeptin, and 1 mM PMSF). The concentration of protein in the cell lysates was estimated using Bio-Rad dye reagent concentrate (Bio-Rad Laboratories, Hercules, CA) in microtiter plates. A standard curve was generated using solutions of 1 μg/μL bovine serum albumin (BSA). Total protein (50 μg/mL) was separated on 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane. The membrane was incubated with 5% skim milk in PBST (0.05% v/v Tween-20 in PBS, pH 7.2) for 1 h. The membranes were incubated with primary antibody (1:5000) at 4 °C overnight and then with secondary antibody (1:5000) for 1 h. The membranes were washed three times in PBST for 10 min each. The signal was detected using the Amersham ECL system (Amersham-Pharmacia Biotech, Arlington Heights, IL). Relative protein expression was quantified by densitometry using LabWorks 4.5 software and calculated relative to the β-actin reference band.

Enzyme-Linked Immunosorbent Assay for Cytokines. The concentrations of TNF-α, IL-1β, and IL-6 in cell medium were determined by commercial mouse enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA), following the manufacturer's protocols.

Transfection and NF-κB-Luciferase Assays. The RAW 264.7 cells (1×10^5 cells/well) were cultured in 6-well plates and transfected the following day using a TransFast transfection reagent (Promega, Madison, WI). After 18 h, the medium was aspirated and replaced with culture medium. To assay the effect of the pNF-κB-Luc, the cells were cotransfected with the pNF-κB-Luc plasmid reporter gene (Stratagene, Jolla, CA) for 1 h and then treated with 10−75 μM GA and 18βGA in the absence or presence of LPS (1 μg/mL) for 1 h. Luciferase activity was determined using a Bright-Glo luciferase system (Promega). The level of induction of luciferase activity was determined as the ratio of cells with and without treatment.

Effects of GA and 18βGA on the PI3 Kinases. The phosphoinositide 3-kinase (PI3K) activity of the two isoforms, p110δ and p110γ, were determined using a commercial PI3 kinase activity/inhibitor assay kit (Millipore, Billerica, MA), following the manufacturer's protocols.

Determination of Intracellular ROS Production. Intracellular ROS levels were measured by detecting the fluorescence intensity of the oxidant-sensitive probe 2′,7′-dichlorofluorescein-diacetate (DCFH-DA). Dichlorofluorescein (DCFH), converted from DCFH-DA by deacetylase within the cells, is oxidized by a variety of intracellular ROS to yield 2′,7′-dichlorofluorescein (DCF), a highly fluorescent compound. The cells were incubated with 10−75 μM GA or 18βGA in the presence or absence of LPS (1 μg/mL) for 18 h. The cells were stained with 20 μM

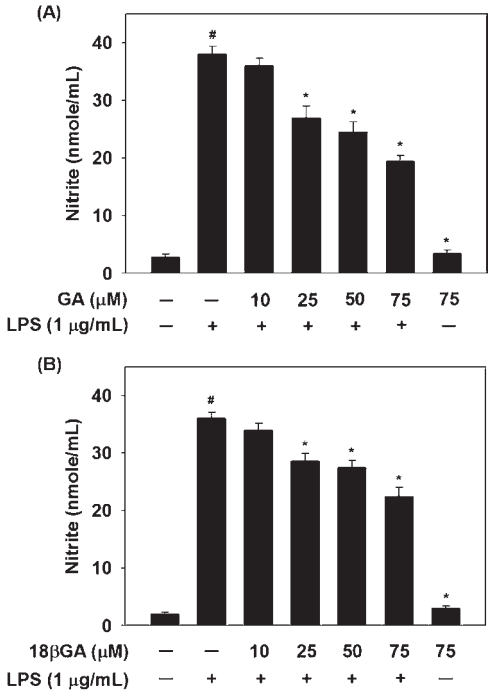

Figure 1. Effects of GA (A) and 18GA (B) on LPS-induced nitrite production in RAW 264.7 cells. The cells were incubated with 10−75 μM GA or 18GA in the presence or absence of LPS (1 μg/mL) for 18 h. The results from a representative replicate are expressed as the mean ± SD ($n = 3$). #, $p < 0.05$ indicates a significant difference from the control group. ∗, $p < 0.05$ indicates a significant difference from the LPS-treated group.

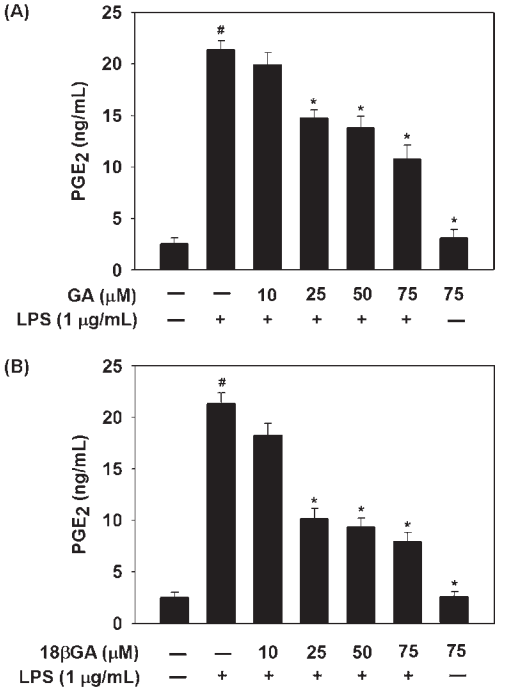

Figure 2. Effects of GA (A) and 18GA (B) on LPS-induced PGE_2 production in RAW 264.7 cells. The cells were incubated with 10−75 μM GA or 18GA in the presence or absence of LPS (1 μg/mL) for 18 h. The results from a representative replicate are expressed as the mean ± SD ($n = 3$). #, $p < 0.05$ indicates a significant difference from the control group. ∗, $p < 0.05$ indicates a significant difference from the LPS-treated group.

DCFH-DA for 15 min at room temperature and subjected to the determination of intracellular ROS production using a FLUOstar galaxy spectrophotometer (BMG Lab Ltd.).

Statistics. All data are presented as the mean ± standard deviation. Statistical analysis was performed using SAS software. ANOVA analysis was conducted using analysis of variance. Significant differences between means were determined by Duncan's multiple-range tests; differences were considered to be significant if $p < 0.05$. All of the samples were measured in triplicate.

RESULTS

Cytotoxic Effects of GA and 18βGA on the RAW 264.7 Cells. The cytotoxic effects of GA and 18βGA on the RAW 264.7 cells were examined using the MTT assay to determine the effective treatment concentrations. Exposing the cells to 10−200 μM GA for 24 h did not reduce the viability of the RAW 264.7 cells (data not shown). However, viability was reduced to 58% of the control when the cells were exposed to 200 μM 18βGA for 24 h. These data indicate that GA and 18βGA do not affect the viability of the RAW 264.7 cells at concentrations lower than 200 and 150 μM, respectively.

Effects of GA and 18GA on NO Production. After 18 h of macrophage stimulation, the nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction. The stimulation of RAW 264.7 cells with LPS (1 μg/mL) caused a significant increase in cellular nitrite levels compared to the unstimulated control. As shown in Figure 1, a dose-dependent and significant decrease in nitrite accumulation was observed in the presence of GA and 18βGA at concentrations of 25−75 μM. Although 18βGA had an inhibitory effect on NO production, the effect was not as strong as that exerted by GA. At a concentration of 75 μM, 51 and 34% reductions in nitrite production were observed in cells treated with GA and 18βGA, respectively. Nevertheless, GA and 18βGA at 10 μM did not interfere with the reaction between nitrite and Griess reagents.

Effects of GA and 18βGA on PGE_2 Production. To determine the potential of GA and 18βGA to inhibit PGE_2 production, the amount of released PGE_2 was measured in LPS-stimulated RAW 264.7 cells using anti-PGE_2-coated ELISA plates. After treatment with LPS (1 μg/mL) for 18 h, the PGE_2 concentration in the medium increased markedly by about 6-fold. When the RAW 264.7 cells were treated with LPS in combination with different concentrations of GA or 18βGA for 18 h, a significant dose-dependent inhibition of PGE_2 production was observed (Figure 2). The levels of released PGE_2 from cells treated with 75 μM GA or 18βGA were 49 and 58%, respectively, of the levels released by cells treated with LPS alone.

Effects of GA and 18βGA on mRNA Expression of iNOS and COX-2. To investigate the mechanism responsible for the observed inhibition of pro-inflammatory mediator (NO and PGE_2) release in response to LPS, the biosyntheses of iNOS and COX-2 were investigated at the transcriptional level by quantifying mRNA levels. As shown in Figure 3, levels of iNOS and COX-2 were significantly increased in LPS-stimulated RAW 264.7 cells compared to unstimulated cells. However, LPS-induced iNOS expression was significantly attenuated by GA and 18βGA treatment in a dose-dependent manner, and inhibition was more pronounced in response to GA. In the same set of experiments, GA and 18βGA showed slight but significant

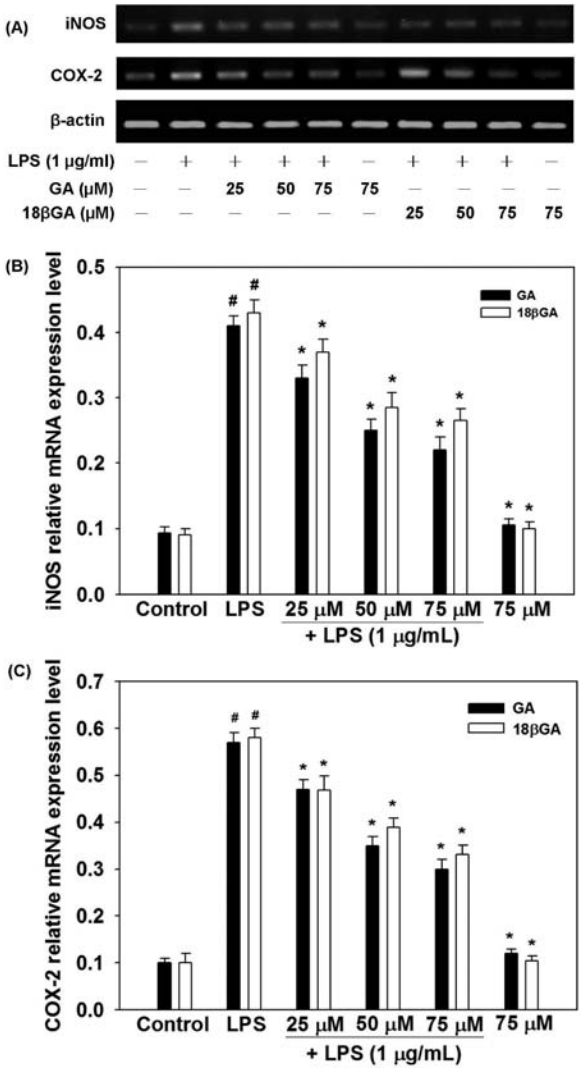

Figure 3. Effects of GA and 18βGA on LPS-induced iNOS and COX-2 mRNA expression in RAW 264.7 cells. The cells were incubated with GA or 18βGA (25, 50, and 75 μM) in the presence or absence of LPS (1 μg/mL) for 18 h. (A) Amplified bands represent iNOS, COX-2, and β-actin in representative samples. β-Actin expression was used as an internal control gene. (B) Quantification of iNOS gene expression levels are expressed as the ratio of iNOS/β-actin and compared to the LPS-induced group. (C) Quantification of COX-2 gene expression levels is expressed as the ratio of COX-2/β-actin and compared to the LPS-induced group. Data are expressed as the mean ± SD ($n = 3$). *, $p < 0.05$ indicates a significant difference from the LPS-treated group.

inhibitory activity on COX-2 expression in response to LPS stimulation for 18 h. In these experiments, β-actin was used as an internal control gene to quantify relative gene expression in LPS-treated cells in the presence or absence of GA and 18βGA.

Effects of GA and 18βGA on Protein levels of iNOS and COX-2. The effects of GA and 18βGA on the protein levels of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells were examined by Western blot analysis. As shown in Figure 4, LPS (1 μg/mL) significantly increased iNOS and COX-2 protein expression compared to the unstimulated control cells. When GA or 18βGA (25, 50, 75 μM) was simultaneously added to the medium with LPS (1 μg/mL), a dose-dependent reduction in iNOS and COX-2 protein levels was observed.

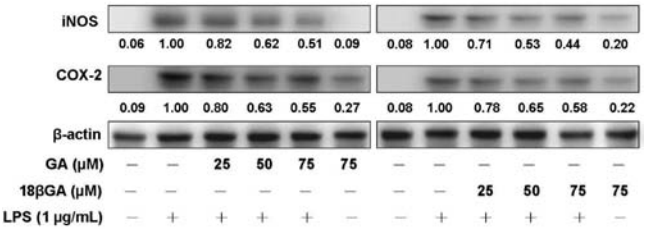

Figure 4. Effects of GA and 18βGA on LPS-induced iNOS and COX-2 protein levels in RAW 264.7 cells. The cells were treated with different concentrations of GA or 18βGA in the presence or absence of LPS (1 μg/mL) for 18 h. The relative protein expression was densitometrically quantified using LabWorks 4.5 software and normalized to the β-actin reference bands.

Inhibitory Effects of GA and 18βGA on LPS-Induced Production of TNF-α, IL-6, and IL-1β The inhibitory effects of GA and 18βGA on the expression of iNOS and COX-2 mRNA in the macrophage cell line prompted us to assess the effects of GA and 18βGA in regulating other pro-inflammatory mediators, such as TNF-α, IL-6, and IL-1β. The amounts of TNF-α, IL-6, and IL-1β in the medium were detected by ELISA after treatment with GA or 18βGA (10−75 μM) in the presence or absence of LPS (1 μg/mL) for 18 h. There was no effect of GA or 18βGA on the production of TNF-α, IL-6, or IL-1β in unstimulated RAW 264.7 cells. As shown in Figure 5, LPS-stimulated RAW 264.7 macrophages exhibited increased TNF-α, IL-6, and IL-1β production. When RAW 264.7 cells were treated with different concentrations of GA and 18βGA, the LPS-stimulated release of TNF-α, IL-6, and IL-1β was inhibited to various degrees in a dose-dependent manner. At a concentration of 75 μM, GA decreased the levels of TNF-α, IL-6, and IL-1β released from RAW 264.7 cells by 46, 42, and 51%, respectively. Similarly, 18βGA treatment caused 34, 35, and 42% decreases in TNF-α, IL-6, and IL-1β release, respectively.

Transcriptional Activity of NF-κB. Because the transcription factor NF-κB induces expression of many immediate pro-inflammatory genes, activation of NF-κB was also analyzed. Transcriptional activity of NF-κB was evaluated to assess whether GA and 18βGA inhibited NF-κB binding activity in LPS-stimulated RAW 264.7 cells. As shown in Figure 6, treatment with GA or 18βGA (25, 50, 75 μM) significantly ($p < 0.05$) inhibited LPS-induced NF-κB transcriptional activity in RAW 264.7 cells. This result indicates that GA and 18βGA block LPS-induced NF-κB activation, and this effect may result in the inhibition of LPS-induced production of NO, COX-2, and TNF-α.

Effects of GA and 18βGA on the PI3 Kinases. To address the mechanism of the anti-inflammatory effects of GA and 18βGA, the ability of these agents to inhibit PI3 kinases was determined. To this end, the activations of PI3K p110δ and p110γ were determined using the PI3 kinase activity/inhibitor assay kit. The inhibitory activity of wortmannin (100 nM), a classical PI3K inhibitor, was set as 100%. The efficacies of GA and 18βGA were determined compared to that of wortmannin as a relative percentage. As depicted in Figure 7, several concentrations of GA or 18βGA (25, 50, 75 μM) dose-dependently inhibited PI3 kinase p110δ and p110γ activity. However, there was no marked inhibitory effect of GA or 18βGA at 10 μM.

Inhibitory Effects of GA and 18βGA on Intracellular ROS Production. Figure 8 presents the effects of GA and 18βGA on intracellular ROS production in LPS-stimulated RAW 264.7

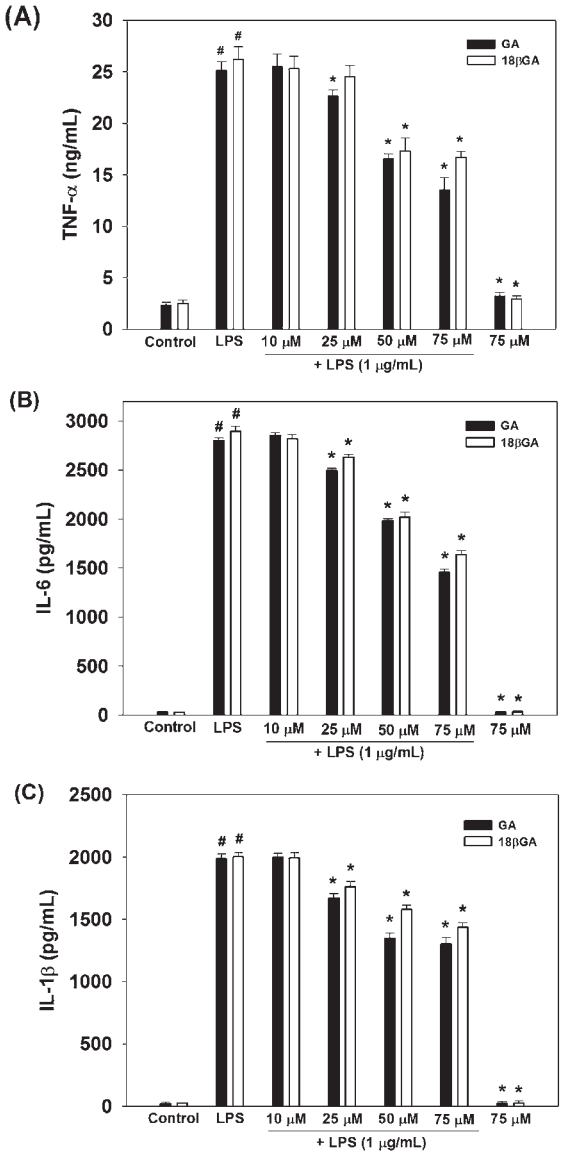

Figure 5. Effects of GA and 18βGA on the production of pro-inflammatory mediators in LPS-induced RAW 264.7 cells: (A) inhibition of TNF-α expression in the cell-free culture medium; (B) inhibition of IL-6 expression in the cell-free culture medium; (C) inhibition of IL-1β expression in the cell-free culture medium. The cells were incubated with GA or 18βGA in the presence or absence of LPS (1 μg/mL) for 18 h. TNF-α, IL-6, and IL-1β concentrations in the medium were assayed by ELISA. Data are expressed as the mean ± SD (n = 3). #, p < 0.05 indicates a significant difference from the control group. ∗, p < 0.05 indicates a significant difference from the LPS-treated group.

cells. The LPS treatment caused an elevation of intracellular ROS, as assessed by cell labeling with the fluorescent probe DCFH-DA. During inflammation, a pronounced increase in oxygen uptake results in the massive release of intracellular ROS in cells treated with LPS (1 μg/mL) compared to untreated control cells. Treatment with 50 μM GA or 18βGA significantly inhibited LPS-induced intracellular ROS generation (approximately 21.1 and 26.4% inhibition, respectively); however, treatment with 10 μM GA or 18βGA did not interfere with ROS production. Within a range of 18βGA concentrations, dose-dependent inhibition of LPS-induced ROS production was observed.

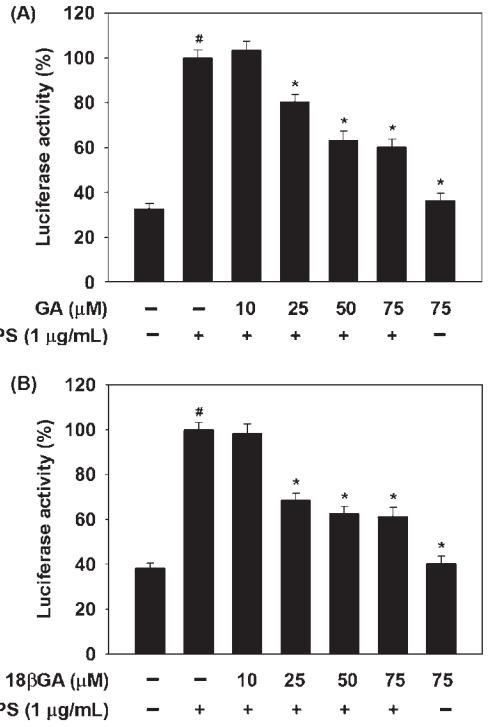

Figure 6. Effects of GA and 18βGA on LPS-induced transcriptional activity of NF-κB in RAW 264.7 cells. The cells were transfected with 1 μg of NF-κB-Luc reporter gene and treated with GA or 18βGA in the presence or absence of LPS (1 μg/mL) for 18 h. Data are expressed as the mean ± SD (n = 3). #, p < 0.05 indicates a significant difference from the control group. ∗, p < 0.05 indicates a significant difference from the LPS-treated group.

DISCUSSION

Epidemiological studies have demonstrated that a diet rich in plant nutraceuticals is negatively correlated with various diseases that are associated with inflammatory disorders.[17] Several components have been isolated from licorice, including triterpene saponins, flavonoids, isoflavonoids, and chalcones, and GA is considered to be the major biologically active component.[7] We have previously reported that GA and 18βGA both manifest anti-inflammatory activities but through different mechanisms, namely, by influencing PI3K/Akt/GSK3β signaling and glucocorticoid receptor (GR) activation.[12] In the present study, we demonstrate that at nontoxic doses (25−75 μM) GA and 18βGA (a) reduce the secretion of NO, PGE_2, TNF-α, IL-6, IL-1β, and ROS; (b) decrease protein levels of iNOS and COX-2; (c) decrease the mRNA levels of iNOS and COX-2; and (d) significantly inhibit LPS-induced NF-κB and PI3K p110δ and p110γ activity in the RAW 264.7 cells. Our findings are consistent with previous studies demonstrating the inhibitory effect of licorice extracts on NO and PGE_2 production.[8,9] Those also indicate a potential anti-inflammatory effect of licorice, as demonstrated by a dose-dependent inhibition of iNOS and COX-2 gene expression. In addition to reporting that licorice extracts reduce levels of IL-6 and TNF-α, IL-1β, and IL-8, another study also demonstrated that NF-κB p65 phosphorylation was inhibited, suggesting that licorice has the potential for the treatment of periodontitis.[18]

Recently, several studies using LPS-stimulated macrophage cell lines have investigated the in vitro anti-inflammatory

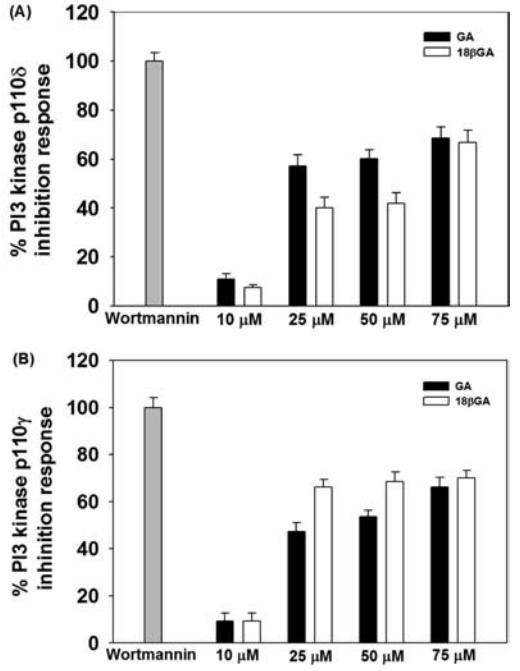

Figure 7. Effects of GA and 18βGA on PI3 kinase activity: (A) inhibition of PI3 kinase p110δ activity; (B) inhibition of PI3 kinase p110γ activity. The value for wortmannin (100 nM) was set as 100%, and the inhibitory activities of GA and 18βGA were referenced to the wortmannin to obtain the relative percentage, which reflects the efficacy of inhibition. Data are expressed as the mean ± SD (n = 3).

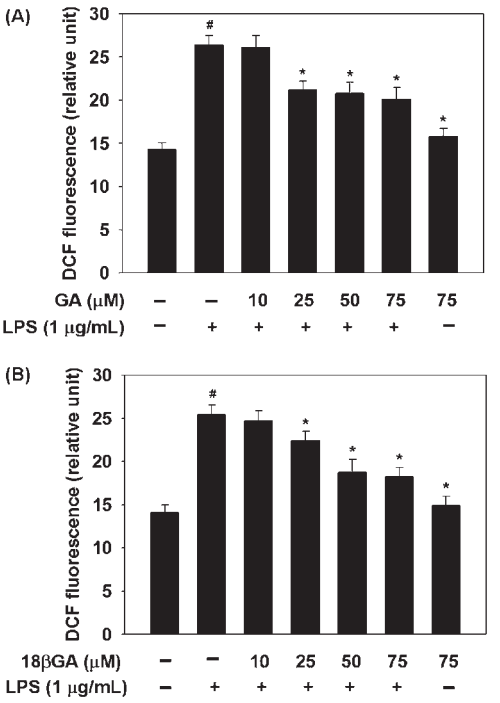

Figure 8. Effects of GA (A) and 18βGA (B) on LPS-induced ROS production in RAW 264.7 cells. The cells were incubated with GA (A) and 18βGA (B) in the presence or absence of LPS (1 μg/mL) for 18 h. Data are expressed as the mean ± SD (n = 3). #, $p < 0.05$ indicates a significant difference from the control group. ∗, $p < 0.05$ indicates a significant difference from the LPS-treated group.

properties of nutraceuticals, including polyphenols, phenolic acids, and flavonoids.[19] In each of these studies, the ability to modulate iNOS gene expression and decrease NO production is important in screening natural compounds for anti-inflammatory properties.[20] In agreement with previous studies, our results confirm that the dose-dependent inhibition of NO production occurs concomitantly with the suppression of iNOS expression at the mRNA and protein levels, as shown by RT-PCR and Western blot analysis (Figures 3 and 4). In addition, we analyzed another important mediator of inflammation, COX-2, which promotes cellular proliferation, suppresses apoptosis, and enhances angiogenesis and oncogenic function.[21] Indeed, selective COX-2 inhibitors, such as celecoxib, NS-398, and sulindac, have been implicated in the prevention of cancer and the treatment of inflammation.[22] Various plant compounds have also been shown to suppress the activation of NF-κB, thereby inhibiting COX-2 gene expression and PGE_2 production in LPS-stimulated RAW 264.7 cells.[23] In this study, GA and 18βGA also inhibit PGE_2 production by suppressing COX-2 mRNA and protein expression, suggesting that GA and 18βGA may also provide therapeutic benefits in the suppression and prevention of inflammatory diseases.

The transcription factor NF-κB is important for the regulation of various genes involved in immune and acute phase inflammatory responses, as well as for cell survival. Most anti-inflammatory drugs have been shown to suppress the expression of pro-inflammatory genes by inhibiting the NF-κB activation pathway.[24] The release of NF-κB from IκB results in the translocation of NF-κB into the nucleus, where it binds to specific sequences in the promoter region of target genes. Numerous studies have suggested that free NF-κB translocates to the nucleus, where it binds to κB-binding sites in the promoter regions of target genes and induces the transcription of pro-inflammatory mediators, such as iNOS and COX-2.[25] We have previously found that cotreatment with GA and 18βGA induced the activation of PI3K/Akt/GSK3β and GR,[12] suggesting that GA and 18βGA inhibit LPS-induced expression of COX-2 and iNOS genes by blocking NF-κB activation, although inhibition of other inflammatory cytokines and ROS may also be involved.

Inflammatory disorders are often characterized by the production of significant levels of free radicals, nitrogen reactive species, and inflammatory cytokines. In response to a pro-inflammatory stimulus, macrophages produce excessive levels of cytokines, such as TNF-α, IL-6, and IL-1β, which can injure cellular biomolecules and functional synergy on the coexistence of TNF-α responsive NF-κB signal transducers and activators of transcription protein binding elements within the gene promoter.[26,27] Because our results show that GA and 18βGA inhibit LPS-induced TNF-α, IL-6, and IL-1β production, it is possible that GA and 18βGA interfere with the transcription of TNF-α, IL-6, and IL-1β and that the activation of NF-κB is followed by decreased levels of these cytokines. In addition, ROS molecules act as mediators of cellular injury and are involved in the onset of cellular damage during endotoxemia. ROS are also believed to be involved in inflammatory gene expression through redox-based activation of the NF-κB signaling pathway.[28] According to the literature, many plant or plant-derived molecules possess antioxidant activity, which could inhibit NF-κB activation by reducing levels of ROS.[4,29] In this study, exposure of RAW 264.7 cells to LPS apparently stimulated the accumulation of intracellular ROS. Pretreatment of cells with GA and 18βGA significantly attenuated LPS-induced ROS production. On the

basis of our previous study, GA and 18βGA can elevate the activity and expression of antioxidant enzymes.[30] It is conceivable that the antioxidant activity of GA and 18βGA is mechanistically responsible for the observed inhibition of NF-κB activation.

PI3K is an intracellular protein that plays a major role in the development of immune response, inflammation, and tumor growth. Recently, the expression pattern and functions of PI3K p110δ and p110γ have generated much interest in developing PI3K inhibitors as a novel class of anti-inflammatory agents.[31] In this study, GA and 18βGA inhibited PI3K p110δ and p110γ activity, consistent with the idea that anti-inflammatory compounds indirectly inhibit PI3K in LPS-stimulated macrophages as a result of reduced TNF-α production and NF-κB inactivation.[32] PI3K activation influences both anti-inflammatory and pro-inflammatory mechanisms. PI3K itself can be a pro-inflammatory and anti-inflammatory regulator in cell biology. Blocking PI3K p110δ and p110γ can prevent Ser phosphorylation of the p65 subunit, an event required for maximal transcriptional activity of NF-κB. This mechanism may culminate in the indirect inhibition of NF-κB activation and the decreased release of downstream inflammatory cytokines.[33] In addition, the activation of NF-κB requires phosphorylation of IκB, which targets IκB for ubiquitination and degradation. Inhibition of p110δ and p110γ, a demonstrated consequence of diminished Akt phosphorylation, caused NF-κB inactivation by decreasing phosphorylation of IκB and attenuating IκB degradation in LPS-stimulated macrophages.[34] Kim et al.[32] also reported that inhibiting LPS-induced PI3K-Akt activation led to reduced NF-κB activation, resulting in suppressed expression of the iNOS and COX-2 genes. These results may explain that PI3K p110δ and p110γ inhibition resulted in blocked NF-κB activation and suggest that GA and 18βGA inhibit inflammatory cytokine production at least in part by inhibiting NF-κB activation, independent of PI3K p110γ and p110δ activity. Furthermore, our results reveal PI3K p110δ and p110γ inhibition may be a new domain for screening phytochemicals that may act as effective anti-inflammatory agents, and cancer chemoprevention agents may also be expected.

In conclusion, we have shown that GA and 18βGA exert protective effects by inhibiting the production of NO, PGE_2, TNF-α, IL-6, IL-1β, and ROS, reducing the expression of pro-inflammatory genes (iNOS and COX-2), and significantly blocking activation of transcription factors such as NF-κB and PI3K p110δ and p110γ. Overall, these data provide another view with our previous studies having demonstrated that GA and 18βGA affect PI3K/Akt/GSK3β signaling and GR activation, respectively.[12] To our knowledge, this is the very first study demonstrating the anti-inflammatory ability of phytochemicals GA and 18βGA, related to the inhibitory effect to PI3K p110δ and p110γ. These data together suggest that GA and 18βGA regulate the expression of iNOS and COX-2 by inhibiting PI3K p110δ and p110γ and NF-κB. These findings have suggested underlying molecular mechanisms for the anti-inflammatory actions of GA and 18βGA, indicating that GA and 18βGA may be clinically useful for the reduction of inflammation and the prevention of related diseases.

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ABBREVIATIONS USED

18βGA, 18β-glycyrrhetinic acid; COX, cyclooxygenase; iNOS, nitric oxide synthase; GA, glycyrrhizic acid; GR, glucocorticoid receptor; LPS, lipopolysaccharide; NF-κB, nuclear factor κ-light-chain-enhancer of activation; PI3K/Akt/GSK3, phosphoinositide-3-kinase/Akt/glycogen synthase kinase-3 β; PG, prostaglandins; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α.

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