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Glycyrrhizic Acid and 18β-Glycyrrhetinic Acid Inhibit Inflammation via PI3K/Akt/GSK3β Signaling and Glucocorticoid Receptor Activation

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Many lung-related diseases, such as asthma and chronic obstructive pulmonary disease, are initiated by airway inflammation, and several studies indicate that glycyrrhizic acid (GA) alleviates inflammatory lung disease. We previously showed that GA and 18β-glycyrrhetinic acid (18βGA), found in licorice, can act as neuroprotective agents by promoting downstream PI3K/Akt signaling. In this study, we investigate the effects of GA and 18βGA on inflammation. We show that both GA and 18βGA reduce inflammatory cytokine production and its resulting anti-inflammation. GA acts via PI3K/Akt/GSK3β to reduce cytokine production, while 18βGA leads to the dissociation of a glucocorticoid receptor (GR)-HSP90 complex to block inflammation. Our data suggest that GA and 18βGA display anti-inflammatory activities but inhibit inflammation via different mechanisms. We propose that GA and 18βGA may be valuable biological inhibitors of lung inflammation. Interestingly, these data may explain why licorice is frequently used to treat inflammatory disease and it might be a promising nutraceutical for remedying inflammation.

KEYWORDS: Glycyrrhizic acid; 18β-glycyrrhetinic acid; PI3K/Akt signaling pathway; GSK3β phosphorylation; glucocorticoid receptor; anti-inflammation

INTRODUCTION

Asthma and chronic obstructive pulmonary disease (COPD) are characterized by progressive airway inflammation (*1*, *2*). Airway inflammation can be stimulated by a number of factors, including lipopolysaccharides and cytokines. These molecules induce inflammation by activating immune cells, including macrophages and neutrophils, and by promoting production of pro-inflammatory cytokines such as TNFα, IL-1, and IL-6. Asthma and COPD have similar clinical features; however, COPD typically leads to irreversible lung damage, including emphysema (*3*).

Glucocorticoids are widely used to alleviate asthma and COPD symptoms (*4*). Glucocorticoids, such as dexamethasone, act by binding and activating glucocorticoid receptor signaling to inhibit NF-κB transcription and downstream inflammation. Constant uptake of glucocorticoids leads to adverse effects, including immunosuppression and infection (*5*), as well as glucocorticoid resistance in asthmatic or COPD patients (*6*).

Licorice, a well-known herb plant with biological properties, has been widely used in food additives (sweetener and flavoring agent), nutraceuticals (liver protection), and traditional Chinese medicine (TCM) for thousands of years. Many studies indicate that licorice may be useful for treating airway and lung disorders. Interestingly, iv injection of glycyrrhizic acid (GA), the primary bioactive compound in licorice, reduces lipopolysaccharide (LPS)-induced acute respiratory distress syndrome (ARDS) lethality in BALB/c mice (*7*). Daily supplementation of GA also alleviates ovalbumin (OVA)-induced allergic asthma in BALB/c mice (*8*). Additionally, GA injection attenuates the development of carrageenan-induced lung injury in CD mice (*9*). Because GA can be converted into 18β-glycyrrhetinic acid (18βGA) before entering the circulatory system (**Figure 1**) (*10*), these findings suggest that GA and 18βGA inhibit airway and lung inflammation. We previously showed that GA and 18βGA promote PI3K/Akt pathway activation (*11*), a pathway known to play a role in inflammation (*12*). However, whether or not the signal through PI3K/Akt which activated by GA and 18βGA affects inflammation remains unclear. In the present study, RAW264.7 cells (macrophage of BALB/c mice) were used to investigate the anti-inflammatory effects of GA and 18βGA and to characterize the signaling pathways that act downstream of GA and 18βGA to affect inflammation. We show that both GA and 18βGA reduce the inflammatory response. GA acts via PI3K/Akt/GSK3β to reduce inflammation, while 18βGA blocks inflammation by promoting the dissociation of a glucocorticoid receptor (GR)-HSP90.

MATERIALS AND METHODS

Chemicals. Glycyrrhizic acid (GA), 18β-glycyrrhenitic acid (18βGA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), sodium bicarbonate, tris(hydroxymethyl)aminomethane (Tris), and LPS from *Escherichia coli* 0111:B4 were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum, DMEM, LY294002, wortmannin, Trizol, and SYBR Safe DNA gel stain were obtained from Invitrogen (Carlsbad, CA). Anti-Akt and antiphosphorylated Akt (Ser473) were purchased from Cell Signaling (Boston, MA). Anti-GSK3β, antiphosphorylated GSK3β, and Catch and Release v2.0

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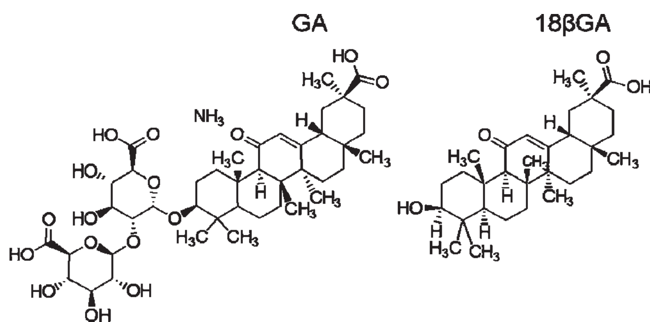

Figure 1. Chemical structures of GA and 18βGA.

reversible immunoprecipitation system were purchased from Millipore (Temecula, CA). All fine chemicals were obtained from Showa Chemical (Tokyo, Japan) and Sigma-Aldrich.

Cell Culture. The mouse BALB/c macrophage cell line (RAW264.7) (BCRC 60001) was obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 5 mg/L penicillin, and 5 mg/L streptomycin at 37 °C in 5% CO_2. All treatments were performed when cells were at 80% confluence.

Cell Viability Assay. The cell toxicity of GA and 18βGA was measured by MTT analysis. Briefly, RAW264.7 cells (5×10^4 cells/mL) were seeded in 96-well microtiter plates. After coincubation with GA or 18βGA, the medium was removed and replaced with fresh medium containing 0.5 mg/mL MTT for 2 h at 37 °C. The violet crystal which converted from yellow MTT by living cells was dissolved by DMSO, and optical density was measured at 570 nm using a BMG LABTECH FLUOstar fluorescence reader (Jena, Germany).

PI Staining for Apoptosis. Cells were treated with various concentrations (0, 10, 25, 50, and 100 μM) of GA or 18βGA for 24 h. The cells were then harvested and washed twice with PBS and then fixed in 80% ethanol for 30 min at 4 °C. The cells were then stained with PI (40 μg/mL) for 15 min at room temperature in the dark and subjected to flow cytometric analysis of DNA content using a Becton Dickinson FACScan flow cytometer (Franklin Lakes, NJ). Approximately 1×10^4 cells were scored/sample. The percentage of cells undergoing apoptosis was calculated using CELL Quest software.

RNA Extraction and Detection. Treated cells were washed three times with PBS, and Trizol (Invitrogen) was used to extract the intracellular RNA. Next, a RevertAid First Strand cDNA synthesis kit (Fermentas) was used to translate RNA into cDNA, and Taq DNA polymerase (Fermemtas) was used to amplify the desired cDNA sequence. The primers used to amplify TNFα, IL-1α, IL-6, and GAPDH were as follows: TNFα, forward = 5′-ACACCGTCAGCCGATTTGC-3′, reverse = 5′-CCCTGAGCCATAATCCCCTT-3′; IL-1α, forward = 5′-ATAACCTGCTGGTGTGTGAC-3′, reverse = 5′-TGCAGACTCAA-ACTCCACTT-3′; IL-6, forward = 5′-TTGTGCAATGGCAATTCT-3′, reverse = 5′-AGAGCATTGGAAATTGGG-3′; GAPDH, forward = 5′-ACCACAGTCCATGCCATCAC-3′, reverse = 5′-TCCACCACCC-TGTTGCTGTA-3′. Primer sequences were performed as described in Huang et al. (*13*). The PCR product was electrophoresed on a 1.5% agarose gel, and DNA was detected using SYBR Safe DNA gel stain (Invitrogen). The gel was then photographed using BioDoc-It system (UVP, Cambridge, UK). Results were expressed as a ratio of the DNA signal after normalization relative to the corresponding GAPDH signal from each sample.

Western Blotting. Treated cells were lysed in RIPA buffer (Millipore, Billerica, MA) and boiled at 100 °C for 10 min with 4× protein loading dye (8% SDS, 0.04% Coomassie Blue R-250, 40% glycerol, 200 mM Tris-HCl (pH 6.8), 10% 2-mercaptoethanol). Samples were then subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto an Immobilon-P PVDF membrane (Millipore) and incubated with primary antibody (phospho-Akt, Akt, phospho-GSK3β, or GSK3β) overnight (1:2000 dilutions). Membranes were then incubated with horseradish peroxide-conjugated secondary antibody (1:5000 dilutions) and analyzed using the Chemiluminescent ECL detection system (Millipore). Protein levels were normalized relative to Akt or GSK3β. Signal intensity was quantified using VisionWorks LS 6.3.3 (UVP).

Immunoprecipitation. RAW264.7 cells were harvested using RIPA lysis buffer after coincubation with GA or 18βGA. Immunoprecipitation of GR-HSP90 complexes was performed using the Catch and Release v2.0 reversible immunoprecipitation system (Millipore), per manufacturer instructions.

Statistical Analysis. All data are expressed as means ± SD. An ANOVA was used to evaluate differences between multiple groups. Significant differences were subjected to Duncan's test to compare the means of two specific groups. A p value < 0.05 was considered to be significant.

RESULTS

GA and 18βGA Do Not Induce Apoptosis in RAW264.7 Cells. To evaluate the cytotoxicity of GA and 18βGA to mouse macrophage RAW264.7 cells, we performed an MTT assay and PI staining. Twenty-four hour incubation in medium containing 0.1−100 μM GA or < 50 μM 18βGA does not decrease cell viability in RAW264.7 cells; however, 18βGA treatment leads to cytotoxicity at concentrations greater than 50 μM (**Figure 2A,B**). These results indicate that GA and 18βGA do not induce apoptosis in RAW264.7 cells at concentrations less than 50 μM and that 18βGA may be more cytotoxic than GA.

GA and 18βGA Decrease LPS-Induced Inflammatory Gene Expression. To investigate the anti-inflammatory effects of GA and 18βGA, pro-inflammatory cytokine gene expression was examined following LPS treatment. RAW267.4 cells were treated with LPS with GA or 18βGA for 6 h. Following treatment, total RNA was isolated and a reverse transcribed. The resulting cDNA was subjected to PCR to assess expression of the pro-inflammatory cytokines TNFα and IL-1α. Dexamethasone (Dex) treatment, which served as the positive control, markedly reduces TNFα and IL-1α expression. At concentrations of 1 and 5 μM, GA could decrease TNFα expression in a dose-dependent mannar, but 18βGA reduced TNFα and IL-1α expression in a dose-dependent manner (**Figure 3A,B**). These results indicate that GA and 18βGA inhibit LPS-induced expression of inflammatory cytokines.

GA Modulates PI3K/Akt/GSK3β Activation in RAW264.7 cells. Next, we examined the anti-inflammatory signaling pathway activated by GA and 18βGA. To determine if GA and 18βGA activate PI3K/Akt signaling, RAW264.7 cells were pretreated with LY294002 or wortmannin for 30 min, then incubated in LPS plus GA or 18βGA for 6 h. LY294002 and wortmannin are two pan-PI3K inhibitors, of which wortmannin is even more potent than LY294002 (*14*). As shown in **Figure 4A**, treatment with LY294002 significantly decreases LPS-induced IL-6 expression while wortmannin treatment increases IL-6 expression. Furthermore, treatment with GA plus LY294002 enhances the anti-inflammatory effects of GA, while wortmannin decreases these effects. Interestingly, these trends were not observed following treatment with 18βGA. Next, we assessed Akt phosphorylation following treatment with GA or 18βGA. As shown in **Figure 4B**, GA is a more potent Akt activator than 18βGA. At 10 and 25 μM concentrations, GA significantly increases Akt phosphorylation (1.3-fold increase), while 18βGA slightly increases Akt phosphorylation at 5 μM but not at other concentrations (10−100 μM). Because GSK3β is a downstream phorphorylated target of PI3K/Akt, we next assessed phospho-GSK3β levels following GA or 18βGA treatment. At 5 and 10 μM, GA increases levels of p-GSK3β (**Figure 4C**), while 18βGA leads to elevated p-GSK3β only at 5 μM. Together, these data suggest that GA acts through PI3K/Akt/GSK3β to inhibit LPS-induced inflammation, while 18βGA inhibits inflammation via a different mechanism.

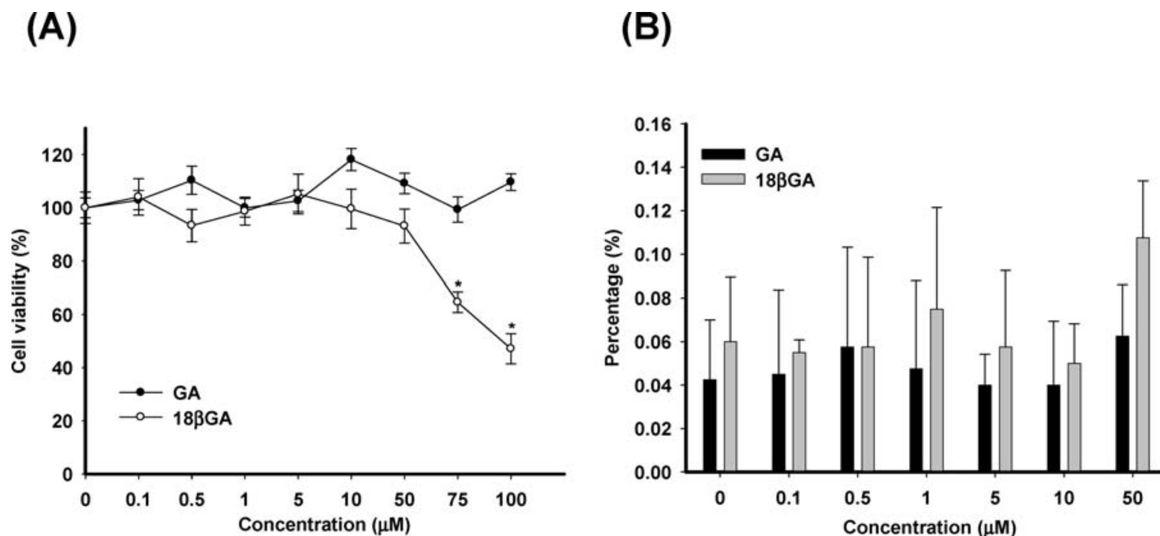

Figure 2. Cytotoxicity of GA and 18βGA in RAW264.7 cells. Cells were coincubated with a range of concentrations of GA or 18βGA for 24 h, and cell viability was measured by MTT assay (**A**). Cells were analyzed by flow cytometry after PI staining, and percent apoptotic cells was calculated using CELL Quest software (**B**). Data are presented as means ± SD (n = 5). * $p < 0.05$ vs control group.

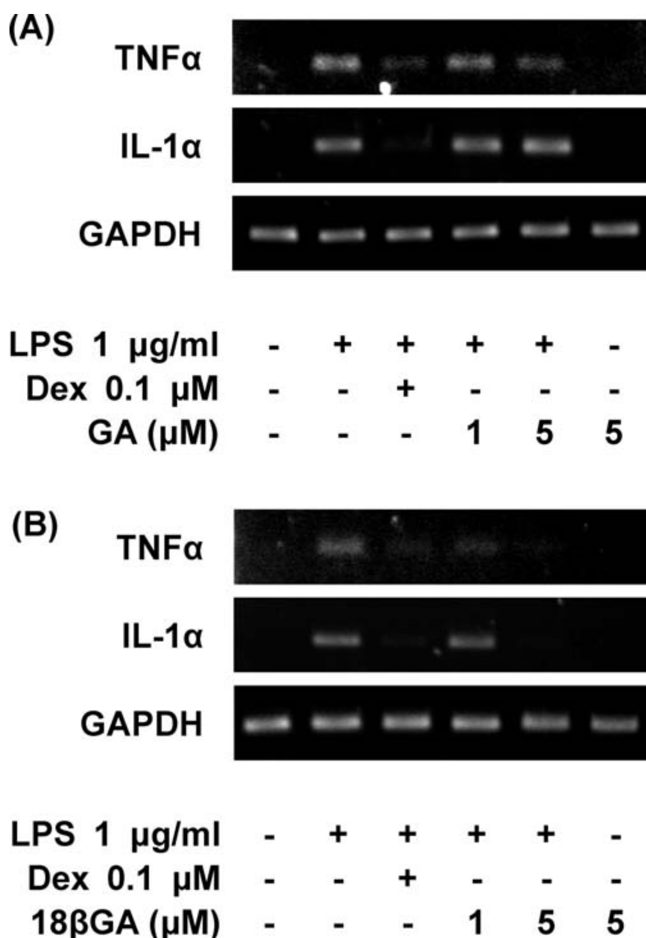

Figure 3. Effects of GA and 18βGA on LPS-induced expression of inflammatory cytokine genes. Cells were coincubated with 1 μM and 5 μM GA (**A**) or 18βGA (**B**) and 1 μg/mL LPS for 6 h. Dexamethasone (Dex, 0.1 μM) treatment was used as positive control.

GA Activates PI3K/Akt/GSK3β Signaling in RAW264.7 Cells. To further determine if GA activates PI3K, we assessed GA-induced activation of Akt and GSK3β in a serum-free environment. RAW264.7 cells were incubated in serum-free DMEM medium for 100 min. Next, the cells were treated with 2 μM wortmannin for 30 min, followed by treatment with GA. At 10 and 25 μM, GA elevates Akt phosphorylation and treatment with 25 μM GA induces GSK3β phosphorylation (**Figure 5**). Interestingly, GA-induced phosphorylation of Akt and GSK3β is inhibited by wortmannin. These data imply that GA directly promotes Akt and GSK3β phosphorylation via PI3K signaling.

GA and 18βGA Influence Glucocorticoid Receptor Activation in RAW267.4 Cells. Finally, we examined the effects of GA (**Figure 6A**) and 18βGA (**Figure 6B**) treatment on glucocorticoid receptor (GR) activation. Dissociation of GR and HSP90 was used as a readout of GR activation. Following coincubation with GA (1 and 5 μM) for 1 h, the ratio of HSP90/GR does not change compared to controls. Alternatively, 18βGA treatment decreases the HSP90/GR ratio at the same concentrations. Although 18βGA treatment leads to GR activation, activation was weaker than Dex activation. We conclude that 18βGA induces GR activation, albeit at a lower level than Dex.

DISCUSSION

Airway inflammation initiates many lung diseases, and numerous factors lead to immune system activation and inflammation. Several studies suggest that bioactive compounds, such as GA, inhibit inflammation (*7*, *8*). We previously demonstrated that GA and 18βGA protect PC12 cells from 6-hydroxydopamine by activating a PI3K/Akt signaling pathway (*11*). In this study, we demonstrate the anti-inflammatory effects of GA and 18βGA in response to LPS-induced inflammation. Additionally, we show that GA activates PI3K/Akt/GSK3β while 18βGA affects glucocorticoid receptor signaling to inhibit inflammation.

Toll-like receptors (TLRs) play a critical role in innate and adaptive immunity. The TLR subfamily consists of 13 members, which recognize distinct microbial patterns such as LPS, flagellin, viral double-stranded RNA, and unmethylated CpG motifs (*15*). Toll-like receptor-4 (TLR4) recognizes LPS and leads to NF-κB activation via PI3K signaling. Phosphoinositide 3-kinase is an intracellular signaling molecule that has been linked to LPS-induced inflammation, and many lines of evidence suggest that PI3K inhibition may inhibit inflammation (*16*). Recently, it has been suggested that PI3K activation may also be important for inflammation inhibition. Inhibitors of PI3K, such as LY294002

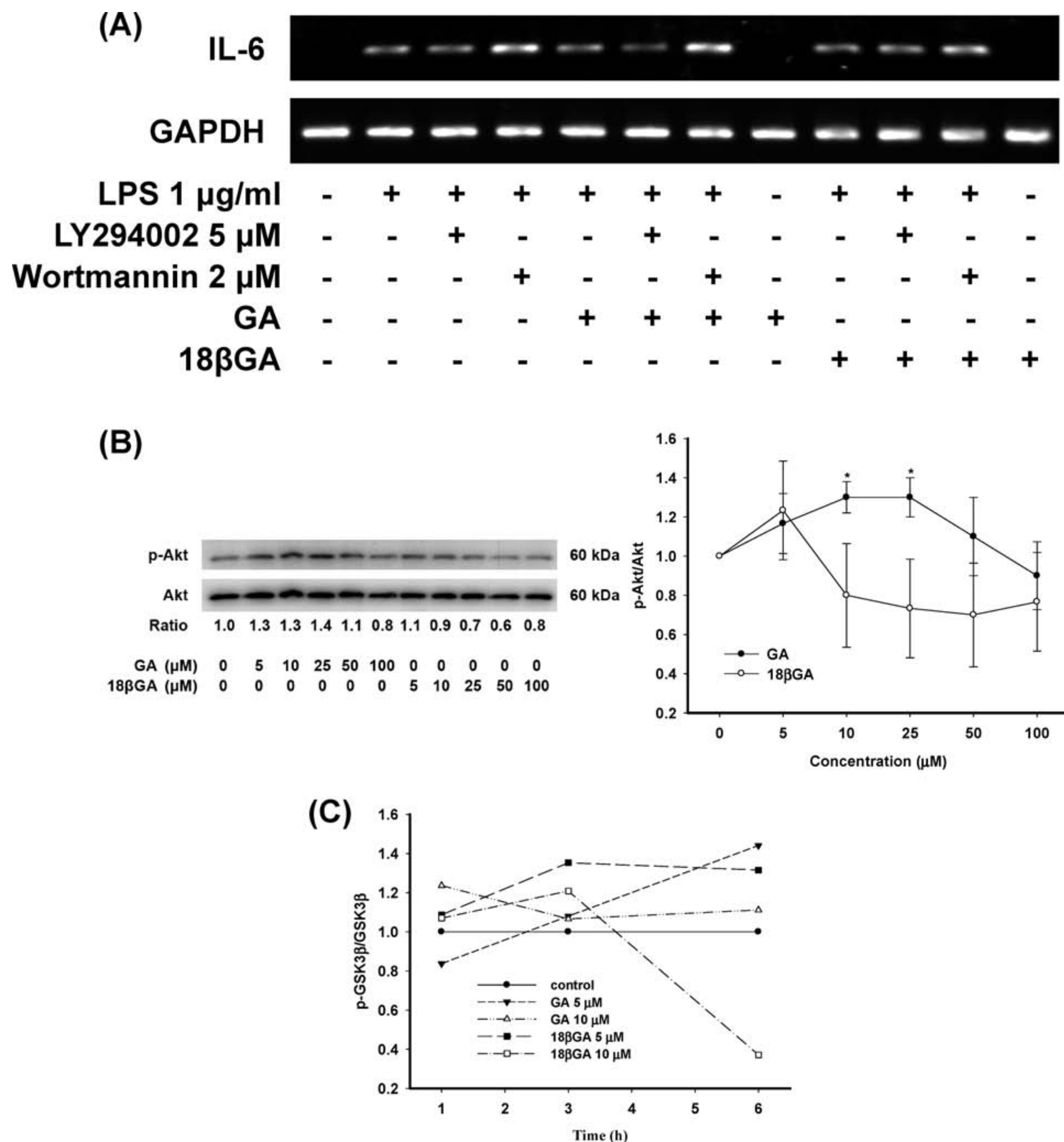

Figure 4. Effects of GA and 18βGA on PI3K/Akt activation in RAW264.7 cells. Cells were pretreated with PI3K inhibitor for 30 min, then coincubated with LPS and GA or 18βGA for 6 h. Next, total RNA was extracted and subjected to RT-PCR to assess gene expression (**A**). Cells were preincubated with serum-free medium for 2 h and treated with GA or 18βGA for 30 min. P-Akt levels were measured by Western blot analysis and normalized to total Akt levels (**B**). Cells were incubated in 5 or 10 μM of GA or 18βGA for 1−6 h in DMEM medium containing 10% FBS. After treatment, GSK3β levels were analyzed by Western blot (**C**). Data were quantified by UVP VisionWorks LS. Data are presented as means ± SD (n = 3). * $p < 0.05$ vs control group.

and wortmannin, have different effects on PI3K-based inflammation. LY294002 inhibits PI3K-induced inflammation, while wortmannin has the opposite effect (*17*), and our results also are consistent with these findings (**Figure 4A**). GA treatment decreases LPS-induced IL-6 expression. This effect is enhanced by LY294002 but decreased by wortmannin. 18βGA decreases IL-6 expression but does not respond to PI3K inhibitors. These findings suggest that GA signals through PI3K while 18βGA does not.

We also evaluated assessed Akt signaling. It is known that PI3K can induce Akt activation, which leads to GSK3β phosphorylation (*12*). Glycogen synthesis kinase-3β induces NF-κB activation, and this pathway can be inhibited by GSK3β phosphorylation. For example, α-lipoic acid has been shown to act as an anti-inflammatory by promoting GSK3β phosphorylation to inhibit NF-κB activity (*18*). It is known that PI3K/Akt/GSK3β signaling inhibits inflammation. In this study, we show that GA induces Akt phosphorylation more efficiently than 18βGA (**Figure 4B**). GA also increases GSK3β phosphorylation to a greater degree than 18βGA (**Figure 4C**). Additionally, we show that GA-induced GSK3β phosphorylation is inhibited by wortmannin (**Figure 5**). On the basis of our findings, we conclude that GA is a more potent activator of PI3K/Akt/GSK3β signaling than 18βGA. It is known that the inhibitory effect of wortmannin is similar to siRNA in terms of mode of PI3Kβ inhibition (*17*). Our data also show that 5 μM LY294002 did not markedly affect

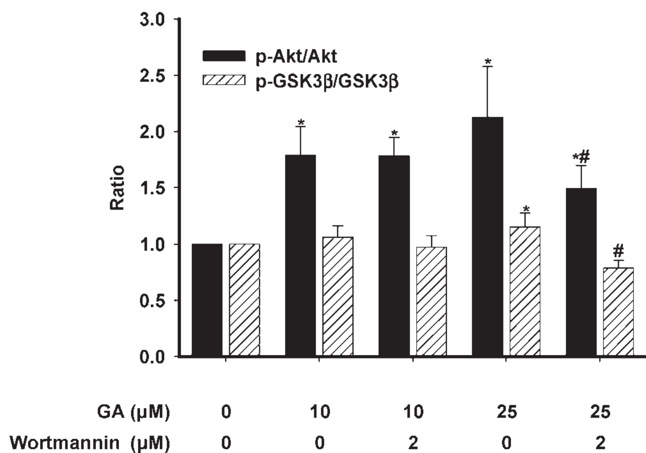

Figure 5. Effect of GA on PI3K/Akt/GSK3β activation. Cells were preincubated with serum-free medium for 100 min, then treated with wortmannin (2 μM) for 30 min prior to incubation with GA (10 and 25 μM) for 30 min. Akt and GSK3β phosphorylation levels were analyzed by Western blot analysis. Data were quantified by UVP VisionWorks LS. * $p < 0.05$ vs control group; # $p < 0.05$ vs GA group.

the PI3Kβ activation (IC_{50} of LY294002 on PI3Kβ: 11 μM), but wortmannin at 2 μM could inhibit PI3Kβ activation (IC_{50} of wortmannin on PI3Kβ: 0.001 μM) (*14*). We speculate that GA induces activation of PI3K signaling by influencing PI3Kβ. A detailed mechanism describing the interaction between GA and PI3Kβ is worth further study.

Because the chemical structures of GA and 18βGA are similar to hormones, it may be worthwhile to evaluate potential interactions between these compounds and hormone receptors. Long-term licorice consumption can lead to hypertension due to transformation of GA in licorice to 18βGA in gastrointestinal tract, which stimulates mineralocorticoid inhibition of 11β-hydroxysteroid dehydrogenase type 2 (*10*, *19*). On the basis of its chemical structure, 18βGA is more similar to sterols than GA. Our results suggest that 18βGA promotes GR-HSP90 dissociation more efficiently than GA (**Figure 6**). Glucocorticoids are steroid hormones that can diffuse easily across the plasma membrane. After glucocorticoids bind GR, GR dissociates from HSP90 (*20*). GR negatively interacts with NF-κB and AP1, two well-characterized inducers of pro-inflammatory cytokine expression (*21*). GA and 18βGA can bind GR and mineralocorticoid receptor, although the binding affinities are much lower than those between GR and

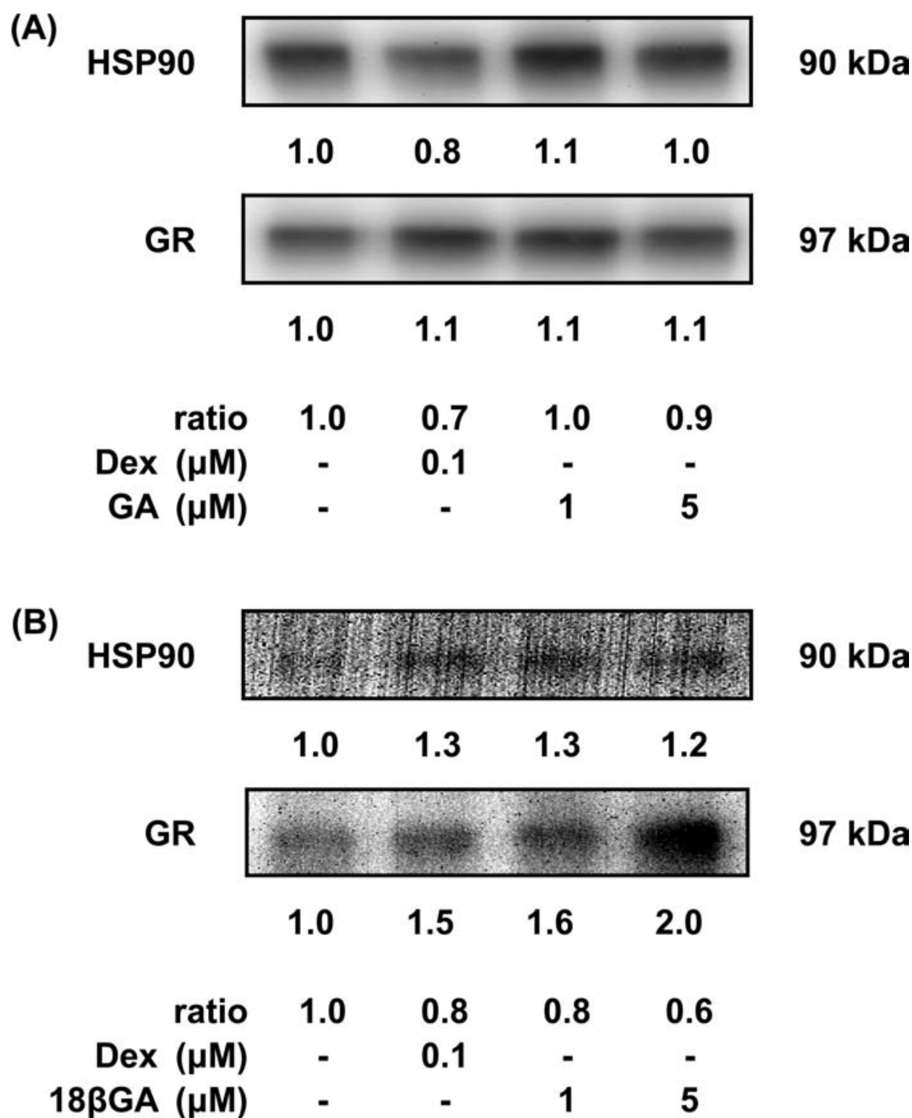

Figure 6. Effects of GA and 18βGA on GR activation. Cells were incubated with GA (**A**) or 18βGA (**B**) for 1 h in DMEM medium containing 10% FBS. After treatment, cell lysate was incubated with anti-GR antibody to isolate intracellular GR-HSP90 complexes. After purification, samples containing GR-HSP90 were subjected to Western blot analysis for GR and HSP90 content.

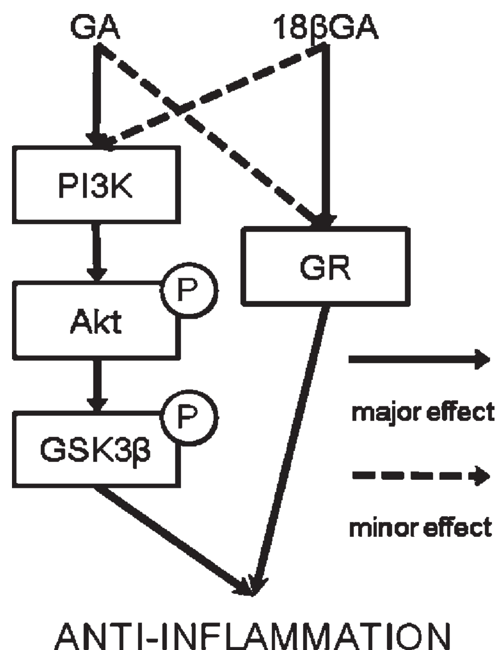

Figure 7. Potential anti-inflammatory mechanisms of GA and 18βGA.

dexamethasone or aldosterone (*22*). Our results support this finding and suggest that 18βGA may be a more potent inflammation inhibitor than GA. According to recent research, raw licorice and roasted licorice extracts can protect against both acute inflammation and chronic inflammatory conditions including rheumatoid arthritis (*23*). Interestingly, roasted licorice extract inhibits acute inflammation more potently than raw licorice extract, and Hwang et al. (*24*) showed that 18βGA levels in licorice are increased after roasting. Our data suggest that 18βGA is a potent inhibitor of acute inflammation.

Schrofelbauer and co-workers showed that 0.5 mM GA interferes with membrane-dependent receptor signaling to attenuate pro-inflammatory responses (*25*). Our data suggest that GA and 18βGA activate PI3K/Akt signaling and GR activation at $1-10$ μM to inhibit the inflammatory response. It is hard to determine the concentration of GA and 18βGA in dry licorice powder that is used to treat inflammation in TCM. However, the relative low working concentration may easily be achieved in the daily usage. Both of these studies indicate that GA is an ideal anti-inflammatory agent that can activate multiple anti-inflammatory mechanisms with no cytotoxicity (**Figure 7**). Additionally, the European Union suggests that daily intake less than 10 mg of GA (equivalent to approximately 5 g of licorice sweets) can be considered very safe, if not consuming more than 100 mg of GA per day (*26*). It means that it might be safe to use licorice powder or GA as a bioactive component in nutraceuticals.

On the basis of our findings, we conclude that GA and 18βGA influence PI3K/Akt/GSK3β signaling and GR activation, respectively. Our results clearly indicate that PI3K signaling is affected more by GA than by 18βGA. However, 18βGA is a more potent activator of GR than is GA. These differences are likely due to differing chemical structures of GA and 18βGA. Furthermore, GA and 18βGA may serve as novel anti-inflammatory agents based on their anti-inflammatory properties and may be used in the nutraceuticals to alleviate numbers of inflammatory syndrome.

ABBREVIATIONS USED

18βGA, 18β-glycyrrhetinic acid; Akt, protein kinase B (PKB); COPD, chronic obstructive pulmonary disease; Dex, dexamethasone; GA, glycyrrhizic acid; GR, glucocorticoid receptorr, GSK3β, glycogen synthase kinase 3β; HSP90, heat shock protein 90; IL-1α, interleukin 1α; IL-6, interleukin 6; LPS, lipopolysaccharide; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; PI3K, phosphoinositide 3-kinase; TCM, traditional Chinese medicine; TNFα, tumor necrosis factor α; TLR, toll-like receptor.

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