Glycyrrhetinic Acid Suppressed NF-κB Activation in TNF-α-Induced Hepatocytes

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ABSTRACT: Tumor necrosis factor-alpha (TNF-α) is a crucial inflammatory cytokine when hepatocytes are damaged. Glycyrrhiza uralensis Fisch. (Chinese licorice) has been widely used in Chinese herbal prescriptions for the treatment of liver diseases and as a food additive. Nuclear factor-kappa B (NF-κB) reporter gene assay in TNF-α-induced HepG2 was used as a screening platform. IκBα phosphorylation and p65 translocation were measured by Western blotting, and nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) gene expression were further confirmed in rat primary hepatocytes. Results showed that TNF-α enhanced NF-κB activity was significantly attenuated by glycyrrhetinic acid in a concentration-dependent manner in the NF-κB reporter gene assay. Glycyrrhetinic acid decreased the gene expression of iNOS through inhibited IκBα phosphorylation and p65 translocation in protein level. Furthermore, NO production and iNOS expression were reduced by glycyrrhetinic acid in TNF-α-induced rat primary hepatocytes. These results suggest that glycyrrhetinic acid may provide hepatoprotection against chronic liver inflammation through attenuating NF-κB activation to alleviate the inflammation.

KEYWORDS: TNF-α, hepatocytes, Glycyrrhiza uralensis, NF-κB, glycyrrhetinic acid

INTRODUCTION

Chronic liver injury, resulting from viral infection, alcohol consumption, chemical substances, drugs, autoimmune diseases, and so on, induces liver fibrogenesis that can lead to the onset of cirrhosis or even hepatocellular carcinoma. When a liver is damaged, hepatocytes live in an inflammatory microenvironment. Liver inflammation can be induced by xenobiotic toxicity (such as alcohol and chemicals) or endogenous stimulation (such as LPS and cytokines). TNF-α, which is mainly released by Kupffer cells in the liver, has emerged as a key cytokine in various liver diseases.1 TNF-α production is one of the earliest events in many types of liver injury, triggering the production of other cytokines that together recruit inflammatory cells, inducing hepatocyte apoptosis and initiating hepatic wound-healing response, including fibrogenesis. The involvement of TNF-α has been demonstrated in both acute and chronic alcoholic hepatitis2 and in type B and C virus hepatitis.3 Liver injury and fibrosis are reduced in TNF-α−/− mice4 and TNF receptor (TNFR)1−/− mice,5 and liver fibrosis induced by carbon tetrachloride (CCL4) is also alleviated in TNFR1−/− mice.6 Therefore, TNF-α has been considered as a crucial inflammatory cytokine for liver injury and subsequent liver fibrosis. NF-κB, a major downstream of TNF-α, is permissive to α1(I) collagen expression and prevents apoptosis in activated hepatic stellate cells, which are key players in liver fibrosis. On the basis of this knowledge, inhibition of NF-κB is one important target for the therapeutic blockade of fibrogenesis.7 NF-κB is a family of heterodimeric proteins including a proteolytic processing the p50 subunit as well as p65 subunit. NF-κB is ordinarily inactivated in a latent cytoplasmic form by association with various inhibitors such as IκBα. Dissociation of p65 from IκBα, through phosphorylation of IκBα followed by proteolysis via ubiquitination, is a prerequisite for p65 translocation.8 NF-κB is a redox-sensitive transcription factor that regulates many genes related to inflammation, such as iNOS, interleukin-8 (IL-8), and cyclooxygenase-2 (COX-2) at the transcription level.9

The dried root of Glycyrrhiza uralensis Fisch. (Chinese licorice or Gan-Cao in Chinese) is widely used in traditional Chinese prescriptions10 and as a flavoring additive in the food industry. Triterpenes such as glycyrrhizin (glycyrrhizic acid) and glycyrrhetic acid, as well as flavonoids, such as neoliquiritin, liquiritigenin, and liquiritin, are two kinds of its major constituents.11 Longynin decoction12 and Sho-saiko-to13 are famous Chinese prescriptions containing licorice for hepatoprotective effects. Glycyrrhizin, the major sweetening component, has been reported to exhibit hepatoprotective effects in different chemical-induced liver injury models. Tsuruoka et al. reported that glycyrrhizin (10.5 mg/kg, ip) suppresses increases in AST and ALT, inhibiting iNOS mRNA expression and protein, cell infiltration, and the degeneration of hepatocytes in the liver of concanavalin A-treated mice.14 Lee et al. reported that glycyrrhizin (200 mg/kg, ip) alleviates CCL4-induced liver injury by diminishing free radical toxic properties and down-regulating pro-inflammatory mediators.15 Both glycyrrhizin and glycyrrhetic acid exhibit protective effects on retnorsine-induced liver damage in rats,16 inhibit Smad-...
mediated type I collagen gene transcription, and suppress experimental murine liver fibrosis.\textsuperscript{17} Flavonoids from licorice have been reported to exhibit potent radical-scavenging activity.\textsuperscript{18} A \( \text{CO}_2 \)-supercritical licorice extract exhibited potent anti-inflammatory properties in LPS-induced IL-1/\( \beta \) -6, and -8 and TNF-\( \alpha \) responses of macrophages.\textsuperscript{19} Furthermore, two isoflavans, licoricidin and licorisoflavlan A, isolated from the above supercritical licorice extract were potent inhibitors of the pro-inflammatory cytokine in the ex vivo human whole blood model, suggesting that these two isoflavans could be potential candidates for the development of a new therapy to prevent and/or treat periodontitis-associated tissue destruction.\textsuperscript{20} However, the effects of licorice against endogenous stimulation such as TNF-\( \alpha \) in hepatocytes are not clear.

The TNF-\( \alpha \)-activated HepG2 cell model has been used for evaluating the translocation of the p65 of NF-\( \kappa \)B subunits mediated by its inhibitor (I\( \kappa \)B\textsuperscript{\alpha}) proteolysis.\textsuperscript{12} The hepatoprotective effects of \( G. \) uralensis in TNF-\( \alpha \)-induced HepG2 and rat primary hepatocyte inflammation were investigated in this study.

\section*{Materials and Methods}

\subsection*{Materials and Equipment.} Silica gel (230–400 mesh) and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) were used for column chromatography. Solvents (analytical grade) were purchased from Merck (Darmstadt, Germany). Nuclear magnetic resonance (NMR) spectra were run in methanol-\( d_4 \) (Sigma-Aldrich Co., St. Louis, MO, USA) on a Varian unity INOVA-500 or VNMRS 600 (Varian, Palo Alto, CA, USA) using standard pulse sequences. Mass spectra (ESIMS) were recorded on a Finnigan MAT LCQ ion trap mass spectrometer system (Thermoquest, San Jose, CA, USA). Dulbecco’s modified Eagle’s medium (DMEM) and nonessential amino acids (NEAA) were purchased from Gibco BRL (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Gibco BRL (Gaithersburg, MD, USA). pNF-\( \kappa \)B-Luc and pCMV-\( \beta \)-gal were purchased from Life Technologies (Grand Island, NY, USA). Antibodies to I\( \kappa \)B\textsuperscript{\alpha}, antiphospho-I\( \kappa \)B\textsuperscript{\alpha}, antiphospho-I\( \kappa \)K\( \alpha \)/\( \beta \), anti-p65, anti-lamin A/C, and anti-\( \alpha \)-tubulin monoclonal antibodies were purchased from Cell Signaling (Danvers, MA, USA). HRP conjugated secondary antibodies were used. Pyrrolidine dithiocarbamate (PDTC) was purchased from Merck. All other chemicals used were of analytical grade and purchased from a commercial supplier (Sigma-Aldrich Co.).

\subsection*{Preparation of \( G. \) uralensis Extract and Isolation of Pure Compounds.} \( G. \) uralensis was purchased from an herbal retailer in Taipei. A voucher herbarium specimen has been deposited at the Herbarium of National Research Institute of Chinese Medicine (NRICM) (NHP00917) and was identified by Dr. I-Jung Lee, herbarium leader of NRICM. The dried slices of \( G. \) uralensis (3 kg) were extracted at 60 °C by aqueous ethanol (80% ethanol/H\textsubscript{2}O) overnight three times. The extract was concentrated with a vacuum rotary evaporator under reduced pressure and dried in a vacuum oven (491 g). The dried sample (GUE, about 16.4% yield based on dry raw materials) was sequentially partitioned with ethyl acetate (EtOAc) and n-butanol (BuOH) to give ethyl acetate- (GUE-EtOAc, 87.8 g, 2.93% of raw materials), n-butanol- (GUE-BuOH, 125.2 g, 4.17% of raw materials), and water- (GUE-H\textsubscript{2}O, 265.3 g, 8.84% of raw materials) soluble fractions. The bioactive fraction, GUE-EtOAc, was chromatographed by silica gel column (5.5 × 60 cm) and eluted with an n-hexane/EtOAc gradient. The active fraction of 30–50% EtOAc elute was further purified over Sephadex LH-20 (3.8 × 40 cm, 50% EtOAc/MeOH elution) and then silica gel columns (2.8 × 30 cm, 30 or 40% EtOAc/n-hexane) to give glycyrrhetinic acid (0.024 g). The other two major components, glycyrrhizin (1.03 g) and liquiritin (1.35 g), were isolated from the BuOH subfraction by repeated Sephadex LH-20 column chromatography (Figure 1). All of the isolated compounds were mainly identified by 1D- and 2D-NMR and MS and further compared with an authentic sample, respectively (see the Supporting Information). For bioassay, the extracts and isolated pure compounds were all dissolved in dimethyl sulfoxide (DMSO) and diluted with a medium to give a final DMSO concentration of <0.1%. The vehicle control group contained 0.1% DMSO. Curcumin, a bioactive phenol compound from the Zingiberaceae family, was used as a positive control.\textsuperscript{22,23}

\subsection*{Cell Culture.} HepG2 cells were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan, no. 60025) and were cultured with DMEM containing 10% heat-inactivated FBS, 2% NEAA, and 2% glutamine in a 5% CO\textsubscript{2} incubator at 37 °C. Cells were

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{structures.png}
\caption{Structures of three major isolated pure compounds from \( G. \) uralensis.}
\end{figure}
Cytotoxicity Assay. The assay of reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was performed to evaluate the cytotoxicity of crude extract, subfractions, and isolated pure compounds. All test samples mentioned above were dissolved in DMSO, and the final concentration of DMSO was <0.1%; 0.1% DMSO was used as the control group.

Transfection and Reporter Assay. Transient transfection was conducted with Genejuice (Merck) according to the instructions of the manufacturer. HepG2 cells were seeded into 24-well plates at a density of 1 × 10^5 cells/well and cotransfected with the pNF-kB-Luc (0.2 μg/well) and pCMV-β-gal (0.02 μg/well). The total DNA amount was normalized with empty vectors in the transfection mixture. After transfection (24 h), the cells were cultured in FBS-free DMEM for the treatment of the test sample for 30 min and then incubated in TNF-α (10 ng/mL) for another 6 h. The total cells were harvested for luciferase assay. The luciferase assay was conducted using a 96-well luminometer with the dual luciferase substrate system (Promega, Madison, WI, USA). Relative luciferase activity was normalized to the β-gal assay and presented as the ratio of cells with and without TNF-α treatment. Each experiment was repeated at least three times.

mRNA Expression of iNOS, COX-2, and IL-8 by Real-Time Polymerase Chain Reaction (PCR). HepG2 cells were seeded into 6-well plates at a density of 5 × 10^5 cells/well. After incubation overnight, cells were washed with PBS, and the medium was replaced with FBS-free DMEM. After 24 h of starvation, cells were pretreated with test samples for 6 h and then stimulated with TNF-α (10 ng/mL) for another 3 h. After washing with cold phosphate-buffered saline (PBS), the total RNA was prepared by TriPure isolation reagent (Roche, Mannheim, Germany) following the manufacturer’s directions. After that, cDNA preparation and PCR were performed by a RevertAid First Strand cDNA synthesis kit (Fermentas, USA) according to the manufacturer’s protocol. For real-time PCR, the amplification was carried out in a total volume of 20 μL containing 0.5 μM of each primer, 4 mM MgCl2, 10 ng/μL of SYBR Green Master (Roche), and 10 μL of 1:10 diluted cDNA. Real-time PCR was performed using the LightCycler480 (Roche), and PCR reactions were performed in duplicate and heated to 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 10 s, and extension at 72 °C for 12 s. The sequences of primers for real-time PCR are listed in Table S1 (Supporting Information). Data are represented as the fold induction of inflammatory-related genes by TNF-α-induced compared to the spontaneous group. PDTC, a specific NF-κB inhibitor, was used as a positive control.24

Protein Preparation and Western Blotting. HepG2 cells were seeded into a 10 cm culture plate at a density of 2 × 10^5 cells/well. After incubation overnight, cells were washed with PBS and the medium was replaced with FBS-free DMEM. After 24 h of starvation, cells were pretreated with test samples for 6 h and then stimulated with TNF-α (10 ng/mL) for another 30 min. The cells were washed twice with ice-cold PBS and then lysed in RIPA lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 500 μM sodium orthovanadate, 10 μg/mL aprotinin, 10 mM NaF, 1% Triton X-100, and 0.1% SDS, pH 7.4) on ice for 20 min. The total cell lysates were clarified by centrifugation at 10000g for 30 min at 4 °C. The supernatants were collected for phosphor-IkBα assay.

For nuclear p65 assay, nuclear extraction was performed by a nuclear extraction kit (Millipore, Billerica, MA, USA). Briefly, HepG2 cells were lysed in ice-cold cytoplasmic lysis buffer and incubated on ice for 15 min. After centrifugation at 10000g for 5 min at 4 °C, the supernatants were discarded and the cell pellets resuspended with ice-cold cytoplasmic lysis buffer. After cytoplasm extraction by syringing with a 27 gauge needle, the supernatants were removed, and the remaining pellet consisted of the nuclear portion of the cell lysates. The nuclear pellet was suspended in ice-cold nuclear extraction buffer and incubated for 1 h. Nuclear proteins were harvested in the supernatant after centrifugation at 10000g for 10 min at 4 °C.

The protein contents of the supernatants were measured with the Bio-Rad Protein Assay Kit. Equal amounts (50 μg) of the samples were subjected to SDS-PAGE using 10% running gels and then transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat milk in TBS-Tween buffer (25 mM Tris, 190 mM NaCl, and 0.5% Tween-20, pH 7.5) for 1 h at room temperature and subsequently incubated overnight at 4 °C with appropriate primary antibodies. After hybridization with the primary antibodies, the membrane was incubated with an HRP-labeled secondary antibody for 1 h. Following washing with TBS-Tween buffer, the membrane was developed with enhanced chemiluminescence (ECL) Western blotting reagents (Amersham Pharmacia Biotech). Immunoblots images were analyzed by ImageJ (NIH, USA).

Isolation and Culture of Primary Rat Hepatocytes. Isolation of rat hepatocytes from Sprague-Dawley (SD) rats was performed using the two-step collagenase perfusion method outlined in a previous study.25 The isolated hepatocytes were cultured in Leibovitz L-15 medium supplemented with 10% FBS, 45 μg/mL penicillin G, and 50 μg/mL streptomycin in collagen-precoated 24-well plates (1 × 10^5 cells/mL) and incubated under 5% CO2 at air at 37 °C.

Assay of NO Release. An NO release assay was carried out according to the method described by Chen et al.26 Briefly, rat primary hepatocytes were seeded at 1 × 10^5 cells/well in a 96-well plate. After a washing with PBS, cells were pretreated with glycyrrehinic acid (40 μM) for 6 h and then incubated with 10 ng/mL TNF-α for another 24 h. The supernatant (100 μL) was mixed with the same volume of Griess reagent (% sulphanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in water) and incubated for 15 min in the dark. The absorbance of the water-soluble purples/red product was read at 570 nm with an enzyme-linked immunosorbent assay (ELISA) reader. The amount of NO was calculated by a calibration curve established with 1.5–100 μM NaNO2.

Statistical Analysis. Results are expressed as the mean ± standard deviation (SD) for three independent experiments. Differences between specific means were analyzed by one-way analysis of variance (ANOVA) using the SPSS system, vers. 11.0 (SPSS, Chicago, IL, USA). Group means were compared using one-way ANOVA followed by S–N–K test. p values of <0.05 were considered significant.

RESULTS

Extracts and Isolated Compounds of G. uralensis Attenuated the NF-κB Activation in TNF-α-Induced HepG2 Cells. The cytotoxicity of the extracts of G. uralensis were measured by MTT assay. Results showed that the ethanolic crude extract (GUE) and its subfractions have no direct cytotoxicity to HepG2 cells at the working concentrations (5–25 μg/mL) (Figure S1 in the Supporting Information). To determine the effect of G. uralensis on TNF-α-induced NF-κB activation, HepG2 cells were transfected with NF-κB cis-reporter plasmid, pNF-kB-Luc, which contains five repeats of NF-κB binding element GGGGAC-TTCC in the enhancer element of the plasmid. Thereafter, cells were pretreated with or without 30 min GUE and its subfractions and then incubated with TNF-α (10 ng/mL) for 6 h. As shown in Figure 2, TNF-α significantly increased the NF-κB activation by a factor of 2.1 over the spontaneous control. GUE (25 μg/mL) treatment clearly attenuated the NF-κB activation induced by TNF-α with an IC50 = 18.1 ± 2.8 μg/mL. In addition, GUE-EtOAc suppressed the activation of NF-κB at a lower concentration (10 μg/mL), but not in other subfractions. This result may imply that bioactive compounds exist in the EtOAc subfraction of GUE.

Under activity-guided fractionation to investigate the bioactive components in G. uralensis, the bioactive components were isolated from GUE-BuOH and GUE-EtOAc. Two other major pure compounds were isolated from GUE-BuOH, named glycyrrehizin and liquiritin, for comparison. Figure 3 shows that the glycyrrehizin acid significantly decreased the activation of NF-
Glycyrrhetinic Acid Suppressed the Gene Expression of iNOS and COX-2 but Not IL-8 in TNF-α-Induced HepG2 Cells. To evaluate the expression of inflammatory genes in TNF-α-induced HepG2 cells, quantitative real-time PCR was performed. After prescreening of the gene expressions in TNF-α-induced HepG2 cells, iNOS, COX-2, and IL-8 were selected as indicators for further experiments. As shown in Figure 4, HepG2 cells were stimulated with TNF-α (10 ng/mL) for 3 h, and the mRNA expressions of iNOS, COX-2, and IL-8 were significantly enhanced compared with the spontaneous control (2.3-, 3.1-, and 4.3-fold, respectively). Glycyrrhetinic acid (40 μM) dramatically suppressed the mRNA expression of iNOS and COX-2 by 57 and 53%, respectively. However, the mRNA expression of IL-8 showed no significant changes in either glycyrrhetinic acid or curcumin in TNF-α-induced HepG2 cells.

Glycyrrhetinic Acid Suppressed the TNF-α-Induced Activation of NF-κB via Decreased Phosphorylation of IκBα and Translocation of p65. To explore whether glycyrrhetinic acid inhibition of iNOS expression is mediated by blocking the IκBα/NF-κB pathway, the protein levels of phospho-IκBα in cytosol and NF-κB p65 in the nucleus were analyzed by Western blotting. With or without pretreatment of glycyrrhetinic acid (40 μM) for 6 h, HepG2 cells were stimulated with TNF-α for 30 min. After TNF-α treatment, phosphorylation of IκBα in cytosol was apparently increased, because IκBα was degraded by ubiquitination. Thereby, NF-κB was activated (Figure 5A). Moreover, the translocation of p65 from cytosol into the nucleus revealed that the transcription factor, NF-κB, was activated in the nucleus (Figure 5B). Glycyrrhetinic acid suppressed the phosphorylation of Akt, inhibited the activation of IKK-α/β, and decreased the phosphorylation of IκBα, thereby inactivating NF-κB activity. PDTC, a specific inhibitor of NF-κB activation, inhibited the nuclear translocation of the NF-κB p65 subunit by reducing IκBα degradation and served as a positive control.

Glycyrrhetinic Acid Has an Anti-inflammatory Effect on TNF-α-Induced Rat Primary Hepatocytes. To clarify the anti-inflammatory effect on the liver ex vivo, the rat primary hepatocytes were stimulated by TNF-α and all samples were tested at noncytotoxic concentration as shown in Figure S2 (Supporting Information). NO is a molecular mediator of many physiological processes, including inflammation. After 24 h of rat primary hepatocyte stimulation by TNF-α, the nitrite concentration in the culture medium was measured as an indicator of NO production according to the Griess reaction. TNF-α (10 ng/mL) caused distinct increase of NO production compared to the spontaneous control (18.1 vs 10.7 μM, p < 0.05). As shown in Figure 6A, glycyrrhetinic acid significantly decreased NO production. Moreover, the biosynthesis gene, iNOS, was investigated at the transcription level by quantifying the mRNA level. TNF-α-induced iNOS expression was significantly reduced by 62% by glycyrrhetinic acid (Figure 6B). Curcumin served as the positive control showing anti-inflammatory effects in both NO production and iNOS expression.

### DISCUSSION

In the present study, the NF-κB reporter gene assay was performed to evaluate the hepatoprotective qualities of *G. uralensis*. We demonstrated that glycyrrhetinic acid (a) attenuated the activation of NF-κB in a concentration-dependent manner (10–40 μM); (b) decreased the mRNA...
levels of iNOS and COX-2; (c) significantly inhibited the phosphorylation of IκBα and nuclear translocation of p65 in TNF-α-induced HepG2 cells; and (d) reduced NO production via suppression of iNOS expression in TNF-α-induced rat primary hepatocytes.

TNF-α is a pleiotropic cytokine triggering pro-inflammatory effects via NF-κB related pathways in various liver injuries. Therefore, therapeutic intervention in TNF-α/NF-κB signaling is technically feasible and could be of potential benefit in inflammatory liver disease.27 Wu et al. reported that a n-hexane/ethanol (9:1; v/v) nonpolar extract of licorice inhibited LPS-induced nitrate oxide production in RAW 264.7 cells and also showed Nrf2-dependent transcriptionally active Nrf2-mediated antioxidant and induced phase II detoxifying agents genes in the Nrf2 knocked out mice.28 Recently, Chen et al. reported that glycyrrhetinic acid (25 mg/kg, ig) protects the liver from CCL4-induced oxidative stress through activation of the Nrf2 signaling, thereby reserving its hepatoprotective effect.29 Furthermore, with chemical acute toxicity, endogenous factors, such as TNF-α and LPS, stimulate hepatocytes, causing chronic liver inflammation.1,30 Ngan et al. reported that Ginkgo biloba revealed anti-inflammatory effects via activation of the PPARs transcriptional activity in TNF-α-induced HepG2 cell.31 In our study, we focused on the role of G. uralensis in TNF-α-induced chronic liver inflammation. GUE and GUE-EtOAc attenuated the activation of NF-κB induced by TNF-α in a concentration-dependent manner with IC50 values of 18.1 ± 2.8 and 8.5 ± 3.2 μg/mL, respectively. Glycyrrhetinic acid, the bioactive compound isolated from GUE-EtOAc, showed suppression effects on NF-κB activation with an IC50 value of 14.3 ± 1.1 μM (~6.73 μg/mL) by reporter gene assay. A previous study demonstrated that glycyrrhetinic acid inhibited NF-κB activation in TNF-α-activated HUVEC cells.32 Our findings provide evidence that glycyrrhetinic acid protects the liver from inflammation via inactivation of NF-κB signaling. Curcumin, as a positive control, has also been reported with hepatoprotective and anti-inflammatory effects via suppression of NF-κB activation both in vivo33 and in vitro.34

The transcription factor NF-κB is important for the regulation of various genes involved in immune and acute phase inflammatory responses.35 Most anti-inflammatory functional foods, such as mulberry leaf,36 Dunaliella salina alga,34 and Magnolia officinalis,35 have been shown to suppress the expression of pro-inflammatory genes by inhibiting the NF-κB activation pathway. The NF-κB signal transduction pathway is directly engaged by the TNFR/Akt/IKK pathway with TNF-α stimulation.36 Our study showed that glycyrrhetinic acid markedly suppressed NF-κB activation through the suppression of Akt phosphorylation and inhibition of IκBα phosphorylation, thereby inhibiting p65 translocation (Figures 3 and 5). These results are coincident with the effect of curcumin on NF-κB signaling.37 The pro-inflammatory-related genes, inducible iNOS, COX-2, and IL-8, are downstream from NF-κB and are related to liver diseases. When a liver is damaged, iNOS is induced, followed by increased NO production, thereby decreasing the liver’s detoxification ability and resulting in fibrosis or hepatitis.38 COX-2 is a pro-inflammatory mediator that is correlated with chronic hepatitis, liver cirrhosis, and hepatocarcinogenesis.39 IL-8, which is produced in response to stimulation by TNF-α, is a potent chemokine that may be implicated in infiltration of neutrophils causing acute hepatitis.40 Our results showed that the mRNA expressions of iNOS, COX-2, and IL-8 were significantly enhanced after being stimulated with TNF-α for 3 h in HepG2 cells. Glycyrrhetinic acid effectively suppressed mRNA expression of iNOS and COX-2, but not that of IL-8 (Figure 4). Various transcription

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Effect of glycyrrhetinic acid on the mRNA expression of iNOS (A), COX-2 (B), and IL-8 (C) in TNF-α-induced HepG2 cells. HepG2 cells were pretreated with glycyrrhetinic acid (40 μM) for 6 h, followed by treatment with TNF-α (10 ng/mL) for 3 h. The expression of iNOS, COX-2, or IL-8 mRNA was analyzed by quantitative real-time PCR. The level of iNOS, COX-2, or IL-8 mRNA expression was normalized to GAPDH. Curcumin and PDTC were used as positive controls. Data are presented as the mean ± SD of three independent experiments. *p < 0.05, compared with the spontaneous control. #p < 0.05, compared with the TNF-α induced group.
factors induced by TNF-α such as NF-κB and AP-1 modulate IL-8 expression. The effect of glycyrrhetinic acid on the regulation of AP-1 in TNF-α-induced hepatocytes is not clear and remains to be further investigated.

Although HepG2 is frequently used to replace primary human hepatocytes in in vitro models for various experiments, cell lines still have some restriction such as lack of phase I enzymes and thereby cannot reflect real organisms. There-fore, we further isolated primary rat hepatocytes to confirm the effects of glycyrrhetinic acid on TNF-α-induced inflammation. Modulation of iNOS gene expression and suppression of NO production are important targets for anti-inflammatory properties in screening natural compounds. Our results showed that glycyrrhetinic acid inhibited NO production and concomitantly suppressed iNOS expression at the mRNA level in primary hepatocytes (Figure 6). These results were also supported by the hepatoprotective property of G. uralensis in animal models.

Glycyrrhizin and glycyrrhetinic acid are two major bioactive components in G. uralensis. Glycyrrhizin, a glucuronide of glycyrrhetinic acid, is much more polar. It may hydrolyze in vivo to glycyrrhetinic acid, which is responsible for most of its pharmacological properties. Lin et al. reported that a 3 day pretreatment with either glycyrrhizin (200 mg/kg, ip) or glycyrrhetinic acid (10 mg/kg, ip) exhibited protective effects on retrorsine-induced liver damage in rats. However, glycyrrhizin may have too high of a polarity to pass through the cell membrane. Therefore, it has not shown an effect on NF-κB activation in the in vitro study (Figure 3). Nonetheless, Wang et al. reported that both glycyrrhizin and glycyrrhetinic acid attenuate the generation of excessive NO, by suppressing the expression of pro-inflammatory genes via inhibition of NF-κB in a LPS-induced RAW 264.7 cell model. This result may imply that glycyrrhizin cannot competitively inhibit the TNF receptor to inactive the NF-κB. Therefore, the suppression of NF-κB activation by glycyrrhizin may go through other pathways, such as the PI3K/Akt/GSK3β pathway. Furthermore, roasted licorice extract inhibits acute inflammation more potently than raw licorice extract, and the glycyrrhetinic acid levels in licorice are increased after roasting. Our results support this finding and suggest that glycyrrhetinic acid, an aglycone triterpene, may be a more potent inflammatory inhibitor than its glycoside, glycyrrhizin. Likewise, liquiritin, a glycoside of liquiritigenin with O-glucopyranosyl at C-4′ of the B-ring, has no effect in the reporter gene assay. Our results suggest that glycyrrhetinic acid in G. uralensis contributed, at least partially, to its anti-inflammatory effect by inactivating NF-κB through decreasing phosphorylation of IκB and thereby inhibiting the translocation of p65. To our knowledge, this is the first study related to the inhibitory effect of TNF-α/NF-κB signaling in hepatocytes demonstrating the anti-inflammatory ability of glycyrrhetinic acid. These findings provide the molecular mechanisms for the hepatoprotective action of glycyrrhetinic acid against TNF-α-induced chronic inflammation and may be beneficial to the prevention of chronic liver injury.
DMSO, dimethyl sulfoxide; DMEM, Dulbecco’s modified Eagle’s medium; (FBS, fetal bovine serum; GUE, ethanolic extracts of Glycyrrhiza uralensis; iNOS, inducible nitric oxide synthase; IL-8, interleukin-8; NO, nitric oxide; NF-κB, nuclear factor-kappa B; PDTC, pyrrolidine dithiocarbamate; TNF-α, tumor necrosis factor-alpha; TNFR, TNF receptor

**REFERENCES**


**ABBREVIATIONS USED**

- DMSO, dimethyl sulfoxide
- DMEM, Dulbecco’s modified Eagle’s medium
- (FBS, fetal bovine serum
- GUE, ethanolic extracts of Glycyrrhiza uralensis
- iNOS, inducible nitric oxide synthase
- IL-8, interleukin-8
- NO, nitric oxide
- NF-κB, nuclear factor-kappa B
- PDTC, pyrrolidine dithiocarbamate
- TNF-α, tumor necrosis factor-alpha
- TNFR, TNF receptor

**ASSOCIATED CONTENT**

- Supporting Information
  Additional experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

- AUTHOR INFORMATION

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**Notes**

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