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The constituents of licorice (*Glycyrrhiza uralensis*) differentially suppress nitric oxide production in interleukin-1β-treated hepatocytes

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ABSTRACT

Licorice (Glycyrrhizae radix) is the roots and stolons of *Glycyrrhiza uralensis* Fischer or *Glycyrrhiza glabra* Linnaeus in the Japanese Pharmacopoeia. Glycyrrhizae radix has been widely used as a sweetener and a traditional medicine. A Glycyrrhizae radix extract contains many constituents and has antispasmodic, antitussive, anti-ulcer, and anti-inflammatory effects. However, reports comparing the anti-inflammatory effects of these constituents are very few. Here, we purified several constituents from the roots and stolons of *G. uralensis* and examined and compared their anti-inflammatory effects by monitoring the levels of the inflammatory mediator, nitric oxide (NO), in interleukin (IL)-1β-treated rat hepatocytes. From the *G. uralensis* extract, we purified the main constituent glycyrrhizin and the constituents that are characteristic of *G. uralensis* (chalcones and flavanones). These constituents suppressed NO production in IL-1β-treated rat hepatocytes, and isoliquiritigenin showed the greatest suppression activity. Isoliquiritigenin, isoliquiritin, and liquiritigenin significantly decreased both protein and mRNA for the inducible nitric oxide synthase. These constituents reduced the levels of mRNAs encoding tumor necrosis factor α and IL-6. In contrast, although glycyrrhizin is abundant, it showed a 100-fold lower potency in NO suppression. Therefore, both glycyrrhizin and the minor constituents (isoliquiritigenin, isoliquiritin, and liquiritigenin) may be responsible for the anti-inflammatory effects of *G. uralensis*. It is also implied that these constituents may have a therapeutic potential for inflammatory hepatic disorders.

1. Introduction

Licorice is as a natural sweetener in foods and a traditional medicine that is used worldwide to treat a variety of diseases, such as peptic ulcer and hepatitis [1]. In the Japanese Pharmacopoeia, licorices are defined as the dried roots and stolons of *Glycyrrhiza uralensis* Fischer and *Glycyrrhiza glabra* Linnaeus and are designated Glycyrrhizae radix (*kanzo* in Japanese) [2]. Glycyrrhizae radix has been used in many formulae of Japanese herbal (Kampo) medicines, such as *kanzoto* and *shakuyakukanzoto*. Glycyrrhizae radix has a variety of pharmacological properties, including antispasmodic, antitussive, anti-ulcer, and anti-inflammatory effects, and attenuates the adverse effects of other components in the Kampo formulae [3].

Glycyrrhizae radix includes many constituents: triterpene glycosides (mainly, glycyrrhizin), chalcones (*e.g.*, isoliquiritigenin and isoliquiritin), and flavanones (*e.g.*, liquiritigenin and liquiritin) [4,5]. Glycyrrhizin (also designated glycyrrhizic acid) is a glycoside of glycyrrhetinic acid (also designated glycyrrhetic acid) (Fig. 1A) and is abundantly present in the roots and stolons. This saponin is the major sweet constituent and the main bioactive compound in Glycyrrhizae radix [1], which displays hepatoprotective properties in humans and mice [6,7]. The contents of isoliquiritin, liquiritigenin, and liquiritin in *G. uralensis* are significantly higher than those of *G. glabra*; therefore, these constituents are characteristic of *G. uralensis* [4,5]. As far as we searched the literatures to date, there are no reports that compare anti-inflammatory activity of several Glycyrrhizae radix constituents using any types of cells.

The inflammatory mediator nitric oxide (NO) plays a pivotal role in innate immunity and pathophysiology of various diseases, and inducible nitric oxide synthase (iNOS) synthesizes NO in hepatocytes and macrophages [8,9]. Similarly in the hepatocytes in the liver, the *iNOS* gene is induced by the inflammatory cytokine

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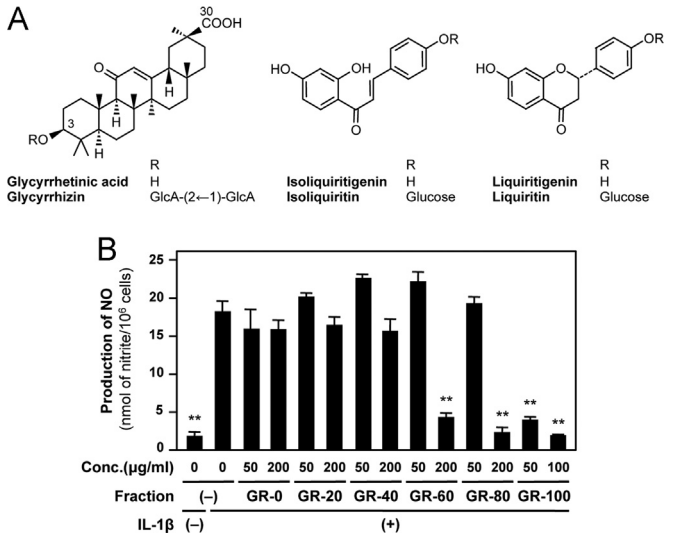

Fig. 1. Identification of the constituents in a *G. uralensis* water extract. (A) The chemical structures of the constituents of *G. uralensis*. GlcA, glucuronic acid. The C-30 of glycyrrhizin may be conjugated with glucuronic acid in the liver [34]. (B) The fractions of a *G. uralensis* extract suppress NO induction in rat hepatocytes. A *G. uralensis* extract was stepwise fractionated by methanol (0–100%) to give fractions (GR-0 to 100). The hepatocytes were treated with IL-1β and/or each fraction for 8 h. The NO levels in the medium (mean ± SD) are shown. $^{**}P < 0.01$ *versus* IL-1β alone.

interleukin-1β (IL-1β) in primary cultured rat hepatocytes, and this induction mimics liver injury, such as acute hepatitis [9,10]. As iNOS protein is induced, the NO level linearly increases from 6 h to 12 h after the addition of IL-1β.

Using the hepatocytes, we demonstrated that NO is a sensitive marker that can be used to monitor inflammatory responses to anti-inflammatory drugs [11], Japanese herbal medicines [12] and their constituents, such as chlorogenic acid (*i.e.*, 5-*O*-caffeoylquinic acid) in the flowers and buds of the Japanese honeysuckle (*Lonicera japonica*) [13] and nobiletin (polymethoxylated flavone) in the peels of *Citrus unshiu* [14]. Only one paper using primary cultured hepatocytes, which described anti-inflammatory effects of glycyrrhizin, was found in the literatures that we have searched to date [15]. This report suggested NO suppression activity of glycyrrhizin in primary cultured hepatocytes that were isolated from Bacillus Calmette-Guérin (BCG)-vaccinated rats. Hepatoprotective herbal medicines suppress the IL-1β-induced expression of iNOS and inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6), in hepatocytes [13,14]. The transcription factor nuclear factor κB (NF-κB) is involved in these processes by regulating the expression of *iNOS* gene and these inflammatory genes [16–18].

iNOS induction is also regulated *via* a post-transcriptional mechanism that is mediated by antisense transcripts (asRNAs) [9]. asRNAs are transcribed from the *iNOS* gene and interact with iNOS mRNA to stabilize iNOS mRNA [9,19–21]. Recently, herbal constituents (*e.g.*, chlorogenic acid [13], nobiletin [14], gomisin N [18], and shisoflavanone A [22]), as well as Kampo formulae (*e.g.*, *inchinkoto* [12] and *ninjinyoeito* [23]), have been reported to decrease the levels of iNOS asRNA, leading to the inhibition of iNOS expression.

In the present study, we purified glycyrrhizin and several constituents that are characteristic of *G. uralensis*. Next, we examined whether the constituents (aglycone–glycoside pairs) suppressed NO production, as well as *iNOS* gene expression, in IL-1β-treated hepatocytes. Finally, we investigated which constituent (s) is responsible for the NO suppression activity of Glycyrrhizae radix.

2. Materials and methods

2.1. Materials

Glycyrrhizin, isoliquiritigenin, and liquiritigenin were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) as standards. Glycyrrhetinic acid was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). These compounds (greater than 95% purity) were dissolved in dimethyl sulfoxide.

2.2. Plant materials and extraction

The roots and stolons of *G. uralensis* Fischer, which were collected from the Inner Mongolia Autonomous Region, China, and identified and authenticated by Dr. Yutaka Yamamoto (Tochimoto Tenkaido Co. Ltd., Osaka, Japan), were purchased from Tochimoto Tenkaido Co. Ltd. A voucher specimen was deposited in the Ritsumeikan Herbarium of Pharmacognosy, Ritsumeikan University, under the Code number RIN-GU-013. The dried roots and stolons of *G. uralensis* (Glycyrrhizae radix; 502.6 g) were pulverized and extracted by hot water under reflux. The solvent was evaporated under reduced pressure to yield a hot-water extract (160.0 g).

2.3. Isolation of the constituents from a G. uralensis extract

The *G. uralensis* extract was fractionated on a Diaion HP20 column (Mitsubishi Chemical Corporation, Tokyo, Japan) by the elution stepwise by methanol–water mixture (0, 20, 40, 60, 80, and 100% methanol) to give the fractions (GR-0, 20, 40, 60, 80, and 100, respectively). The fraction GR-80 showing high NO suppression activity was further purified by Wakogel C-200 chromatography (Wako Pure Chemical Industries Ltd.) or Cosmosil 75 C_{18}-Prep octadecylsilyl chromatography (Nacalai Tesque, Kyoto, Japan). Finally, we purified glycyrrhizin (3850 mg), isoliquiritigenin (4.3 mg), isoliquiritin (234 mg), liquiritigenin (16.4 mg), and liquiritin (8.8 mg). To determine the structures, the ^{1}H- and ^{13}C nuclear magnetic resonance (NMR) spectra, infrared (IR) spectra, and ultraviolet (UV) spectra were analyzed (Supplementary data).

2.4. Preparation of primary cultured rat hepatocytes

Male Wistar rats were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan), housed at 21–23 °C and acclimatized. Hepatocytes were isolated from the rat livers by collagenase perfusion [24]. The isolated cells were resuspended in Williams' E (WE) medium (Sigma-Aldrich Corp., St. Louis, MO, USA), seeded at 1.2×10^6 cells/dish, and incubated at 37 °C for 2 h, after which the medium was replaced. The hepatocytes were incubated at 37 °C overnight and analyzed the next day. All of the animal care and experimental procedures were carried out in accordance with the guidelines and laws of the Japanese government and were approved by the Animal Care Committee of Ritsumeikan University, Biwako-Kusatsu Campus.

2.5. Determination of the NO levels and LDH activity

The hepatocytes were each treated with fraction or a constituent in the presence of 1 nM rat IL-1β (PeproTech, Rocky Hill, NJ, USA) for 8 h. If required, 1400W (Abcam plc., Cambridge, Cambridgeshire, United Kingdom), a selective iNOS inhibitor [25], was added to the medium to a final concentration of 50 nM. The levels of nitrite (a stable metabolite of NO) in the medium were measured using the Griess method [26]. Briefly, 150 μl of the medium or sodium nitrite (standard) was mixed with 150 μl of the Griess reagent [0.5% sulfanilamide, 0.05% *N*-(1-napthyl)

ethylenediamine, and 2.5% phosphoric acid]. After 5 min at room temperature, absorbance at 540 nm was measured. To monitor the cytotoxicity, the LDH activities in the medium were measured using LDH Cytotoxicity Detection Kits (Takara Bio Inc., Otsu, Shiga, Japan). Unless cytotoxicity was observed, the half-maximal inhibitory concentrations (IC_{50}) were determined [11].

2.6. Measurement of direct NO quenching activity of constituents

Each constituent was added to the WE medium containing 20 μM $NaNO_2$ and incubated at 37 °C for 1.5 h. This medium (150 μl) was mixed with the Griess reagent (150 μl) and incubated at room temperature for 5 min. Absorbance at 540 nm was measured in triplicate to monitor the decrease of nitrite by the constituent. The levels of nitrite using the medium containing $NaNO_2$ alone was set to 100%.

2.7. Western blot analyses

The hepatocytes were treated with 1 nM IL-1β and each constituent for 8 h, and whole-cell lysates were prepared [18]. Briefly, the hepatocytes were lysed, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted onto a Sequi-Blot membrane (Bio-Rad, Hercules, CA, USA). Immunostaining was performed using primary antibodies that were raised against rat iNOS (Thermo Fisher Scientific, Waltham, MA, USA) and rat β-tubulin (Cell Signaling Technology Inc., Danvers, MA, USA), followed by visualization with an Enhanced Chemiluminescence Blotting Detection Reagent (GE Healthcare Biosciences Corp., Piscataway, NJ, USA).

2.8. Reverse transcription-polymerase chain reactions (RT-PCR)

The hepatocytes were treated with 1 nM IL-1β and/or each constituent for 4 h, and the total RNA was prepared [9]. The cDNA was reverse-transcribed in a strand-specific manner with an oligo (dT) primer for mRNAs and a sense primer for iNOS asRNA, and PCR was performed with paired primers [9]. The primer sequences CCAACCTGCAGGTCTTCGATG and GTCGATGCACAACTGGGTGAAC ($5'->3'$) were used to detect the iNOS mRNA by cDNA amplification. The primer sequences that were used for the iNOS asRNA detection were TGCCCCTCCCCCACATTCTCT (RT), ACCAGGAGGCGCCATCCCGCTGC and CAAGGAATTATACACGGAAGGGCC (PCR). The primer sequences for PCR were TCCCAACAAGGAGGAGAAGTTCC and GGCAGCCTTGTCCCTTGAAGAGA for the TNF-α mRNA; GAGAAAAGAGTTGTGCAATGGCA and ATAGGCAAATTTCCTGGTTATATCC for the IL-6 mRNA; and TCTGGTTGGAATGGTGACAACATGC and CCAGGAAGAGCTTCACTCAAAGCTT for the elongation factor 1α (EF) mRNA as internal controls. The mRNA levels were estimated in triplicate by quantitative PCR with the Thermal Cycler Dice Real Time System (Takara Bio Inc.), and the obtained values were normalized to EF mRNA [9].

2.9. Statistical analyses

The results that are presented in the figures are representative of at least three independent experiments that yielded similar results. The values are presented as the mean ± standard deviation (SD). The differences were analyzed using Student's *t*-test. The statistical significance was set at $P<0.05$ and $P<0.01$.

3. Results and discussion

3.1. The fractions of a G. uralensis extract suppress the NO induction in hepatocytes

We extracted the dried roots and stolons of *G. uralensis* (Glycyrrhizae radix) using hot water, and this extract (GR extract) was subjected to fractionation by hydrophobicity. Because the high content of saponins (mostly glycyrrhizin) inhibited phase separation, the GR extract was not successfully fractionated by the standard ABC method [13] (data not shown). Therefore, we performed Diaion HP-20 chromatography to fractionate the GR extract into six fractions.

Then, we investigated the effects of the resultant fractions on NO induction in IL-1β-treated rat hepatocytes. When each fraction was simultaneously added to the medium with IL-1β, all of the fractions significantly decreased the levels of NO production (Fig. 1B). Three fractions GR-60 (21.2 g), GR-80 (22.0 g) and GR-100 (3.7 g), which were eluted with 60%, 80%, and 100% methanol, respectively, effectively suppressed NO production. The LDH activity in the medium was very low (data not shown), suggesting that all of the fractions of the GR extract were not toxic to hepatocytes at the concentrations that are indicated in the figure. Together, these data indicate that the three fractions GR-60, GR-80, and GR-100 may include active compounds that may suppress NO induction.

3.2. Several constituents in a GR extract suppress NO induction in hepatocytes

Next, we tried to isolate the constituents that effectively suppressed NO induction from the fractions showing NO suppression activity. Because the amount of the fraction GR-100 was much smaller, the fractions GR-60 and GR-80 were subjected to thin layer chromatography. This analysis showed that the major spots of both fractions were similarly developed (data not shown). Therefore, we selected the middle fraction GR-80 and then purified five constituents from this fraction, as described in Supplementary data. Finally, we identified glycyrrhizin and two aglycone–glycoside pairs (isoliquiritigenin and isoliquiritin; and liquiritigenin and liquiritin) by analyzing the NMR and UV spectra (Supplementary data). These constituents except for glycyrrhizin are characteristic of *G. uralensis* [4,5].

To examine whether the constituents in a GR extract affect *iNOS* gene expression, we added each constituent to the medium for hepatocytes. Among these constituents, isoliquiritigenin significantly suppressed NO induction in the presence of IL-1β in a dose-dependent manner (Fig. 2A). Similarly, isoliquiritin and liquiritigenin significantly suppressed NO induction. When evaluating the LDH activity in the medium, these constituents displayed no cytotoxicity at the concentrations that are indicated in Fig. 2A. When the IC_{50} values were calculated (Table 1), isoliquiritigenin showed the highest potency in NO suppression activity, with an IC_{50} value of 11.9 μM. In another assay system using a mouse RAW264.7 macrophage line, an IC_{50} value of isoliquiritigenin of 7.8 μM was reported [27].

In contrast, the main constituent glycyrrhizin suppressed NO induction only at a high concentration, showing an IC_{50} value of 1176 μM, which is approximately 100-fold higher than that of isoliquiritigenin. Then, we examined the degradation of glycyrrhizin by incubating the WE medium containing 1000 μM glycyrrhizin at 37 °C for 8 h. Glycyrrhizin was purified by Diaion HP-20 chromatography and analyzed by high-performance liquid chromatography (HPLC). The content of glycyrrhizin did not change by the incubation (data not shown). Furthermore, when the WE medium containing 1000 μM glycyrrhizin was similarly incubated in the presence of hepatocytes, the content of glycyrrhizin was almost the same as that of the medium without incubation (data not shown). These results suggest that the

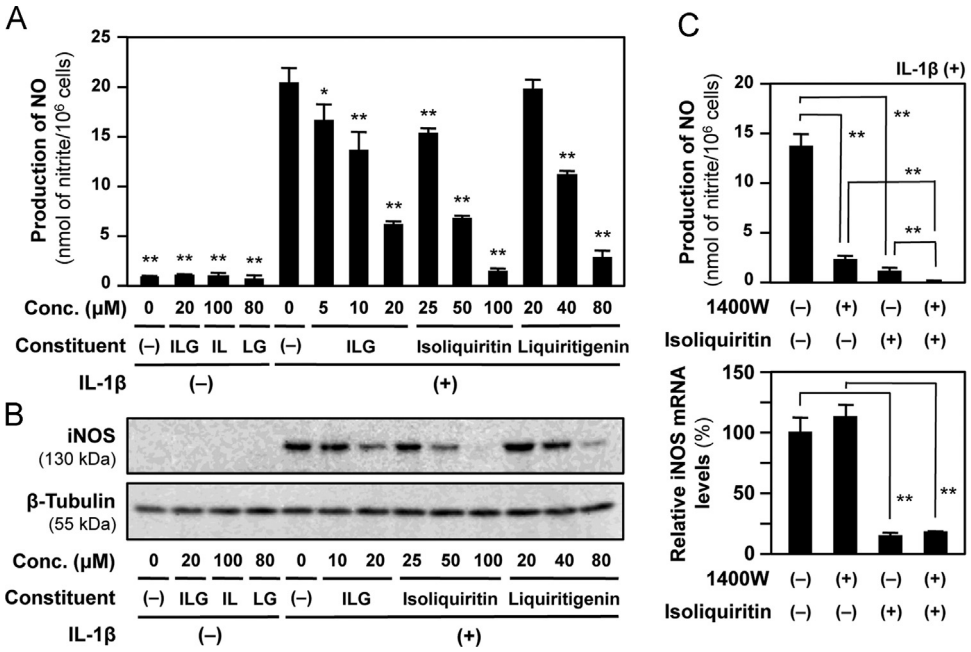

Fig. 2. The *G. uralensis* constituents suppress iNOS expression in hepatocytes. (A) The effects of the constituents in a GR extract on the induction of NO production. The hepatocytes were treated with IL-1β and/or each constituent for 8 h. The NO levels in the medium were measured in triplicate (mean ± SD). $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* IL-1β alone. ILG, isoliquiritigenin; IL, isoliquiritin; LG, liquiritigenin. (B) The effects of the constituents on the induction of iNOS protein expression. The hepatocytes were treated with IL-1β and/or each constituent for 8 h. The cell extracts were resolved using SDS-PAGE and immunoblotted with an anti-iNOS or anti-β-tubulin antibody (internal control). (C) The effects of 1400W, a selective iNOS inhibitor, on the levels of NO (upper panel) and iNOS mRNA (lower panel). The hepatocytes were treated with 50 nM 1400W and/or 100 μM isoliquiritin in the presence of 1 nM IL-1β for 8 h (NO) or 4 h (iNOS mRNA). The NO levels in the medium were measured in triplicate (mean ± SD). The total RNA from the cells was analyzed using quantitative RT-PCR to estimate the levels of iNOS mRNA. The mRNA levels were measured in triplicate (mean ± SD), the obtained values were normalized to EF mRNA (internal control), and the value in the presence of IL-1β alone was set at 100%. $^{**}P < 0.01$.

Table 1
The effects of the *G. uralensis* constituents on NO induction in rat hepatocytes.

Compound	Sugar	Content [%][a]	IC_{50} [μM][b]	LogP[c]
Triterpenes:				
Glycyrrhetinic acid	None	ND	NA	5.45
Glycyrrhizin	Glucuronic acid	3.37 ± 1.57	1176 ± 482	2.74
Chalcones:				
Isoliquiritigenin	None	0.05–0.65	11.9 ± 1.5	3.04
Isoliquiritin	Glucose	0.32 ± 0.22	40.4 ± 7.5	0.82
Flavanones:				
Liquiritigenin	None	0.11 ± 0.12	41.2 ± 5.9	2.79
Liquiritin	Glucose	1.68 ± 1.06	NA	0.43

ND, not determined; NA, not applicable due to high cytotoxicity (glycyrrhetinic acid) or low NO suppression activity (liquiritin).

[a] The contents of isoliquiritigenin (range) [5] and the others (mean ± SD; $n=87$) [4] in a *G. uralensis* extract.

[b] The half-maximal inhibitory concentration of nitric oxide (NO) production in hepatocytes (mean ± SD; $n=3$–5).

[c] *n*-Octanol/water partition coefficient as predicted using the ALOGPS 2.1 program [29].

degradation of glycyrrhizin did not occur during the incubation at 37 °C for 8 h regardless of the presence of hepatocytes, thereby not affecting its IC_{50} value.

To investigate whether the constituents in a GR extract had direct NO quenching activity, assays to measure the NO quenching were performed, as described in Section 2. We added constituents to final concentrations around their IC_{50} values (glycyrrhizin, 1000 μM; isoliquiritigenin, 10 μM; isoliquiritin, 40 μM; and liquiritigenin, 40 μM) to the medium containing 20 μM $NaNO_2$. The decreases of NO levels were less than 5% and were statistically not significant ($P > 0.05$), comparing with the NO levels of the medium containing $NaNO_2$ alone. These data suggest that any of these constituents did not directly quench NO.

3.3. The G. uralensis constituents inhibit iNOS gene expression in hepatocytes

To further investigate the effect of the active *G. uralensis* constituents on *iNOS* gene expression, we examined the expression of the iNOS protein in hepatocytes. As shown in Fig. 2B, western blot analyses indicated that isoliquiritigenin, isoliquiritin, and liquiritigenin decreased the iNOS protein expression that was induced by IL-1β.

Then, we examined a possibility that these constituents inhibited enzyme activity of iNOS protein. Therefore, we added isoliquiritin and/or 1400W [25], which is a selective iNOS inhibitor and does not affect the expression of iNOS mRNA, to the medium and measured the levels of both NO and iNOS mRNA. As shown in Fig. 2C, the addition of 1400W significantly decreased the NO levels, and isoliquiritin also decreased the NO levels, suggesting that 1400W and isoliquiritin showed an additive effect. The addition of 1400W, however, did not decrease in the levels of iNOS mRNA, whereas isoliquiritin significantly reduced the levels of iNOS mRNA (lower panel). Isoliquiritin also decreased iNOS protein (Fig. 2B), although isoliquiritin did not inhibit enzyme activity of iNOS protein (Fig. 2C). Therefore, isoliquiritin may suppress the *iNOS* gene expression by reducing the iNOS mRNA, resulting in the decrease of iNOS protein.

Quantitative RT-PCR analyses revealed that the constituents including isoliquiritin significantly reduced iNOS mRNA levels in a dose-dependent manner (Fig. 3A), suggesting that these constituents suppressed the induction of iNOS mRNA. Taken together, isoliquiritigenin, isoliquiritin, and liquiritigenin decreased the levels of both iNOS mRNA and protein. These results indicate that the three constituents inhibited the IL-1β-induced expression of the *iNOS* gene at the transcriptional level.

It is possible that the constituents suppress iNOS expression at the post-transcriptional level because iNOS asRNA interacts with

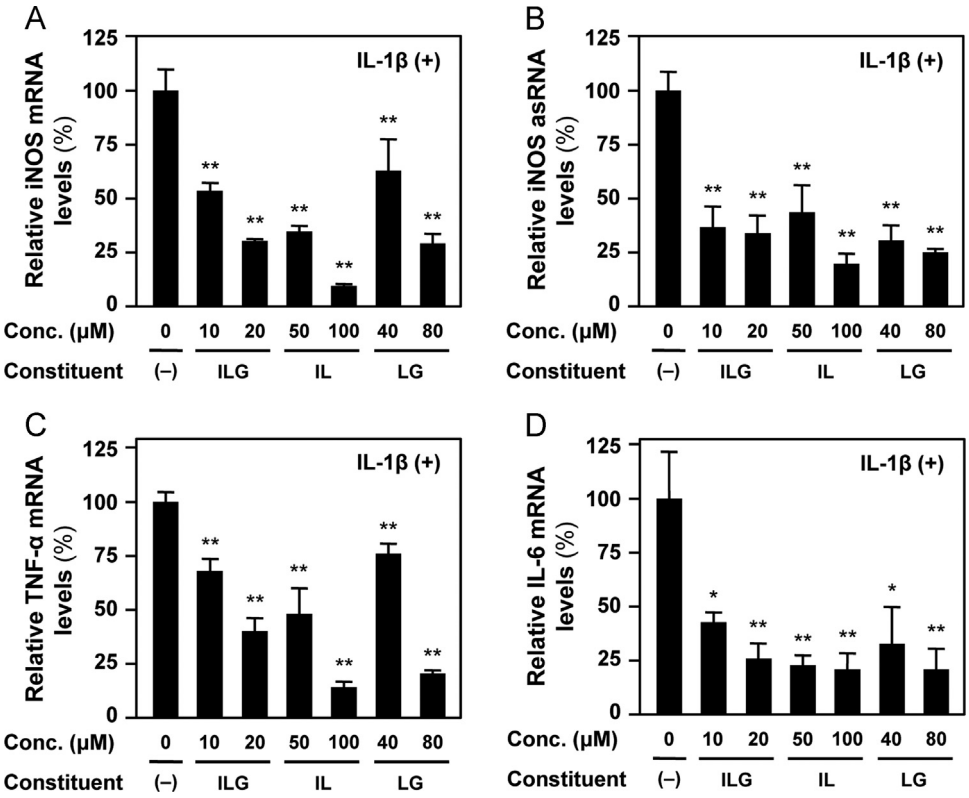

Fig. 3. Three constituents reduced the expression of the *iNOS* gene and inflammatory genes. The hepatocytes were treated with IL-1β and/or each constituent for 4 h. The total RNA from the cells was analyzed using quantitative RT-PCR to estimate the levels of mRNAs or iNOS asRNA. The mRNA levels were measured in triplicate (mean ± SD), the obtained values were normalized to EF mRNA, and the value in the presence of IL-1β alone was set at 100%. The relative levels of iNOS mRNA (A), iNOS asRNA (B), TNF-α mRNA (C), and IL-6 mRNA (D) are shown. A negative control PCR using total RNA without RT did not give amplification (data not shown). ILG, isoliquiritigenin; IL, isoliquiritin; LG, liquiritigenin. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* IL-1β alone.

and stabilizes iNOS mRNA [9,21]. Therefore, we estimated the levels of iNOS asRNA by quantitative RT-PCR. Isoliquiritigenin, isoliquiritin, and liquiritigenin significantly reduced iNOS asRNA levels (Fig. 3B). Because decreases in iNOS asRNA lead to decreased iNOS mRNA stability, these results indicate that the three constituents may post-transcriptionally regulate iNOS mRNA levels by reducing iNOS asRNA stability.

Both transcriptional and post-transcriptional mechanisms to regulate iNOS expression may be targets of herbal medicines. The Kampo formula *inchinkoto* and herbal constituents, such as gomisin N and nobiletin, affect the nuclear translocation and activation of NF-κB, leading to the suppression of *iNOS* gene expression [12,14,18].

3.4. The G. uralensis constituents suppress inflammatory genes

We examined whether the constituents that were isolated from a *G. uralensis* extract affect the expression of inflammatory genes in hepatocytes. As shown in Figs. 3C and D, the *G. uralensis* constituents decreased the levels of the mRNAs encoding TNF-α and IL-6, whereas the inflammatory cytokine IL-1β induced the expression of these mRNAs. After tissue damage or bacterial infection, IL-6 is an important mediator of an acute phase response in the hepatocytes [28]. Because the constituents down-regulated IL-6 mRNA, these results support that the *G. uralensis* constituents may have anti-inflammatory effects.

NF-κB is believed to regulate the mRNA expression of these inflammatory genes through the NF-κB-binding site(s) in their promoters [16,29]. Indeed, liquiritigenin suppressed iNOS expression by inhibiting NF-κB activation in RAW264.7 macrophages [30,31]. NF-κB also regulates the transcription of iNOS asRNA [9], and the *G. uralensis* constituents decreased the levels of iNOS asRNA (Fig. 3B). Therefore, it is likely that the constituents in a *G. uralensis* extract reduce NF-κB activity, resulting in the inhibition of expression of NF-κB-regulating genes, including not only *iNOS* but also inflammatory genes in hepatocytes.

3.5. The NO suppression activity of the G. uralensis constituents

We further compared the effects of the aglycone–glycoside pairs in the *G. uralensis* constituents. An aglycone of glycyrrhizin is glycyrrhetinic acid, which is believed to be an active metabolite [32]. When glycyrrhizin is orally administered, intestinal bacteria hydrolyze it to glycyrrhetinic acid, which is then absorbed in the intestine and transferred to the liver [32]. When glycyrrhetinic acid was added to the medium, it showed toxicity to rat hepatocytes (*i.e.*, LDH activity in the medium) at a concentration of more than 40 μM (data not shown). In contrast, glycyrrhizin did not display cytotoxicity at 1000 μM (data not shown).

The contents of isoliquiritigenin, isoliquiritin, or liquiritigenin were much lower than glycyrrhizin [4,5] but displayed high NO suppression activities, with IC_{50} values of 11.9–41.2 μM (Table 1). In contrast, although glycyrrhizin is highly abundant (3.37%) in *G. uralensis*, it possessed an IC_{50} value of 1176 μM in NO suppression. Together with these data, the NO suppression activity of a GR extract can be attributed to the activities of both glycyrrhizin and the three constituents. Because NO is an inflammatory mediator, these constituents may be responsible for the anti-inflammatory effects of *G. uralensis*.

3.6. Lipophilicity of the G. uralensis constituents

The mechanism how the *G. uralensis* constituents affect intracellular signal transduction remains unclear. If a constituent is

relatively hydrophobic, the plasma membrane may be permeable to the constituent. As a marker of lipophilicity (hydrophobicity), we predicted the *n*-octanol/water partition coefficient (logP) using the ALOGPS 2.1 program [33]. The LogP values of the *G. uralensis* compounds had IC_{50} values that ranged from 0.82 to 3.04 (Table 1). In contrast, glycyrrhetinic acid, which is a hydrolyzed metabolite of glycyrrhizin and shows toxicity to hepatocytes, had a high logP value, suggesting that glycyrrhetinic acid may be easily absorbed by the cell. Glycyrrhetinic acid, which is hydrolyzed from glycyrrhizin, is almost completely metabolized through glucuronidation and/or sulfation in the liver [34]. Therefore, the concentration of glycyrrhetinic acid in the liver is assumed to be very low; thus, hepatotoxicity may not appear *in vivo*. Once a constituent enters into the cell, it may interact with various proteins, such as protein kinases. Alternatively, flavonoids from *G. uralensis* may bind to mRNA or asRNA because flavonoids (*i.e.*, catechins and apigenin) bind to RNA [35,36]. It is possible that these intracellular interactions may affect gene expression.

3.7. The potential use of the G. uralensis constituents for an anti-inflammatory therapy

We compared the NO suppression potency of the *G. uralensis* constituents with that of other herbal constituents in hepatocytes. The IC_{50} value of isoliquiritigenin was much lower than that of nobiletin (51 μM) [14], suggesting that isoliquiritigenin has high potency in NO suppression. Furthermore, isoliquiritigenin, isoliquiritin, and liquiritigenin decreased the mRNA levels of inflammatory cytokines, including TNF-α and IL-6 (Fig. 3C, D). In contrast, an IC_{50} value of glycyrrhizin was higher than that of chlorogenic acid (652 μM) [13]. Although abundant glycyrrhizin showed much lower potency in NO suppression in hepatocytes (Table 1), its hepatoprotective activities have been reported [6,7]. Taken together, the anti-inflammatory activity of *G. uralensis* in hepatocytes may be attributed to the activities of not only glycyrrhizin but also the three constituents. Each constituent may be differentially responsible for a variety of pharmacological effects of *G. uralensis*.

The *G. uralensis* constituents have potential values to treat inflammatory diseases, especially viral hepatitis, alcoholic liver diseases, and non-alcoholic steatohepatitis (NASH). Similarly to herbal medicines for prostate cancer [37], future clinical trials are required to examine whether the *G. uralensis* constituents are used as a complementary and alternative medicine for an anti-inflammatory therapy for inflammatory hepatic disorders.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.06.004.

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