International Immunopharmacology 28 (2015) 917–924

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Flavonoids derived from liquorice suppress murine macrophage activation by up-regulating heme oxygenase-1 independent of Nrf2 activation

Rui Wang [a], Cheng Yue Zhang [b], Li Ping Bai [a], Hu Dan Pan [a], Li Min Shu [b], Ah-Ng Tony Kong [b], Elaine Lai-Han Leung [a], Liang Liu [a,*], Ting Li [a,*]

[a] State Key Laboratory of Quality Research in Chinese Medicine/Macau Institute for Applied Research in Medicine and Health, Macau University of Science and Technology, Macau, China
[b] Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA

ARTICLE INFO

Article history:
Received 23 March 2015
Accepted 28 March 2015
Available online 11 April 2015

Keywords:
Liquiritigenin
Isoliquiritin
Isoliquiritigenin
Nrf2
HO-1

ABSTRACT

Liquiritigenin (LQG), isoliquiritin (ILQ) and isoliquiritigenin (ILG) are flavonoids derived from liquorice and all possess a similar chemical structural backbone. In the current study, we found that ILQ and ILG had suppressive effects on lipopolysaccharide (LPS)-induced inflammatory responses in murine macrophage by suppressing the iNOS and COX-2 proteins and mRNA expression. A mechanistic study indicated that the effect was associated with an induction of antioxidant and detoxification enzymes, including UGT1A1, NQO1, and heme oxygenase-1 (HO-1) mRNA expression. The regulator of these enzymes, nuclear factor-erythroid 2-related factor 2 (Nrf2), which plays a critical role in LPS-induced inflammatory responses, could be activated by ILQ and ILG. Additionally, ILQ and ILG promoted Nrf2 signaling activation by inhibiting the Kelch-like ECH-associated protein 1 (Keap1) and increasing Nrf2 translocation, inducing the expression of these antioxidant enzymes. We further found that ILQ and ILG induced HO-1 expression independent of Nrf2 expression. With respect to the effect of these compounds on NF-κB signaling, ILG was found to markedly inhibit IκBα degradation and phosphorylation, while LQG and ILQ had no significant effects. These results indicate that there are correlations between the anti-inflammatory responses and the chemical structural properties of these flavonoids.

1. Introduction

Liquiritigenin (LQG), isoliquiritin (ILQ) and isoliquiritigenin (ILG) are derived from the roots of *Glycyrrhizae uralensis*, better known as liquorice or licorice, which has been used in herbal medicine for treating injuries, swelling and detoxification for hundreds of years [1] and has been widely used globally as a dietary supplement due to its multiple pharmacological properties [2,3]. Glycyrrhizin, oleane triterpenoids, glucose and flavonoids are the main components of liquorice, and among these, the liquorice flavonoids are known for a variety of biological activities. A number of reports indicate that these naturally occurring flavonoids possess anti-depressant, anti-tumor-promoting and anti-inflammation properties [4–6]. According to a recent report, LQG (Fig. 1A), ILQ (Fig. 1B) and ILG (Fig. 1C) are the three major active flavonoids in liquorice exhibiting antioxidant effects [7]. However, the molecular mechanisms responsible for the antioxidant and anti-inflammatory effects remain unclear.

Various pathological conditions and diseases such as inflammation and cancer are linked with oxidative stress, which induces biochemical alterations in cellular components such as proteins, lipids and nucleic acids [8]. The activation of Nrf2 exhibits cytoprotective effects by inhibiting pro-inflammatory cytokines [9]. Under normal conditions, Nrf2 is anchored in the cytoplasm through binding to Keap1 and can be activated by diverse stimuli including oxidants, antioxidants and chemopreventive agents. Oxidative or covalent modification of thiols in certain cysteine residues results in the dissociation of Nrf2 from Keap1 and its subsequent translocation to the nucleus [10]. In the nucleus, Nrf2 dimerizes with a small Maf protein and binds to the cis-acting antioxidant responsive element (ARE) DNA sequences of Phase II antioxidant genes, including the classical conjugating enzymes such as UDP-glucuronosyltransferases (UGTs), reduction enzymes such as NAD(P)H dehydrogenases (NQOs) and stress response enzymes such as HO-1, to activate their transcription [11,12].

Among the redox-sensitive inducible enzymes, HO-1 serves as a protective gene due to its antioxidant abilities and anti-inflammatory properties [17,18]. Accumulating studies have proven that both mouse and human deficiencies in HO-1 expression have the phenotype of an increased inflammatory state [19,20]. In addition, several studies reported that HO-1 induction negatively regulates nitric oxide (NO)

* Corresponding authors at: State Key Laboratory of Quality Research in Chinese Medicine/Macau Institute for Applied Research in Medicine and Health, Macau University of Science and Technology, Avenida Wai Long, Taipa, Macau, China. Tel.: +853 8897 2401; fax: +853 2882 5886.
E-mail addresses: lliu@must.edu.mo (L. Liu), tli@must.edu.mo (T. Li).

http://dx.doi.org/10.1016/j.intimp.2015.03.040
1567-5769/

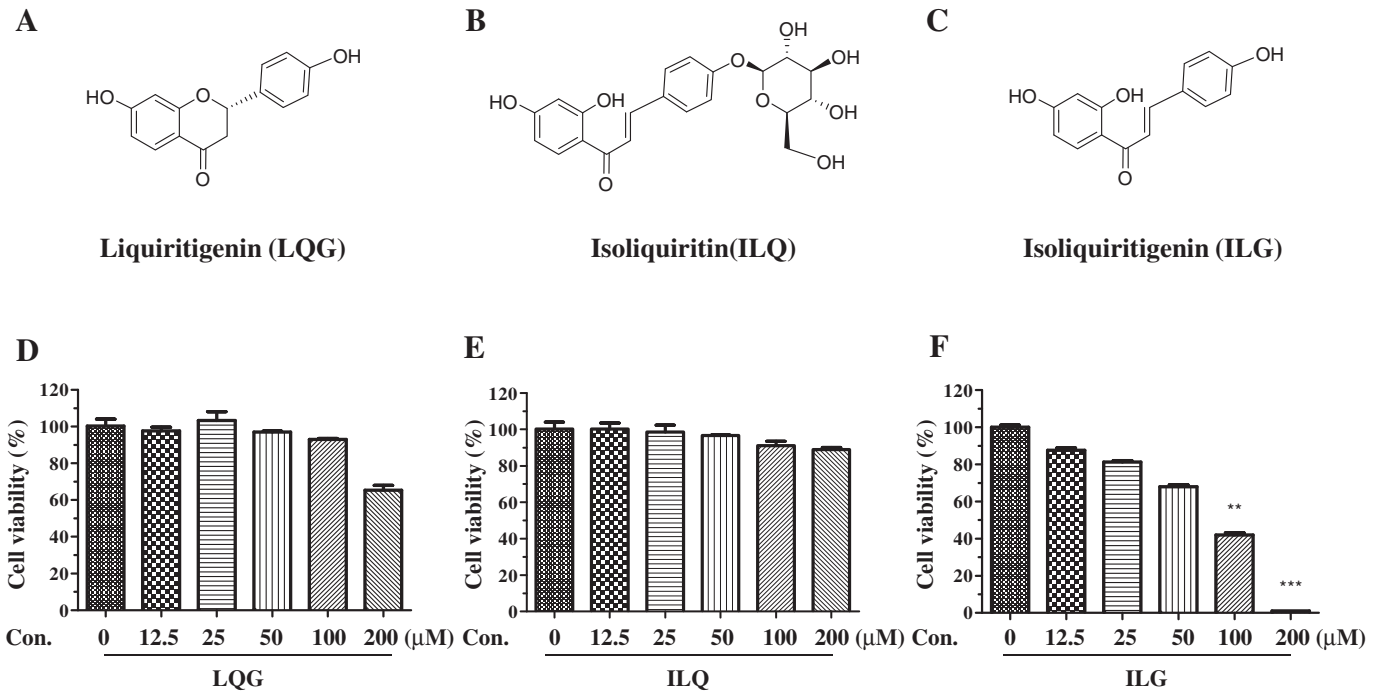

Fig. 1. The chemical structures and cytotoxicity of liquiritigenin, isoliquiritin and isoliquiritigenin. (A–C) The chemical structures of LQG, ILQ and ILG. (D–F) The cytotoxicity of LQG, ILQ and ILG. RAW 264.7 cells were incubated with the indicated dosages of LQG, ILQ and ILG, respectively, for 24 h, and the growth conditions were determined by the MTT assay. Data were obtained from three independent experiments and are expressed as mean ± SEM. $^{**}p < 0.01$;$^{***}p < 0.001$, respectively, indicating significant differences between the treatment and control groups.

expression [21,22]. As the rate-limiting enzyme, HO-1 catabolizes excess heme and generates biliverdin, iron and carbon monoxide (CO) [23], which contribute to the antioxidant effect of HO-1. Extracts from liquorice are thought to be the potential chemicals that activate ARE and induce the expression of ARE-driven genes [7], but the underlying mechanism has not been clearly elucidated.

Among the various transcriptional factors regulating immune and inflammatory responses, NF-κB is one of the set of ubiquitous and well-characterized factors in cellular signaling [24]. Under normal circumstances, NF-κB is bound to IκBα in the cytoplasm, present as a heterodimer composed of p65 and p50 proteins. When cells are activated by stimuli, such as LPS or TNF-α, IκB kinase (IKK) and IκBα are phosphorylated. Consecutively, the phosphorylated form of IκBα is ubiquitinated and then degraded. NF-κB is then released and translocated into the nucleus, where it binds to the κB enhancer element of DNA and induces transcription of many inflammatory mediators [25].

In the current study, the anti-inflammatory effects of the three compounds derived from liquorice on activated murine macrophages induced by LPS were examined. In addition, due to the key role of NF-κB and Nrf2 signaling in regulating the immune response, we performed a mechanistic study to demonstrate the influence of these compounds on these two signaling pathways. The results showed that LQG, ILQ and ILG could inhibit LPS-induced NF-κB activation and up-regulate HO-1 expression, which contributed to the anti-inflammatory responses.

2. Materials and methods

2.1. Cell and reagents

RAW 264.7 and HEK293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in DMEM (Gibco, Paisley, UK) supplemented with 10% (*v*/*v*) heat-inactivated fetal bovine serum (FBS; Gibco), 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were cultured at 37 °C in a humidified atmosphere including 5% CO_2. The HepG2-C8 cell line was a gift from Prof. Ah-Ng Tony Kong, Rutgers University. Lipopolysaccharide (LPS) was obtained from Sigma. Primary antibodies against iNOS, COX-2, IκBα, P-IκBα, P-IKKα/β, P-p65, HO-1, Nrf2 and Keap1 were obtained from Cell Signaling, and a primary antibody against β-actin was obtained from Santa Cruz. LQG, ILQ and ILG compounds (>98% purity, verified by HPLC) were obtained from the Nanjing Zelang Medical Technology Co. (Nanjing, China), dissolved in DMSO and stored at −40 °C as a stock solution.

2.2. Cytotoxicity assay

RAW 264.7 cells were plated into 96-well plates at a density of 5000 cells/well for overnight, and the cells were incubated with LQG, ILQ and ILG at various concentrations (12.5, 25, 50, 100 and 200 μM) for 24 h. MTT (5 mg/mL) was added to the cells for 4 h incubation, followed by addition of solvent (10% sodium dodecyl sulfate (SDS), 50% *N*,*N*-dimethyl formamide, pH 7.2) to dissolve the purple precipitate. A_{570nm} was determined from each well on the following day. The percentage of cell viability was calculated using the following formula: Cell viability (%) = $A_{treated} / A_{control} \times 100$. The data reported represented three independent experiments.

2.3. Luciferase reporter activity assay

The stably transfected HepG2-C8 cells expressing the ARE-luciferase vector were used to study the effects of different compounds on the Nrf2–ARE pathways. The luciferase assay kit was used according to the manufacturer's instructions (Promega, Madison, WI). Briefly, HepG2-C8 cells (1×10^5 cells/well) were plated in 12-well plates in 1 mL/well. After attaching, the cells were incubated with various concentrations of the different compounds. Afterwards, the cells were lysed using the reporter lysis buffer, and 10 μL of the cell lysate supernatant was analyzed for luciferase activity by a Sirius luminometer (Bertold Detection System GmbH, Pforzheim, Germany). Normalization of the luciferase activity was performed based on protein concentrations, which were determined by a BCA protein assay (Pierce Biotech, Rockford, IL, USA).