Inflamm. res. 58 (2009) 257–262
1023-3830/09/0500257-6
DOI 10.1007/s00011-008-8183-6

Inflammation Research

Isoliquiritigenin, from *Dalbergia odorifera*, up-regulates anti-inflammatory heme oxygenase-1 expression in RAW264.7 macrophages

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Received 13 August 2008; returned for revision 15 September 2008; returned for final revision 28 October 2008; accepted by G. Wallace 24 November 2008

Published Online First: 24.01.2009

Abstract. *Objectives:* Isoliquiritigenin (ISL), one of the major constituents of *Dalbergia odorifera* T. Chen (Leguminosae), is reported to exert anti-inflammatory effects, but the relevant anti-inflammatory mechanisms are not completely understood. Heme oxygenase-1 (HO-1) has been proven to be involved in the resolution of inflammatory responses. In this study, we investigated whether ISL could induce HO-1 expression in RAW264.7 macrophages, and if so, whether HO-1 could mediate the anti-inflammatory effects of ISL.
Methods: The protein expression of inducible nitric oxide synthase and HO-1 was analyzed by western blot analysis. The production of nitric oxide (NO) and interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) was assayed by Griess and ELISA, respectively. The TNF-α and HO-1 mRNA expression was analyzed by northern blot analysis.
Results: ISL markedly suppressed LPS-induced NO, IL-1β, and TNF-α production. ISL induced HO-1 expression through the extracellular signal-regulated kinase1/2 pathway in RAW264.7 macrophages. The effects of ISL on LPS-induced NO and TNF-α production were reversed by the HO-1 inhibitor, tin protoporphyrin.
Conclusions: ISL is an effective HO-1 inducer capable of inhibiting macrophage-derived inflammation.

Key words: Isoliquiritigenin – Nitric oxide – Tumor necrosis factor-α – Heme oxygenase-1 – Extracellular signal-regulated kinase1/2

Introduction

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in the catabolism of excess heme and generation of iron, carbon monoxide, and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase [1]. Recently, HO-1 has been proven to be involved in the resolution of inflammatory responses [2]. HO-1 and its enzymatic by-products are the critical regulators of inflammation with macrophages acting as the critical targets [3]. An increasing number of therapeutic agents have been reported to induce HO-1 expression and exert their anti-inflammatory effects through HO-1 induction [4–8].

Isoliquiritigenin (ISL, 4,2',4'-trihydroxychalcone), one of the major constituents of *Dalbergia odorifera* T. Chen (Leguminosae) [9], is a flavonoid with a chalcone structure. Previous reports demonstrated that *D. odorifera* showed anti-inflammatory activity and inhibited prostaglandin biosynthesis as well as platelet aggregation induced by arachidonic acid [9, 10]. Moreover, several reports demonstrated that ISL, isolated from licorice (*Glycyrrhiza uralensis*), decreased the pro-inflammatory mediators in RAW264.7 macrophages via suppression of nuclear factor-κB activation and mitogen-activated protein kinases (MAPK) signal pathways [11, 12]. However, the relevant anti-inflammatory mechanisms of ISL are not completely understood. Recently, we have demonstrated that ISL can induce HO-1 expression in hepatic stellate cells [13]. In the present study, we investigated whether ISL could also induce HO-1 expression in RAW264.7 macrophages, and if so, whether HO-1 could mediate the anti-inflammatory effects of ISL.

Materials and Methods

Plant materials and isolation of ISL

The heartwood of *D. odorifera* was purchased from the herbal medicine co-operative association of Jeonbuk Province, Korea, in October 2002, and the voucher specimen (No. WP02-008) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Korea).

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Dried heartwoods of *D. odorifera* (60 g) were extracted twice with EtOH (1 l) under ultrasonication for 3 h. After evaporation of the solvent under vacuum, the extract (8 g) was treated with EtOAc to give EtOAc-soluble and EtOAc-insoluble portions. The EtOAc-soluble fraction (6 g) was chromatographed on silica gel column using a n-hexane-EtOAc (gradient) to obtain five fractions (Fr. A-E). Fraction D (2.8 g) was subjected to Sephadex LH-20 column chromatography with MeOH-H_2O (9:1) to give four subfractions (Fr. D1–D4). Fr. D2 (928 mg) was further separated on a silica gel column with $CHCl_3$-MeOH (10:1) to give compound 1 (32 mg, 0.053 w/v%). Compound 1 was identified as ISL by comparison with reported spectral data (MS, ^{1}H- and ^{13}C-NMR) [14] and previously reported in the literature [15].

Reagents and cell culture

All reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Liquiritigenin (LIQ) was from ChromaDex, Inc. (Santa Ana, CA). Inhibitors of p38 (SB203580), of ERK1/2 (U0126), and of JNK (SP600125) were from Calbiochem (San Diego, CA). Tin protoporphyrin (SnPP), an inhibitor of HO-1 activity, was from Porphyrin Products Inc. (Logan, UT, USA). Primary antibodies, including iNOS and HO-1, and responsible secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Enzyme-linked immunosorbent assay (ELISA) kits for IL-1β and TNF-α were from R&D Systems, Inc. (Minneapolis, MN).

RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured at 37 °C under 5 % CO_2 in DMEM supplemented with 2 mM glutamine, antibiotics (100 U/ml of penicillin A and 100 µg/ml of streptomycin), and 10 % heat-inactivated fetal bovine serum (Gibco-BRL, Gaithersburg, MD, USA).

Nitrite quantification

NO_2^- accumulation was used as an indicator of nitric oxide (NO) production in the medium, as described previously [16]. The isolated medium were mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. $NaNO_2$ was used to generate a standard curve and nitrite production was determined by measuring optical density at 550 nm.

RT-PCR

Total RNA (5 µg) was reverse transcribed using the Moloney Murine Leukemia Virus reverse transcriptase (Gibco BRL), and the resultant cDNAs were diluted 10-fold for PCR. The primers used were as follows: inducible nitric oxide synthase (iNOS) sense [16], 5´-TCT GCG CCT TTG CTC ATG AC-3´, and antisense, 5´-TAA AGGC TCC GGG CTC TG-3´ (254 bp). PCR reactions were carried out in the presence of 1.5 mM $MgCl_2$ for 30 cycles at the following temperatures and times: denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 10 s, followed by a final extension at 72 °C for 10 min. The amplified PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining. The integrity of cDNA samples was confirmed using primers specific for GAPDH.

Northern blot analysis

Total RNA was separated electrophoretically on a 1 % agarose gel containing 5.4 % formaldehyde, transferred to nylon membranes (Hybond-N; Amersham Pharmacia Biotech, Uppsala, Sweden) by electroblotting, and fixed with UV irradiation. The RNA was hybridized with randomly primed [^{32}P]-cDNA-specific for tumor necrosis factor-α (TNF-α), HO-1 or GAPDH. Specific cDNA probes were amplified by RT-PCR using the selective primers and cloned in a TA vector (Promega, Madison, WI, USA). The primers used were as follows: TNF-α sense [16], 5´-TAC TGA ACT TCG GGG TGA TCG GTC C-3´, and antisense, 5´-CAG CCT TGT CCC TTG AAG AGA ACC-3´ (295 bp); and HO-1 sense [17], 5´-CGC AAC AAG CAG AAC CCA-3´, and antisense, 5´-TGA CGC CAT CTG TGA GGG-3´ (507 bp). Prehybridization and hybridization were performed as described previously [16, 17].

Western blot analysis

Whole-cell lysate were separated by 10 % SDS-PAGE, and electrotransferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). Nitrocellulose membranes were then incubated with specific antibodies against iNOS or HO-1. Immunoreactive bands were detected by incubating with anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Measurement of IL-1β and TNF-α secretion

RAW 264.7 cells were preincubated at 37 °C for 1 h in medium containing ISL and then further incubated with LPS (200 ng/ml). Culture supernatants of culture medium were collected and the concentration of interleukin-1β (IL-1β) and TNF-α was determined using a Quantikine® Immunoassay kit (R&D Systems, Inc., Minneapolis, MN), according to the manufacturer's instructions.

Statistical analysis

Data were analyzed with Student's *t*-test where appropriate or by one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests when comparing more than three means. Calculations were performed with GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

Results

ISL inhibits NO production and iNOS expression in LPS-stimulated RAW264.7 macrophages

To compare the potent anti-inflammatory activity of ISL, ISL and LIQ (Fig.1), a structurally related major flavonoid found in *D.odorifera* [9], were examined for their effects on LPS-induced NO production and iNOS gene expression in RAW 264.7 macrophages. NO production was monitored in RAW 264.7 macrophages stimulated by LPS in the presence or absence of these two compounds for 24 h. LPS (200 ng/ml) evoked a 10-fold induction of nitrite production versus the naïve control. Incubation with LPS and ISL (1, 2, 5 and 10 µM) resulted in dose-dependent inhibition of nitrite generation (25, 37, 66 and 86 %, respectively), whereas such an inhibitory effect was not observed with LIQ-pretreated cells (Fig. 2A).

We also examined the effects of ISL and LIQ on iNOS mRNA and protein expression by RT-PCR and western blot analyses, respectively. Treatment with ISL caused concentration-dependent inhibition of iNOS mRNA and protein expression in LPS-stimulated RAW264.7 macrophages (Fig. 2B). However, this inhibitory effect was not observed with LIQ. These results indicate that inhibition of iNOS expression by ISL occurred in parallel with the comparable inhibition of NO production. Examination of the cytotoxicity of

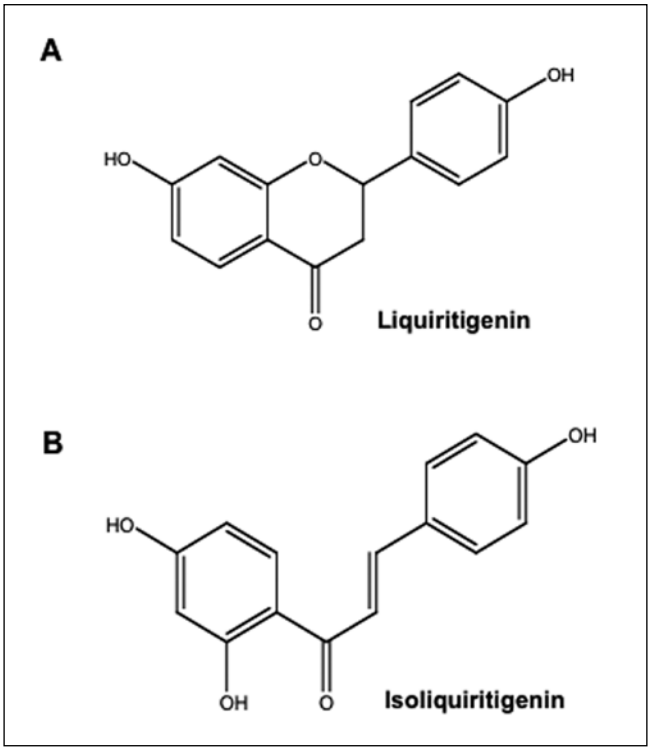

Fig. 1. Chemical structures of liquiritigenin (**A**) and isoliquiritigenin (**B**).

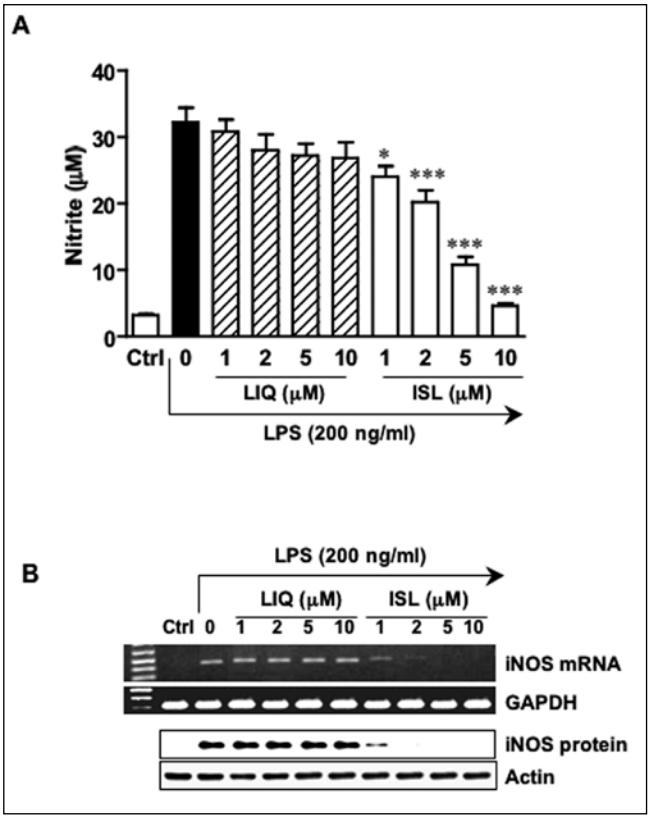

Fig. 2. Effects of LIQ and ISL on NO production and iNOS expression in LPS-stimulated RAW 264.7 macrophages. (**A**) RAW 264.7 macrophages were pretreated with the indicated concentrations of LIQ or ISL for 1 h before being incubated with LPS (200 ng/ml) for 24 h. Control cells were incubated with vehicle alone. The culture supernatants were subsequently collected and analyzed for nitrite production. Each column shows the mean ± SD of triplicate determinations. Statistical significance: $^{*}p<0.01$ and $^{***}p<0.001$ vs. LPS alone. (**B**) The levels of iNOS mRNA (*upper panel*) and protein (*lower panel*) were monitored 12 and 24 h after treatment of cells with LPS (200 ng/ml), respectively, with or without LIQ or ISL. The expression of iNOS mRNA and protein in cells was analyzed by RT-PCR and western blot, respectively. GAPDH and actin are shown as the loading controls.

ISL by MTT assay indicated that, even at 20 μM, ISL did not affect the viability of RAW 264.7 macrophages (data not shown). Therefore, inhibition of LPS-induced NO production by ISL was not the result of a cytotoxic effect on these cells. Since these data showed that ISL markedly decreased the pro-inflammatory mediator, NO, we further investigated the effects and mechanisms of ISL in the LPS-induced inflammation in RAW264.7 macrophages.

ISL suppresses the production of pro-inflammatory cytokines in LPS-stimulated RAW264.7 macrophages

We examined the effect of ISL on IL-1β production in RAW 264.7 macrophages stimulated with LPS. Fig. 3A shows that the increased production of IL-1β in LPS-stimulated RAW 264.7 macrophages is inhibited by ISL treatment in a dose-dependent manner. Treatment with a high dose of ISL (10 μM) caused 79 % inhibition of the production of IL-1β by LPS. The production of TNF-α was also measured in the medium of RAW264.7 macrophages cultured with LPS (200 ng/ml) in the presence or absence of ISL for 6 h. At the indicated concentrations, ISL inhibited TNF-α production in LPS-treated macrophages in a dose-dependent manner (Fig. 3B). Northern blot analysis was used to verify whether the inhibition of TNF-α production by ISL accompanied suppression of TNF-α mRNA. ISL also inhibited the increase in TNF-α mRNA by LPS (Fig. 3C). These findings suggest that the inhibition of TNF-α production by ISL is due to the suppression of the LPS-induced expression of TNF-α mRNA.

ISL induces HO-1 expression via ERK1/2 pathway in RAW264.7 macrophages

We examined whether ISL induces HO-1 expression in RAW264.7 macrophages. As shown in Fig. 4A, the treatment of ISL for 12 h resulted in an increase in HO-1 expression in a dose-dependent manner at both the mRNA and protein levels. At 10 μM ISL, the time course of HO-1 induction revealed that protein was already detectable 4 h after treatment and that its level continued to increase steadily even at 12 h (Fig. 4B). Next, to examine the possible upstream signaling pathway involved in the ISL-mediated HO-1 expression, RAW 264.7 macrophages were exposed to the ERK1/2 inhibitor U0126, the JNK inhibitor SP600125, or the p38 inhibitor SB203580. As shown in Fig. 4C, the induction of HO-1 by ISL was inhibited by U0126 in a dose-dependent manner. In contrast, the selective JNK or p38 inhibitors failed to affect the induction activity of HO-1 by ISL.

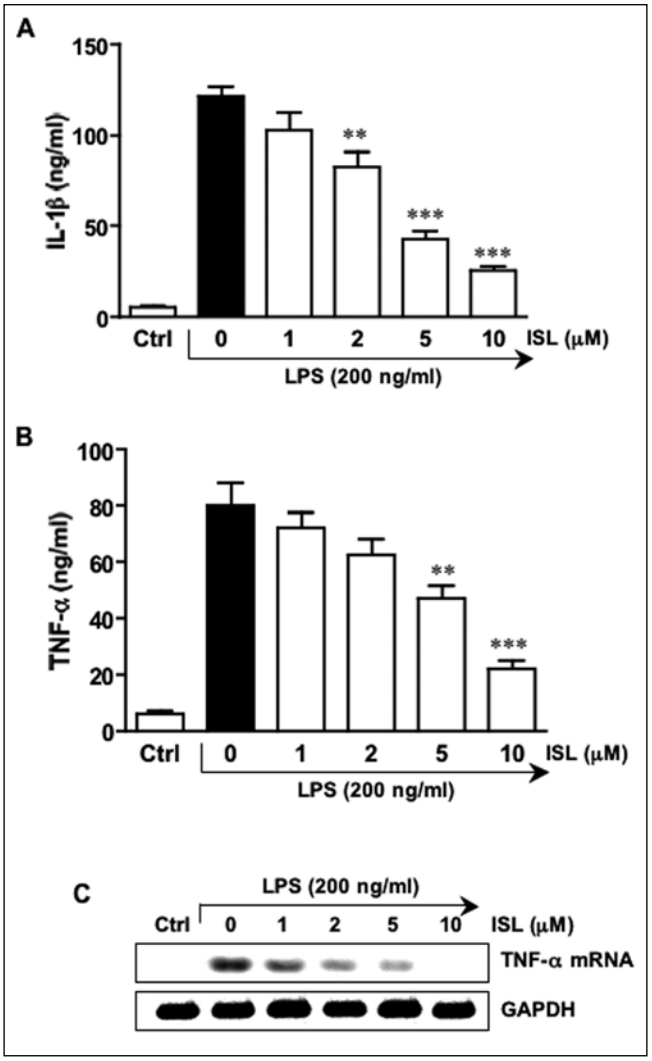

Fig. 3. Inhibition of IL-1β and TNF-α production by ISL in LPS-stimulated RAW 264.7 macrophages. RAW 264.7 cells were pretreated with the indicated concentrations of ISL for 1 h before being incubated with LPS (200 ng/ml) for 16 h. The culture supernatants were subsequently collected and analyzed for IL-1β (**A**) and TNF-α (**B**). Control cells were incubated with vehicle alone. Each column shows the mean ± SD of triplicate determinations. Statistical significance: $^{**}p<0.05$ and $^{***}p<0.001$ vs. LPS alone. (**C**) TNF-α mRNA was monitored by northern blot analysis in cells cultured with LPS (200 ng/ml) in the presence or absence of ISL for 3 h. The amount of RNA loaded in each lane was confirmed by rehybridization of the stripped membrane with a ^{32}P-labeled probe complementary to GAPDH.

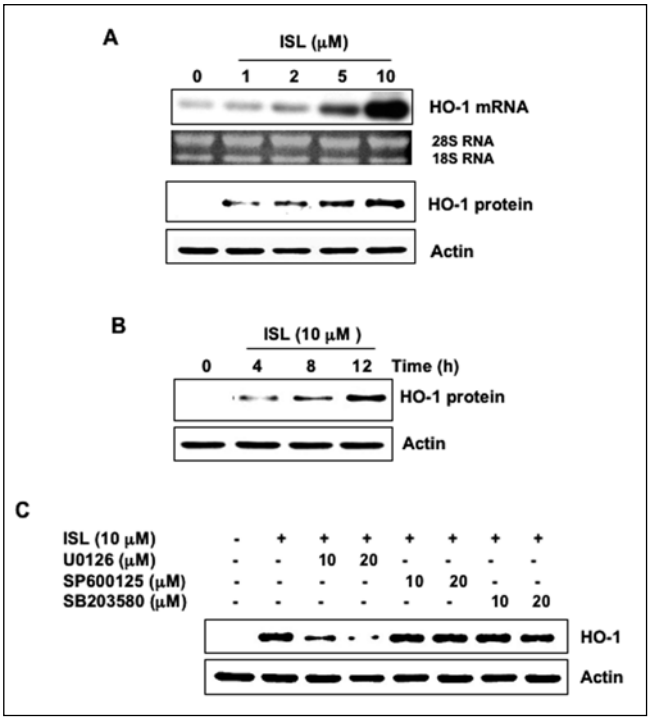

Fig. 4. ISL induces HO-1 expression. (**A**) Dose-dependent induction of HO-1 mRNA and protein by ISL. Cells were treated with the indicated concentrations of ISL for 12 h, and the expression of mRNA (*upper panel*) and protein (*lower panel*) were examined by northern and western blot analyses, respectively. Ethidium bromide staining of ribosomal RNA (18S and 28S) and actin are shown as the loading controls. (**B**) Time-dependent induction of HO-1 protein by ISL. Cells were treated with ISL (10 μM) for the indicated periods (4, 8, or 12 h), and the levels of HO-1 protein in cells were analyzed by western blotting. Actin is shown as the loading control. (**C**) Effect of MAPK inhibition on ISL-induced HO-1 expression in RAW 264.7 macrophages. Cells were pretreated with the indicated concentrations of U0126, SP600125 or SB203580 for 1 h and then treated with 10 μM ISL for 12 h. The levels of HO-1 protein were detected by western blotting.

HO-1 mediates the suppressive effect of ISL on LPS-stimulated pro-inflammatory mediators production

To assess the potential role of HO-1 in inhibiting LPS-stimulated NO and TNF-α production, macrophages were pretreated with 10 μM ISL for 4 h in the presence or absence of SnPP, a competitive inhibitor of HO-1 [18], before the LPS stimulation. As shown in Fig. 5, SnPP treatment significantly attenuated their inhibitory activity of ISL on LPS-stimulated NO and TNF-α production.

Discussion

Liquiritigenin (LIQ) and isoliquiritigenin (ISL) are structurally related major flavonoid found in *D. odorifera*. In the present study, we found that ISL suppressed the LPS-induced production of NO and abolished the LPS-induced expression of both iNOS mRNA and protein in a parallel, concentration-dependent manner. However, these inhibitory effects were not observed with LIQ. Because ISL is a flavonoid with chalcone structure (2,4,4´-trihydroxychalcone), this result support a previous study demonstrating that chalcone derivatives show potent anti-inflammatory activity and are lead compounds for novel anti-inflammatory drugs [19, 20]. In addition to pro-inflammatory enzyme, iNOS, inhibition by ISL, this compound also inhibited LPS-induced secretion of pro-inflammatory cytokines including IL-1β and TNF-α.

ISL, an active component present in plants like *Glycyrrhiza* and *Dalbergia*, has been evaluated for its various biological activities including anti-platelet aggregation [21] and anti-allergic activities [22]. Recently, ISL isolated from licorice (*Glycyrrhiza uralensis*) has been reported to exert anti-

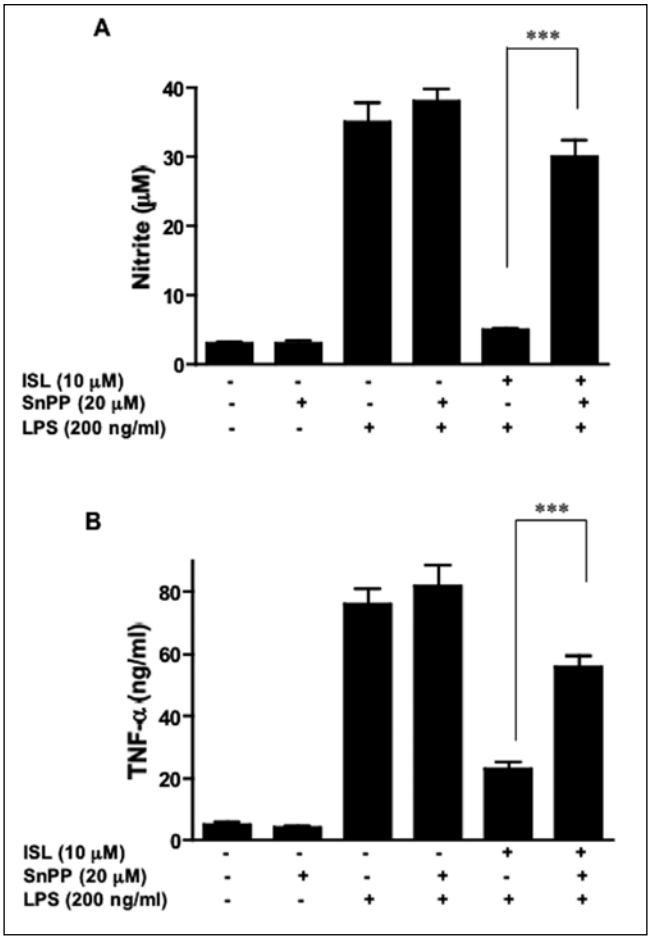

Fig. 5. Inhibition of HO-1 activity suppresses the inhibitory effects of ISL on NO (**A**) and TNF-α (**B**) production in LPS-stimulated RAW 264.7 macrophages. Cells were pretreated for 4 h with 10 μM ISL in the presence or absence of 20 μM SnPP, and then stimulated for 16 h with LPS. The productions of NO and TNF-α were determined as described in Materials and methods. Each column shows the mean ± S.D. of three independent experiments. $^{***}p<0.001$ *vs*. LPS + ISL, as analyzed by Student's *t*-test.

inflammatory properties [11, 12]. However, the underlying mechanisms that could explain the anti-inflammatory effect of ISL remain obscure.

In the present study, we found that ISL induces HO-1 expression in RAW264.7 macrophages in time- and concentration-dependent manners. The induction of HO-1 is widely recognized as an effective cellular strategy to counteract a variety of cellular damage and inflammation [2, 3]. Moreover, recent data reveal that the anti-inflammatory and protective effects of chalcones are strongly associated with and depend on the expression of HO-1 [19, 20, 23, 24]. Previously, we also have shown that 2', 4', 6'-tris(methoxymethoxy) chalcone, a synthetic chalcone derivative, display s potent anti-inflammatory effect in murine macrophages [17] and murine colitis model [25] via HO-1-dependent pathway. Thus, we suggest that induction of HO-1 by ISL could partly underlie the beneficial effect exerted by chalcone.

Modulation of gene transcription is principal mechanism for the induction of HO-1 protein expression. Several pathways have been implicated in transmitting the extracellular signals to the nuclei for HO-1 gene expression. In general, HO-1 gene expression can be induced through signaling pathway such as the MAPKs [26–28].

Here, by using different MAPK inhibitors, our result show clearly that specific inhibitor of ERK1/2 MAPK, U0126, abolished the induction effect of ISL on HO-1 expression. Thus, this data support the assertions that activation of ERK1/2 MAPK is involved in the HO-1 induction by ISL.

In addition, we confirmed whether the inhibition of LPS-induced NO and TNF-α production by ISL was related to its ability to induce HO-1 expression in RAW 264.7 macrophages. Our results show that inhibitor of HO-1, SnPP, decrease the suppressive effect of ISL on LPS-induced NO and TNF-α production. This data suggest that expression of the HO-1 participate in the inhibitory mechanism of ISL on LPS-stimulated pro-inflammatory mediators production. Moreover, these data demonstrate that HO-1 might mediate the anti-inflammatory effects of ISL in RAW 264.7 macrophages.

In summary, we have demonstrated that ISL, from *D. odorifera*, induces HO-1 expression by activation of ERK1/2 MAPK and ISL-induced HO-1 expression is considerably associated with the LPS-induced NO and TNF-α production in murine macrophages. Thus, induction of HO-1 by ISL may be important in the understanding of a novel mechanism for the anti-inflammatory activity of ISL.

Acknowledgements: This work was supported by grant No. RTI05-03-02 from the Regional Technology Innovation Program of the Ministry of Commerce Industry and Energy (MOCIE).

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