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Inhibitory Effects of Licochalcone A and Isoliquiritigenin on Monocyte Adhesion to TNF-α-activated Endothelium*

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Numerous natural herbal compounds have been reported to inhibit adhesion and migration of leukocytes to the site of inflammation. Licorice extracts, which have been widely used in traditional Chinese medicinal preparation, possess various pharmacological effects. Isoliquiritigenin, a biogenetic precursor of flavonoids with various pharmacological effects, is a natural pigment present in licorice. We attempted to explore whether licorice extracts and isoliquiritigenin mitigate monocyte adhesion to tumor necrosis factor-α (TNF-α)-activated human umbilical vein endothelial cells (HUVEC). In addition, it was tested whether the inhibition of monocyte adhesion to the activated HUVEC accompanied a reduction in vascular cell adhesion molecule-1 expression (VCAM-1). Dry-roasted licorice extracts in methylene chloride but not in ethanol markedly interfered with THP-1 monocyte adhesion to TNF-α-activated endothelial cells. Licochalcone A compound isolated from licorice extract in methylene chloride appeared to modestly inhibit the interaction of THP-1 monocytes and activated endothelium. In addition, isoliquiritigenin abolished the monocyte adhesion with attenuating VCAM-1 protein expression on HUVEC induced by TNF-α. These results demonstrated that non-polar components from dry-roasted licorice extracts containing licochalcone A as well as isoliquiritigenin were active in blocking monocyte adhesion to cytokine-activated endothelium, which appeared to be mediated most likely through the inhibition of VCAM-1 expression on HUVEC. Therefore, licorice may hamper initial inflammatory events on the vascular endothelium involving induction of endothelial cell adhesion molecules.

Key words : *Glycyrrhizae radix* extract, Isoliquiritigenin, Licochalcone A, Tumor necrosis factor-α, Vascular cell adhesion molecule-1

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INTRODUCTION

The inflammatory cascades include the interaction of pro-inflammatory and anti-inflammatory cytokines within the arterial wall. Elevated levels of a particular cytokine such as tumor necrosis factor-α (TNF-α) affect the arterial wall.[1-3] The expression of cell adhesion molecules is a common feature in inflammatory environments and occurs in the early development of atherosclerosis.[4] CAM proteins have been observed in atherosclerotic lesions and at sites pre-disposed to lesion formation in human coronary atherosclerotic plaques.[5,6] It has been documented that some flavonoids act as anti-inflammatory agents to inhibit the expression of cell adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1).[7-11]

Plants produce a variety of antioxidants against molecular damage from reactive oxygen species, and phenolic compounds compose the major class of plant-derived antioxidants. Licorice root contains flavonoids from the flavan and chalcone subclasses.[12] Licorice root derived from the plant *Glycyrrhiza glabra* or *Glycyrrhiza Radix* is one of the most ancient medical plants which have long been used in the traditional Chinese, Tibetan and Indian medicines for the treatment of pulmonary diseases and inflammatory processes. Licorice root extract was added to medicines for the improvement of tastes and the intensification of their action.[13-15] However, the precise mechanisms by which licorice root may reduce inflammatory adversity are not completely defined.

Among several flavonoids that were isolated and purified from licorice root extract, isoliquiritigenin, a flavonoid with a chalcone structure (Fig. 1), is reported

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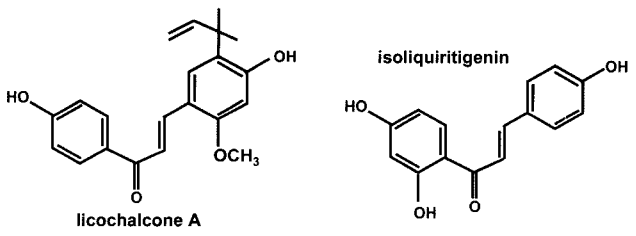

Fig. 1 Chemical structures of licochalcone A and isoliquiritigenin

to have vasorelaxant effect, anti-platelet action, anti-allergic activity, and anti-atherosclerotic effect.[15-18] As LDL oxidation is a key event in the formation of the early atherosclerotic lesion, the use of these natural antioxidants may be proven beneficial to attenuate atherosclerosis.[18] Licochalcone A is another hydroxy chalcone compound isolated from licorice (Fig. 1), which has anti-tumor activity in various malignant human cell lines, anti-bacterial and anti-parasitic activity, and immunomodulatory effects.[19-21]

This study examined novel effects of dry-roasted *Glycyrrhizae radix* licorice extract on monocyte adhesion to pro-inflammatory cytokine-activated human umbilical vein cells (HUVEC), and further investigated the anti-inflammatory mechanisms of the licorice components, licochalcone A and isoliquiritigenin, with respect to VCAM-1 expression.

MATERIALS AND METHODS

1. Materials

M199 medium chemicals, RPMI 1640 medium chemicals, isoliquiritigenin and 3-(4, 5-dimetylthiazol-yl)-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich Co. (St. Louis, MO), as were all other reagents, unless specifically stated elsewhere. Fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, bovine brain extract, human epidermal growth factor and hydrocortisone were purchased from Cambrex Corporation (East Rutherford, NJ).

2. Preparation of Crude Ethanol and Methylene Chloride Extracts of Glycyrrhiza Inflata

The dry-roasted roots of plant *Glycyrrhiza inflata* Bat., a particular plant species of licorice, were purchased from Dea-Guang Ltd. (Seoul, Korea). The dry-roasted roots of licorice (0.5 kg) were extracted three times (3×30 min), each with 3 L ethanol–water (95:5, vol/vol) by sonication using a powersonic 420 ultrasonic cleaning instrument (Hwashin Instrument Co., Seoul, Korea). The extracts were combined and then evaporated *in vacuo* to yield 125 g of residue (ethanol extract). A part of the residue (50.0 g) was extracted with methylene chloride (CH_2Cl_2) at room temperature to give 6.5 g of CH_2Cl_2-soluble fraction (methylene chloride fraction).

3. Isolation and Identification of Licochalcone A from *Glycyrrhiza Inflata*

A part of fraction of methylene chloride (6.2 g) was fractionated by silica gel SiO_2 column chromatography eluted with *n*-hexane-ethyl acetate (100:0–0:100, vol/vol). The major fraction (1.2 g) was separated by Recycle HPLC (JAI analytical company, Japan, SiO_2, *n*-hexane-ethyl acetate, 3:2, vol/vol) to give 80 mg of compound X. The compound X was identified as licochalcone A on the basis of their UV, ^1H NMR spectra and ^{13}C NMR spectra, and electron ionization MS analyses. Licochalcone A was a major component containing ≈11.0 mg/g in the fraction of methylene chloride of *Glycyrrhiza inflate* licorice, whereas another licorice component isoliquiritigenin was recognized below the limit of detection of 3 ng on column. Isoliquiritigenin was not a major component in non-polar methylene chloride extracts.

Dry-roasted *Glycyrrhiza inflata* extract, licochalcone A, and isoliquiritigenin (Sigma-Aldrich Co., Fig. 1) were dissolved in dimethyl sulfoxide for culturing with cells; the final culture concentration of dimethyl sulfoxide was ≤0.5%.

4. Preparation and Culture of Human Endothelial Cells

HUVEC were isolated from umbilical cords using collagenase as described elsewhere.[22] Cultures were maintained at 37 ℃ in humidified atmospheres of 5% CO_2 in the air. Cells were cultured in 25 mM HEPES-buffered M199 containing 10% FBS, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin supplemented with 0.75 mg/mL human epidermal growth factor and 0.075 mg/mL hydrocortisone. Cells were passaged at confluence and used within 10 passages. Endothelial cells were confirmed by their cobblestone morphology and uptake of fluorescent 1.1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labeled acetylated LDL.[23]

HUVEC were plated at 90-95% confluence in all experiments. Cells were incubated overnight with methylene chloride fraction from dry-roasted licorice extract or with licorice components of licochalcone A and isoliquiritigenin in the absence and presence of 10 ng/mL TNF-α (Roche Molecular Biochemicals, Mannheim, Germany). In experiments for the TNF-α-induced VCAM-1 expression, HUVEC were incubated with 25 μM licochalcone A and isoliquiritigenin of licorice components prior to the exposure to TNF-α.

5. Cell Adhesion Assay

Human monocytic leukemic cell line THP-1 was obtained from American Type Culture Collection (Rockville, MD). HUVEC were grown in 25 mM HEPES-buffered at density of 1.0×10^5 cells on 4-well glass chamber slides. Cells were pre-treated with methylene chloride fraction from dry-roasted licorice extract or with licorice components of licochalcone A and isoliquiritigenin prior to the 6 h exposure to 10 ng/mL TNF-α.[24] THP-1 cells were labeled with 5 μM calcein AM (Molecular Probes Inc., Eugene, OR) in RPMI 1640 medium containing 10% FBS. In the co-culture system, the labeled THP-1 (5.0×10^5) were seeded onto confluent monolayer of HUVEC treated with methylene chloride licorice fraction, of licochalcone A, or isoliquiritigenin and/or TNF-α and were incubated for 2 h. The co-cultured cells were thoroughly washed and images were obtained at 485 nm excitation and 538 nm emission using a SPOT II digital camera-attached fluorescence microscope with Spot II data acquisition software (Diagnostic Instrument, Livingston, Scotland).

6. Western Blot Analysis

Whole cell extracts were prepared from HUVEC in 1 M Tris-HCl (pH 6.8) lysis buffer containing 10% SDS, 1% β-glycerophosphate, 0.1 M Na_3VO_4, 0.5 M NaF and protease inhibitor cocktail. Cell lysates containing equal amounts of total protein were fractionated by electrophoresis on 8% SDS-PAGE gels and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by soaking the membrane in TBS-T buffer [0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl, and 0.1% Tween 20] containing 5% nonfat dry milk for 3 h. The membrane was incubated for 3 h with a primary antibody [polyclonal rabbit anti-human VCAM-1 antibody (1:1,000), Santa Cruz Biotechnologies, Santa Cruz, CA]. After three washes with TBS-T buffer, the membrane was then incubated for 1 h with goat anti-rabbit IgG horseradish peroxidase (1:10,000, Jackson ImmunoResearch Laboratories, West Grove, PA). The level of VCAM-1 protein was determined by using Supersignal West Pico chemiluminescence detection reagents (Pierce Biotech. Inc., Rockford, IL) and Konica X-ray film (Konica Co., Tokyo, Japan). Incubation with monoclonal mouse β-actin antibody (1:5,000) was also performed for the comparative control.

7. Data Analysis

The results were presented as mean±SEM. Statistical analyses were conducted using Statistical Analysis Systems statistical software package version 6.12 (SAS Institute Inc., Cary, NC). The ANOVA was followed by Duncan's test for multiple comparisons. Differences were considered significant at $P<0.05$.

RESULTS AND DISCUSSION

1. Inhibition of TNF-α-induced Monocyte Adhesion by Dry-roasted Licorice

It has been shown that flavonoids block monocyte adhesion and transmigration to the activated endothelium.[11] The present study tested the hypothesis that licorice components containing flavonoids with a chalcone structure inhibit mononuclear leukocyte recruitment on the TNF-α-induced vascular endothelium. The *in vitro* adhesion assay of monocytes to HUVEC using a calcein-AM staining technique supported this hypothesis. A small number of monocytes were adhered to quiescent HUVEC free of TNF-α (Fig. 2). There was heavy staining on the TNF-α-alone-exposed HUVEC, indicative of a marked increase in the THP-1 adherence to the activated HUVEC. However, the treatment of TNF-α-exposed cells with 25 μg/mL methylene chloride fraction markedly inhibited monocyte adherence (Fig. 2). In contrast, the ethanol extract of dry-roasted *Glycyrrhiza inflata* licorice did not have such effect. Accordingly, non-polar components of dry-roasted licorice extracts appeared to be responsible for the mononuclear leukocyte recruitment on the cytokine-stimulated vascular endothelium.

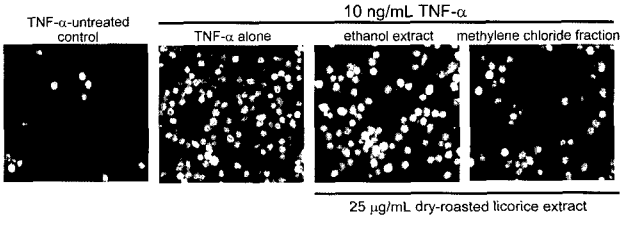

Fig. 2 Inhibition of THP-1 monocyte adhesion to TNF-α-activated HUVEC by methylene chloride fraction of dry-roasted licorice. HUVEC were pre-treated with 25 μg/mL dry-roasted licorice extract overnight and then activated with 10 ng/mL TNF-α for 6 h. Endothelial cells were co-cultured with calcein AM-labeled THP-1 monocytes for 2 h. Microphotographs (3 independent experiments) were obtained using a fluorescence microscopy. Magnification ×200

The methylene chloride fraction was further fractionated and the major component was separated and confirmed as licochalcone A. This study examined the anti-inflammatory effect of the purified licochalcone A on

THP-1 cell adhesion to TNF-α-exposed HUVEC monolayer. When HUVEC were treated with 25 μM licochalcone A in the presence of 10 ng/mL TNF-α, the staining was strikingly attenuated, suggesting that licochalcone A- and TNF-α-exposed cells revealed a substantial inhibition of the TNF-α-induced THP-1 adhesion (Fig. 3).

Western blot analysis was used to address whether the methylene chloride fraction and licochalcone A obtained from dry-roasted licorice block the TNF-α-activated expression of VCAM-1 (Fig. 4). As expected, there was relatively weak expression of VCAM-1 in TNF-α-untreated quiescent cells. Expression of VCAM-1 protein was markedly enhanced in TNF-α-stimulated cells over the quiescent cells. TNF-α-exposed cells treated with 25 μg/mL methylene chloride fraction proved near-full inhibition of expression of VCAM-1, while the ethanol extract at 25 μg/mL concentration did not inhibit their expression (Fig. 4A). In addition, licochalcone A- and TNF-α-exposed cells exhibited a marked inhibition of VCAM-1 expression in a dose-dependent manner (Fig. 4B). TNF-α-induced VCAM-1 expression was attenuated by treatment with ≥10 μM licochalcone A and this expression was fully abolished at ≥ 20 μM licochalcone A (Fig. 4B). The methylene chloride fraction at 25 μg/mL comprised 0.81 μM licochalcone A. When compared to the blocking effect of 20 μM licochalcone A on TNF-α-induced VCAM-1 expression, the methylene chloride fraction at concentrations 25 times as low revealed comparable expression blockade. Thus, other components in the methylene chloride fraction appeared to synergically abolish the expression.

Since atherosclerosis is a chronic inflammatory disease associated with increased oxidative stress in the vascular endothelium, it would be conceivable that the anti-atherogenic effect of licorice root is mainly due to anti-oxidative properties. Polyphenolics are effective scavengers of reactive oxygen species involved in the regulation of adhesion molecule expression.[25] This study did not examine the antioxidant activity of licorice components. However, it is speculated that some methylene chloride components inhibiting monocyte adherence to the activated HUVEC act as potent antioxidant agents. A recent *in vivo* study has shown that licochalcone A and licorisoflavan A with anti-nephritis activity showed weak scavenging activity against superoxide anion radical in mice with glomerular disease.[26] Accordingly, the anti-inflammatory activity of licochalcone A inhibiting monocyte adhesion is unlikely due to its antioxidant activity.

Licochalcone A has anti-tumor activity in various malignant human cell lines, and anti-bacterial and anti-parasitic activity.[19,20] In addition, licochalcone A has been shown to be a potent echinocytogenic agent that modifies the erythrocyte membrane.[27] This oxygenated chalcone has been shown to have immunomodulatory effects inhibiting the proliferation of lymphocytes and modulating the production of pro- and anti-inflammatory cytokines from monocytes and T cells.[21] The present study showed novel activity of licochalcone A inhibiting monocyte adhesion to pro-inflammatory cytokine-activated endothelium fundamentally occurred in the initial events of inflammatory atherosclerosis.

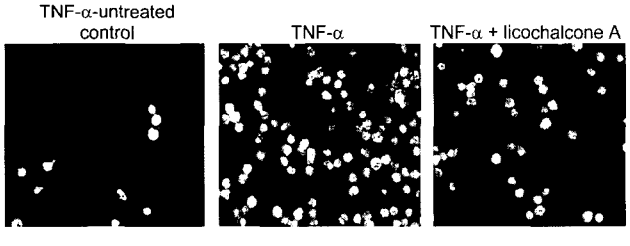

Fig. 3 Inhibitory effects of licochalcone A on THP-1 adherence to TNF-α-activated HUVEC. HUVEC were incubated in the absence and presence of 25 μM licochalcone A purified in methylene chloride fraction and then activated with 10 ng/mL TNF-α for 6 h. Endothelial cells were co-cultured with calcein AM-labeled THP-1 monocytes for 2 h. Microphotographs (3 separate experiments) were obtained using a fluorescence microscopy. Magnification ×200

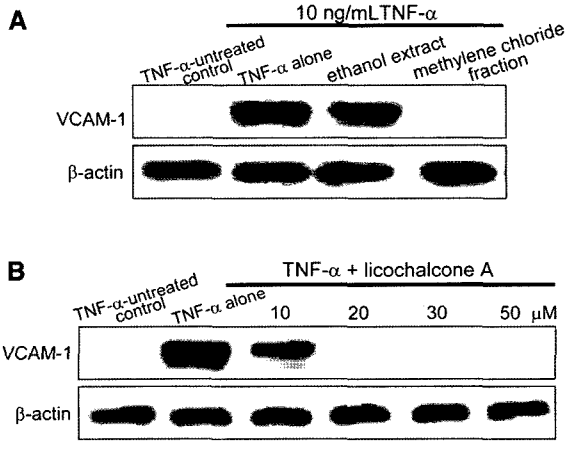

Fig. 4 Western blot data showing effects of licorice extracts on (A) and dose response of licochalcone A to (B) the expression level of VCAM-1 in TNF-α-stimulated HUVEC. After culturing HUVEC with each extract of 25 μg/mL or 10-50 μM licochalcone A, and 10 ng/mL TNF-α, cell extracts were subjected to 8% SDS-PAGE and Western blot analysis with a primary antibody against VCAM-1. β-Actin protein was used as an internal control.

2. Inhibition of TNF-α-induced Expression of VCAM-1 by Isoliquiritigenin

This study also attempted to explore whether other components of licorice are capable of inhibiting monocyte adherence to activated endothelium. Isoliquiritigenin, another chalcone-type component present in licorice, was tested in TNF-α-treated cells (Fig. 5). There was weak staining in isoliquiritigenin- and TNF-α-treated HUVEC, suggesting that isoliquiritigenin mitigated the monocyte adhesion to endothelial cells augmented by TNF-α. Western blot analysis was used to address that the inhibition of THP-1 adhesion by isoliquiritigenin may be as a result of down-regulation of TNF-α-activated VCAM-1 expression (Fig. 6). As expected, TNF-α markedly increased the VCAM-1 expression and isoliquiritigenin abolished the enhanced VCAM-1 expression. When isoliquiritigenin was added in concentrations between 1 and 50 μM VCAM-1 induction by TNF-α, TNF-α-induced VCAM-1 expression was decreased in a dose-dependent manner with inhibitory dose requiring only with ≥10 μM (Fig. 6). Thus, to achieve the full inhibitory effect of isoliquiritigenin in the VCAM-1 expression model, doses of ≥25 μM were required.

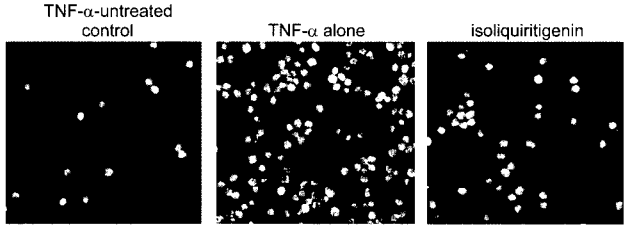

Fig. 5 Microphotographs showing blockade of enhanced THP-1 monocyte adhesion to isoliquiritigenin- and TNF-α-exposed HUVEC. HUVEC were pre-treated with 25 μM isoliquiritigenin and then activated with 10 ng/mL TNF-α for 6 h. Endothelial cells were co-cultured with calcein AM-labeled THP-1 monocytes for 2 h. Microphotographs (3 independent experiments) were obtained using a fluorescence microscopy. Magnification ×200

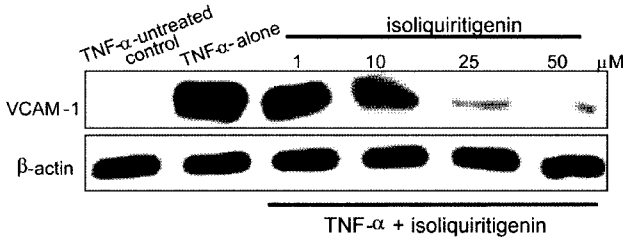

Fig. 6 Inhibitory dose responses of isoliquiritigenin to VCAM-1 induction in TNF-α-stimulated HUVEC. After culturing HUVEC with 1-50 μM isoliquiritigenin and 10 ng/mL TNF-α, cell extracts were subjected to 8% SDS-PAGE and western blot analysis with VCAM-1 primary antibody. β-Actin protein was used as an internal control.

Isoliquiritigenin has been shown to have vasorelaxant effect, anti-platelet action, anti-allergic activity, and anti-atherosclerotic effect.[15-18] Isoliquiritigenin at a concentration of 30 μM highly inhibited 2,2'-azobis (2-amidinopropane) dihydrochloride-induced LDL oxidation, suggesting that this compound is a very potent antioxidant toward LDL oxidation.[28] As LDL oxidation is a key event in the formation of early atherosclerotic lesion, the use of this natural antioxidant from the plant licorice root may be proven beneficial to attenuate atherosclerosis. On the other hand, it was shown that isoliquiritigenin was capable of lowering the levels of ICAM-1 and VCAM-1, both of which are crucial in the regulation of immune response and inflammation, on murine endothelial cells and mouse myeloid leukemia cells.[29] The structure-activity relationships study on chalcone derivatives suggested that the inhibitory activity of these chalcones in the expression of cell adhesion molecules is attributable to the 4-hydroxy group as well as the possible co-planarity between the phenyl ring and the adjacent conjugated ketone.[29]

In summary, the current study has demonstrated that nonpolar licorice components were capable of preventing the early processes of atherosclerosis involving inducible VCAM-1 expression. Licochalcone A isolated from methylene chloride fraction of dry-roasted licorice blocked monocyte adhesion on the TNF-α-activated endothelium most likely via the down-regulation of VCAM-1 expression. In addition, isoliquiritigenin had similar effects to licochalcone A. This observation might have clinical implications for therapeutic strategies preventing and attenuating inflammatory diseases. Although definite mechanisms underlying the protection of licorice components against early atherogenic process are not fully understood in this study, their selective inhibitory effects for the VCAM-1 expression may argue for the major target of anti-atherogenic action of licorice root.

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