Blockade of Cytokine-Induced Endothelial Cell Adhesion Molecule Expression by Licorice Isoliquiritigenin Through NF-κB Signal Disruption

HYANG-MI KWON,* YEAN-JUNG CHOI,* JUNG-SUK CHOI,* SANG-WOOK KANG,* JI-YOUNG BAE,* IL-JUN KANG,* JONG-GAB JUN,† SANG-SOO LEE,‡ SOON SUNG LIM,§ AND YOUNG-HEE KANG§,[1]

*Department of Food and Nutrition and Institute of Korea Nutrition Research, Hallym University; †Department of Chemistry, Hallym University, Chuncheon; ‡Department of Orthopaedic Surgery, Chunchon Sacred Heart Hospital, Chuncheon; and §Regional Research Center, Hallym University, Chuncheon, Republic of Korea

Numerous polyphenolic compounds have been found to inhibit adhesion and migration of leukocytes to sites of inflammation that are partly regulated by the expression of cell adhesion molecules (CAM) such as vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and platelet endothelial cell adhesion molecule-1 (PECAM-1). Licorice root extracts have been used in traditional Chinese, Tibetan, and Indian medicine for the treatment of pulmonary diseases and inflammatory processes. Expression of CAM proteins was examined in human umbilical vein endothelial cells (HUVEC) treated with a licorice component (isoliquiritigenin, 18β-glycyrrhetinic acid, glycyrrhizin, formononetin, or ononin) and exposed to TNF-α. The involvement of NF-κB in the transcriptional control of CAM proteins was assessed by degradation of IκBα and nuclear translocation of NF-κB using Western blotting techniques and immunocytochemical staining. At nontoxic ≥10 μ*M*, isoliquiritigenin blocked the induction of VCAM-1 and E-selectin on activated HUVEC and markedly interfered with THP-1 monocyte adhesion to TNF-α-activated endothelial cells. Isoliquiritigenin abolished TNF-α-induced mRNA accumulation of VCAM-1 and E-selectin. Additionally, immunocytochemical staining revealed that isoliquiritigenin attenuated PECAM-1 expression induced by TNF-α. In contrast, other components recognized in licorice, 18β-glycyrrhetinic acid, glycyrrhizin, formononetin, and ononin did not downregulate the expression of VCAM-1 and/or PECAM-1 activated by TNF-α, implying that these components are inactive in modulating adhesion of leukocytes to stimulated endothelial cells. Isoliquiritigenin downregulated CAM proteins in TNF-α-activated HUVEC at the transcriptional levels by blocking degradation of IκBα and nuclear translocation of NF-κB. These results demonstrate that the induction blockade of VCAM-1 and E-selectin by isoliquiritigenin was directly mediated by its interference with the CAM mRNA transcription through NF-κB-dependent mechanisms under inflammatory conditions. Exp Biol Med 232:235–245, 2007

Key words: atherosclerosis; cell adhesion molecules; isoliquiritigenin; nuclear factor-κB; tumor necrosis factor-α

Introduction

Overexpression of cell adhesion molecules (CAM), such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), endothelial-leukocyte adhesion molecule-1 (E-selectin), and platelet endothelial cell adhesion molecule-1 (PECAM-1), is a common feature in inflammatory environments, which are one of the first events in the development of atherosclerosis (1). CAM proteins have been observed in atherosclerotic lesions and at sites predisposed to lesion formation in human coronary atherosclerotic plaques (2, 3). These CAM proteins are induced by pro-inflammatory cytokines such as interleukin-1 and tumor necrosis factor-α (TNF-α), growth factors, platelet activator, and chemotactic factors in the blood (4–6). It has been previously shown that some flavonoids act as anti-inflammatory agents to inhibit expression of CAM and matrix proteases (7–10). In our previous study (8), natural flavones, apigenin, and luteolin

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[1] To whom correspondence should be addressed at Department of Food and Nutrition, Hallym University, Chuncheon, Kangwon-do, 200–702, Republic of Korea. E-mail: yhkang@hallym.ac.kr

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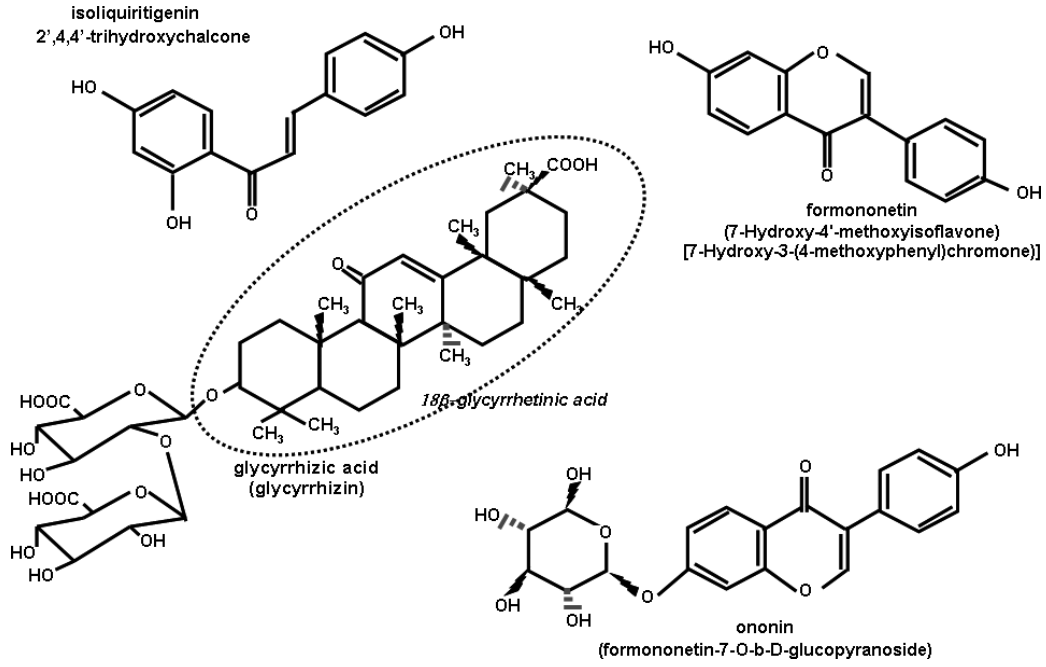

Figure 1. Chemical structures of isoliquirtigenin, 18β-glycyrrhetinic acid, glycyrrhizin, formononetin, and ononin.

inhibited TNF-α-induced CAM proteins, VCAM-1, ICAM-1, and E-selectin at transcriptional levels.

Licorice root, derived from the plant *Glycyrrhiza glabra* or *G. radix*, one of the most ancient medical plants, has long been used in traditional Chinese, Tibetan, and Indian medicine for the treatment of pulmonary diseases and inflammatory processes (11). Licorice root extracts were added to medicines to improve their taste as a sweetener or a spice and to intensify their action (12). Licorice root extract contains many different subclasses of flavonoids, flavans, and chalcones (13–16). Among several flavonoids isolated from licorice root extract, the isoflavan glabridin exhibits estrogen-like activity in human osteoblasts and skeletal tissues (16) and improves survival of mice in an experimental model of septic shock (15). Glycyrrhizin, a major bioactive triterpene glycoside of licorice root extracts, and its aglycone 18β-glycyrrhetinic acid possess a wide range of pharmacological properties (11, 17, 18). In addition, isoliquiritigenin, a flavonoid with a chalcone structure in licorice root extract, is regarded as a promising cancer chemopreventive agent (19, 20).

The present study assessed a novel anti-inflammatory property of licorice root components (Fig. 1) with respect to the monocyte trafficking on the activated endothelium. Accordingly, it was determined whether cytokine-induced monocyte adhesion could be alleviated by isoliquiritigenin, 18β-glycyrrhetinic acid, glycyrrhizin, formononetin, and ononin in human umbilical vein endothelial cells (HUVEC). We observed that isoliquiritigenin could act as an anti-inflammatory agent by downregulating CAM expression in the leukocyte-endothelium interaction system activated by pro-inflammatory cytokines. The positive regulatory domains required for cytokine induction of CAM have been defined in their promoters. DNA binding studies reveal a requirement for nuclear factor-κB (NF-κB), a pivotal transcription factor in chronic inflammatory diseases (8, 10). This study further elucidated possible NF-κB-dependent mechanisms of isoliquiritigenin-mediated downregulation of CAM expression.

Materials and Methods

Materials. Isoliquiritigenin, glycyrrhizin, 18β-glycyrrhetinic acid, formononectin, ononin, M199 medium chemicals, RPMI 1640 medium chemicals, and 3-(4,5-dimetylthiazol-yl)-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemical (St. Louis, MO), as were all other reagents, unless otherwise stated. Collagenase was purchased from Worthington Biochemicals (Lakewood, NJ). 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). Human monocytic leukemic cell line THP-1 was obtained from American Type Culture Collection (Manassas, VA). TNF-α was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Antibodies against human VCAM-1, human β-actin, and human NF-κB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human E-selectin and PECAM-1 antibodies were obtained from R & D Systems (Minneapolis, MN). Human IκBα and phospho-IκBα antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG were obtained from Jackson ImmunoResearch Laboratories

(West Grove, PA). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG was provided from Sigma Chemical. Reverse transcriptase and *Taq* DNA polymerase were purchased from Promega (Madison, WI).

All licorice components were dissolved in dimethyl sulfoxide (DMSO) for culturing with cells (21), and the final culture concentration of DMSO was $\leq$0.5%.

Cell Culture. HUVEC were isolated from human umbilical cords using collagenase, as described elsewhere (22, 23). Cells were cultured in 25 m*M* HEPES-buffered M199 containing 10% FBS, 2 m*M* glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and growth supplements (0.9 mg/ml bovine brain extract, 0.75 mg/ml human epidermal growth factor, and 0.075 mg/ml hydrocortisone) at 37°C in a humidified atmosphere of 5% CO_2 in air. HUVEC were identified by their cobblestone morphology and uptake of acetylated LDL (24).

HUVEC were plated at 90%–95% confluence in all experiments. The concentrations of all licorice components used for 24-hr culture experiments were $\leq$50 μ*M*. It was tested whether during 6 hrs incubation in the presence of licorice components 10 ng/ml TNF-α affected HUVEC viability using MTT assay (25, 26). TNF-α/licorice component had no measurable effect on cell viability (data not shown). Accordingly, cells were pretreated overnight with $\leq$50 μ*M* of each licorice compound and exposed to 10 ng/ml TNF-α for 6 hrs. It was shown that TNF-α-activated HUVEC exhibited marked induction in VCAM-1 in a time-dependent manner, with peak expression at 6 hrs (23).

Western Blot Analysis. Western blot analysis was performed using whole cell extracts prepared from HUVEC, as previously described (23). Cell extracts were fractionated by electrophoresis on 8% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by soaking the membrane in a buffer (0.5 *M* Tris-HCl [pH 7.5], 1.5 *M* NaCl, and 1 mg/ml Tween 20) containing 5% nonfat milk. The membrane was incubated with polyclonal rabbit anti-human VCAM-1 (1:1000), or polyclonal goat anti-human E-selectin (1:500), polyclonal rabbit anti-human IκBα (1:500), or polyclonal rabbit anti-human phospho-IκBα (ser 32, 1:500). The membrane was then incubated with a goat anti-rabbit IgG (1:7500) or rabbit anti-goat IgG (1:5000) conjugated to horseradish peroxidase. The protein levels were 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 polyclonal rabbit anti-human β-actin antibody (1:1000) was performed for comparative control.

Immunocytochemistry. After HUVEC grown on 4-well chamber slides were thoroughly washed with PBS containing 0.05% Tween 20, cells were fixed with 4% ice-cold formaldehyde for 30 mins and treated for 2 mins with 0.1% Triton-X100 and 0.1% citric acid in PBS. For blocking any nonspecific binding, cells were incubated for 3 hrs with 1% bovine serum albumin (BSA).

In Situ Detection of PECAM-1 Expression. After washing fixed cells with PBS, polyclonal goat anti-human PECAM-1 antibody (1:50 dilution in PBS) in 0.1% BSA was sufficiently added to cells and incubated overnight at 4°C. Cells were incubated with Cyanine 3–OSu conjugate-anti-goat IgG (1:750 dilution; Rockland Co., Gilbertville, PA) as a secondary antibody. Fluorescent images were obtained by a fluorescence microscopy with an Olympus BX51 fluorescent microscope (Olympus, Tokyo, Japan) with differential interference contrast and reflected light fluorescence.

Cell Adhesion Assay. HUVEC were cultured at a density of 7.0×10^4 cells on a 4-well glass chamber slide containing 25 m*M* HEPES-buffered M199 with 10% FBS. THP-1 cells were grown in RPMI-1640 medium containing 10% FBS. HUVEC were pretreated with each test licorice component overnight prior to the 6 hrs exposure to 10 ng/ml TNF-α. THP-1 cells were labeled with 5 μ*M* calcein-AM (Molecular Probes Inc., Eugene, OR) for 30 mins. The labeled THP-1 (7.0×10^5) were seeded onto confluent HUVEC treated with isoliquiritigenin, 18β-glycyrrhetinic acid, formononectin, ononin, and/or TNF-α and incubated for 2 hrs. Co-cultured cells were washed, and the 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). For the adhesion quantification, the calcein-AM fluorescent intensity was measured at 485 nm excitation and 538 nm emission by a Fluoroskan ELISA plate reader (Labsystems Oy, Helsinki, Finland).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis. Following culture protocols, total RNA was isolated from HUVEC using a commercially available Trizol reagent kit (Invitrogen, Carlsbad, CA). The RNA (5 μg) was reversibly transcribed with 200 units of reverse transcriptase and 0.5 μg/μl oligo-$(dT)_{15}$ primer (Bioneer Co., Korea). The expressions of the mRNA transcripts of VCAM-1 (forward primer: 5′-ATGCCTGG-GAAGATGGTCGTGA-3′, reverse primer: 5′-TGGAGCTGGTAGACCC TCGCTG-3′), E-selectin (forward primer: 5′-ATCATCCTGCAACTTCACC-3′, reverse primer: 5′-ACACCTCACCAAACCCTTC-3′), and β-actin (forward primer: 5′-GACTACCTCATGAAG ATC-3′, reverse primer: 5′-GATCCACATCTGCTGGAA-3′) were evaluated by RT-PCR as previously described, with slight modification (23). The PCR was performed in 25 μl of 10 m*M* Tris-HCl (pH 9.0), 25 m*M* $MgCl_2$, 10 m*M* dNTP, 5 units of *Taq* DNA polymerase, and 10 μ*M* of each primer and terminated by heating at 94°C for 10 mins. After thermocycling and electrophoresis of the PCR products (25 μl) on 1 % agarose gel, the bands were visualized using a TFX-20M model-UV transilluminator (Vilber-Lourmat, France), and gel photographs were obtained. The absence of contaminants was routinely checked by the RT-PCR assay of negative control samples without a primer addition.

Nuclear Extract Preparation. Cytosolic protein fraction and nuclear protein extract were prepared by a detergent lysis procedure to assay the translocation of NF-κB (23, 27). HUVEC were lysed in a buffer of 20 m*M* HEPES (pH 7.9), 1 m*M* EDTA, 10 m*M* NaCl, 1 m*M* dithiothreitol, 1 mg/ml Nonidet P40, 0.4 m*M* phenylmethylsulfonyl fluoride, 0.01 ng/ml leupeptin, and 200 units aprotinin and incubated on ice for 10 mins. Proteins were extracted from nuclear pellets by incubation with a high-salt buffer containing 420 m*M* NaCl, 1 m*M* EDTA, 20 m*M* HEPES (pH 7.9), 25% glycerol, 1 m*M* dithiothreitol, 0.4 m*M* phenylmethylsulfonyl fluoride, 0.01 ng/ml leupeptin, and 200 units of aprotinin with vigorous shaking. The nuclear debris was pelleted by a brief centrifugation, and the supernatant was stored at −70°C.

NF-κB Localization. *Immunocytochemistry.* After washing fixed cells with PBS, rabbit polyclonal anti-human NF-κB (1:200) was added and incubated overnight at 4°C. Cells were washed with TBS and incubated with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:5000). Fluorescent images were obtained by a fluorescence microscopy with an Olympus BX51 fluorescent microscope.

Western Blot Analysis. For the determination of NF-κB localization, Western blot analysis was carried out with cytosolic and nuclear protein fractions using anti-human NF-κB primary antibody (1:1000). The Western blot analytical procedures were described above.

Data Analysis. The results are presented as mean ± SEM for each treatment group in each experiment. Statistical analyses were conducted using Statistical Analysis Systems statistical software package version 6.12 (SAS Institute Inc., Cary, NC). Significance was determined by one-way ANOVA followed by Duncan multiple range test for multiple comparisons. P values <0.05 were considered statistically significant.

Results

TNF-α-Induced CAM Protein Expression. Western blot analysis was used to address whether licorice components block the TNF-α-activated expression of VCAM-1 and E-selectin (Fig. 2A and 2B). There was weak expression of all three CAM in TNF-α-untreated quiescent cells ($P < 0.05$). Expression of these CAM proteins was significantly enhanced in TNF-α-treated cells. TNF-α-exposed cells treated with 50 μ*M* isoliquiritigenin proved significant inhibition of expression of VCAM-1 and E-selectin, whereas other components, including 18β-glycyrrhetinic acid and its glycoside glycyrrhizin, did not inhibit their expression. When isoliquiritigenin was added in concentrations between 1 and 50 μ*M*, VCAM-1 induction by TNF-α was decreased in a dose-dependent manner, with inhibitory doses being ≥1 μ*M* ($P < 0.05$); doses ≤10 μ*M* could be attainable *in vivo* (Fig. 2C). Thus, to achieve the near-complete inhibition of VCAM-1 expression by isoliquiritigenin in this model, micromolar doses of ≥10 μ*M* were required. To address the fact that isoliquiritigenin and 18β-glycyrrhetinic acid block the TNF-α-activated expression of PECAM-1 protein, PECAM-1 specific antibody was used for immunostaining assay. Figure 3 compares the effects of isoliquiritigenin on the expression level of PECAM-1 elevated by TNF-α. There was a slight staining in the untreated control, while a heavy PECAM-1 staining in TNF-α-alone-exposed cells was observed ($P < 0.05$), indicative of increased expression of PECAM-1 protein at single-cell level. The isoliquiritigenin- and TNF-α-exposed cells revealed significant inhibition of PECAM-1 expression. In contrast, the 18β-glycyrrhetinic acid-treated cells did not show any inhibitory effect on PECAM-1 expression enhanced by TNF-α.

Adhesion of THP-1 Monocytes to TNF-α-Activated Endothelial Cells. The observed inhibition of CAM expression by licorice components suggested that treatment of these components might inhibit mononuclear leukocyte recruitment on the TNF-α-induced vascular endothelium. *In vitro* adhesion assay of monocytes to HUVEC using a calcein-AM staining technique supported this notion. Few monocytes were adhered to unstimulated HUVEC free of TNF-α (Fig. 4). There was heavy staining on the TNF-α-alone-exposed HUVEC ($P < 0.05$), indicative of a marked increase in the THP-1 adherence to the activated HUVEC. However, the treatment of TNF-α-exposed cells with 50 μ*M* isoliquiritigenin inhibited monocyte adherence dramatically and significantly. In contrast, adding other components of 18β-glycyrrhetinic acid, formononetin, and ononin did not have such effect on HUVEC (Fig. 4). Accordingly, the Western blot data and immunostaining results (Figs. 2 and 3) supported the *in vitro* adhesion results (Fig. 4). Consistently, the THP-1 adhesion was dose-dependently attenuated in TNF-α-exposed cells treated with ≥1 μ*M* isoliquiritigenin and significantly inhibited at ≥10 μ*M* (Fig. 5).

TNF-α-Induced CAM Transcription. There were weak signals for the basal mRNA expression of VCAM-1 and E-selectin in quiescent cells (Fig. 6). In contrast, their CAM mRNA was greatly increased in TNF-α-stimulated HUVEC. However, RT-PCR data showed that the mRNA levels of VCAM-1 and E-selectin in isoliquiritigenin-treated cells were relatively low (Fig. 6). The PECAM-1 mRNA level was also found to be greatly downregulated in cells exposed to isoliquiritigenin (data not shown). These observations were consistent with a substantial attenuation of expression of CAM proteins by isoliquiritigenin (Fig. 2). These results imply that isoliquiritigenin inhibits CAM expression by directly modulating gene transcription.

Inhibition of TNF-α-Induced IκBα Degradation by Isoliquiritigenin. Western blot analysis revealed that TNF-α-activated HUVEC exhibited marked decrease in IκB level and increase in phospho-IκBα level in a time-dependent manner, demonstrating maximum degradation of IκBα from NF-κB complex at 1 to 2 hrs after incubation

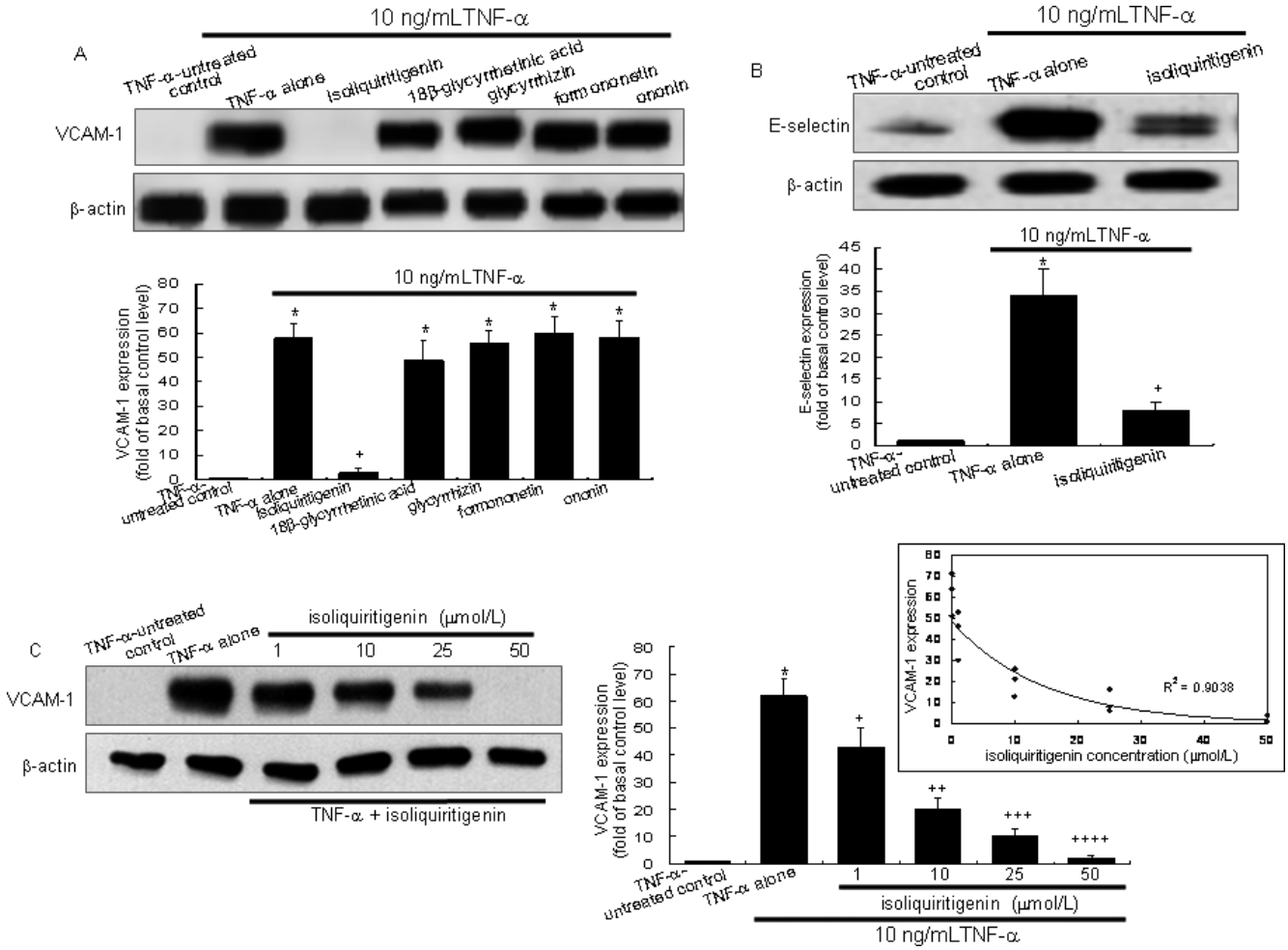

Figure 2. Western blot analyses showing effects of isoliquiritigenin, 18β-glycyrrhetinic acid, glycyrrhizin, formononetin, and ononin on expression levels of VCAM-1 (A) and of isoliquiritigenin on E-selectin (B) in TNF-α-stimulated HUVEC. Cells were incubated with 50 μ*M* isoliquiritigenin, 18β-glycyrrhetinic acid, glycyrrhizin, formononetin, or ononin overnight and exposed to 10 ng/ml TNF-α for 6 hrs. After HUVEC culture protocols, cell extracts were subjected to 8% SDS-PAGE and Western blot analysis with a primary antibody against VCAM-1 or E-selectin (3 separate experiments). Inhibitory dose response of the induction of VCAM-1 to isoliquiritigenin in TNF-α-stimulated HUVEC was shown (C). After HUVEC culture protocols with 1–50 μ*M* isoliquiritigenin and 10 ng/ml TNF-α, cell extracts were subjected to Western blot analysis (three separate experiments). β-Actin protein was used as an internal control. The box insert in panel C shows a nonlinear inverse correlation between isoliquiritigenin concentrations in culture and VCAM-1 expression. Nonlinear curve fit ($R^2 = 0.9038$) was a logistic dose-response relationship with the following coefficients: $y = 48.419e^{-0.0688x}$. $^{*}P < 0.05$, relative to TNF-α-untreated control incubation. $^{+}P < 0.05$, relative to TNF-α-alone incubation; $^{++}P < 0.05$, relative to incubation with 1 μ*M* isoliquiritigenin; $^{+++}P < 0.05$, relative to incubation with 10 μ*M* isoliquiritigenin; $^{++++}P < 0.05$, relative to incubation with 25 μ*M* isoliquiritigenin.

with TNF-α (data not shown). This study attempted to address whether licorice components block the TNF-α-induced IκBα phosphorylation to activate NF-κB. There were relatively low levels of phospho-IκBα in TNF-α-untreated quiescent cells. The phospho-IκBα levels were markedly enhanced in TNF-α–stimulated cells. When isoliquiritigenin was added in concentrations between 1 and 50 μ*M*, IκBα phosphorylation by TNF-α was decreased in a dose-dependent manner (Fig. 7B). TNF-α-exposed cells treated with ≥10 μ*M* isoliquiritigenin showed substantial inhibition of phosphorylation of IκB. Thus, to achieve inhibitory effect of isoliquiritigenin on NF-κB translocation, micromolar doses of isoliquiritigenin were required.

Cellular Localization of NF-κB. It was tested whether isoliquiritigenin inhibits TNF-α-induced stimulation of CAM expression by interfering with the translocation of the transcription factor NF-κB. Intracellular localization of NF-κB p65 in HUVEC was evaluated by a fluorescent microscopy using specific NF-κB p65 antibody (Fig. 8A). Cytoplasmic immunofluorescence-staining was observed in TNF-α-free HUVEC, while heavy nuclear staining in TNF-α-alone-exposed cells was observed, indicative of nuclear localization of activated NF-κB p65 at single-cell level. However, isoliquiritigenin- and TNF-α-treated cells had the diminished staining level of nuclear p65. In addition, Western blot analysis revealed that an increase in nuclear NF-κB p65 was observed following exposure to 10 ng/ml TNF-α (Fig. 8B). It was shown that the marked increase in nuclear NF-κB p65 appeared following 4 hrs exposure to TNF-α (data not shown). When

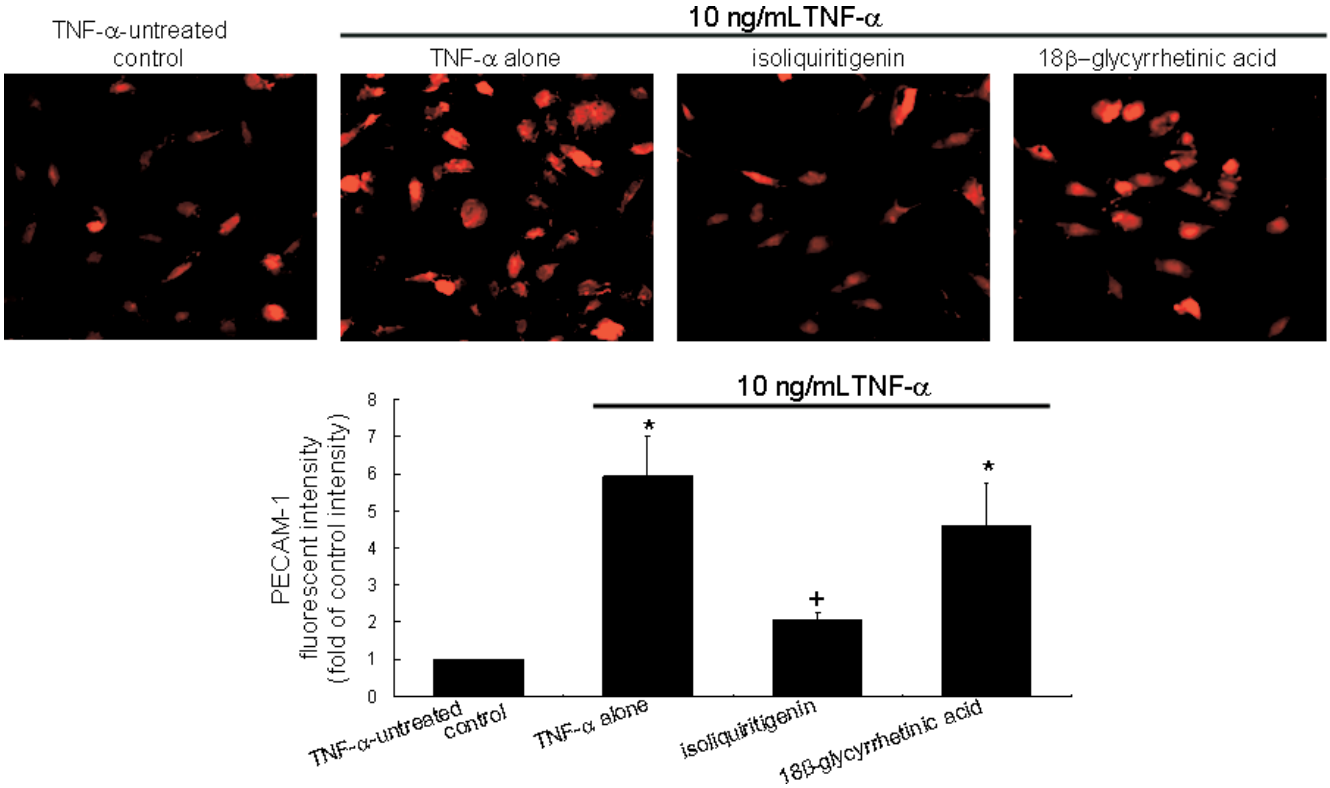

Figure 3. Effect of isoliquiritigenin and 18β-glycyrrhetinic acid on expression of PECAM-1 protein in cultured HUVEC treated with TNF-α. Cells were incubated with 50 μ*M* isoliquiritigenin and 18β-glycyrrhetinic acid overnight and exposed to 10 ng/ml TNF-α for 6 hrs. After HUVEC culture protocols with 10 ng/ml TNF-α, cells were fixed and then incubated with goat anti-human PECAM-1 for immunocytochemical staining. Antibody localization was detected with Cy-3 conjugated anti-goat IgG. Microphotographs (six independent experiments) were obtained using a fluorescence microscope. Magnification ×200. $^{*}P < 0.05$, relative to TNF-α-untreated control incubation; $^{+}P < 0.05$, relative to TNF-α-alone incubation. Color version of figure available in the on-line journal.

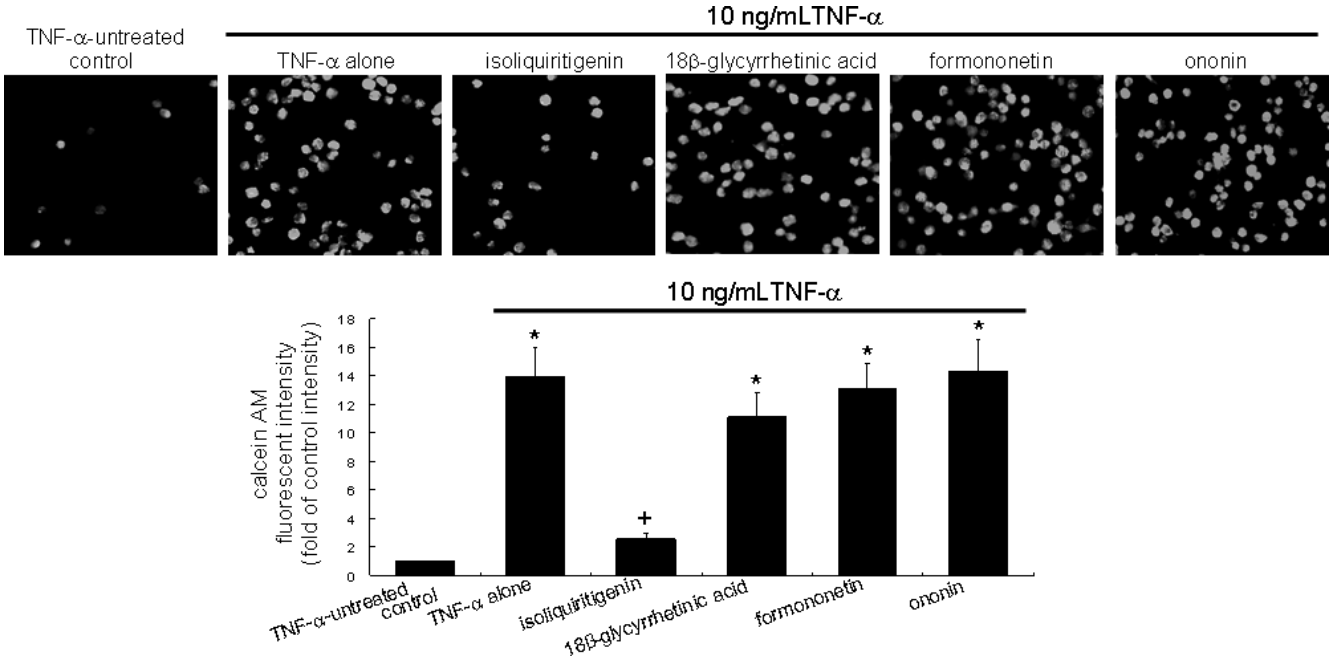

Figure 4. Inhibition of THP-1 monocyte adhesion to the TNF-α-activated HUVEC by isoliquiritigenin, 18β-glycyrrhetinic acid, formononetin, and ononin, HUVEC were pretreated with and without 50 μ*M* of each component overnight and then activated with 10 ng/ml TNF-α for 6 hrs. Endothelial cells were co-cultured with calcein AM-labeled THP-1 monocytes for 2 hrs. Microphotographs (three independent experiments) were obtained using a fluorescence microscope. Magnification ×200. $^{*}P < 0.05$, relative to TNF-α-untreated control incubation; $^{+}P < 0.05$, relative to TNF-α-alone incubation.

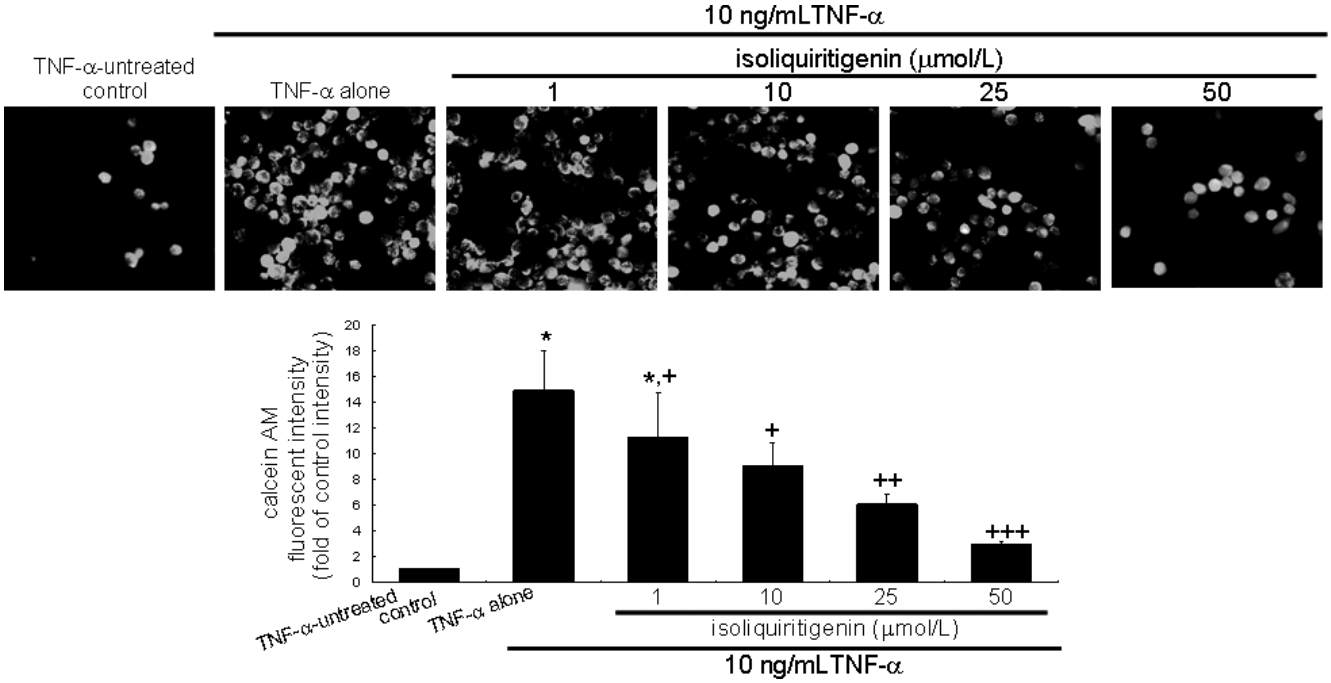

Figure 5. Inhibitory dose response of THP-1 monocyte adhesion to the TNF-α-activated HUVEC by isoliquiritigenin. HUVEC were pretreated with 1–50 μ*M* isoliquiritigenin overnight and then activated with 10 ng/ml TNF-α for 6 hrs. Endothelial cells were co-cultured with calcein AM-labeled THP-1 monocytes for 2 hrs. Microphotographs (three independent experiments) were obtained using a fluorescence microscope. Magnification ×200. $^{*}P < 0.05$, relative to TNF-α-untreated control incubation; $^{+}P < 0.05$, relative to TNF-α-alone incubation; $^{++}P < 0.05$, relative to incubation with 10 μ*M* isoliquiritigenin; $^{+++}P < 0.05$, relative to incubation with 25 μ*M* isoliquiritigenin.

TNF-α-exposed cells were treated with 50 μ*M* isoliquiritigenin, a marked decrease in nuclear NF-κB p65 protein was detected. In contrast, NF-κB p65 protein in the cytosolic extract modestly increased following treatment with isoliquiritigenin (Fig. 8B), suggesting that isoliquiritigenin can inhibit the expression of endothelial CAM specifically by preventing the translocation of NF-κB.

Discussion

Five major observations were extracted from this study.

I. Isoliquiritigenin at the nontoxic dose of ≥10 μ*M* nearly abolished expression of VCAM-1 and E-selectin proteins *via* a direct modulation at their gene transcriptional levels.

2. Isoliquiritigenin also substantially attenuated expression of PECAM-1 at 50 μ*M*.

3. The major components of licorice, 18β-glycyrrhetinic acid and its glycoside glycyrrhizin, did not have such inhibitory activity even at 50 μ*M*.

4. Isoliquiritigenin at ≥1 μ*M,* but not 18β-glycyrrhetinic acid, formononetin, or ononin, dose-dependently attenuated THP-1 adhesion in TNF-α-exposed cells.

5. The isoliquiritigenin appeared to inhibit the CAM expression by blunting the degradation of IκB and translocation of NF-κB stimulated by TNF-α.

These overall observations demonstrate that isoliquiritigenin has the potential capability to prevent the early events in atherosclerosis (Fig. 9). The ability to block the TNF-α-induced activation of CAM expression argues for the major target of action of isoliquiritigenin possibly mediated *via* the transcriptional mechanisms.

Licorice root extract contains many different subclasses of flavonoids, for instance isoflavans, isoflavones, and chalcones (12–14). Isoliquiritigenin, a flavonoid with a simple chalcone structure 4,2′,4′-trihydroxychalcone in licorice root extract, is regarded as a promising lead as a potential cancer chemopreventive agent (19, 20). Isoliquiritigenin has previously been reported to suppress cyclooxygenase-2, known to play an important role in inflammation, and to induce apoptosis in mouse colon cancer cells (28). The present study revealed that isoliquiritigenin blocked pro-inflammatory cytokine-induced protein expression of VCAM-1, E-selectin, and PECAM-1 at transcriptional levels and blunted THP-1 monocyte-endothelial cell interaction. The reduction in VCAM-1 noted in this study suggests an effect of isoliquiritigenin on endothelial function. It should be pointed out that co-culture of HUVEC with isoliquiritigenin and TNF-α did not cause toxicity (data not shown), as determined by MTT assay. In a previous study (29), it was shown that isoliquiritigenin was capable of lowering the levels of both ICAM-1 and VCAM-1 on mouse myeloid leukemia cells. On the other hand, the present study showed that the isoflavone formononetin and its glycoside, ononin, were inactive in inhibiting CAM expression of activated endothelial cells and in hindering monocyte-endothelial cell interaction. We have reported that

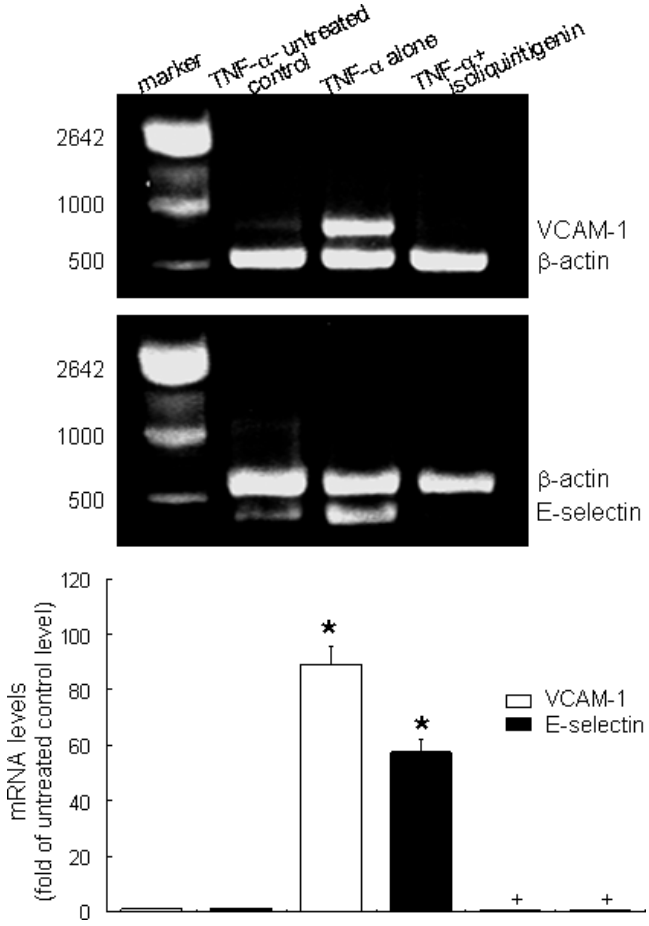

Figure 6. Reverse transcriptase-polymerase chain reaction analyses showing the steady state mRNA transcriptional levels of VCAM-1 and E-selectin in isoliquiritigenin-treated and TNF-α-stimulated HUVEC. Confluent HUVEC were incubated with 50 μ*M* isoliquiritigenin overnight and exposed to 10 ng/ml TNF-α for 6 hrs. β-Actin gene was used as an internal control for the co-amplification with VCAM-1 and E-selectin (three separate experiments). $^{*}P < 0.05$, relative to respective TNF-α-untreated control incubation; $^{+}P < 0.05$, relative to respective TNF-α-alone incubation.

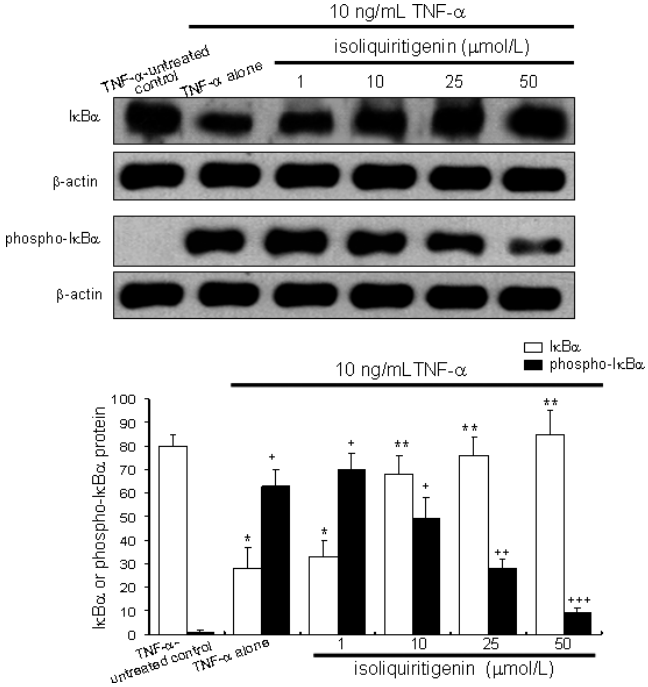

Figure 7. Inhibition of TNF-α-induced Inhibitory protein IκBα degradation from NF-κB complex by respective TNF-α-alone incubation in the presence of TNF-α. HUVEC extracts pretreated with 1–50 μ*M* isoliquiritigenin and exposed to 10 ng/ml TNF-α for 2 hrs were electrophoresed on 8% SDS-PAGE gel, followed by Western blot analysis with a primary antibody against human IκBα or human phospho-IκBα (ser 32) (three independent experiments). $^{*}P < 0.05$, relative to TNF-α-untreated control level of IκBα; $^{**}P < 0.05$, relative to respective TNF-α-alone incubation for IκBα; $^{+}P < 0.05$, relative to TNF-α-untreated control level of phospho-IκBα; $^{++}P < 0.05$, relative to respective TNF-α-alone incubation for phospho-IκBα; $^{+++}P < 0.05$, relative to respective incubation with 25 μ*M* isoliquiritigenin for phospho-IκBα.

the potential capability to prevent cytokine-induced CAM protein expression differs among individual flavonoid subclasses (8). Quercetin and flavones such as luteolin and apigenin prevented the activation of CAM expression and hence blocked monocyte adhesion on the TNF-α-activated endothelium (8). Thus, it is most likely that the expression inhibition of CAM by these chalcone-type and isoflavone-type flavonoids may stem from the difference of their chemical structures. The structure-activity relationships study on chalcone and flavone derivatives related to isoliquiritigenin suggested that the inhibitory activity of the chalcone derivatives is attributable to the 4-hydroxy group as well as the possible coplanarity between the phenyl ring and the adjacent conjugated ketone (29).

Glycyrrhizin, a major bioactive triterpene glycoside of aqueous licorice root extracts, and its aglycone, 18β-glycyrrhetinic acid, did not inhibit the expression of VCAM-1 and PECAM-1 induced by pro-inflammatory TNF-α and hence monocyte adhesion to the activated endothelium. However, glycyrrhizin attenuated elevation of ICAM-1 expression and apoptosis in the immunologic cytotoxicity of rat hepatocytes (30). A carbon-fucosylated derivative of 18β-glycyrrhetinic acid hampered E-selectin-, L-selectin-, and P-selectin-dependent eosinophil and neutrophil adhesion to interleukin-1β-stimulated HUVEC, whereas no effect was seen with this compound in the neutrophil adhesion to immobilized ICAM-1 (31). In addition, glycyrrhizin blocked selectin and attenuated renal ischemia-reperfusion injury when given after the onset of reperfusion in a rabbit model (32). We cannot explain the discrepancy between the results from this study and others differentially showing effects of glycyrrhizin on the endothelial function in CAM expression. It is deemed that the ability of this glycomimetic glycyrrhetinic acid to interfere with the function of selectins but not VCAM-1 and PECAM-1 makes it a desirable candidate as an anti-inflammatory agent. However, the mechanism underlying the selectin-responsive anti-inflammatory activity of glycyrrhizin is poorly understood.

The ability of isoliquiritigenin to block TNF-α-induced

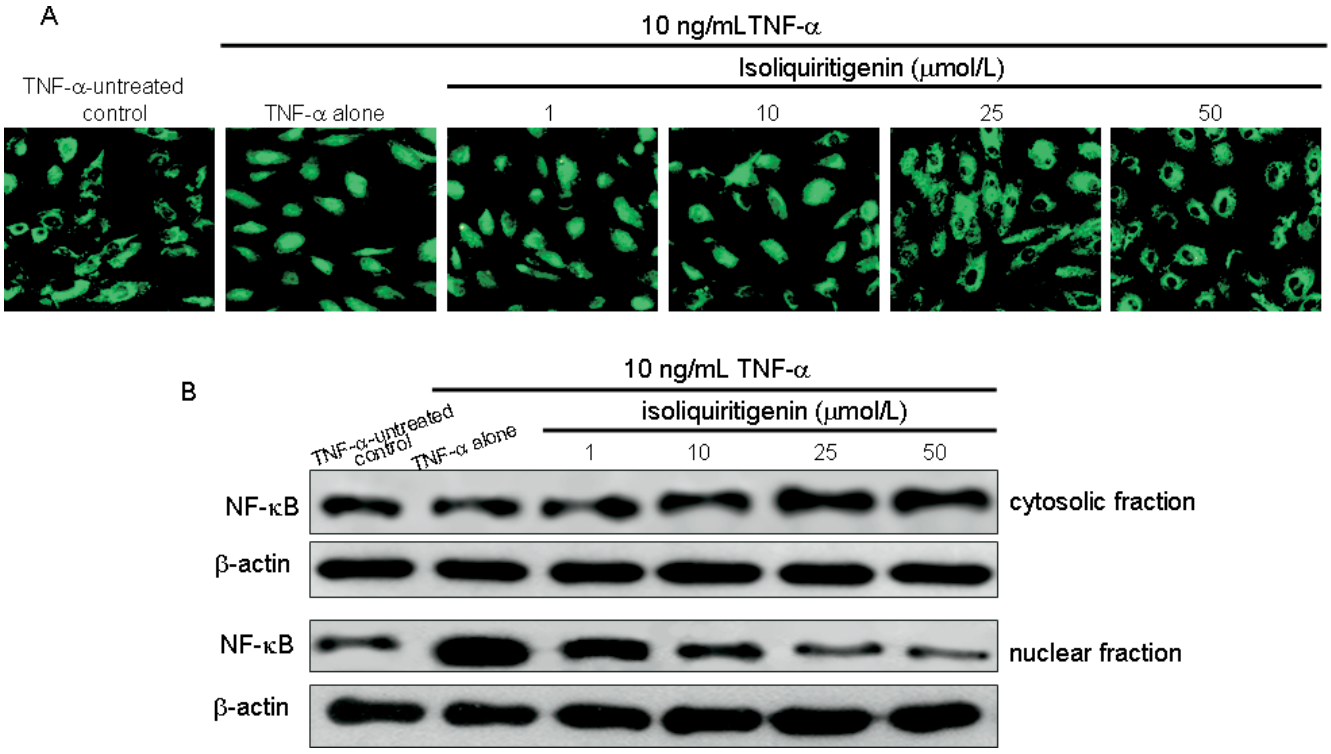

Figure 8. Representative microphotographs (A) and Western blot analyses (B) showing effect of isoliquiritigenin on translocation of NF-κB p65 in TNF-α-treated HUVEC. Cells were incubated with 50 μ*M* isoliquiritigenin overnight and exposed to 10 ng/ml TNF-α for 6 hrs. The NF-κB localization was visualized by binding with a fluorescein isothiocyanate-conjugated secondary antibody. Microscopic images were obtained using a fluorescence microscopy (three independent experiments). Magnification ×200. For Western blot analysis, HUVEC extracts (cytosolic and nuclear protein fractions) pretreated with 50 μ*M* isoliquiritigenin and exposed to 10 ng/ml TNF-α for 4 hrs were electrophoresed on 8% SDS-PAGE gel, followed by Western blot analysis with a primary antibody against human NF-κB p65 (three independent experiments). Color version of this figure is available in the on-line journal.

CAM expression could be due to its antioxidant capacity. It has been demonstrated that oxidative stress upregulates VCAM-1 expression *via* redox-sensitive transcriptional activation and is inhibited by the antioxidants (33). Classical antioxidant vitamin E has been shown to inhibit expression of CAM and adhesion of monocytes to endothelial cells (34). Chalcones, including isoliquiritigenin, have been shown to be potent antioxidants, indicative of the relation

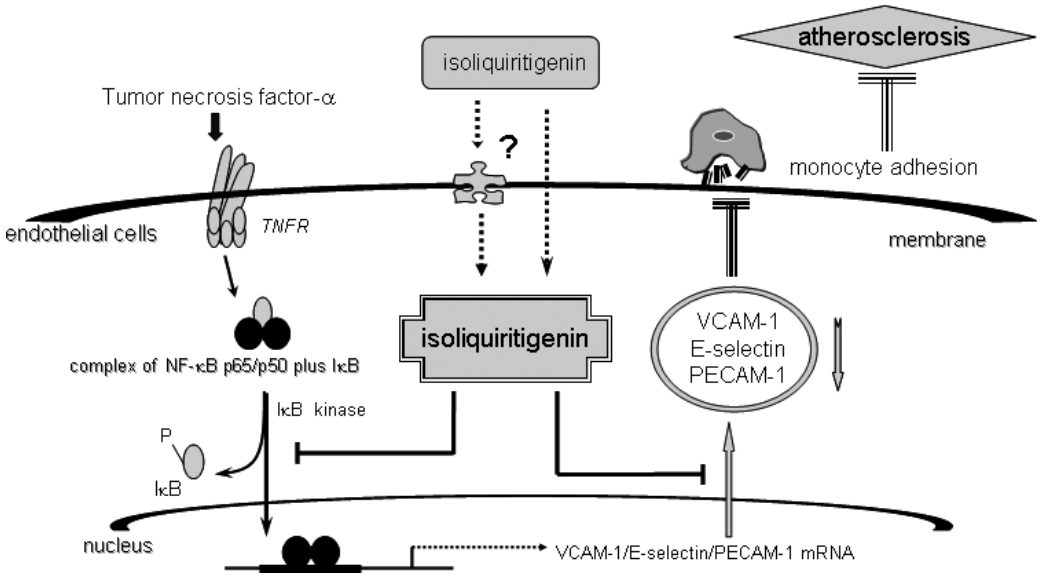

Figure 9. Schematic diagram showing antiatherogenic and anti-inflammatory features of isoliquiritigenin in TNF-α-induced endothelial CAM expression. The symbol —| indicates inhibition or blockade.

of their structures to their potency as tyrosinase inhibitors (35). It is not yet established which signaling pathways are involved in the modulation of CAM proteins. Clearly, the activation of endothelial expression of VCAM-1 and E-selectin by TNF-α was blocked, possibly by novel mechanism(s) responsive to isoliquiritigenin. The inhibitory mechanism(s) of isoliquiritigenin were inferred from the possibility that chalcones may interrupt a signaling cascade involving transcription activation of NF-κB, which plays an important role in the inducible regulation of cellular inflammatory genes (36, 37). This study revealed that isoliquiritigenin resulted in diminished degradation of the NF-κB-IκB complex, leading to inhibition of NF-κB translocation into the nucleus and transcriptional activity. It was shown that 2′-hydroxychalcone inhibited the adhesion of peripheral neutrophils to the endothelial cells by inhibiting the TNF-α- and lipopolysaccharide-induced expression of CAM proteins through interfering activation of NF-κB (38). The mechanism(s) whereby isoliquiritigenin blocks endothelial CAM expression through inhibiting activation of NF-κB are not fully understood. A chalcone derivative, 3′,4′,5′,3,4,5-hexamethoxy-chalcone, induced heme oxygenase-1 in murine macrophages and down-regulated an NF-κB-dependent inflammatory pathway with a mechanism of action different from antioxidant chalcones (36).

The initial signal of TNF-α in term of the interaction of ligand with its receptor could be affected by isoliquiritigenin. We assume that isoliquiritigenin enables endothelial cells to be resistant to the TNF-α-induced expression of VCAM-1. The access of isoliquiritigenin to putative binding proteins in HUVEC may modulate TNF-α-mediated activation of NF-κB signaling cascades through interrupting activation of mitogen-activated protein kinases (MAPK) by TNF-α. Cardamomin, 2′,4′-dihydroxy-6′-methoxychalcone, inhibited LPS-induced degradation and phosphorylation of IκBα and suppressed transcriptional activity and phosphorylation of RelA/p65 subunit of NF-κB *via* impaired activation of Akt and p38 MAPK (37). Alternatively, isoliquiritigenin may regulate the nuclear activity of NF-κB through other signaling pathways, that is, protein tyrosine kinase and/or protein kinase C-mediated pathways, and independent of NF-κB activation signaling pathway. Butein, a chalcone derivative, was shown to be a specific protein tyrosine kinase inhibitor (39). The blockade of formyl peptide-induced respiratory burst by 2′,5′-dihydroxy-2-furfurylchalcone involves phospholipase D signaling in neutrophils *via* the blockade of protein kinase Cα, ADP-ribosylation factor, and Rho A membrane association (40).

In summary, our studies have demonstrated that isoliquiritigenin, but not the other major licorice components, 18β-glycyrrhetinic acid and its glycoside glycyrrhizin, blocks the early atherogenic process involving endothelial expression of inducible adhesion molecules. Isoliquiritigenin blocked monocyte adhesion on the TNF-α-activated endothelium and the activation of CAM expression (Fig. 9). The selective inhibition of CAM expression by isoliquiritigenin was at least in part mediated *via* the regulation of translocation of NF-κB. This effect of isoliquiritigenin might have implications for strategies preventing and attenuating inflammatory diseases. The isoliquiritigenin-responsive mechanism(s) appear to be dependent of NF-κB-sensitive transcriptional regulatory mechanism(s) and may argue for transcriptional mechanisms as the major target of the antiatherogenic action of isoliquiritigenin.

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