



Hesperidin, hesperidin methyl chalone and phellopterin from *Poncirus trifoliata* (Rutaceae) differentially regulate the expression of adhesion molecules in tumor necrosis factor- α -stimulated human umbilical vein endothelial cells

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Received 15 October 2007; received in revised form 18 December 2007; accepted 11 January 2008

KEYWORDS

Hesperidin;
Hesperidin methyl chalone;
MAP kinases;
Phellopterin;
Tumor necrosis factor- α ;
Vascular cell adhesion molecule-1

Abstract

The fruits of *Poncirus trifoliata* (L.) are widely used in Oriental medicine to treat allergic inflammation. Recently, several active compounds including hesperidin, hesperidin methyl chalone and phellopterin from *P. trifoliata* (Rutaceae) were isolated and characterized. The goal of this study was to investigate the differential effect of hesperidin, hesperidin methyl chalone and phellopterin derived from *P. trifoliata* (Rutaceae) on the induction of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) by TNF- α and the possible molecular mechanisms by which they differentially regulate ICAM-1 and VCAM-1 expressions. Stimulation of human umbilical vein endothelial cells (HUVECs) with TNF- α resulted in the increase of ICAM-1 and VCAM-1 expressions, while pretreatment with the three components completely inhibited VCAM-1 expression in a dose-dependent manner but had no effect on ICAM-1 expression. All three compounds failed to block TNF- α -induced phosphorylation of ERK1/2, which

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ECAM, endothelial cell adhesion molecule; ECGS, endothelial cell growth supplements; ECL, enhanced chemoluminescence; ERK, extracellular regulated kinase; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor κ B; SDS, sodium dodecyl sulfate; PKC, protein kinase C; PVDF, polyvinylidene difluoride; TBS-T, Tris-buffered saline/Tween 20; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

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is involved in regulating ICAM-1 production by TNF- α . Furthermore, they efficiently inhibited the phosphorylation of Akt and PKC, suggesting that Akt or PKC pathways are an important target by which these compounds regulate TNF- α -induced VCAM-1 but not ICAM-1. Additionally, treatment with these chemicals also inhibited U937 monocyte adhesion to HUVECs stimulated with TNF- α . Interestingly, the inhibitory effect of hesperidin, hesperidin methyl chalone and phellopterin on monocyte adhesion to HUVECs was recapitulated by transfecting cells with VCAM-1 siRNA. Taken together, hesperidin, hesperidin methyl chalone and phellopterin reduce TNF- α -induced VCAM-1 expression through regulation of the Akt and PKC pathway, which contributes to inhibit the adhesion of monocytes to endothelium.

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1. Introduction

Vascular endothelial cells (ECs) lining the vascular wall normally release vasoactive substances to regulate vascular functions. However, following activation by inflammatory cytokines, ECs express chemotactic protein(s) to attract monocytes. Monocytes interact with ECs and subsequently extravasate into inflamed tissue to become foam cells leading to atherosclerosis [1]. Thus, cytokines are suggested to play a key role in monocyte/EC interaction and atherosclerosis [2–4]. The proinflammatory cytokine, tumor necrosis factor (TNF)- α , is a prototype “activation agonist” that modulates leukocyte adhesion and transmigration in vascular inflammatory diseases including atherosclerosis. The observed biological activities of TNF- α are mediated in part by the induction of a suite of genes that regulate leukocyte-endothelial cell interactions, such as adhesions (intercellular adhesion molecule-1; ICAM-1 and vascular cell adhesion molecule-1; VCAM-1), procoagulants (tissue factor), and additional inflammatory cytokines and chemokines in the EC [5].

ICAM-1, a membrane glycoprotein that belongs to the immunoglobulin superfamily mediates the adhesion of leukocytes to activated endothelium by establishing strong bonds with its ligands and inducing firm, leukocyte-specific β 2 integrins (e.g., lymphocyte function-associated antigen 1 and α L β 2 integrin), which induce the arrest of inflammatory cells at the vascular surface [6]. VCAM-1 is a 110-kDa cell surface glycoprotein that belongs to the immunoglobulin superfamily, and adheres to certain blood leukocytes and tumor cells that bear α 4 integrins. In the vascular system, VCAM-1 is expressed on activated ECs, smooth muscle cells, and fibroblasts in a variety of pathologic conditions including atherosclerosis [7–9]. Therefore, it has been hypothesized that a blockade of the binding between ICAM-1 or VCAM-1 and its ligands should reduce the severity of inflammation.

In the context of adhesion molecule expression by TNF- α , there is involvement of mitogen-activated protein (MAP) kinases, extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 [10]. Activation of MAP kinases in turn activates transcription factors, such as activator protein-1 and nuclear factor- κ B (NF- κ B), which may promote adhesion molecule surface expression and subsequent atherosclerosis [10,11]. In a previous study, we clearly demonstrated that inhibitors of p38 (SB203580) and ERK (PD98059), but not JNK, downregulated the ICAM-1 expression mediated by TNF- α [12]. Several pieces of evidence suggested that the PI3K/Akt signaling pathway is involved in the induction of adhesion molecules in ECs [13,14]. Moreover, a

role for protein kinase C in the induction of VCAM-1 expression was determined [15,16].

Traditionally, *Poncirus trifoliata* (Rutaceae) has been used in folk medicine as a remedy for the treatment of diverse chronic inflammatory diseases including gastritis, dysentery, inflammation, digestive ulcers, etc. In addition, its respective major components were reported to have various biological effects against inflammation, bacteria, and anaphylaxes activities [17,18]. Recent *in vitro* studies suggested *P. trifoliata* to have an anticancer activity, as it inhibits colon cancer cell growth and possesses antioxidant activity and may be a potent anti-leukemic agent by promoting apoptosis of cancer cells [19,20]. Recently, active compounds including hesperidin, hesperidin methyl chalone and phellopterin were isolated from *P. trifoliata*. There is a considerable interest in various health-promoting benefits of these drugs, although their effects on EC function have not been elucidated. In this study, we found that active compounds from *P. trifoliata*, especially the three drugs hesperidin, hesperidin methyl chalone and phellopterin, differentially suppressed ICAM-1 and VCAM-1 expression in TNF- α -treated HUVECs. Here, we investigated our hypothesis that this differential effect may be due to inhibition of PI3K/Akt and PKC signaling pathways.

2. Materials and methods

2.1. Extraction and isolation

All active compounds including hesperidin, hesperidin methyl chalone and phellopterin from *P. trifoliata* (Rutaceae) were provided by Dr. Seoung-Ho Lee, Yeungnam University (Kyongsan, Korea). These drugs were isolated and purified as described below. The purity of drugs (\geq 99%) was validated by HPLC.

The dried fruits of *P. trifoliata* (L.) Rafinesque (10 kg) were extracted three times with MeOH at room temperature for 7 days. The MeOH solution was concentrated under reduced pressure to give a residue (500 g) and it was partitioned between H₂O and CH₂Cl₂.

The CH₂Cl₂ extract (160 g) was loaded on a silica gel column (80 \times 12 cm) and eluted with *n*-hexane/EtOAc (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10, each 4 l) in a gradient mode to give 10 fractions (PF1–10). The fraction PF6 (5.8 g) was chromatographed on a silica gel column (70 \times 6 cm) and eluted with *n*-hexane/CH₂Cl₂/acetone (0.1:5:5, 0.3:5:5, 0.5:5:5, 1.0:5:5, 1.5:5:5, 2:5:5, each 4 l) to give 6 fractions (PF61–6). The fraction PF62 was chromatographed over a reverse phase column (50 \times 4 cm) eluting with MeOH/H₂O (5:5, 6:4, 7:3, 8:2, 9:1, each 4 l) to give 4 fractions (PF621–4), the fraction PF622 was rechromatographed over a C18 reverse phase column (50 \times 4 cm) eluting with MeOH/H₂O (6:4, 7:3, 8:2, each 3 l) to afford 1 (30 mg). The H₂O extract (80 g) was chromatographed on a

MCI column (60×5 cm) and eluted with a gradient of MeOH/H₂O (2:8, 3:7, 4:6, 5:5, 9:1, each 4 l) to give 5 fractions (PFW1–5). The fraction PFW2 (8.5 g) and PFW4 (18.5 g) were recrystallized in MeOH/CHCl₃ (1:1) to afford 2 (100 mg) and 3 (80 mg), respectively.

2.1.1. Phellopterin

Compound 1 was obtained as yellow needles and had the molecular formula C₁₇H₁₆O₅ as determined by FABMS, ¹H NMR, ¹³C NMR and DEPT spectral data. The ¹H NMR and ¹³C NMR indicated that it was a C-5 OMe-substituted linear furanocoumarin. The presence of unsubstituted pyrone and furan rings was recognizable from two pairs of doublets at δ 8.09 (1H, d, *J*=9.7 Hz, H-4)/6.25 (1H, d, *J*=9.7 Hz, H-3) and δ 7.60 (1H, d, *J*=2.3 Hz, H-2')/6.96 (1H, d, *J*=2.3 Hz, H-3'). The ¹H NMR spectrum of 1 also showed other four signals at δ 5.57 (1H, t, *J*=7.3 Hz, H-2''), 4.81 (2H, d, *J*=7.3 Hz, H-1''), 1.71, 1.67 (3H each, s, 2×CH₃), which were due to the prenyloxy group. The assignments of C-5 and C-8 were confirmed by HMBC linkage of H-1''/C-8 and 5-OMe/C-5. On the basis of the comparison of these data with those of literature, compound 1 was identified as phellopterin.

2.1.2. Hesperidin

Compound 2 was obtained as white to yellow powder. Its molecular formula C₂₈H₃₄O₁₅ was determined by FABMS, ¹H NMR, ¹³C NMR and DEPT spectral data. The ¹H NMR spectrum of 2 showed three typical flavanone skeleton signals at δ 5.50 (1H, dd, *J*=12.1, 3.3 Hz, H-2), 3.25 (1H, m, H-3b), 2.78 (1H, dd, *J*=17.1, 3.3 Hz, H-3a), a pair of *meta*-coupled protons of ring A at δ 6.14 (1H, d, *J*=2.1 Hz, H-8)/6.12 (1H, d, *J*=2.1 Hz, H-6), three protons of 1,3,4-trisubstituted ring B at δ 6.91 (3H, m, H-2',5',6'), two anomeric protons of glucose and rhamnose at δ 4.97 (1H, d, *J*=7.3 Hz, H-1'') and 4.53 (1H, s, H-1'''), one methoxy signal at δ 3.77 (3H, s, 4'-OCH₃), one methyl signal at δ 1.08 (3H, d, *J*=6.2 Hz, H-6''), and two aromatic hydroxy signals at δ 12.01 (1H, s, 5-OH) and 9.12 (1H, s, 3'-OH). Acid hydrolysis of compound 2 gave the sugar moieties and identified as glucose and rhamnose by TLC. The methoxy group and sugar moiety linkage were confirmed by HMBC. By comparing its ¹H and ¹³C NMR spectral data with reported literature, compound 2 was concluded to be hesperetin-7-*O*-rutinoside (-Glc⁶-Rha).

2.1.3. Hesperidin methyl chalone

Compound 3 was obtained as yellow powder. Its molecular formula C₂₉H₃₆O₁₅ was determined by FABMS, ¹H NMR, ¹³C NMR and DEPT spectral data. In the ¹H NMR spectrum, a set of *trans*-olefinic proton at δ 7.51 (d, *J*=2.6 Hz, H-β)/6.42 (d, *J*=6.1 Hz, H-α), a set of *meta*-coupled proton at δ 6.33 (d, *J*=2.1 Hz, H-3')/6.22 (d, *J*=2.1 Hz, H-5'), and three ABX system protons based on 1,3,4-trisubstituted benzene ring at δ 7.49 (dd, *J*=8.5, 1.9 Hz, H-6)/7.21 (d, *J*=1.5 Hz, H-2)/6.89 (d, *J*=8.5 Hz, H-5) were observed. The ¹H NMR spectrum also exhibited two anomeric protons of glucose and rhamnose at δ 5.27 (d, *J*=7.3 Hz, H-1'') and 4.85 (d, *J*=1.5 Hz, H-1'''), two methoxy signals at δ 3.93 and 3.86, and one methyl signal at δ 0.99 (d, *J*=6.3 Hz, H-6''). Acid hydrolysis of compound 3 gave the sugar moiety and identified as glucose and rhamnose by TLC. The methoxy group and sugar moiety linkage (-Glc⁶-Rha) were confirmed by HMBC. Its ¹H and ¹³C NMR spectral data were in good agreement with those reported in the literature and the structure of compound 3 was determined as hesperidin methyl chalone.

2.1.4. Phellopterin

¹H NMR (250 MHz, CDCl₃) δ: 8.09 (1H, d, *J*=9.7 Hz, H-4), 7.60 (1H, d, *J*=2.3 Hz, H-2'), 6.96 (1H, d, *J*=2.3 Hz, H-3'), 6.25 (1H, d, *J*=9.7 Hz, H-3), 5.57 (1H, t, *J*=7.3 Hz, H-2''), 4.81 (2H, d, *J*=7.3 Hz, H-1''), 4.14 (3H, s, 5-OMe), 1.71, 1.67 (3H each, s, 2×Me); ¹³C NMR (63 MHz, CDCl₃) δ: 160.5 (C-2), 150.7 (C-7), 145.0 (C-2'), 144.3 (C-5), 144.3 (C-9), 139.5 (C-3''), 139.3 (C-4), 126.8 (C-8), 119.8 (C-2''), 114.5 (C-6), 112.7 (C-3), 107.5 (C-10), 105.0 (C-3'), 70.3 (C-1'), 60.7 (5-OMe), 25.7 (C-4''), 18.0 (C-5'').

2.1.5. Hesperidin

¹H NMR (250 MHz, DMSO-*d*₆) δ: 12.01 (1H, s, 5-OH), 9.12 (1H, s, 3'-OH), 6.91 (3H, m, H-2',5',6'), 6.14 (1H, d, *J*=2.1 Hz, H-8), 6.12 (1H, d, *J*=2.1 Hz, H-6), 5.50 (1H, dd, *J*=12.1, 3.3 Hz, H-2), 4.97 (1H, d, *J*=7.3 Hz, H-1''), 4.53 (1H, s, H-1'''), 3.77 (3H, s, 4'-OCH₃), 3.25 (1H, m, H-3b), 2.78 (1H, dd, *J*=17.1, 3.3 Hz, H-3a), 1.08 (3H, d, *J*=6.2 Hz, H-6''); ¹³C NMR (63 MHz, DMSO-*d*₆) δ: 197.3 (C-4), 165.4 (C-7), 163.4 (C-5), 162.8 (C-9), 148.2 (C-4'), 146.7 (C-3'), 131.4 (C-1'), 118.2 (C-6'), 114.5 (C-2'), 112.3 (C-5'), 103.6 (C-10), 100.9 (C-1''), 99.65 (C-1''), 96.62 (C-6), 95.79 (C-8), 78.12 (C-2), 75.74 (C-3''), 75.30 (C-5''), 72.98 (C-2''), 71.83 (C-4''), 70.25 (C-4''), 69.95 (C-2''), 69.32 (C-3''), 68.11 (C-5''), 65.69 (C-6''), 55.91 (OCH₃), 42.02 (C-3), 18.11 (C-6'').

2.1.6. Hesperidin methyl chalone

¹H NMR (250 MHz, CD₃OD) δ: 7.51 (d, *J*=2.6 Hz, H-β), 7.49 (dd, *J*=8.5, 1.9 Hz, H-6), 7.21 (d, *J*=1.5 Hz, H-2), 6.89 (d, *J*=8.5 Hz, H-5), 6.42 (d, *J*=6.1 Hz, H-α), 6.33 (d, *J*=2.1 Hz, H-3'), 6.22 (d, *J*=2.1 Hz, H-5'), 5.27 (d, *J*=7.3 Hz, H-1''), 4.85 (d, *J*=1.5 Hz, H-1'''), 3.93 (s, OCH₃), 3.86 (s, OCH₃), 3.21–3.95 (m, protons of sugar), 0.99 (d, *J*=6.3 Hz, H-6''); ¹³C NMR (63 MHz, CD₃OD) δ: 192.4 (C-β'), 168.4 (C-4'), 166.0 (C-2'), 162.1 (C-6'), 150.7 (C-3), 149.2 (C-4), 142.4 (C-β), 129.4 (C-1), 125.9 (C-α), 122.4 (C-6), 113.5 (C-2), 111.9 (C-5), 103.1 (C-1'), 100.5 (C-1''), 99.36 (C-1''), 96.26 (C-3'), 95.38 (C-5'), 75.74 (C-3''), 75.30 (C-5''), 72.98 (C-2''), 71.83 (C-4''), 70.25 (C-4''), 69.95 (C-2''), 69.32 (C-3''), 68.11 (C-5''), 65.69 (C-6''), 56.51 (C4-OCH₃), 56.45 (C6'-OCH₃), 17.92 (C-6'').

2.2. Materials

Tissue culture medium 199, fetal bovine serum (FBS), antibiotics (penicillin/streptomycin), glutamine and collagenase were supplied by Gibco-BRL (Rockville, MD). VCAM-1 small interfering RNA (siRNA), siRNA transfection kit, anti-ICAM-1, anti-VCAM-1, and anti-phospho-ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-p-Akt and anti-p-PKC antibodies were obtained from Cell Signaling Technology (Beverly, MA). Enhanced chemiluminescence (ECL) Western blotting detection reagent was from Amersham (Buckinghamshire, UK). All other chemicals including endothelial cell growth supplements (ECGS) and heparin were supplied by Sigma-Aldrich (St. Louis, MO).

2.3. Cell culture

HUVECs were isolated from umbilical cord veins by collagenase treatment [21] and grown in medium 199 supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 5 U/ml heparin, 100 IU/ml penicillin, 10 μg/ml streptomycin and 50 μg/ml ECGS. Cells were cultured in 100 mm dishes and grown in a humidified 5% CO₂ incubator. HUVECs were plated at a density of 1×10⁷ cells per 100 mm dish. Cells were used between passage number 3 and 6. U937 human monocytes were obtained from Korea Cell Line Bank (KCLB, Seoul, Korea) and grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 25 mM HEPES, 25 mM NaHCO₃, 100 IU/ml penicillin, and 10 μg/ml streptomycin.

2.4. Western blot analysis

For isolation of total cell extracts, cells were lysed in PRO-PREP protein extract solution. The sample was centrifuged at 13,000 rpm×20 min at 4 °C. Protein concentration was determined by the Bradford method. An equal volume of 2×SDS sample buffer (0.1 M Tris-Cl, 20% glycerol, 4% SDS, and 0.01% bromophenol blue) was added to an aliquot of the supernatant fraction from the lysates, and the samples were boiled for 5 min. 30 μg of protein was subjected to 10% SDS-polyacrylamide gel electrophoresis for 1 h 30 min at 110 V. The separated proteins were transferred to PVDF

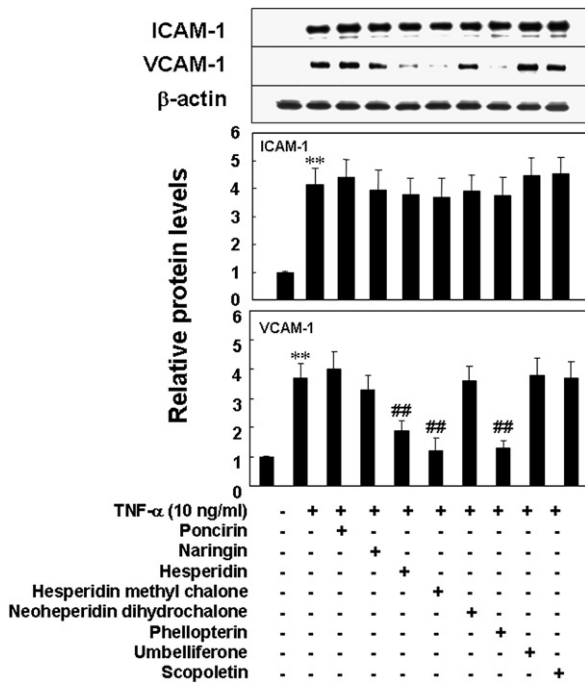


Figure 1 Differential effect of components of *Poncirus trifoliata* on the expression of ICAM-1 and VCAM-1 in TNF-α-treated HUVECs. Cells were pretreated with components of *P. trifoliata* at 50 μM concentration for 24 h and then treated with TNF-α (10 ng/ml) for 6 h. After treatment, protein was extracted from the cells and ICAM-1 and VCAM-1 protein levels were determined by Western blot analysis as described in Materials and methods. The band intensities were assessed by scanning densitometry. Data are presented as means ± SEM of three independent experiments. One-way analysis of variance was used to compare the multiple group means followed by Newman–Keuls test (significance compared with control, ***P* < 0.01; significance compared with TNF-α, ##*P* < 0.01).

membrane for 2 h at 20 mA with SD Semi-dry Transfer Cell® (Bio-Rad). The membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T) for 2 h at room temperature. Next, the membranes were incubated with anti-ICAM-1, anti-VCAM-1, anti-p-ERK1/2, anti-p-Akt and anti-p-PKC antibodies at a 1:500 concentration (4 μg/ml) in 5% skim milk in TBS-T overnight at 4 °C, and the bound antibody was detected by horseradish peroxidase-conjugated anti-rabbit IgG. The membranes were washed and then developed using a Western blotting Luminol Reagent system (Amersham).

2.5. Monocytic cell adhesion assay

HUVECs were seeded into two-well chamber slides 48 h prior to experiments. The medium was refreshed before stimulation with TNF-α. U937 monocytes (3 × 10⁷) were incubated in RPMI 1640 medium containing 2% FBS and 10 mg/ml of the fluorescent dye BCECF/AM (Boehringer, Mannheim, Germany) at 37 °C for 30 min. Fluorescence-labeled cells were pelleted and resuspended (7.5 × 10⁵/ml) in medium 199 with 10 mM HEPES buffer (M199H). HUVECs were washed three times with M199H prior to the addition of loaded cells, and were then incubated at 37 °C. After 30 min, cell suspensions were withdrawn and the HUVECs were gently washed with M199H. Fluorescent images were selected using a high-resolution video camera (DXC-960MD; Sony) mounted on a BH-2 Olympus microscope (Melville, NY). Images of 0.2 mm in width within these first selected areas were picked and the immunoreactivity of these images was measured using SigmaGel 1.0 (Jandel Scientific, Germany). Analyses were repeated three times over the same region and the results shown are the means of three independent experiments.

2.6. Statistical evaluations

Scanning densitometry was performed using an Image Master® VDS (Pharmacia Biotech Inc., San Francisco, CA). Treatment groups were compared using one-way analysis of variance (ANOVA) and the Newman–Keuls test was used to locate any significant differences identified in the ANOVA. *P* < 0.05 or *P* < 0.01 was accepted as significant.

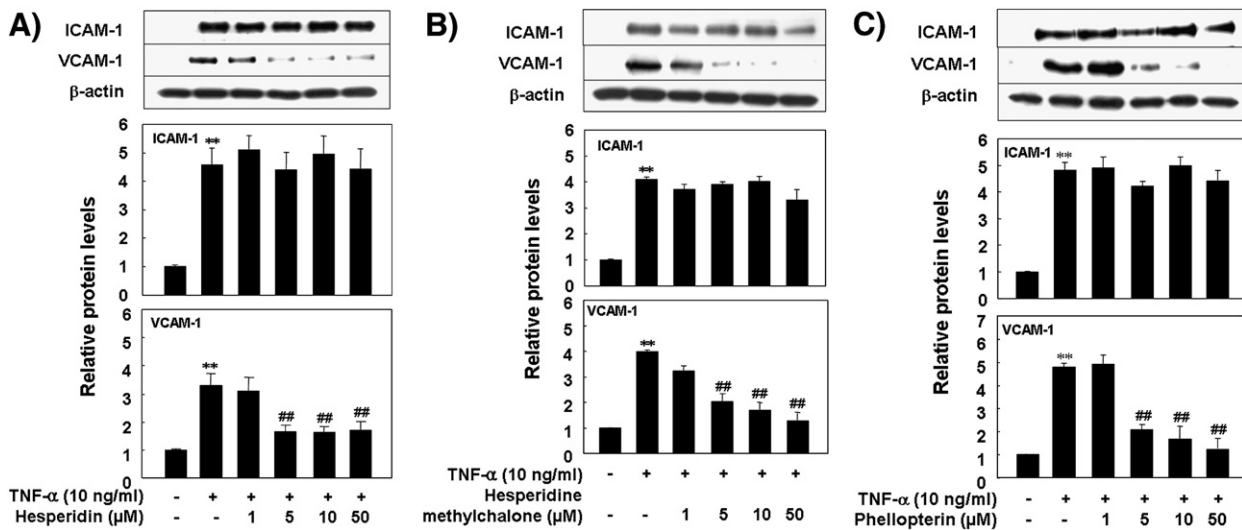


Figure 2 Inhibitory effect of hesperidin, hesperidin methyl chalone and phellopterin on VCAM-1 but not ICAM-1 in a dose-dependent manner in TNF-α-stimulated HUVECs. Cells were pretreated with hesperidin (A), hesperidin methyl chalone (B) and phellopterin (C) at a concentration of 1, 5, 10 and 50 μM for 24 h, and then treated with TNF-α for 6 h. Protein levels were detected by Western blot analysis as described in Materials and methods. The band intensities were assessed by scanning densitometry. Data are presented as means ± SEM of three independent experiments. One-way analysis of variance was used to compare the multiple group means followed by Newman–Keuls test (significance compared to control, ***P* < 0.01; significance compared with TNF-α, ##*P* < 0.01).

3. Results

3.1. Differential effect of components of *P. trifoliata* on the expression of ICAM-1 and VCAM-1 in TNF- α -treated HUVECs

We tested the effect of poncirin, naringin, hesperidin, hesperidin methyl chalone, neoheperidin dehydrochalone, phellopterin, umbelliferone and scopoletin, which are derived from *P. trifoliata* on the ICAM-1 and VCAM-1 expressions in HUVECs stimulated with TNF- α . The cells were pretreated with drugs at high concentration (50 μ M) for 24 h and then co-treated with TNF- α (10 ng/ml) for 6 h. The results show that TNF- α -induced ICAM-1 induction was not affected by any compounds. Interestingly, the induction of VCAM-1 by TNF- α was significantly inhibited by only three compounds (hesperidin, hesperidin methyl chalone and phellopterin) of the active compounds from *P. trifoliata* (Fig. 1).

3.2. Inhibitory effect of hesperidin, hesperidin methyl chalone and phellopterin on TNF- α -mediated VCAM-1 induction in HUVECs in a concentration dependent manner

To confirm the inhibitory effect of these three compounds on VCAM-1 expression, we pretreated cells with hesperidin, hesperidin methyl chalone and phellopterin in a concentration dependent manner (1, 5, 10 and 50 μ M) for 24 h. Next, cells were treated with TNF- α for 6 h. Fig. 2A shows that hesperidin failed to inhibit ICAM-1 expression whereas it showed a significant inhibition of VCAM-1 expression from 5 to 50 μ M. Hesperidin methyl chalone also exhibited an inhibition effect on VCAM-1 expression by TNF- α from 1 μ M but significantly from 5 μ M (Fig. 2B). Likewise, phellopterin did not inhibit TNF- α -induced ICAM-1 expression, whereas it inhibited VCAM-1 expression from 5 μ M, and completely

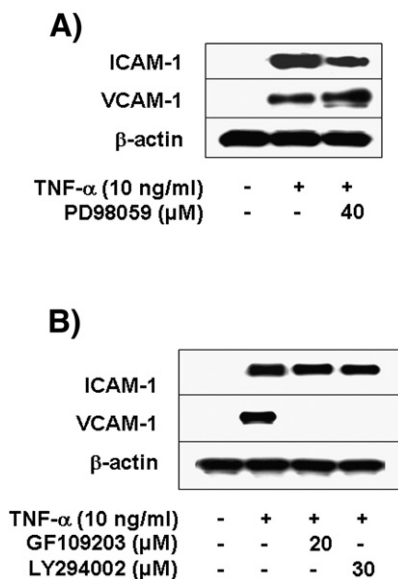


Figure 3 Differential role of ERK1/2, PKC and PI3 kinase in the production of ICAM-1 and VCAM-1 induced by TNF- α . Cells were treated with a specific ERK1/2 inhibitor (PD98059) at 40 μ M, a specific PKC inhibitor (GF109203) at 20 μ M and a specific PI3 kinase inhibitor (LY294002) at 30 μ M for 1 h and then co-treated with TNF- α (10 ng/ml) for 6 h. Protein level was detected by western blot analysis as described in Materials and methods. Data were confirmed by two independent experiments.

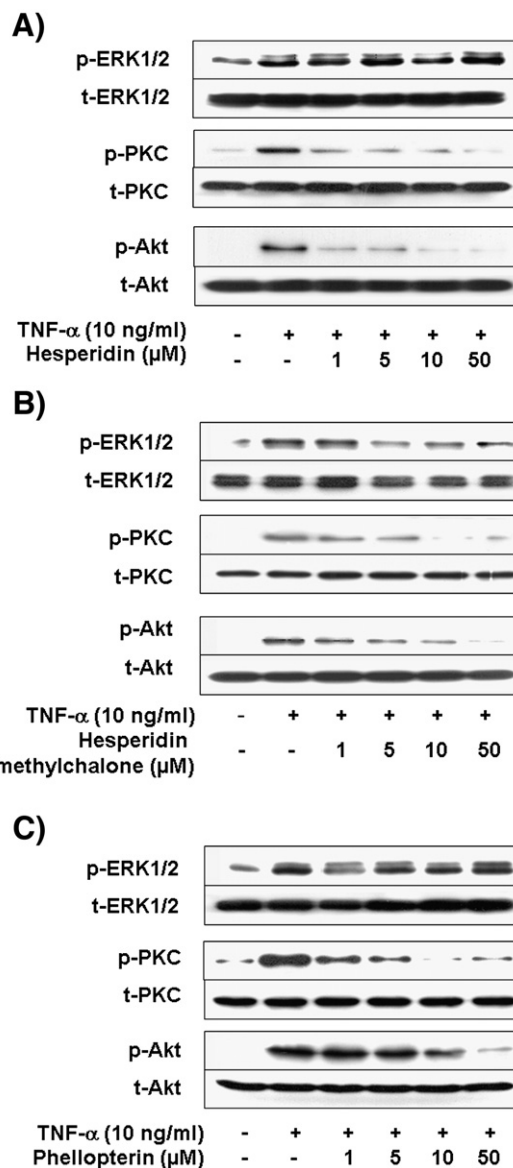


Figure 4 Effects of hesperidin, hesperidin methyl chalone and phellopterin on ERK1/2, Akt and PKC activation by TNF- α in HUVECs. Cells were pretreated with hesperidin (A), hesperidin methyl chalone (B) and phellopterin (C) in a concentration dependent manner (1, 5, 10 and 50 μ M) for 24 h, and then treated with TNF- α for 10 min for detection of phosphor-ERK1/2 and PKC or for 30 min for detection of phosphor-Akt. Cells were extracted and protein level was detected by Western blot analysis. Data were confirmed by two independent experiments.

suppressed it at 50 μ M concentration (Fig. 2C). Compared to the other two compounds, phellopterin slightly increased VCAM-1 expression by TNF- α at 1 μ M, although this was not significant.

3.3. Effects of hesperidin, hesperidin methyl chalone and phellopterin on ERK1/2, Akt and PKC activation by TNF- α in HUVECs

Previously we determined that specifically inhibiting ERK1/2 (PD98059) affects TNF- α -stimulated ICAM-1 expression [12]. We also tested ERK1/2's effect on VCAM-1 induction. Our result

suggested that treating with the ERK1/2 inhibitor had no effect on VCAM-1 expression in TNF- α -treated HUVECs (Fig. 3A). Moreover, we verified the effect of PI3K (LY294002) and PKC (GF109203) inhibitors on ICAM-1 and VCAM-1 (Fig. 3B). Interestingly, these inhibitors also showed differential effects on ICAM-1 and VCAM-1 expression. Two inhibitors were able to suppress VCAM-1, but not ICAM-1. Therefore, to determine whether the effects of hesperidin, hesperidin methyl chalone and phellopterin on VCAM-1 expression were due to inhibition of PI3K/Akt and PKC signaling pathways, HUVECs were exposed to 10 ng/ml TNF- α for 30 min to detect Akt and PKC phosphorylation, and for 10 min to detect ERK1/2 phosphorylation, based on our previous data [12]. Our results show that three compounds (hesperidin, hesperidin methyl chalone and phellopterin) have no effect on ERK1/2 phosphorylation, whereas they significantly inhibited the phosphorylation of Akt and PKC in dose-dependent manner (Fig. 4A, B and C). These findings suggest that TNF- α -induced PI3K/Akt and PKC activations are differentially involved in expression of VCAM-1 from ICAM-1, which pathways can be regulated by hesperidin, hesperidin methyl chalone and phellopterin.

3.4. Inhibition of TNF- α stimulated adhesion of monocyte to ECs by hesperidin, hesperidin methyl chalone and phellopterin

The monocyte/EC interaction is in part regulated by the expression of specific adhesion molecules. Among these, VCAM-1 expression plays an important role in the adhesion of monocyte (U937) cells to ECs [22,23]. We found that incubation of HUVECs with hesperidin, hesperidin methyl chalone or phellopterin significantly attenuated the level of VCAM-1. Therefore, we investigated the effect of the three drugs on adhesion of monocytes to HUVECs after stimulation with TNF- α . Adhesion of human monocyte U937 cells to HUVECs increased by 4-fold after stimulation with TNF- α at 10 ng/ml for 6 h (Fig. 5B), whereas very few U937 cells were adhered to inactivated HUVECs (Fig. 5A). In contrast, when HUVECs were pretreated with hesperidin, hesperidin methyl chalone and phellopterin for 24 h, the results showed significant reduction of adherent cells to ECs from 4-fold to 2.5-fold (Figs. 5A and 4B), indicating that they effectively prevent the adhesion of monocytes to ECs stimulated with TNF- α through regulation of VCAM-1 expression.

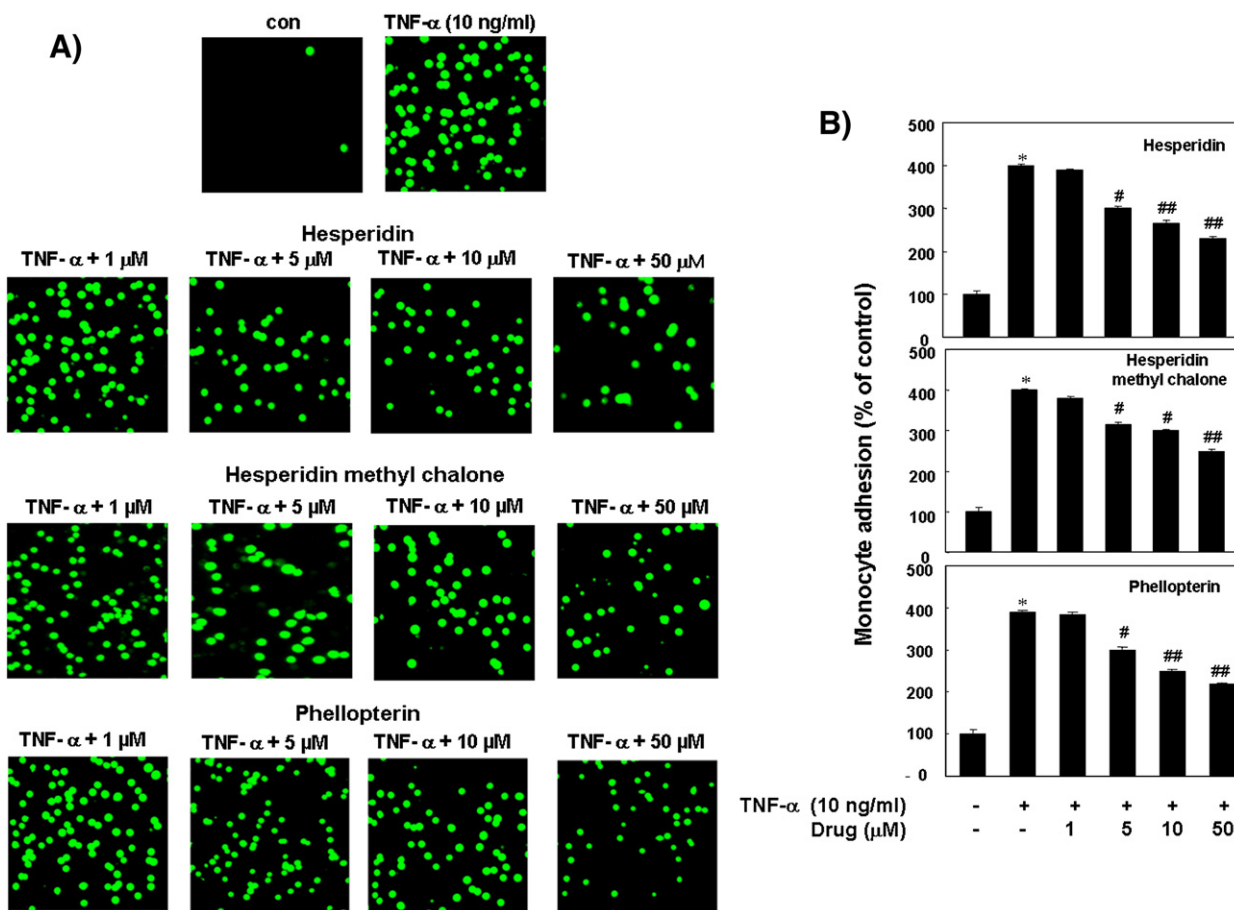


Figure 5 Inhibition of TNF- α stimulated monocyte adhesion to ECs by hesperidin, hesperidin methyl chalone and phellopterin. HUVECs were stimulated with TNF- α for 6 h after pretreatment with or without hesperidin, hesperidin methyl chalone and phellopterin (1, 5, 10 and 50 μ M) for 24 h. Then, cells were coincubated with fluorescent labeled monocytic cells for 30 min at 37 $^{\circ}$ C, and monocyte adhesion to ECs was presented as images (A) and a percentage of U937 cells bound to TNF- α -untreated cells (control) (B). Data represent mean \pm SEM of three separate experiments performed in triplicate. One-way analysis of variance was used to compare the multiple group means followed by Newman-Keuls test (significance compared to control, * P <0.05; significance compared with TNF- α , # P <0.05, ## P <0.01).

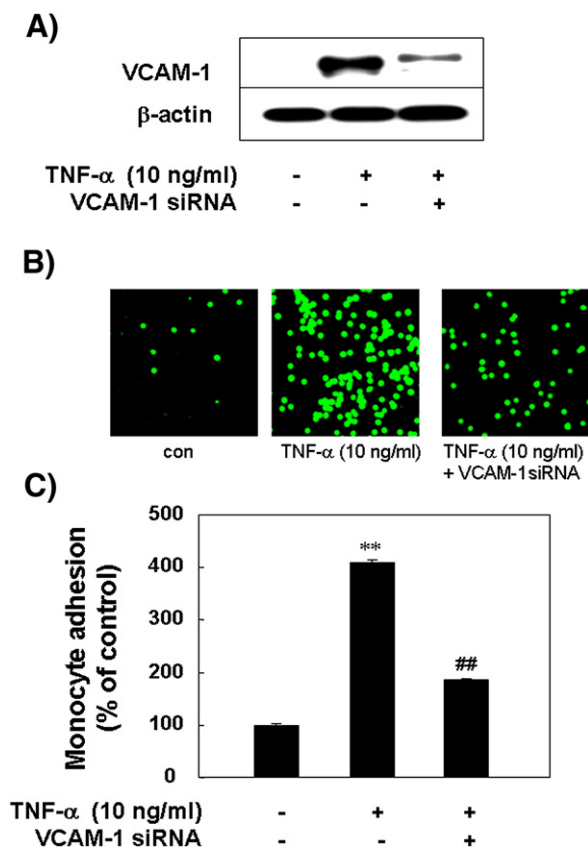


Figure 6 Attenuation of adhesion of U937 cells to ECs stimulated with TNF- α by knockdown of VCAM-1. Cells were transiently transfected with VCAM-1 siRNA, and then stimulated with 10 ng/ml TNF- α for 6 h. Thereafter, cells were coincubated with fluorescent labeled monocytic cells for 30 min at 37 °C. (A) Western blot analysis showed that VCAM-1 was effectively knockdowned in ECs transfected with VCAM-1 siRNA. Monocyte adhesion was presented as images (B) and a percentage of U937 cells bound to TNF- α -untreated cells (control) (C). Data represent mean \pm SEM of three separate experiments performed in triplicate. One-way analysis of variance was used to compare the multiple group means followed by Newman–Keuls test (significance compared to control, ** $P < 0.01$; significance compared with TNF- α , ## $P < 0.01$).

3.5. Attenuation of adhesion U937 cells to HUVECs stimulated with TNF- α by knockdown of VCAM-1

To verify that the reduction of adherent monocytes to ECs by hesperidin, hesperidin methyl chalone and phellopterin is due to the downregulation of VCAM-1 expression, we treated HUVECs with siRNA against VCAM-1, and then stimulated the cells with TNF- α for 6 h. The efficiency of knockdown of VCAM-1 was tested by Western blot analysis and it was over 80% (Fig. 6A), and VCAM-1 knockdown resulted in a marked (>55%) inhibition in the adhesion of monocytes to ECs (Fig. 6B and C). Thus, our findings suggest that the effect of TNF- α on U937 monocyte adhesion is mediated through the induction of VCAM-1 on ECs. Additionally, we conclude that among all components of *P. trifoliata*, hesperidin, hesperidin methyl chalone and phellopterin effectively inhibit monocyte adhesion to ECs by downregulation of TNF- α -mediated VCAM-1 expression in HUVECs.

4. Discussion

The functional state of ECs is the major determinant of leukocyte extravasation in both non-pathological and pathological contexts. Various agonists including cytokines such as TNF- α and chemokines induce EC activation. TNF- α stimulation of ECs activates the cell surface expression of adhesion molecules such as ICAM-1 and VCAM-1 [24,25]. Here we demonstrated that TNF- α (10 ng/ml) significantly induced the expressions of ICAM-1 and VCAM-1 in HUVECs. Specifically, we found that hesperidin, hesperidin methyl chalone and phellopterin which are derived from *P. trifoliata* dramatically inhibit TNF- α -induced VCAM-1 protein expression, but not ICAM-1 in HUVECs. Hesperidin, hesperidin methyl chalone and phellopterin have been known to have anti-inflammatory properties. Several anti-inflammatory agents diminish leukocyte adhesion by inhibiting cytokine-induced endothelial cell adhesion molecule (ECAM) expression at the transcriptional level. However, not all of these compounds exert the same effect on cytokine-induced ECAM expression. For example, lactacystin can reduce the cytokine-induced expression of E-selectin, ICAM-1 and VCAM-1 [26], whereas other compounds appear to be selective for one particular ECAM (e.g., VCAM-1) [27,28]. Because the leukocyte adhesion cascade is documented to have receptor–ligand functional overlap, as VCAM-1 has been shown to support tethering and rolling of lymphocytes [29], compounds that suppress the VCAM-1 expression may be effective in blocking leukocyte adhesion in a variety of inflammation settings. In contrast, reducing the expression of all ECAMs may cause detrimental side effects, and such a broad effect may not be necessary to achieve a therapeutic effect. For example, VCAM-1 is present on aortic endothelium that covers early foam cell lesions [30]. Three components of *P. trifoliata*, hesperidin, hesperidin methyl chalone and phellopterin, show selective reduction of TNF- α -induced VCAM-1 expression and consequent monocytic cell adhesion to HUVECs. Further animal studies investigating the ability of hesperidin, hesperidin methyl chalone and phellopterin to reduce pathological inflammation, in particular atherosclerosis, are clearly warranted and are currently ongoing in our laboratory.

NF- κ B activity can be modulated through phosphorylation of MAPK. In our previous study, we have shown that p38 and ERK1/2 but not JNK are upstream regulators of NF- κ B activation in TNF- α -stimulated HUVECs [12]. We also found that ERK1/2 is not involved in VCAM-1 expression. Therefore, it was reasonable to test the effect of hesperidin, hesperidin methyl chalone and phellopterin on the phosphorylation of ERK1/2. Our results showed that these three compounds have no inhibitory effect on ERK1/2 (Fig. 3). Thus, we hypothesize that they inhibit VCAM-1 expression in TNF- α -stimulated HUVECs via a NF- κ B-independent mechanism, even though this hypothesis is required to determine further. Our results showed that PI3K/Akt and PKC signaling pathways are involved in VCAM-1 induction but not ICAM-1. In this study, we showed that hesperidin, hesperidin methyl chalone and phellopterin significantly inhibit phosphor-Akt and PKC in HUVECs (Fig. 3).

We also found that these drugs significantly reduced monocyte adhesion in part by inhibiting TNF- α -stimulation of VCAM-1. Experiments with siRNA against the VCAM-1 gene showed that VCAM-1 knockdown dramatically decreased U937

cell adhesion to HUVECs (Fig. 5), suggesting that hesperidin, hesperidin methyl chalone and phellopterin inhibit monocyte adhesion to endothelial cells by reducing VCAM-1 expression.

In conclusion, we have found that hesperidin, hesperidin methyl chalone and phellopterin 1) dramatically inhibited TNF- α -induced VCAM-1 expression and have no effect on ICAM-1 expression in HUVECs, 2) significantly reduced TNF- α -induced phosphorylation of Akt and PKC, but not ERK1/2, and 3) markedly decreased TNF- α -induced monocytic cell adhesion to HUVECs, which is due to inhibition of VCAM-1 expression. In this respect, these drugs have potential as a therapeutic for the treatment of pathological inflammation, especially diseases involving VCAM-1 (e.g., atherosclerosis and malignancy).

Acknowledgements

This work was supported by Korea Food and Drug Administration (S-06-02-2-CHM-230-0-C) and by the MRC program of MOST/KOSEF (R13-2005-012-01003-0).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.intimp.2008.01.011.

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