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ARTICLE

Acteoside and 6-O-Acetylacteoside Downregulate Cell Adhesion Molecules Induced by IL-1β through Inhibition of ERK and JNK in Human Vascular Endothelial Cells

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Acteoside, an active phenylethanoid glycoside of many medicinal plants and bitter tea, displays anti-inflammatory properties *in vitro*. However, it is unclear whether acteoside and similar compounds may inhibit the expression of cell adhesion molecules (CAMs), which plays a role in the pathogenesis of atherosclerosis and inflammation. Here, we found that acteoside, isoacteoside, and 6-*O*-acetylacteoside inhibited IL-1β-activated expression of intercellular CAM-1 (ICAM-1) and vascular CAM-1 (VCAM-1) in human umbilical vein endothelial cells (HUVECs); the inhibitory potency was as follows: 6-*O*-acetylacteoside > acteoside > isoacteoside. Acteoside and 6-*O*-acetylacteoside also dose-dependently inhibited VCAM-1 gene promoter activity in IL-1β-activated HUVECs. The inhibition of acteoside and 6-*O*-acetylacteoside on IL-1β-activated expression of CAMs was manifested by decreased phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). These results indicate that acteoside and 6-*O*-acetylacteoside may exert anti-inflammatory activities in vascular endothelium by inhibiting the expression of CAMs, primarily through decreased phosphorylation of ERK and JNK.

KEYWORDS: Acteoside; 6-*O*-acetylacteoside; cell adhesion molecules; IL-1β; MAPKs; human vascular endothelial cells

INTRODUCTION

Acteoside was originally isolated from the flowers of *Syritnga vulgaris* (*1*). Acteoside and its derivatives such as 6-*O*-acetylacteoside have also been found in many other plants such as *Paulownia tomentosa* (*2*), *Leucoseptrum japonicum* (*3*), *Forsythia viridissima* (*4*), *Rehmannia glutinosa* (*5*) and *Harpagophytum procumbens* (*6*). The stem bark of *P. tomentosa* has been used to treat infective diseases such as gonorrhea and erysipelas in Chinese medicine (*7*). *H. procumbens* was originally used to treat fevers, arthritis, rheumatism, and digestive disorders in the northwestern parts of Southern Africa. Indeed, recent studies have demonstrated the anti-inflammatory and antirheumatic activities of *H. procumbens* (*8*, *9*). In addition to medicinal plants, bitter tea, which is a popular beverage in southern China, is brewed from the leaves of 10 plant species including *Ligustrum purpurascens* which contains acteoside as the major physiologically active component of the plant (*10*). Bitter tea has been claimed to have several biological effects such as activation of the central nervous system, improvement of sore throat and hypertension (*11*, *12*). During brewing bitter tea, hot water can convert acteoside into the isomer isoacteoside (*7*).

Acteoside is also named verbascoside, and its structure contains several chemical groups, such as caffeic acid, 3,4-dihydroxyphenylethanol, glucose, and rhammose (**Figure 1**). Several studies have indicated that acteoside and isoacteoside exhibit antioxidative, anti-inflammatory, and neuronal protective activities both *in vitro* and *in vivo*. For instance, acteoside inhibits nitric oxide and TNF-α productions through blocking of AP-1 activation in lipopolysaccharide-stimulated macrophage (*13*, *14*). Lee et al. (*15*) reported that acteoside inhibites histamine release and prostaglandin E_2 production induced by melittin in RBL-2H3 mast cells. In animal experiments, Hausmann et al. (*16*) further demonstrated that acteoside reduces intestinal inflammation in dextran sulfate sodium-induced colitis and thus may be a potential therapeutic drug for inflammatory bowel disease. In addition, acteoside was found to effectively reduce 1,1-diphenyl-2-picrylhydrazyl radical (*17*, *18*) and hydroxyl radical (*19*, *20*), and the free radical-scavenging activity might be associated with the sequestration of iron ions. Acteoside also protects bovine pulmonary endothelial cells against free radical-induced oxidative stress (*21*). With regard to neuronal protective activity, acteoside has been shown to inhibit glutamate-induced neurotoxicity

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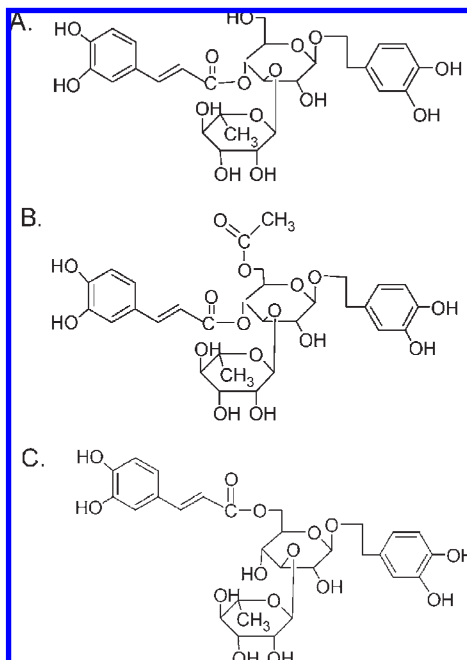

Figure 1. Chemical structures of (**A**) acteoside, (**B**) 6-*O*-acetylacteoside, and (**C**) isoacteoside.

through blocking intracellular Ca^{2+} influx (*22*, *23*), and Lee et al. (*24*) have reported that acteoside reverses scopolamine-induced memory deficits and may have antiamnesic activity. A recent report demonstrated that acteoside exerts antitumor activity on human HL-60 promyelocytic leukemia cells and that this action may be mediated by decreasing G1 cyclin-dependent kinase (CDKs) activities, which then induces cell cycle arrest at the G0/G1 phase (*25*).

The endothelial cells play a critical role in regulation of vascular tone, homeostasis, and inflammation. Increased numbers of monocytes adhering to the vascular endothelium and their subsequent recruitment into the artery wall are initial events in the pathogenesis of atherosclerosis and inflammation (*26*). The surface membrane proteins of intercellular cell adhesion molecule-1 (ICAM-1) and the vascular cell adhesion molecule-1 (VCAM-1) are the two most important adhesion molecules involved in leukocytes recruitment onto endothelial and smooth muscle cells (*27*). The leukocyte CD11/CD18 (beta 2 integrins) adhesion molecules and the very late antigen-4 (VLA-4) are the ligands for ICAM-1 and VCAM-1 respectively (*28*). The expression of ICAM-1 and VCAM-1 is elevated at sites of inflammation in response to various stimuli, such as the pro-inflammatory cytokines interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ). The induction of adhesion molecules in endothelial cells by these pro-inflammatory cytokines is regulated at the level of gene transcription and requires the transcription factor activating protein-1 (AP-1) and the nuclear factor-κB (NF-κB) (*29*, *30*). The mammalian mitogen-activated protein kinase (MAPK) family has three members including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and the p38 kinases. IL-1β is able to activate these three MAPK members and leading to activation of AP-1 (*31*), but depending on the cell type, the pathways do not necessarily lead to NF-κB activation. Therefore, it may be beneficial in attenuating the inflammation by blocking the activity of AP-1 and NF-κB in order to decrease the expression of ICAM-1 and VCAM-1 on endothelial cells so as to inhibit the interaction between leukocytes and endothelial cells (*32*, *33*).

The expression of CAMs on vascular endothelium is a critical event in attracting leukocytes into damaged tissues to induce inflammatory response. Acteoside and similar compounds display anti-inflammatory properties in several *in vitro* assay models. However, it is unclear whether acteoside and similar compounds may inhibit the expression of CAMs and how they may do so mechanistically. In the present study, we examined the inhibitory effects of acteoside, isoacteoside and 6-*O*-acetylacteoside on leukocyte adhesion to human umbilical vein endothelial cells (HUVECs) and the expression of ICAM-1 and VCAM-1 induced by a pro-inflammatory cytokine, IL-1β. In addition, we investigated the effect of acteoside and 6-*O*-acetylacteoside on phosphorylation of activation of ERK, JNK, and p38, all of which participate in the expression of ICAM-1 and VCAM-1.

MATERIALS AND METHODS

Materials and Cell Culture. Acteoside (isolated from *P. tomentosa*), isoacteoside (from *H. procumbens*), and 6-*O*-acetylacteoside (from *H. procumbens*) were purchased from Equl Corporation (Shanghai, China), and the purities of each of these three compounds exceeded 98%. Human umbilical vein endothelial cells (BCRC H-UV001) and human promyelocytic leukemia HL-60 (BCRC 60027) were obtained from Food Industry Research and Development Institute (Hsinchu, Taiwan) and were cultured in commercial Endothelial Cells Growth Medium (Cell Application Inc., San Diego, CA) and RPMI 1640 medium containing 10% fetal bovine serum (Invitrogen Taiwan Ltd., Taiwan), respectively. Cultures were maintained at 37 °C with a gas mixture of 5% CO_2 and 95% air.

MTT Assay. HUVECs were cultured in a 24-well plate and treated with acteoside, isoacteoside, or 6-*O*-acetylacteoside for 24 h. The cell viability was determined by MTT assay as described previously (*34*).

Western Blotting. Equal amounts of total cellular protein (25−50 μg) were resolved by SDS−polyacrylamide gel electrophoresis (SDS−PAGE), and transferred onto Immobilon-P membranes (Millipore, Bedford, MA), as described previously (*35*). The membrane was then incubated with anti-α-tubulin (Zymed Laboratories Inc., South San Francisco, CA), anti-IκB, anti-ERK1, antiphospho-ERK, anti-JNK, antiphospho-JNK, anti-p38, antiphophso-p38, anti-VCAM-1 or anti-ICAM-1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and subsequently probed with secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and visualized using Enhanced Chemiluminescence kits (Santa Cruz Biotechnology). The density of the bands was quantified using a computerized densitometer (Alpha Innotech IS-1000 Digital Imaging System).

Endothelial Cell−Leukocyte Adhesion Assay. HL-60 cells were labeled with [^{3}H]-thymidine by incorporating [^{3}H]-thymidine into its DNA for 2 h, after which cells were washed by centrifugation. HUVECs treated with acteoside, isoacteoside, or 6-*O*-acetylacteoside in 6-well plates were washed thrice, and [^{3}H]-thymidine-labeled HL-60 cells were added to each well of the HUVECs. HL-60 cells were allowed to adhere to HUVECs by incubation at 37 °C for 30 min, after which unbound HL-60 cells were removed by washing. HL-60 cells bound to HUVECs were determined by lysis of total cells with 50 mM Tris, pH 8.0, and 1% SDS, and the radioactivity (cpm) was counted using a Beckman L-600 scintillator.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was isolated, and the RNA (2 μg) was reverse-transcribed at 42 °C for 1 h by adding 5 μM oligo dT, 200 units of reverse transcriptase (Promega Corporation, Madison, WI), 2.5 mM deoxyribonucleotide triphosphates (dNTP) (Promega), and 10 mM dithiothreitol to obtain the cDNA, as described previously (*35*). The semiquantitative PCR was carried out with 2 μL of cDNA and 23 μL of PCR mix buffer containing each primer (0.2 μM), dNTP (2.5 mM), and Taq DNA polymerase (1.25 units) (Promega). After the PCR, 10 μL of the reaction mixture was subjected to electrophoresis on a 1.5% agarose gel, and the PCR products were visualized by SYBR green staining. The density of the bands was quantified using a computerized densitometer (Alpha Innotech IS-1000 Digital Imaging System). Semiquantitative PCR primers for the human VCAM-1, ICAM-1, and GADPH cDNA were synthesized according to the following oligonucleotide sequences: VCAM-1, forward primer 5′-AGATAGATAGTCCACTGAATG-3′, reverse primer 5′-ACAAGTCACTGTGCACAGATA-3′; ICAM-1, forward primer

5′-AGCAATGTGCAAGAAGATAGC-3′, reverse primer 5′-AAGATCTCGAGTGACAGTCAC-3′; and GAPDH, forward primer 5′-ACCACAGTCCATGCCATCAC-3′, reverse primer 5′-TCCACCACCCTGTTGCTGTA-3′.

Transition Transfection. The VCAM-1/chloramphenicol acetyltransferase (VCAM-1/CAT) reporter plasmid was generously provided by Dr. Douglas Dean (Washington University, St. Louis, MO) and has been characterized previously (*36*). HUVECs were seeded in 6-well plates. When cells were confluent, the cells were transfected with the VCAM-1/CAT reporter plasmid or pNF-κB-Luc reporter plasmid (Stratagene, La Jolla, CA), and phRL-TK (Promega, Madison, WI) as an internal control plasmid using LipofectAMINE2000 (Invitrogen Taiwan Ltd., Taiwan). After transfection, cells were pretreated with test compounds for 30 min and then treated with IL-1β (2 ng/mL) for another 8 or 18 h. Each well of the cell lysate was used to assay chloramphenicol acetyltransferase (for VCAM-1/CAT) or luciferase (for pNF-κB-Luc and phRL-TK) activities by a CAT ELISA kit (Roche Diagnostics Ltd., Taiwan) and a Dual-Luciferase reporter assay kit (Promega Corporation, Madison, WI), respectively. Luminescence was measured using a Plate Chameleon Multilabel plate reader (HIDEX OY, Turku, Finland). CAT or luciferase activity of reporter plasmids was normalized to luciferase activity of the internal control plasmid.

Statistical Analysis. Cell experimental data were analyzed by one-way ANOVA and following by Tukey's multiple comparison test (GraphPad Prism Software, San Diego, CA). The data were expressed as the mean ± SD, and differences were considered to be significant at $p < 0.05$.

RESULTS

Acteoside, Isoacteoside, and 6-*O*-Acetylacteoside Inhibit Leukocyte Adhesion to IL-1β-Activated HUVECs. We first examined whether acteoside, isoacteoside, and 6-*O*-acetylacteoside block leukocyte adhesion to endothelial cells HUVECs activated by IL-1β. As shown in **Figure 2A**, IL-1β markedly increased leukocyte adhesion, and 100 μM acteoside, isoacteoside, and 6-*O*-acetylacteoside significantly inhibited leukocyte adhesion to IL-1β-activated HUVECs. The inhibition of leukocyte adhesion to HUVECs was in the following order: 6-*O*-acetylacteoside > acteoside > isoacteoside. Moreover, acteoside and 6-*O*-acetylacteoside inhibited leukocyte adhesion to HUVECs in a dose-dependent manner (**Figures 2B** and **2C**). The results suggest that these three test compounds possess anti-inflammatory activity against IL-1β action in HUVECs. The effects of the three test compounds are not attributed to their inhibition of cell viability, because HUVECs incubated with acteoside, isoacteoside, or 6-*O*-acetylacteoside at 50−200 μM for 24 h did not significantly affect the cell viability, as determined using the MTT assay (**Figure 3**).

Acteoside, Isoacteoside, and 6-*O*-Acetylacteoside Inhibit IL-1β-Induced Expression of ICAM-1 and VCAM-1 in HUVECs. Western blot, RT-PCR, and transition transfection experiments were conducted to examine whether the blockade of leukocyte adhesion to HUVECs was mediated through inhibiting IL-1β-induced ICAM-1 and VCAM-1 expression. As expected, IL-1β significantly induced protein expressions of ICAM-1 and VCAM-1, and 100 μM of acteoside, isoacteoside, or 6-*O*-acetylacteoside markedly inhibited the protein expressions of ICAM-1 and VCAM-1 induced by IL-1β (**Figure 4A**). 6-*O*-Acetylacteoside exhibited the highest inhibitory effects, followed by acteoside and isoacteoside (in this order), on adhesion molecule expression. Because isoacteoside showed the lowest effects on leukocyte adhesion to HUVECs (**Figure 2A**) and on adhesion molecule expression (**Figure 4A**), we chose acteoside and 6-*O*-acetylacteoside for the following experiments. When acteoside and 6-*O*-acetylacteoside were added at concentrations between 25 and 150 μM, the protein expressions of ICAM-1 and VCAM-1 by IL-1β were inhibited in a dose-dependent manner (**Figures 4B** and **4C**).

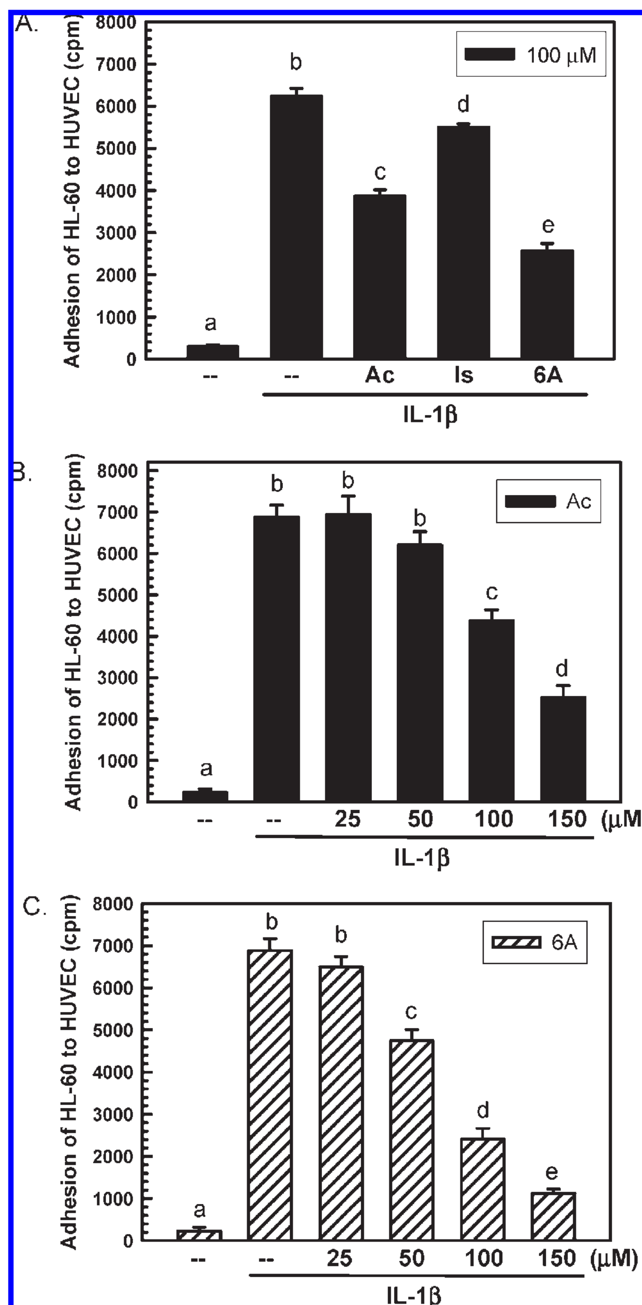

Figure 2. Effects of acteoside (Ac), isoacteoside (Is), and 6-*O*-acetylacteoside (6A) on leukocyte adhesion to HUVECs induced by IL-1β. (**A**) Cells were pretreated with 100 μM Ac, Is or 6A for 30 min, then treated with IL-1β (2 ng/mL) for 24 h, and [^{3}H]-thymidine-labeled HL-60 cells were added for additional 30 min. (**B**) Cells were pretreated with Ac (25−150 μM) for 30 min before incubation with IL-1β and [^{3}H]-thymidine-labeled HL-60 cells, as described above. (**C**) Cells were pretreated with 6A (25−150 μM) for 30 min, then treated with IL-1β and [^{3}H]-thymidine-labeled HL-60 cells, as described above. At the end of incubation, cells were washed, and the adhesion of HL-60 to HUVECs was determined. Values (means ± SD of triplicate tests) not sharing the same superscript letter are significantly different ($P < 0.05$).

To examine whether acteoside and 6-*O*-acetylacteoside inhibited IL-1β-induced ICAM-1 and VCAM-1 expression at the transcription level, we used semiquantitative RT-PCR to determine the mRNA levels of ICAM-1 and VCAM-1. **Figure 5** shows that 100 μM aceteoside (**Figure 5A**) and 100 μM 6-*O*-acetylacteoside (**Figure 5B**) markedly inhibited the IL-1β-induced mRNA

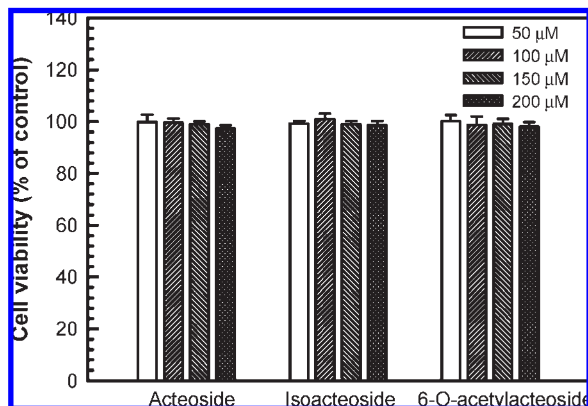

Figure 3. Effects of acteoside (Ac), isoacteoside (Is), and 6-*O*-acetylacteoside (6A) on cell viability in HUVECs. Cells were incubated with various concentrations of Ac, Is, or 6A for 24 h, and the cell viability was determined by MTT assay. The values are presented as means ± SD of triplicate tests.

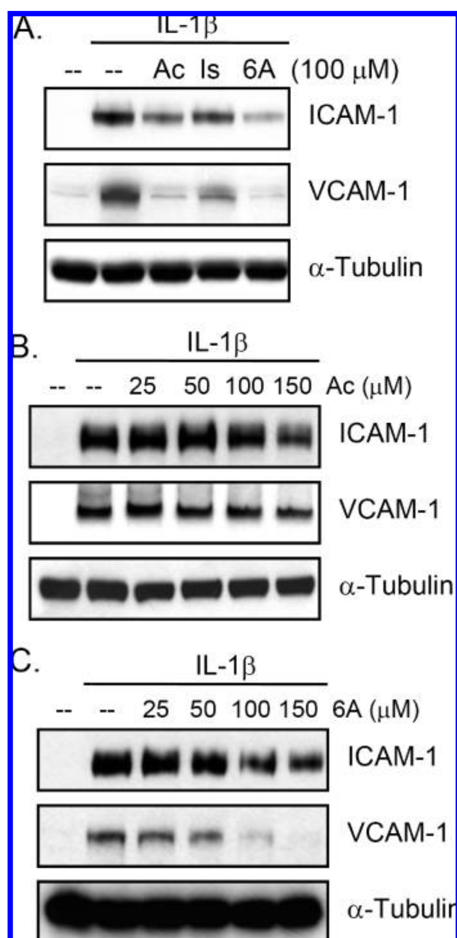

Figure 4. Effects of acteoside (Ac), isoacteoside (Is), and 6-*O*-acetylacteoside (6A) on the protein expression of ICAM-1 and VCAM-1 induced by IL-1β (2 ng/mL) in HUVECs. (**A**) Cells were pretreated with Ac, Is or 6A (100 μM) for 30 min, then treated with IL-1β for 24 h. (**B**) Cells were pretreated with Ac (25−150 μM) for 30 min, then treated with IL-1β for 24 h. (**C**) Cells were pretreated with 6A (25−150 μM) for 30 min, then treated with IL-1β for 24 h. The protein levels of ICAM-1 and VCAM-1 were detected by Western blot analysis.

expression of ICAM-1. Quantification of the data in **Figures 5A** and **5B** using computerized densitometer (IS-1000 Digital Imaging System) indicated that 100 μM acteoside significantly

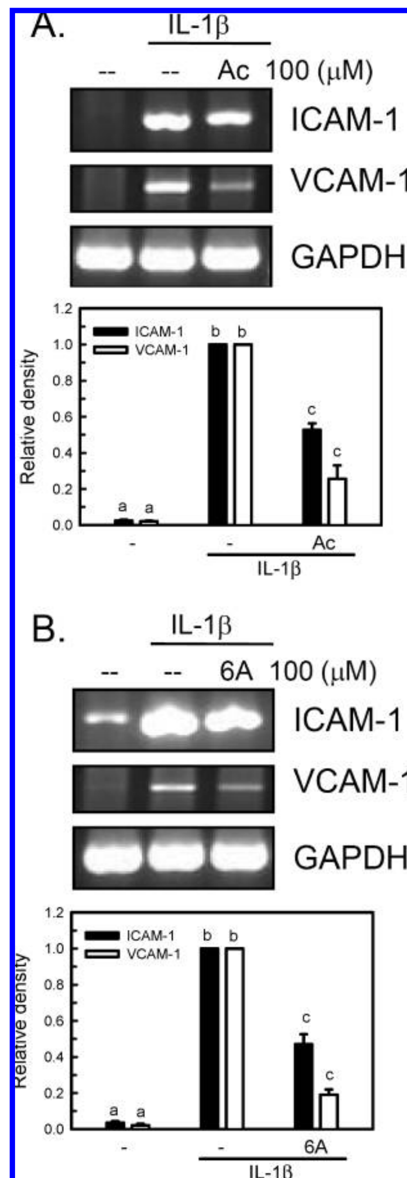

Figure 5. Effects of acteoside (Ac) and 6-*O*-acetylacteoside (6A) on mRNA expression of ICAM-1 and VCAM-1 in HUVECs induced by IL-1β (2 ng/mL). Cells were pretreated with 100 μM of Ac (**A**) or 6A (**B**) for 30 min, then treated with IL-1β for 8 h. The mRNA levels of ICAM-1 and VCAM-1 were detected by RT-PCR analysis and quantified by a computerized densitometer. Values (means ± SD of triplicate tests) not sharing the same superscript letter are significantly different ($P < 0.05$).

inhibited ICAM-1 and VCAM-1 by 47% and 74%, respectively. Similarly, 100 μM 6-*O*-acetylacteoside significantly inhibited ICAM-1 and VCAM-1 by 53% and 81.0%, respectively.

In a transition transfection experiment, HUVECs cells were transfected with VCAM-1 reporter plasmid containing VCAM-1 promoter (−288 to +12) and CAT reporter genes, and these cells showed a significant induction of CAT activity by IL-1β. Similar to the VCAM-1 mRNA levels, acteoside and 6-*O*-acetylacteoside significantly inhibited the VCAM-1 promoter activity induced by IL-1β in a dose-dependent manner (**Figure 6**). These results suggest that acteoside and 6-*O*-acetylacteoside are able to inhibit ICAM-1 and VCAM-1 expression at the transcription level.

Acteoside and 6-*O*-Acetylacteoside Inhibit IL-1β-Induced Activation of ERK and JNK in HUVECs. Because the activation of the transcription factors AP-1 and NF-κB is critical for induction of ICAM-1 and VCAM-1 expression by IL-1β, we determined

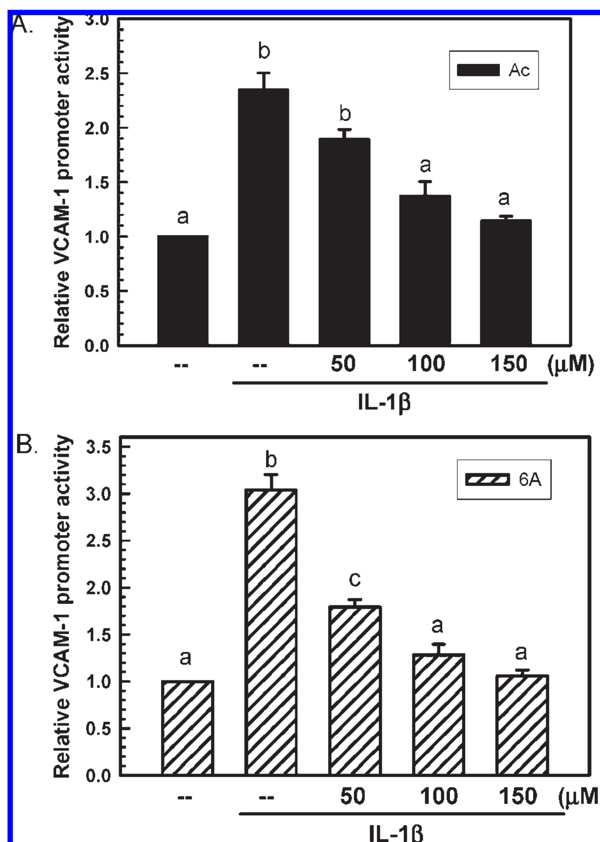

Figure 6. Effects of acteoside (Ac) and 6-*O*-acetylacteoside (6A) on the activity of VCAM-1 gene promoter induced by IL-1β (2 ng/mL) in HUVECs. Transfected cells were pretreated with various concentrations of (**A**) Ac or (**B**) 6A for 30 min, then treated with IL-1β for another 18 h. The total protein was subsequently assayed for chloramphenicol acetyltransferase and luciferase activities, as described in Materials and Methods. Values (means $\pm$ SD of triplicate tests) not sharing the same superscript letter are significantly different ($P < 0.05$).

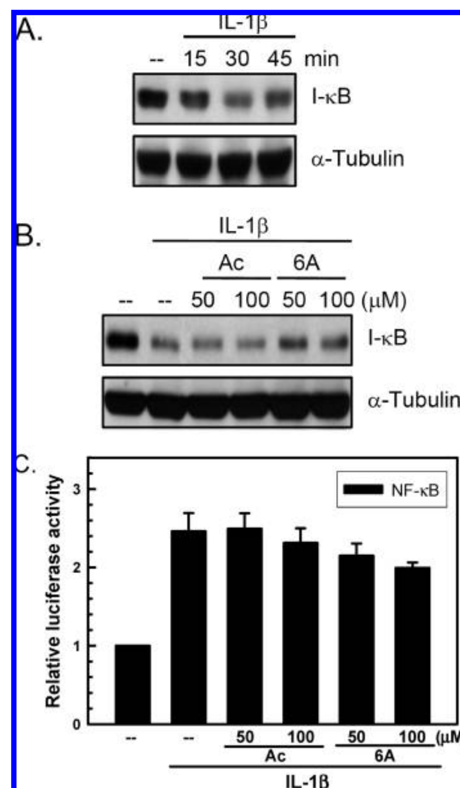

Figure 7. Effects of acteoside (Ac) and 6-*O*-acetylacteoside (6A) on IκB degradation and NF-κB transcriptional activity induced by IL-1β (2 ng/mL) in HUVECs. (**A**) Cells were treated with IL-1β for the indicated time points, and IκB protein level was detected by Western blot analysis. (**B**) Cells were pretreated with Ac or 6A for 30 min, then treated with IL-1β for 30 min, after which IκB protein level was detected by Western blot analysis. (**C**) Cells were transfected with pNF-κB-Luc reporter plasmid and phRL-TK plasmid as an internal control. After transfection, cells were pretreated with Ac or 6A for 30 min, then treated with IL-1β for another 8 h. The values (means $\pm$ SD of triplicate tests) in (**C**) are not significantly different among the five groups treated with IL-1β.

whether acteoside and 6-*O*-acetylacteoside suppress the AP-1 and NF-κB signal pathways in IL-1β-activated HUVECs. As shown in **Figure 7A**, IL-1β induced the transient degradation of IκB, and the effect was strongest at 30 min of stimulation. Under this experimental condition (i.e., 30 min induction with IL-1β), acteoside and 6-*O*-acetylacteoside failed to prevent the degradation of IκB, although there was a slight recovery of IκB by 6-*O*-acetylacteoside (**Figure 7B**). In addition, neither acteoside nor 6-*O*-acetylacteoside significantly inhibited NF-κB activity by transient transfection with pNF-κB-Luc report plasmid (**Figure 7C**). These results suggest that inhibition of ICAM-1 and VCAM-1 expression by acteoside and 6-*O*-acetylacteoside might not be mediated through inhibiting the NF-κB signal pathway.

Because the activation of AP-1 depends primarily on the phosphorylation of the MAPK signal pathways, we then determined the effect of acteoside and 6-*O*-acetylacteoside on the phosphorylation of ERK, JNK, and p38 in IL-1β-activated HUVECs. We found that IL-1β markedly increased the phosphorylation of ERK, JNK, and p38, which peaked at 30 min, 30 min, and 60 min, respectively (**Figure 8A**). We then found that acteoside and 6-*O*-acetylacteoside inhibited the levels of p-ERK and p-JNK (30 min induction with IL-1β), but did not affect that of p-p38 (60 min induction with IL-1β) (**Figure 8B**). Neither acteoside nor 6-*O*-acetylacteoside affected the protein levels of ERK, JNK or p38. Quantification of the data in **Figure 8B** using computerized densitometer (IS-1000 Digital Imaging System) indicated that both acteoside and 6-*O*-acetylacteoside significantly inhibited the phosphorylation levels of ERK and JNK, respectively (**Figure 8C**).

To further demonstrate the importance of the activation of ERK and JNK to ICAM-1 and VCAM-1 expression in IL-1β-activated HUVECs, PD98059 and SP600125 were used to inhibit ERK and JNK, respectively. As shown in **Figure 8D**, 1.0 μM PD98059 and 5 μM SP600125 markedly inhibited the protein expression of ICAM-1 and VCAM-1 in IL-1β-activated HUVECs. The results suggest that both ICAM-1 and VCAM-1 expression are partially mediated by the activation of ERK and JNK, and that acteoside and 6-*O*-acetylacteoside may inhibit ICAM-1 and VCAM-1 expression through suppressing ERK and JNK phosphorylation.

DISCUSSION

Upregulation of CAMs on vascular endothelium is a key step in the pathogenesis of atherosclerosis and inflammation. Although previous studies have demonstrated the anti-inflammatory activity of acteoside and similar compounds in several cell models, there is little or no information about their effects on the expression of CAMs in HUVECs. The present study clearly demonstrated that acteoside, isoacteoside and 6-*O*-acetylacteoside were effective in reducing ICAM-1 and VCAM-1 expression in

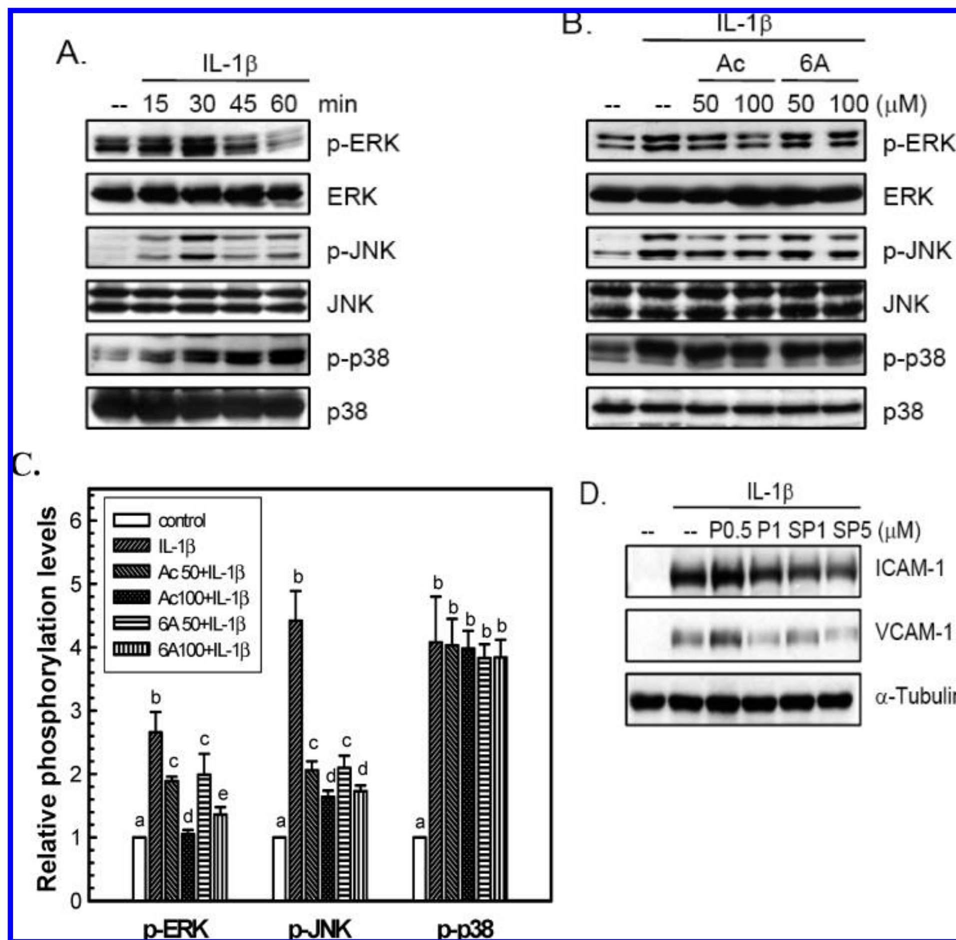

Figure 8. Effects of acteoside (Ac) and 6-*O*-acetylacteoside (6A) on the expression of MAPK proteins induced by IL-1β (2 ng/mL) in HUVECs. (**A**) Representative Western blots of ERK, JNK, and p38 and their phosphorylated levels (p-ERK, p-JNK and p-p38) in cells treated with IL-1β for indicated time points. The numbers below the bands indicate the intensities relative to the individual IL-1β control levels. (**B**) Cells were pretreated with Ac or 6A for 30 min, and then treated with IL-1β for 30 min (for ERK and JNK) or 60 min (for p38). (**C**) Quantification of data from panel **B** by computerized densitometer; data are means $\pm$ SD of triplicate tests. (**D**) Cells were pretreated with ERK inhibitor PD98059 (0.5 μM and 1 μM) or JNK inhibitor SP600125 (1 μM and 5 μM) for 30 min, and then treated with IL-1β for 24 h.

HUVECs, with inhibition potency in the order 6-*O*-acetylacteoside > acteoside > isoacteoside. This inhibition was associated with the inhibition of leukocyte adhesion to the endothelial cells, thereby reducing recruitment of leukocytes and, as a result, inhibiting the inflammatory responses. Mechanistically, acteoside and 6-*O*-acetyl acteoside may inhibit ICAM-1 and VCAM-1 expression through the inhibition of the ERK and JNK signal pathways (**Figure 8**).

Reactive oxygen species (ROS) can lead to vascular pathology through several mechanisms, including the promotion of low-density lipoprotein oxidation (*37*, *38*), cytotoxicity to endothelial and vascular smooth muscle cells (*39*, *40*), and increased expression of ICAM-1 and VCAM-1 (*41*), all of which are pertinent to atherogenesis. As with other pro-inflammatory cytokines, IL-1β also stimulates production of ROS in various cell types (*42*−*45*). The activation of MAPKs by IL-1β occurs in an ROS sensitive manner (*46*). In this study, we found that acteoside and 6-*O*-acetylacteoside inhibited the phosphorylation of ERK and JNK, and this effect might be associated with their capacity as ROS scavengers. Indeed, previous studies (*10*, *47*) have shown that acteoside and its derivatives exhibit antioxidative activity in different assay systems. Epidemiological studies have also indicated that natural antioxidative products, such as flavonoids, are associated with a reduced risk of cardiovascular diseases (*48*). Our results suggest that acteoside and its derivatives also have the potential to decrease the risk for cardiovascular diseases.

It is well-known that NF-κB activation is first mediated by the phosphorylation and degradation of the inhibitor of κB (IκB), which then releases NF-κB from IκB/NF-κB complexes. In the present study, we found that acteoside and 6-*O*-acetylacteoside did not prevent IκB degradation induced by IL-1β (**Figure 7B**). Using transition transfection assay, we further demonstrated that acteoside and 6-*O*-acetylacteoside did not inhibit IL-1β-induced NF-κB transactivation activity (**Figure 7C**). These results suggest that the inhibition of ICAM-1 and VCAM-1 expression by acteoside and 6-*O*-acetylacteoside may not be mediated through the inhibition of NF-κB transactivation activity. However, estimation of NF-κB transactivation activity by pNF-κB-Luc reporter plasmid may not represent the true action of NF-κB in the gene promoter of ICAM-1 and VCAM-1. In addition, the gene promoter of ICAM-1 and VCAM-1 contains additional responsive elements for the binding of other transcription factors which may cooperate with NF-κB to regulate NF-κB activity. Therefore, we cannot exclude the possibility that acteoside and 6-*O*-acetylacteoside may inhibit IL-1β-induced adhesion molecule expression through decreased NF-κB transactivation activity of other transcription factors.

In summary, we have demonstrated that, of the three test compounds, 6-*O*-acetylacteoside exhibits the strongest inhibition on the expression of CAMs and leukocyte adhesion to HUVECs, while isoacteoside has the lowest inhibition (**Figure 2A** and

Figure 4A). The results suggest that the inhibitory effects of these acteoside derivatives are dependent on its structure, i.e., the side chain position of caffeic acid has a major effect, since the caffeic acid moiety of isoacteoside is at the 6 position of the glucose moiety, whereas that of acteoside and 6-*O*-acetylacteoside is at the 4 position (**Figure 1**). In addition, the acetyl group of 6-*O*-acetylacteoside appears to be helpful for the anti-inflammatory activity, possibly by increasing lipid solubility leading to enhanced cell uptake, as 6-*O*-acetylacteoside was somewhat stronger than acteoside in the inhibition of leukocyte adhesion to HUVECs and in the downregulation of VCAM-1 and ICAM-1 protein expression in HUVECs (**Figure 2A** and **Figure 4A**). The possible relation between the structural properties of acteoside and anti-inflammatory activity deserves further investigation.

ABBREVIATIONS USED

ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen-4; HUVECs, human umbilical vein endothelial cells; AP-1, activating protein-1; NF-κB, nuclear factor-κB; IκB, inhibitor of κB; GADPH, glyceraldehyde-3-phosphate dehydrogenase; MAPKs, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; DPPH, 1,1-diphenyl-2-picryl-hydrazyl; CAT, chloramphenicol acetyltransferase; Ac, acteoside; Is, isoacteoside; 6A, 6-*O*-acetylacteoside; CDK, cyclin-dependent kinase; RT-PCR, reverse-transcription polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

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