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Caffeic Acid Disturbs Monocyte Adhesion onto Cultured Endothelial Cells Stimulated by Adipokine Resistin

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ABSTRACT: Adipokines have been implicated in the pathogenesis of atherosclerosis via pro-inflammatory mechanisms contributing to insulin resistance. The adipokine resistin causes endothelium dysfunction, which plays an important role in sustaining atherogenesis. This study investigated whether resistin induced expression of cell adhesion molecules and integrins in endothelial cells and THP-1 monocytes and whether such induction was attenuated by 1−20 μM caffeic acid. Resistin enhanced endothelial expression of vascular cell adhesion molecule 1 (VCAM-1), intercellular cell adhesion molecule 1 (ICAM-1), and E-selectin and monocyte expression of β1, β2, and α4 integrins. The enhancement of these proteins was diminished by caffeic acid with reduced THP-1 cell adhesion on activated endothelium. Caffeic acid at $\leq$20 μM demoted resistin-stimulated interleukin 8 (IL-8) production responsible for ICAM-1 and β2 integrin induction. The endothelial up-regulation of IL-8 secretion by resistin entailed toll-like receptor 4 (TLR4) activation, but caffeic acid diminished IL-8 production and TLR4 induction. Furthermore, caffeic acid encumbered resistin-activated nuclear factor κB (NF-κB) signaling. These results demonstrate that caffeic acid blocked monocyte trafficking to resistin-activated endothelium via disturbing NF-κB signaling responsive to IL-8. Therefore, caffeic acid may have therapeutic potential in preventing obesity-associated atherosclerosis.

KEYWORDS: *atherosclerosis, caffeic acid, endothelial cells, monocytes, resistin*

INTRODUCTION

Atherosclerosis is a chronic and progressive inflammatory disease that leads to advanced cardiovascular diseases.[1,2] Vascular inflammation causes leukocyte transmigration responsible for vessel remodeling and atherosclerotic plaque formation.[3] Endothelial migration of leukocytes begins with a multistep adhesion process that recruits leukocytes from circulation.[4,5] The vascular endothelial cells are a major target of pro-inflammatory cytokines that modulate expression of many genes involved in inflammation, thrombosis, and coagulation.[6,7] Tight adhesion of leukocytes to activated endothelium is mediated by the leukocyte integrins and their endothelial cell counterreceptors.[4,8] Adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular cell adhesion molecule 1 (ICAM-1) play an important role in inducing inflammation.[5,8] In addition, chemokines induce integrins on rolling leukocytes that bind to vascular ligands.[9] One of these integrins is the heterodimeric lymphocyte function-associated antigen 1 (LFA-1, αL/β2 integrin) that interacts with ICAM-1, an endothelial cell counterreceptor, upon inflammation.[10]

Obesity is a main risk factor for various diseases such as atherosclerosis, type 2 diabetes, and cancer. Adipose tissue is an active organ that secretes several proteins known as adipokines that participate in the regulation of physiological and pathological processes including inflammation and metabolism.[11] Resistin is a novel adipokine secreted by fat cells that elevates glucose tolerance and insulin resistance.[12] Rodent resistin is produced in adipocytes, whereas macrophages are a major source of human resistin.[13] It has been found that resistin participates in the inflammatory response.[12,13] Resistin increases transcriptional events leading to an increased expression of several pro-inflammatory cytokines including interleukin 1 (IL-1) and tumor necrosis factor α (TNF-α).[14] In addition, resistin up-regulates ICAM-1, VCAM1, and monocyte chemotactic protein (MCP) 1, all of which are involved in chemotactic pathways responsible for leukocyte recruitment to sites of inflammation.[15] Resistin per se can be up-regulated by IL and also by the endotoxin lipopolysaccharide (LPS).[16] Accordingly, diabetes-associated resistin may be a cross-link in the close relationship between inflammation and insulin resistance.[17]

Caffeic acid (Figure 1A), which is unrelated to caffeine, is a naturally occurring phenolic compound especially abundant in coffee. It is also found in all plants due to a key intermediate in the biosynthesis of lignin. Caffeic acid has been shown to be an antioxidant and to exhibit immunomodulatory and anti-inflammatory activity.[18] Caffeic acid protects skin cells through exerting anti-inflammatory and anticancer properties when exposed to ultraviolet radiation.[19] In addition, caffeic acid reduces DNA methylation in human cancer cell lines that contributes to tumor growth and regulates epigenetics.[20] The phenolic caffeic acid may prevent atherosclerosis via inhibition of both low-density lipoprotein (LDL) oxidation and reactive oxygen species (ROS) production.[21] Oral supplementation of caffeic acid phenethyl ester attenuates the atherosclerotic process and reduces nuclear factor κB (NF-κB) activity and expression of NF-κB-related genes.[22] Accordingly, these anti-inflammatory and antiatherogenic actions of caffeic acid might be involved in resistin-associated atherosclerosis. Caffeic acid phenethyl ester was shown to suppress the production of leptin

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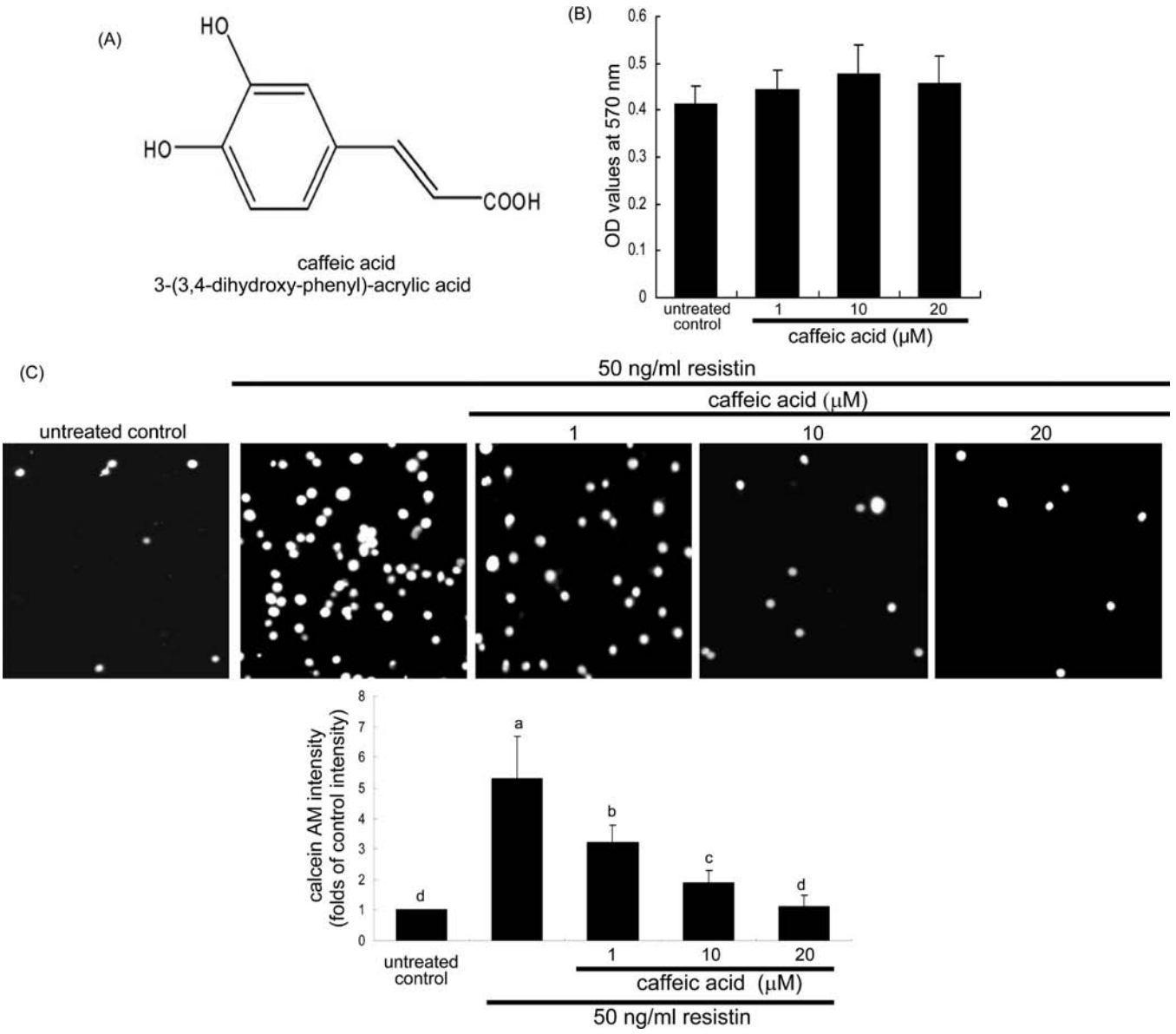

Figure 1. (A) Structure of caffeic acid and (B) viability of HUVEC after incubation with caffeic acid. Cells were treated with 1−20 μM caffeic acid for 24 h. HUVEC viability was measured by using MTT assay after the 24 h culture. Values are mean ± SEM (n = 4) and expressed as percent cell survival relative to caffeic acid-untreated controls (viability = 100%). (C) Microphotographs showing inhibition of THP-1 monocyte adhesion onto resistin-exposed HUVEC by caffeic acid. Photograph images (three independent experiments) were obtained by fluorescence microscopy with fluorescein blue filter. Magnification: 200×. The bar graphs (bottom panel, n = 3) represent quantitative results obtained by use of a Fluoroscan ELISA plate reader at λ = 485 nm excitation and λ = 538 nm emission. Means without a common letter differ, $P < 0.05$.

and resistin during differentiation to adipocytes in 3T3-L1 cells.[23]

On the basis of the evidence that caffeic acid possesses anti-inflammatory activity, this study investigated inhibitory effects of caffeic acid on obesity-associated early atherosclerosis by examining adipokine resistin-induced monocyte recruitment onto endothelial cells. Induction of endothelial cell adhesion molecules and monocyte integrins was determined in caffeic acid-treated human umbilical vein endothelial cells (HUVEC) and THP-1 monocytic cells exposed to resistin. In addition, IL-8 secretion and toll-like receptor 4 (TLR4) expression was measured in caffeic acid-supplemented and resistin-treated HUVEC. Furthermore, this study explored NF-κB-associated mechanisms of caffeic acid's actions. This study found that caffeic acid antagonized resistin-activated monocyte trafficking to endothelium via disturbing NF-κB signaling responsive to inflammatory IL-8.

MATERIALS AND METHODS

Chemicals. M199 medium chemicals, RPMI1640 medium chemicals, and 3-(4, 5-dimetylthiazolyl)diphenyltetrazolium bromide (MTT) were obtained from Sigma−Aldrich Chemical Co. (St. Louis, MO), as were all other reagents, unless specifically stated elsewhere. Fetal bovine serum (FBS), penicillin−streptomycin, trypsin−ethylenediaminetetraacetic acid (EDTA), human epidermal growth factor (hEGF), and hydrocortisone were purchased from Cambrex (East Rutherford, NJ). Human monocytic leukemic cell line THP-1 was obtained from American Type Culture Collection (Manassas, VA). Resistin was obtained from Phoenix Phamaceuticals

(Burlingame, CA). Antibodies against human VCAM-1, human ICAM-1, human platelet endothelial cell adhesion molecule (PECAM) 1, and human integrins $\beta 2$, $\alpha 4$, and $\beta 1$ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Human IL-8 and human E-selectin were purchased from R&D Systems (Minneapolis, MN). Goat anti-rabbit IgG and goat anti-mouse IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Preparation and Culture of Human Umbilical Vein Endothelial Cells. HUVEC were isolated from human umbilical cords by use of collagenase, as previously described elsewhere.[24] Human umbilical cords were obtained from the Department of Obstetrics and Gynecology, Chuncheon Sacred Heart Hospital (Chuncheon, Korea). Cells were cultured in 25 mM *N*-(2-hydroxyethylpiperazine)-*N*′-2 ethanesulfonic acid (HEPES)-buffered M199 containing 10% FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/mL streptomycin supplemented with 0.075 mg/mL hEGF and 0.075 mg/mL hydrocortisone. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. Endothelial cells were passaged at confluence and used for experiments within 10 passages. HUVEC were plated at 90−95% confluence in all experiments. Cells were pretreated for 2 h with 1−20 μM caffeic acid and exposed to 50 ng/mL resistin or 10 ng/mL IL-8 for different times.

At the end of the incubation with 1−20 μM caffeic acid for 24 h, MTT assay was carried out to test cellular toxicity. HUVEC were incubated in fresh medium containing 1 mg/mL MTT for 3 h at 37 °C. The purple formazan product was dissolved in 0.5 mL of 2-propanol with gentle shaking. Absorbance of formazan was measured at λ = 570 nm by use of a Bio-Rad Model 550 microplate reader (Hercules, CA). In this study, nontoxic doses of caffeic acid were ≤20 μM (Figure 1B).

Culture of THP-1 Monocytes and in Vitro Cell Adhesion Assay. HUVEC were grown in 25 mM HEPES-buffered M199 at density of 7.0×10^4 cells on 24-well glass chamber slides. THP-1 cells were labeled with 5 μM calcein-AM (Molecular Probes, Eugene, OR) in RPMI 1640 medium containing 10% FBS. HUVEC were pretreated for 2 h with 1−20 μM caffeic acid and exposed to 50 ng/mL resistin for 8 h. Calcein-AM-labeled THP-1 cells were seeded at a density of 5.0×10^5 cells/mL on caffeic acid-treated and resistin-activated HUVEC and incubated for 1 h. After cocultured cells were thoroughly washed with phosphate-buffered saline (PBS), photograph images were obtained at λ = 485 nm excitation and λ = 538 nm emission by use of a SPOT II digital camera-attached fluorescence microscope (Diagnostic Instrument, Livingston, Scotland).

Protein Isolation and Western Blot Analysis. After culture protocols, whole HUVEC lysates were prepared in 1 M Tris-HCl (pH 6.8) lysis buffer containing 10% sodium dodecyl sulfate (SDS), 1% β-glycerophosphate, 0.1 M Na_3VO_4, 0.5 M NaF, and protease inhibitor cocktail. Cell lysates containing equal protein amounts were electrophoresed on SDS−8−10% polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by soaking the membrane in TBS-T buffer [0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl, and 0.1% Tween 20] containing 5% nonfat dry milk for 3 h. The membrane was incubated overnight with primary antibodies of specific proteins. After three washes with TBS-T buffer, the membranes were then incubated for 1 h with goat anti-rabbit IgG, donkey anti-goat IgG, or goat anti-mouse IgG conjugated to horseradish peroxidase. The protein levels were determined by using Supersignal West Pico chemiluminescence detection reagents (Pierce Biotechnology, Rockford, IL) and Agfa medical X-ray film (Gevaert, Belgium). Incubation with anti-human β-actin was performed for the comparative control.

Enzyme-Linked Immunoadsorbent Assay. To measure the secretion of IL-8 from HUVEC, collected culture media were assayed by use of enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) according to the manufacturer's instructions.

Immunocytochemistry. After challenge with 50 ng/mL resistin, HUVEC were thoroughly washed with PBS containing 0.2% Tween 20 (PBS-T), fixed with 4% ice-cold formaldehyde for 30 min, and incubated for 1 h with 20% FBS to block any nonspecific binding. After the cells were washed with PBS-T, rabbit antihuman NF-κB p65 (Santa Cruz Biotechnology) was added and the cells were incubated overnight at 4 °C. Cells were washed with TBS-T and incubated with a Cy3-conjugated goat anti-rabbit IgG as a secondary antibody. Fluorescent images were obtained by an Axiomager Optical fluorescent microscope (Zeiss, Germany).

IL-8 siRNA Transfection. The study investigated the involvement of inflammatory IL-8 in resistin-induced monocyte adhesion. A human specific small interfering RNA (siRNA) kit (Santa Cruz Biotechnology) was used to specifically inhibit IL-8 expression. Nucleofection of HUVEC was performed for the gene delivery according to the optimized protocols provided by the manufacturer (Amaxa Biosystem, Cologne, Germany). Briefly, HUVEC [$(0.5-1) \times 10^6$ cells] were pelleted, gently resuspended in an Amaxa cuvette containing 100 μL of nuclerofector solution (Amaxa nucleofector, HUVEC transfection kit), mixed with 300 nM IL-8 siRNA, and pulsed in the nucleofector device (program A-34 for endothelial cells). Cells were immediately transferred into 6-well plates with 500 μL of prewarmed fresh M199 medium. After nucleofection for 24 h, transfected cells were exposed to 50 mg/mL resistin and cultured for 8 h.

Data Analysis. The results are presented as mean ± SEM for each treatment group in each experiment. Statistical analyses were conducted with Statistical Analysis Systems statistical software package (SAS Institute, Cary, NC). Significance was determined by one-way analysis of variance (ANOVA) followed by Duncan multiple range test for multiple comparisons. Differences were considered significant at $P < 0.05$.

RESULTS

Inhibition of Resistin-Induced Monocyte Adhesion by Caffeic Acid. In vitro adhesion data for the interaction of THP-1 cells with HUVEC are shown in Figure 1C. There were small numbers of monocytes adhered to quiescent HUVEC free of 50 ng/mL resistin. Increased staining was observed in HUVEC exposed to resistin alone, indicating enhanced adherence of THP-1 cells to resistin-activated HUVEC (Figure 1C, ≈5-fold). However, adding caffeic acid at nontoxic doses of 1−20 μM to resistin-exposed HUVEC markedly attenuated the monocyte adherence in a dose-dependent manner. Notably, ≥10 μM caffeic acid was effective in inhibiting leukocyte adhesion to resistin-activated endothelium.

Suppression of Expression of Cell Adhesion Molecules and Integrins by Caffeic Acid. Western blot analysis was performed to address whether resistin induced expression

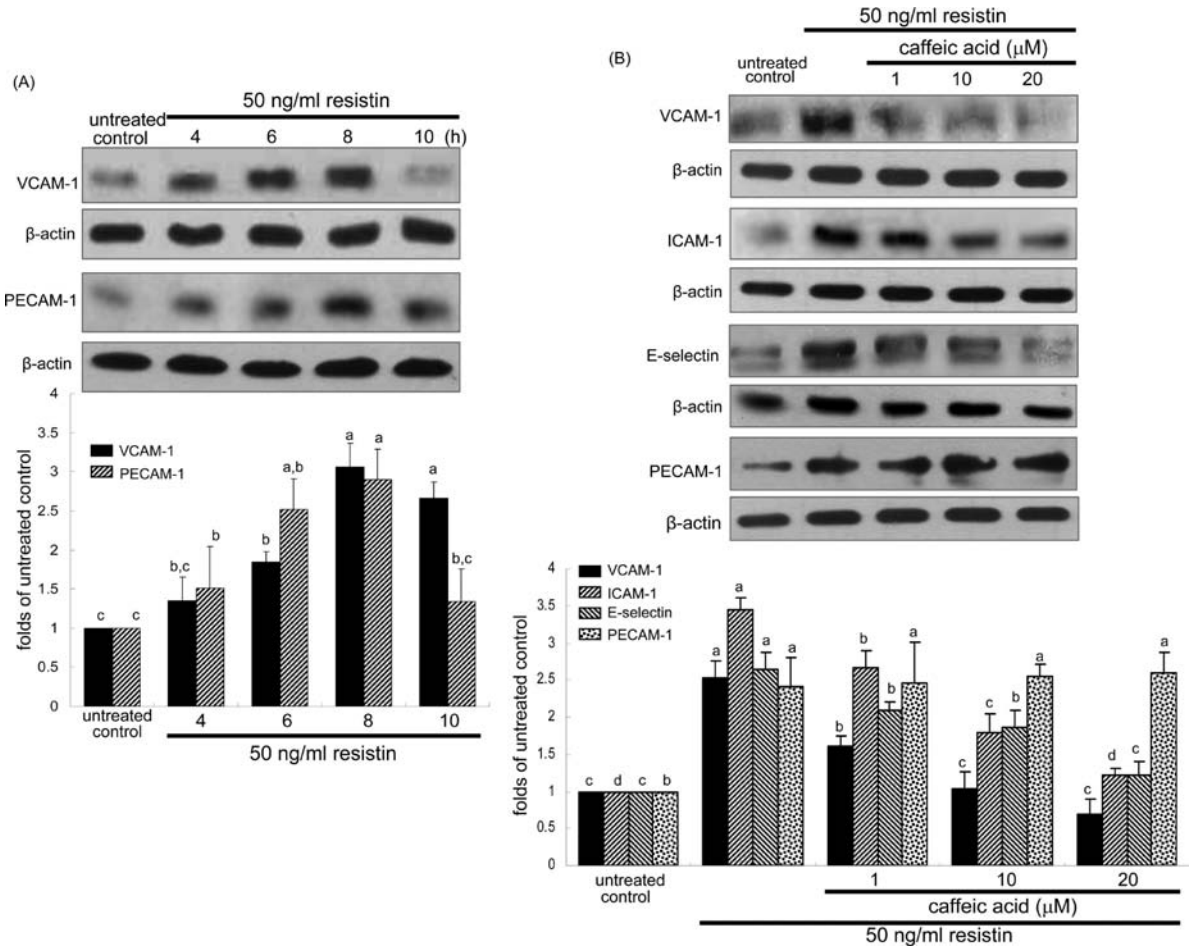

Figure 2. Western blot analyses showing (A) time course responses of expression of VCAM-1 and PECAM-1 and (B) inhibitory effects of caffeic acid on expression of cell adhesion molecules in resisin-stimulated HUVEC. Cells were incubated with 50 ng/mL resistin for 4−10 h. After HUVEC culture protocols, cell lysates were subjected to SDS−8% PAGE and Western blot analysis with each primary antibody of respective adhesion molecules. Representative blot data were obtained from three independent experiments, and β-actin protein was used as an internal control. The bar graphs (mean ± SEM) in the bottom panels represent quantitative results of blots obtained from a densitometer. Means without a common letter differ, $P < 0.05$.

of VCAM-1, ICAM-1, E-selectin, and PECAM-1 and whether such induction was inhibited by caffeic acid. Expression of VCAM-1 and PECAM-1 was elevated within 4 h by resistin treatment of HUVEC and remained high up to 8 h (Figure 2A). When 1−20 μM caffeic acid was added to resistin-exposed HUVEC for 8 h, the enhanced expression of VCAM-1, ICAM-1, and E-selectin was significantly and dose-dependently diminished (Figure 2B). These results imply that resistin hampers leukocyte recruitment to endothelial cells caused by obesity-associated inflammation through inhibiting induction of endothelial adhesion molecules. In contrast, resistin-elevated PECAM-1 expression was not influenced by caffeic acid treatment (Figure 2B). PECAM-1, concentrated in the junctions between endothelial cells, mediates adhesive interactions required during leukocyte transmigration.[25] Accordingly, caffeic acid did not antagonize the tight adhesion of endothelial cells required for leukocyte transmigration.

This study also examined whether resistin induced expression of monocyte integrins and whether this induction was attenuated by caffeic acid. When THP-1 cells were exposed to resistin for 8 h, the expression of β1, β2, and α4 integrins was enhanced (Figure 3). Pretreatment with 1−20 μM caffeic acid and reduced the enhanced expression of these integrins. Thus, caffeic acid encumbered adipokine-triggered induction of the α4/β1 integrin very late antigen-4 (VLA-4) and the αL/β2 integrin LFA-1 in activated monocytes.

Blockade of IL-8 Effect by Caffeic Acid. Resistin promoted secretion of the chemokine IL-8 from endothelial cells (≈3.8 fold, $P < 0.05$), as quantified by ELISA (Figure 4A). The inhibitory effect on secretion of IL-8 was observed in ≥10 μM caffeic acid-treated HUVEC. This study hypothesized that IL-8 was involved in the leukocyte adhesion to endothelial cells exposed to resistin. As expected, IL-8 enhanced the expression of endothelial ICAM-1 and monocyte integrin β2 (Figure 4B). Caffeic acid suppressed IL-8-induced expression of ICAM-1 in HUVEC and integrin β2 in THP-1 cells. The hypothesis was further proved by using 100 nM IL-8 siRNA (Figure 4C). When IL-8 siRNA-transfected HUVEC were exposed to 50 nM resistin for 8 h, the resistin-stimulated ICAM-1 expression was completely abolished (Figure 4C).

Caffeic Acid Inhibition of TLR4 Induction by Resistin. It was shown that resistin competes with LPS for binding to TLR4 and activation of TLR4 leads to downstream release of inflammatory modulators including TNF-α and IL-1.[26] This study attempted to investigate that TLR4 served as an endothelial receptor to resistin for atherosclerotic process.

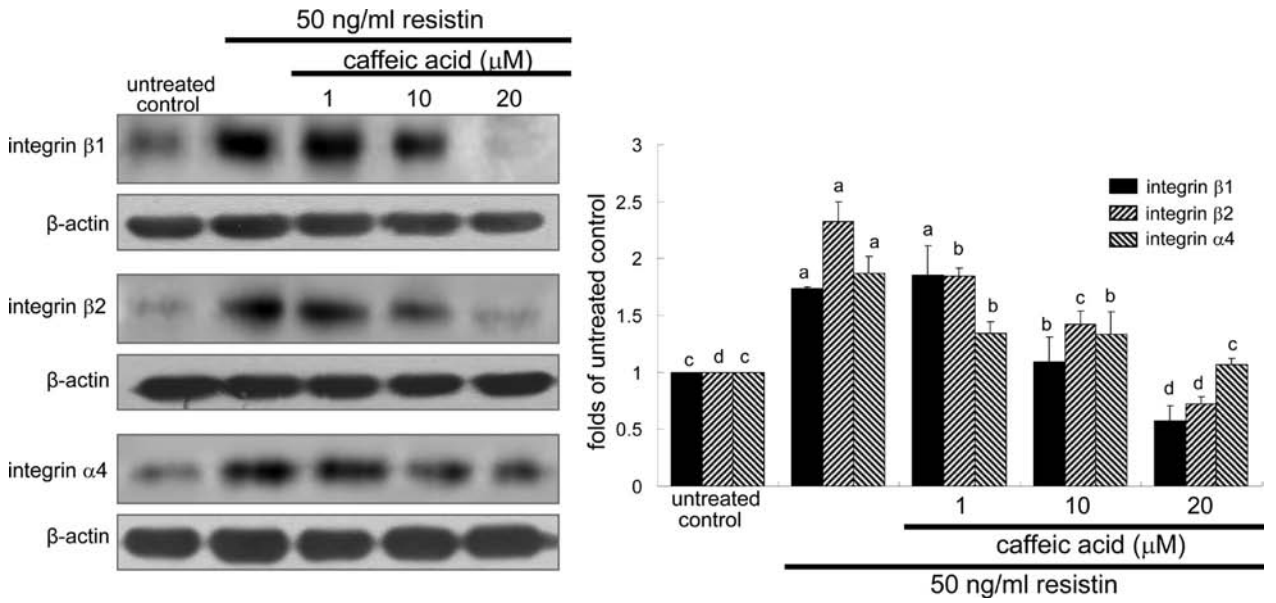

Figure 3. Inhibitory effects of caffeic acid on expression levels of integrins in resistin-stimulated THP-1 monocytes. After cells were cultured with 1−20 μM caffeic acid and 50 ng/mL resistin, cell extracts were subjected to SDS−8% PAGE and Western blot analysis with a primary antibody against each integrin (three separate experiments). β-Actin protein was used as an internal control. The bar graphs (mean ± SEM) in the right panel represent quantitative results of blots obtained from a densitometer. Means without a common letter differ, $P < 0.05$.

Resistin induced the membrane receptor TLR4 in HUVEC, suggesting that TLR4 was the membrane receptor of resistin (Figure 5A). Caffeic acid at ≥1 μM reversed TLR4 induction enhanced by resistin, which can explain the anti-inflammatory role of caffeic acid in early atherosclerosis. When 30 μg/mL oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (OxPAPC), a TLR4 inhibitor, was treated to endothelial cells, the increased IL-8 secretion by resistin was significantly attenuated by ≈40% (Figure 5B). This result indicated that TLR 4 may be involved, in part, for Il-8 production elicited by resistin.

Disturbance of NF-κB Signaling by Caffeic Acid. This study tested whether caffeic acid inhibited resistin-promoted expression of cell adhesion molecules by disturbing NF-κB transactivation. Following the exposure of HUVEC to 50 ng/mL resistin, the IκBα phosphorylation increased with a decrease in total IκBα level (Figure 6A). When cells were treated with resistin in the presence of 1−20 μM caffeic acid, caffeic acid inhibited IκBα phosphorylation dose-dependently. Furthermore, intracellular localization of NF-κB in HUVEC was evaluated with immunocytochemical analysis using specific NF-κB p65 antibody (Figure 6B). Cytoplasmic immunofluorescence staining was observed in resistin-untreated HUVEC, while notable nuclear staining in cells exposed to resistin occurred, indicating nuclear localization of NF-κB. However, nuclear staining was diminished in caffeic acid-treated and resistin-exposed cells. These results demonstrate that caffeic acid inhibited resistin-activated NF-κB signaling in HUVEC, leading to induction of inflammatory resistin-responsive gene proteins.

This study further tested whether the atherogenic activity of IL-8 protein in resistin-treated HUVEC was mediated through its stimulation of NF-κB signaling. When HUVEC were activated with 10 ng/mL IL-8, the IκBα phosphorylation was enhanced concurrently with a decrease in total IκBα level (Figure 7A). Caffeic acid at ≥10 μM significantly inhibited the activation of IκBα. Transfection of HUVEC with 100 nM IL-8 siRNA abrogated the IκBα phosphorylation elevated by resistin (Figure 7B).

DISCUSSION

Five observations were extracted from this study. (1) Nontoxic caffeic acid at ≤20 μM attenuated THP-1 monocyte adhesion to resistin-activated endothelial cells. (2) Resistin induced endothelial expression of VCAM-1, ICAM-1, and E-selectin, all of which were reduced by caffeic acid. (3) Caffeic acid attenuated the induction of the monocyte counterreceptors of β1, β2, and α4 integrins by resistin. (4) Caffeic acid at ≥10 μM diminished resistin-stimulated endothelial IL-8 secretion responsible for induction of ICAM-1 and β2 integrin through disturbing NF-κB signaling. (5) Endothelial TLR4 induction by resistin was demoted by treating caffeic acid. These observations demonstrate that caffeic acid exerts antiatherogenic activity in obesity-associated atherosclerosis via an inhibition of NF-κB-dependent transcription of gene proteins involved in endothelial interaction of leukocytes. Interestingly, TLR4 appeared to be a novel endothelial receptor to resistin.

Obesity contributes to causing various diseases such as atherosclerosis, diabetes, and cancer. Adipokines secreting from adipose tissue have been implicated in pathophysiological processes.[11] Caffeic acid phenethyl ester suppressed the production of leptin and resistin during differentiation to adipocytes in 3T3-L1 cells.[23] The adipokine resistin elevates glucose tolerance and insulin resistance and participates in the inflammatory responses.[12,13] Accordingly, resistin may be a cross-link in the close relationship between diabetes-associated insulin resistance and inflammatory atherosclerosis.[17,27] Atherogenic mechanisms of resistin may involve up-regulation of inflammatory cytokines, matrix proteases, and cell adhesion molecules. Resistin increases expression of pro-inflammatory cytokines IL-1, IL-6, and TNF-α through activating transcriptional events.[14] In addition, resistin promotes endothelin-1 release, up-regulating adhesion molecules and MCP-1 responsible for leukocyte recruitment to the sites of inflammation.[15]

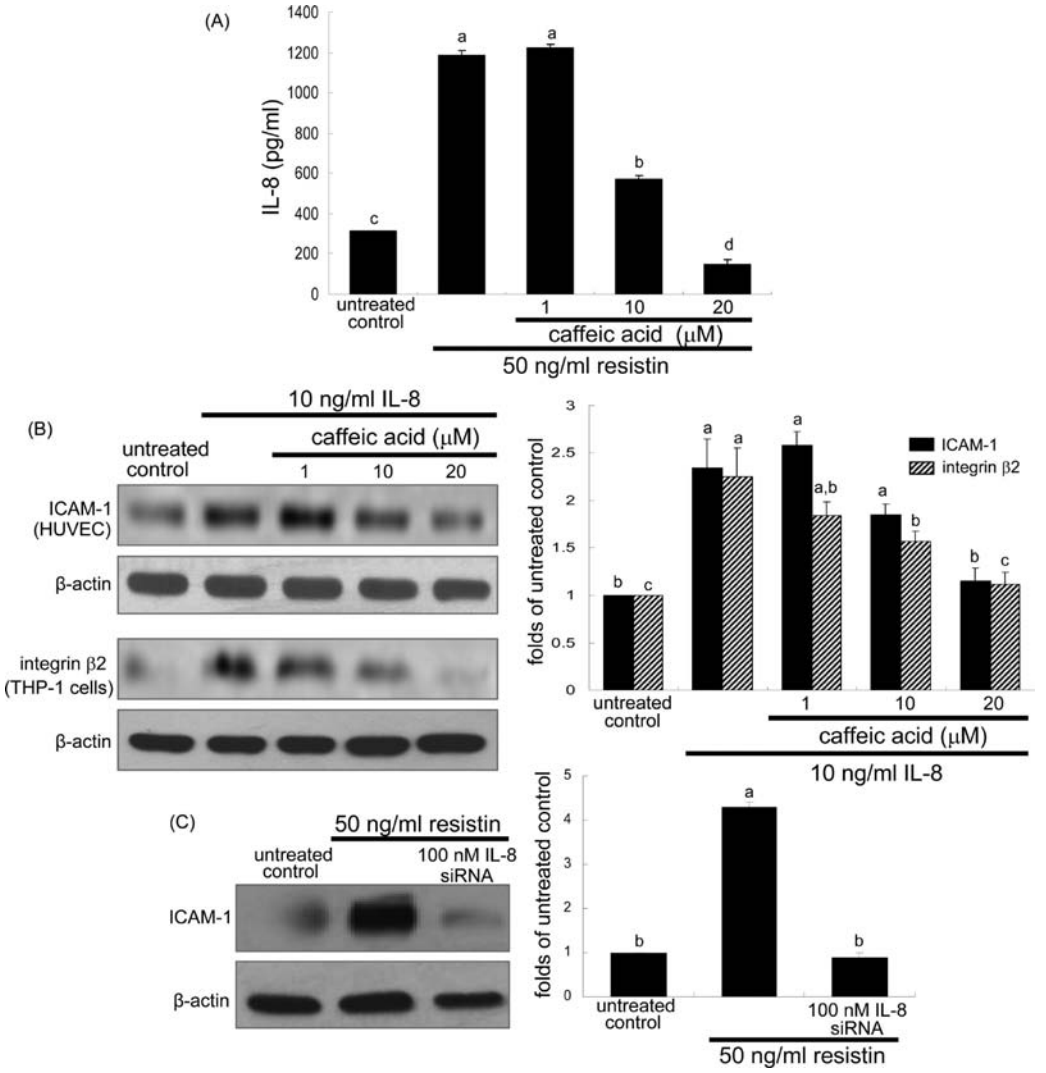

Figure 4. Inhibition of (A) IL-8 secretion and (B) its expression of ICAM-1 and integrin β2 by caffeic acid. Cells were pretreated with 1−20 μM caffeic acid for 2 h and exposed to 50 ng/mL resistin for 8 h. Cell culture media were collected for measurement of IL-8 secretion by use of an ELISA kit. After cells were cultured with 1−20 μM caffeic acid and 10 ng/mL IL-8, lysates of HUVEC and THP-1 cells were subjected to SDS−8% PAGE and Western blot analysis with a primary antibody against ICAM-1 or β2 integrin (three separate experiments). (C) Blockade of resistin-induced ICAM-1 expression in the presence of 100 nM IL-8 siRNA. β-Actin protein was used as an internal control. The bar graphs (mean ± SEM) in the right panels represent quantitative results of blots obtained from a densitometer. Means without a common letter differ, $P < 0.05$.

Circulating VCAM-1 and resistin may participate in the vascular damage in hypertensive type 2 diabetic patients.[28] Thus, it appears plausible that resistin may be a potential target for repealing obesity-related metabolic diseases. In this report employing the leukocyte"endothelium interaction system, resistin enhanced temporary interactions by E-selectin, causing THP-1 monocytes to "roll" along the activated endothelium. Caffeic acid was highly effective in blocking resistin-promoted leukocyte recruitment to the activated endothelium.

Caffeic acid is a naturally occurring phenolic compound abundant in coffee beans and other plant foods and exhibits immunomodulatory and anti-inflammatory activity.[18] Chlorogenic acids such as caffeic acid easily reach a millimolar level in the gastrointestinal tract because of its high concentration in coffee and fruits. It has been shown that caffeic acid might be an antiatherogenic modulator. Caffeic acid may prevent atherosclerosis via inhibition of both LDL oxidation and ROS production.[21] Oral supplementation of caffeic acid phenethyl ester attenuates the atherosclerotic process.[22] Unfortunately, the present study did not investigate the antiatherogenic effects of caffeic acid metabolites circulating in plasma. This study showed that caffeic acid per se suppressed the leukocyte−endothelium interaction by encumbering the binding of VLA-4-VCAM-1 and LFA-1-ICAM-1. However, it should be noted that caffeic acid preserved resistin-induced expression of PECAM-1 that is involved in the process of leukocyte transmigration. Notably, the inhibition of integrin induction by caffeic acid is thought to be its novel effect. However, the definite mechanisms underlying caffeic acid protection against early atherogenic processes are not fully understood.

The dynamic response of leukocytes to the CXC chemokine IL-8 is of central interest in inflammation. Increased ICAM-1 expression dependent on β2 integrin can take place rapidly after initial contact with IL-8.[10] The goal of this study was to identify signaling events that are critical for increased expression of ICAM-1 and β2 integrin responsive to resistin. Little has been reported about the effect of caffeic acid on chemokine-dependent cell adhesion. Resistin elevated endothelial

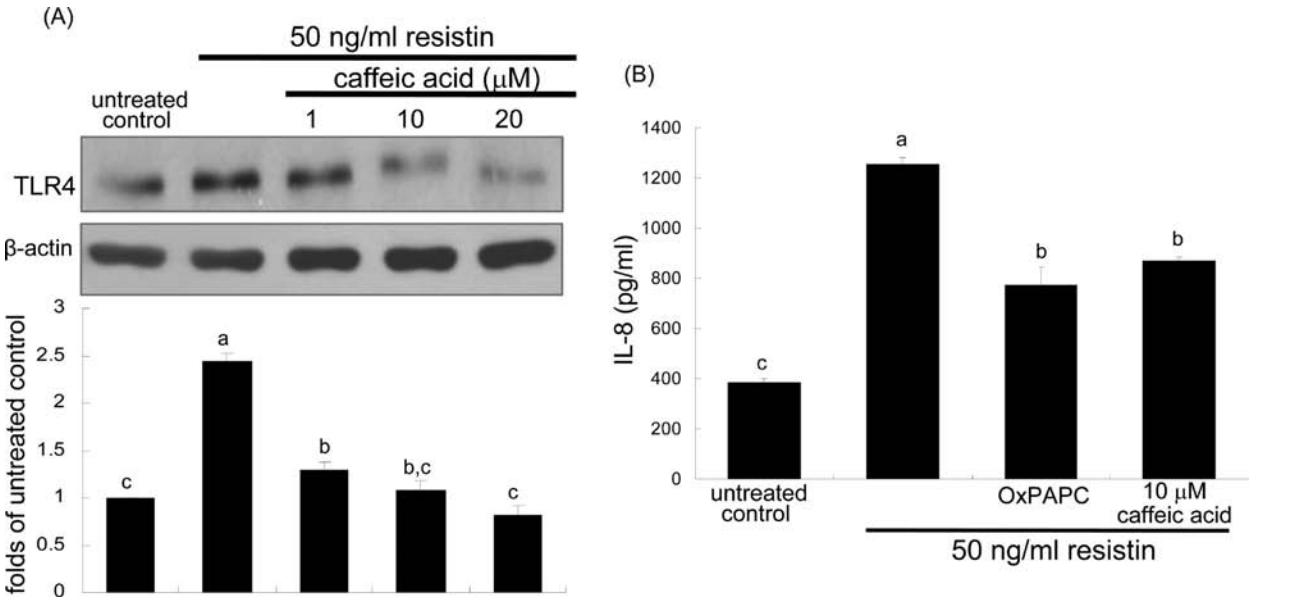

Figure 5. (A) Inhibitory effects of caffeic acid on expression levels of TLR4 and (B) effect of TLR4 inhibition on IL-8 secretion in resistin-stimulated HUVEC. After cells were cultured with 1−20 μM caffeic acid and 50 ng/mL resistin, cell extracts were subjected to SDS−8% PAGE and Western blot analysis with a primary antibody against human TLR4 (three separate experiments). β-Actin protein was used as an internal control. The bar graphs (mean ± SEM) in the bottom panel represent quantitative results of blots obtained from a densitometer. Cells were treated with 10 μM caffeic acid or 30 μg/mL OxPAPC and exposed to 50 ng/mL resistin for 8 h. Cell culture media were collected for measurement of IL-8 secretion by use of an ELISA kit. Means without a common letter differ, $P < 0.05$..

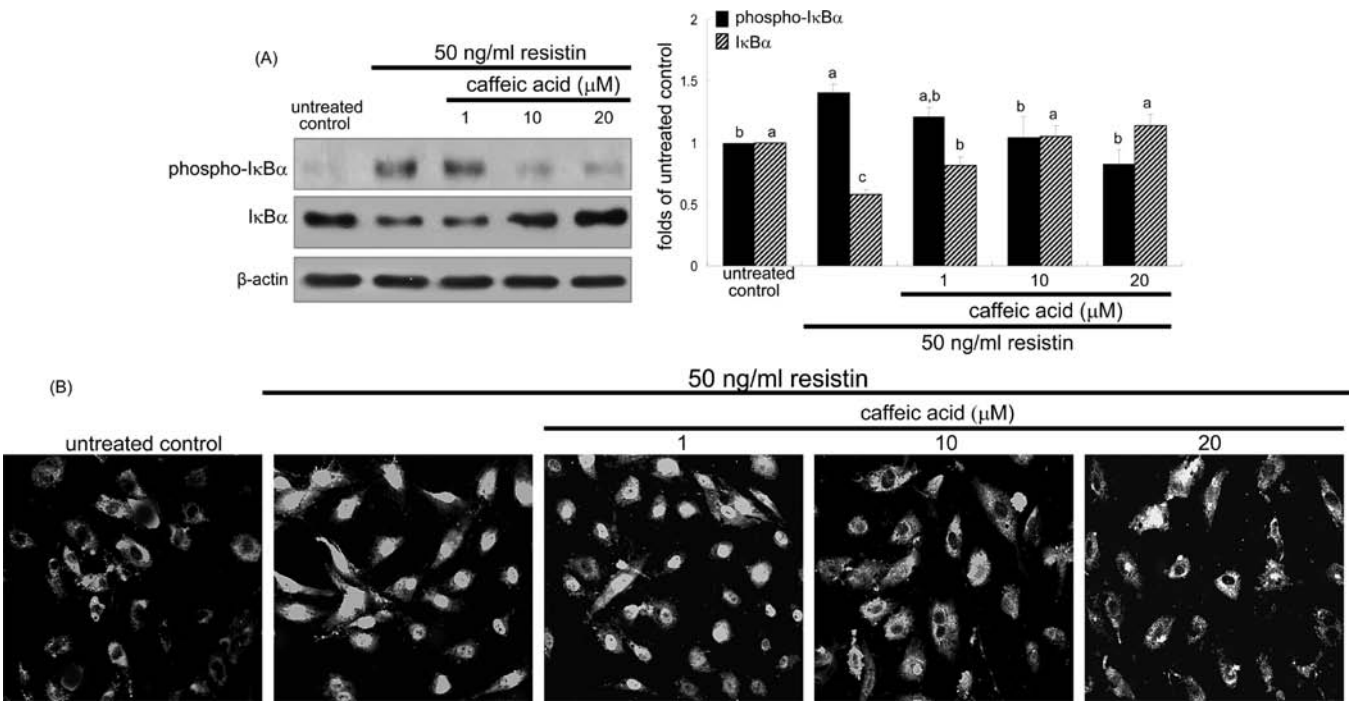

Figure 6. (A) Suppression of resistin-induced phosphorylation of IκBα and (B) blockade of nuclear translocation of NF-κB by caffeic acid. Endothelial cells were pretreated with 1−20 μM caffeic acid for 2 h and exposed to 50 ng/mL resistin for 8 h. (A) Cell lysates were subjected to SDS−8% PAGE and Western blot analysis with a primary antibody of phospho-IκB or total IκB. β-Actin protein was used as an internal control. Bands are representative of three separate experiments. The bar graphs (mean ± SEM) in the right panel represent quantitative results of blots obtained from a densitometer. Means without a common letter differ, $P < 0.05$. (B) Immunocytochemical analysis was performed with rabbit anti-human NF-κB p65 and Cy3-conjugated anti-rabbit IgG. Fluorescent images were obtained by a fluorescent microscope. Original magnification: 200×.

production of IL-8 responsible for the induction of ICAM-1 and β2 integrin. The resistin-promoted ICAM-1 induction was fully ascribed to IL-8 production, as proved by IL-8 siRNA treatment. Accordingly, resistin-associated early atherogenesis may be ascribed to inflammatory activity of IL-8 in endothelial cells and monocytes. The expression of Mac-1 (αMβ2 integrin) and the induction of the surface receptors CXCR1 and CXCR2 are up-regulated during adhesion and transendothelial migra-

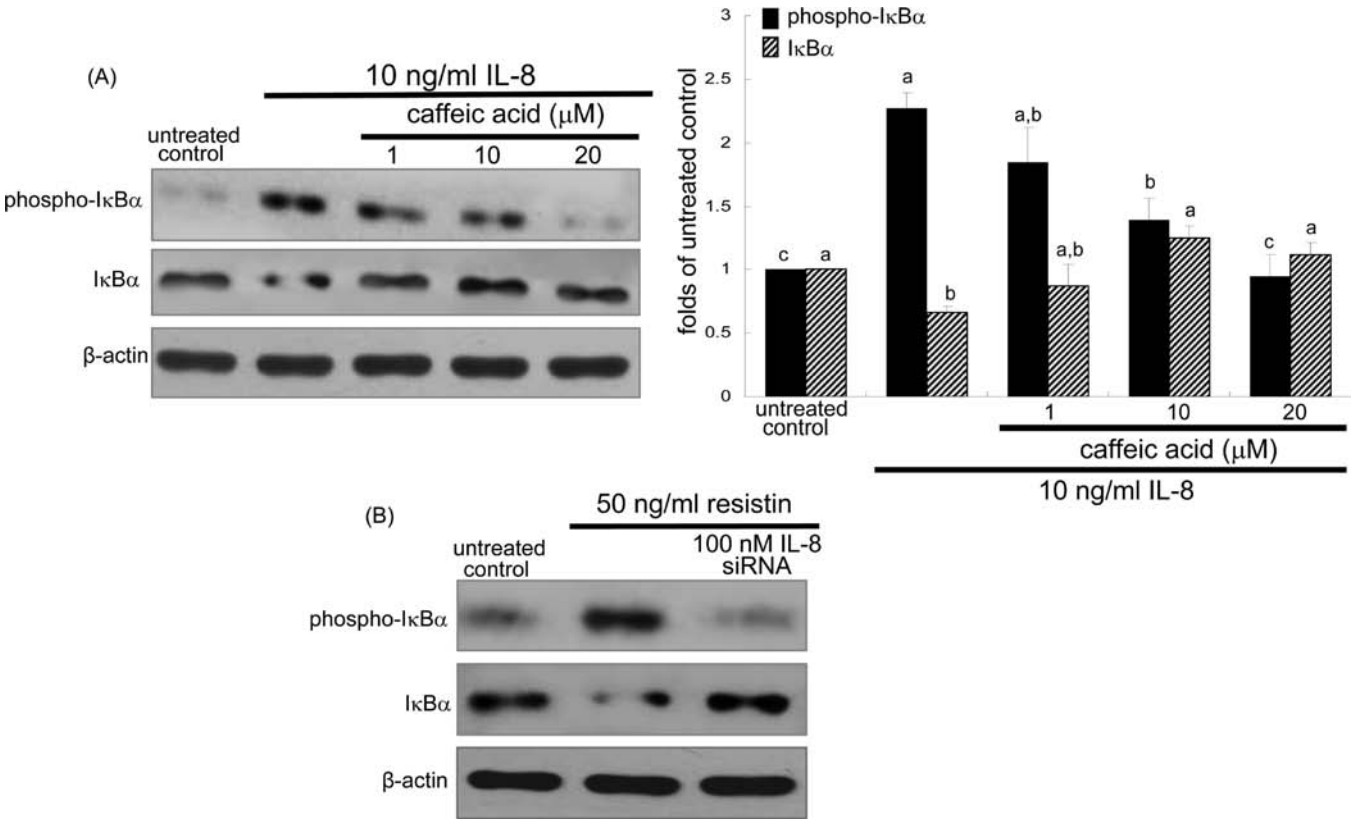

Figure 7. Western blot data showing (A) inhibition of IL-8-induced IκB activation by caffeic acid and (B) abolishment of resistin-induced IκB phosphorylation by the presence of IL-8 siRNA. Endothelial cells were pretreated with 1−20 μM caffeic acid or 100 nM siRNA and exposed to 50 ng/mL resistin. Cell lysates were subjected to SDS−8% PAGE and Western blot analysis with a primary antibody of phospho-IκB or total IκB. β-Actin protein was used as an internal control. Bands are representative of three separate experiments. The bar graphs (mean ± SEM) in the right panel represent quantitative results of blots obtained from a densitometer. Means without a common letter differ, $P < 0.05$.

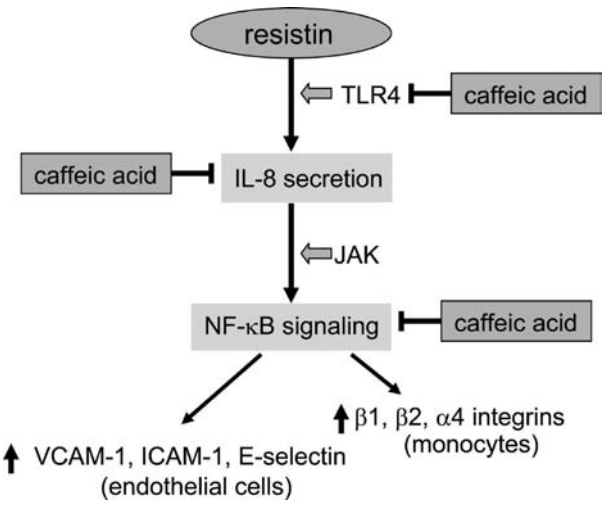

Figure 8. Schematic diagram showing antiatherogenic and anti-inflammatory actions of caffeic acid in resistin-induced monocyte−endothelial interaction. Inhibition or blockade by caffeic acid is indicated..

tion of neutrophils.[29] Although this study did not examine the induction of CXCR1 and CXCR2 by resistin, it is deemed that these IL-8 receptors were up-regulated by resistin. Nevertheless, resistin elevated endothelial expression of TLR4, which is a novel finding. Resistin competes with LPS for binding to TLR4, and activation of TLR4 leads to downstream release of inflammatory modulators including TNF-α and IL-1.[26] Thus, TLR4 was thought to serve as a receptor for the pro-inflammatory effects of resistin in endothelial cells, which may explain the inflammatory role of resistin in early atherosclerosis. Caffeic acid retarded the resistin induction of TLR4 partially responsible for IL-8 production.

How the cell adhesion molecule genes are selectively modulated in response to resistin and which signaling pathways are involved in the selective regulation of these genes remain unknown. It is assumed that several inflammation-responsive pathways may be involved in antiatherogenic activity of caffeic acid. Clearly, the activation of endothelial expression of VCAM-1, ICAM-1, and E-selectin by resistin is retarded, possibly by novel mechanism(s) responsive to caffeic acid. Resistin-up-regulated IL-8 activates the NF-κB-responsive signaling pathway in the leukocyte-endothelium interaction system. Similarly, resistin and high glucose induce the up-regulation of P-selectin and fractalkine and increase the ensuing increased monocyte adhesion by a mechanism involving oxidative stress and NF-κB and AP-1 activation.[30] Caffeic acid disturbs NF-κB-responsive signaling through reducing IL-8 induction via resistin−TLR4 signaling. TLR4 is induced during adipocyte differentiation, and fatty acid-induced resistin secretion is mediated via NF-κB and TLR4.[31] However, definite mechanisms underlying the atheroprotection of caffeic acid are not yet fully understood in obesity-associated atherosclerosis.

In summary, our study demonstrated that caffeic acid disturbed resistin-stimulated early atherosclerotic process involving the induction of endothelial cell adhesion molecules

and monocyte integrins. However, definite mechanisms underlying the atheroprotection of caffeic acid against the early atherogenic process remain unclear. The resistin-promoted leukocyte−endothelium interaction required the IL-8-triggered NF-κB-dependent pathway, which was disturbed by caffeic acid (Figure 8). In addition, resistin-induced IL-8 production entailed TLR4 induction that was also blunted by caffeic acid. Accordingly, these observations may have clinical implications for therapeutic strategies of caffeic acid preventing obesity-associated inflammatory diseases including atherosclerosis.

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Notes

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ABBREVIATIONS

HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular cell adhesion molecule 1; IL, interleukin; IκB, inhibitory κB; LFA-1, lymphocyte function-associated antigen 1; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein 1; NF-κB, nuclear factor κB; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor α; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late antigen 4

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