Arch Pharm Res Vol 30, No 10, 1318-1327, 2007

Archives of Pharmacal Research
http://apr.psk.or.kr

Anti-inflammatory Mechanisms of Apigenin: Inhibition of Cyclooxygenase-2 Expression, Adhesion of Monocytes to Human Umbilical Vein Endothelial Cells, and Expression of Cellular Adhesion Molecules

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(Received July 24, 2007)

The aim of this study was to clarify the anti-inflammatory mechanism of apigenin. Apigenin inhibited the collagenase activity involved in rheumatoid arthritis (RA) and suppressed lipopolysaccharide (LPS)-induced nitric oxide (NO) production in a dose dependent manner in RAW 264.7 macrophage cells. Pretreatment with apigenin also attenuated LPS-induced cyclooxygenase-2 (COX-2) expression. In addition, apigenin profoundly reduced the tumor necrosis factor-α (TNF-α)-induced adhesion of monocytes to HUVEC monolayer. Apigenin significantly suppressed the TNF-α-stimulated upregulation of vascular cellular adhesion molecule-1 (VCAM-1)-, intracellular adhesion molecule-1 (ICAM-1)-, and E-selectin-mRNA to the basal levels. Taken together, these results suggest that apigenin has significant anti-inflammatory activity that involves blocking NO-mediated COX-2 expression and monocyte adherence. These results further suggest that apigenin may be useful for therapeutic management of inflammatory diseases.

Key words: Apigenin, Collagenase, Hyaluronidase, Nitric oxide, COX-2, Adhesion, VCAM-1, ICAM-1, E-selectin

INTRODUCTION

Flavonoids are diphenyl propanoids that exist abundantly in plant foods and are important constituents of the human diet. Flavonoids can scavenge reactive oxygen species (ROS) (Antonella *et al.*, 1995; Yukiko *et al.*, 1994), chelate iron ions (which catalyze many processes leading to the appearance of free radicals) (Afanas'av *et al.*, 1989; Morel *et al.*, 1993), and inhibit lipid oxidation (Elliott and Kandaswami, 1986). Several dietary flavonoids provide modest protection against cardiovascular diseases (Fisher and Hollenberg, 2005; Arts and Hollenberg, 2005), and improve endothelial function and reduce blood pressure in humans (Vita, 2005). Flavonoids also inhibit the development of atherosclerosis in animal models (Waddington *et al.*, 2004).

Apigenin, a 4',5,7 trihydroxy flavone, belongs to flavone subclass of flavonoids. It is ubiquitously distributed in leaves, vegetables, stems, and fruits of several plants (Singh *et al.*, 2004). Apigenin, unlike the flavonoids quercetin and kaempferol, is non-toxic and non-mutagenic (Maruta *et al.*, 1982; Stoewsand *et al.*, 1984), and blocks the development of mouse skin tumors (Wei *et al.*, 1990) and the proliferation of human breast-cancer cells (Hirano *et al.*, 1989). Apigenin is a potent antioxidant (Nielsen *et al.*, 1999), cyclooxygenase inhibitor (Kelm *et al.*, 2000), cell cycle inhibitor (Lepley and Pelling, 1997), protein kinase C inhibitor (Lin *et al.*, 1997), and apoptosis inducer (Gupta *et al.*, 2001).

Derivatives of arachidonic acid (AA) are the potent mediators of inflammation, and cyclooxygenase (COX) and 5-lipoxygenase (LOX) are involved in metabolisms of AA. Flavonoids may inhibit COX and 5-LOX, and thereby modulate metabolism of arachidonic acid (Kato *et al.*, 1983; Baumann *et al.*, 1980) and attenuate inflammation

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(Huang *et al.*, 1991). Cyclooxygenase-2 (COX-2) regulates proliferation, angiogenesis, inflammation, and tumorigenesis. Extracellular stimuli, including cytokines, ultra-violet light (UV-light), and tumor promoters, induce COX-2 expression (Van Dross *et al.*, 2005). COX expression is elevated in the synovial lining layer, the subsynovial synoviocytes, the vascular endothelial cells, and mononuclear inflammatory cells in patients with rheumatoid arthritis (RA) or osteoarthritis (OA) (Goldenberg, 1999; Crofford, 1997).

Monocyte and neutrophil adherence to human umbilical vein endothelial cell (HUVEC) monolayer is elevated in early RA. Cellular adhesion molecules (CAMs) are stimulated by inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and interleukins. Up-regulated expression of CAMs increases monocyte and neutrophil adherence to HUVEC monolayer, and is involved in RA and atherosclerosic responses (Amrani *et al.*, 2000; Broide *et al.*, 1992; Silverman *et al.*, 2001).

To elucidate the mechanism for anti-inflammatory activities of apigenin, we investigated the antioxidant activity and the inhibitory effects on enzymes involved in RA, the production of nitric oxide (NO), COX-2 expression, and cellular adhesion.

MATERIALS AND METHODS

Chemicals and reagents

Dimethyl sulfoxide (DMSO), calcein *O,O'*-diacetate tetrakis (acetoxymethyl) ester (Calcein-AM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), heparin, lipopolysaccharide (LPS, *Escherichia coli* serotype 0127:B8), Dulbecco's modified Eagle's medium (DMEM), 2-amino-5-mercapto-1,3,4-thiadiazole (AMT), hyaluronidase (EC 3.2.1.35), Collagenase (EC 3.4.24.3, *Clostridium histolyticum* type H), and PZ-peptide (4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg monohydrate) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Cell culture medium and reagents, such as F-12K, RPMI-1640, fetal bovine serum (FBS), penicillin/streptomycin, trypsin-EDTA, and endothelial cell growth supplement (ECGS) were obtained from GIBCO (Invitrogen Inc., NY, U.S.A.). Antibodies against COX-2 and horseradish peroxidase-conjugated (HRPC) mouse anti-goat were purchased from Santa Cruz Biotech (Santa Cruz, CA, U.S.A.). TNF-α was purchased from BD Science (CA, U.S.A.). Ethanol and other reagents were commercial grade.

Cell culture

RAW 264.7 murine macrophage cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were cultured in DMEM containing 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) in a 5% CO_2 humidified incubator at 37°C. The monocytic cell line, THP-1, was obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). THP-1 was cultivated in RPMI-1640 medium containing 10% FBS and 100 units/mL of penicillin/streptomycin at 37°C in a 5% CO_2 incubator under controlled moisture. THP-1, a floating cell line, was subcultured after being collected by centrifugation at 2,090×*g* for 2 min. THP-1 was used for cell-cell adhesion assay at passage numbers 30-40. Human umbilical vein endothelial cells (HUVECs, CRL-2480, ATCC) were cultured with F-12K nutrient mixture (Kaighn's modification, GIBCO) containing 10% FBS, 100 units/mL penicillin/streptomycin, 0.1 mg/mL of heparin, and 0.03 mg/mL of ECGS. HUVECs were cultured at 37°C in a humidified 5% CO_2 incubator. For subculture, HUVEC monolayer was rinsed twice with phosphate buffered saline (PBS, pH 7.4) to remove all traces of serum (which can inhibit trypsin) and were subdivided using 0.05% trypsin with 0.53 mM EDTA. HUVECs were used at passage numbers 20-30.

Radical scavenging activity

The scavenging activity of apigenin on 2,2-diphenyl-1-picrylhydrazyl (DPPH)-induced radicals was monitored according to the method described by Yen and Chen (1995). A methanolic solution (0.2 mL) containing extracts was mixed with 4 mL of methanol, and a methanolic solution of DPPH (1 mmol/L, 0.5 mL) was added. The mixture was vortexed for 15 sec, left to stand at room temperature for 30 min, and the absorbance read at 517 nm using UV-spectrophotometry (Agilent Technologies Inc., CA, U.S.A.).

Reducing power

The reducing power of apigenin was determined according to the method of Oyaizu (1986) and Amarowicz *et al.*(2005). A stock solution of apigenin was prepared in DMSO. Apigenin was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50°C for 20 min. After that, 2.5 mL of 10% trichloroacetic acid was added and centrifuged at 2,090×*g* for 10 min. The supernatant layer (2.5 mL) was added to 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. The absorbance of the mixture was measured at 700 nm using UV-spectrophotometry.

Hyaluronidase (HAase) inhibition assay

The HAase inhibition assay was performed according to the Morgan-Elson method (Lee *et al.*, 2005; Lee *et al.*, 1993). HAase solution (7,900 units/mL in 0.1 M acetate buffer, pH 3.5) was mixed with 100 μL of sample and incubated at 37°C in a water bath incubator for 20 min. The HAase activator (0.1 mL of 12.5 mM $CaCl_2$) was added and incubated further for 20 min. For the HAase reaction,

250 μL of hyaluronic acid (1.2 mg/mL), the substrate, in 0.1 M acetate buffer (pH 3.5) was added and incubated at 37°C in a water bath incubator for 40 min. For termination of the HAase reaction, 0.1 mL of 0.4 N NaOH and 0.1 mL of 0.4 M potassium tetraborate were added and warmed in boiling water for 3 min. After cooling completely, 3 mL of DMAB reagent (4 g of r-demethylaminobenaldehyde, 350 mL of glacial acetic acid, and 50 mL of 10 N HCl) was added to the reactant and incubated at 37°C in a water bath incubator for 20 min. HAase reaction product, 4-acetyl-glucosamine, was measured by UV-spectrophotometry at 585 nm wavelength. HAase inhibition activity was expressed by the decrease compared to the control; % Hyaluronidase inhibition = $[(A_c - A_s)/A_c] \times 100$, where A_c and A_s were the absorbance of control and sample, respectively. The control was 1% DMSO solution instead of apigenin.

Collagenase inhibition assay

The collagenase assay was performed by Sawabe's procedure (Sawabe *et al.*, 1998; Park *et al.*, 2006) with minor modification. Collagenase (EC 3.4.24.3, 5 μg) was added to PZ-peptide (0.5 mg), a substrate of collagenase, in 0.1 M Tris buffer (pH 7.4), with or without apigenin. Total volume was 1.7 mL. The mixture was incubated at 37°C in a water bath for 30 min, and 1 mL of 25 mM citric acid solution was added to terminate the enzyme reactions. After mixing with 5 mL of ethylacetate, the absorbance of the organic layer was measured by UV-spectrophotometry at 320 nm. Collagenase inhibition was calculated by following equation: % Collagenase inhibition = $[(A_c - A_s)/A_c] \times 100$, where A_c was (the absorbance of control with collagenase – the absorbance of control without collagenase), and A_s was (the absorbance of sample with collagenase – the absorbance of sample without collagenase).

Cell viability assay

The effect of apigenin on viability of RAW 264.7 macrophage cells was evaluated using the cell counting kit (CCK-8, Dojindo Laboratories, Tokyo, Japan). In brief, RAW 264.7 cells were plated at 1×10^4 cells/well in 96-well tissue culture plates, and incubated at 37°C for 24 h. The cells were treated with various concentrations of apigenin. After 24 h further incubation, 10 μL of the CCK-8 solution was added to the wells and incubated for 3 h. The resulting color was measured at 450 nm using a microplate reader (Molecular Devices, Emax, Sunnyvale, CA). The cytotoxicity of apigenin for HUVECs was examined using the MTT assay. HUVECs were seeded at 1×10^4 cells/well in 96-well culture plates (Corning Inc., U.S.A.), and were cultured for 24 h at 37°C in a humidified 5% CO_2 incubator. Apigenin was added to the plate and incubated for 24 h. MTT was added at 0.5 mg/mL final concentration and incubated for 4 h at 37°C. After discarding all medium from the plates, 100 μL of DMSO was added to all wells. The plates were placed for 5 min at room temperature with shaking, so that complete dissolution of formazan was achieved. The absorbance of the MTT formazan was determined at 540 nm by UV-spectrophotometric plate reader (Emax, Molecular Devices Inc., U.S.A.). Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells.

Nitric oxide (NO) assay

NO production was assayed by the measurement of nitrite, a stable NO oxidation product, from RAW 264.7 macrophage cells. RAW 264.7 cells were plated in 24-well culture plates at 1×10^5 cells/well and incubated for 24 h in a humidified 5% CO_2 incubator at 37°C. Cells were pre-treated with various concentrations of apigenin for 2 h and stimulated by 1 μg/mL of LPS for 18 h. LPS-stimulated nitrite-production from RAW 254.7 cells was measured by the Griess reation (Chattopadhyay *et al.*, 2002; Dawson and Dawson, 1995). Briefly, 100 μL of each supernatant was mixed with 100 μL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride in distilled water), and the absorbance of the mixture was determined with a microplate reader (Molecular Devices, Emax, Sunnyvale, CA) at 540 nm. In this experiment, AMT (1 μM), a inducible nitric oxide synthetase (iNOS) inhibitor, was used as a positive control (Rairigh *et al.*, 1998; Liao *et al.*, 2006).

Western blot analysis of COX-2

RAW 264.7 macrophage cells were treated with apigenin for 2 h and stimulated by 1 μg/mL LPS for 18 h to induce COX-2. Celecoxib (20 μg/mL), a selective COX-2 inhibitor, was used as a positive control (Goldenberg, 1999). The cells were then rinsed with ice-cold PBS and lysed with 80 μL of lysis buffer (3 mM $CaCl_2$, 2 mM $MgCl_2$, 1% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail in 10 mM Tris-Cl, pH 7.4) by incubation on ice for 10 min. Lysates were vortexed for 5 min, and the supernatant was collected by centrifugation at 12,000×*g* for 30 min. The protein concentration was determined using the Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA). Lysates (5 μg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to a nitrocellulose membrane (Whatman GmbH, Germany). Non-specific binding was inhibited by exposing the membranes to TBST (150 mM NaCl, and 0.1% Tween 20 in 50 mM Tris-HCl, pH 7.5) containing 5% skim milk for 2 h at room temperature. Then the membranes were incubated overnight with a primary antibody at 4°C with gentle shaking. Blots were washed five times in TBST for 40 min, followed by incubation with HRP-conjugated secondary antibodies for 1 h at room temperature. Blots were washed

five times in TBST for 40 min and developed for visualization using ECL reagent (LabFrontier, Seoul, Korea).

Cell-cell adhesion assay

HUVECs were seeded at 1×10^5 cells/well in 96-well tissue culture plates (Corning 3603, Corning Inc., NY, U.S.A.). After 24 h incubation at 37°C, HUVEC monolayer was treated with apigenin for 24 h, and then were stimulated with 5 ng/mL TNF-α for 24 h. The HUVEC monolayer was washed three times with PBS before cell-cell adhesion assay (Ludwig *et al.*, 2004). Calcein-AM labeled monocytes, THP-1, were cocultured at 5×10^5 cells/well with HUVEC monolayer for 1 h in a humidified 5% CO_2 incubator at 37°C. Non-adherent THP-1 cells were removed by washing four times with PBS. Adherence of calcein-AM labeled THP-1 cells was determined by fluorescent intensity, measured using a fluorescent plate reader (FL600, Bio-Tek Instruments, Inc., Winooski, VT, U.S.A.). The excitation and emission wavelengths for the calcein-AM molecule are 485 and 530 nm, respectively.

For photographs, HUVECs were seeded on 24-well culture plates. Calcein-AM labeled THP-1 cells attached on the HUVEC monolayer were photographed at a magnification of ×100 using an inverted fluorescence microscope (IX 71, OLYMPUS Inc., Tokyo, Japan) connected to an OLYMPUS DP50 camera with Imaging software (ViewfinderLite, Ver. 1.0.134, Pixera Corporation, U.S.A. and OLYSIA BioAutoCell Ver. 3.2, Soft Imaging System, Tokyo, Japan).

RT-PCR analysis for transcription of cellular adhesion molecules

Total RNA was isolated from HUVECs using the RNeasy kit (Qiagen Inc. Valencia, CA) after treatment with apigenin, RT-PCR was performed using a One-Step RT-PCR kit (Qiagen and Bioneer Corp.) and primers at a final concentration of 1 μM. For PCR, human primers for CAMs were used as follows: vascular cellular adhesion molecule-1 (VCAM-1) forward primer: 5'-ATGCCTGGGAAGATGGTCGTGA-3', VCAM-1 reverse primer: 5'-TGGAGCTGGTAGACCCTCGCTG-3', intracellular adhesion molecule-1 (ICAM-1) forward primer: 5'-GGTGACGCTGAATGGGGTTCC-3', ICAM-1 reverse primer: 5'-GTCCTCATGGTGGGGCTATGACTC-3', E-selectin forward primer: 5'-ATCATCCTGCAACTTCACC-3', and E-selectin reverse primer: 5'-ACACCTCACCAAACCCTTC-3'. Total RNA (1 μg/μL) of basal HUVECs was used as a control, and primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a house-keeping gene (Corpe *et al.*, 2005), were used to determine PCR efficiency: GAPDH forward primer: 5'-ATGACAACAGCCTCAAGATCATCAG-3', GAPDH reverse primer: 5'-CTGGTGGTCCAGGGGTCTTACTCCT-3'. Thermal cycling was performed according to a modified method (Park *et al.*, 2003; Choi *et al.*, 2004). For cDNA synthesis and predenaturation, 1 cycle of 50°C for 30 min and 95°C for 15 min was performed on total RNA. For PCR amplification, 30 cycles of 95°C for a 1 min denaturation, 55°C for 2 min annealing, 72°C for a 3 min extension, and 1 cycle of 72°C for a 10 min final extension were performed using a Bio-Rad thermal cycler (MJ Mini, Bio-Rad Inc., U.S.A.). RT-PCR products were stored at 4°C until agarose gel separation. Transcriptional changes were calculated using an electrophoresis image quantification program (Bio-Rad Inc., U.S.A.).

Protein assay

For standardization of cell-cell adhesion assay data, the amount of plated HUVEC monolayer was measured by BCA protein assay (Pierce Inc., IL, U.S.A.) using bovine serum albumin as a standard, following cell solubilization using 0.1 N NaOH and 1% of 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate.

Statistical analysis

The Student's t-test was used to determine the statistical significance between values. Data is expressed as mean ± standard errors (SEM) of at least triplicate values.

RESULTS AND DISCUSSION

Antioxidant activities of apigenin

Although many flavonoids are ROS scavengers (Antonella *et al.*, 1995; Yukiko *et al.*, 1994), apigenin did not scavenge DPPH radicals or possess reducing power (Table I). These results were consistent with the data reported by Nuutila *et al.* (2003). Generally the antioxidant property causes the cytoprotective and neuroprotective function by biochemical ROS quenching (Moosmann and Behl, 1999). However, apigenin is cytoprotective against doxorubicin (DOX)-

Table I. Antioxidant activities of apigenin

	Antioxidant activities	
	DPPH scavenging effect (%)	Reducing Power (Absorbance at 700 nm)
Apigenin		
500 μM	ND[1)]	0.006 ± 0.003*
1,000 μM	ND[1)]	0.018 ± 0.004**
Ascorbic acid[2)]		
1,000 μM	66.07 ± 0.68	
Pyrogallol[2)]		
125 μg/mL		1.75 ± 0.044

*P<0.5, compared to the control, **P<0.05, compared to the control.
[1)]N.D.: not detected.
[2)]Ascorbic acid and pyrogallol were used as positive control for DPPH radical scavenge and reducing power assay, respectively.

induced injury of cardiomyocytes (Psotová *et al.*, 2004), and *tert*-butyl hydroperoxide-induced injury of hepatocytes (Joyeux *et al.*, 1995), which may be mediated by its transition metal chelation (Van Acker *et al.*, 1996).

Inhibitory effects of apigenin on hyaluronidase and collagenase activity

Cartilage contains collagen and aggrecans such as hyaluronic acid and chondroitin sulfate (Aigner and Stöve, 2003; Schauss *et al.*, 2007). Abnormal breakdown of connective tissue contributes to pathological conditions such as RA and atherosclerosis. HAase (EC 3.2.1.35) is involved in inflammatory reactions (Sawabe *et al.*, 1992; Edelstam *et al.*, 1992; Noble *et al.*, 1996), and collagenase (EC 3.4.24.7), a matrix metalloproteinase-1 (MMP-1), mainly breaks down collagen type I and type III (Park *et al.*, 2006). MMP-1 degrades collagen and proteoglycan matrices in joints (Stenman *et al.*, 2005). Collagenases are expressed by chondrocytes and secreted into the synovial fluid (Tchetina *et al.*, 2007; Johansson *et al.*, 1997). Apigenin only weakly inhibited hyaluronidase activity, but inhibited collagenase activity by 85.3% at 500 μM (Table II).

Inhibition of LPS-stimulated NO production and COX-2 expression by apigenin in RAW 264.7 macrophage cells

NO is involved in the pathophysiology of inflammatory joint disease and plays a key role in the cartilage catabolism mediated by inflammation (Belmont *et al.*, 1997). COX, which converts arachidonic acid to prostaglandins (PGs), is a critical inflammatory enzyme (Dibois *et al.*, 1998). An inducible isoform, COX-2, is expressed during inflammatory disease in many cells, including fibroblasts and macrophages, and mediates the release of large quantities of proinflammatory PGs at the site of inflammation (Xie *et al.*, 1991; Lee *et al.*, 1992). Thus, COX-2 and NO are key regulatory molecules in the inflammatory process in RA. Furthermore, exogenously-generated NO induces COX-2 expression in synovial cells (Honda *et al.*, 2000).

Apigenin was cytotoxic to RAW 264.7 cells at 20 μM (Fig. 1), so doses from 0.1 μM to 10 μM were used for the inhibition of NO production and COX-2 expression assay. Apigenin partially inhibited LPS-stimulated NO production in RAW 264.7 cells (Fig. 2), whereas AMT (1 μM), a selective iNOS inhibitor, inhibited LPS-induced NO production completely. LPS also increased COX-2 expression levels significantly, and pretreatment with apigenin attenuated

Table II. Inhibitory effects of apigenin on hyaluronidase and collagenase

	Inhibition (%)	
	Hyaluronidase	Collagenase
Apigenin		
500 μM	–[1)]	85.3 ± 0.9*
1,000 μM	–[1)]	92.9 ± 2.2*

[1)]Less than 10% inhibition,
*P<0.001, compared to the control.

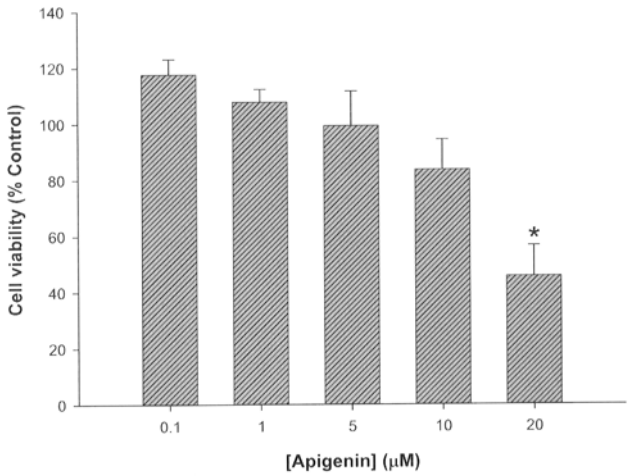

Fig. 1. Effect of apigenin on cell viability of RAW 264.7 macrophage cells. RAW 264.7 cells were cultured with 0-20 μM of apigenin at 37°C in a 96-well plate for 24 h. Cell viability was evaluated by a cell counting kit (Dojindo Laboratories, Tokyo, Japan), and expressed as a percentage of the control (without apigenin). Values are expressed as the mean ± SEM of three individual experiments. *P<0.05, compared to the control.

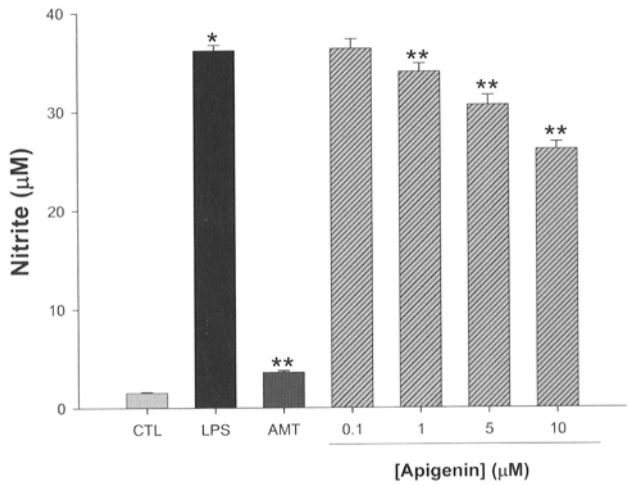

Fig. 2. Effect of apigenin on LPS-stimulated NO production in RAW 264.7 macrophage cells. RAW 264.7 cells were treated with the indicated concentrations of apigenin for 2 h and then treated with LPS (1 μg/mL). After 18 h incubation, the amount of nitrite, a stable NO oxidation product, was measured by the Griess reaction assay. Values were obtained from three independent experiments and are expressed as the mean ± SEM. *P<0.05, compared to the control; **P<0.05, compared to the LPS-treated group. CTL, control; LPS, lipopolysaccharide; AMT (1 μM), 2-amino-5-mercapto-1,3,4-thiadiazole.

these increases in a dose-dependent manner (Fig. 3). COX-2 was not expressed at all in RAW 264.7 cells without stimulation (Fig. 3A), and LPS-stimulated COX-2 expression was also suppressed by celecoxib (20 μM), a selective inhibitor of COX-2 (Goldenberg MM, 1999) (Fig. 3B). Apigenin (10 μM) showed greater inhibiton of COX-2 induction than celecoxib. These results are consistent with a report that apigenin decreased NO production by down-regulating COX-2 expression in the same cells (Chun *et al.*, 2004). Cellular ROS generation, for example by 12-O-tetradecanoylphorbol-13-acetate (TPA), stimulates the expression of COX-2 (Fischer and Adam, 1985; Ahn *et al.*, 2002), but the reduction of free radicals by treatment with N-acetyl cysteine (NAC) does not decrease COX-2 expression (Van Dross *et al.*, 2005). As mentioned above, apigenin has minimal antioxidant activity compared to structurally related flavonoids, such as quercetin, mortin, and myricetin (Safari and Sheikh, 2003; McPhail *et al.*, 2003). Nevertheless, apigenin suppressed LPS-induced COX-2 expression in RAW 264.7 cells (Fig. 3). This suppression could be caused by inhibition of Akt activation, or by inhibition of AA release causing suppression of PGs synthesis (Van Dross *et al.*, 2005). As a whole, our results suggest that apigenin may retard inflammatory processes in rheumatoid synovium by suppressing NO production and COX-2 expression.

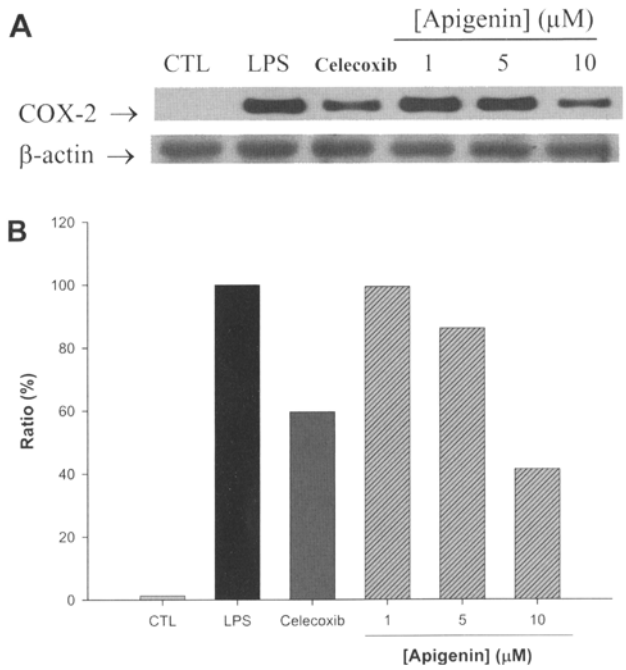

Fig. 3. Effect of apigenin on LPS-stimulated COX-2 expression levels in RAW 264.7 macrophages. RAW 264.7 cells were treated with the indicated concentrations of apigenin for 2 h and then treated with LPS (1 μg/mL). After 18 h incubation, equal amounts of total protein were subjected to 10% SDS-PAGE. (A) Expression of COX-2 was determined by western blots. β-Actin was used as an internal control. Celecoxib (20 μg/mL) was used as a positive control. (B) Quantitation of western blots results was performed by a densitometer. Results are represented as the relative ratio (%) by normalizing to β-actin signals.

Inhibition of cell-cell adhesion by apigenin

Apigenin was cytotoxic to HUVECs at concentrations greater than 100 μM concentration (Fig. 4), so lower concentrations (12.5 μM, 25 μM, and 50 μM) were used for the cell adhesion assay, which measured the adhesion of THP-1 to HUVEC monolayer. TNF-α-stimulation of HUVEC monolayer significantly increased THP-1 adhesion (Fig. 5), and apigenin reduced the adhesion of THP-1 to HUVEC monolayer in a concentration-dependent fashion, with significance at 50 μM (Fig. 5B). Fig. 5A shows representative images of cell-cell adhesion, treated with or without TNF-α and apigenin.

Exposure of HUVECs in the vascular system to inflammatory mediators, such as interleukin-1 and TNF-α, induces CAMs expression for monocyte attachment (Amrani *et al.*, 2000; Broide *et al.*, 1992). We expected apigenin would suppress the induction of TNF-α-upregulated CAMs in HUVECs. There was no basal mRNA transcription of CAMs in HUVECs (Fig. 6). TNF-α significantly increased the transcription of VCAM-1-, ICAM-1-, and E-selectin, and apigenin decreased this induction. These results are consistent with apigenin-mediated inhibition of the cell adhesion (Fig. 5). RT-PCR results revealed over 85% suppression of VCAM-1, ICAM-1, and E-selectin transcription at 12.5 μM apigenin, in spite of only a 20% inhibition of adhesion at the same concentration of apigenin (Fig. 5B).

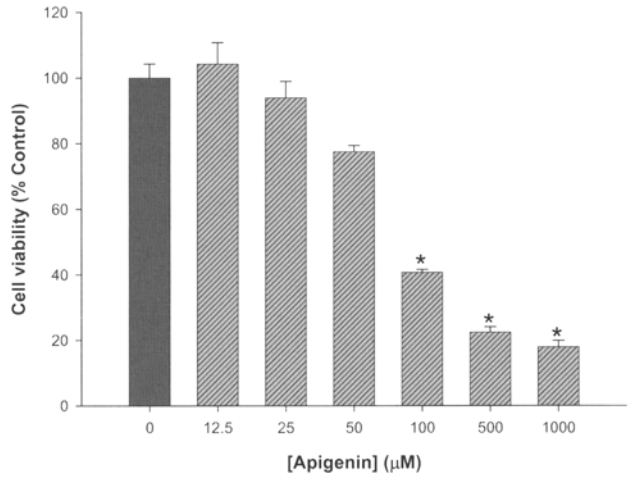

Fig. 4. Effect of apigenin on cell viability of HUVEC monolayer. Apigenin (0-1,000 μM) was added to HUVEC monolayer and incubated in a humidified 5% CO_2 incubator for 24 h at 37°C. Cell viability was performed by MTT assay. Intensity of MTT formazan was determined at 540 nm on a plate reader (Emax, Molecular Devices Inc., U.S.A.). Cell viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells. The IC_{50} value was 65.4 μM. *P<0.05, compared to the control.

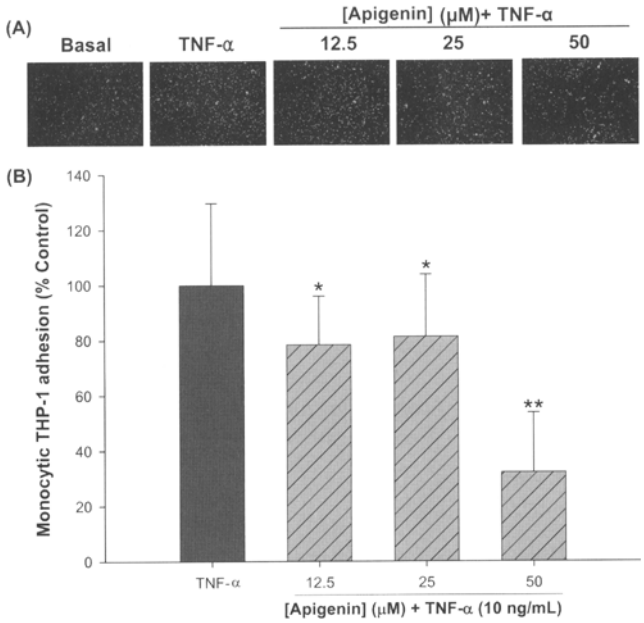

Fig. 5. Effect of apigenin on cell-cell adhesion of monocytic THP-1 to HUVEC monolayer. Prior to cell-cell adhesion assay, THP-1 was fluorescently labeled with calcein-AM (5 ng/mL) for 30 min. (A) Representative images of adhesion of THP-1 to HUVEC monolayer. HUVECs were seeded in 24-well culture plates and incubated for 24 h. Apigenin was added to HUVEC monolayer and pre-incubated for 30 min. TNF-α was added to stimulate adhesion to HUVEC monolayer, and incubated for 6 h. Fluorescently labeled THP-1 cells were photographed by a fluorescence microscope with a WB filter at 100×magnification. (B) Apigenin (0-50 μM) inhibited the adhesion of THP-1 to HUVEC monolayer. HUVEC monolayer was stimulated with 10 ng/mL of tumor necrosis factor-α (TNF-α) for 6 h. Calcein-AM labeled monocytes and HUVEC monolayer were co-incubated in a humidified 5% CO_2 incubator for 1 h at 37°C. Adhesion of monocytic THP-1 to HUVEC monolayer is expressed as fluorescent intensity at 485 and 530 nm. Values are the average of at least triplicate determinations. *P<0.5, **P<0.01, compared to TNF-α treatment.

We assume that this finding was due to the involvement of other components, such as monocyte platelet-activating factor (PAF), involved in cell-cell adhesion (Mayer *et al.*, 2002; Zimmerman *et al.*, 1997).

TNF-α and the interleukin family up-regulate CAMs expression in HUVEC monolayer (Maksimowicz-McKinnon *et al.*, 2004), and mediates monocyte and neutrophil adherence to blood vessel walls, especially HUVEC monolayer. These responses relate to several inflammatory diseases, particularly RA (Ludwig *et al.*, 2004) and atherosclerosis (Koskinen and Lemstrom, 1997). VCAM-1, ICAM-1, and E-selectin are constitutively expressed and further induced by inflammatory agents (Silverman *et al.*, 2001). The expression of CAMs protein is also regulated by mitogen-activated protein kinase (MAPKs) and nuclear factor-κB (NF-κB) pathways (Lee *et al.*, 2006). We are planning to study the effect of apigenin on these pathways

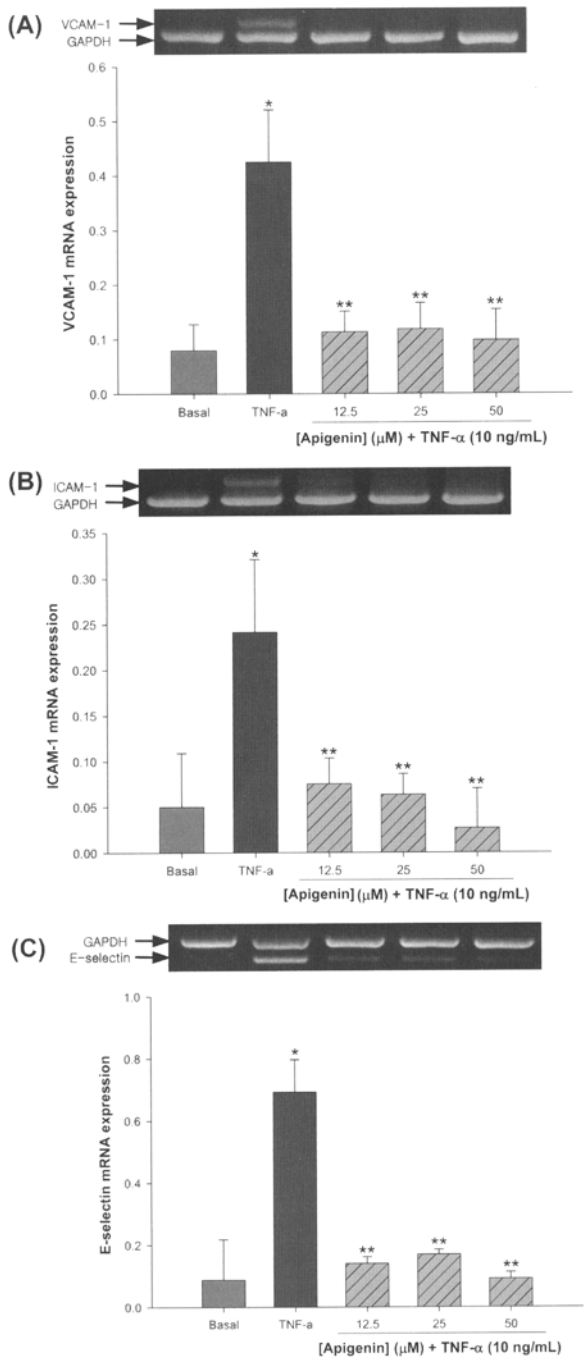

Fig. 6. Suppression of TNF-α-stimulated CAMs transcription in HUVECs by apigenin. HUVEC monolayer was incubated with TNF-α (10 ng/mL) and apigenin (0-50 μM) for 6 h. Isolated RNA samples were analyzed by RT-PCR using primers specific for VCAM-1, ICAM-1, E-selectin, and GAPDH. The intensity of PCR product bands was quantitated by scanning densitometry and standardized to equivalent GAPDH mRNA levels. Data are expressed as mean ± SEM of three independent experiments. (A) VCAM-1 transcription. *P<0.001, compared to the basal level; **P<0.005, compared to TNF-α treatment group. (B) ICAM-1 transcription. *P<0.05, compared to the basal level; **P<0.05, compared to TNF-α treatment group. (C) E-selectin transcription. *P< 0.005, compared to the basal level; **P<0.05, compared to TNF-α treatment group.

in the future.

In this study, we showed apigenin inhibited NO production and COX-2 expression, and suppressed the cell adhesion and CAMs transcription in HUVEC monolayer. NO production and COX-2 expression were inhibited at 10 μM apigenin, whereas suppression of CAMs expression appeared at 50 μM apigenin. We expect that the relatively low concentration of apigenin (10-50 μM) needed for these effects could be obtained via oral administration. Otherwise, the inhibition of the collagenase-reaction occurred at 500 μM apigenin, which is cytotoxic to RAW 264.7 cells and HUVECs. Direct injection of apigenin (500 μM) to inflammatory joints might allow inhibition of collagenase without toxicity to other cell types. The inhibitory activities if apigenin on the inflammatory responses suggests that it may be useful as a functional food component or an alternative medicine to help treat inflammatory symptoms.

ACKNOWLEDGEMENTS

This work was supported by a grant of the Korea Research Foundation Grant funded by the Korean Government (MOEHRD)(KRF-2005-005-J13002).

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