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Anti-inflammatory Inhibition of Endothelial Cell Adhesion Molecule Expression by Flavone Derivatives

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Endothelial expression of cell adhesion molecules (CAM) including VCAM-1, E-selectin, and PECAM-1 plays a leading role in atherosclerosis. Phenolic flavones have been shown to have an anti-inflammatory property. This study examines whether 3′,4′-dimethoxy-7-hydroxyflavone (methoxyflavone) and 2′,3′,7-trihydroxyflavone (hydroxyflavone) inhibited monocyte adhesion to TNF-α-activated endothelium via reduction of CAM expression in human umbilical vein endothelial cells (HUVEC). In stimulated HUVEC the expression of VCAM-1 and E-selectin was enhanced with increasing mRNA levels. Methoxyflavone markedly interfered with the THP-1 monocyte adhesion to TNF-α-stimulated HUVEC. At concentrations of ≥25 μM, methoxyflavone blocked the induction of VCAM-1 but not that of E-selectin on the activated HUVEC. Immunocytochemical staining showed that methoxyflavone modestly inhibited PECAM-1 expression induced by TNF-α. In contrast, hydroxyflavone minimally inhibited TNF-α-stimulated E-selectin expression without affecting VCAM-1 level. The inhibitory effect of methoxyflavone on THP-1 adhesion to HUVEC appears to be greater than that of hydroxyflavone, most likely due to a greater inhibition of CAM expression. Thus, some flavone derivatives containing methoxy groups may have therapeutic potential attenuating inflammatory response-related atherosclerosis.

KEYWORDS: Flavone derivatives; tumor necrosis-α; cell adhesion molecules; endothelium

INTRODUCTION

There is increasing evidence that an increased intake of polyphenolic phytochemicals such as flavonoids naturally found in fruits and vegetables may contribute to the low incidence of cardiovascular diseases (French paradox) (*1*−*4*). Flavonoids have been shown to modify eicosanoid biosynthesis (*5*), prevent platelet aggregation (*6*, *7*), and promote relaxation of cardiovascular smooth muscle (*8*, *9*). In addition, flavonoids and related polyphenolics have a great potential to delay low-density lipoprotein (LDL) oxidation with the radical scavenging capacity (*10*, *11*), and wine flavonoids protect against atherosclerosis by inhibiting the accumulation of oxidized LDL in atherosclerotic lesions (*12*). This observation implies that flavonoids confer protection against early events in atherogenic lesion formation.

Cell adhesion molecules (CAM) such as vascular cell molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), endothelial-leukocyte adhesion molecule-1 (E-selectin), and platelet endothelial cell adhesion molecule-1 (PECAM-1) have been observed in atherosclerotic lesions and at sites predisposed to lesion formation in human coronary atherosclerotic plaques (*13*−*16*). Overexpression of CAM is a common feature in inflammatory environments, which is one of the first events in the development of atherosclerosis (*17*, *18*). These CAM proteins are induced by proinflammatory cytokines such as interleukin-1 and tumor necrosis factor-α (TNF-α) (*17*−*21*). In addition, growth factors, platelet activator, and chemotactic factors in the blood are involved in the CAM induction. It has been documented that some flavonoids act as anti-inflammatory agents to inhibit the expression of CAM (*22*−*26*).

In our previous study (*26*), apigenin and luteolin, flavones naturally found, nearly completely inhibited TNF-α-induced CAM proteins, VCAM-1, ICAM-1, and E-selectin, at transcriptional levels. To test the hypothesis that the flavone inhibition of CAM induction virtually involved in the atherogenic lesion formation stems from chemical structures of flavones, the present study elucidated the capability of flavone derivatives with different structural features (**Figure 1**) in inhibiting TNF-α-induced CAM expression in human umbilical vein endothelial cells (HUVEC). This allowed the correlation between structural elements and potential capacity inhibiting CAM expression to be defined. This study further investigated the anti-inflammatory activity of flavone derivatives at transcriptional levels with respect to CAM expression.

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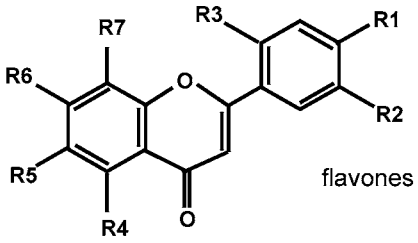

	R1	R2	R3	R4	R5	R6	R7
11N	OCH_3	OCH_3	H	H	H	OH	H
4N	OH	H	H	H	H	OH	H
2N	H	H	OH	H	H	OH	H
5C	OCH_3	H	H	H	H	H	OH
5N	OCH_3	H	H	H	H	OH	H
21N	OH	H	OH	H	H	OH	H

Figure 1. Flavone backbone and flavone derivatives used in the experiments.

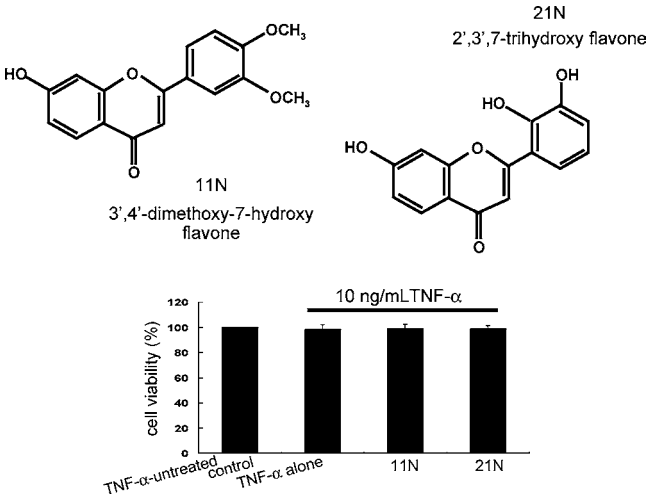

Figure 2. Chemical structures of 3′,4′-dimethoxy-7-hydroxyflavone (11N) and 2′,3′,7-trihydroxyflavone (21N): HUVEC viability after coculture with both 11N or 21N and TNF-α, as determined by MTT assay (in the bottom panel). Values are mean ± SEM, $n = 4$, expressed as percent cell survival relative to TNF-α- and 11N- or 21N-untreated controls (viability = 100%).

MATERIALS AND METHODS

Materials. M199 medium chemicals, RPMI 1640 medium chemicals, 7,8-dimethoxyflavone, and 3-(4,5-dimethylthiazolyl)diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO), as were all other reagents, unless specifically stated elsewhere. Collagenase was purchased from Worthington Biochemicals (Lakewood, NJ). Fetal bovine serum (FBS), penicillin−streptomycin, trypsin−EDTA, bovine brain extract, human epidermal growth factor, and hydrocortisone were purchased from Cambrex Corp. (East Rutherford, NJ). Human monocytic leukemic cell line THP-1 was obtained from American Type Culture Collection (Rockville, MD). TNF-α was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Reverse transcriptase, *Taq* DNA polymerase, and dNTP were purchased from Promega Co. (Madison, WI).

Preparation of Flavone Derivatives. The tested flavones were synthesized according to the method described in our previous paper (*27*). The hydroxy groups of the flavone derivatives were protected with methoxymethyl groups using chloromethylmethyl ether (*28*). The structures of flavone derivatives, 3′,4′-dimethoxy-7-hydroxyflavone (11N) and 2′,3′,7-trihydroxyflavone (21N), were confirmed by IR, UV, ^{1}H NMR, and ^{13}C NMR spectra (**Figure 2**). 11N has been isolated from *Launaea asplenifolia* Hook (*29*). All flavone derivatives were solubilized by dimethyl sulfoxide (DMSO) for culturing with cells; the final culture concentration of DMSO was ≤0.5%.

Preparation of HUVEC. HUVEC were isolated from human umbilical cords using collagenase as described elsewhere (*30*). Primary cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were cultured in 25 mM HEPES-buffered M199 containing 10% FBS, 2 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin supplemented with 0.75 mg/mL human epidermal growth factor, and 0.075 mg/mL hydrocortisone. Primary-cultured cells were passaged at confluence and used within 10 passages. Endothelial cells were confirmed by their cobblestone morphology and uptake of fluorescent 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled acetylated LDL (Molecular Probes Inc., Eugene, OR; ref *31*).

Cell Culture Experiments. HUVEC were plated at 90−95% confluence in all experiments. Cells were incubated for 16−18 h with flavone derivatives in the absence and presence of 10 ng/mL TNF-α (Roche). In experiments for the dose response to the TNF-α-induced VCAM-1 expression, HUVEC were incubated with 1−50 μM flavone derivatives prior to an exposure to TNF-α.

In a special protocol, it was tested whether during 24 h of incubation ≥25 μM flavone derivatives affected HUVEC viability. At the end of the 24 h incubation period, the MTT assay was performed to quantitate cellular viability (*32*). HUVEC were incubated in a fresh medium containing 1 mg/mL MTT for 3 h at 37 °C. After removal of unconverted MTT, the purple formazan product was dissolved in 1 mL of acidic 2-propanol with gentle shaking for 10 min. The purple formazan product was measured colorimetrically at $\lambda = 570$ nm with background subtraction at $\lambda = 690$ nm.

Western Blot Analysis for CAM Protein Expression. For the measurements of CAM protein expression, whole cell extracts were prepared from HUVEC in 1 M Tris-HCl (pH 6.8) lysis buffer containing 10% SDS, 1% β-glycerophosphate, 0.1 M Na_3VO_4, 0.5 M NaF, and protease inhibitor cocktail. Cell lysates containing equal amounts of total protein were fractionated by electrophoresis on 8% SDS-PAGE gels 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 for 3 h with a primary antibody [polyclonal rabbit anti-human VCAM-1 antibody (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA)] and polyclonal goat anti-human E-selectin antibody (1:500, Santa Cruz Biotechnology). After three washes with TBS-T buffer, the membrane was then incubated for 1 h with goat anti-rabbit IgG horseradish peroxidase (1:10000 dilution, Jackson ImmunoResearch Laboratory, West Grove, PA) or rabbit anti-goat IgG conjugated to horseradish peroxidase (1:1000 dilution, Jackson ImmunoResearch Laboratory). The

levels of VCAM-1 and E-selectin proteins were determined by using Supersignal West Pico chemiluminescence detection reagents (Pierce Biotechnology Inc., Rockford, IL) and Konica X-ray film (Konica Co., Tokyo, Japan). Incubation with monoclonal mouse β-actin antibody (1:5000 dilution, Sigma Chemical) was also performed for the comparative control.

Immunocytochemistry for in Situ Detection of PECAM-1 Expression. After HUVEC grown on eight-well chamber slides were thoroughly washed with PBS containing 0.05% Tween 20 (PBS/T), cells were fixed with 4% ice-cold formaldehyde for 30 min and treated for 2 min with 0.1% Triton-X100 and 0.1% citric acid in PBS. For blocking any nonspecific binding, cells were incubated for 3 h with 1% bovine serum albumin (BSA) and 0.3% Triton-X100 in PBS. Cells were washed with PBS/T, and polyclonal goat anti-human PECAM-1 antibody (1:50 dilution in PBS; Santa Cruz Biotechnology) in 0.1% BSA was sufficiently added to cells and incubated overnight at 4 °C. Cells were incubated with cyanine 3-OSu conjugate−anti-goat IgG (1:750 dilution; Rockland Co., Gilbertville, PA) as a secondary antibody. Fluorescent images were obtained by a fluorescence microscopy with an Olympus BX51 fluorescent microscope with differential interference contrast and reflected light fluorescence.

Coculture and in Vitro Monocyte Adhesion Assay. The treatment with flavone derivatives might inhibit monocyte recruitment on the TNF-α-stimulated vascular endothelium. HUVEC were grown in 25 mM HEPES buffer at a density of 1.0×10^5 cells on four-well glass chamber slides. Cells were pretreated with flavone derivatives for 16−18 h prior to the 6 h exposure to 10 ng/mL TNF-α (*33*). Human monocytic leukemic cell line THP-1 cells (American Type Culture Collection) were labeled with 5 μM calcein AM (Molecular Probes Inc.) in RPMI 1640 medium containing 10% FBS. In the coculture system, the labeled THP-1 cells were seeded at a density of 5.0×10^5 cells onto a nearly confluent monolayer of HUVEC treated with the flavone derivative and/or TNF-α and were incubated for 2 h. The cocultured cells were thoroughly washed, and images were obtained at 485 nm excitation and 538 nm emission using a SPOT II digital camera-attached fluorescence microscope with Spot II data acquisition software (Diagnostic Instrument, Livingston, Scotland). The quantitative results were obtained by using a Fluoroscan enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Rad Laboratories, Hercules, CA) at $\lambda = 485$ nm excitation and $\lambda = 538$ nm emission.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis. To determine whether the inhibitory effects of 11N on activated CAM expression reflect a decrease in the steady-state mRNA levels of CAM proteins, TNF-α-induced mRNAs of VCAM-1 and E-selectin in HUVEC were examined by RT-PCR analysis. Total RNA was isolated from HUVEC using a commercially available Trizol reagent kit (Gibco BRL, Gainsberg, MD) after culture protocols. The RNA (5 μg) was reversibly transcribed with 10000 units of reverse transcriptase and 0.5 mg/mL oligo-$(dT)_{15}$ primer (Bioneer Co., Daejeon, Korea). The expressions of the mRNA transcripts of VCAM-1 (forward primer, 5′-ATGCCTGGGAAGATGGTCGTGA-3′; reverse primer, 5′-TGGAGCTGGTAGACCCTCGCTG-3′), E-selectin (forward primer, 5′-ATCATCCTGCAACTTCACC-3′; reverse primer, 5′-ACACCTCACCAAACCCTTC-3′), and β-actin (forward primer, 5′-GACTACCTCATGAAGATC-3′; reverse primer, 5′-GATCCACATCTGCTGGAA-3′) were evaluated by RT-PCR as previously described (*33*). The PCR was performed in 50 μL of 10 mM Tris-HCl (pH 8.3), 25 mM $MgCl_2$, 10 mM dNTP, 100 units of *Taq* DNA polymerase, and 0.1 μM each primer and was terminated by heating at 70 °C for 15 min. After thermocycling and electrophoresis of the PCR products (5 μL) on 1% agarose−formaldehyde gel containing ethidium bromide (0.5 μg/mL) for 1 h at 100 V, the bands were visualized using a UV transilluminator (Amersham Pharmacia Biotechnology, Piscataway, NJ), and gel photographs were obtained using Polaroid Type 667 positive/negative films. The absence of contaminants was routinely checked by the RT-PCR assay of negative control samples without a primer addition.

Data Analysis. The results are presented as mean ± SEM. Statistical analyses were conducted using Statistical Analysis Systems statistical software package version 6.12 (SAS Institute Inc., Cary, NC). One-way ANOVA was used to determine anti-inflammatory effects of flavone derivatives onto the activated endothelium. The differences among flavone treatment groups were analyzed with Duncan's multiple-range test and were considered to be significant at $P < 0.05$.

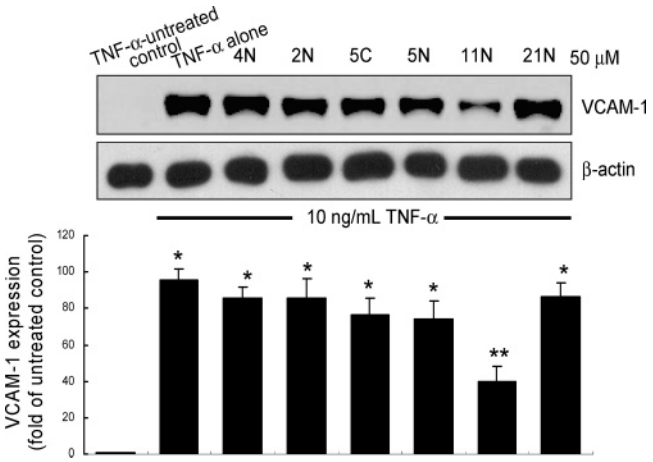

Figure 3. Western blot data showing effects of various flavone derivatives on expression levels of VCAM-1 in TNF-α-stimulated HUVEC. After HUVEC culture protocols with 50 μM of each tested flavone and 10 ng/mL TNF-α, cell extracts were subjected to 8% SDS-PAGE and Western blot analysis with a primary antibody against VCAM-1. β-Actin protein was used as an internal control. The bar graphs (mean ± SEM, $n = 3$) in the bottom panel represent quantitative results obtained from a densitometer. *, $P < 0.05$, compared to TNF-α-untreated control (value = 1). **, $P < 0.05$, compared to TNF-α-alone.

RESULTS AND DISCUSSION

Inhibition of TNF-α-Induced Expression of VCAM-1 and E-Selectin by 11N. Flavonoids may inhibit early events in the atherosclerotic process by modulating monocyte adhesion and transmigration (*26*). Although definite mechanisms underlying the flavonoid protection against early atherogenic process are not fully understood, they may involve down-regulation of inflammatory chemokines and cytokines, matrix proteases, and CAM (*22*, *34*, *35*). Many in vitro studies have revealed that TNF-α and interleukin-1β induce CAM expression in human vascular endothelial cells and smooth muscle cells (*21*, *33*, *36*, *37*). In the present study Western blot analysis was used to address blockage of the TNF-α-activated expression of VCAM-1 and E-selectin by flavone derivatives (**Figures 3** and **4**). Coculture of HUVEC with 11N and TNF-α did not cause toxicity (**Figure 2**), as determined by MTT assay. As expected, there was undetectable or relatively weak expression of VCAM-1 and E-selectin in TNF-α-untreated quiescent cells. Expression of these CAM proteins was greatly enhanced in TNF-α-stimulated cells over the quiescent cells. TNF-α-exposed cells treated with 50 μM 11N proved marked inhibition of expression of VCAM-1 (**Figure 3**). When HUVEC were treated with other test flavone derivatives including 21N, the expression of VCAM-1 was feebly or barely decreased. We also attempted to quantify the E-selectin expression in experimental protocols using 50 μM flavones (**Figure 4**). TNF-α-induced E-selectin expression was substantially inhibited by 50 μM 21N. However, 11N did not influence the E-selectin induction by TNF-α. In addition, 4′-methoxyflavone and 2′-hydroxyflavone containing one methoxy group or one hydroxy group in ring B (see **Figure 1**) appeared to intensify the E-selectin expression activated by TNF-α. Accordingly, it is assumed that there is a linkage between the presence of methoxy groups in the flavone benzoyl system and CAM expression in HUVEC.

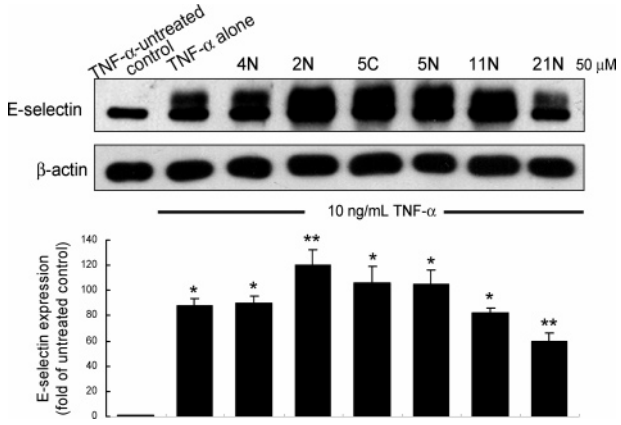

Figure 4. Effects of flavone derivatives on TNF-α-stimulated E-selectin expression in HUVEC. After HUVEC culture protocols with 50 μM of each flavone and 10 ng/mL TNF-α, cell extracts were subjected to 8% SDS-PAGE and Western blot analysis with a primary antibody against E-selectin. β-Actin protein was used as an internal control. The bar graphs (mean ± SEM, $n = 3$) in the bottom panel represent quantitative densitometric results of upper bands. *, $P < 0.05$, compared to TNF-α-untreated control (value = 1). **, $P < 0.05$, compared to TNF-α-alone.

To address blockage of the TNF-α-activated expression of PECAM-1 protein by 11N, PECAM-1-specific antibody was used for immunostaining assay. **Figure 5A** compares the effects of 11N on expression level of PECAM-1 elevated by TNF-α. As expected, there was a lack of staining in the untreated control, whereas a heavy PECAM-1 staining in TNF-α-alone-exposed cells was observed, indicative of increased expression of PECAM-1 protein at single cell level. The 11N- and TNF-α-exposed cells reveal substantial inhibition of PECAM-1 expression. We also attempted to quantify the PECAM-1 protein expression in our Western blot experimental protocols. The Western blot data (**Figure 5B**) supported the staining results (**Figure 5A**), suggesting that 11N is capable of attenuating TNF-α-induced PECAM-1 expression in the vascular endothelium.

When 11N was added in concentrations between 1 and 50 μM, VCAM-1 induction by TNF-α decreased in a dose-dependent manner with inhibitory doses requiring only ≥25 μM (**Figure 6**). On the other hand, TNF-α-induced E-selectin expression was not affected by 11N within the test range of doses. To achieve the substantial inhibitory effect of 11N on TNF-α-induced monocyte adhesion in the VCAM-1 expression model, micromolar doses of ≥25 μM were required.

It has been demonstrated that there is structure−activity relationship of natural flavonoids in free radical scavenging effects (*38*, *39*). In addition, we have reported that the potential capability to prevent inflammation-induced CAM protein expression differs among individual flavonoid subclasses (*26*). In particular, quercetin and flavones such as luteolin and apigenin prevented the activation of CAM expression and hence blocked monocyte adhesion on the TNF-α-activated endothelium (*26*). It is speculated that the expression inhibition of CAM by these flavonoids may stem from the differences of their chemical structures. **Figure 7** compares the effects of 7,8-dimethoxyflavone on protein expression level of VCAM-1, E-selectin, and PECAM-1. Consistently, there was undetectable or low expression of all three CAM in quiescent cells and relatively substantial expression in TNF-α-treated HUVEC. However, this flavone did not affect the CAM protein expression stimulated by TNF-α.

The ability of 11N to block TNF-α-induced CAM expression could be due to their antioxidant capacity. It has been demonstrated that oxidative stress up-regulates VCAM-1 and E-selectin expression via redox-sensitive transcriptional activation, which is inhibited by the known antioxidants pyrrolidine dithiocarbamate and *N*-acetylcysteine (*40*, *41*). Also, the classical

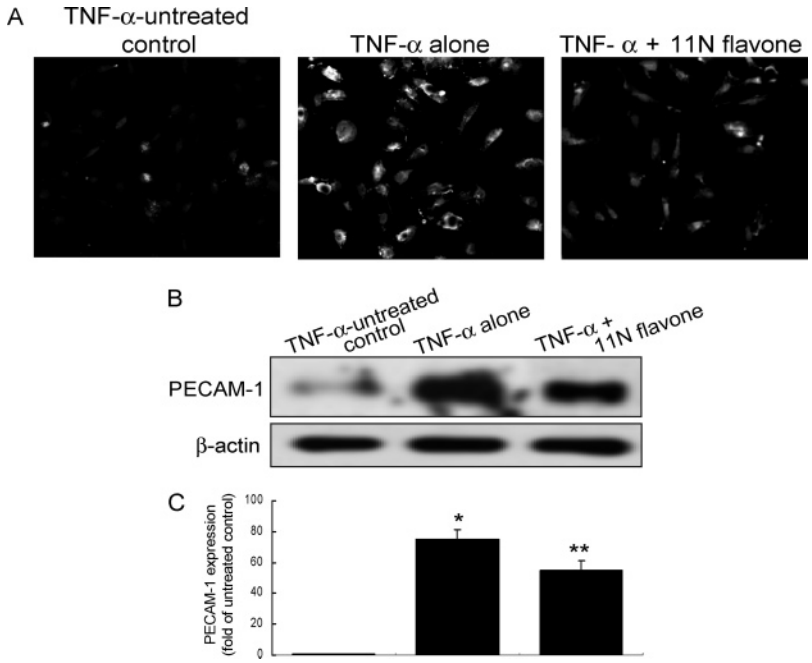

Figure 5. Effect of 11N on expression of PECAM-1 protein in cultured HUVEC treated with TNF-α. Cells were incubated with 50 μM 11N for 30 min and exposed to 10 ng/mL TNF-α for 6 h. After HUVEC culture protocols with 50 μM of each flavone and 10 ng/mL TNF-α, cells were fixed and then incubated with goat anti-human PECAM-1 for immunocytochemical staining (**A**). Antibody localization was detected with Cy-3-conjugated anti-goat IgG using a fluorescence microscopy with rhodamine green filter. Magnification: 200-fold. In another set of experiments for PECAM-1 expression, cell extracts were subjected to 8% SDS-PAGE and Western blot analysis with a primary antibody against PECAM-1 (**B**). The bar graphs (mean ± SEM, $n = 3$) represent quantitative densitometric results of upper bands (**C**). *, $P < 0.05$, compared to TNF-α-untreated control (value = 1). **, $P < 0.05$, compared to TNF-α-alone.

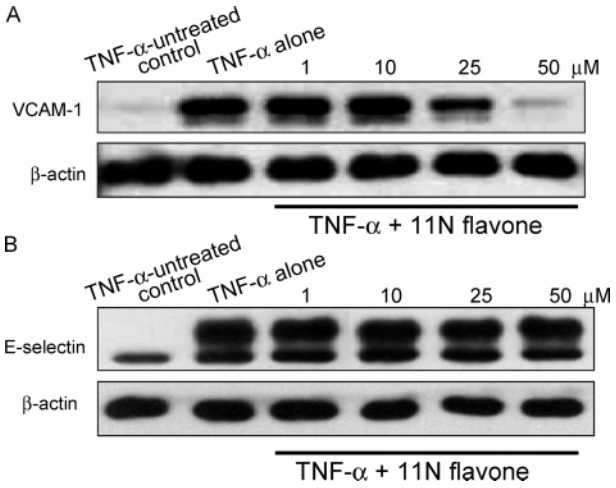

Figure 6. Inhibitory dose responses of 11N to induction of VCAM-1 (**A**) and E-selectin (**B**) in TNF-α-stimulated HUVEC. After HUVEC culture protocols with 1–50 μM 11N and 10 ng/mL TNF-α, cell extracts were subjected to 8% SDS-PAGE and Western blot analysis with VCAM-1 or E-selectin primary antibody (three separate experiments). β-Actin protein was used as an internal control.

antioxidant vitamin E has been shown to inhibit expression of CAM and adhesion of monocytes to endothelial cells (*42*). The natural flavones, luteolin and apigenin, were relatively inactive at micomolar concentrations in scavenging 1,1-diphenyl-2-picrylhydrazyl radical in cell-free systems (*43*). In addition, submillimolar *N*-acetylcysteine did not down-regulate TNF-α-induced VCAM-1 expression (unpublished data). Accordingly, it is unlikely that the antioxidative potential of 11N contributes to their blockade of endothelial CAM induction by TNF-α.

It is not established whether the property of anti-inflammatory flavones to mitigate endothelial CAM induction in vitro translates into true anti-atherogenicity in vivo. Whether flavones act in vivo as anti-inflammatory agents appears to depend on their bioavailability. It has been shown that small intestinal absorption of dietary flavonoids ranges from 0 to 60% of the intake dose, and the removal half-lives range from 2 to 28 h (*44*, *45*). Absorbed flavonoids undergo extensive first-pass metabolism in epithelial cells of the small intestine and in the liver (*46*). The small intestine appears to be the organ primarily responsible for glucuronidation and to play a role in methylation, and flavonoid metabolites conjugated with methyl group, glucuronate, and sulfate groups are the predominant forms present in plasma (*44*). Accordingly, it is speculated that flavone derivatives used in this study would be metabolized in vivo and that the metabolites may cause the anti-inflammatory effects on endothelial CAM expression. The possible metabolite conjugates could be chemically distinct from their parent flavone derivatives, differing in size, polarity, ionic form, and, most likely, their physiologic actions.

Inhibition of TNF-α-Induced Monocyte Adhesion by 11N. It has been shown that flavonoids block monocyte adhesion and transmigration to the activated endothelium (*26*). The observed inhibition of CAM expression by 11N suggested that this flavone treatment might inhibit mononuclear leukocyte recruitment on the TNF-α-induced vascular endothelium. In vitro adhesion assay of monocytes to HUVEC using calcein AM staining technique supported this notion. A small number of monocytes were adhered to quiescent HUVEC free of TNF-α (**Figure 8**). There was heavy staining on the TNF-α-alone-exposed HUVEC, indicative of a marked increase in the monocytic THP-1 adherence to the activated HUVEC. However, the treatment of TNF-α-exposed cells with 50 μM 11N markedly inhibited monocyte adherence. It should be noted that the blockade of TNF-α-stimulated monocyte adherence by 11N was most likely mediated mainly via inhibition of VCAM-1 protein expression. In contrast, 50 μM 21N moderately but insignificantly inhibited the TNF-α-induced THP-1 adhesion, possibly through the fairly small inhibition of E-selectin expression upregulated by TNF-α (**Figure 4**). Accordingly, the Western blot data (**Figure 3**) supported the in vitro adhesion results (**Figure 8**). The effect of 11N on THP-1 monocyte adhesion to the HUVEC appears to be much greater than that of 21N.

The flavone luteolin has been recently reported to reduce lipopolysaccharide-induced lethal toxicity, possibly by inhibiting TNF-α and ICAM-1 expression in vivo (*47*). It should be pointed out that the treatment with TNF-α and/or 11N or 21N did not cause cytotoxicity, thus implying that blunting the adhesion of monocytes to the activated endothelial cells by these flavones is not attributed to cytotoxicity. The inhibition of the expression of CAM, especially VCAM-1, appeared to be responsible for the adhesion blockade.

Effect of 11N on TNF-α-Induced CAM Transcription. There were weak signals for the basal mRNA expression of VCAM-1 and E-selectin in quiescent cells (**Figure 9**). In contrast, their CAM mRNA was greatly increased in TNF-α-stimulated HUVEC. The RT-PCR data showed that the TNF-α-induced mRNA accumulation of VCAM-1 in 11N-treated

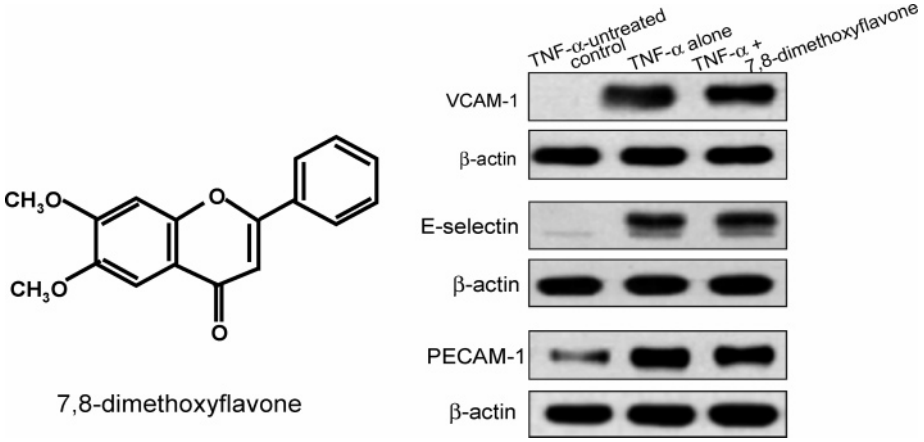

Figure 7. Chemical structures of 7,8-dimethoxyflavone and Western blot data showing effects of this flavone derivative (50 μM) on expression of HUVEC adhesion molecules stimulated by 10 ng/mL TNF-α. Cell extracts were subjected to 8% SDS-PAGE and Western blot analysis with a primary antibody against VCAM-1, E-selectin, or PECAM-1. β-Actin protein was used as an internal control.

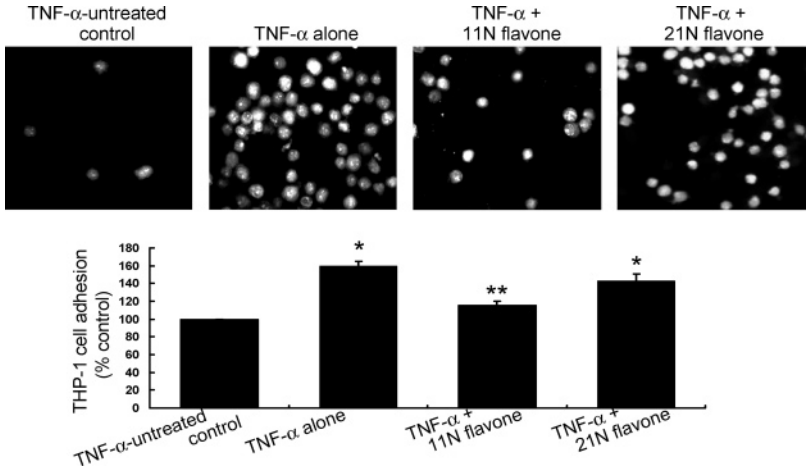

Figure 8. Inhibition by 11N of THP-1 monocyte adhesion to the TNF-α-activated HUVEC. HUVEC were pretreated with and without 11N or 21N at 50 μM for 30 min and then activated with 10 ng/mL TNF-α for 6 h. Endothelial cells were cocultured with calcein AM-labeled THP-1 monocytes for 2 h. Microphotographs (three independent experiments) were obtained using a fluorescence microscopy with fluorescein blue filter (upper panel). Magnification: ×200. The bar graphs (bottom panel) represent quantitative results obtained by using a Fluoroscan ELISA plate reader at λ = 485 nm excitation and λ = 538 nm emission.*, $P < 0.05$, compared to TNF-α-untreated control. **, $P < 0.05$, compared to TNF-α-alone.

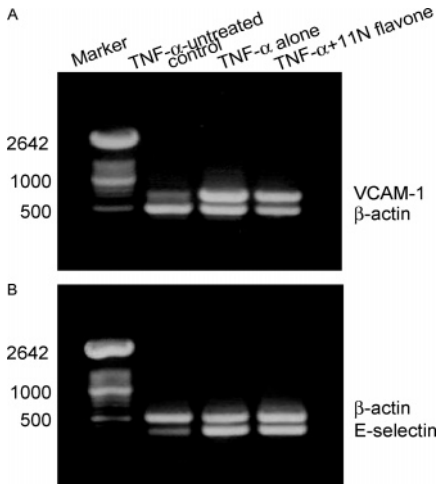

Figure 9. Reverse transcriptase Polymerase Chain Reaction data showing the steady-state mRNA transcriptional levels of VCAM-1 and E-selectin. Confluent human umbilical vein endothelial cells were incubated with 50 μM 11N for 30 min and exposed to 10 ng/mL TNF-α for 6 h. β-Actin protein was used as an internal control for the coamplification with VCAM-1 and E-selectin.

cells was sustained. This was conflicted with a substantial attenuation of expression of VCAM-1 protein by 11N shown in **Figure 3**. These results imply that this flavone inhibited VCAM-1 expression independent of transcriptional modulation of VCAM-1 protein. As expected, 21N did not reduce the up-regulated mRNA level of E-selectin induced by TNF-α (data not shown), which was consistent with no attenuation of expression of E-selectin protein by 21N.

It is not yet established how CAM proteins are selectively modulated in response to pro-inflammatory cytokines and which signaling pathways are involved in the modulation of these proteins. Indeed, endothelial expression of VCAM-1 and PECAM-1 activated by TNF-α was blocked possibly through mechanism(s) responsive to 11N but not to 21N. Nevertheless, 11N-triggered signaling mechanisms are still uncertain from this study. The initial signal of TNF-α in terms of the interaction of ligand with its receptor could be affected by 11N. This speculation is inferred from a previous study demonstrating that flavopiridol, a flavonoid currently in cancer clinical trials, sensitizes cholangiocarcinoma cells to cytotoxicity of TNF-related apoptosis-inducing ligand (*48*). We assume that 11N enables endothelial cells to be resistant to the TNF-α-induced expression of VCAM-1. In addition, it is assumed that the access of 11N to putative binding proteins in the endothelial cells may modulate the TNF-α-mediated activation of signaling cascades through interruption of the activation of extracellular signal-regulated kinases by TNF-α.

Regulation of the inducibility of nitric oxide synthase and cyclooxygenase 2 involves transcriptional regulation and post-transcriptional mRNA stabilization (*49*, *50*). Indeed, 11N did not mitigate the TNF-α induction of VCAM-1 by transcriptional step-down regulation. We propose that 11N can affect CAM expression by altering mRNA stability with a minimal effect on the constitutive levels of VCAM-1 mRNA. Genes encoding numerous proto-oncogenes, cytokines, and a number of G-protein-coupled receptors are regulated post-transcriptionally at the level of mRNA stability (*51*, *52*). Recent evidence points to an important role for mitogen-activated kinase in regulating the turnover of cytokine mRNA such as TNF-α (*52*, *53*). The present study did not perform additional experiments for the VCAM-1 mRNA stability. It has been shown that the addition of flavopiridol does not affect the transcriptional activation of a vascular endothelial growth factor (VEGF) promoter−luciferase construct but dramatically diminishes the mRNA stability of VEGF (*54*). Resveratrol, a polyphenolic phytoalexin found in grapes and wine, up-regulated endothelial nitric oxide synthase (eNOS) mRNA expression in a time- and concentration-dependent manner by stabilizing eNOS mRNA (*50*).

In summary, our studies have demonstrated that 11N is capable of preventing the early processes of atherosclerosis involving inducible VCAM-1 expression. 11N blocked the activation of expression of VCAM-1 and PECAM-1 but not that of E-selectin and prevented monocyte adhesion on the TNF-α-activated endothelium. This observation might have implications for strategies preventing and attenuating inflammatory diseases. The selective inhibitory mechanisms of 11N for the CAM expression appear to be antioxidant-insensitive and may argue against transcription-related mechanisms as the major target of the antiatherogenic action of 11N.

LITERATURE CITED

(1) Kris-Etherton, P. M.; Keen, C. L. Evidence that the antioxidant flavonoids in tea and cocoa are beneficial for cardiovascular health. *Curr. Opin. Lipidol.* **2002**, *13*, 41−49.

(2) Geleijnse, J. M.; Launer, L. J.; Van der Kuip, D. A.; Hofman, A.; Witteman, J. C. Inverse association of tea and flavonoid intakes with incident myocardial infarction: the Rotterdam Study. *Am. J. Clin. Nutr.* **2002**, *75*, 880−886.

(3) Vinson, J. A.; Mandarano, M.; Hirst, M.; Trevithick, J. R.; Bose, P. Phenol antioxidant quantity and quality in foods: beers and the effect of two types of beer on an animal model of atherosclerosis. *J. Agric. Food Chem.* **2003**, *51*, 5528−5533.

(4) Mennen, L. I.; Sapinho, D.; de Bree, A.; Arnault, N.; Bertrais, S.; Galan, P.; Hercberg, S. Consumption of foods rich in flavonoids is related to a decreased cardiovascular risk in apparently healthy French women. *J. Nutr.* **2004**, *134*, 923−926.

(5) Polagruto, J. A.; Schramm, D. D.; Wang-Polagruto, J. F.; Lee, L.; Keen, C. L. Effects of flavonoid-rich beverages on prostacyclin synthesis in humans and human aortic endothelial cells: association with ex vivo platelet function. *J. Med. Food* **2003**, *6*, 301−308.

(6) Sheu, J. R.; Hsiao, G.; Chou, P. H.; Shen, M. Y.; Chou, D. S. Mechanisms involved in the antiplatelet activity of rutin, a glycoside of the flavonol quercetin, in human platelets. *J. Agric. Food Chem.* **2004**, *52*, 4414−4418.

(7) Dell'Agli, M.; Busciala, A.; Bosisio, E. Vascular effects of wine polyphenols. *Cardiovasc. Res.* **2004**, *63*, 593−602.

(8) Hnatyszyn, O.; Moscatelli, V.; Rondina, R.; Costa, M.; Arranz, C.; Balaszczuk, A.; Coussio, J.; Ferraro, G. Flavonoids from *Achyrocline satureioides* with relaxant effects on the smooth muscle of guinea pig corpus cavernosum. *Phytomedicine* **2004**, *11*, 366−369.

(9) Ajay, M.; Gilani, A. U.; Mustafa, M. R. Effects of flavonoids on vascular smooth muscle of the isolated rat thoracic aorta. *Life Sci.* **2003**, *74*, 603−612.

(10) Hwang, J.; Wang, J.; Morazzoni, P.; Hodis, H. N.; Sevanian, A. The phytoestrogen equol increases nitric oxide availability by inhibiting superoxide production: an antioxidant mechanism for cell-mediated LDL modification. *Free Radical Biol. Med.* **2003**, *34*, 1271−1282.

(11) Chung, S. K.; Kim, Y. C.; Takaya, Y.; Terashima, K.; Niwa, M. Novel flavonol glycoside, 7-*O*-methyl mearnsitrin, from *Sageretia theezans* and its antioxidant effect. *J. Agric. Food Chem.* **2004**, *52*, 4664−4668.

(12) Aviram, M.; Fuhrman, B. Wine flavonoids protect against LDL oxidation and atherosclerosis. *Ann. N. Y. Acad.* Sci. **2002**, *957*, 146−161.

(13) Iiyama, K.; Hajra, L.; Iiyama, M.; Li, H.; DiChiara, M.; Medoff, B. D.; Cybulsky, M. I. Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation. *Circ. Res.* **1999**, *5*, 199−207.

(14) Lessner, S. M.; Prado, H. L.; Waller, E. K.; Galis, Z. S. Atherosclerotic lesions grow through recruitment and proliferation of circulating monocytes in a murine model. *Am. J. Pathol.* **2002**, *160*, 2145−2155.

(15) Van der Wal, A. C.; Das, P. K.; Tigges, A. J.; Becker, A. E. Adhesion molecues on the endothelium and mononuclear cells in human atherosclerotic lesions. *Am. J. Pathol.* **1992**, *141*, 161−168.

(16) O'Brien, K. D.; Allen, M. D.; McDonald, T. O.; Chait, A.; Harlan, J. M.; Fishbein, D.; McCarty, J.; Furgerson, M.; Hudkins, K.; Benjamin, C. D.; Lobb, R.; Alpers, C. E. Vascular cell adhesion molecule-1 is expressed in human coronary atherosclerotic plaques. *J. Clin. Invest.* **1993**, *92*, 945−951.

(17) Scalia, R.; Appel, J. Z., III; Lefer, A. M. Leukocyte-endothelium interaction during the early stages of hypercholesterolemia in the rabbit. Role of P-selectin, ICAM-1, and VCAM-1. *Arterioscler. Thromb. Vasc. Biol.* **1998**, *18*, 1093−1100.

(18) Schonbeck, U.; Mach, F.; Libby, P. CD154 (CD40 ligand). *Int. J. Biochem. Cell Biol.* **2000**, *32*, 687−693.

(19) Osborn, L.; Hessian, C.; Tizard, R.; Vassalio, C.; Luhowskyj, S.; Chi-Rosso, S.; Lobb, R. Direct expression cloning of vascular cell adhesion molecule-1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell* **1989**, *59*, 1203−1211.

(20) Cybulsky, M. L.; Gimbrone, M. A., Jr. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science* **1991**, *251*, 788−791.

(21) Dustin, M. L.; Rothlein, R.; Bhan, A. K.; Dinarello, C. A.; Springer, T. A. Induction by IL-1 and interferon γ: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J. Immunol.* **1986**, *137*, 245−254.

(22) Bito, T.; Roy, S.; Sen, C. K.; Shirakawa, T.; Gotoh, A.; Ueda, M.; Ichihashi, M.; Packer, L. Flavonoids differentially regulate IFN γ-induced ICAM-1 expression in human keratinocytes: molecular mechanisms of action. *FEBS Lett.* **2002**, *520*, 145−152.

(23) Gerritsen, M. E.; Carley, W. W.; Ranges, G. E.; Shen, C. P.; Phan, S. A.; Ligon, G. F.; Perry, C. A. Flavonoids inhibit cytokine-induced endothelial cell adhesion protein gene expression. *Am. J. Pathol.* **1995**, *147*, 278−292.

(24) Murase, T.; Kume, N.; Hase, T.; Shibuya, Y.; Nishizawa, Y.; Tokimitsu, I.; Kita, T. Gallates inhibit cytokine-induced nuclear translocation of NF-κB and expression of leukocyte adhesion molecules in vascular endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **1999**, *19*, 412−1420.

(25) Kobuchi, H.; Roy, S.; Sen, C. K.; Nguyen, H. G.; Packer, L. Quercetin inhibits inducible ICAM-1 expression in human endothelial cells through the JNK pathway. *Am. J. Physiol.* **1999**, *277*, C403−C411.

(26) Choi, J. S.; Choi, Y. J.; Park, S. H.; Kang, J. S.; Kang, Y. H. Flavones mitigate tumor necrosis α-induced adhesion molecule upregulation in cultured human endothelial cells: Role of nuclear factor-κB. *J. Nutr.* **2004**, *134*, 1013−1019.

(27) Lim, S. S.; Jung, S. H.; Ji, J.; Shin, K. H.; Keum, S. R. Synthesis of flavonoids and their effects on aldose reductase and sorbitol accumulation in streptozotocin-induced diabetic rat tissue. *J. Pharm. Pharmacol.* **2001**, *53*, 653−668.

(28) Greene, T. W.; Wuts, P. G. M. *Protective Groups in Organic Synthesis*, 2nd ed.; Wiley: New York, 1991; Chapter 3, Section 2, pp 149−150.

(29) Gupta, D. R.; Dhiman, R. P.; Ahmed, B. Constituents of *Launaea asplenifolia* Hook. *Pharmazie* **1985**, *40*, 273−274.

(30) Jaffe, E. A.; Nachman, R. L.; Becker, C. G.; Minick, C. R. Culture of human endothelial cells derived from umbilical veins: Identification by morphologic and immunologic criteria. *Clin. Invest.* **1973**, *52*, 2745−2756.

(31) Voyta, J. C.; Via, D. P.; Butterfield, C. E.; Zetter, B. R. Identification and isolation of endothelial cells based on their increased uptake of acetyl-low-density lipoprotein. *J. Cell Biol.* **1984**, *99*, 2034−2040.

(32) Denizot, F.; Lang, R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Method*s **1986**, *89*, 271−277.

(33) Park, S. H.; Park, J. H. Y.; Kang, J. S.; Kang, Y. H. Involvement of transcription factors in plasma HDL protection against TNF-α-induced vascular cell adhesion molecule-1 expression. *Int. J. Biochem. Cell Biol.* **2003**, *35*, 168−182.

(34) Sartor, L.; Pezzato, E.; Dell'Aica, I.; Caniato, R.; Biggin, S.; Garbisa, S. Inhibition of matrix-proteases by flavonoids: chemical insights for anti-inflammatory and anti-invasion drug design. *Biochem. Pharmacol.* **2002**, *64*, 229−237.

(35) Sato, T.; Koike, L.; Miyata, Y.; Hirata, M.; Mimaki, Y.; Sashida, Y.; Yano, M.; Ito, A. Inhibition of activator protein-1 binding activity and phosphatidylinositol 3-kinase pathway by nobiletin,

a polymethoxy flavonoid, results in augmentation of tissue inhibitor of metalloproteinases-1 production and suppression of production of matrix metalloproteinases-1 and -9 in human fibrosarcoma HT-1080 cells. *Cancer Res.* **2002**, *62*, 1025−1029.

(36) Gamble, J. R.; Bradley, S.; Noack, L.; Vadas, M. A. TGF-β and endothelial cells inhibit VCAM-1 expression on human vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* **1995**, *15*, 949−955.

(37) Wang, X.; Feuerstein, G. Z.; Gu, J.-L.; Lysko, P. G.; Yue, T.-L. Inerleukin-1β induces expression of adhesion molecules in human vascular smooth muscle cells and enhances adhesion of leukocyte to smooth muscle cells. *Atherosclerosis* **1995**, *115*, 89−98.

(38) Rajendran, M.; Manisankar, P.; Gandhidasan, R.; Murugesan, R. Free radicals scavenging efficiency of a few naturally occurring flavonoids: a comparative study. *J. Agric. Food Chem.* **2004**, *52*, 7389−7394.

(39) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure−antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.

(40) Marui, N.; Offermann, M. K.; Swerlick, R.; Kunsch, C.; Rosen, C. A.; Ahmad, M.; Alexander, R. W.; Medford, R. M. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J. Clin. Invest.* **1993**, *92*, 1866−1874.

(41) Kokura, S.; Rhoads, C. A.; Wolf, R. E.; Yoshikawa, T.; Granger, D. N.; Aw, T. NF κ B signaling in posthypoxic endothelial cells: relevance to E-selectin expression and neutrophil adhesion. *J. Vasc. Res.* **2001**, *38*, 47−58.

(42) Martin, A.; Foxall, T.; Blumberg, J. B.; Meydani, M. Vitamin E inhibits low-density lipoprotein-induced adhesion of monocytes to human aortic endothelial cells *in vitro*. *Arterioscler. Thromb. Vasc. Biol.* **1997**, *17*, 429−436.

(43) Choi, Y. J.; Kang, J. S.; Park, J. H. Y.; Lee, Y. J.; Choi, J. S.; Kang, Y. H. Polyphenolic flavonoids differ in their antiapoptotic efficacy in hydrogen peroxide-treated human vascular endothelial cells. *J. Nutr.* **2003**, *133*, 985−991.

(44) Manach, C.; Donovan, J. L. Pharmacokinetics and metabolism of dietary flavonoids in humans. *Free Radical Res.* **2004**, *38*, 771−785.

(45) Walle, T. Absorption and metabolism of flavonoids. *Free Radical Biol. Med.* **2004**, *36*, 829−837.

(46) Williamson, G. The use of flavonoid aglycones in *in vitro* systems to test biological activities: based on bioavailability data, is this a valid approach? *Phytochem Rev.* **2002**, *1*, 215−222.

(47) Kotanidou, A.; Xagorari, A.; Bagli, E.; Kitsanta, P.; Fotsis, T.; Papapetropoulos, A.; Roussos, C. Luteolin reduces lipopolysaccharide-induced lethal toxicity and expression of proinflammatory molecules in mice. *Am. J. Respir. Crit. Care Med.* **2002**, *165*, 818−823.

(48) Taniai, M.; Grambihler, A.; Higuchi, H.; Werneburgm, N.; Bronk, S. F.; Farrugia, D. J.; Kaufmann, S. H.; Gores, G. J. Mcl-1 mediates tumor necrosis factor-related apoptosis-inducing ligand resistance in human cholangiocarcinoma cells. *Cancer Res.* **2004**, *64*, 3517−3524.

(49) Igwe, O. J.; Murray, J. N.; Moolwaney, A. S. Interleukin 1-induced cyclooxygenase and nitric oxide synthase gene expression in the rat dorsal root ganglia is modulated by antioxidants. *Neuroscience* **2001**, *105*, 971−985.

(50) Wallerath, T.; Deckert, G.; Ternes, T.; Anderson, H.; Li, H.; Witte, K.; Forstermann, U. Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. *Circulation* **2002**, *106*, 1652−1658.

(51) Headley, V. V.; Tanveer, R.; Greene, S. M.; Zweifach, A.; Port, J. D. Reciprocal regulation of β-adrenergic receptor mRNA stability by mitogen activated protein kinase activation and inhibition. *Mol. Cell Biochem.* **2004**, *258*, 109−119.

(52) Ma, P.; Cui, X.; Wang, S.; Zhang, J.; Nishanian, E. V.; Wang, W.; Wesley, R. A.; Danner, R. L. Nitric oxide post-transcriptionally up-regulates LPS-induced IL-8 expression through p38 MAPK activation. *J. Leukoc. Biol.* **2004**, *76*, 278−287.

(53) Chung, Y. J.; Zhou, H. R.; Pestka, J. J. Transcriptional and posttranscriptional roles for p38 mitogen-activated protein kinase in upregulation of TNF-α expression by deoxynivalenol (vomitoxin). *Toxicol. Appl. Pharmacol.* **2003**, *193*, 188−201.

(54) Melillo, G.; Sausville, E. A.; Cloud, K.; Lahusen, T.; Varesio, L.; Senderowicz, A. M. Flavopiridol, a protein kinase inhibitor, down-regulates hypoxic induction of vascular endothelial growth factor expression in human monocytes. *Cancer Res.* **1999**, *59*, 5433−5437.

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