Dietary Flavonoids Attenuate Tumor Necrosis Factor α-induced Adhesion Molecule Expression in Human Aortic Endothelial Cells

STRUCTURE-FUNCTION RELATIONSHIPS AND ACTIVITY AFTER FIRST PASS METABOLISM

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Flavonoids have been suggested to exert human health benefits by anti-oxidant and anti-inflammatory mechanisms. In this study, we investigated whether and by what mechanisms dietary flavonoids inhibit expression of cellular adhesion molecules, which is relevant to inflammation and atherosclerosis. We found that the capacity of flavonoids to inhibit tumor necrosis factor α-induced adhesion molecule expression in human aortic endothelial cells was dependent on specific structural features of the flavonoids. The 5,7-dihydroxyl substitution of a flavonoid A-ring and 2,3-double bond and 4-keto group of the C-ring were the main structural requirements for inhibition of adhesion molecule expression. In striking contrast, hydroxyl substitutions of the B- and C-rings but not the A-ring were essential for antioxidant activity. Hence, only hydroxyl flavones, such as apigenin and chrysin, and flavonoids, such as galangin, kaempferol, and quercetin, were able to inhibit endothelial adhesion molecule expression, whereas flavone, chromone, the flavanone, narigenin, and the flavanol, (−)-epicatechin, were ineffectual. At low concentrations, the active flavonoids significantly attenuated expression of E-selectin and intercellular adhesion molecule 1 but not vascular cell adhesion molecule 1. In addition, exposure of apigenin and kaempferol to cultured hepatocytes, mimicking first pass metabolism, greatly diminished the inhibitory effect of flavonoids on endothelial intercellular adhesion molecule 1 expression. We conclude that the effect of dietary flavonoids on endothelial adhesion molecule expression depends on their molecular structure, concentration, and metabolic transformation but not their antioxidant activity.

Epidemiological studies indicate that an increased intake of dietary flavonoids is associated with a decreased risk of cardiovascular diseases (1,2). Oxidative stress has been suggested to play an important role in the pathogenesis of cardiovascular diseases, mainly through oxidative modification of low density lipoprotein, which initiates vascular inflammation and atherosclerotic lesion formation (3). Because of the high antioxidant capacity of flavonoids in vitro, it has been suggested that flavonoids act as antioxidants in human plasma and other extracellular fluids and protect low density lipoprotein from oxidation. However, studies of ex vivo oxidation of plasma and low density lipoprotein obtained from human subjects before and after short or long term consumption of flavonoid-rich foods have yielded conflicting results (4). Furthermore, flavonoids are poorly absorbed and extensively metabolized (5), which may limit or alter their antioxidant and biological activities (6,7). Therefore, it is unlikely that the health benefits of dietary flavonoids are explained merely by their antioxidant activity in plasma or low density lipoprotein.

Beyond the traditional view of atherosclerosis as a build-up of oxidized lipids in the arterial wall, it is now well established that activation of the vascular endothelium plays a key role in the initiation and progression of the disease. Arterial leukocyte recruitment is an important initiating step in atherosclerosis (8). Leukocyte-endothelial interactions and leukocyte emigration to the subendothelium in response to cytokines and chemokines are mediated by adhesion molecules expressed on endothelial cells, such as E- and P-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) (9). The expression of these adhesion molecules is regulated by reduction/oxidation (redox)-sensitive transcription factors, in particular NF-κB (10). Hence, the purported cardiovascular benefits of dietary flavonoids may be explained by a local antioxidant effect on endothelial cells and subsequent modulation of intracellular redox environment, cell signaling, and gene expression.

Therefore, in this work we investigated whether and by what mechanisms flavonoids attenuate the expression of endothelial adhesion molecules. In particular, we characterized: (i) the effect of different flavonoids on adhesion molecule expression in human aortic endothelial cells (HAEC); (ii) the relation of this effect to the antioxidant capacity of the flavonoids; (iii) the dependence on the flavonoid concentration; and (iv) the effect on HAEC of flavonoids that had been previously incubated with primary hepatocytes. We found that the attenuation of adhesion molecules by flavonoids was strictly dependent on their metabolism.
molecular structure and was not related to their reducing activity. The dose-response analysis suggested a saturable mechanism for the attenuation of adhesion molecule expression, following a hyperbole-type dependence with the concentration, in contrast to the linear response with respect to NF-κB inhibition. Furthermore, we observed that exposure of flavonoids to hepatocytes, a simulation of first pass metabolism, strongly attenuated the subsequent inhibitory effect of the flavonoids on adhesion molecule expression in HAEC.

**EXPERIMENTAL PROCEDURES**

**Materials**—Flavonoids (apigenin, chrysin, quercetin, (−)-epicatechin, kaempferol, and galangin) and caffeic acid were purchased from Sigma-Aldrich. Chromone and flavone were obtained from Indofine (Hillsborough, NJ). TNFα was purchased from Roche Applied Science. All of the other chemicals were of the highest grade available.

**Endothelial Cells**—Human aortic endothelial cells were obtained from Clonetics (Walkersville, MD) at third passage. Upon receipt, the cells were seeded at a ratio of 1:3 in 75-cm² flasks ( precoated with 1% bovine gelatin; Sigma) and grown at 37°C, under 5% CO₂, and in a humidified atmosphere in endothelial cell growth medium (Clonetics-Cambrex) containing bovine brain extract, human epithelial growth factor, hydrocortisone, amphotericin B, gentamicin sulfate, and 2% fetal bovine serum. The medium was periodically renewed until the cells reached 70–90% confluence, at which point they were treated with trypsin-EDTA (Sigma). Subsequently, the cells were expanded in 75-cm² precoated flasks at a ratio of 1:5 until passages 5 and 6, when they were plated, and the experiments were carried out.

**Experiments**—Human aortic endothelial cells were plated in 96-well plates ( precoated with 1% gelatin) at an average density of 5 × 10⁴ cells/ml medium. The medium consisted of Medium 199 (Sigma) supplemented with 20% fetal bovine serum (Invitrogen), 1 mM glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, 0.1 µg/ml amphotericin B (Sigma), and 1 ng/ml human basic fibroblast growth factor (Roche Applied Science). The HAEC were allowed to attach to the plates overnight (18 h), after which they were washed with Hanks’ salt-balanced solution (Sigma), and the medium was renewed. The cells were incubated at 37°C, under 5% CO₂, and in a humidified atmosphere until they reached confluence, typically 24–48 h after seeding.

For experiments, HAEC were incubated with medium (100 µl) containing different concentrations of the various flavonoids (5–50 µM) for 18 h prior to the inflammatory challenge (100 units/ml TNFα). The solutions were freshly prepared by dissolving the flavonoids in Me₂SO and subsequent dilution in culture medium containing 20% serum. The final concentration of Me₂SO in the medium did not exceed 0.1%. Proper controls with the vehicle Me₂SO were carried out. In addition, to eliminate potential effects of artificially generated reactive oxygen species, catalase (0.1 µM) and/or superoxide dismutase (3 µM) were added, and phenol red-free medium was used. For comparative purposes, we also preincubated cells for 30 min before TNFα addition with the irreversible inhibitor of IkBα phosphorylation (IC₅₀ = ~10 µM), BAY 11–7821 (Tocris, Ellisville, MI), which leads to decreased NF-κB activation and subsequent decreased expression of adhesion molecules in endothelial cells (11).

The cells were examined regularly using an inverted optical microscope. No changes in cell morphology were observed with any of the treatments. Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, using a Cell Proliferation Kit I, according to the manufacturer’s instructions (Roche Applied Science).

**Adhesion Molecule Expression**—Surface expression of adhesion molecules (E-selectin, VCAM-1, and ICAM-1) was determined by ELISA performed on HAEC monolayers in flat-bottomed 96-well plates. Following treatment, the cells were fixed in phosphate-buffered saline containing 0.1% glutaraldehyde. For cell ELISA, the plates were blocked at 37°C for 1 h with 5% skim milk powder in phosphate-buffered saline and then incubated overnight at 4°C with a primary antibody to either E-selectin, ICAM-1, and VCAM-1 was assessed by the addition of α-phenylendiamine-dihydrochloride (Sigma). The absorbance at 492 nm was recorded in a plate reader spectrophotometer (Spectromax 190, Molecular Devices, Sunnyvale, CA).

**Hepatocyte-conditioned Medium**—Rat hepatocytes (Clonetics, Walkersville, MD) were seeded in 12-well collagen-coated plates, and the cultures were established for 48 h. Subsequently, the cells were incubated for 18–24 h with apigenin or kaempferol, as follows. The flavonoids were freshly prepared in Me₂SO and further diluted in hepatocyte medium, consisting of hepatocyte basic medium (Clonetics) supplemented with human epithelial growth factor, gentamicin, ascorbic acid, and insulin, according to the manufacturer’s instructions (Clonetics). The hepatocytes were maintained at 37°C and 5% CO₂ in 1 ml of medium or incubated with 1 ml of medium containing 30 or 100 µM apigenin or kaempferol. After 18–24 h, this “hepatocyte-conditioned medium” was removed and immediately frozen until further analysis or use in experiments. The cells were washed and replenished with 1 ml of fresh medium, and their viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. None of the treatments affected cell viability.

As control, 1 ml of medium, or 1 ml of medium containing 30 or 100 µM apigenin or kaempferol was incubated at 37°C and 5% CO₂ in the absence of hepatocytes. After 18–24 h, this “control conditioned medium” was removed and immediately frozen until further analysis. The final concentration of Me₂SO was ≤0.1% in all of the incubations.

**Experiments Using Hepatocyte-conditioned Medium**—HAEC were plated in 96-well gelatin-coated plates as described above. The cells were maintained for 8 h in medium consisting of hepatocyte basic medium (supplemented with epithelial growth factor, ascorbic acid, gentamicin, and insulin) diluted 1:1 with complete Medium 199 (phenol red-free Medium 199 supplemented with 20% fetal bovine serum, glutamine, and antibiotics, as described above). Subsequently, HAEC were incubated
Anti-inflammatory and Anti-oxidant Activities of Flavonoids

overnight (15 h) with hepatocyte-conditioned medium (prepared as described above) diluted 1:1 with complete Medium 199 (100 μl of total volume/well). As control, HAEC were incubated with control conditioned medium (prepared as described above) diluted 1:1 with complete Medium 199. The final concentration of fetal bovine serum in these incubations was 10%. Following these overnight incubations, 100 units/ml TNFα was added, the cells were incubated for 5 h, and surface expression of ICAM-1 was determined by ELISA.

**Ferric-reducing Antioxidant Parameter**—The antioxidant capacity of flavonoids and culture media was evaluated by their iron reducing capacity. In this assay, antioxidants act as reductants of Fe^{3+} to Fe^{2+}, which is chelated by tripyridyltriazine to form a colored complex (12). Briefly, 40 μl of flavonoid-containing solutions or culture medium were mixed in a 96-well plate with 300 μl of reagent solution containing 1.7 mM FeCl₃ and 0.8 mM tripyridyltriazine in 300 mM sodium acetate, pH 3.6. The samples were incubated for 15 min at 37 °C, and the absorbance at 593 nm was recorded in a plate reader spectrophotometer (Spectromax 190). The results were compared with a standard curve prepared with different concentrations of the antioxidant trolox and are expressed as “trolox equivalents.”

**Measurement of Kaempferol**—The concentration of kaempferol in medium was determined by HPLC with electrochemical detection, using a C-18 column (150 × 4.6 mm) (Sigma). The mobile phase, delivered at a flow rate of 1 ml/min, consisted of solvent A (0.1% acetic acid in water, pH 3) and solvent B (methanol), using a linear gradient from 40% to 70% B in 10 min. Kaempferol was quantitated by amperometric detection at +0.6 V (BAS-LC 4; Bioanalytical Systems Inc., West Lafayette, IN).

**Statistical Analysis**—The results shown are the means ± S.E. of at least three independent experiments (including three to five observations for each treatment), using HAEC from at least three different donors and three different sets of freshly isolated rat hepatocytes. Statistical analysis was performed by one-way analysis of variance. If statistical significance was reached for analysis of variance (p < 0.05), Tukey-Kramer was applied as post-hoc test. For paired samples, the Student’s t test was applied.

**RESULTS**

**Structure-related Effect of Flavonoids on Endothelial Adhesion Molecule Expression**—To identify flavonoids that can affect endothelial function, we screened various structurally related flavonoids (Fig. 1) for their effectiveness to inhibit adhesion molecule expression in primary human aortic endothelial cells. Confluent HAEC were incubated with flavonoids (10–50 μM) for 18 h in medium containing 20% fetal bovine serum and then incubated with 100 units/ml (1 ng/ml) TNFα for another 7 h. Cell surface expression of E-selectin, ICAM-1, and VCAM-1 was assessed by ELISA. We found that hydroxyl flavonoids and flavonols, i.e. apigenin, chrysin, kaempferol, and galangin, significantly inhibited TNFα-induced adhesion molecule expression (Fig. 2), as did quercetin (see Fig. 5A).

These results suggest that the basic structure required for inhibition of adhesion molecule expression is 5,7-dihydroxyflavone (chrysin), regardless of substitutions of the B-ring (Fig. 1). Interestingly, naringenin, which is structurally identical to apigenin except for the absence of the 2,3-double bond in the C-ring (Fig. 1), was ineffectual at inhibiting adhesion molecule expression (data not shown). Caffeic acid, whose structure partially resembles that of a flavone or flavonol containing the 2,3-double bond and 4-keto group of the C-ring and a catechol group in the B-ring (Fig. 1), also did not show any inhibitory activity (data not shown). In addition, flavanol, (−)-epicatechin, which is structurally related to quercetin but lacks the C-ring double bond and keto group, and flavone, which lacks any hydroxyl substitutions of the A- and C-rings, and the related compound, chroomone (Fig. 1), were all ineffectual (data not shown). Taken together, these data indicate that the C-ring double bond and keto group and the A-ring 5,7-dihydroxy groups but not the B-ring per se are required for flavonoids to inhibit TNFα-induced adhesion molecule expression in HAEC.

With respect to the dose dependence of the effects of the active flavonoids, statistically significant inhibition of the expression of at least one type of adhesion molecule was observed at concentrations ≥10 μM for chrysin and galangin, ≥30 μM for apigenin, and 50 μM for kaempferol (Fig. 2). In general, expression of ICAM-1 and E-selectin was more strongly inhibited than VCAM-1. In fact, only 50 μM apigenin (Fig. 2) significantly inhibited VCAM-1 expression.

**Structure-related Antioxidant Capacity of Flavonoids**—To evaluate whether the above observed anti-inflammatory effect of specific flavonoids was related to their antioxidant capacity, the ferric-reducing antioxidant parameter was determined. As shown in Fig. 3, the flavones apigenin and chrysin and the flavanone naringenin exerted no antioxidant activity, in contrast to flavonoids substituted with a hydroxyl group in the 3-position of the C-ring, i.e., flavonols and flavanols (Fig. 1). Flavonols were differentially active, with quercetin exhibiting the strongest antioxidant capacity (2.53 ± 0.31 μM trolox equivalents) followed by kaempferol (1.40 ± 0.17 μM) and then galangin (0.95 ± 0.02 μM). These data indicate that the presence of hydroxyl groups in the B-ring (Fig. 1) enhances the antioxidant capacity of the flavonoid. This conclusion is buttressed by the antioxidant capacity of (−)-epicatechin (2.24 ± 0.09 μM), which is similar to that of quercetin (Fig. 3). Caffeic acid exhibited an antioxidant capacity (1.58 ± 0.07 μM) similar to that of kaempferol. Thus, the reducing activity of the tested compounds appears to be determined by the hydroxyl substitutions of the C- and B-rings but not the A-ring (Fig. 1). Importantly, no correlation was found between the reducing activity of the various flavonoids and their inhibitory effect on endothelial adhesion molecule expression (compare Figs. 2 and 3).

**Effect of Flavonoid Concentration on Endothelial Adhesion Molecule Expression**—The dose dependence of the effect of flavonoids on adhesion molecule expression was carefully evaluated in the physiologically relevant concentration range of 5–30 μM. The HAEC were incubated without (control) or with flavonoids and challenged with TNFα as described above, and the results were analyzed as the percentage of inhibition of E-selectin or ICAM-1 expression (Fig. 4). We found that the response was nonlinear in the concentration range of the flavonoids tested. The experimental data of inhibition of E-selectin and ICAM-1 expression by apigenin and kaempferol (Fig. 4, A and C) were best fitted by a hyperbola-type function of the following
equation: % Inhibition = % Maximal Inhibition [Flavonoid]/ ([Flavonoid] + K). Expression of E-selectin was inhibited by 30–40% by 30 μM kaempferol and apigenin, and ICAM-1 expression was inhibited by ~30 and 60%, respectively (Fig. 4, A and C). A similar pattern was observed for the inhibition of E-selectin and ICAM-1 expression by the structurally related flavonoids chrysin and galangin (Fig. 4, B and D), with the hyperbola-type dose-response curve particularly evident for E-selectin (Fig. 4B). VCAM-1 expression was not inhibited by any of the flavonoids at concentrations ≤30 μM (Fig. 2).

In the same set of experiments, we also tested the effect of 5–30 μM of the flavonol, quercetin, on endothelial adhesion molecule expression. Quercetin strongly and dose-dependently inhibited E-selectin and ICAM-1 expression (Fig. 5A). The dose-response curve for the inhibition of ICAM-1 expression followed the hyperbola-type function observed for other flavonoids (Fig. 4). Quercetin concentrations as low as 5–10 μM inhibited ICAM-1 expression by as much as 50–60%, which was close to the maximal inhibition described by the function (Fig. 5A). In contrast, a linear dose-response curve was observed for the effect of the irreversible inhibitor of TNFα-stimulated IκBα phosphorylation, BAY 11–7821 (5–20 μM), on the expression of E-selectin, ICAM-1 (Fig. 5B), and VCAM-1 (data not shown).

Effect of Hepatocyte-exposed Flavonoids on Endothelial Adhesion Molecule Expression—To investigate the effect of first pass metabolism on the biological activity of flavonoids, apigenin and kaempferol were incubated with hepatocytes prior to evaluating the antioxidant capacity of the flavonoids and the inhibitory effect on TNFα-induced endothelial adhesion molecule expression. As shown in Fig. 6A, after exposure to hepatocytes, medium originally containing 30 μM kaempferol exhibited a significantly lower ferric-reducing activity (9.1 ± 6.2 μM) than control medium containing the same concentration of kaempferol but incubated in the absence of hepatocytes (31.8 ± 6.6 μM). Similar results were obtained when the medium originally contained 100 μM kaempferol (56.6 ± 12.2 μM versus 99.6 ± 12.9 μM in the presence and absence of hepatocytes, respectively) (Fig. 6A). After only 6 h of incubation with hepatocytes, the concentration of kaempferol in the medium was not detectable (from originally 30 μM) or greatly diminished (from 100 μM), as measured by HPLC with electrochemical detection (Fig. 6, B and C, respectively).

FIGURE 1. Structure of the flavonoids and related compounds investigated in this paper. (Active) indicates that the compound dose-dependently inhibited TNFα-induced adhesion molecule expression in human aortic endothelial cells.
HAEC were then incubated for 15 h with either 1:1 diluted hepatocyte-conditioned medium or 1:1 diluted control conditioned medium, with both media originally containing 30 or 100 μM apigenin or kaempferol. Subsequently, the cells were incubated for 5 h with 100 units/ml TNFα, and ICAM-1 expression was measured by ELISA. The values are expressed as percentages of TNFα stimulation. Control cells incubated in the absence of flavonoids and TNFα are also shown. The data are means ± S.E. of at least three independent experiments. Analysis of variance was used to analyze the dose-response trend. * significantly different from 0 μM (Tukey-Kramer, post-hoc analysis).

Flavonoids were previously reported to prevent adhesion molecule expression in endothelial cells (13–16). In general, recent observations in human umbilical vein endothelial cells agree with the early data of Gerritsen and colleagues (13). These investigators showed that hydroxyl flavones and flavonols are the most effectual flavonoids in inhibiting cytokine-induced expression of ICAM-1 by 46 or 36%, respectively (Fig. 7). In contrast, hepatocyte-conditioned medium originally containing 30 μM apigenin or kaempferol failed to inhibit ICAM-1 expression in HAEC (Fig. 7). Furthermore, control conditioned medium containing 100 μM apigenin or kaempferol attenuated TNFα-induced ICAM-1 expression by 63 and 54%, whereas hepatocyte-conditioned medium only and nonsignificantly inhibited by 36 and 30%, respectively. These data indicate that the inhibitory effect of flavonoids on endothelial adhesion molecule expression is greatly diminished by prior exposure of the flavonoids to hepatocytes, suggesting substantial first pass hepatic metabolism.

DISCUSSION

In this work, we characterized the in vitro effect of several dietary flavonoids on adhesion molecule expression in human aortic endothelial cells, a cell type relevant to vascular homeostasis and atherosclerosis. We found that only hydroxyl flavones and flavonols were able to inhibit adhesion molecule expression, in contrast to flavanones and flavanols. The 5,7-dihydroxyl substitution of a flavonoid A-ring and the 2,3-double bond and 4-keto group of the C-ring appeared to be the main structural requirements for this activity (Fig. 8). On the other hand, hydroxyl substitutions of the B- and C-rings did not play a role. In striking contrast, a hydroxyl group in the 3-position of the C-ring made an important contribution to the antioxidant capacity of a flavonoid, as did the presence and number of hydroxyl groups in the B-ring but not the A-ring (Fig. 8). Thus, the structural requirements for flavonoids to inhibit endothelial adhesion molecule expression and exert antioxidant activity were mutually exclusive.
Anti-inflammatory and Anti-oxidant Activities of Flavonoids

We also noted some differences between our data and that of and Gerritsen et al. (13), which may be due to differences in the source of endothelial cells (umbilical vein versus aorta) and experimental conditions (serum concentrations, cytokines, and incubation times). In our study, quercetin was 10 times more potent at inhibiting ICAM-1 expression, with an IC₅₀ of 5 μM compared with 50 μM reported by Gerritsen et al. (13). Also, in contrast to Gerritsen et al. (13), we did not observe significant inhibition of VCAM-1 expression by flavonoid concentrations ≤30 μM. However, our data are in agreement with those of Choi et al. (15), who found inhibition of VCAM-1 expression by quercetin only at concentrations greater than 25 μM. It is conceivable that the mechanism by which low concentrations of quercetin inhibit ICAM-1 and E-selectin expression is different from the one by which higher concentrations inhibit VCAM-1 expression (15). Interestingly, when used at concentrations of 50 μM or higher, quercetin was toxic to HAEC in our experiments. It is also noteworthy that the effect of quercetin on HAEC was not abolished by the addition of catalase or superoxide dismutase, excluding a possible role for reactive oxygen species generated in the extracellular medium.

It is widely recognized that induction of endothelial adhesion molecules by inflammatory cytokines strongly depends on activation of the transcription factor, NF-κB (10). Regulation of NF-κB and several other transcription factors is redox-sensitive because of modification of critical cysteine residues in upstream signal transducers, particularly protein kinases and phosphatases, and the transcription factors themselves, which may differentially affect their DNA binding capacity (17). The notion that antioxidants may inhibit NF-κB and other redox-sensitive transcription factors and hence act as anti-inflammatory agents has been extensively studied (18–21). However, the mechanism by which flavones and flavonols inhibit endothelial adhesion molecule expression has remained elusive. Although Choi et al. (15) observed inhibition of nuclear translocation and DNA binding of NF-κB by flavones and flavonols, Gerritsen et al. (13) and Kobuchi et al. (14) did not find such inhibition, despite very similar experimental conditions. In our experiments, the dose-response curve for the inhibition of adhesion molecule expression by a NF-κB inhibitor markedly differed from the dose-response curve with the active flavonoids. Our data therefore suggest a more complex mechanism than inhibition of NF-κB activation alone for the effect of flavones and flavonols on adhesion molecule expression. In contrast to Choi et al. (15), who reported strong inhibition of endothelial NF-κB activation by 50 μM apigenin or quercetin, we did not see significant inhibition of p65 nuclear translocation and DNA binding by 15 or 30 μM quercetin. Nevertheless, we observed strong inhibition of E-selectin and ICAM-1 expression with 5–30 μM quercetin. It is possible that the inhibition of NF-κB activation is observed only at

FIGURE 4. Dose-response effect of flavones and flavonols on E-selectin and ICAM-1 expression in human aortic endothelial cells. The HAEC were incubated for 18 h without or with 5–30 μM apigenin (closed circles), kaempferol (open circles) (A and C), chrysin (closed triangles), or galangin (open triangles) (B and D) and then challenged with 100 units/ml TNFα. E-selectin and ICAM-1 were measured by ELISA. The percentage of inhibition of the expression of E-selectin (A and B) and ICAM-1 (C and D) was calculated for each experiment as follows: 100 − [(treated − control)/(100 − control)] × 100. The data are the means ± S.E. of at least three independent experiments.

FIGURE 5. Dose-response effect of quercetin (A) and a NF-κB inhibitor (B) on E-selectin and ICAM-1 expression in human aortic endothelial cells. A, the HAEC were incubated for 18 h without or with 5–30 μM quercetin and then challenged with 100 units/ml TNFα. B, the HAEC were incubated for 30 min without or with 5–20 μM BAY 11–7821 and then challenged with 100 units/ml TNFα. E-selectin and ICAM-1 were measured by ELISA. The percentage of inhibition of the expression of E-selectin (closed diamonds) and ICAM-1 (open diamonds) was calculated as explained in the legend to Fig. 4. The data are the means ± S.E. of at least three independent experiments.
higher concentrations of flavonoids, and other mechanism(s) mediate(s) the inhibition of E-selectin and ICAM-1 expression at lower, more physiologically relevant concentrations of the flavonoids.

Considering that many signaling pathways are involved in the regulation of adhesion molecule expression and are redox-sensitive, we studied whether the active flavonoids could act as reducing agents and thus potentially modulate the cellular redox environment. Interestingly, we did not find any relationship between the reducing capacity of the flavonoids and their effect on adhesion molecule expression. Thus, only the flavonols galangin, kaempferol, and quercetin acted both as antioxidants and inhibitors of adhesion molecule expression. In contrast, compounds with comparatively high reducing activity, such as (-)-epicatechin or caffeic acid, failed to attenuate TNFα-induced adhesion molecule expression. On the other hand, apigenin and chrysin, which exhibited no antioxidant activity, effectively inhibited adhesion molecule expression, whereas naringenin, another nonantioxidant flavonoid, was ineffective. Thus, the structural requirements of flavonoids for reducing or antioxidant activity are very different from the structural requirements for anti-inflammatory effects.

The observation that inhibition of adhesion molecule expression was strictly dependent on the flavonoid structure suggests an interaction with specific cellular target(s). Flavonoids have been shown to affect the activity of many mammalian enzymes in vitro (22), and several structure-activity relationships have been noted. Many ATP-dependent...
enzymes and signaling proteins are inhibited by flavonoids, including protein kinase C (23), myosin light chain kinase (24), protein tyrosine kinases (25), lipid kinases such as phosphoinositol 3-kinase (26), Na/K ATPase (27), and sarcoplasmic reticulum Ca-ATPase (28, 29). Many of the ATP-dependent enzymes and kinases have been implicated in inflammation. A common mechanism of the ATP-utilizing enzymes seems to be competitive binding of the flavonoid to the ATP-binding site.

The importance of the pattern of A-ring hydroxyl substitution and C-ring 2,3 unsaturation and 4-keto group was recognized as strongly affecting the inhibitory activity (30, 31), in striking parallel to the structural elements identified in our study for the inhibition of adhesion molecule expression (Fig. 8).

The modulatory activity of flavonoids is not restricted to ATP-dependent enzymes; inhibition of phospholipases, lipoxygenases, and cyclooxygenases by flavonoids has also been described (32, 33). More recently, apigenin, chrysin, and kaempferol have been identified as efficient agonists of the transcription factor, PPARγ, whereas flavanones and flavonols were inefficient (34). Again, the substitution of 5- and 7-hydroxyl residues of the A-ring was an important factor for the activity.

It is likely that a combination of mechanisms is responsible for the anti-inflammatory effect of flavonoids in HAEC. This notion is supported by the saturable effect of flavonoids on adhesion molecule expression, with a hyperboles-type function for the inhibition at relatively low concentrations, which was not observed at higher concentrations. Thus, the plateau or maximal effect in the lower concentration range was ~60–70% inhibition of ICAM-1 expression by apigenin and quercetin and 40–50% inhibition of E-selectin expression by kaempferol and quercetin. This type of response supports the hypothesis that physiologically relevant concentrations of these flavonoids may affect specific cellular targets, e.g. inhibit or saturate an enzyme, carrier, or receptor, that directly or indirectly affects adhesion molecule expression.

Human endothelial cells are exposed to flavonoids via the blood stream. An important consideration is that flavonoids are extensively altered during first pass metabolism, and the molecular forms reaching the circulation and peripheral tissues are different from those present in foods (5). After absorption, the flavonoids are metabolized by Phase II drug-metabolizing enzymes, resulting in formation of glucuronide and sulfate conjugates, with or without methylation in the catechol group, if present. Importantly, the biological activities of the conjugates are likely to be different from those of the native compound. For example, Koga and Meydani (35) reported that quercetin and in vivo metabolites of (+)-catechin decreased the adhesion of monocytes to HAEC, whereas the in vivo metabolites of quercetin and (+)-catechin aglycon did not.

In our work, we observed that the effect on adhesion molecule expression was strongly dependent on the chemical structure of the flavonoids. Because dietary flavonoids are subject to first pass metabolism, we studied whether the characterized activity of the flavonoids apigenin and kaempferol on adhesion molecule expression in HAEC was affected by prior exposure to hepatocytes. We created a model of first pass metabolism with cultured primary rat hepatocytes. This in vitro system has several limitations, including the fact that the activity of some of the hepatic detoxifying enzymes rapidly declines in culture. In addition, several differences between humans and rats have been described in the metabolism and biotransformation of flavonoids (36). Nevertheless, we found that after exposure to rat hepatocytes, the activity of apigenin and kaempferol in HAEC was abolished or greatly diminished. Although we did not attempt to identify the metabolites of these flavonoids, glucuronides of apigenin and kaempferol have been described as the main biotransformed compounds (37, 38). Our results suggest that first pass metabolism may significantly decrease the biological effect of flavonoids on endothelial cells because of hepatic uptake or biotransformation. Further work is needed to determine the biological activity of each conjugate, although obtaining sufficient amounts of purified conjugates for experiments will be a formidable challenge.

Although it has been shown that flavonoids such as apigenin, quercetin, tea catechins, and procyanidins exhibit anti-inflammatory effects in vivo, including inhibition of adhesion molecule expression (13, 39–43), the underlying mechanisms of action remain unclear. The present knowledge of the human metabolism of flavonoids supports the concept that the metabolites of the flavonoids, not their dietary forms (usually glycosides), are responsible for the observed in vivo effects. However, our data indicate that first pass metabolism of apigenin lowers, rather than enhances, its inhibitory effect on endothelial adhesion molecule expression and therefore suggest that the anti-inflammatory effect of apigenin observed in vivo (13, 39) is unlikely to be mediated by its hepatic metabolites. It is possible that free flavonoids (i.e. their aglycones) are locally generated in vivo from their metabolites at sites of inflammation, for example by the action of glucuronidases and sulfatases (44, 45). Alternatively, flavonoids could exert their anti-inflammatory effect via induction of Phase II enzymes, as is the case for other compounds (46). These hypotheses, which have yet to be examined, could provide alternative explanations for the in vivo anti-inflammatory effect of flavonoids (13, 39–43).

In summary, this in vitro study showed that attenuation of adhesion molecule expression in human aortic endothelial cells by dietary flavonoids is strictly dependent on their chemical
Anti-inflammatory and Anti-oxidant Activities of Flavonoids

structure but not their antioxidant activity. Thus, only hydroxy-flavonones and flavonones were biologically active in endothelial cells; at low concentrations, this activity was restricted to inhibition of E-selectin and ICAM-1 expression. In addition, hepatic metabolism may substantially attenuate the anti-inflammatory effect of flavonoids on endothelial function. We conclude that data from in vitro studies using cultured cells exposed to relatively high concentrations of unmetabolized flavonoids cannot be extrapolated to the in vivo situation.

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