The Flavonoid Phloretin Suppresses Stimulated Expression of Endothelial Adhesion Molecules and Reduces Activation of Human Platelets

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ABSTRACT  Atherosclerosis is a chronic inflammatory disease accompanied by the expression of endothelial adhesion molecules. Phloretin is a plant-derived phytochemical that is mainly present in apples. Because phloretin is reported to promote antioxidative activities, we investigated the effects of phloretin on cytokine-induced expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (E-selectin) in human umbilical vein endothelial cells (HUVECs). Phloretin prevented TNF-α-stimulated upregulation of VCAM-1, ICAM-1, and E-selectin expression in a concentration-dependent manner. To the same extent as for TNF-α, phloretin also inhibited IL-1β-induced upregulation in expression of all 3 adhesion molecules. Inhibition of cytokine-induced adhesion molecule expression for VCAM-1, ICAM-1, and E-selectin was detected already at the level of mRNA. Preincubation with phloretin dose-dependently attenuated TNF-α-stimulated adhesion of monocyte THP-1 cells to HUVECs and human aortic endothelial cells. Phloretin did not affect TNF-α-stimulated activation of nuclear factor κB (NF-κB) but inhibited activation of interferon regulatory factor 1, a transcription factor involved in the regulation of endothelial cell adhesion molecule expression. In human platelets, phloretin diminished adenosine diphosphate (ADP) and thrombin receptor-activating peptide–stimulated expression of the activated form of the GPIIb/IIIa complex and reduced platelet aggregation stimulated by ADP. Thus phloretin may have beneficial effects in the onset and progression of cardiovascular diseases.

KEY WORDS: • phloretin • adhesion molecules • platelets • cytokines • atherosclerosis

Dietary compounds have attracted considerable attention during the past few years. A great number and variety of phytochemicals are present in the human nutrition. Among them, the flavonoids account for the largest group and contain >5000 different natural substances. They are notable for their anticarcinogenic properties (1). Recent epidemiological studies and experimental data also indicate beneficial effects of plant-derived compounds on cardiovascular health (2–4).

Phloretin and its glucoside phloridzin are abundantly present in apples, especially in the peel (5,6). For many years, the occurrence of phloretin was considered restricted to apples. A recent study, however, reported the identification and isolation of phloridzin in strawberries, which extends the knowledge of the natural sources of this polyphenolic compound (7). It can be expected that phloretin will be found in additional plant species in the future. The main biological action of phloretin described in the literature is the inhibition of glucose cotransporter 1 (8,9). Phloretin furthermore possesses antioxidative properties. Studies have revealed that apples exert antioxidative activities, attributed to phytochemicals present in the skin (10). Phloretin accounts in part for the antioxidative capacity of apples (11). Other studies have established the pharmacophore responsible for the antioxidative activity of phloretin (12,13).

The generation and abundance of reactive oxygen species are closely associated with the development and progression of atherosclerosis, a disease accompanied by a chronic inflammatory process. This process involves activation of the vascular endothelium and a concomitant increase in adhesion of mononuclear cells as well as platelets to the injured endothelial layer. Endothelial cells recruit leukocytes by selectively expressing adhesion molecules: e.g., vascular cell adhesion molecules (VCAM-1),2 intercellular adhesion molecules (ICAM-1), and endothelial leukocyte adhesion molecules (E-selectin) (14). Proinflammatory cytokines such as TNF-α and IL-1β, commonly found in atherosclerotic lesions, can induce chemotactic factors and other cytokines that contribute to the expression of cell adhesion molecules (15).

Despite the established antioxidative properties of phlor-
Materials and methods

Materials. All reagents and media were purchased from Sigma Chemical, unless otherwise specified. Recombinant human TNF-α was obtained from BD Pharmingen and IL-1β from R&D Systems.

Cell isolation and culture. Human umbilical vein endothelial cells (HUVECs, perterm birth) were isolated by collagenase type II (Biochrom KG) digestion of human umbilical veins by standard techniques and were cultured in EC medium (MCDB 131, Gibco BRL, Life Technologies GmbH), as described previously (16). We performed all experiments with HUVECs from passages 1 to 3 and seeded cells at 1 × 10⁴ cells/well in 96-well plates. Human aortic endothelial cells (HAECs) were purchased from Promocell and grown in MV medium of the manufacturer, supplemented with 5% fetal calf serum (FCS), 10 g/L epidermal growth factor, 12 μg/L endothelial cell growth supplement, 1 μg/L hydrocortisone, 50 μg/L amphotericin B, 50 μg/L gentamicin. THP-1 cells constitute a human myelomonocytic cell line that is widely used to study monocyte/macrophage biology in cell culture systems (17). These cells were used for our cell attachment studies with endothelial cells. Cells were isolated from the American Type Culture Collection, cultured in RPMI 1640 (Gibco BRL), and supplemented with 10% heat-inactivated FCS, 2 mmol/L l-glutamine, 100 μg/mL penicillin, and 100 μg/mL streptomycin.

Determination of adhesion molecule expression by ELISA. HUVECs were precoated for 1 h with varying concentrations (1–100 μmol/L) of phloretin or solvent dimethyl sulfoxide, followed by treatment with TNF-α (5 μg/L) or IL-1β (8 μg/L) in the presence of phloretin for 4 h. The expression of VCAM-1, ICAM-1, and E-selectin was measured by cellular ELISA as described previously (18).

RT-PCR. The total cellular RNA was isolated from confluent endothelial monolayers using a RNaseasy Total RNA kit (Qiagen). RT-PCR was performed as described previously (16). The following primers were used:

- Human GAPDH-F (5’-ATGACACCAAGCTCAAGATCATCAG-3’).
- Human GAPDH-R (5’-CTGGTGGTGCAGGGTCTTACTCC-3’).
- VCAM-1-FF (5’-CCGAATCCTGATATCCTGCTC-3’).
- VCAM-1-RF (5’-CAGGCCTGCTAAGGTTATGC-3’).
- ICAM-1-FF (5’-AACGGGAAGGTTGATGACGT-3’).
- ICAM-1-RF (5’-CGAAGGTGTTTCAAGACGTCT-3’).
- E-selectin-FF (5’-AGAAATATGGTGTTCCAGATGA-3’).
- E-selectin-RF (5’-AAACTGGAGATTCCTTTGGAATTG-3’).
- PCR products were separated on polyacrylamide gels (3%) and the gels were stained with 0.1% silver nitrate.

Assay for THP-1 cell adhesion to endothelial cells. Adhesion studies were performed with the human monocyctic cell line THP-1 as described previously (18).

Measurement of transcription factor activity. Nuclear extracts were prepared as described previously (19). Protein concentrations were determined by Bradford reagent. For analysis of transcription factor activity, the TransAM transcription factor family Kits were used (Active Motif). Samples were processed according to the manufacturer’s protocol as described previously (18). A total of 10 μg of nuclear extract was used in each experiment for nuclear factor κB (NF-κB) and 5 μg for all other transcription factors. The oligonucleotides contained the following consensus binding sequences: for NF-κB 5’-GGGACCTTCC-3’, AP-1 5’-TGAGTCA-3’, SP-1 5’-GGGGCGGCGG-3’, GATA-2 5’-AGATATAA-3’, interferon regulatory factor-1 (IRF-1) 5’-GAAACGTGAACT-3’. To reveal specificity of binding, a competitor for transcription factor binding (corresponding wild-type consensus oligonucleotide) was added in a molar excess prior to the probe where indicated.

Results

Endothelial adhesion molecule expression. The expression of VCAM-1, ICAM-1, and E-selectin was very low in unstimulated HUVECs (Fig. 1), and preincubation with phloretin did not affect these basal expressions (data not shown). Treatment of HUVECs with phloretin at concentrations up to 100 μmol/L was not cytotoxic as determined by trypan blue exclusion (data not shown). Exposure of cells to TNF-α (5 μg/L) for 4 h induced strong upregulation of surface expression of VCAM-1, ICAM-1, and E-selectin (Fig. 1). Figure 1A–C depicts the effects on TNF-α-induced adhesion molecule expression after preincubation (1 h) of HUVECs with various concentrations of phloretin (1–100 μmol/L). Phloretin at doses ranging from 30 to 100 μmol/L induced significant dose-dependent inhibition of VCAM-1 protein expression (Fig. 1A). At a concentration of 80 μmol/L, phloretin completely prevented TNF-α-induced upregulation and reduced VCAM-1 expression to basal levels. Likewise, the TNF-α-induced expression of ICAM-1 was dose-dependently inhibited by preincubation with phloretin at concentrations of 20 to 100 μmol/L (Fig. 1B). In similar concentrations (10 to 100 μmol/L), TNF-α-induced upregulation of E-selectin was inhibited by preincubation of HUVECs with phloretin (Fig. 1C).

In addition to TNF-α, we tested whether phloretin could also diminish upregulation of adhesion molecules by another proinflammatory stimulus. IL-1β (8 μg/L) strongly increased expression of all adhesion molecules after 4 h (Fig. 2). Pre-
treatment with phloretin at doses of 5 to 100 μmol/L 1 h prior to IL-1β-stimulation significantly prevented upregulation of VCAM-1 (Fig. 2A). For ICAM-1 and E-selectin, higher concentrations of phloretin were necessary for suppression of stimulated adhesion molecule expression. IL-1β-stimulated expression of both adhesion molecules was significantly and dose-dependently inhibited by pretreatment with phloretin at 50–100 μmol/L (Fig. 2B and C).

Next, we were interested in determining whether the interference of phloretin with cytokine-induced adhesion molecule expression also occurs at the transcriptional level. We determined gene transcription levels of mRNAs. Preincubation of HUVECs with 100 μmol/L phloretin for 1 h dimin-

![Figure 1](https://academic.oup.com/jn/article-abstract/135/2/172/4663638)

**FIGURE 1** Phloretin inhibits TNF-α-stimulated upregulation of VCAM-1, ICAM-1, and E-selectin expression. HUVECs were preincubated for 1 h with the indicated doses of phloretin and expression of VCAM-1 (A), ICAM-1 (B), and E-selectin (C) was induced by stimulation with TNF-α (5 μg/L) for 4 h. Data are expressed as a percentage of TNF-α-induced adhesion molecule expression. Values are means ± SEM, n = 5–6. Different from TNF-α alone, *P < 0.05.

![Figure 2](https://academic.oup.com/jn/article-abstract/135/2/172/4663638)

**FIGURE 2** Suppression of IL-1β-stimulated adhesion molecule expression by phloretin. HUVECs were preincubated for 1 h with the indicated doses of phloretin, followed by stimulation with IL-1β (8 μg/L) for 4 h. Expression of VCAM-1 (A), ICAM-1 (B), and E-selectin (C) was measured. Data are expressed as a percentage of IL-1β-induced adhesion molecule expression. Values are means ± SEM, n = 5–6. Different from stimuli alone, *P < 0.05.
ished TNF-α-induced upregulation of VCAM-1, as well as ICAM-1 and E-selectin mRNA (Fig. 3).

Transcription factor activation. To test whether the inhibitory effect of phloretin on cytokine-induced adhesion molecule expression is mediated via NF-κB, we measured the nuclear translocation of 2 members of the NF-κB family of transcription factors. Whereas phloretin alone had no effect, incubation of HUVECs with TNF-α (5 μg/L) for 1 h induced the nuclear translocation of p65 and p50 (Fig. 4A). However, preincubation of HUVECs with 100 μmol/L phloretin for 1 h prior to TNF-α stimulation did not prevent nuclear translocation of p65 and p50 (Fig. 4A). As a positive control, preincubation of HUVECs for 3 h with 50 μmol/L of the proteasome inhibitor MG132, a known inhibitor of NF-κB activation (20), prevented the nuclear translocation of p65 and p50 (data not shown).

Because phloretin did not exert any inhibitory effect on nuclear translocation of NF-κB, we studied the influence of phloretin on other transcription factors known to be involved in gene expression of adhesion molecules, e.g., AP-1, SP-1, GATA-2, and IRF-1 (21–26). Stimulation with TNF-α did not induce nuclear translocation of c-Fos and c-Jun (AP-1), SP-1, and GATA-2 (data not shown). However, the nuclear translocation of IRF-1 was strongly induced by TNF-α (Fig. 4B). Preincubation with 100 μmol/L phloretin for 1 h completely prevented TNF-α-stimulated nuclear translocation of IRF-1.

Monocyte adhesion to endothelial cells. To assess the functional relevance of phloretin-mediated suppression of TNF-α-induced upregulation of adhesion molecules, we examined the influence of phloretin on both basal and TNF-α-stimulated adhesion of THP-1 cells to endothelial cells. To apply the results from HUVECs to endothelial cells of the arterial tree, we used HAECs and HUVECs. Human monocytic THP-1 cells demonstrated very low basal adhesion to unstimulated endothelial cell monolayers (Fig. 5). Preincubation with phloretin for 1 h had no influence on basal adhesion (data not shown). In response to TNF-α, however, we observed a significant increase of THP-1 adherence to the endothelial monolayer. Cytokine-induced cell adhesion was dose-dependently reduced by 1 h of preincubation with phloretin (20 to 100 μmol/L) prior to TNF-α stimulation in both cell types. The highest dose of phloretin (100 μmol/L) reduced the stimulated adhesion to basal levels.

Activation of human blood platelets. We next determined whether phloretin has an effect on the stimulation and activation of human platelets. ADP (50 μmol/L) and TRAP (15 μmol/L) stimulated the expression of CD62P (P-selectin) and the activated form of the GPIIb/IIIa complex, 2 surface receptors involved in platelet activation and aggregation, in whole blood after 10 min (Fig. 6). Preincubation of blood samples with phloretin (0.1 to 10 μmol/L) for 30 min had no influence on the expression levels of CD62P after stimulation with either agonist (data not shown). In contrast, at concentrations of 1 and 10 μmol/L, phloretin significantly inhibited ADP- and TRAP-induced upregulation of the activated GPIIb/IIIa complex (Fig. 6A and B). To test the functional relevance of diminished expression of the activated GPIIb/IIIa complex by phloretin, we measured platelet aggregation in platelet-rich plasma. ADP-stimulated aggregation of blood platelets was significantly reduced by preincubation with 10 and 50 μmol/L phloretin (Fig. 7A). Figure 7B shows an original registration demonstrating the inhibitory effects of phloretin on the aggregation response to ADP (1.25 μmol/L).

![Figure 3](https://academic.oup.com/jn/article-abstract/135/2/172/4663638)

**FIGURE 3** Phloretin inhibits cytokine-induced mRNA expression of all 3 adhesion molecules. HUVECs were preincubated with 100 μmol/L phloretin for 1 h and stimulated with TNF-α (5 μg/L) for 4 h. Levels of mRNAs for VCAM-1, ICAM-1, and E-selectin were determined. GAPDH served as a housekeeping gene. A representative gel from 3 independent experiments is shown.

**FIGURE 4** Effect of phloretin on TNF-α-induced activation of transcription factors. HUVECs were stimulated with TNF-α (5 μg/L) for 1 h and analyzed for nuclear translocation of NF-κB family members p65 and p50 after preincubation with 100 μmol/L phloretin for 1 h (A). (B) HUVECs were treated as in A and nuclear translocation of IRF-1 was measured. (means ± SEM, n = 3). C: A consensus oligonucleotide was added in a molar excess as a competitor for transcription factor binding.

**FIGURE 5** PHLORETIN AND ADHESION

**DISCUSSION**

Cardiovascular diseases are the leading cause of mortality in Western countries. Recent years have seen an increasing interest in the health benefits of dietary compounds contained in...
The growing list of phytochemicals and their biological activities has become the focus of intensive research. Numerous studies have established the protective and preventive effects of phytochemicals (4,27,28). The main underlying physiological mechanism is believed to be their antioxidative activities (29). In addition, a number of phytochemicals can stimulate the production of nitric oxide, thereby contributing to improved vascular function in humans (30).

Phloretin, a dihydrochalcone flavonoid, has until now been found in apples and strawberries (5,7). In the present study phloretin dose-dependently prevented cytokine-induced upregulation of VCAM-1, ICAM-1, and E-selectin in HUVECs. We already detected the inhibitory effect of phloretin on the expression of adhesion molecules in HUVECs on the level of mRNA for all 3 adhesion molecules. Phloretin furthermore significantly reduced the adhesion of monocytes to endothelial cells of different vascular trees, demonstrating the functional relevance of diminished adhesion molecule expression. In addition, the functional importance of the diminished expression of the activated GPIIb/IIIa complex in platelets was demonstrated by suppressed platelet aggregation.

Plasma concentrations of phloretin in nonsupplemented humans lie in the order of magnitude of 1 μmol/L (31). In phloretin-supplemented rats, however, they reach 55 to 65 μmol/L after 10 h (32). This implies that the concentrations of phloretin used in our studies can be achieved in plasma after supplementation, at least in rats.

We thus established, in addition to its major biological action as inhibitor of glucose transporters (8), a cardiovascular relevant protective effect of phloretin. A study in Finland found that the intake of flavonoids was inversely associated with coronary mortality and that the protective effects were associated with a diet high in apples and onions (33). A French study likewise evidenced that women were at lower risk from cardiovascular diseases after consumption of flavonoid-rich foods (34). Reports on the cardioprotective effects of flavonoids are, however, not consistent. A recent U.S. study revealed no significant influence of total flavonoid or selected flavonol and flavone intake on the risk of cardiovascular disease, although a slight nonsignificant inverse association for apples was found (35).

For a number of different plant-derived substances, an inhibitory effect on the expression of adhesion molecules has been described in vivo and in vitro. Polyphenols from olive oil and red wine suppressed VCAM-1 expression in HUVECs (36). Formononetin-enriched isoflavones reduced the circulating levels of VCAM-1 in humans (37), and in hypercholesterolemic rabbits naringenin inhibited the expression of ICAM-1 in endothelial cells (38). Two flavones, luteolin and apigenin, inhibited TNF-α-induced upregulation of adhesion molecules and adhesion of THP-1 cells to HUVECs (39).

Regulation of adhesion molecule expression is coupled to oxidative stress through the transcription factor NF-κB (40). In our study, however, we found no effect on the nuclear translocation of NF-κB by pretreatment of HUVECs with...
phloretin. For maximum induction of transcription by cytokines, however, a combination of different transcription factors known to be involved in adhesion molecule promoter activation—including AP-1, SP-1, GATA-2, and IRF-1—together with NF-κB—is necessary (21–26). We accordingly found that pretreatment of HUVECs with phloretin inhibits the nuclear translocation of IRF-1. In another study, the induction of VCAM-1 expression by cytokines in HUVECs was accompanied by the interaction of p65/p50 proteins of the NF-κB precursor protein and the activation of NF-κB by TNF-α (19). The ubiquitin-proteasome pathway is required for processing the NF-κB1 phloridzin in strawberries (Fragaria x ananassa Duch.) by HPLC-PDA-MS/MS and NMR spectroscopy. J. Agric. Food Chem. 51: 6516–6520.

A

B

FIGURE 7 Phloretin suppresses ADP-stimulated aggregation of human platelets in platelet rich plasma. (A) Platelets were pretreated with the indicated doses of phloretin for 30 min and then activated with 1.25 or 2.5 μmol/L ADP. Aggregation was recorded for 10 min. Data are presented as a percentage of maximum aggregation without preincubation with phloretin. *P < 0.05 compared to control (means ± SEM, n = 9). (B) Original traces of a representative experiment demonstrating the dose-dependent reduction of maximum aggregation by preincubation with the indicated doses of phloretin.

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Bibliography


