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Dietary flavonoid apigenin inhibits high glucose and tumor necrosis factor α-induced adhesion molecule expression in human endothelial cells

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Abstract

Diabetes mellitus is associated with increased endothelial dysfunction and development of atherosclerotic vascular diseases. In contrast, an increased intake of dietary flavonoids is associated with a decreased risk of cardiovascular diseases. Here we demonstrate that high glucose (HG) and tumor necrosis factor α (TNFα) result in the expression of adhesion molecules and junctional molecules on endothelial cells (EC) within a short time. Simultaneously, we examined the regulatory effects of several dietary flavonoids. We demonstrated the short-term expression of adhesion molecules in a human EC line cultured with normal glucose (5.5 mM), HG (30 mM) and TNFα (10 ng/ml) by reverse transcription–polymerase chain reaction (RT-PCR), immunocytochemistry and adhesion assay. The expression of intercellular adhesion molecule-1 (ICAM1) and vascular cell adhesion molecule-1 (VCAM1) increased, but that of occludin decreased. Apigenin strongly inhibited the expression of VCAM1, IκB kinase (IKK) α and IKKε/IKKi, and suppressed the adhesion of U937 cells. From the structure and inhibitory activity of several dietary flavonoids, it was recognized that a double bond between apigenin and the third hydroxyl group was required for inhibition of gene expression. HG and TNFα induced the expression of cell adhesion molecules and reduced that of occludin in EC. These flavonoids modified the expression of cloudin 5 and occludin. These results demonstrated that apigenin inhibits HG- and TNFα-induced adhesion molecule expression and that flavonoids regulate the expression of junctional molecules in human EC. It is suggested that apigenin inhibited the expression of several genes through inhibition of IKKs.

Keywords: Apigenin; Adhesion molecule; Human endothelial cells; High glucose; TNF

1. Introduction

Chronic hyperglycemia induces the development of diabetic microvascular disease [1], but the short-term regulatory mechanisms responsible for the influence of high glucose (HG) concentrations on vascular cell function and their implications for cell–cell interaction in the microcirculation remain unclear. Augmented HG triggered acute reversible changes in cellular metabolism, leading to increased aldose reductase [1] and protein kinase C activation [2]. The deleterious effect of HG on cell function is additionally mediated by advanced glycosylation end products (AGEs) [3]. AGEs are highly reactive as a result of prolonged exposure to HG. Cao et al. [4] indicated that acute hyperglycemia during myocardial infraction is an indicator of adverse short-term results and mortality in diabetic patients. However, while HG induces the denaturation of vascular cells, the details are unclear. HG may contribute to the angiopathy of diabetes at the early stages of vascular denaturation [5,6].

Diabetes mellitus is associated with endothelial dysfunction characterized by compromised endothelium-dependent responses [7]. Acute inflammation causes leakage of plasma proteins and increased adhesion of circulating leukocytes to the endothelial cells (EC) luminal surface [8]. This recruitment of leukocytes is a critical early event in the development of atherosclerosis. Early during atherosclerosis, activated cell surface molecules such as intercellular adhesion molecule-1 (ICAM1), vascular cell adhesion molecule-1 (VCAM1) and platelet/endothelial cell adhesion molecule-1, and migration factors such as monocyte chemoattractant protein-1 (MCP1) are expressed on the surface of EC [9]. A recent report indicated that a moderate increase in the expression of

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ICAM1 reduces the barrier function of EC, and that higher levels of ICAM1 affect cell junctions and the cytoskeleton [10]. The early changes underlying vascular leakiness were indicated to occur with alterations in EC shape and cell junctions. In addition, activated leukocytes preferentially migrate across an endothelium that has been stimulated by cytokine during inflammation. These effects of diabetic plasma on leukocytes were found to be triggered by increased plasma levels of tumor necrosis factor α (TNFα) [11]. Furthermore, TNFα released during an inflammatory insult regulates tight junction (TJ) molecules and alters the permeability of the blood–brain barrier (BBB) [12]. The EC is permeable to TJ molecules such as cloudin and occludin [13,14]. These early changes represent a response by TNFα, and the expression of leukocyte adhesion molecules on leukocytes may be initiated by more subtle alterations on the EC surface [15]. Moreover, since the trigger of the BBB is destruction, the expression of TJ proteins and adhesion molecules might be closely related in the short term [16]. Actually, diabetes-related alterations in the function of the BBB are a major contributor to central nervous system dysfunction in diabetic patients [17]. The brain occludin content in diabetic rats was significantly decreased compared to that in control rats [18]. Increased BBB permeability and altered TJs might present a novel target for the prevention of neurological complications in diabetes.

An increased intake of dietary flavonoids is associated with a decreased risk of cardiovascular disease [19]. Flavonoids act as antioxidants in human plasma and other extracellular fluids, and protect low-density lipoproteins from oxidation [20]. Furthermore, flavonoids reduced the cytokine-induced expression of E-selection, ICAM1 and VCAM1 in umbilical vein EC [21]. The beneficial effects of dietary flavonoids might be explained by antioxidative capacity and subsequent modulation of intercellular redox maintenance, cell signaling and gene expression [20]. Although dietary flavonoids are reported to be effective against the inflammatory stress-induced expression of adhesion molecules in EC, the precise mechanisms of expression in HG- and TNFα-treated cells have yet to be elucidated. Particularly, the mechanism of early change in vascular leakiness has not been clarified. Therefore, we were interested in whether there is a modifying effect on the HG- and TNFα-induced expression of adhesion molecules in EC caused by several dietary flavonoids. In this study, we investigated the expression of adhesion molecules following HG and TNFα stimulation in cultured human EC. Furthermore, we examined whether flavonoids can modulate early changes in gene expression.

2. Materials and methods

2.1. Materials

Recombinant human TNFα was purchased from Roch Applied Science USA. VCAM1 polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). An immunocytochemical assay kit was purchased from Dako Japan (Kobe, Japan). Apigenin, caffeic acid, chrysin, quercetin, epicatechin and kaempferol were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

2.2. Cell culture

The human EC line ISO-HAS, derived from a patient with angiosarcoma, was obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). ISO-HAS cells were cultured in 50% Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Gibco Invitrogen, Grand Island, NY, USA) and conditioned medium from the angiosarcoma cell line ISO1 (DMEM/ISO1) [22,23]. ISO-HAS cells were identified to constitutively express CD31, von Willebrand factor, Flt-1, kinase insert domain receptor (KDR), and vascular endothelial growth factor (VEGF) [24,25]. The cells were seeded at 1:1 ratio in 75-cm^2 flasks (Becton Dickinson, Bedford, MA, USA) and grown at 37°C under 5% CO_2 in a humidified atmosphere in DMEM/ISO1. The medium was periodically renewed until the cells had reached 70–90% confluence, at which point they were treated with 0.25% trypsin (Sigma-Aldrich, Inc.).

Human monocytic U-937 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). They were maintained in RPMI-1640 medium supplemented with 5% FBS. U937 cells were grown in suspension cultures and subcultured 1:4 three times per week in 75-cm^2 culture flasks.

2.3. Treatment of cultures

The human EC line ISO-HAS cells were plated in 10-mm cell culture dishes (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) or 96-well plates (Becton Dickinson). The cells were grown at 37°C under 5% CO_2 in a humidified atmosphere until confluent, typically 24–48 h after seeding. They were then incubated with control glucose (5.5 mM) or test concentration (30 mM), and with TNFα and different concentrations of the various flavonoids (10, 30 and 50 μM) for 0–24 h.

2.4. Total RNA extraction and cDNA

Total RNA was extracted from cultured neurons using Trizol reagent (Gibco Invitrogen). DNase I (Gibco Invitrogen) was used to treat the RNA sample at room temperature for 15 min to remove genomic DNA. DNase I was heated for 15 min at 65°C for its inactivation. First-strand cDNA synthesis was performed with Superscript II (Gibco Invitrogen).

2.5. Reverse transcription–polymerase chain reaction

PCR was performed for adhesion molecules, TJ proteins and IκB kinases (IKKs). Primers were selected from GenBank and designated using the primer design software Primer Express (Applied Biosystems, Foster City, CA).

Table 1 summarizes the primer sets used. The reaction mixture (50 μl) contained 200 ng of the cDNA sample; 1.25 U of Ampli-Taq DNA polymerase; 1× PCR reaction buffer; 200 mM of each primer; 200 μM dATP, dCTP, dGTP and dTTP; and 1.5 mM $MgCl_2$ (Applied Biosystems). The thermal cycling conditions included 5 min at 95 °C, then 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. After amplification, 10 μl of the reaction mixture was electrophoresed on a 2% NuSive/agarose (3:1) (FMC Product, Rockland, ME, USA) gel and visualized with UV illumination after staining with ethidium bromide. The amount of mRNA expressed was calculated relative to that per 18S ribosomal RNA. The PCR conditions were confirmed to be the same as described in the report (with no problems) and comprised the standard composition and conditions generally used.

2.6. Real-time quantitative PCR

Quantitative PCR was carried out using real-time *TaqMan* technology [26], and the results were analyzed with a Model 7700 Sequence Detector System (Applied Biosystems). Quantitative PCR was performed for IKKi and to monitor the expression of a housekeeping gene, 18S ribosomal RNA (*TaqMan* ribosomal RNA control reagents). An 18S ribosomal RNA amplicon was used as internal control for quantitation of the total amount of cDNA. Serial dilutions of cDNA were used to create a standard curve for the quantitation of IKKε/IKKi. The amount of mRNA expressed was calculated relative to that per 18S ribosomal RNA. Primers and the *TaqMan* probes were selected from GenBank (IKKε/IKKi; accession number AB016590) and designed using the primer design software Primer Express (Applied Biosystems). The sequence of the *TaqMan* probe was 5′-CTGCCCTTCATCCCCTTTGG-TGGG-3′ for IKKκ/IKKi. The amplification reaction mixture (50 μl) contained 25 ng of the cDNA sample; 1.25 U of Ampli-Taq DNA polymerase; 1× PCR buffer; 200 nM of each primer; 200 μM dATP, dCTP and dGTP; 400 μM dUTP; 5.5 mM $MgCl_2$; and 100 nM *TaqMan* probe (Applied Biosystems). The thermal cycling conditions included 2 min at 50°C and 10 min at 95°C. Thermal cycling proceeded with 40 cycles of 95°C for 15 s and 60°C for 1 min.

2.7. Human EC–U937 adhesion assay

For the adhesion assay, ISO-HAS cells were resuspended in DMEM/ISO1 CM medium with 10% FBS at the desired density and plated in either 24-well or 96-well plates. Human monocyte-like U937 cells (1×10^6 cells /ml) were added and incubated with EC at 37°C for 3 h. At the end of the incubation, the wells were filled with culture medium and aspirated three times to remove unbound U937 cells. The adhesion of U937 cells to EC was measured using quantitative monolayer adhesion assays, similar to those previously described [27]. Briefly, the number of bound U937 cells per square millimeter was determined by direct microscopy. One randomly selected central field and four peripheral fields of intact endothelial monolayers were examined with an ocular grid using phase-contrast microscopy (×200). The total number of bound U937 cells was determined by a hemacytometer. Residual U937 cells not removed from empty wells by the washing procedure represented −2% of bound U937 cells in a typical experiment.

2.8. Immunocytochemical analysis

The EC for the immunocytochemical assay were resuspended in DMEM/ISO1 CM medium with 10% FBS to the desired density for culture and plated in immunohistochemical plates (Costar). The cells were fixed in 4% paraformaldehyde (Sigma-Aldrich, Inc.) at room temperature for 15 min and washed with 0.01% Tween 20–phosphate-buffered saline (PBS) three times. After wash with PBS, blocking reagents (Dako, Tokyo, Japan) were added. After further washing, the antibody to VCAM1 was added at room temperature for 30 min. The secondary antibody was then added at room temperature for 45–60 min. Finally, the cells were treated with a color development agent (Dako) at room temperature for 15 min and, after more washing, observed with a microscope.

2.9. Statistical analysis

Data are expressed as mean±S.D. The significance of differences was determined using Fisher's protected least significant difference method following analysis of variance.

3. Results

3.1. HG- and TNFα-induced expression of adhesion molecules and effects of flavonoids on cultured human EC lines

Confluent human EC lines were incubated with TNFα (10 ng/ml) and 5.5 mM glucose or HG (30 mM) for 2, 4 and

Table 1
Sequences of primers for RT-PCR

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	Amplicon (bp)	Accession number
ICAM1	GTAGCAGCCGCAGTCATAATG	TTGTGTGTTCGGTTTCATGG	126	J03132
MCP1	AAACTGAAGCTCGCACTCTCG	TGAAGGTGGCTGCTATGAGCA	81	NM_002982
VCAM1	CAAAGGCAGAGTACGCAAACA	GGATTTTCGGAGCAGGAAAG	142	M60335
Cloudin 5	AGGCGTGCTGTACCTGTTTTG	TCGTAAAACTCGCGGACGA	84	CR456420
Occludin	ATAAATCCACGCCGGTTCC	TAACGAGGCTGCCTGAAGTCA	79	U49184
IKKα	CACAGACCTTCAGATCACTCCTACA	ACACGGTCCTGACTCTGCAC	83	AF080157
IKKε/IKKi	TACCATGCAGCCACTGGC	ATCTCCTTGTTCCGCCGT	65	AB016590

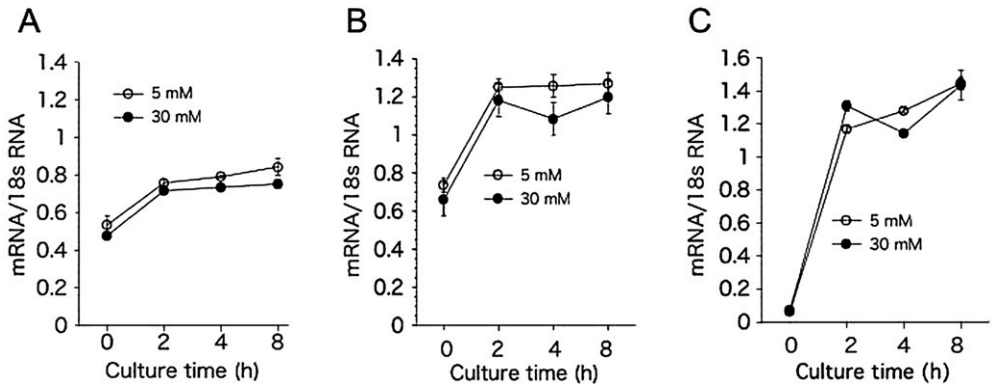

Fig. 1. Effects of incubation times on HG- and TNFα-induced ICAM1, MCP1 and VCAM1 gene expression in human EC. A, ICAM 1; B, MCP 1; C, VCAM 1. The human EC line ISO-HAS was cultured for 2, 4 and 8 h with 5.5 mM glucose, HG (30 mM) and TNFα (10 ng/ml). The data are expressed as mean±S.D (n=4).

8 h. The gene expression of ICAM1, VCAM1 and MCP1 was investigated by reverse transcription–polymerase chain reaction (RT-PCR). As shown in Fig. 1, HG and TNFα augmented the expression of ICAM1, VCAM1 and MCP1, compared with 5 mM glucose without TNFα in 2 h. The increase in expression reached a plateau in 2 h. The gene expression of

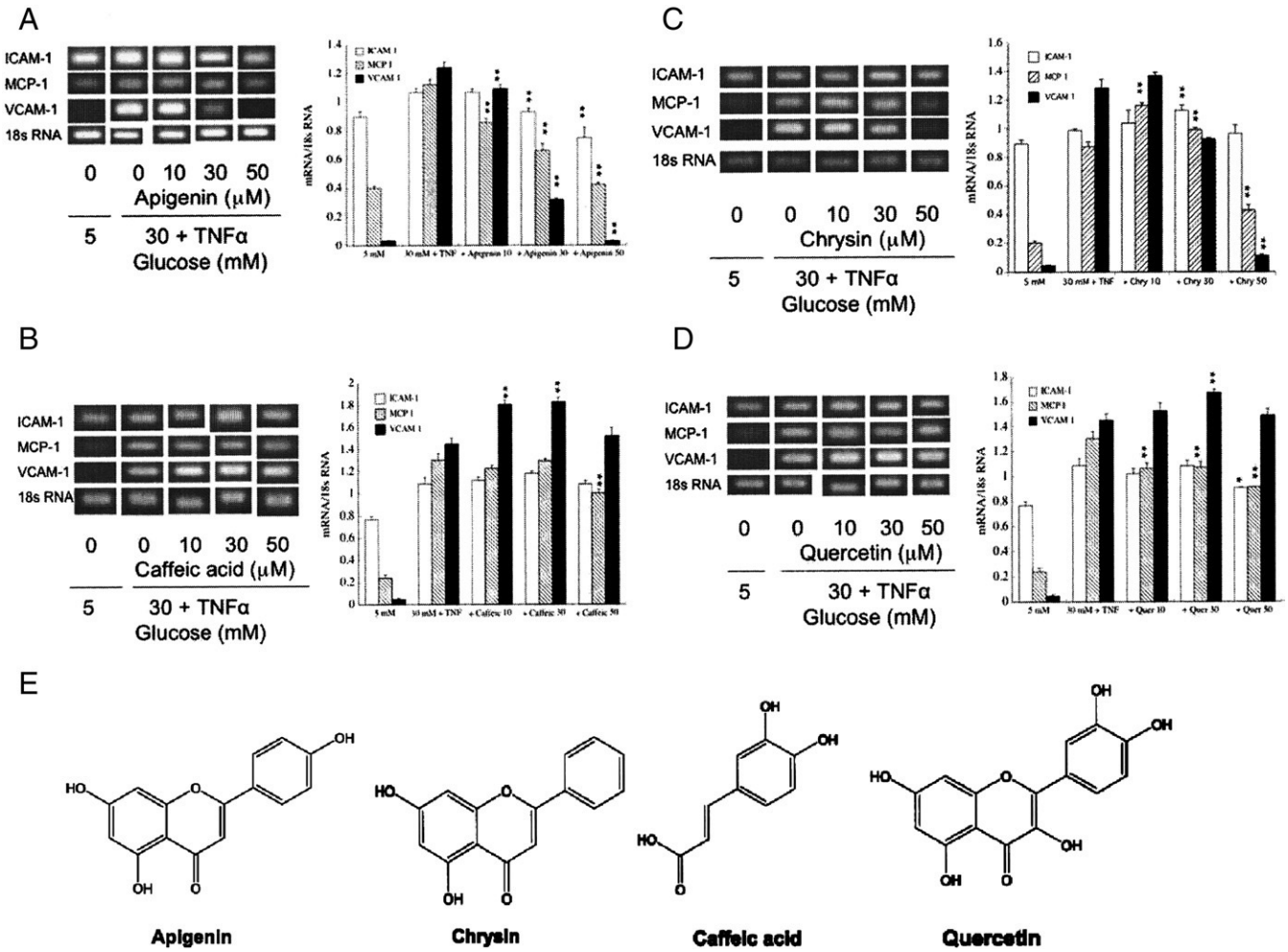

Fig. 2. Effects of flavonoid concentrations on ICAM1, MCP1 and VCAM1 gene expression in human EC. The human EC line ISO-HAS was cultured for 4 h without and with 10, 30 and 50 μM apigenin (A), caffeic acid (B), chrysin (C) and quercetin (D), and then challenged with HG (30 mM) and TNFα (10 ng/ml). The structures of apigenin, caffeic acid, chrysin and quercetin are indicated (E). The data are expressed as mean±S.D (n=4). *P<.05, significantly different from 0 μM. **P<.01, significantly different from 0 μM.

VCAM1 showed the most remarkable increase. Moreover, we examined the effects of several dietary flavonoids. Confluent human EC lines were incubated with several flavonoids (10, 30 and 50 μM) in TNFα (10 ng/ml) and HG (30 mM) for 4 h. HG and TNFα augmented the gene expression of ICAM1, MCP1 and VCAM1 compared with 5 mM glucose without TNFα (Fig. 2). On the other hand, several flavonoids inhibited the HG- and TNFα-induced gene expression in EC. In particular, apigenin and chrysin strongly inhibited the gene expression of VCAM1 and MCP1 at 50 μM. Caffeic acid inhibited the expression of MCP1, but greatly enhanced the expression of VCAM1. Similarly, quercetin significantly inhibited the expression of ICAM1 and MCP1 (50 μM), but enhanced that of VCAM1 (30 μM). Kaempferol inhibited the expression of MCP1 and VCAM1. Chromone, epicatechin, flavone and naringenin did not affect the expression of adhesion molecules (data not shown). Next, we reviewed the activity–structure relation of several flavonoids. Naringenin is structurally identical to apigenin, except for the absence of the 2,3-double bond in the C-ring (Fig. 2E). In addition, flavone lacks any hydroxy substitution in the A-ring, as does the related compound chromon. From the structure and inhibitory activity of these dietary flavonoids, it was recognized that a double bond (in the C-ring) of flavonoids and the third hydroxyl group (A-ring) were required for the inhibition of gene expression. Conversely, quercetin and kaempferol enhanced ICAM1 expression at 10 and 30 μM, respectively. Similarly, quercetin enhanced VCAM1 expression at 30 μM.

3.2. HG- and TNFα-induced cloudin 5 and occluding expression and effects of flavonoids on cultured human EC lines

Similarly, the gene expression of cloudin 5 and occludin by induced TNFα and HG was investigated by RT-PCR. HG and TNFα augmented the expression of cloudin 5 (1.2-fold) compared with 5 mM glucose without TNFα. On the other hand, the expression of occludin was reduced by HG and TNFα (0.89-fold) (data not shown). Flavonoids modified the gene expression of cloudin 5 (Table 2). Kaempferol and quercetin enhanced the expression of cloudin 5. However, apigenin dose-dependently decreased the expression of cloudin 5. Moreover, we examined the influence of flavonoids on the gene expression of occludin (Table 3). Caffeic acid, galangin, kaempferol, naringenin and quercetin enhanced the expression of occludin. Flavone decreased the expression of occludin. No common structural feature for the gene expression of occludin and cloudin 5 was identified.

3.3. Inhibitory effects of apigenin on HG- and TNFα-induced VCAM1 expression

We investigated the effects of apigenin on HG- and TNFα-induced VCAM1 protein expression in EC by immunocytochemistry. VCAM1 protein was observed upon treatment with HG and TNFα in human EC. On the other hand, apigenin inhibited the expression of VCAM1 protein at 30 mM glucose and 10 ng/ml TNFα (data not shown).

3.4. Inhibitory effects of apigenin on U937 cell adhesion in cultured human EC

The adhesion of U937 cells to EC was measured using quantitative monolayer adhesion assays. Pretreatment of human EC monolayers for 20 h with 30 mM glucose and 10 ng/ml TNFα resulted in a 21.3-fold increase in the adhesion of U937 cells (Fig. 3E). Under similar conditions, 50 μM apigenin strongly inhibited the adhesion of U937 cells. The rate of inhibition by apigenin was 81.2% with 30 mM glucose and 10 ng/ml TNFα.

3.5. HG- and TNFα-induced IKKs and effects of flavonoids on cultured human EC lines

We investigated the TNFα- and HG-induced gene expression of the IKK subtypes IKKα and IKKε/IKKi by RT-PCR and real-time quantitative PCR . Furthermore, we analyzed the effects of 50 μM apigenin, chrysin, caffeic acid

Table 2
Effects of flavonoids concentration on induced cloudin 5 gene expression in human EC

Flavonoids (mM)	Occludin			
	0	10	30	50
Apigenin	0.37±0.01 (100%)	0.36±0.012 (97.3%)	0.30±0.012** (81.1%)	0.26±0.013** (70.3%)
Caffeic acid	0.32±0.027 (100%)	0.45±0.043** (140.6%)	0.49±0.021** (153.1%)	0.305±0.038 (95.3%)
Chrysin	0.35±0.055 (100%)	0.44±0.021* (125.7%)	0.451±0.035* (128.6%)	0.40±0.030 (114.5%)
Epicatechin	0.44±0.034 (100%)	0.51±0.015 (115.9%)	0.50±0.012 (113.6%)	0.41±0.037 (93.2%)
Flavone	0.35±0.055 (100%)	0.42±0.070* (120.0%)	0.43±0.010* (122.9%)	0.39±0.033 (111.4%)
Galangin	0.27±0.007 (100%)	0.34±0.030** (125.9%)	0.28±0.020 (103.9%)	0.27±0.036 (100%)
Kaempferol	0.27±0.007 (100%)	0.27±0.009 (100%)	0.38±0.021** (140.7%)	0.37±0.025** (137.0%)
Naringenin	0.37±0.010 (100%)	0.39±0.034 (105.4%)	0.42±0.007 (113.5%)	0.35±0.023 (94.6%)
Quercetin	0.32± 0.027 (100%)	0.46±0.024** (143.8%)	0.58±0.005** (181.3%)	0.607±0.044** (189.7%)

ISO-HAS cells were cultured for 4 h without and with 10, 30 and 50 μM of several flavonoids and then challenged with HG (30 mM) and TNFα (10 ng/ml). The data are expressed as mean±S.D (n=4).

* P<.05, significantly different from 0 μM.

** P<.01, significantly different from 0 μM.

Table 3
Effects of flavonoids concentration on induced occludin gene expression in human EC

Flavonoids (mM)	Occludin			
	0	10	30	50
Apigenin	0.17±0.024 (100%)	0.20±0.014 (116.5%)	0.24±0.011** (140.6%)	0.19±0.006 (108.8%)
Caffeic acid	0.09±0.007 (100%)	0.33±0.024** (366.7%)	0.39±0.016** (433.3%)	0.22±0.009** (244.4%)
Chrysin	0.15±0.026 (100%)	0.094±0.023** (62.7%)	0.19±0.028** (123.3%)	0.25±0.028** (166.9%)
Epicatechin	0.14±0.040 (100%)	0.23±0.016** (164.3%)	0.25±0.028** (175.0%)	0.15±0.033 (109.3%)
Flavone	0.15±0.026 (100%)	0.074±0.041** (49.3%)	0.10±0.019** (68.0%)	0.09±0.008** (60.0%)
Galangin	0.09±0.007 (100%)	0.13±0.041** (144.4%)	0.13±0.019** (144.4%)	0.18±0.008** (200%)
Kaempferol	0.09±0.007 (100%)	0.10±0.013 (111.1%)	0.13±0.001** (144.4%)	0.18±0.011** (200%)
Naringenin	0.17±0.024 (100%)	0.16±0.003 (94.1%)	0.22±0.009** (129.4%)	0.30±0.019** (176.5%)
Quercetin	0.09±0.007 (100%)	0.14±0.013** (155.6%)	0.38±0.019** (422.2%)	0.30±0.011** (333.3%)

ISO-HAS cells were cultured for 4 h without and with 10, 30 and 50 μM of several flavonoids and then challenged with HG (30 mM) and TNFα (10 ng/ml). The data are expressed as mean±S.D (n=4).

* P<.05, significantly different from 0 μM.

** P<.01, significantly different from 0 μM.

and kaempferol on gene expression. As shown in Fig. 4, the gene expression of IKKα and IKKκ/IKKi was induced by TNFα and HG. The expression of IKKα induced by HG and TNFα was inhibited by apigenin, caffeic acid and quercetin. Similarly, IKKε/IKKi expression was strongly inhibited by apigenin, caffeic acid, chromon kaempferol and quercetin, respectively. Apigenin strongly inhibited both IKKα and IKKε/IKKi. For the expression of IKKε/IKKi, the results of real-time quantitative PCR (data not shown) were confirmed to be similar to those of RT-PCR.

4. Discussion

Diabetes mellitus is associated with increased endothelial dysfunction and development of atherosclerotic vascular diseases. Based on the premise that HG and TNFα induce the expression of adhesion molecules and junctional molecules on EC within a short period of time, we investigated the HG- and TNFα-induced expression of ICAM1, VCAM1 and MCP1, and the regulation of cloudin 5 expression on EC lines. Moreover, we examined the regulatory effects of several dietary flavonoids.

HG and TNFα directly influenced the adhesion of EC to leukocytes in the short term. Acute short-term hyperglycemia is becoming an important risk factor for several diseases such as atherosclerotic vascular diseases. Acute hyperglycemic stimulation can affect the onset of diabetic complications and the development of atherosclerosis. In human aortic EC, HG induced monocyte–EC adhesion and transmigration by increasing VCAM1 and MCP1 expression at 4 h [6]. Treatment with HG and TNFα at 4 h was indicated to increase significantly the expression of ICAM1, VCAM1 and MCP1, and cell adhesion in the human monocyte cell line U937. In contrast, several dietary flavonoids inhibited the HG- and TNFα-induced expression of ICAM1, VCAM1 and

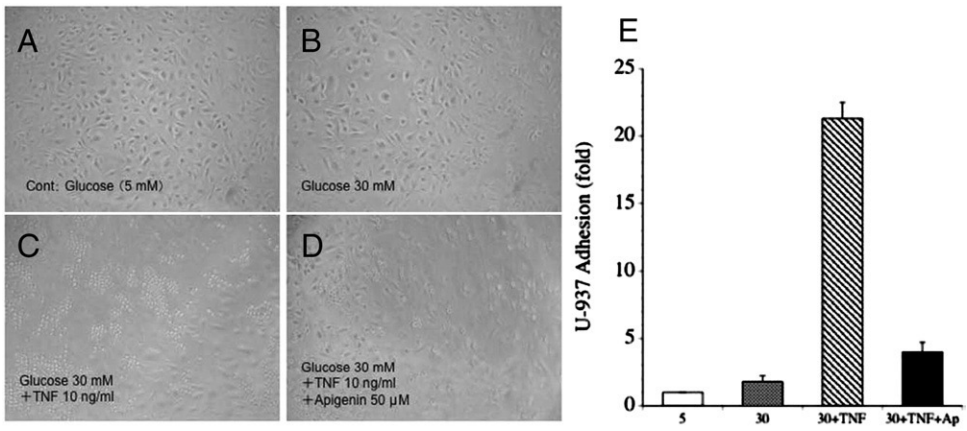

Fig. 3. Inhibitory effects of apigenin against adhesion of U937 cells by cultured human EC. The human EC line ISO-HAS was cultured for 24 h without and with 50 μM apigenin and then challenged with HG (30 mM) and TNFα (10 ng/ml). The U937 cells (1×10^6 cells/ml) were added and incubated with EC at 37°C for 3 h. The adhesion of U937 cells to EC was measured using quantitative monolayer adhesion assays, similar to those previously described [27]. Glucose 5.5 mM (A); glucose 30 mM (B); glucose 30 mM+TNFα 10 ng/ml (C); and glucose 30 mM+TNFα 10 ng/ml+apigenin 50 μM (D). The inhibitory effects of apigenin are indicated (fold) (E). The number of bound U937 cells per square millimeter was determined by direct microscopy. The data are expressed as mean±S.D (n=6).

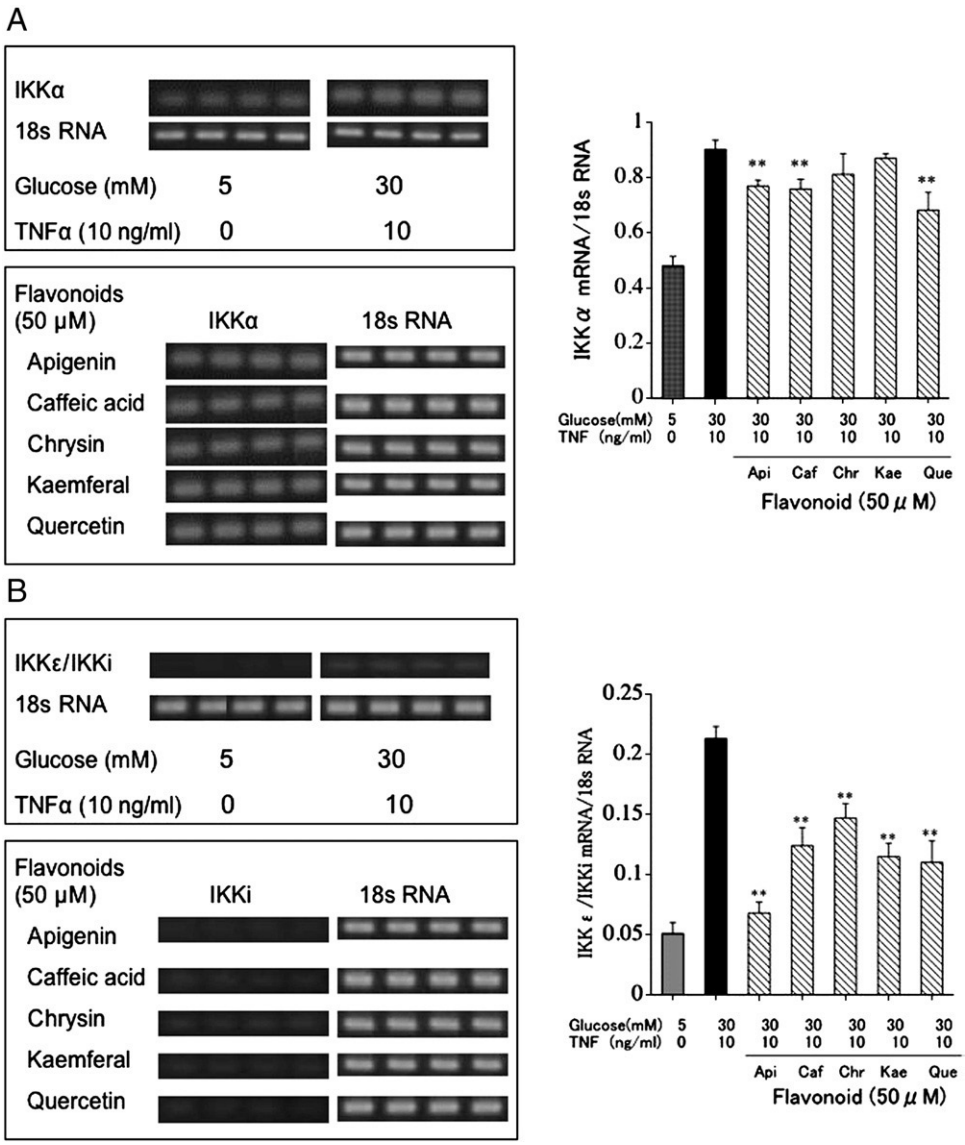

Fig. 4. Effects of several flavonoids on IKKα and IKKε/IKKi gene expression in human EC. ISO-HAS cells were cultured for 4 h without and with 50 μM apigenin, caffeic acid, chrysin and quercetin, and then challenged with HG (30 mM) and TNFα (10 ng/ml). IKKα (A) and IKKε/IKKi (B). Ap, apigenin; Ca, caffeic acid; Chr, chrysin; Kae, kaempferol; Que, quercetin. The data are expressed as mean±S.D (n=4). *P<.05, significantly different from 30 mM glucose+10 ng/ml TNFα. **P<.01, significantly different from 30 mM glucose+10 ng/ml TNFα.

MCP1. Flavonoids regulate the HG- and TNFα-induced expression of adhesion molecules such as VCAM1 in human EC. Notably, apigenin and chrysin strongly inhibited HG- and TNFα-induced VCAM1 gene expression. A report indicated that TNFα-induced ICAM1, MCP1 and VCAM1 expression was inhibited to the same degree by apigenin [26]. In our experiments, apigenin strongly inhibited the expression of VCAM1, but weakly inhibited that of ICAM1 and MCP1. The difference between our results and those of Lotito and Frei [28] may be due to the source of EC and experimental conditions. We believe that timing of treatment and glucose level influence expression. By studying structure and inhibition, it was recognized that the double bond of flavonoid and the third-place hydroxyl group were required for the inhibition of gene expression. Among flavonoids, apigenin most strongly inhibited gene expression and simultaneously blocked the adhesion of U937 cells. The TNFα-induced expression of adhesion molecules on leukocytes may be initiated by more subtle alterations to the surface of EC early on [15].

The expression of adhesion molecules on EC induced by inflammatory cytokines such as TNFα strongly depends on the activation of the transcription factor NF-κB [29]. Virtually all inducers of NF-κB cause the activation of the signal IKK complex. In fact, NF-κB was activated by the phosphorylation of IκB by the IKKs IKKα, IKK/βγ and IKKε/IKKi (humans, IKKε; mice, IKKi), which resulted in IκB degradation [29,30]. We demonstrated that HG and

TNFα induced the gene expression of IKK subtypes. The expression of IKKα and IKKε/IKKi was significantly induced by HG and TNFα. On the other hand, apigenin strongly attenuated the HG- and TNFα-induced IKKα and IKKε/IKKi expression in EC. IKKε/IKKi was first identified as an IKK-like protein with a 30% amino acid identity to IKKα and IKKβ in the kinase domain [31]. IKKε/IKKi directly phosphorylates only serine 36 of IκB and significantly stimulates NF-κB activation during inflammatory responses [31]. A recent report demonstrated that apigenin does not alter NF-κB–DNA binding activity in human monocytes [32]. Our results and these reports suggest that apigenin inhibits the production of inflammatory mediators by blocking NF-κB activation and, as a consequence, suppression of IKK activity and degradation of IκB.

Gerritsen et al. [21] indicated that apigenin inhibited the TNFα-induced production of interleukin (IL) 6 and IL-8, and the IL-1α-induced production of prostaglandin. In human umbilical vein EC, apigenin did not inhibit the TNFα-induced nuclear translocation of NF-κB or p50 (NFKB1)/p65 (RelA). Meiler et al. [33] demonstrated that the dominant-negative mutant of IKKβ in TNFα stimulated EC expression induction of the surface adhesion molecules E-selectin, ICAM1 and VCAM1. In this study, the gene expression of IKKε/IKKi was strongly inhibited. TNFα induced a number of proinflammatory changes that increase leukocyte adhesion, transendothelial migration and vascular leakage, and promote thrombosis [34]. The regulatory actions of the flavonoids appear to be distal to the activation of NF-κB, since the IKKs were blocked by apigenin. Thus, dietary flavonoids may prevent diabetic complications such as arteriosclerotic vascular disorder by regulating the activation of NF-κB.

The early changes underlying vascular leakiness were indicated to occur concomitant with alterations in EC shape and in cell junctions such as TJs. We demonstrated that HG and TNFα reduced levels of occludin in the short term. Dietary flavonoids modified the gene expression of cloudin 5 and occludin. In flavonoids, the third hydroxyl group in the C-ring may be necessary for an increase in cloudin 5. Also 3,4-dihydroxyl groups of the A-ring may be necessary for an increase in occludin expression. Spoerri et al. [35] showed that HG induces the expression of VEGF and insulin-like growth factor-I in human retinal EC. These three receptors are involved in the effect of HG-induced changes on occludin levels in human retinal EC. The reduced expression of occludin by human retinal EC leads to structural failure and breakdown of the blood–retinal barrier [1,7]. We demonstrated that changes in the TJ permeability of brain capillary EC are associated with IL-1β nitric oxide and polyunsaturated fatty acids [36,37]. Dietary flavonoid might regulate the changes in TJ permeability in brain EC. In this study, the HG-induced expression of occludin was reduced in EC in the short term. HG stimulation regulated the expression — and might have decreased the amount — of occludin. On the other hand, the gene expression of cloudin 5 did not change upon HG and TNFα stimulation. The details are unclear, but it may not be markedly regulated by HG and TNFα stimulation in the short term.

In conclusion, we have presented evidence that the expression of ICAM1, MCP1 and VCAM1 was increased by HG and TNFα in vitro. Furthermore, our finding suggest that dietary flavonoids regulate HG- and TNFα-induced expression of adhesion and functional molecules differently. Dietary flavonoid might regulate the HG- and TNFα-induced expression of adhesion molecules in human EC. In particular, apigenin strongly inhibited the expression of VCAM1, IKKα and IKKε/IKKi, and suppressed the adhesion of U937 cells. Thus, apigenin may prevent diabetic complications such as arteriosclerotic vascular disorder by regulating the activation of NF-κB.

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