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Fisetin Induces Nrf2-Mediated HO-1 Expression Through PKC-δ and p38 in Human Umbilical Vein Endothelial Cells

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ABSTRACT

Fisetin is a natural flavonoid from fruits and vegetables that exhibits antioxidant, neurotrophic, anti-inflammatory, and anti-cancer effects in various disease models. Up-regulation of heme oxygenase-1 (HO-1) expression protects against oxidative stress-induced cell death, and therefore, plays a crucial role in cytoprotection in a variety of pathological models. In the present study, we investigated the effect of fisetin on the up-regulation of HO-1 in human umbilical vein endothelial cells (HUVECs). Small interfering RNA and pharmacological inhibitors of PKC-δ and p38 MAPK attenuated HO-1 induction in fisetin-stimulated HUVECs. Fisetin treatment resulted in significantly increased NF-E2-related factor 2 (Nrf2) nuclear translocation, and antioxidant response element (ARE)-luciferase activity, leading to up-regulation of HO-1 expression. In addition, fisetin pretreatment reduced hydrogen peroxide (H_2O_2)-induced cell death, and this effect was reversed by ZnPP, an inhibitor of HO-1. In summary, these findings suggest that induction of HO-1 expression via Nrf2 activation may contribute to the cytoprotection exerted by fisetin against H_2O_2-induced oxidative stress in HUVECs. J. Cell. Biochem. 112: 2352–2360, 2011.

KEY WORDS: FISETIN; HO-1; NRF2; ENDOTHELIAL CELLS; OXIDATIVE STRESS

Heme oxygenase-1 (HO-1) is one of the cytoprotective enzymes that catalyze the degradation of heme to generate biliverdin, free iron, and carbon monoxide [Ghattas et al., 2002]. Numerous stresses induce HO-1 and HO-1 induction plays a role in the maintenance of homeostasis in the face of oxidative injury. In addition, HO-1 has been therapeutically implicated in several diseases such as vascular injury, acute renal injury, hypertension, and others [Morse and Choi, 2002]. Lack of HO-1 causes profound changes in cellular homeostasis in genetically deficient mice and humans [Poss and Tonegawa, 1997], and is associated with susceptibility to oxidative stress and an increased pro-inflammatory state [Poss and Tonegawa, 1997; Yachie et al., 1999]. Numerous studies have suggested that HO-1 has important roles in cellular protection, such as anti-inflammatory [Lee and Chau, 2002], anti-proliferative [Choi et al., 2004], and anti-apoptotic effects [Liu et al., 2003].

It was recently shown that HO-1 is a potential therapeutic target in the treatment of vascular disease [Durante, 2010]. HO-1, which is highly expressed in tissues such as the heart and blood vessels, protects against vascular diseases, and has a cytoprotective effect in the circulation. Induction of HO-1 expression in the endothelial cells inhibits platelet aggregation and smooth muscle cell proliferation [Sato et al., 2001; Zhang et al., 2002]. HO-1 deficiency in mice results in severe vascular damage, which is presumably mediated by a transcriptional response to injury, with specific effects on cell cycle regulation, coagulation, thrombosis, and redox homeostasis [True et al., 2007]. These results indicate that HO-1 plays an important role in the vascular system as a potent protector against atherogenesis.

Flavonoids are polyphenolic compounds that are widely distributed in plants, and are regularly consumed in the human diet. Recent studies suggest that flavonoids will be useful in the prevention of various diseases, including atherosclerosis, because of their antioxidant and anti-inflammatory properties [Shukla et al., 2006; Onozuka et al., 2008]. In addition, various flavonoids induce HO-1 expression in a variety of cell types; in particular, dietary flavonoids that have a beneficial effect in vascular disease may also augment HO-1 expression [Meng et al., 2009].

Fisetin (3, 7, 3′, 4′-tetrahydroxyflavone) is a flavonoid present in various types of vegetables and fruits, including strawberry, apple, persimmon, grape, onion, and cucumber, at concentrations of 2–160 μg/g [Arai et al., 2000] (Fig. 1). Fisetin reportedly exhibits

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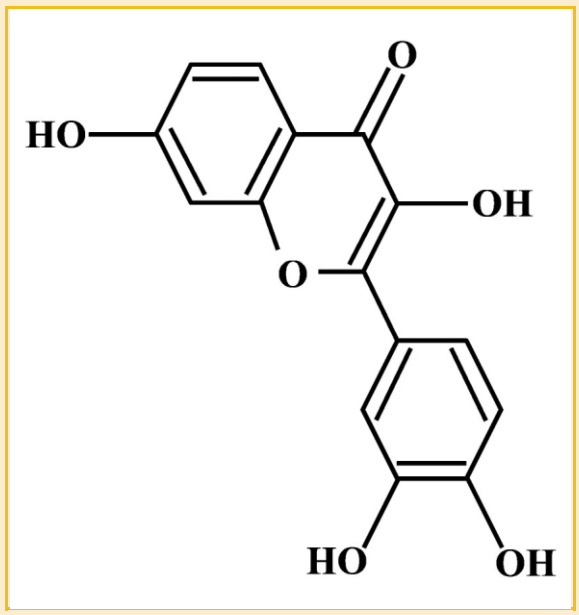


Fig. 1. Chemical structure of fisetin.

anti-cancer, antioxidant, neurotrophic, and anti-inflammatory activities [Hou et al., 2001; Higa et al., 2003; Maher, 2006; Khan et al., 2008]. Several studies indicated that fisetin has immunomodulatory effects [Higa et al., 2003], including inhibition of TH2-type cytokine production by activated human basophils and production of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-8 in human mast cells. Furthermore, fisetin has been associated with therapeutic effects in several diseases, including pulmonary inflammatory and neuroinflammatory diseases [Zheng et al., 2008; Geraets et al., 2009]. A recent study suggested that fisetin plays a role in ameliorating atherosclerosis by preventing LDL oxidation and blocking oxidized low-density lipoprotein (oxLDL) uptake by macrophages [Lian et al., 2008]. The above results show that fisetin exhibits many physiological effects, but the detailed molecular mechanism and effect of fisetin-induced HO-1 expression in the endothelial cells have never been reported.

In the present study, we investigated whether fisetin exerts a cytoprotective effect via upregulation of HO-1 expression in the human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

MATERIALS

Fisetin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT), and zinc protoporphyrin (ZnPP) were obtained from Sigma (St. Louis, MO), and TRIzol reagent was supplied by Invitrogen (Carlsbad, CA). TransPass R2 Transfection Reagent was obtained from New England Biolabs (Hercules, CA). Medium 199 (M199), fetal bovine serum (FBS), and tissue culture reagents were obtained from WelGENE Co. (Daegu, Korea). The following antibodies were used; anti-Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Lamin B (Santa Cruz), anti-HO-1 (Epitomics, Burlingame, CA), anti-p38 (Cell Signaling Technology, Beverly, MA), anti-PKC-δ (Cell Signaling Technology), and anti-GAPDH (AbFrontier, Seoul, Korea). Wortmannin, PD98059, SB203580, rottlerin, and SP600125 were purchased from Calbiochem (La Jolla, CA). Nrf2 (SC-37049) and PKC-δ (SC-36253) small interfering RNAs (siRNAs) were obtained from Santa Cruz Biotechnology, and p38 (#6564) siRNA was purchased from Cell Signaling Technology. All other chemicals and reagents were of analytical grade.

CELL CULTURE

We purchased HUVECs from StemCell Technologies (Vancouver, Canada). Briefly, cells were cultured in M199 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml human fibroblast growth factor, and 5 U/ml heparin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and passaged every 2–3 days. HUVECs were cultured to approximately 80% confluency, and further incubated with fresh medium containing the above reagents. Cells were used within passages 4–9 for all the experiments.

WESTERN BLOT ANALYSIS

After treatment, cells were washed with phosphate-buffered saline (PBS) and mixed with RIPA buffer containing 1 mM EDTA, 5 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF), followed by centrifugation at 14,000 g for 15 min. Cell lysates separated by polyacrylamide gel electrophoresis were transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Boston, MA) at 100 V for 1 h. The blots were incubated for 1 h at room temperature with a monoclonal antibody against human HO-1 or a monoclonal antibody against GAPDH. Horseradish peroxidase-conjugated anti-IgG antibodies were used as the secondary antibody for detection of HO-1 and GAPDH protein bands by enhanced chemiluminescence WESTSAVE Up™ (AbFrontier, Seoul, Korea).

RNA ISOLATION AND REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Total RNA was isolated from HUVECs using TRIzol®. A total of 1 μg of RNA was used for the cDNA synthesis using random primers. Reverse transcription was performed with 200 U/μl M-MLV reverse transcriptase, 10 mM dNTPs, 5× reaction buffer, 0.02 μg/μl oligo (dT) primers, and 40 U/μl RNase inhibitor at 42°C for 1 h. The reaction was stopped at 94°C for 3 min, and the cDNA products were stored at −20°C. PCR was performed using synthesized cDNA as a template and specific primers for HO-1 or GAPDH as a loading control. The primer sequence for human HO-1 was 5′-ACATCTATGTGGCCCTGGAG-3′ (forward) and 5′-TGTTGGGGAAGGTGAAGAAG-3′ (reverse). Amplified products were resolved by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

ASSAY FOR HO ACTIVITY

HO enzyme activity was measured as described previously [Lee et al., 2010b]. Briefly, microsomes from harvested cells were added to a reaction mixture containing NADPH, rat liver cytosol as a source of

biliverdin reductase, and the substrate hemin. The reaction was carried out in the dark for 1 h at 37°C, and the amount of extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm.

NRF2, p38, AND PKC-δ SILENCING BY siRNA

HUVECs were plated in six-well plates at a density of 2.0×10^5 cells/well and transfected with PKC-δ, p38, or Nrf2-siRNA or scrambled siRNA for 18 h. For each transfection, 1,200 μl of the transfection medium was added to 0.25–1 μg or 10–30 nM of the siRNA duplex/transfection reagent mix (TransPass R2 solution A + B), and the entire volume was added gently to the cells.

IMMUNOFLUORESCENCE STAINING

HUVECs were cultured in a glass culture chamber slide (Falcon Plastics Inc., London Ontario, Canada), and processed for histological and immunofluorescence analyses. Cells were fixed, permeabilized, and stained using each antibody (rabbit monoclonal anti-HO-1 antibody at a 1:500 dilution or polyclonal rabbit anti-Nrf2 antibody at a 1:200 dilution in PBS containing 1% bovine serum albumin). The images were observed under a fluorescence microscope (Eclipse 50i; Nikon, Japan) and a laser scanning confocal microscope (LSM510 META; Zeiss, Oberkochen, Germany).

PREPARATION OF NUCLEAR PROTEINS

After treatment with fisetin for 4 h, the cells were washed with PBS and centrifuged at 3,300 g for 5 min at 4°C. Pellets were resuspended in ice-cold hypotonic buffer A [10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT)], and a protease inhibitor cocktail containing 0.3 μM aprotinin, 0.5 mM PMSF, and 2 mM leupeptin. Cells were incubated for 15 min on ice, vortexed vigorously for 10 s, and recentrifuged at 7,000 g for 2 min at 4°C. Pellets were resuspended in ice-cold buffer B containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, and protease inhibitors, followed by incubation at 4°C for 30 min with periodic vortexing. The mixture was centrifuged at 12,000 g for 30 min at 4°C. The supernatant was collected and stored at −70°C for protein assay and Western blot analyses.

MEASUREMENT OF PROMOTER ACTIVITY

ARE-luciferase (ARE-Luc) reporter plasmid was a generous gift from Dr. Park, R. K. (Wonkwang University, Korea). HEK 293 cells at 60% confluency were transfected with plasmids using ExGen™ 500 reagent (Fermentas, Hanover, MD). Briefly, a transfection complex containing 250 ng of ARE-luciferase plasmid DNA, 200 ng of pcDNA3-β-gal, and 3.3 μl of ExGen™ 500 was added to the cells [Lee et al., 2010a]. After 24 h of transfection, cells were treated with various concentrations of fisetin for 16 h. Cells were lysed using reporter lysis buffer (Promega, Madison, WI), and luciferase activities were measured using the luciferase assay system (Promega). The luciferase activity was normalized to β-gal activity (#75705; Pierce, Rockford, IL).

DETECTION OF CELL DEATH

Cell death was detected by annexin V-fluorescein isothiocyanate (annexin V-FITC; BD PharMingen, San Diego, CA) and propidium iodide (PI) staining of necrotic and apoptotic cells. Cells were washed in PBS, resuspended in 100 μl of binding buffer containing 5 μl of annexin V-FITC and 1 μg/ml PI, and incubated for 10 min at room temperature (RT) in the dark. Cells were analyzed using a FACScan (Becton Dickinson, San Jose, CA). Data were analyzed using CELLQuest software (Becton Dickinson).

TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE-MEDIATED dUTP NICK END-LABELING (TUNEL) ASSAY

To measure DNA fragmentation, the commercially available in situ death detection kit (Roche Diagnostics, Mannheim, Germany) was utilized. HUVECs were cultured in a glass culture chamber slide and fixed for 30 min in 10% neutral buffered formalin solution at room temperature. A TUNEL assay system was used, according to the manufacturer's instructions, for examination under a fluorescence microscope (Eclipse 50i; Nikon, Japan), with excitation at 488 nm and emission at 525 nm.

STATISTICAL ANALYSIS

Statistical significance was evaluated with student's *t*-test or estimated by ANOVA followed by Bonferroni's test. The results are expressed as mean ± standard deviation (SD).

RESULTS

FISETIN INDUCES HO-1 EXPRESSION AND ACTIVITY IN A CONCENTRATION- AND TIME-DEPENDENT MANNER

Induction of HO-1 expression is responsible for protecting cells against toxicity caused by oxidative insults. Therefore, we investigated whether fisetin altered the expression of HO-1 in HUVECs. Treatment of HUVECs with fisetin for 16 h induced a concentration-dependent enhancement in HO-1 protein expression (Fig. 2A). Additionally, treatment with 10 μM fisetin increased HO-1 protein expression in a time-dependent manner (Fig. 2B). Cells treated with fisetin showed concentration- and time-dependent increases in HO-1 mRNA expression (Fig. 2C,D). After treatment with 5 and 10 μM fisetin, HO-1 localization was determined in fixed cells by immunofluorescence staining with an anti-HO-1 antibody followed by a fluorescence-tagged secondary antibody. Immunofluorescence analysis revealed that HO-1 protein levels were significantly increased in HUVECs treated with 5 and 10 μM fisetin [Fig. 3A (control) versus Fig. 3B,C (fisetin-treated cells)].

We further examined the effects of fisetin on HO-1 activity by determining HO-1 activity in fisetin-treated HUVECs. Exposure of HUVECs to various concentration of fisetin for 16 h resulted in significantly increased HO-1 activity compared to the control cells (Fig. 3D) (untreated cells, $^*P < 0.05$), confirming that up-regulation of HO-1 mRNA and protein is accompanied by increased HO-1 activity in fisetin-stimulated HUVECs.

INVOLVEMENT OF PKC-δ AND MAPK SIGNALING PATHWAYS IN FISETIN-INDUCED HO-1 EXPRESSION

Several signaling pathways are involved in the induction of HO-1 expression [Lee et al., 2009]. To determine the upstream signaling pathway involved in fisetin-mediated HO-1 induction, we examined the effects of specific inhibitors of several pathways (MAPKs, PI3K,

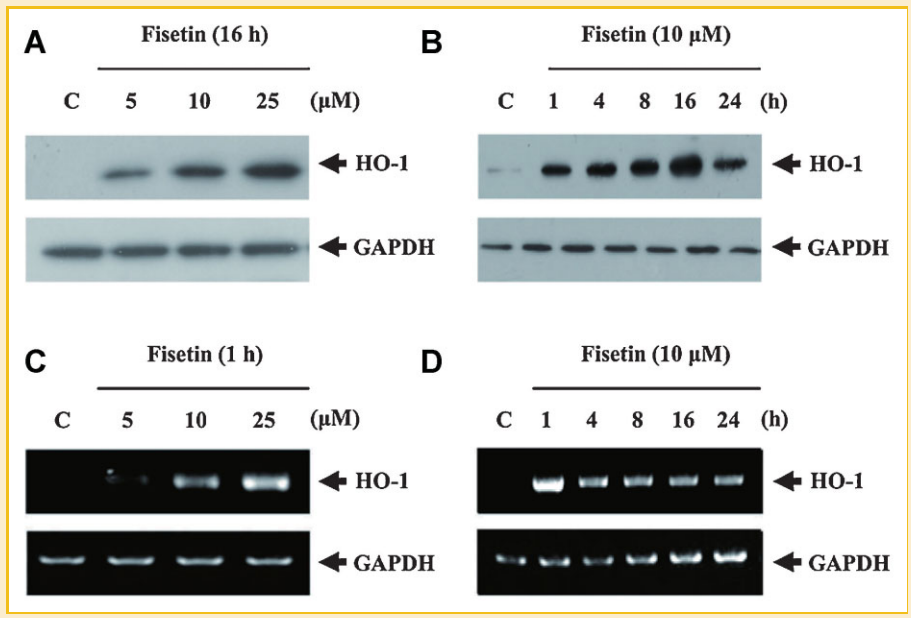


Fig. 2. Up-regulation of HO-1 by fisetin in HUVECs. Fisetin increased HO-1 protein levels in a dose-dependent manner (A) and in a time-dependent manner (B), cells lysates were prepared, and protein samples (20 μg) were subjected to Western blot by using anti-HO-1 antibody and anti-GAPDH antibody (loading control). RT-PCR analysis of mRNA transcripts of HO-1 induced in HUVECs exposed to various concentration of fisetin (C) and different time intervals (D). Total RNA was extracted and analyzed by RT-PCR and the amplified RT-PCR product was visualized on a 1.5% agarose gel.

PKC), on HO-1 expression. Among them, inhibitors of the PKC-δ and p38 pathways significantly reduced fisetin-induced HO-1 protein and mRNA expression (Fig. 4A,B). We confirmed the involvement of PKC-δ and p38 signaling in fisetin-induced HO-1 expression by using specific siRNAs for PKC-δ and p38. PKC-δ and p38 proteins were decreased after silencing with PKC-δ and p38 siRNA compared to negative controls (Fig. 4C,D). PKC-δ and p38 siRNA abolished fisetin-mediated induction of HO-1 protein (Fig. 4E,F), indicating that PKC-δ and p38 signaling play a role in fisetin-mediated HO-1 induction.

FISETIN-INDUCED EXPRESSION OF HO-1 IS MEDIATED BY Nrf2

Nrf2 is a redox-sensitive basic-leucine zipper transcription factor that translocates to the nucleus in response to oxidative or electrophilic stress. The induction of phase II detoxifying and antioxidant enzymes, including HO-1, is regulated by the Nrf2/Keap1 transcription factor system [Kobayashi and Yamamoto, 2005]. Therefore, we assessed whether fisetin could activate Nrf2 in association with HO-1 up-regulation. To determine whether fisetin stimulates Nrf2 translocation in HUVECs, cells were treated with various concentrations of fisetin for 4 h, and the nuclear fractions were extracted for preparation of nuclear proteins. Nrf2 proteins in the cellular nuclear compartments were detected by Western blot. Treatment with fisetin resulted in a concentration-dependent increase in the nuclear levels of Nrf2 (Fig. 5A). To further evaluate the fisetin-induced nuclear translocation of Nrf2, an immunofluorescence assay was used to detect the distribution of Nrf2 in cells before and after fisetin treatment. Nrf2 fluorescence was evenly distributed throughout the cytoplasm and nucleus of untreated cells. After treatment with 10 μM fisetin for 4 h, Nrf2 fluorescence was primarily concentrated in the nuclei (Fig. 5C).

We also examined the effect of Nrf2 siRNA transfection on fisetin-induced HO-1 expression. Nrf2 proteins decreased after silencing with Nrf2 siRNA compared to negative controls in total cell lysates (data not shown). The fisetin-induced increase in HO-1 protein was abolished by transfection with Nrf2 siRNA (Fig. 5B), suggesting that fisetin-mediated induction of HO-1 expression requires activation of Nrf2.

The enhancer region of the majority of genes that encode phase II detoxifying and antioxidant enzymes, including HO-1, contain an ARE sequence, and several studies have demonstrated that the Nrf2 transcription factor has a major role in the regulation of ARE-derived expression of target genes [Huang et al., 2002]. Thus, we also assessed ARE promoter activity in fisetin-treated cells. Cells were transiently transfected with an ARE luciferase reporter plasmid, treated with various concentrations of fisetin for 16 h, and luciferase activity was determined. As expected, treatment of cells with fisetin markedly increased expression of the ARE-Luc reporter gene in a concentration-dependent manner (Fig. 6, $^{*}P < 0.01$). This data clearly supports the hypothesis that Nrf2-ARE is an important transcription factor responsible for fisetin-mediated HO-1 induction.

PHARMACOLOGICAL INHIBITION OF HO-1 INCREASES CELL DEATH IN HYDROGEN PEROXIDE-STIMULATED HUVECs

A recent study suggested that HO-1 has the cytoprotective effect of inhibiting apoptosis in the failing heart [Wang et al., 2010]. To further determine whether the increased level of HO-1 activity enhanced by fisetin confers cytoprotection against oxidative stress,

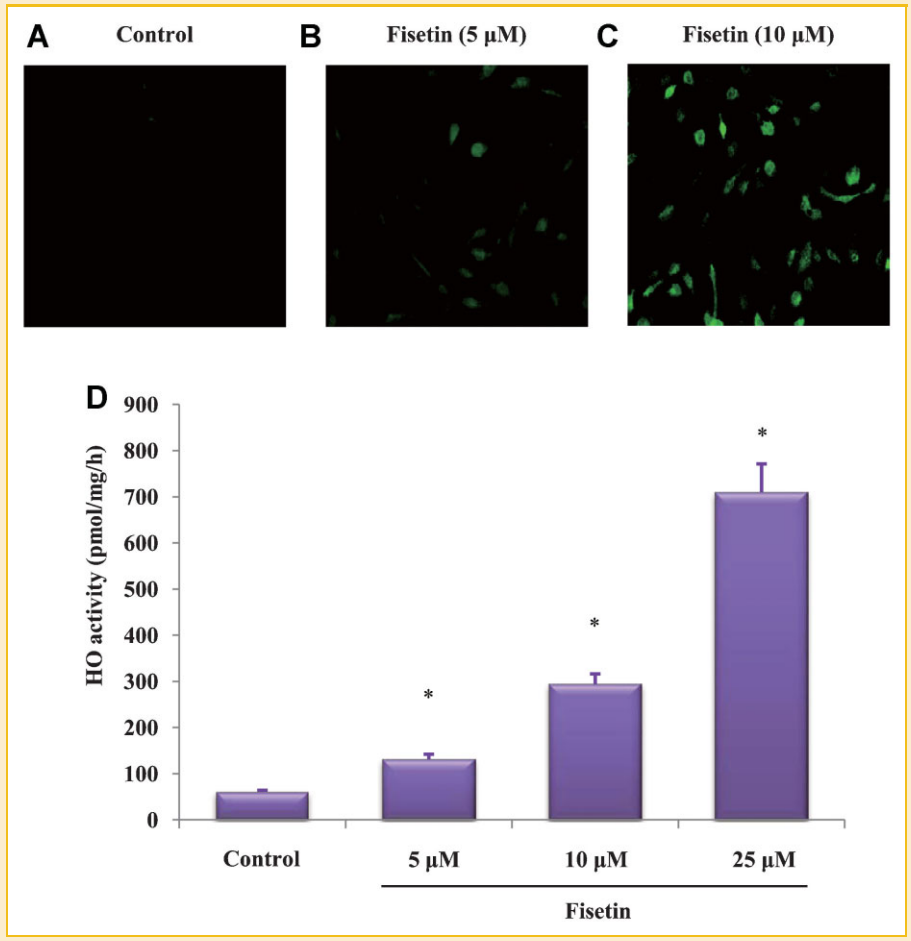


Fig. 3. Induction of HO-1 expression in fisetin-stimulated HUVECs. Immunofluorescence staining for HO-1 showed increased expression of HO-1 in HUVECs treated for 16 h with 5 and 10 μM fisetin compared to that in the non-treated HUVECs (A–C). Cells were fixed, and HO-1 localization was determined by immunofluorescence staining with an anti-HO-1 antibody followed by a fluorescence-tagged secondary antibody. HO-1 activity was measured in HUVECs 16 h after treatment with various concentrations of fisetin (D). Each bar represents the mean ± SD of three independent experiments. $^{*}P < 0.05$ versus control.

HUVECs were pretreated with a specific HO-1 inhibitor, ZnPP. Hydrogen peroxide (H_2O_2)-stimulated cells were pre-incubated with or without 1 μM ZnPP and fisetin and cell death was detected by FACS analysis after 16 h. Annexin V/PI staining followed by FACS analysis showed that while there was minimal spontaneous death of cells in untreated cultures, ZnPP-pretreated cells displayed increased cell death following treatment with fisetin and H_2O_2, compared to cells treated with fisetin and H_2O_2 alone (Fig. 7A). To confirm this data, we assessed the presence of dead cells by in situ terminal nick end-labeling (TUNEL staining), which is widely used in detecting DNA fragmentation in situ. This histochemical technique detects the appearance of intensely stained nuclei, which indicates incorporation of labeled dUTP into the 3′-end of fragmented DNA derived from apoptotic nuclei. H_2O_2 (300 μM) treatment significantly increased the proportion of TUNEL-positive cells, which was reduced by fisetin treatment. Fisetin-induced reduction of the proportion of TUNEL-positive cells was abrogated by pretreatment with ZnPP (Fig. 7B). These results demonstrated that the cytoprotective effect of fisetin is presumably mediated by induction of HO-1.

DISCUSSION

Flavonoids that are able to scavenge free radicals and protect cells from oxidative damage have recently attracted attention. Accumulating evidence has confirmed the cytoprotective effect and molecular mechanisms of several flavonoids, such as baicalein, quercetin, and kaempferol [Lin et al., 2003; Hirose et al., 2009]. Several studies suggested that fisetin can serve as a pharmaceuticals or nutraceuticals or functional food for patients with various diseases [Lian et al., 2008; Geraets et al., 2009]. However, the effects and mechanisms of HO-1 induction by fisetin have not been extensively investigated. In this study, we demonstrated that the flavonoid fisetin induced HO-1 protein expression via PKC-δ-p38-Nrf2-dependent pathways in endothelial cells.

Numerous studies have indicated that the induction of phase II enzymes by natural or synthetic compounds, including flavonoids, is sufficient for the acquisition of antioxidant and protective activities [Wagner et al., 2008; Kim et al., 2009]. HO-1, a phase II cytoprotective enzyme and stress-inducible protein, is widely distributed in mammalian tissues. The antioxidant and anti-

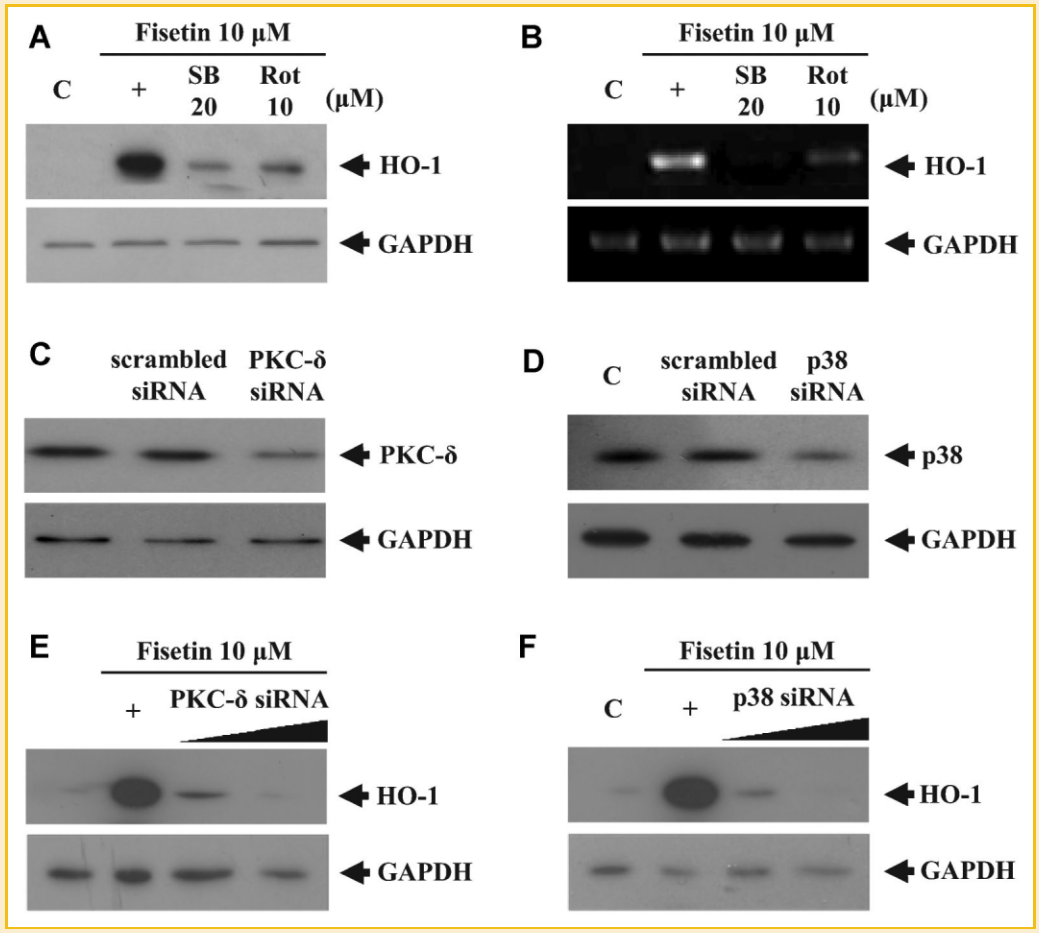


Fig. 4. Involvement of PKC-δ and p38 signaling pathways in fisetin-induced HO-1 expression. Cells were pretreated with SB 203580 (p38 inhibitor) or Rot (PKC-δ inhibitor) for 1 h, followed by incubation with 10 μM fisetin for 16 h. Whole cell lysates were prepared and subjected to Western blot analysis with antibodies against anti-HO-1 and GAPDH, as indicated (A). HUVECs were pretreated with SB 203580 or Rot at the indicated concentrations for 1 h, followed by incubation with 10 μM of fisetin for 1 h. Total RNA was prepared and subjected to RT-PCR for HO-1 and GAPDH (B). Transient transfection of HUVECs with PKC-δ or p38 siRNA inhibited PKC-δ or p38 protein expression (C,D). PKC-δ or p38 siRNA (20 and 30 nM) abrogated up-regulation of fisetin-induced HO-1 protein (E,F). Representative Western blots of three independent experiments are shown. +, fisetin alone treated group.

inflammatory effects of HO-1 result in cytoprotective actions in the tissues where it is expressed, including a variety of pathological models [Nath et al., 1992; Choi and Alam, 1996; Ohnishi et al., 2010].

Protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), ER-localized pancreatic endoplasmic reticulum kinase (PERK), and MAPK are reportedly involved in HO-1 expression and in Nrf2-dependent transcription [Bloom and Jaiswal, 2003; Cullinan and Diehl, 2004]. Several lines of evidence have demonstrated that multiple kinase signaling pathways are involved in induction of HO-1 expression. PKC-δ and MAPK play a key role in the activation of Nrf2 in association with HO-1 expression [Rushworth et al., 2005]. In human monocytes, curcumin induces HO-1 expression by activation ARE via PKC-δ, p38 and Nrf2 pathways [Rushworth et al., 2006], whereas 15-deoxy-Δ12, 14-prostaglandin J2 induces HO-1 expression via the ERK and Akt/PI3K pathway but not the p38 and JNK pathway [Kim et al., 2008]. Therefore, signaling mechanisms involved in HO-1 induction may depend on cell types and inducers. Our results revealed that the PKC-δ and p38 pathways are required for fisetin-induced expression of HO-1 and accumulation of Nrf2 in the nucleus (Fig. 4). In addition, the effect of Nrf2 siRNA on HO-1 supports the notion that HO-1 is strongly associated with the Nrf2 transcription factor (Fig. 5). ARE is a regulatory sequence that participates in the coordinated transcriptional activation of genes encoding various antioxidant enzymes and phase II detoxifying enzymes such as HO-1. Enhanced expression of the ARE-Luc reporter gene by fisetin indicates that fisetin can stimulate ARE transcriptional activity (Fig. 6). Taken together, these results imply that fisetin-induced HO-1 expression requires activation of Nrf2-ARE in HUVECs. Keap1/Nrf2/ARE signaling is thought to play a significant role in protecting cells from endogenous and exogenous stress [Kensler et al., 2007].

Numerous studies have suggested that induction of HO-1 expression prevents oxidative stress-induced cell death [Jang et al., 2009; Morse et al., 2009]. We hypothesized that induction of HO-1 by fisetin would have cytoprotective effects against oxidative stress that would result in suppression of cell death in H_2O_2-stimulated HUVECs. In this study, we examined whether

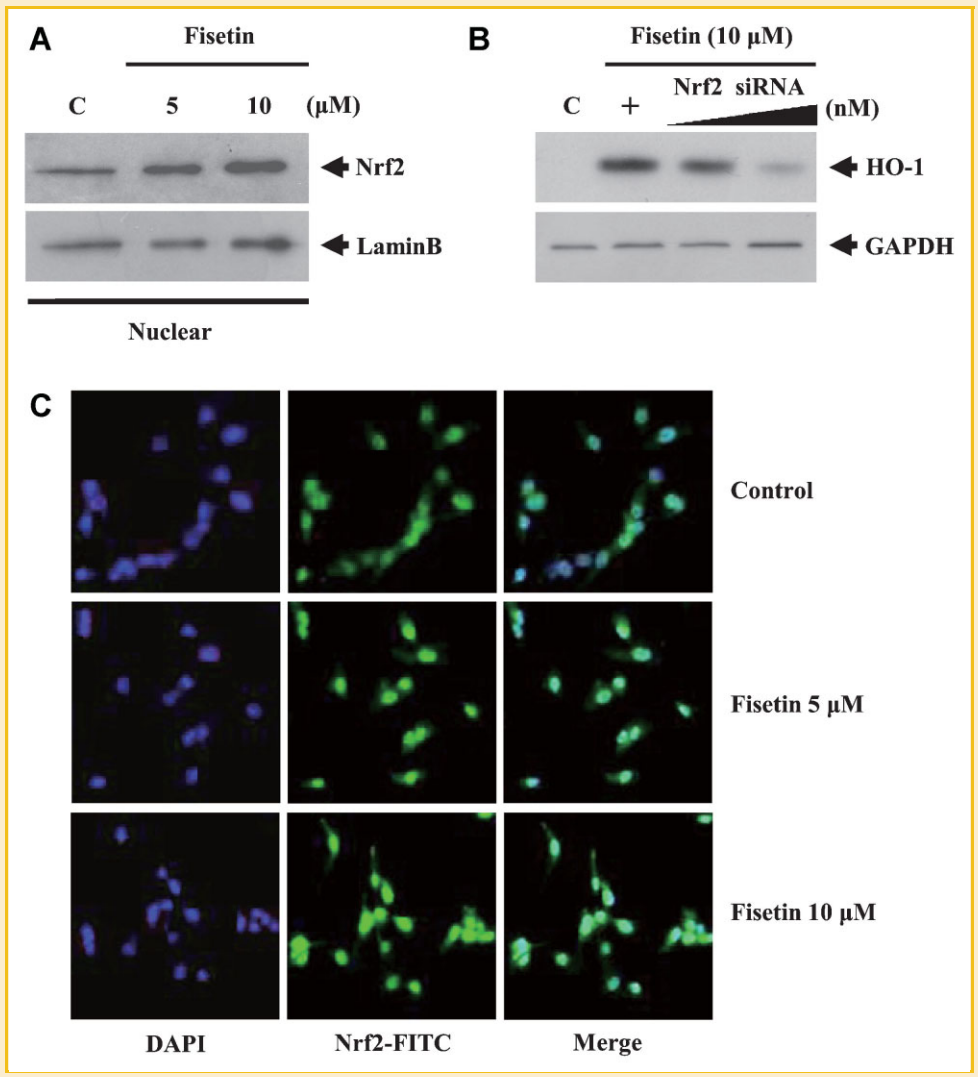
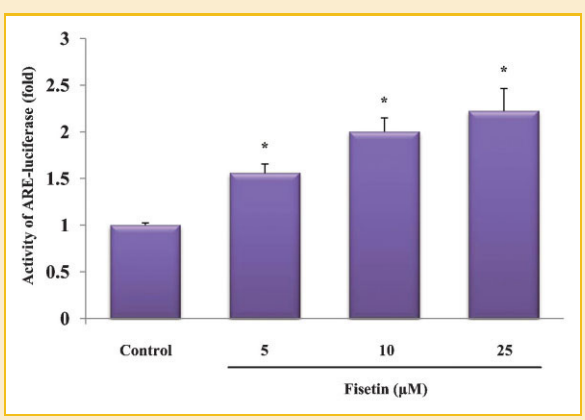


Fig. 5. Fisetin-induced expression of HO-1 is mediated by Nrf2. Cells were treated with fisetin at the indicated concentrations for 6 h. Nuclear extracts were prepared, and protein samples (40 μg) were subjected to Western blotting by using an anti-Nrf2 antibody or an anti-Lamin B (a nuclear protein marker) antibody (A). Transient transfection of HUVECs with Nrf2 siRNA inhibited expression of the HO-1 protein. Nrf2 siRNA abrogated up-regulation of fisetin-induced HO-1 protein (B). Cells were stained with DAPI and an anti-Nrf2 antibody, and immunofluorescence was observed under a confocal microscope (C). +, fisetin alone treated group.



suppression of fisetin-mediated HO-1 expression by treatment with the HO-1 specific inhibitor ZnPP increased cell death in H_2O_2-stimulated HUVECs (Fig. 7). We observed that cell death increased in ZnPP-treated cells compared to cells treated with fisetin alone, indicating that fisetin-mediated HO-1 expression serves as a mechanism for protecting cells from oxidative stress.

Induction of HO-1 is an important mechanism for cellular protection against oxidative stress, and investigation of the

Fig. 6. Activation of ARE-Luc reporter by fisetin. Cells transfected with an ARE-luciferase construct were treated with various concentrations of fisetin for 16 h, and the lysates were mixed with a luciferase substrate. A luminometer was used to measure luciferase activity. Data represent the mean ± SD of four independent experiments. $^{*}P < 0.001$ versus control.

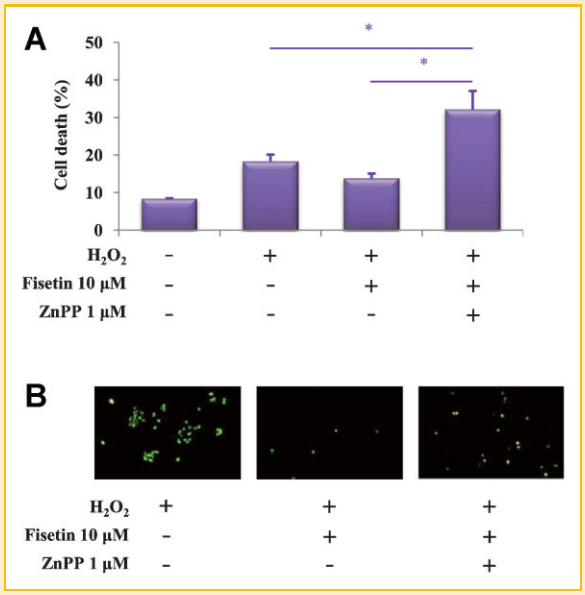


Fig. 7. Effect of fisetin-induced HO-1 inhibition on cell death in H_2O_2-stimulated HUVECs. Cells were incubated in the absence or presence of ZnPP for 16 h before the indicated tests were performed. H_2O_2-stimulated HUVECs were pretreated for 1 h with 1 μM ZnPP and then treated with 10 μM fisetin. Quantitative data are presented as the percentage of dead cells after treatment with annexin V/PI staining (A). Results represent three independent experiments. $^*P < 0.05$ versus control. Protective effect of fisetin on H_2O_2-induced cell death in HUVECs as determined by in situ terminal nick end-labeling (TUNEL) (B).

molecular mechanism of fisetin-mediated HO-1 induction will suggest strategies for consumption and treatment of flavonoids. Herein, we demonstrated that fisetin, which is a flavonoid in many fruits and vegetables, induced expression of HO-1 in HUVECs through activation of the PKC-δ, p38, and Nrf2-ARE signaling pathways. Our results also strongly support our hypothesis that HO-1 expression induced by flavonoids such as fisetin protects against oxidative stress-induced cell death in endothelial cells. This study provides biological evidence that a potential therapeutic mechanism of fisetin is its ability to protect against oxidative stress-related diseases such as atherosclerosis.

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