International Immunopharmacology 75 (2019) 105769

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Naringenin-induced HO-1 ameliorates high glucose or free fatty acids-associated apoptosis via PI3K and JNK/Nrf2 pathways in human umbilical vein endothelial cells

Jian Feng[a,b,1], Jing Luo[a,1], Li Deng[c], Yi Zhong[a], Xing Wen[a], Ying Cai[a], Jiafu Li[a,b,*]

[a] Department of Cardiology, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan, People's Republic of China
[b] Key Laboratory of Medical Electrophysiology, Ministry of Education, Institute of Cardiovascular Research of Southwest Medical University, Luzhou, Sichuan, People's Republic of China
[c] Department of Rheumatology, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan, People's Republic of China

ARTICLE INFO

Keywords:
Naringenin
Endothelial cells
Heme oxygenase-1
NF-E2-related factor 2
Phosphatidylinositol 3-kinase
C-Jun N-terminal kinase

ABSTRACT

Naringenin (NG), a flavanone extracted from various plants, has potent vasoprotective effects likely related to the induction of heme oxygenase-1 (HO-1). In the current study, we investigated mechanisms underlying the effect of NG on HO-1 expression and high glucose (HG)- or free fatty acids (FFA)-induced apoptosis in human umbilical vein endothelial cells (HUVECs). First, we found that HUVECs exposed to NG exhibited enhanced HO-1 expression in a concentration- and time-dependent manner. Moreover, HUVECs treated with NG exhibited activation of phosphoinositide 3 kinase (PI3K)/Akt, extracellular-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK). LY294002 (a PI3K inhibitor) and SP600125 (a JNK inhibitor) reduced NG-induced HO-1 expression, whereas BIRB796 (a p38 inhibitor) and PD98059 (an ERK inhibitor) had no effect. The cytoprotective effects of NG were correlated with activation of the transcription factor NF-E2-related factor 2 (Nrf2), a critical regulator of HO-1 expression. Indeed, the results of our experiments using LY294002 and SP600125 indicated that NG may stimulate Nrf2 through PI3K/Akt and JNK pathway activation. Moreover, treatment of HUVECs with Nrf2 siRNA decreased NG-induced HO-1 expression. Finally, pretreatment of HUVECs with NG remarkably reduced HG- or FFA-induced cell apoptosis, and this effect was greatly abrogated in the presence of SnPP (an HO-1 inhibitor). Above all, our data show that NG increased HO-1 expression and reduced HG- or FFA-induced cell apoptosis in HUVECs by upregulating PI3K, JNK, and Nrf2 pathways, which may confer an adaptive survival response in diabetes-induced vascular injury.

1. Introduction

Usually arising from structural and functional abnormalities of endothelium (including damage at the cellular level), endothelial dysfunction plays a critical part in the pathogenesis of various cardiovascular diseases [1]. A number of studies have shown that endothelial damage is an early indicator of diabetic macrovascular complications [2]. Diabetes mellitus is associated with long-term complications such as structural and functional abnormalities of heart, brain, and kidney, as well as increased risk for the development of cardiovascular diseases. Although the pathophysiological basis of these complications remains uncertain, hyperglycemia appears to play a central role [3]. Indeed, damage of vascular endothelial cells elicited by hyperglycemia impairs endothelial function, resulting in the initiation and propagation of further vascular complications [4]. Convincing evidence suggests that hyperglycemia-induced endothelial dysfunction is often caused by overproduction of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide radicals [5].

Heme oxygenase-1 (HO-1) is a representative cytoprotective enzyme that catalyzes the rate-limiting steps of heme degradation [6]. Multiple lines of evidence from both in vitro and in vivo systems have revealed that HO-1 can provide anti-oxidation and cytoprotection [7]. Moreover, compelling evidence indicates that HO-1 can protect the vasculature against remodeling and endothelial dysfunction [8]. Yang et al. [9] demonstrated an anti-oxidant role of HO-1 in vitro for reducing endogenous ROS production in human umbilical vein endothelial cells (HUVECs) grown in serum from rats exposed to cigarette smoke. Subsequently, Chang et al. [10] highlighted the anti-inflammatory role

* Corresponding author at: Department of Cardiology, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan, People's Republic of China.
E-mail address: lijiafu198948@swmu.edu.cn (J. Li).
[1] These authors contributed equally to this paper.

https://doi.org/10.1016/j.intimp.2019.105769
Received 12 May 2019; Received in revised form 4 July 2019; Accepted 17 July 2019
Available online 25 July 2019
1567-5769/ © 2019 Elsevier B.V. All rights reserved.

of HO-1 in HUVECs treated with contrast media as an inflammatory model. They revealed that HO-1 induction reduced expression of intercellular cell adhesion molecule-1 and adhesion molecule receptors. Moreover, Abraham et al. [11] reported that HO-1 overexpression in human dermal microvessel endothelial cells prevented both the slowdown of cell cycle progression and apoptosis in response to high glucose (HG). Therefore, HO-1 may be a potential target for hyperglycemia-induced endothelial dysfunction.

Epidemiological studies revealed an association between increased consumption of dietary flavonoids and a reduced risk of many human diseases, such as cardiovascular diseases, cancer, and chronic inflammation [12]. Naringenin (NG), an active flavanone extracted from citrus fruits and tomatoes, reportedly exhibits a wide range of biological properties including anti-inflammatory, antioxidant, antibacterial, and anti-apoptotic activities [13]. With regard to its pharmacokinetic properties, orally administered NG is poorly absorbed by the human gastrointestinal tract, with only 15% oral bioavailability [14] and an elimination half-life of 2.31 ± 0.40 h [15]. After absorption in humans and rats, NG is mainly metabolized into 7- or 4′-glucuronides by uridine 5′-diphospho-glucuronosyltransferase and then excreted into the urine within 5 days (in humans) [16].

Previous studies reported that NG attenuates obesity and leads to prevention of dyslipidemia, insulin resistance, and atherosclerosis [17,18]. Although many studies have shown that NG can induce HO-1 expression in spleen, pancreatic, and pulmonary tissues, as well as SH-SY5Y cells [19–21], whether HO-1 is involved in the vascular endothelial protective effect of NG and, if so, the related molecular mechanisms remain unknown. Therefore, objectives of the present study were to investigate the underlying mechanism by which NG affects HO-1 expression and its role in the protective effect of NG against HG- or free fatty acids (FFA)-induced apoptosis in HUVECs.

2. Materials and methods

2.1. Reagents

NG (powder, purity: ≥98%, HPLC) extracted from citrus was purchased from Meilun Biotechnology (Dalian, China). The NG was endotoxin-free. Dulbecco's Modified Eagle Medium (DMEM) was obtained from HyClone (Logan, UT). Fetal bovine serum (FBS) was purchased from Biological Industries (Cromwell, CT), whereas antibiotic solutions (100 U/mL penicillin, 100 μg/mL streptomycin) were obtained from Gibco (Grand Island, NY). Three-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and antibodies against HO-1, NF-E2-related factor 2 (Nrf2), Akt, phospho-Akt, c-Jun N-terminal kinase (JNK), phospho-JNK, extracellular-related kinase (ERK), phospho-ERK, p38 mitogen-activated protein kinase (p38 MAPK), and phospho-p38 MAPK were acquired from Abcam (Cambridge, UK). All secondary antibodies were supplied by Cell Signaling Technology (Danvers, MA). LY294002, SP600125, PD98059, and BIRB796 were obtained from Sigma (St. Louis, MO). SnPP, Control siRNA, and Nrf2 siRNA were obtained from Santa Cruz Biotechnology (Dallas, TX). An Annexin-V-fluorescein isothiocyanate (FITC) apoptosis analysis kit was obtained from Sungene Biotech (Tianjing, China).

2.2. Cell culture and treatment

HUVECs originally purchased from American Type Culture Collection were kindly provided by Dr. Fang Xie (Department of Oncology, The Affiliated Hospital of Southwest Medical University, Luzhou, China). Cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were allowed to reach 60%–80% confluency at 37 °C under an atmosphere of 5% CO_2 before performing experiments. Cells were treated with concentrations of NG ranging from 0 to 100 μM in culture medium. For HG treatment, a concentration of 33 mM glucose is commonly used to induce endothelial cell activation, thus mimicking diabetic conditions [11,22]. Therefore, cells were treated with 33 mM HG for 24 h in the presence or absence of NG. Controls were exposed to NG in DMEM containing a normal glucose concentration (5.5 mM). FFA was prepared as a mixture of oleate and palmitate (2:1; Sigma), as described in a previous study [23]. Cells were exposed to 1-mM FFA for 72 h in the presence or absence of NG. At the end of the exposure time, cells were immediately processed for further analysis as described below.

2.3. Measurement of cell viability

MTT is used as a conventional indicator of cell viability by determining its mitochondrial-dependent reduction to formazan [24]. Briefly, HUVECs were seeded into 96-well plates at a density of 3000 cells/well and final volume of 100 μL, and then incubated for 24 h. Cells were subsequently treated with various concentrations of chemicals and evaluated for viability at different time points, as indicated. After these experimental procedures, 20 μL of MTT (5 mg/mL) was added to each well for an additional 4-h incubation at 37 °C in a CO_2 incubator. Intercellular reduction of soluble yellow MTT into insoluble purple formazan crystals was allowed to proceed. The medium was then removed and formazan crystals were solubilized in 150 μL of dimethyl sulfoxide. Absorbance values were then measured at 490 nm using a microplate reader (Bio-Rad, Hercules, CA). Cell viability data are shown as a percentage of control.

2.4. Cytosolic and nuclear extract preparation

Cytosolic and nuclear extracts were prepared using a Nuclear and Cytoplasmic Extraction Reagent (Beyotime Biotechnology, Shanghai, China). Briefly, cells were washed with cold 1× phosphate-buffered saline (PBS) and lysed on ice in buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. After incubation in an ice bath for 15 min, cells were centrifuged at 12,000 ×*g* for 5 min at 4 °C. Next, the supernatant was harvested as the cytosolic extract, while pellets were resuspended in ice-cold buffer C [20 mM HEPES (pH 7.9), 20% glycerol, 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF] followed by incubation in an ice bath for 30 min. After vortexing, resulting suspensions were centrifuged at 12,000 ×*g* for 10 min at 4 °C, and supernatants were harvested as nuclear extracts. Proteins were stored at −80 °C, and protein contents were determined by bicinchoninic acid assay (Beyotime).

2.5. Western blot analysis

After treatment and/or co-treatment, cells were harvested, washed twice with cold 1× PBS, and lysed on ice. Equal amounts of protein from each sample were denatured in 5× SDS-PAGE reducing sample buffer and subjected to SDS-PAGE on 10% polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (Hybond-P PVDF; Amersham Bioscience, Little Chalfont, UK). After blocking with 5% dried skim milk in Tris-buffered saline containing Tween (TBST; 10 mM Tris, 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature, membranes were incubated with primary antibodies at 4 °C overnight. The following day, membranes were washed three times for 15 min each with TBST, and then incubated with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody at 37 °C for 60 min. Immunoreactive bands were visualized using an enhanced chemiluminescence western blotting detection kit (Amersham), and images were captured using a ChemiDoc XRS + Imaging System with Image Lab Software (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or histone was used as an internal control. Protein expression quantified by densitometry is reported as densitometric values of HO-1 or Nrf2 normalized to GAPDH or histone, respectively.

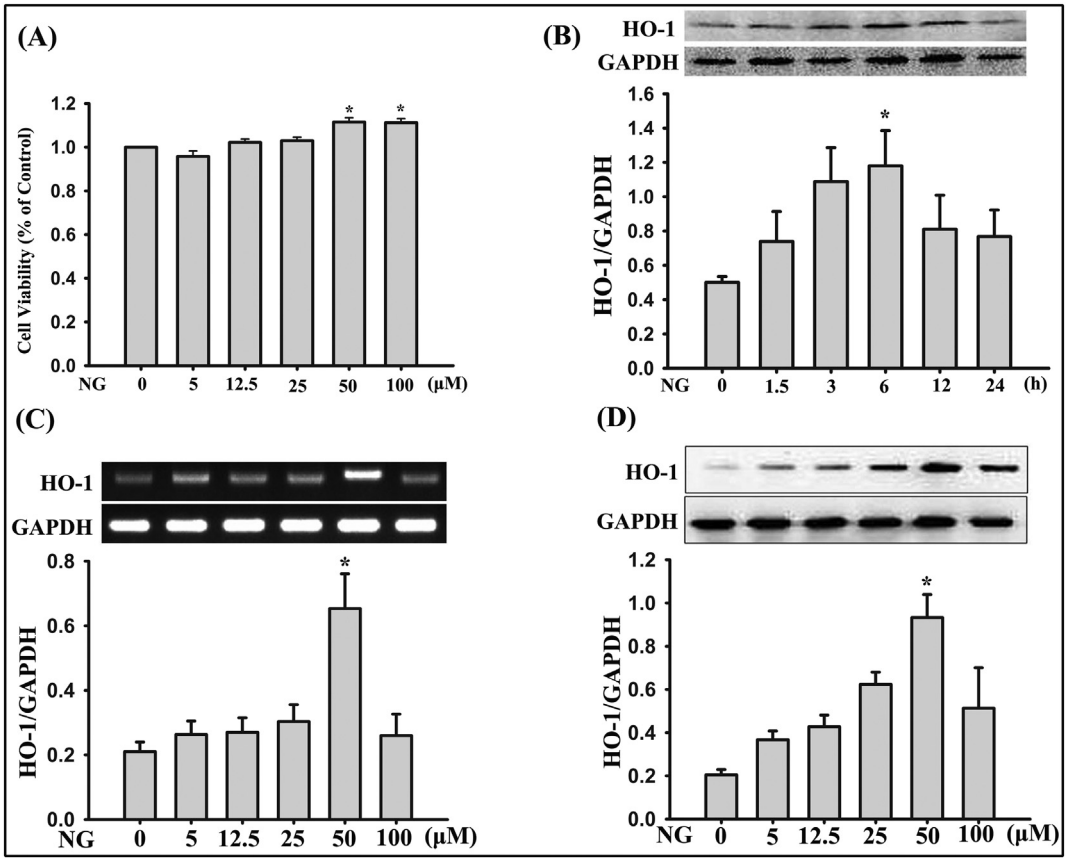

Fig. 1. NG induced HO-1 expression in HUVECs. (A) HUVECs were treated with various concentrations of NG for 24 h, and cell viability was evaluated by MTT assay. $^*P < 0.05$ versus 0 μM NG. (B) Western blot analysis of HO-1 and GAPDH expression in HUVECs treated with 25 μM NG for indicated times (0, 1.5, 3, 6, 12, or 24 h). Representative image from one of three independent experiments. $^*P < 0.05$ versus 0 h. (C, D) Cells were incubated with indicated concentrations of NG (0, 5, 12.5, 25, 50, or 100 μM) for 6 h and HO-1 expression was determined by RT-PCR or western blot analysis. GAPDH was used as a control for equal loading. $^*P < 0.05$ versus 0 μM NG.

2.6. Reverse transcriptase polymerase chain reaction (RT-PCR)

After experimental treatments, total RNA was isolated from HUVECs with TRIzol reagent (Ultrapure RNA Kit; Applied Biosystems, Foster City, CA) and reverse-transcribed using a ReverTra Ace® qPCR RT Master Mix kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol. After reverse transcription (RT), cDNA was amplified using a Maxime RT PreMix kit (Beyotime) and specific primers sets for target genes as follows: HO-1, forward, 5′-CTT TGA GGA GTT GCA GGA GC-3′, reverse, 5′-TGT AAG GAC CCA TCG GAG AA-3′; GAPDH, forward, 5′-CCA CTC CTC CAC CTT TG-3′, reverse, 5′-CAC CAC CCT GTT GCT GT-3′. All primers were synthesized from Sangon Biotech (Shanghai, China). PCR conditions for HO-1 were as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s; 54 °C for 30 s; and 72 °C for 60 s. Amplification products were resolved by 2% agarose gel electrophoresis, stained with ethidium bromide, and imaged with Quantity One Software (Bio-Rad). GAPDH was chosen as a housekeeping gene because it exhibited low variability in expression levels between different treatments. Relative mRNA expression of HO-1 was normalized to the amount of GAPDH.

2.7. Hoechst 33342/PI staining

Apoptotic cells were characterized by nuclear condensation of chromatin and/or nuclear fragmentation using a Hoechst 33342/PI staining kit from Solarbio Science & Technology (Beijing, China) according to the manufacturer's instructions. In brief, after indicated treatments, cells were fixed with 1 mL staining buffer. Subsequently, 5 μL of Hoechst 33342 and 5 μL of PI were added. The mixture was incubated at 4 °C or in an ice bath for 20–30 min. After incubation, cells were washed once with PBS and mounted onto slides for microscopy.

2.8. Transient transfection of small interfering RNA (siRNA)

Control siRNA (sc-37007) or Nrf2 siRNA (sc-37030) acquired from Santa Cruz Biotechnology were transfected into HUVECs using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. HUVECs were plated in six-well plates at density of 5000 cells/well in antibiotic-free normal growth medium and placed at 37 °C in a CO_2 incubator. After washing once with 2 mL of siRNA transfection medium, cells were incubated with 100 nM of target siRNA or control siRNA for 6 h at 37 °C in a CO_2 incubator. After incubation, the transfection medium was removed and replaced with fresh normal growth medium. Transfected cells were then incubated for an additional 48 h under normal culture conditions. Finally, transfected cells were treated with or without NG for 6 h.

2.9. Flow cytometry with Annexin V/PI double staining

Cell apoptosis was measured by flow cytometry. Briefly, HUVECs were trypsinized, harvested, washed twice with cold PBS, and centrifuged. Following removal of the supernatant, cells were resuspended in 1 mL of 1× banding buffer. For each sample, 100 μL of staining solution was added to the tube resuspend 1×10^5 cells. After gently vortexing and incubating samples for 10 min at room temperature (protected from light), 5 μL of Annexin V-FITC solution was added to

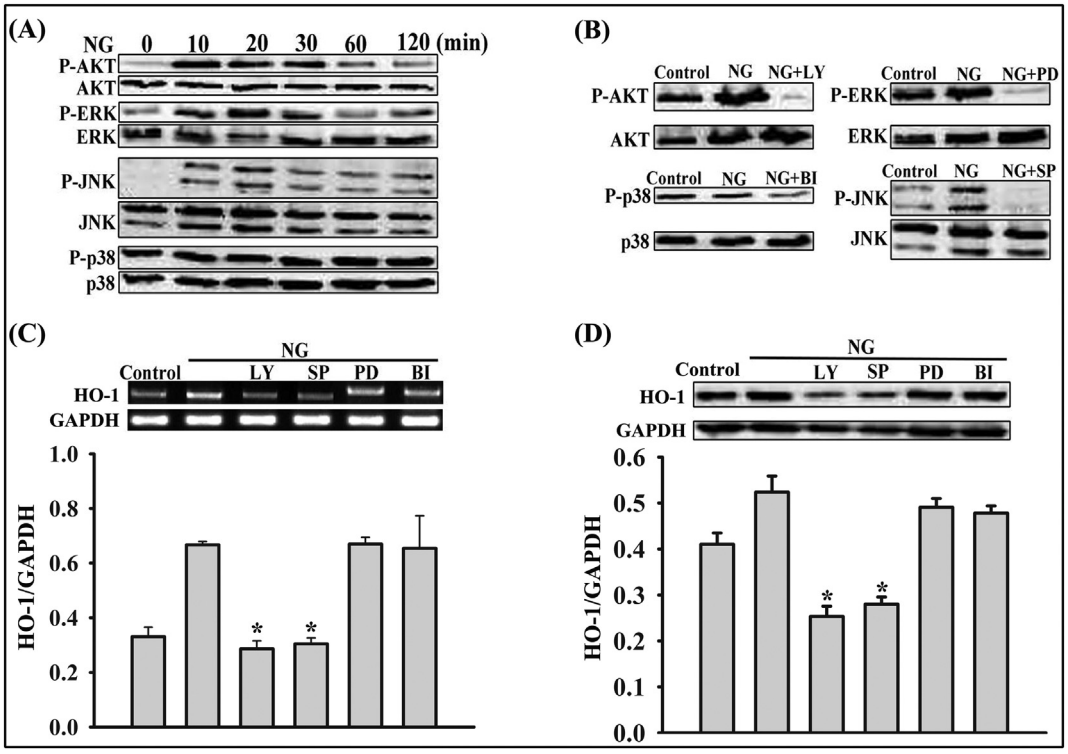

Fig. 2. Effect of NG on phosphorylation of Akt and MAPK signaling pathway elements in HUVECs. (A) Cells were treated with NG for indicated times and then P-Akt, Akt, P-ERK, ERK, P-JNK, JNK, P-p38, and p38 were detected using specific antibodies. Protein expression was analyzed by western blot analysis. (B) Effects of signaling inhibitors on signaling protein phosphorylation by NG. Cells were treated with 50 μM NG in the presence or absence of 30 μM LY294002 (LY), 10 μM SP600125 (SP), 40 μM PD98059 (PD), or 20 μM BIRB796 (BI). (C, D) Cells were preincubated with LY, SP, PD, or BI for 1 h and then treated with NG for an additional 6 h. HO-1 mRNA or protein expression was then examined by RT-PCR or western blot analysis, respectively. The results are expressed as mean ± SEM of three independent experiments. *$P < 0.05$ versus NG alone.

each tube. Next, 5 μL of PI solution was added to each tube for incubation at room temperature for 5 min (protected from light). Finally, 500 μL of PBS was added to each sample, which were gently vortexed and analyzed by flow cytometry within 1 h. FITC and PI fluorescence were detected using 520-nm and 636-nm band-pass filters and excitation at a 494-nm wavelength. Percentages of cells exhibiting a specific apoptosis were calculated by subtracting the percentage of spontaneously apoptotic cells in relevant controls from the total percentage of apoptotic cells in samples.

2.10. Statistics

All data are expressed as mean ± SEM. Statistical significance among multiple groups was assessed by analysis of variance (ANOVA) followed by Bonferroni post hoc test using SPSS 17 (IBM, Armonk, NY). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. NG induced HO-1 expression in HUVECs

We first assessed whether NG induced cytotoxicity in HUVECs with an MTT assay. Incubation of HUVECs with indicated concentrations of NG did not reduce cell viability (Fig. 1A). Next, we tested the effect of NG on HO-1 mRNA and protein expression in HUVECs. HUVECs treated with NG for the indicated time periods exhibited increasing HO-1 expression for 6 h in a time-dependent manner; thereafter, it decreased (Fig. 1B). Moreover, treatment of cells with NG at various concentrations for 6 h resulted in elevated expression of HO-1 mRNA and protein in a dose-dependent manner (Fig. 1C and D). These results suggest that NG enhanced HO-1 expression.

3.2. Effect of NG on phosphorylation of Akt and MAPK signaling pathway elements in HUVECs

Activation of several upstream kinases can reportedly induce nuclear translocation of Nrf2 and, subsequently, increase HO-1 expression [25,26]. To explore upstream signaling pathways responsible for NG-induced Nrf2 activation and upregulation of HO-1 expression, we examined the effect of NG on phosphorylation of Akt and MAPKs. Treatment with NG significantly increased phosphorylation of Akt, ERK, and JNK at both 10 min and 20 min, whereas protein expression of total p38 and phosphorylated p38 remained unchanged (Fig. 2A). LY294002 (a PI3K inhibitor), SP600125 (a JNK inhibitor), and PD98059 (an ERK inhibitor) suppressed NG-induced activation of Akt, JNK, and ERK, respectively (Fig. 2B). Next, we incubated HUVECs with LY294002, BIRB796, SP600125, or PD98059 for 1 h before treatment with NG to investigate the effect of each inhibitor on HO-1 expression. NG-induced changes in HO-1 mRNA and protein expression were reversed by LY294002 and SP600125 (Fig. 2C and D). These data suggest that PI3K/Akt and JNK are involved in NG-induced HO-1 upregulation in HUVECs.

3.3. Role of Akt and JNK in NG-induced Nrf2 activation in HUVECs

Nrf2 plays an important role in the induction of several cytoprotective phase II detoxifying enzymes, including HO-1 [27]. Therefore, we investigated whether Nrf2 is involved in NG-induced HO-1 expression in HUVECs. As shown by western blotting of nuclear extract fractions, nuclear translocation of Nrf2 increased in a dose- and time-dependent manner in cells exposed to various concentrations of NG for 4 h and 50 μM NG for different durations (Fig. 3A and B). Furthermore, as illustrated in Fig. 3C, pharmacological inhibition of Akt or JNK

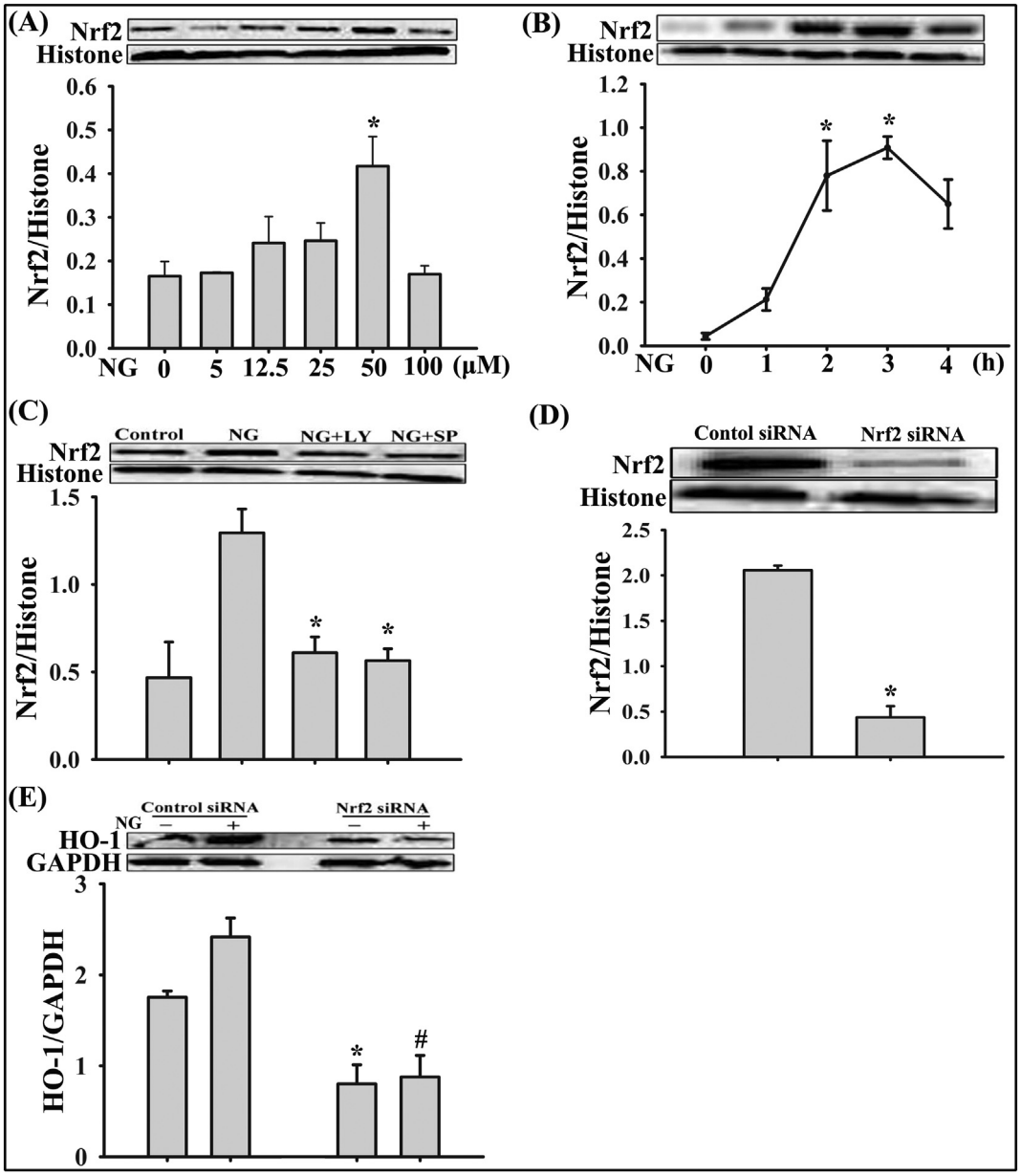

Fig. 3. Role of Akt and JNK in NG-induced Nrf2 activation in HUVECs. (A, B) HUVECs were pretreated with the indicated concentration of NG for 4 h, or NG (50 μM) for the indicated times. Protein levels of Nrf2 were determined by western blot analysis. Histone was used as internal controls. The experiment was repeated three times, and similar results were obtained. *$P < 0.05$ versus 0 μM (A) or 0 h (B). (C) Effects of Akt and JNK inhibitors on NG-induced Nrf2 activation. Nuclear extracts were prepared from HUVECs treated with NG in the presence or absence of LY or SP. *$P < 0.05$ versus NG. (D) HUVECs were transfected with Control siRNA or Nrf2 siRNA for 48 h, nuclear extracts were isolated, and levels of Nrf2 was detected by western blot analysis. *$P < 0.05$ versus Control siRNA. (E) After 48-h transfection, cells were treated with NG (50 μM) for 6 h and HO-1 protein expression was examined by western blot analysis. Data are expressed as mean ± SEM of three independent experiments. *$P < 0.05$ versus Control siRNA; $^{\#}P < 0.05$ versus Control siRNA + NG.

attenuated NG-induced nuclear localization of Nrf2, suggesting that PI3K/Akt and JNK are also upstream activators of Nrf2. HUVECs transiently transfected with Nrf2 siRNA exhibited significantly inhibited Nrf2 protein expression compared with controls (Fig. 3D). To confirm the requirement of Nrf2 for NG-induced HO-1 expression, NG-induced upregulation of HO-1 was significantly reduced by transfection with Nrf2-specific siRNA, whereas there was no change in HUVECs transfected with control siRNA (Fig. 3E). These results further suggest that NG induced HO-1 via activation of Akt/Nrf2 or JNK/Nrf2 pathways.

3.4. Effect of NG on HG- or FFA-induced cell apoptosis

To ascertain the protective role that NG exerts against HG-induced apoptosis in HUVECs, we employed a Hoechst 33342 assay to observe nuclear condensation after treatment with NG or HG in the presence or absence of NG or SnPP (an HO-1 inhibitor). NG reversed HG-induced apoptosis (Fig. 4A). Analysis of apoptosis by flow cytometry showed that percentages of apoptotic cells were significantly increased in HUVECs cultured in HG conditions compared with the negative control group (Fig. 4B). However, percentages of apoptotic HUVECs cultured in HG were significantly reduced after NG treatment (Fig. 4B). To further emphasize the anti-apoptotic effect of HO-1 induction, HUVECs were treated with SnPP. SnPP reversed the protective effects of NG, as

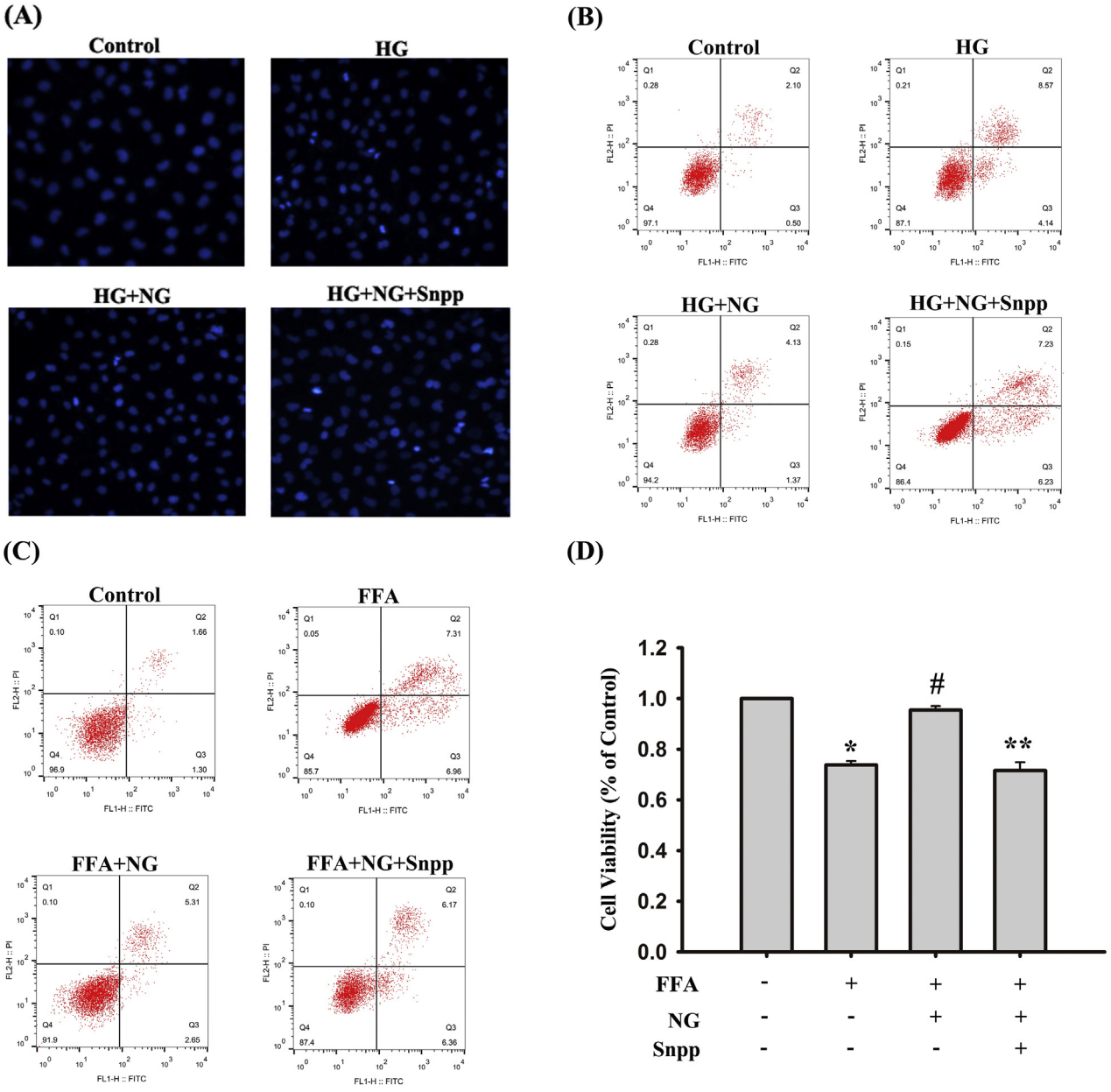

Fig. 4. Effect of NG on HG- or FFA-induced apoptotic death. (A) HUVECs were treated with 50 μM NG in the presence or absence of SnPP (15 μM) for 6 h, followed by the addition of HG to cells for a further 24 h. Cell apoptosis was detected by Hoechst 33342 assay. Cells with condensed nuclei or fragmented nuclei were defined as apoptotic cells. Control indicates normal HUVECs without any treatment. (B) HUVECs were treated with 50 μM NG in the presence or absence of SnPP (15 μM) for 6 h, followed by addition of HG to cells for a further 24 h. Assessment of apoptosis by flow cytometry using Annexin V and propidium iodide (PI) staining. (C) HUVECs were treated with 50 μM NG in the presence or absence of SnPP (15 μM) for 6 h, followed by the addition of FFA (1 mM) to cells for a further 72 h. Assessment of apoptosis by flow cytometry using Annexin V and PI staining. (D) HUVECs were treated with 50 μM NG in the presence or absence of SnPP (15 μM) for 6 h, followed by the addition of FFA (1 mM) to cells for a further 72 h. Cell viability was evaluated by MTT assay. $^{*}P < 0.05$ versus Control; $^{\#}P < 0.05$ versus FFA; $^{**}P < 0.05$ versus FFA + NG.

indicated by a significant increase in the percentage of apoptotic cells. As inappropriate elevation of FFA is also involved in diabetes [23], we also examined the effect of NG on FFA-induced apoptosis of HUVECs. As shown in Fig. 4C and D, NG attenuated FFA-induced cell apoptosis in HUVECs, similar to the results for HG. Moreover, SnPP reversed the protective effects of NG, as indicated by a significant increase in the percentage of apoptotic cells. These results further indicated that NG exerts cytoprotection via upregulation of HO-1 expression.

4. Discussion

Consumption of NG, a citrus-derived flavanone, reportedly improves vascular diseases [18,28,29]. However, the mechanism by which NG exerts this protective effect is not fully understood. HO-1 is an important intracellular defense against oxidative stress and inflammation associated with vascular diseases [30]. In this study, we found that the protective functions of NG against HG- or FFA-induced apoptosis of HUVECs were mediated by HO-1 upregulation via PI3K/Akt and JNK pathways (Fig. 5). To our knowledge, we demonstrated for the first time that in HUVECs, NG alleviated HG- or FFA-induced apoptosis by increasing HO-1 expression via PI3K/Nrf2 and JNK/Nrf2 signaling pathways.

PI3K/Akt is involved in maintaining the endothelial homeostasis of blood vessels and serves as a crucial signaling pathway for HO-1

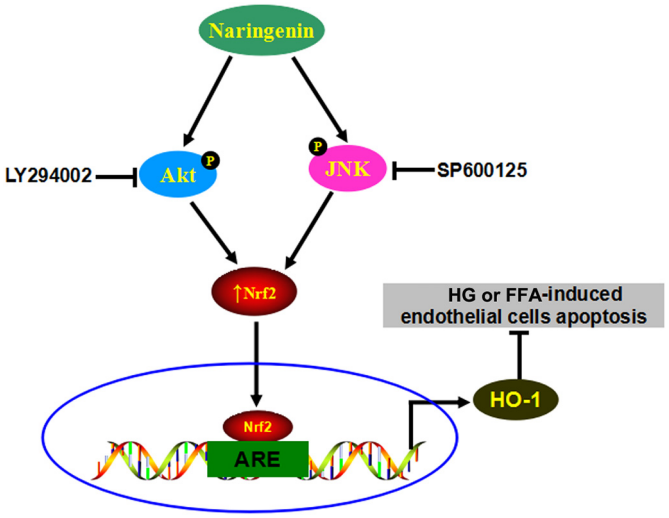

Fig. 5. Proposed pathway for the preventive effect of NG against HG- or FFA-induced apoptosis in HUVECs. NG exerts cytoprotection via activation of Akt, JNK, and Nrf2, which are involved in upregulation of HO-1 expression.

induction [27,31,32]. It was previously reported that LY294002, a PI3K inhibitor, significantly downregulated PM2.5-induced HO-1 mRNA expression, indicating the involvement of PI3K/Akt in the signaling pathway leading to nuclear translocation of Nrf2 and subsequent Nrf2-mediated HO-1 transcription in human lung alveolar epithelial (A549) cells [33]. Immenschuh et al. [34] also found that HO-1 gene expression is upregulated by anti-nonstructural glycoprotein-1 antibodies via activation of a redox-dependent PI3K/Akt-mediated pathway in HUVECs. In the present study, we observed that culture with NG caused rapid activation of Akt protein in HUVECs. Further, as expected, the Akt inhibitor LY294002 abrogated NG-mediated HO-1 expression in HUVECs. These data indicate that NG induced HO-1 expression through PI3K/Akt pathways.

In addition to PI3K/Akt, MAPKs are involved in the most important signaling pathways for HO-1 induction. However, the effect of NG on activation of MAPKs such as JNK, ERK, and p38 remains controversial. In the human vaginal epithelial cell line VK2/E6E7, phosphorylation of p38 and JNK, but not ERK, was involved in NG-induced apoptosis [35]. However, a recent study showed that NG-induced ROS production was dependent on activation of ERK and p38, but not JNK, in a choriocarcinoma (JAR) cell line [36]. These inconsistent observations may arise from different experimental conditions and cell types. In the present study, we found that exposure of HUVECs to NG increased the activation of MAPKs, including ERK and JNK, but not p38. However, JNK and ERK pathways did not contribute equally to NG-induced the expression of HO-1 in HUVECs. Pretreatment of HUVECs with inhibitors specific for JNK (SP600125) significantly suppressed the effect of NG on HO-1 expression, whereas an ERK inhibitor (PD98059) did not inhibit NG-induced HO-1 expression. These results suggest that the JNK pathway is involved in NG-induced HO-1 expression, but not ERK or p38. Meanwhile, Yang et al. [37] showed that ERK and p38 pathways, but not JNK, mediate the epigallocatechin-3-gallate-induced HO-1 expression in HUVECs. Therefore, members of the MAPK family may differ in their mediation of HO-1 induction in HUVECs according to the nature of the stimulus.

Nrf2 plays a pivotal role in HO-1 expression. Recent studies suggest that Nrf2 activation occurs by a coordinated process involving signaling of various upstream kinases such as protein kinase C (PKC), MAPKs, and PI3K [38]. Shen et al. [39] showed that transcriptional activity of the Nrf2 transactivation domain was stimulated by ERK and JNK signaling pathways, while p38 plays a negative role. Hepatocellular carcinoma (HepG2) cells pretreated with MAPK inhibitors reduced pyrrolidine dithiocarbamate-induced nuclear translocation of Nrf2 and subsequent expression of glutamate cysteine ligase modulatory gene, indicating that MAPK-directed phosphorylation is required for Nrf2 activation [40]. In this study, we showed that NG enhanced translocation of Nrf2 into the nucleus, and pretreatment of HUVECs with MAPK inhibitors, such as PI3K and JNK, significantly reduced Nrf2 nuclear translocation, indicating the involvement of PI3K/Akt and JNK pathways in NG-induced Nrf2 activation. Moreover, NG increased HO-1 protein expression in HUVECs, which could be blocked by knockdown using Nrf2 siRNA. These results further suggest that NG induced HO-1 upregulation via PI3K and JNK/Nrf2 pathways.

HG or FFA can induce apoptosis of endothelial cells and damage the integrity of vascular endothelia, which results in endothelial dysfunction [23,41]. In this experiment, we focused on the effect of NG on apoptosis of HUVECs, the first barrier of vessels, as endothelial dysfunction leads to the initiation and propagation of further vascular complications. HUVECs treated with NG exhibited enhanced resistance to HG- or FFA-induced injury. This result is consistent with a recent study demonstrating that NG confers protection against oxidative stress in H9c2 cardiomyoblasts by upregulating Nrf2 target genes [42]. Among various endogenous antioxidants, HO-1 is considered to be a “therapeutic funnel” against diseases associated with oxidative stress based on its potent physiology-regulating properties [43]. In this study, we found that NG meaningfully increased HO-1 mRNA and protein expression in HUVECs. Interestingly, SnPP, an HO-1 inhibitor, significantly attenuated the anti-apoptotic activity of NG in HUVECs, indicating that the cytoprotection imparted by NG was partially mediated by HO-1.

Although we provide evidence indicating mechanisms underlying the effect of NG on HO-1 expression and its protective role against HG- or FFA-induced apoptosis in HUVECs, the major limitation of this study is a lack of in vivo experiments. Thus, caution should be taken in extrapolating our results for cultured endothelial cells to the endothelium of intact animals, as cultured methods cannot fully mimic the complex natural environment of these cells in blood vessels, nor the complexity of in vivo biochemical and biophysical regulatory mechanisms. However, in support of our in vitro findings, it has been reported that NG can ameliorate vascular dysfunction in type 2 diabetic rats [29]. In the future, we will confirm our observations with in vivo animal studies.

In summary, NG induced upregulation of HO-1 expression through activation of Nrf2, which conferred protection against HG- or FFA-induced apoptosis of HUVECs. One of the most salient features of our present study is that PI3K/Akt or JNK was involved in HO-1 induction via Nrf2 activation in NG-treated HUVECs. Moreover, pharmacological inhibition of PI3K/Akt or JNK suppressed Nrf2 nuclear localization and subsequent HO-1 expression. Additionally, enhanced expression of HO-1 by NG may protect HUVECs from cellular injury in response to HG or FFA-induced injury. Thus, the results of this study provide compelling support for the identification of more potent pharmacological agents to add to the arsenal of treatments for diabetes-induced vascular injury, especially atherosclerosis and coronary heart disease, at a time when the aging population is increasing worldwide.

Funding

This work was supported by the National Natural Science Foundation of China (No. 31300946), and Luzhou Municipal People's Government - Sichuan Medical University Science and Technology Strategic Cooperation [2015LZCYD-S03 (7/7)].

Declaration of Competing Interest

None.

Acknowledgements

We are most grateful to Dr. Fang Xie from the Department of Oncology at The Affiliated Hospital of Southwest Medical University for her generous gift of the HUVECs. We thank Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

References

[1] H. Maamoun, M. Zachariah, J.H. McVey, F.R. Green, A. Agouni, Heme oxygenase (HO)-1 induction prevents endoplasmic reticulum stress-mediated endothelial cell death and impaired angiogenic capacity, Biochem. Pharmacol. 127 (2017) 46–59.
[2] Q. Li, Y. Lin, S. Wang, L. Zhang, L. Guo, GLP-1 inhibits high-glucose-induced oxidative injury of vascular endothelial cells, Sci. Rep. 7 (1) (2017) 8008.
[3] DCCT/EDIC Research Group, L.P. Aiello, W. Sun, A. Das, S. Gangaputra, S. Kiss, et al., Intensive diabetes therapy and ocular surgery in type 1 diabetes, N. Engl. J. Med. 372 (18) (2015) 1722–1733.
[4] J.E. Reusch, C.C. Wang, Cardiovascular disease in diabetes: where does glucose fit in, J. Clin. Endocrinol. Metab. 96 (8) (2011) 2367–2376.
[5] F. Folli, D. Corradi, P. Fanti, A. Davalli, A. Paez, A. Giaccari, et al., The role of oxidative stress in the pathogenesis of type 2 diabetes mellitus micro- and macrovascular complications: avenues for a mechanistic-based therapeutic approach, Curr. Diabetes Rev. 7 (5) (2011) 313–324.
[6] J. Kundu, I.G. Chae, K.S. Chun, Fraxetin induces heme oxygenase-1 expression by activation of Akt/Nrf2 or AMP-activated protein kinase α/Nrf2 pathway in HaCaT cells, J. Cancer Prev. 21 (3) (2016) 135–143.
[7] R. Gozzelino, V. Jeney, M.P. Soares, Mechanisms of cell protection by heme oxygenase-1, Annu. Rev. Pharmacol. Toxicol. 50 (2010) 323–354.
[8] W. Durante, Heme oxygenase-1 in growth control and its clinical application to vascular disease, J. Cell. Physiol. 195 (3) (2003) 373–382.
[9] G. Yang, Y. Li, W. Wu, B. Liu, L. Ni, Z. Wang, et al., Anti-oxidant effect of heme oxygenase-1 on cigarette smoke-induced vascular injury, Mol. Med. Rep. 12 (2) (2015) 2481–2486.
[10] C.F. Chang, X.M. Liu, K.J. Peyton, W. Durante, Heme oxygenase-1 counteracts contrast media-induced endothelial cell dysfunction, Biochem. Pharmacol. 87 (2) (2014) 303–311.
[11] N.G. Abraham, T. Kushida, J. McClung, M. Weiss, S. Quan, R. Lafaro, et al., Heme oxygenase-1 attenuates glucose-mediated cell growth arrest and apoptosis in human microvessel endothelial cells, Circ. Res. 93 (6) (2003) 507–514.
[12] E.E. Mulvihill, M.W. Huff, Antiatherogenic properties of flavonoids: implications for cardiovascular health, Can. J. Cardiol. 26 (Suppl A) (2010) 17A–21A.
[13] K. Patel, G.K. Singh, D.K. Patel, A review on pharmacological and analytical aspects of naringenin, Chin. J. Integr. Med. 24 (7) (2018) 551–560.
[14] R. Joshi, Y.A. Kulkarni, S. Wairkar, Pharmacokinetic, pharmacodynamic and formulations aspects of naringenin: an update, Life Sci. 215 (2018) 43–56.
[15] F.I. Kanaze, M.I. Bounartzi, M. Georgarakis, I. Niopas, Pharmacokinetics of the citrus flavanone aglycones hesperetin and naringenin after single oral administration in human subjects, Eur. J. Clin. Nutr. 61 (4) (2007) 472–477.
[16] T. Isobe, S. Ohkawara, S. Ochi, T. Tanaka-Kagawa, H. Jinno, N. Hanioka, Naringenin glucuronidation in liver and intestine microsomes of humans, monkeys, rats, and mice, Food Chem. Toxicol. 111 (2018) 417–422.
[17] E.E. Mulvihill, E.M. Allister, B.G. Sutherland, D.E. Telford, C.G. Sawyez, J.Y. Edwards, et al., Naringenin prevents dyslipidemia, apolipoprotein B overproduction, and hyperinsulinemia in LDL receptor-null mice with diet-induced insulin resistance, Diabetes 58 (10) (2009) 2198–2210.
[18] E.E. Mulvihill, J.M. Assini, B.G. Sutherland, A.S. DiMattia, M. Khami, J.B. Koppes, et al., Naringenin decreases progression of atherosclerosis by improving dyslipidemia in high-fat-fed low-density lipoprotein receptor-null mice, Arterioscler. Thromb. Vasc. Biol. 30 (4) (2010) 742–748.
[19] M.R. de Oliveira, C. Andrade, C.R. Fürstenau, Naringenin exerts anti-inflammatory effects in paraquat-treated SH-SY5Y cells through a mechanism associated with the Nrf2/HO-1 axis, Neurochem. Res. 43 (4) (2018) 894–903.
[20] N. Abdel-Magied, S.M. Shedid, The effect of naringenin on the role of nuclear factor (erythroid-derived 2)-like2 (Nrf2) and haem oxygenase 1 (HO-1) in reducing the risk of oxidative stress-related radiotoxicity in the spleen of rats, Environ. Toxicol. 34 (7) (2019) 788–795.
[21] Y. Li, Y. Pan, L. Gao, J. Zhang, X. Xie, Z. Tong, et al., Naringenin protects against acute pancreatitis in two experimental models in mice by NLRP3 and Nrf2/HO-1 pathways, Mediat. Inflamm. 2018 (2018) (3232491).
[22] D. Chai, B. Wang, L. Shen, J. Pu, X.K. Zhang, B. He, RXR agonists inhibit high-glucose-induced oxidative stress by repressing PKC activity in human endothelial cells, Free Radic. Biol. Med. 44 (7) (2008) 1334–1347.
[23] S. Piro, D. Spampinato, L. Spadaro, C.E. Oliveri, F. Purrello, A.M. Rabuazzo, Direct apoptotic effects of free fatty acids on human endothelial cells, Nutr. Metab. Cardiovasc. Dis. 18 (2) (2008) 96–104.
[24] Q. Hao, X. Lu, N. Liu, X. Xue, M. Li, C. Zhang, et al., Posttranscriptional deregulation of Src due to aberrant miR34a and miR203 contributes to gastric cancer development, BMB Rep. 46 (6) (2013) 316–321.
[25] J.S. Lee, Y.J. Surh, Nrf2 as a novel molecular target for chemoprevention, Cancer Lett. 224 (2) (2005) 171–184.
[26] Y.J. Surh, J.K. Kundu, H.K. Na, Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals, Planta Med. 74 (13) (2008) 1526–1539.
[27] J. Feng, P. Zhang, X. Chen, G. He, PI3K and ERK/Nrf2 pathways are involved in oleanolic acid-induced heme oxygenase-1 expression in rat vascular smooth muscle cells, J. Cell. Biochem. 112 (6) (2011) 1524–1531.
[28] W. Qin, B. Ren, S. Wang, S. Liang, B. He, X. Shi, et al., Apigenin and naringenin ameliorate PKCβII-associated endothelial dysfunction via regulating ROS/caspase-3 and NO pathway in endothelial cells exposed to high glucose, Vasc. Pharmacol. 85 (2016) 39–49.
[29] B. Ren, W. Qin, F. Wu, S. Wang, C. Pan, L. Wang, et al., Apigenin and naringenin regulate glucose and lipid metabolism, and ameliorate vascular dysfunction in type 2 diabetic rats, Eur. J. Pharmacol. 773 (2016) 13–23.
[30] R.M. Ogborne, S.A. Rushworth, M.A. O'Connell, Alpha-lipoic acid-induced heme oxygenase-1 expression is mediated by nuclear factor erythroid 2-related factor 2 and p38 mitogen-activated protein kinase in human monocytic cells, Arterioscler. Thromb. Vasc. Biol. 25 (10) (2005) 2100–2105.
[31] I. Shiojima, K. Walsh, Role of Akt signaling in vascular homeostasis and angiogenesis, Circ. Res. 90 (12) (2002) 1243–1250.
[32] S.H. Seo, G.S. Jeong, Fisetin inhibits TNF-α-induced inflammatory action and hydrogen peroxide-induced oxidative damage in human keratinocyte HaCaT cells through PI3K/AKT/Nrf-2-mediated heme oxygenase-1 expression, Int. Immunopharmacol. 29 (2) (2015) 246–253.
[33] X. Deng, W. Rui, F. Zhang, W. Ding, PM2.5 induces Nrf2-mediated defense mechanisms against oxidative stress by activating PIK3/AKT signaling pathway in human lung alveolar epithelial A549 cells, Cell Biol. Toxicol. 29 (3) (2013) 143–157.
[34] S. Immenschuh, P. Rahayu, B. Bayat, H. Saragih, A. Rachman, S. Santoso, Antibodies against dengue virus nonstructural protein-1 induce heme oxygenase-1 via a redox-dependent pathway in human endothelial cells, Free Radic. Biol. Med. 54 (2013) 85–92.
[35] S. Park, W. Lim, F.W. Bazer, G. Song, Naringenin induces mitochondria-mediated apoptosis and endoplasmic reticulum stress by regulating MAPK and AKT signal transduction pathways in endometriosis cells, Mol. Hum. Reprod. 23 (12) (2017) 842–854.
[36] S. Park, W. Lim, F.W. Bazer, G. Song, Naringenin suppresses growth of human placental choriocarcinoma via reactive oxygen species-mediated P38 and JNK MAPK pathways, Phytomedicine 50 (2018) 238–246.
[37] G.Z. Yang, Z.J. Wang, F. Bai, X.J. Qin, J. Cao, J.Y. Lv, et al., Epigallocatechin-3-gallate protects HUVECs from PM2.5-induced oxidative stress injury by activating critical antioxidant pathways, Molecules 20 (4) (2015) 6626–6639.
[38] J.W. Kim, M.H. Li, J.H. Jang, H.K. Na, N.Y. Song, C. Lee, et al., 15-Deoxy-delta (12,14)-prostaglandin J(2) rescues PC12 cells from H_2O_2-induced apoptosis through Nrf2-mediated upregulation of heme oxygenase-1: potential roles of Akt and ERK1/2, Biochem. Pharmacol. 76 (11) (2008) 1577–1589.
[39] C.P. Shen, Y. Tsimberg, C. Salvadore, E. Meller, Activation of Erk and JNK MAPK pathways by acute swim stress in rat brain regions, BMC Neurosci. 5 (2004) 36.
[40] L.M. Zipper, R.T. Mulcahy, Erk activation is required for Nrf2 nuclear localization during pyrrolidine dithiocarbamate induction of glutamate cysteine ligase modulatory gene expression in HepG2 cells, Toxicol. Sci. 73 (1) (2003) 124–134.
[41] I.A. van den Oever, H.G. Raterman, M.T. Nurmohamed, S. Simsek, Endothelial dysfunction, inflammation, and apoptosis in diabetes mellitus, Mediat. Inflamm. 2010 (2010) 792393.
[42] T. Ramprasath, M. Senthamizharasi, V. Vasudevan, S. Sasikumar, S. Yuvaraj, G.S. Selvam, Naringenin confers protection against oxidative stress through upregulation of Nrf2 target genes in cardiomyoblast cells, J. Physiol. Biochem. 70 (2) (2014) 407–415.
[43] P. Yao, A. Nussler, L. Liu, L. Hao, F. Song, A. Schirmeier, et al., Quercetin protects human hepatocytes from ethanol-derived oxidative stress by inducing heme oxygenase-1 via the MAPK/Nrf2 pathways, J. Hepatol. 47 (2) (2007) 253–261.