Involvement of heme oxygenase-1 induction in anti-vascular inflammation effects of Xanthoceras sorbifolia in human umbilical vein endothelial cells

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Abstract

OBJECTIVE: To define the effects of Xanthoceras sorbifolia (EXS) on vascular inflammation and the mechanisms in endothelial cells.

METHODS: Vascular protective effects of an ethanol extract of seeds from EXS (1-50 μg/mL) against tumor necrosis factor-α (TNF-α)-induced vascular inflammation were examined in human umbilical vein endothelial cells (HUVECs).

RESULTS: EXS significantly decreased TNF-α-induced expression of cell adhesion molecules, such as intracellular adhesion molecule-1, vascular cell adhesion molecule-1, and endothelial cell selectin, in a dose-dependent manner. Pre-treatment with EXS significantly inhibited translocation and transcriptional activity of nuclear factor-kB (NF-kB) increased by TNF-α. EXS also significantly inhibited formation of intracellular reactive oxygen species (ROS). Moreover, the vascular protective effects of EXS were linked to up-regulation of heme oxygenase-1 (HO-1) and nuclear factor E2-related factor-2 (Nrf-2) expression. EXS-induced HO-1 expression was significantly decreased in SnPP (HO-1 inhibitor)- and HO-1 siRNA-treated cells, whereas an increase was found in cobalt protoporphyrin IX (CoPP) (HO-1 inducer)-treated cells. In addition, pre-treatment with EXS increased HO-1 and Nrf-2 expression under TNF-α stimulation with or without N-acetyl-L-cysteine. Furthermore, the inhibitory effects of EXS on TNF-α-induced vascular inflammation were partially reversed in SnPP- and of HO-1 siRNA-treated cells but increased by CoPP.

CONCLUSION: These results suggest that EXS may have important implications for prevention of vascular complications associated with vascular inflammation by inhibition of the NF-kB/ROS pathway and activation of the Nrf-2/HO-1 pathway.

Keywords: Sapindaceae; Vascular disease; inflam-
INTRODUCTION

Vascular inflammation is closely associated with the pathogenesis of many human diseases including atherosclerosis and cardiovascular diseases. Endothelial cells are important participants in vascular inflammation, and activation of these cells after changes induced by proinflammatory cytokines promotes the expression of adhesion molecules, following by proliferation and migration of vascular smooth muscle cells, resulting in vascular remodeling. The inflammatory mediator tumor necrosis factor-α (TNF-α) has been implicated in the pathogenesis of cardiovascular diseases including atherosclerosis. Upregulated expression of cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and endothelial cell selectin (E-selectin), alters the adhesive properties of endothelial cells in the vasculature. This process is one of the key events leading to indiscriminate migration of leukocytes across blood vessels, thereby inducing vascular inflammation. TNF-α also stimulates production of reactive oxygen species (ROS) in a variety of cell types, although ROS can serve as signaling molecules that facilitate activation of nuclear factor-κB (NF-κB). The transcription factor NF-κB is involved in activating large numbers of genes in response to infections, inflammation, and other stressful situations requiring rapid reprogramming of gene expression.

Heme oxygenase (HO)-1 is an inducible isoform of HO, which degrades heme to biliverdin, Fe²⁺, and carbon monoxide. A study has demonstrated that HO-1 expression is primarily regulated at the transcriptional level, and its induction is linked to the transcription factor nuclear erythroid 2-related factor-2 (Nrf-2). Under basal conditions, Nrf-2 is sequestered in the cytoplasm through binding to Kelch-like ECH-associated protein 1 (Keap1). Upon disruption by electrophilic antioxidants, Nrf-2 is released from Keap1 and translocates to the nucleus. Previous studies have reported that HO-1 expression plays a role in several pathological states such as atherosclerosis and inflammatory vascular disorders. HO-1 regulates endothelial functions by inhibiting TNF-α-dependent activation of NF-κB in endothelial cells. Nrf-2-deficient mice exhibit greater induction of proinflammatory genes regulated by NF-κB, such as interleukins (ILs) and TNF-α.

Xanthoceras sorbifolia (EXS) Bunge belongs to the Sapindaceae family and is used as a Traditional Chinese Medicine to cure arterial sclerosis, hyperlipemia, hyper-piesia, chronic hepatitis, rheumatism, and enuresis in children. Moreover, according to a recent study, the leaves of EXS affect vascular relaxation through Akt and SOCE-eNOS-cGMP pathways. Triterpenoid saponins, the main constituents of EXS, have been previously obtained from this plant and shown to have anti-tumor activities. However, to the best of our knowledge, the effects of an ethanol extract of seeds from EXS on vascular inflammation have not yet been defined. The purpose of the present study was to define the effects of EXS on vascular inflammation and the mechanisms in endothelial cells.

MATERIALS AND METHODS

Chemicals and reagents

RPMI 1640, fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL Co., (Grand Island, NY, USA). TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). 2', 7'-Bis (2-carboxyethyl)-5(6)-carboxyfluorescein ace-toxymethyl ester (BCECF-AM) and CM-H₂DCFDA were purchased from Invitrogen (Eugene, OR, USA). Primary antibodies against HO-1, ICAM-1, VCAM-1, E-selectin, NF-κB, p-IκB-α, and Nrf2, and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

EXS preparation and extraction

EXS Bunge seeds (Herbarium voucher No. HBE101-01) were obtained from Professor Song Nan Jin, Institute of Materia Medica, Taishan Medical University. After incubation at 25 °C for 24 h, the seeds were homogenized, soaked in 80% ethanol (100 g homogenate in 800 mL ethanol) for 30 min, and then boiled for 2 h. The extract was filtered through Whatman No. 3 filter paper and centrifuged at 990 × g for 20 min at 4 °C. The supernatant was concentrated using a rotary evaporator, and the resulting extract (3.6 g) was lyophilized using a freeze drier and stored at –70 °C until required.

Cell culture

Primary cultured human umbilical vein endothelial cells (HUVECs) and endothelial cell growth medium-2 (EGM-2) were purchased from Cambrex (East Rutherford, NJ, USA), which was supplemented with 2.5% FBS, recombinant epidermal growth factor, human basic fibroblast growth factor, vascular endothelial growth factor, ascorbic acid, hydrocortisone, human recombinant insulin-like growth factor, heparin, gentamicin, and amphotericin. HUVECs at passages 3 and 8 were maintained in EGM-2 medium in a humidified atmosphere containing 5% CO₂ at 37 °C.

Assessment of cell viability

To determine cell viability, 20 μL MTT was added to a cell suspension for 4 h. After three washes with phosphate-buffered saline (PBS), the insoluble formazan precipitate was determined with a microplate reader.
product was dissolved in dimethyl sulfoxide. The optical density (OD) of each culture well was measured using a microplate reader (Multiskan; Thermo Labsystems, Franklin, MA) at 590 nm. The OD in control cells was considered as 100% viable.

**Cell-based enzyme-linked immunosorbent assay (ELISA)**

A cell-based ELISA was performed as previously reported,17 with minor modifications. Briefly, HUVECs in 96-well plates were pretreated with or without EXS for 18 h, followed by TNF-α treatment for 6 h at 37 °C. After the treatments, the cells were fixed in 1% paraformaldehyde and incubated with mouse anti-human ICAM-1, VCAM-1, or E-selectin antibodies at 1:1000 dilutions in PBS containing 1% bovine serum albumin for 2 h at room temperature. The cells were washed and incubated with a horseradish peroxidase-conjugated secondary antibody. Finally, ICAM-1, VCAM-1, and E-selectin expression levels were quantified by adding a peroxidase substrate solution and measuring the absorbance of each well at 490 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

**Monocyte-HUVEC adhesion assay**

HUVECs were grown to confluency in 24-well culture plates, pretreated with EXS for 18 h, and then stimulated with TNF-α for 6 h. Then, the HL-60 cells were labeled with 10 μM BCECF-AM for 1 h at 37 °C. After washing with medium twice, 2.5 x 10^5 labeled HL-60 cells were added to the HUVECs, followed by incubation for 1 h. Non-adherent HL-60 cells were removed by washing with PBS, and HL-60 cells bound to the HUVECs were analyzed using fluorescence microscopy, followed by lysis in 50 mM Tris-HCl (pH 8.0) containing 0.1% SDS. The fluorescent intensity was measured using a spectrofluorometer (F-2500; Hitachi, Tokyo, Japan) at excitation and emission wavelengths of 485 and 535 nm, respectively. Adhesion data are presented as a percentage of the TNF-α control.

**Western blot analysis**

Cell homogenates (40 μg protein) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Blots were then washed with H2O, blocked with 5% skim milk powder in Tris-buffered saline with Tween-20 (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween-20) for 1 h, and incubated with the appropriate primary antibody at dilutions recommended by the supplier. Then, the membrane was washed, and primary antibodies were detected with goat anti-rabbit or rabbit anti-mouse IgGs conjugated to horseradish peroxidase. Bands were visualized with enhanced chemiluminescence (Amersham Bioscience, Buckinghamshire, UK). Protein expression levels were determined by measuring the signals captured on the nitrocellulose membranes using a ChemiDoc image analyzer (Bio-Rad Laboratories).

**Preparation of cytoplasmic and nuclear extracts**

Cells were collected in cold PBS and centrifuged at 9500 x g for 10 min at 4 °C. Nuclear and cytoplasmic extracts were prepared on ice as described previously by Dschietzig et al.18 Cells were washed with 1 mL buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, and 19 mM KCl) for 5 min at 600 x g. The cells were then resuspended in buffer A with 0.1% NP 40, placed on ice for 10 min for lysis, and then centrifuged at 600 x g for 3 min. The supernatant was collected as the cytosolic extract. The nuclear pellet was then washed in 1 mL buffer A at 4200 x g for 3 min, resuspended in 30 μL buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, and 0.2 mM EDTA), rotated for 30 min at 4 °C, and then centrifuged at 14 300 x g for 20 min. The supernatant was used as the nuclear extract.

**RNA isolation and reverse-transcriptase polymerase chain reaction (RT-PCR)**

Total RNA was isolated from HUVECs using TRIzol reagent and frozen at −70 °C until use. Two micrograms of total RNA were used to synthesize first-strand cDNA using Moloney murine leukemia virus reverse transcriptase (Promega) in a final volume of 20 μL containing 5 mM MgCl₂, 1 mM of each deoxynucleotide triphosphate, 2.5 mM oligo(dT) primers, and 40 U RNase inhibitor. PCR was conducted in a thermocycler in a reaction volume of 50 μL including 20 μL cDNA, BioTaq PCR buffer, 4 mM MgCl₂, 1 U BioTaq DNA polymerase (BioLine), and 6 pmol forward and reverse primers. Oligonucleotide primers were as follows: ICAM-1 (forward, 5′-TGA AGG ACA CCA CAG AGG ACA AC-3′; reverse, 5′-CCC ATT ATG ACT GCG GCT GCT GCT ACC-3′), VCAM-1, (forward, 5′-CAA TTC TAT GCT ACT CAT GCT CAT C-3′; reverse, 5′-TTG ACT TCT TGC TGA CCA CAG C-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward, 5′-CCA TCA CCA TCT TCC AGG AG-3′; reverse, 5′-CCT GCT TCA CCA CCT TCT TG-3′). Amplification was achieved when samples were heated to 95 °C for 5 min and then immediately cycled 39 times through a 1 min denaturing step at 95 °C, a 1 min annealing step at 55 °C, and a 2 min elongation step at 72 °C. The GAPDH mRNA level was used as the internal standard. PCR products were resolved in a 1% agarose gel and analyzed using Opticon MJ Research Instrument Software (Bio-Rad, Hercules, CA, USA).

**Intracellular ROS production assay**

The fluorescent probe CMH,DCFDA was used to determine intracellular generation of ROS. Briefly, 100% confluent HUVECs in 24-well culture plates were pre-treated with EXS for 1 h. After removing from the wells, the HUVECs were incubated with 20 μM CM-H,DCFDA for 1 h and then stimulated with TNF-α. The fluorescence intensity was measured by...
flow cytometry on a FACSCalibur (BD, San Diego, CA) and spectrofluorometer.

**Determination of superoxide dismutase (SOD) activity**

HUVECs were seeded into a 60 mm culture dish to determine SOD activity. At confluency, the cells were incubated with or without TNF-α and EXS. SOD activity was then analyzed in the samples. The total SOD activity in cell lysates was assayed using a kit (Item No. 706002; Cayman Chemical Co., Ann Arbor, MI), according to the manufacturer’s recommendations. SOD activity was measured in a microplate reader at OD₄₅₀, calculated using a concurrently run SOD standard curve, and expressed as U/mg protein.

**Immunofluorescence**

To analyze localization of NF-κB, HUVECs were grown on a Lab-Tek II chamber. The cells were then fixed in formalin and permeabilized with cold acetone. Then, the cells were probed with an anti-NF-κB antibody, followed by a fluorescein isothiocyanate-labeled secondary antibody (Santa Cruz Biotechnology, CA, USA). To visualize nuclei, cells were stained with 1 μg/mL DAPI for 30 min. The cells were finally washed three times with PBS, mounted with Dako Fluorescent mounting medium, and examined under a fluorescence microscope (Axiovision 4; Zeiss, Germany).

**Statistical analysis**

Results are expressed as the mean ± standard error of mean. Data were analyzed using one-way analysis of variance, followed by Student’s t-test using Sigma Plot ver 10.0 to determine any significant differences. Significance was set at P < 0.05.

**RESULTS**

**Cell viability**

To examine the cytotoxic potential of EXS, its effect on HUVEC viability was determined using the MTT assay. Cells were treated with various concentrations of EXS (1-200 μg/mL), and an MTT assay was performed after 24 h of incubation as described in the Methods and Materials. As shown in Figure 1A, all concentrations had no cytotoxic effects. In addition, HUVECs were assessed using a Countess™ Automated Cell Counter (Invitrogen, Carlsbad, CA). Consistent with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay results, the number of endothelial cells was not changed by EXS treatments (1-100 μg/mL) (Figure 1B). Thus, in further experiments, HUVECs were treated with EXS in the concentration range of 1-50 μg/mL.

**TNF-α-induced cell adhesion molecules**

Increased expression of cell adhesion molecules on endothelial cells promotes adhesion of monocytes, which is regarded as the molecular basis for the inflammatory response observed in various diseases. To investigate the regulatory effect of EXS on monocyte adhesion to TNF-α-stimulated HUVECs, cells were pretreated with EXS (1-50 μg/mL) for 18 h and then treated with TNF-α (10 ng/mL) for 6 h, which was followed by an adhesion process by applying fluorescent HL-60 cells and then quantifying the adhered fluorescent monocytes. As shown in Figure 2, HL-60 cells almost never adhered to HUVECs without TNF-α. There was bright fluorescence on TNF-α-treated HUVECs, which was indicative of a marked increase in HL-60 cell adherence to the activated HUVECs. However, pre-treatment with EXS significantly suppressed monocyte adherence in a dose-dependent manner. As detected by the ELISA, EXS reduced TNF-α-induced ICAM-1, VCAM-1 and E-selectin expression. Western blot analysis also showed that EXS inhibited TNF-α-induced expression of these adhesion molecules (Figure 3A). The inhibitory effect of EXS was found to be concentration-dependent (1-50 μg/mL), which was consistent with the ELISA results (Figure 3B). Thus, EXS had anti-inflammatory effects by inhibiting cell adhesion molecule expression and monocyte adhesion.

**NF-κB/ROS signaling**

Extensive studies have demonstrated that activation of NF-κB is essential for transcriptional regulation of che-

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**Figure 1 Effect of EXS on cell viability**

A: cell viability was determined by MTT assays; B: a Countess™ Automated Cell Counter. Cells were treated with various concentrations (1-200 μg/mL) of EXS for 24 h. EXS: Xanthoceras sorbifolia; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Results are expressed as the mean ± standard error of mean of three independent experiments.
motactic cytokines and vascular adhesion molecules that are critically involved in leukocyte adhesion to the endothelium. Therefore, we determined whether EXS suppressed NF-κB activation and translocation into the nuclei of HUVECs. As shown in Figure 4A, western blot analysis revealed that NF-κB in nuclear fractions was increased by TNF-α and inhibited by pretreatment with EXS. In addition, phosphorylation of IkBα induced by TNF-α was attenuated by pretreatment with EXS (Figure 4A). Similar results were obtained by immunocytochemical analysis that showed an increase in nuclear translocation of NF-κB as demonstrated by the intense green fluorescence localized in cell nuclei superimposed with the blue fluorescence of DAPI-stained nuclei. Stimulation with TNF-α increased the number of nuclei containing NF-κB, whereas EXS inhibited nuclear translocation of NF-κB (Figure 4B). These results suggest that EXS may inhibit inflammation by suppression of NF-κB signaling.

As shown in Figure 5A, intracellular ROS levels after TNF-α treatment were higher than in untreated cells (control). However, after EXS treatment, ROS production showed no difference compared with the control. In addition, EXS effectively suppressed TNF-α-induced ROS levels at 50 μg/mL to a level similar to pretreatment with NAC (50 μM).

As shown in Figure 5B, SOD activity was significantly decreased in HUVECs exposed to TNF-α compared with the control ($P < 0.05$, $21.2 \pm 2.1 \mu$ vs $13.2 \pm 1.3$). These changes were significantly attenuated by treatment with EXS at $10 \mu$g/mL ($P < 0.05$, $21.2 \pm 2.1 \mu$ vs $25.5 \pm 3.8$) and $50 \mu$g/mL ($P < 0.05$, $21.2 \pm 2.1 \mu$ vs $41.4 \pm 12.8$). Thus, EXS may also function as an antioxidant.

**HO-1 protein expression and Nrf-2 nuclear localization**

HO-1 is well known for its cytoprotective effect against oxidative stress and plays a critical role in the...
resolution of inflammation.$^{21}$ HO-1 expression is primarily regulated by the Keap1-Nrf-2 system that is sensitive to oxidative and/or electrophilic stresses. We examined whether EXS affected HO-1 protein expression by treating HUVECs with EXS (1-50 μg/mL) for 24 h. As shown in Figure 6A, EXS caused a dose-dependent increase in HO-1 protein levels. The induction of HO-1 by EXS reached a peak at 50 μg/mL. HO-1 expression showed an increase at 12 h and peaked at around 24 h (Figure 6A).
Several studies have reported that nuclear translocation of activated Nrf-2 is an important upstream contributor to the mechanism of HO-1 expression in a wide variety of cell types including endothelial cells. Thus, we examined whether treatment of HUVECs with EXS induced translocation of Nrf-2 to the nucleus. We tested the presence of Nrf-2 protein in nuclear compartments of HUVECs by western blot analysis. As a result, Nrf-2 protein in nuclear fractions was increased by EXS in dose-dependent manner, which showed an increase at 2 h and peaked at around 6 h (Figure 6B). Nrf-2 plays an important role in the induction of HO-1 in many cell types including macrophages. Therefore, we determined whether EXS-mediated HO-1 induction was related to activation of Nrf-2. As a result, induced HO-1 and Nrf-2 protein expression by EXS was significantly inhibited in HO-1 siRNA-treated cells (Figure 6C). As shown in Figure 6D, increased HO-1 and Nrf-2 protein expression by EXS was significantly decreased in SnPP (HO-1 inhibitor)-treated cells but increased in CoPP (HO-1 inducer)-treated cells (Figure 6D). These results suggest that EXS induces HO-1 protein expression in concentration- and time-dependent manners.

EXS-induced HO-1 upregulation on TNF-α-induced vascular inflammation

A recent study has shown that, when overexpressed in endothelial cells, HO-1 inhibits the expression of adhesion molecules associated with endothelial cell activation, including ICAM-1, VCAM-1, and E-selectin. Therefore, we examined whether upregulation of HO-1 induced by EXS mediated these vascular protective effects. The involvement of HO-1 in the inhibition of TNF-α-induced adhesion molecule expression was investigated using HO-1 siRNA (Figure 7A). Figure 7A shows that TNF-α significantly increased VCAM-1, ICAM-1, and E-selectin protein expression, whereas their expression was decreased by pretreatment with EXS. Interestingly, HO-1 knockdown by siRNA reduced the ability of EXS to downregulate VCAM-1, ICAM-1, and E-selectin protein expression in HUVECs.

Similarly, ICAM-1 and VCAM-1 mRNA expression induced by TNF-α was also decreased by pretreatment with EXS (Figure 7B). Interestingly, HO-1 overexpression mediated by CoPP, a HO-1 inducer, reduced the ability of TNF-α to upregulate cell adhesion molecule expression in HUVECs. However, treatment with SnPP, a HO-1 inhibitor, increased ICAM-1 and VCAM-1 mRNA levels. These results suggest that EXS regulated TNF-α-induced cell adhesion molecule expression, and the regulation was associated with the antioxidant system induced by HO-1 (Figure 7A and B). HO-1 overexpression induced by EXS reduced the ability of TNF-α to upregulate cell adhesion molecule expression in HUVECs.

NF-κB has been implicated in activation of specific target genes in HUVECs, including ICAM-1 and VCAM-1. As shown in Figure 7C, there was significantly more IkB-α phosphorylation, and nuclear translocation of NF-κB p65 was inhibited after treatment with EXS than after exposure to TNF-α alone (Figure 7C). CoPP effectively inhibited TNF-α-induced NF-κB in the nuclear fraction, whereas SnPP reduced the ability of EXS to regulate IkB-α phosphorylation as well as nuclear translocation of NF-κB p65 (Figure 7C).

DISCUSSION

In this study, we identified EXS as a potent inhibitor of inflammation and explored the mechanisms involved in the inhibition of TNF-α-activated expression of adhesion molecules such as VCAM-1 and ICAM-1, important inflammatory factors in HUVECs. Our results showed that EXS effectively inhibited TNF-α-in
Figure 6: Effect of EXS on HO-1 and Nrf-2 expression

A-D: cells were incubated for 24 h with the indicated concentrations of EXS (1-50 μg/mL) and periods with 50 μg/mL EXS. Western blot analysis of HO-1 expression and representative blots of three independent experiments are shown; E-H: cells were incubated for 6 h with the indicated concentrations of EXS (1-50 μg/mL) and periods with 50 μg/mL EXS. Cytoplasmic and nuclear fractions were extracted, and Nrf-2 protein levels were determined by western blot analysis; I-K: effect of HO-1 siRNA on Nrf-2 and HO-1 protein expression: L-N: effect of EXS on HO-1 protein expression and Nrf2 translocation into the nucleus under TNF-α stimulation. Each electrophoretogram is representative of results from three individual experiments. EXS: Xanthoceras sorbifolia; HO-1: heme oxygenase-1; Nrf-2: nuclear factor E2-related factor-2; TNF-α: tumor necrosis factor-α; HUVECs: human umbilical vein endothelial cells. Results are presented as the mean ± standard error of mean of four individual experiments. *P < 0.05, **P < 0.01 vs control.

Reduced inflammatory responses in HUVECs, which was likely associated with upregulation of Nrf-2-dependent HO-1 expression and downregulation of the NF-κB/ROS signaling pathway.
Figure 7 Effect of EXS-induced HO-1 upregulation on TNF-α-induced cell adhesion molecules and NF-κB/p65 expression. Cells were incubated with EXS for 18 h and then treated with TNF-α for 6 h.

A-D: cell adhesion molecules in total protein were analyzed by western blotting. E-G: total RNA was subjected to RT-PCR. H-I: effect of EXS on TNF-α-induced NF-κB/p65 translocation into nuclei of HUVECs. Cells were pretreated with EXS and then stimulated with TNF-α. Cytoplasmic (CE) and nuclear (NE) fractions were extracted, and protein levels were determined by western blot analysis. Results are presented as the mean ± standard error of mean of four individual experiments. *P < 0.01 vs control; **P < 0.05 and ***P < 0.01 vs TNF-α alone. EXS: Xanthochromas sorbifolia; TNF-α: tumor necrosis factor-α; HO-1: heme oxygenase-1; NF-κB: nuclear factor-κB; Nrf-2: nuclear factor E2-related factor-2.

Vascular inflammation plays a key role in the pathogenesis of atherosclerosis. The importance of adhesion molecules in atherosclerosis has been examined in some studies. Proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 have been reported to be involved in various inflammatory diseases. Therefore, inhibition of
these proinflammatory cytokines and mediator production is a key factor to evaluate the efficacy of anti-inflammatory agents. In this study, the ELISA results showed that TNF-α increased ICAM-1, VCAM-1, and E-selectin compared with the control, and pretreatment with EXS (1-50 μg/mL) inhibited their expression in a dose-dependent manner. Moreover, the adhesion of monocytes to HUVECs was markedly inhibited by pretreatment of HUVECs with EXS. These results suggest that the anti-inflammatory effect of EXS on vascular inflammation may be partially mediated by inhibition of adhesion molecules.

Among the signaling molecules associated with vascular inflammation, NF-κB is a key signaling node connecting to arrays of downstream molecules important for endothelial cell activation. Inhibiting the NF-κB pathway in endothelial cells has potential therapeutic value in treating inflammatory diseases. Generally, NF-κB is retained in the cytoplasm through an interaction with its inhibitor, IkB, which exerts its inhibitory effects by masking the nuclear localization signal. Thus, degradation of IkB by the proteasome is an essential early step in the NF-κB activation pathway. In the present study, we found that TNF-α induced NF-κB p65 translocation into the nucleus of HUVECs by western blot and immunofluorescence. EXS pretreatment inhibited TNF-α-induced nuclear translocation of NF-κB p65 in a dose-dependent manner. In addition, EXS suppressed TNF-α-induced phosphorylation of IkB-α in the cytoplasm. This result is supported by our previous report showing that medicinal plant extracts such as rhubarb suppress NF-κB p65 expression during vascular endothelial inflammation. EXS potently suppressed NF-κB activation through inhibition of IkB phosphorylation and degradation, and p65 nuclear translocation following TNF-α stimulation. These data suggest that the anti-inflammatory effect of EXS in HUVECs is at least partially regulated via an NF-κB-mediated mechanism.

Formation of oxygen-derived radicals might lead to activation of NF-κB, and induction of ICAM-1 and ROS-mediated NF-κB activation plays an important role in the pathogenesis of atherosclerosis. SOD regulates ROS production, and increased SOD protects cells from excessive ROS generation. Thus, we investigated the effect of EXS on ROS production and SOD activity under TNF-α stimulation. Our results revealed that EXS blocked ROS production, and enhanced cellular defenses prevented TNF-α-induced vascular inflammation. HO-1 and its by-products (biliverdin and CO) have cytoprotective properties including antioxidant, anti-inflammatory, and anti-apoptotic activities. Many phytochemicals are HO-1 inducers and traditionally used in therapeutic strategies for chronic inflammatory diseases. The role of HO-1 in inflammation has been demonstrated in HO-1 knockout mice in which HO-1 deficiency results in elevated production of pro-inflammatory cytokines. We examined whether EXS affected HO-1 protein expression by treating HUVECs with TNF-α. The results showed that EXS caused a dose-dependent increase in HO-1 protein levels. EXS-induced HO-1 expression was significantly decreased in SnPP (HO-1 inhibitor)- and of HO-1 siRNA-treated cells but increased in CoPP (HO-1 inducer)-treated cells. EXS alone induced HO-1 expression in dose- and time-dependent manners. In addition, pre-treatment with EXS increased HO-1 and Nrf-2 expression in TNF-α-stimulated cells treated with or without NAC (Figure 6). These results suggest that EXS serves as an antioxidant in cytokine-induced vascular inflammation. It is clear that antioxidants prevent inflammation in various cell types including HUVECs. Previous studies have shown no evidence that NAC reduces HO-1 expression. We could not rule out that EXS prevented vascular inflammation via Nrf-2/HO-1 activation, although some studies have reported that NAC reduces HO-1 expression.

The transcription factor Nrf-2 plays a considerable role in transcriptional activation of HO-1. Activation of Nrf-2 is regulated by the cytosolic protein Keap1 that negatively modulates nuclear translocation of Nrf-2 and facilitates degradation of Nrf-2 via the proteasome. Upon activation, Nrf-2 enters the nucleus where it binds to AU-rich elements in the HO-1 promoter to trigger gene expression. In the present study, Nrf-2 protein expression and nuclear translocation were increased by EXS in a dose-dependent manner. Thus, EXS might regulate Nrf-2 activation and facilitate its accumulation in the nucleus to promote HO-1 gene expression. Our data demonstrated that the HO system was closely involved in the inhibitory activities of EXS against inflammation. Moreover, EXS significantly inhibited TNF-α-induced ICAM-1, VCAM-1, and E-selectin protein and mRNA expression, which was further decreased by CoPP, an inducer of HO, and reversed by SnPP, an inhibitor of HO. HO-1 induction has also been shown to inhibit expression of proinflammatory mediators through inactivation of NF-κB. Reports show that NF-κB p65 represses Nrf-2 transcription activity. In this study, CoPP decreased TNF-α-induced IkB-α phosphorylation and NF-κB p65 nuclear translocation, whereas SnPP reversed such effects. These results are in agreement with the expression data of cell adhesion molecules, demonstrating that EXS-induced activation of HO-1 was regulated NF-κB activation and suppression of NF-κB-mediated proinflammatory gene expression.

In conclusion, our data indicated that EXS may have important implications in prevention of vascular complications associated with vascular inflammation by inhibition of the NF-κB/ROS pathway and activation of the Nrf-2/HO-1 pathway. These findings may provide a molecular basis for the ability of EXS to suppress vascular inflammation and prevent atherosclerosis.
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