MDG-1 inhibits H_2O_2-induced apoptosis and inflammation in human umbilical vein endothelial cells

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Abstract. MDG-1, a water-soluble polysaccharide extracted from *Ophiopogon japonicus*, has been reported to serve a role in antimyocardial ischemia by protecting cardiomyocytes from hypoxia/reoxygenation-induced damage. However, it remains unknown whether MDG-1 protects human umbilical vein endothelial cells (HUVECs) against oxidative stress-induced damage. In the present study, HUVECs were treated with hydrogen peroxide (H_2O_2) to establish an oxidative stress-induced cell injury model. Treatment of HUVECs with different concentrations of H_2O_2 significantly attenuated cell viability and increased cell apoptosis in a time and dose-dependent manner. Pretreatment with MDG-1 markedly reduced H_2O_2-induced cell death, ROS generation and inflammatory factor secretion. In addition, pretreatment with MDG-1 decreased the expression levels of proapoptotic proteins BCL2 associated X (Bax) and caspase-3, while it increased the expression levels of the antiapoptotic protein BCL2 apoptosis regulator (Bcl-2), compared with H_2O_2 treatment alone. Taken together, the present data suggest that MDG-1 protected HUVECs against H_2O_2-induced apoptosis and inflammation through inhibition of Bax/Bcl-2 protein ratio, caspase-3 expression, and inflammatory factor secretion. This study provides a potential application for MDG-1 in the treatment of cardiovascular disease.

Introduction

Oxidative stress is a key factor in the development of cardiovascular disease (1). A growing body of evidence indicates the close correlation between oxidative stress and the abnormalities in endothelial function. Increased oxidative stress is a major cause of endothelial dysfunction by prolonging cell proliferation (2), disturbing cell cycle (3), increasing the production of reactive oxygen species (ROS) (2), promoting inflammatory responses (4), and activating various intracellular signal transduction pathways (5). Furthermore, systemic and vascular ROS generation modulates inflammatory responses that contribute to microvascular and macrovascular damage (6). Hydrogen peroxide (H_2O_2), one of the ROS, activates protein tyrosine kinases, resulting in stimulation of downstream signaling events that regulate gene expression and in subsequent modification of cardiovascular cells.

Ophiopogon japonicus is a plant, used in traditional Chinese medicine, and widely distributed in Southeast Asia (7). Previous studies have revealed that *O. japonicus* exhibit-santi-inflammatory properties and beneficial cardiovascular effects, including anti-ischemia and anti-arrhythmic effects, inhibiting platelet aggregation, protecting endothelium from apoptosis, and improving microcirculation (8,9). MDG-1, a water-soluble polysaccharide extracted from *O. japonicus*, has been reported to protect cardiomyocytes from hypoxia/reoxygenation-induced damage (10). In addition, MDG-1 presents remarkable anti-ischemic activity and protects cardiomyocytes and human microvascular endothelial cells (HMEC-1) from ischemia-induced cell damage through the sphingosine-1-phosphate/basic fibroblast growth factor/Akt/extracellular signal-regulated kinase (ERK)/endothelial nitric oxide synthase signaling pathway (11).

Human umbilical vein endothelial cells (HUVECs) are the most widely used cell line to study the mechanisms of cardiovascular diseases. Pyruvate protects HUVECs from H_2O_2-induced dysfunction and improves survival following oxidative stress via blocking the mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB pathways (12). Resveratrol protects HUVECs from H_2O_2-induced oxidative stress and senescence via sirtuin1 activation (13). However, although multiple biological functions of MDG-1 have been identified to date, the potential effect of MDG-1 to the H_2O_2-induced endothelial injury has not been explored.

The specific aim of the present study was to evaluate the potential protective effect of MDG-1 in HUVECs under oxidative stress. H_2O_2 was used to induce oxidative stress in HUVECs and to explore the effect of MDG-1 on the endothelium following oxidative stress. The present findings demonstrated that MDG-1 protected HUVECs against H_2O_2-induced apoptosis and inflammation, by inhibiting capspase-3 and BCL2 associated X (Bax)/BCL2 apoptosis regulator (Bcl-2) ratio expression, as well as inhibiting the secretion of inflammatory factors.

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Materials and methods

Cultivation of HUVECs. HUVECs were purchased from the American Type Culture Collection (Manassas, VA, USA). HUVECs were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained in a 37˚C incubator under a humidified atmosphere of 5% CO_2/95% air.

H_2O_2-induced HUVECs injury. Cultured HUVECs were pre-incubated with MDG-1 (5, 10 or 50 mM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 12, 24 and 48 h prior to H_2O_2 treatment (100, 300 or 500 μM). Following the indicated time, the medium was removed, and the cells were subjected to the subsequent experiments.

Cell viability assay. The Cell Counting Kit-8 (CCK-8; Dojindo Molecular technologies, Inc., Rockville, MD, USA) was used to assess cell viability in HUVECs. In brief, HUVECs in the logarithmic growth-phase were collected, and $5x10^4$ cells/well were dispensed into 96-well culture plates with 100 μl culture medium. After 24 h of culture, different concentrations of MDG-1 (5, 10 or 50 mM) were added to each well prior to H_2O_2 treatment (100, 300 or 500 μM). Each of the concentrations above was regarded as one treatment group. Culture plates were then incubated for 4, 6 and 12 h, and cell viability was evaluated by CCK-8, following the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The optical density of indicated groups was used as a surrogate measurement for cell viability.

Apoptosis assay. HUVECs were treated with different concentrations of MDG-1 (5, 10 or 50 mM) prior to H_2O_2 treatment (100, 300 or 500 μM) for 12 h. Cell apoptosis was then analyzed using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. In brief, HUVECs following treatment were washed three times with PBS, trypsinized, centrifuged (400 x g at room temperature) for 10 min, and resuspended to a final concentration of $5x10^4$ cells/ml in binding buffer containing Annexin V-FITC and PI. Apoptotic cells were then analyzed using a BD Accuri C6 flow cytometer equipped with BD Accuri C6 software (version 1.0.264) (both from BD Biosciences).

Detection of ROS. HUVECs ($3x10^3$ cells/well) were cultured on a slide in DMEM and the indicated treatments were performed. Following treatment, the cells were washed three times with PBS, trypsinized, centrifuged (400 x g at room temperature) for 10 min and resuspended to a final concentration of $5x10^4$ cells/ml. The dihydroethidium (DHE; 50 μM; Beyotime Institute of Biotechnology) probe was then added to the cells at 37˚C for 30 min, following which the cells were analyzed by flow cytometry (BD Biosciences).

ELISA. HUVECs ($5x10^4$ cells/well) were seeded in 6-well culture plates and the indicated treatments were performed. The concentration of each secreted inflammatory factor in the cell supernatant was measured by ELISA, according to the manufacturer's protocol. ELISA kits were purchased as following: Human tumor necrosis factor (TNF-α; 070133h; Shanghai WuHao Trading Co., Ltd., Shanghai, China); human interleukin 1β (IL-1β; DL-IL1b-Hu; Wuxi DonglinSci & Tech Development Co., Ltd., Wuxi, China); human IL-6 (ab46042; Abcam, Cambridge, MA, USA); human cyclooxygenase-2 (Cox-2; ESK5229-48T; Sangon Biotech Co., Ltd., Shanghai, China). ELISA kits were used according to the manufacturer's protocol.

Western blot analysis. HUVECs were harvested, washed twice with PBS, and lyzed with radio immuno precipitation assay buffer (Beyotime Institute of Biotechnology) with freshly added 0.01% protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA) on ice for 30 min. Cell lysates were centrifuged at 1,000 x g for 10 min at 4˚C. The supernatant (20-30 μg of protein) was separated on 10% SDS-PAGE and transferred electrophoretically to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The blots were blocked with 5% skim milk overnight at 4˚C, followed by incubation with primary antibodies overnight at 4˚C. Rabbit polyclonal antibodies against Bcl-2 (cat. no. sc-492; 1:150) and Bax (cat. no. sc-493; 1:100) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). A rabbit polyclonal antibody against caspase-3 (cat. no. ab2302; 1:500) was purchased from Abcam. A rabbit monoclonal antibody against GAPDH (cat. no. 5174; 1:1,500) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Blots were then incubated with goat anti-rabbit secondary antibody (Beyotime Institute of Biotechnology) for 1 h at 37˚C and visualized using enhanced chemiluminescence reagents (EMD Millipore). Quantity One 4.62 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to quantitatively analyze protein expression levels.

Statistical analysis. Data were expressed as the mean ± standard deviation of triplicate experiments. One-way analysis of variance, followed by Tukey's post hoc test, was used to analyze the significance of differences between groups with SPSS 19.0 (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

H_2O_2 induces cell death and apoptosis in HUVECs. To identify whether H_2O_2 may cause cytotoxicity *in vitro*, the effect of H_2O_2 on HUVEC viability was determined using the CCK-8 assay. HUVECs were treated with H_2O_2 at concentrations of 100, 300 or 500 μM, and exhibited significantly decreased cell viability in a dose-dependent manner at 6 and 12 h (Fig. 1A). A flow cytometry assay was then used in order to assess the effect of H_2O_2 on HUVEC apoptosis. HUVECs were treated with increasing concentrations of H_2O_2 for 12 h and exhibited a significant dose-dependent increase in the % of apoptotic cells over total, compared with untreated cells (Fig. 1B and C).

MDG-1 protects from H_2O_2-induced cell death and apoptosis in HUVECs. To investigate the effect of MDG-1 on

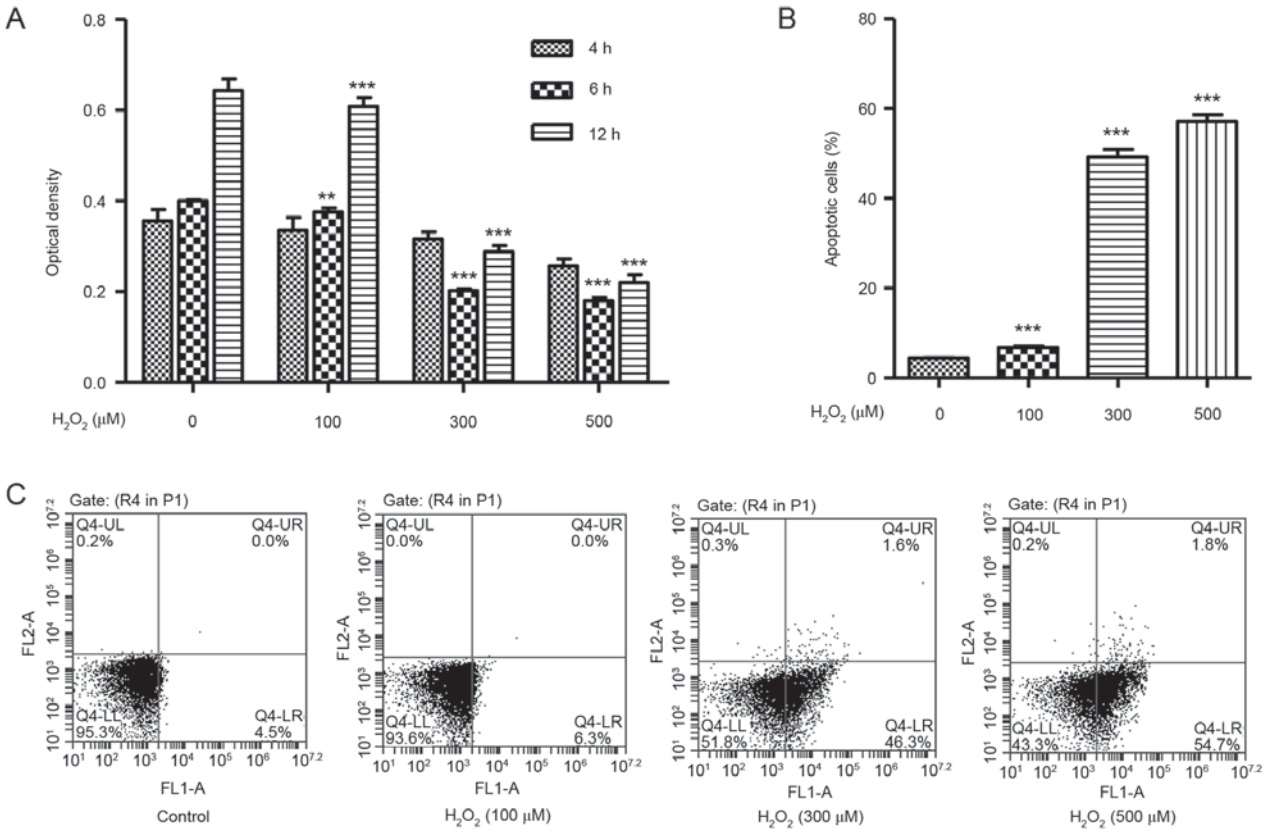

Figure 1. H_2O_2 induces cell death and apoptosis in HUVECs. (A) HUVECs were treated with H_2O_2 at indicated concentrations for 4, 6 and 12 h and cell viability was evaluated using a Cell Counting Kit-8 assay. (B and C) HUVECs were treated with H_2O_2 at indicated concentrations for 12 h and cell apoptosis was assessed by Annexin V-fluorescein isothiocyanate/propidium iodide staining and flow cytometry analysis. $^{**}P<0.01$ and $^{***}P<0.001$ vs. untreated control. H_2O_2, hydrogen peroxide; HUVECs, human umbilical vein endothelial cells.

H_2O_2-induced cytotoxicity, HUVECs were pretreated with MDG-1 at different concentrations prior to exposure to 300 μM H_2O_2 for 12 h. As presented in Fig. 2A, pretreatment of HUVECs with 5, 10 or 50 μM MDG-1 for 12, 24 or 48 h significantly increased cell viability in a dose-dependent manner, compared with cells treated with H_2O_2 alone. In addition, pretreatment of HUVECs with MDG-1 at concentrations of 5, 10 or 50 mM for 24 h significantly decreased cell apoptosis in a dose-dependent manner, compared with cells treated with H_2O_2 alone (Fig. 2B and C). These results indicate that MDG-1 pretreatment protected HUVECs against H_2O_2-induced toxicity.

MDG-1 inhibits H_2O_2-induced ROS generation in HUVECs. To elucidate the possible mechanisms by which MDG-1 prevented H_2O_2-induced HUVEC apoptosis, ROS generation was measured in HUVECs that were treated with H_2O_2 and/or MDG-1. Exposure of HUVECs to 300 μM H_2O_2 for 12 h significantly enhanced ROS generation compared with untreated cells (Fig. 3). However, pretreatment with MDG-1 for 24 h (at concentrations of 5, 10 or 50 mM) significantly attenuated the H_2O_2-induced increase in ROS generation in a dose-dependent manner (Fig. 3). These findings demonstrated that the protective function of MDG-1 on HUVEC H_2O_2-induced toxicity may be associated through its antioxidant effect.

MDG-1 inhibits the H_2O_2-induced secretion of inflammatory factors in HUVECs. To determine the effect of MDG-1 in the inflammatory response of endothelial cells, secretion of the inflammatory factors TNF-α, IL-1β, IL-6 and Cox-2 was measured by ELISA in HUVECs that were treated with H_2O_2 and/or MDG-1. The concentration of TNF-α, IL-1β, IL-6 and Cox-2 was significantly increased in H_2O_2-induced HUVECs, compared with untreated cells (Fig. 4). However, pretreatment with MDG-1 for 24 h resulted in a significant and dose-dependent decrease in TNF-α, IL-1β, IL-6 and Cox-2 secretion, compared with cells induced with H_2O_2 alone (Fig. 4). These results indicated that MDG-1 pretreatment protected against H_2O_2-induced inflammatory responses in HUVECs.

Effect of MDG-1 on expression of apoptosis-related proteins. Western blot analysis was performed to detect the protein expression levels of Bax, Bcl-2 and caspase-3 in HUVECs treated with 300 μM H_2O_2 for 12 h. H_2O_2 treatment decreased the expression of the antiapoptotic protein Bcl-2, while it increased the expression of the proapoptotic proteins caspase-3 and Bax, compared with untreated cells (Fig. 5). Notably, MDG-1 pretreatment at concentrations of 5, 10 and 50 mM markedly reversed the effects induced by H_2O_2 treatment on apoptosis-related protein expression, compared with cells treated with H_2O_2 alone (Fig. 5).

Discussion

Hypoxia-induced injury occurs in many diseases, including sickle cell disease (14), cardiovascular disease (15) and

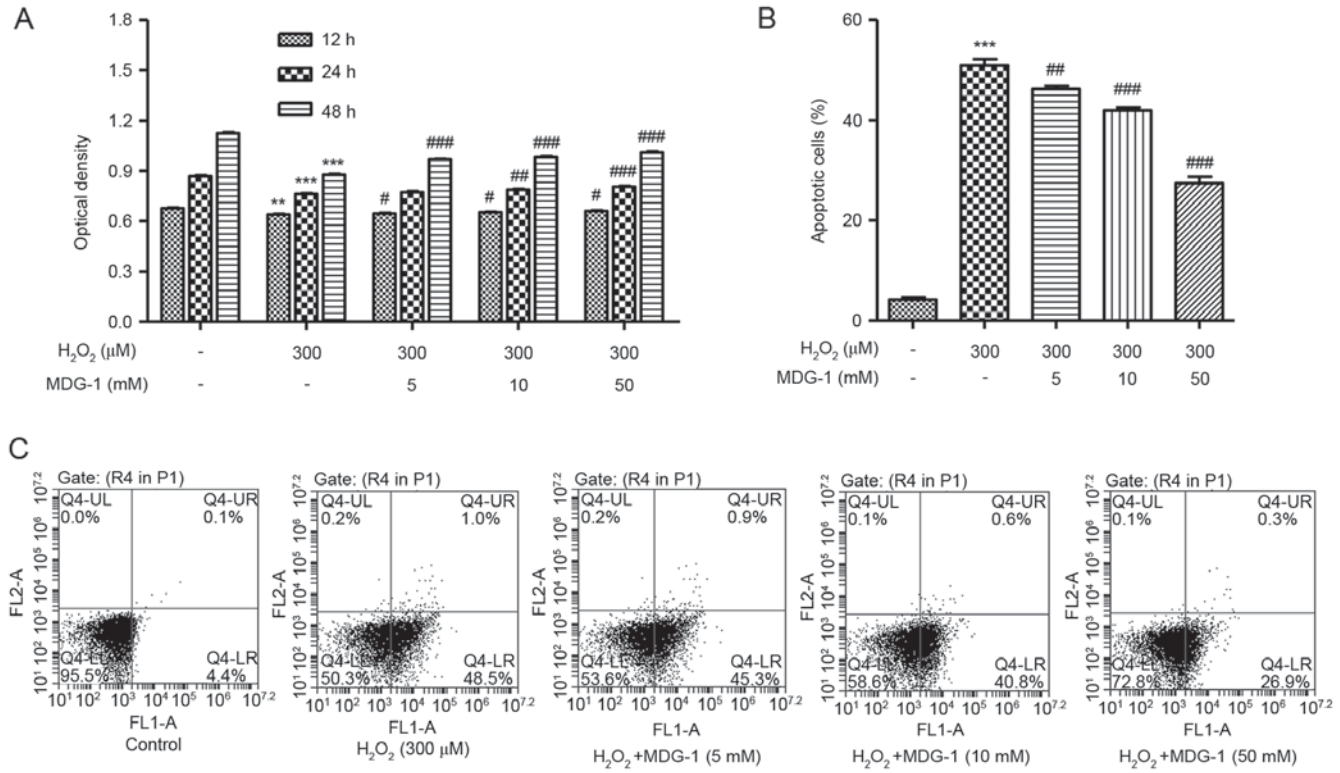

Figure 2. MDG-1 protects HUVECs against H_2O_2-mediated cell death and apoptosis. HUVECs were pretreated with MDG-1 at indicated concentrations for 12, 24 and 48 h prior to treatment with H_2O_2 at 300 μM for 12 h. (A) Cell viability was evaluated using a Cell Counting Kit-8 assay. (B and C) Cell apoptosis was assessed by Annexin V-fluorescein isothiocyanate/propidium iodide staining and flow cytometry analysis. **P<0.01 and ***P<0.001 vs. untreated control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. H_2O_2 treatment alone. HUVECs, human umbilical vein endothelial cells; H_2O_2, hydrogen peroxide.

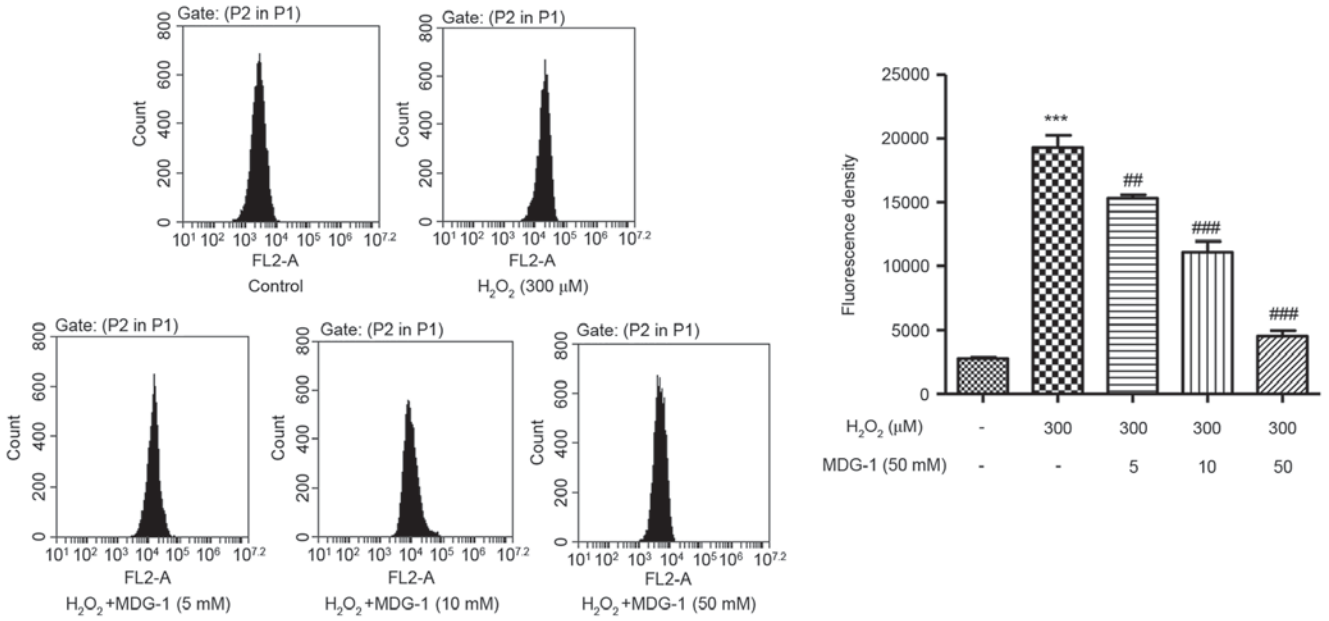

Figure 3. MDG-1 reduces H_2O_2-mediated ROS generation in HUVECs. HUVECs were pretreated with the indicated concentrations of MDG-1 for 24 h prior to treatment with 300 μM H_2O_2 for 12 h. Fluorescence intensity resulting from a ROS probe was measured by flow cytometry. ***P<0.001 vs. untreated control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. H_2O_2 treatment alone. H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; HUVECs, human umbilical vein endothelial cells.

cerebrovascular disease (16). Oxidative stress and increased inflammatory response are two key risk factors for these diseases. MDG-1, a drug extracted from *O. japonicus*, exerts various effects *in vivo*, including anti-ischemic properties (17), cytoprotective and proangiogenic effects (11). Therefore, the present study tested the hypothesis that MDG-1 may confer protective effects against H_2O_2-induced vascular injuries.

To investigate whether MDG-1 mayprotect HUVECs against H_2O_2-induced cytotoxicity, HUVECs were pretreated with MDG-1 at concentrations 5-50 mM for 24 h prior to

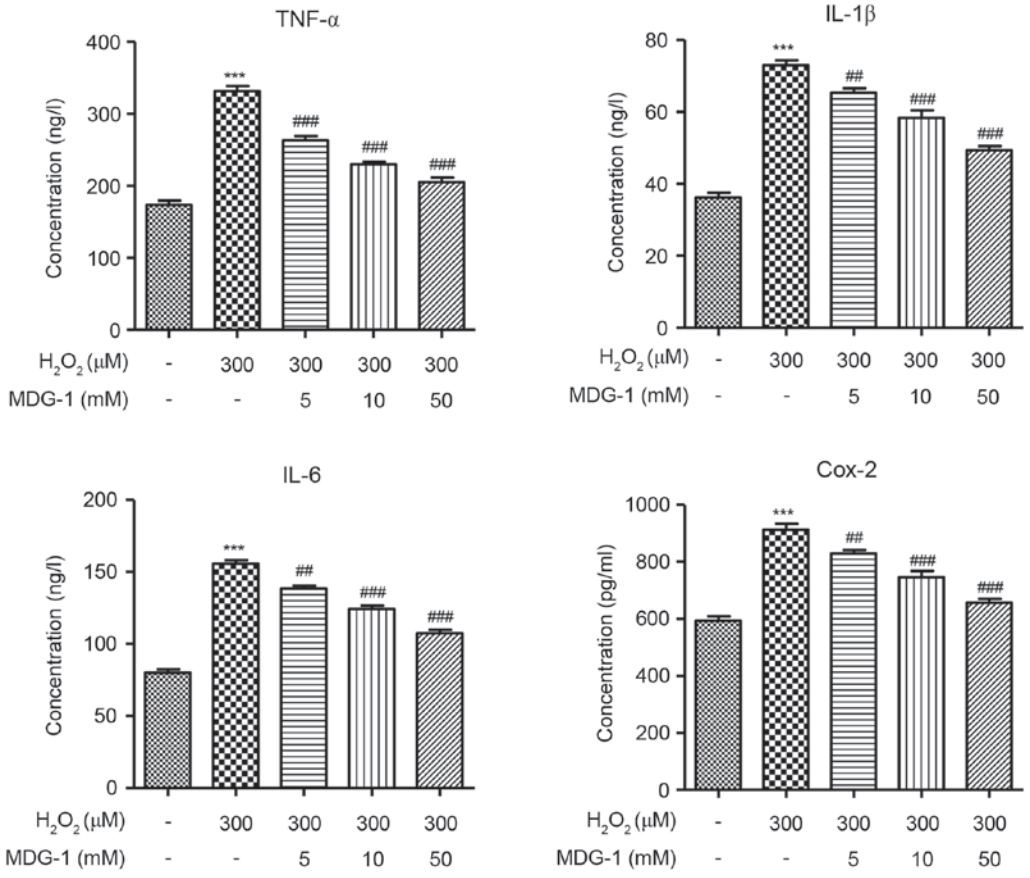

Figure 4. MDG-1 reduces H_2O_2-mediated secretion of inflammatory factors in HUVECs. HUVECs were pretreated with the indicated concentrations of MDG-1 for 24 h prior to treatment with 300 μM H_2O_2 for 12 h. The concentration of TNF-α, IL-1β, IL-6 and Cox-2 was measured in cell supernatants by ELISA. ***P<0.001 vs. untreated control; $^{##}$P<0.01 and $^{###}$P<0.001 vs. H_2O_2 treatment alone. H_2O_2, hydrogen peroxide; HUVECs, human umbilical vein endothelial cells; TNF, tumor necrosis factor; IL, interleukin; Cox, cyclooxygenase.

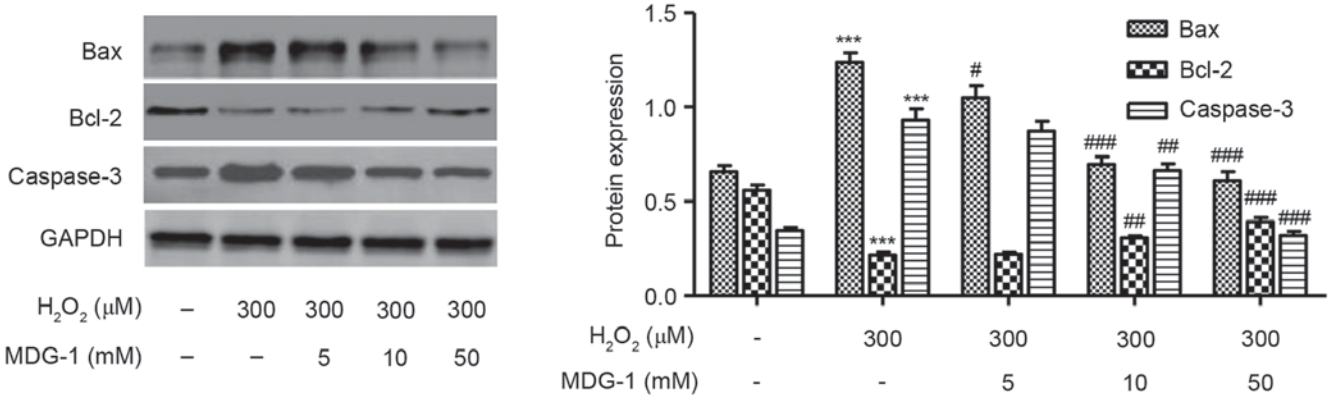

Figure 5. Effect of MDG-1 on expression of apoptosis-related proteins. HUVECs were pretreated with the indicated concentrations of MDG-1 for 24 h prior to treatment with 300 μM H_2O_2 for 12 h. Protein expression levels of Bax, Bcl-2 and caspase-3 were measured by western blot analysis. Representative blot images and quantitative results are depicted, with GAPDH used as a normalization control. ***P<0.001 vs. untreated control; $^{#}$P<0.05, $^{##}$P<0.01 and $^{###}$P<0.001 vs. H_2O_2 treatment alone. HUVECs, human umbilical vein endothelial cells; H_2O_2, hydrogen peroxide; Bax, BCL2 associated X; Bcl-2, BCL2 apoptosis regulator.

exposure to 300 μM H_2O_2. Notably, the results demonstrated that pretreatment with MDG-1 significantly enhanced HUVEC viability and attenuated HUVEC apoptosis induced by H_2O_2. The present results also indicated that the protective effects of MDG-1 were time and dose-dependent. The anticytotoxic and antiapoptotic effect of MDG-1 has also been previously reported in high-fat diet-induced obese C57BL/6 mice (18) and ischemia-induced HMEC-1 cells (11).

Another important finding of the present study was that MDG-1 inhibited oxidative stress induced by H_2O_2 in HUVECs. Treatment with H_2O_2 elicited a marked increase in ROS generation in HUVECs and this increased ROS production was significantly abrogated by pretreatment with MDG-1. The oxidative stress response may be a potential mechanism by which MDG-1 affects the viability of HUVECs, as it has been recently recognized as a mediator of cell apoptosis.

Scavenging of intracellular ROS, such as the hydroxyl radical, or increasing the intracellular levels of reduced glutathione with membrane-permeable antioxidants, significantly blocks apoptosis in endothelial cells (19). Intracellular ROS has been demonstrated to function as a second messenger activating a set of MAPK family members, including ERK1/2 (20), c-Jun N-terminal kinase (21) and p38 MAPK (22). Further studies will be required to elucidate other pathways mediating the inhibition of ROS generation by MDG-1, such as the phosphoinositide 3-kinase/Akt pathway (23).

The inflammatory response is an important contributing factor in H_2O_2-induced injury. Several inflammatory cytokines are induced by H_2O_2 in endothelial cells (24). Ophiopogonin D inhibits H_2O_2-induced secretion of inflammatory factors, such as TNF-α and IL-6, in HUVECs (25). Therefore, H_2O_2 is a powerful proinflammatory mediator in endothelial cells. In the present study, besides cytotoxicity and oxidative stress, H_2O_2 treatment also affected the inflammatory response in HUVECs, as evidenced by an increase in the secretion of TNF-α, IL-1β, IL-6 and Cox-2. Of note, MDG-1 pretreatment significantly attenuated this H_2O_2-stimulated increase in TNF-α, IL-1β, IL-6 and Cox-2 secretion in HUVECs, suggesting that MDG-1 protected HUVECs against H_2O_2-induced inflammatory response. Cox-2 is a potent proinflammatory mediator that promotes the production of multiple inflammatory factors (26). The Huang Qi herb, which is frequently used in traditional Chinese medicine, has been reported to reduce ischemia/reperfusion-induced injury partly by inhibition of Cox-2 (27). In addition, Cox-2 inhibition by NS-398 can confer anti-inflammatory effects, decreasing IL-6 secretion and increasing IL-10 secretion in a liver damage rat model (28). These data suggest that IL-6 and Cox-2 are proinflammatory factors. However, IL-6 confers cytoprotective effects by diminishing oxidant-mediated endothelial cell injury, mediated partly by a signal transducer and activator of transcription 3 and MAPK kinase 1 signaling (29). These results imply that different cell type, disease model and experimental conditions may affect the precise function of IL-6. Thus, the biological effects of IL-6 on H_2O_2-induced inflammation require further research *in vitro* and *in vivo*.

Pretreatment of HUVECs with catalpol increases expression of Bcl-2, decreases expression of Bax, induces Akt activation and BCL2 associated agonist of cell death (Bad) phosphorylation, and ultimately results in reduced H_2O_2-induced apoptosis (30). Among the Bcl-2 family, several members, such as Bcl-2 and Bcl-extra large induce cell survival, while other members, such as Bad and Bax, promote cell death (31). Furthermore, it has been demonstrated that members of the Bcl-2 family, which are located on the mitochondrial membrane, can alter mitochondrial membrane permeability and trigger apoptosis (32). H_2O_2 has been reported to induce cell death in U937 myeloid cells by decreasing the Bcl-2/Bax ratio (33). H_2O_2 has also been reported to induce apoptosis in PC12 rat adrenal pheochromocytoma cells via activation of caspase-3 (34). In the present study, H_2O_2 treatment decreased the expression of Bcl-2, while it increased the expression of the proapoptotic proteins caspase-3 and Bax in HUVECs, compared with untreated cells. Notably, MDG-1 pretreatment markedly reversed these H_2O_2-induced effects.

In conclusion, the present results indicated that MDG-1 may be a potential candidate for preventing oxidative stress-induced damage to endothelial cells. Further studies are required to fully elucidate the potential utility of MDG-1 in protection against cardiovascular dysfunction.

Acknowledgements

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