Halofuginone protects HUVECs from \( \text{H}_2\text{O}_2 \)-induced injury by modulating VEGF/JNK signaling pathway

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Abstract

Background: Halofuginone, which is the main active ingredient of Dichroa fabrifuga, was used to inhibit the synthesis of type I collagen and played increasingly important roles in tumor therapy. This study aims to investigate the protective effects of halofuginone on human umbilical endothelial cells (HUVECs) from \( \text{H}_2\text{O}_2 \)-induced apoptosis and oxidative stress.

Methods: Propidium iodide and Annexin-V double staining assay was used to measure the apoptosis. Cell viability assay, the measurements of reactive oxygen species (ROS) parameters malondialdehyde and superoxide dismutase, western-blot assays, and quantitative PCR were used to elucidate the effects and mechanisms of halofuginone in protecting \( \text{H}_2\text{O}_2 \)-induced injury.

Results: The results showed that halofuginone counteracted \( \text{H}_2\text{O}_2 \)-induced cell viability decline and PCNA downregulation. Furthermore, halofuginone decreased ROS levels and protected HUVECs from \( \text{H}_2\text{O}_2 \)-induced apoptosis. In detail, it showed that \( \text{H}_2\text{O}_2 \) induced a transient activation of Mitogen-activated protein kinases members ERK1/2 and p38, whereas induced a sustained activation of C-Jun N-terminal kinase (JNK), which play dominant roles in triggering apoptosis. Inhibition of JNK activation also inhibited \( \text{H}_2\text{O}_2 \)-mediated apoptosis. Finally, it was shown that halofuginone upregulated VEGF expressions, which functioned by inhibiting sustained JNK activation, thus protecting HUVECs.

Conclusion: Halofuginone has powerful effects in protecting HUVECs from \( \text{H}_2\text{O}_2 \)-induced apoptosis, via upregulating VEGF and inhibiting overactivated JNK signaling pathway. Halofuginone might be a promising preventive drug for cardiovascular diseases.

Keywords: Apoptosis; C-Jun N-terminal kinase; Halofuginone; Oxidative stress; Vascular endothelial cell

1. INTRODUCTION

Cardiovascular diseases (CVDs) have become the major cause of death and illness worldwide.1,2 Vascular endothelial cells have a series of complicated physiological function and play important role in the maintenance of vascular homeostasis.3 Among these diseases, injury of endothelial cells is the first initiating step of pathogenesis and endothelial dysfunction is a major factor that contributes to CVDs.4,5 Oxidative stress has been demonstrated to play important roles in the pathogenesis of CVDs, consisting of superoxides, peroxides, and free radicals.6 Normally, reactive oxygen species (ROS) function as important signaling transducers, whereas overproduction of them lead to the malfunction of various tissues or organs.7,8 Especially in atherosclerosis, lipid peroxidation damage endothelial cells and their functions, inducing endothelial cell apoptosis, stimulating endothelial cell synthesis of platelet activating factor, causing platelet and neutrophils aggregation and promoting inflammation, etc.7,9

Dichroa fabrifuga is a traditional Chinese medicine that has been used in China for hundreds of years with significant antimarial efficacy. Halofuginone (C16H17BrClN3O3) is a halogenated derivative of febrifugine, which is the main active ingredient of Dichroa fabrifuga. Halofuginone has the advantages of increasing the drug efficacy and reducing the gastrointestinal toxicity of febrifugine. Previous reports indicated that halofuginone could regulate cell growth and differentiation, apoptosis, cell migration, and immunity.10,11 Meanwhile, halofuginone has been shown to exhibit promising antioxidant effect.12 Currently, halofuginone has been studied extensively as promising drugs for antifibrosis and antitumor effects.12,13 However, little is known about the effects of halofuginone on cardiovascular system, especially on endothelial cells. This study mainly investigated the protective effects of halofuginone on \( \text{H}_2\text{O}_2 \)-induced apoptosis in vascular endothelial cell and preliminarily explored its molecular mechanisms.

2. METHODS

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Institute of Biochemistry and Cell Biology, CAS (Shanghai, China) and cultured in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA USA) medium containing 10% fetal bovine serum (FBS) and antibiotics at 37ºC under 5% CO2 environment.

2.2. Determination of cytotoxicity by MTT assay

Halofuginone was purchased from Sigma (St. Louis, MO, USA), the purity of halofuginone was ≥95.0% by HPLC. The MTT assay is based on the principle that viable cells convert MTT into an insoluble formazan salt. Briefly, HUVECs were cultured in 96-well plates over night at the density of 1 × 104 per well and were treated with indicated concentrations of halofuginone and/or \( \text{H}_2\text{O}_2 \), (0.5 mmol/l) for indicated time. The reason for using this \( \text{H}_2\text{O}_2 \) concentration was referred to previous reports.11,16 This
concentration will induce a significant cell injury and apoptosis in HUVECs, which will testify the protective effects of halofuginone obviously. Subsequently, the original culture medium was replaced by MTT reagent dissolved in DMEM medium according to the manufacturer’s protocol. Then the plate was incubated for 2 h at 37°C. Finally, the optical density was measured by Fluoroskan Ascent Fluorometer (ThermoFisher, Helsinki, Finland) reader at a wavelength of 490 nm. The percentage growth inhibition was calculated using the following formula:

cell viability (%) = the absorbance of experimental group/ the absorbance of blank untreated group × 100%.

2.3. Malondialdehyde and superoxide dismutase assay
HUVECs were cultured at a density of 2 × 10⁵ per well in six-well plates and then treated for 24 hours with halofuginone (200 nmol/l) before stimulated with H₂O₂ (0.5 mmol/l) for 4 hours. Then assay kits (Jiancheng Bioengineering Institute, Nanjing, China) were used to measure the concentrations of malondialdehyde (MDA) and superoxide dismutase (SOD) in the cell lysates, according to the manufacturer’s protocols.

2.4. Intracellular ROS quantification
The levels of intracellular ROS were determined by the fluorescent probe dihydroethidium (DHE) (ThermoFisher Scientific, Waltham, MA, USA). Briefly, 2 × 10⁵ HUVECs were cultured into six-well plates and were treated with halofuginone and/or H₂O₂ for indicated time, then cells were washed with PBS, then incubated with 10 μmol/l DHE dissolved in DMEM medium for 30 min at 37°C. Subsequently, cells were washed with PBS twice and analyzed by Fluoroskan Ascent Fluorometer (ThermoFisher, Helsinki, Finland).

2.5. Annexin V/PI staining
Cell apoptosis was assessed by measuring membrane redistribution of phosphatidylserine using an Annexin V-FITC apoptosis detection kit (BD Bioscience, Franklin Lakes, NJ, USA) according to the manufacturer’s protocol. Briefly, after treatment, HUVECs were washed twice with PBS, resuspended in 250 μl of binding buffer, and stained with staining solution containing Annexin V-FITC and PI. After incubation in the dark for 30 min, cells were analyzed by FACSCalibur flow cytometer.

2.6. Western blotting assay
Western blots were performed as previously reported. After treatment, the cells were lysed to extract the whole proteins, which were separated in 12% SDS-PAGE and then transferred to a PVDF membrane; the target proteins were detected with different antibodies (4°C overnight). The following primary antibodies were used: antiproliferating cell nuclear antigen (PCNA), anti-Caspase3, anti-Bax, anti-VEGF, anti-p-akt (Abcam, China), anti-p-ERK1/2 anti-ERK1/2, anti-p-P38, anti-P38, anti-p-JNK and anti-JNK (Cell Signaling Technology, Boston, MA, USA), and anti-Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing off the primary antibodies, the PVDF membrane was incubated with HRP-conjugated secondary antibody (Zhongshan, Beijing, China) for 1 hour at 37°C; ECL kit was used to develop the immunoreactive bands. Band intensities in the immunoblots were quantified by densitometry using the Image J software.

2.7. Quantitative real-time PCR
The mRNA levels of human VEGF in HUVECs were detected using SYBR Green Mix (Bio-Rad, Hercules, CA, USA) and an iQTM5 system (Bio-Rad). The experiment has been repeated for three times and been normalized to GAPDH mRNA. The primers used are as follows: VEGF: Forward (5’-ACTTGGACCCGGCTTACTGCT-3’) and Reverse (5’-TGATCCCGCATGCTGACTGGTG-3’) and GAPDH: Forward (5’-AAGGCCGGGGCCACTTGAA-3’) and Reverse (5’-GAACCTGTGGCATGAGCCTTCCA-3’).

2.8. Statistical analysis
All data were normalized to control values of each assay and were presented as mean ± standard error of mean (SEM). Data were analyzed by one-way ANOVA followed by a Bonferroni’s post hoc tests by Graphpad Prism 6 (La Jolla, CA, USA). A two-sided p < 0.05 was considered as statistically significant.

3. RESULTS
3.1. Halofuginone protected HUVECs from H₂O₂-induced injury
The chemical structure of halofuginone was shown in Figure 1A, to show the extent of lipoperoxidation on cell membrane, which could interfere normal cell function by interacting with phospholipid protein and depositing inside the cells. SOD is an antioxidant enzyme to prevent injuries from ROS. It showed that halofuginone intervention significantly attenuated H₂O₂-induced decrease of cellular viability (Figure 1C, p < 0.001). Moreover, the expression levels of another parameter, PCNA, was used, which could also reflect the levels of cell proliferation and viability. It was demonstrated that H₂O₂ induced a significant decrease of PCNA expression level, whereas halofuginone treatment partially attenuated H₂O₂-mediated down-regulation of PCNA (Figure 1D). These findings suggested that halofuginone protected HUVECs from H₂O₂-induced injury.

3.2. Halofuginone protected HUVECs by counteracting H₂O₂-induced apoptosis
Then, we investigated the mechanisms of halofuginone-mediated protection of HUVECs. At first, we measured the ROS levels and corresponding parameters of ROS. MDA is an index of the extent of lipid peroxidation on cell membrane, which could interfere normal cell function by interacting with phospholipid protein and depositing inside the cells. SOD is an antioxidant enzyme to prevent injuries from ROS. It showed that halofuginone intervention significantly decreased ROS levels indicated by DHE. Meanwhile, halofuginone protected HUVECs by decreasing the levels of MDA and upregulating the levels of SOD (Figure 2A-C). Furthermore, we measured the apoptosis rates by flow cytometry of HUVECs upon these treatments. It showed in Figure 2D that H₂O₂ treatment induced a significant increase of apoptosis rates, which meant increased cell injury and cell death of HUVECs, while halofuginone intervention reduced the apoptosis rates, indicating protection against ROS-induced injury. In addition, we detected the expression levels of several important proteins in apoptosis pathway by Western blotting. It showed in Figure 2E that H₂O₂ treatment induced upregulation of proapoptotic cIaspase 3 and Bax, while induced downregulation of anti-apoptotic Bcl-2 protein.

3.3. Halofuginone protected HUVECs by inhibiting JNK overactivation-induced apoptosis by H₂O₂
Next, we determined the detailed mechanisms of halofuginone- conferred protection. Mitogen-activated protein kinases (MAPKs) have been shown to play important roles in the maintenance of cellular functions, especially in cell survival and death. It has been shown in Figure 3A that H₂O₂ treatment caused increased phosphorylations of extracellular regulated kinase 1/2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK) in a relative short time (0.5 and 2 hours), while at the time of 24-hour treatment, the phosphorylations of JNK increased and ERK1/2 and p38 decreased. Meanwhile, a ROS scavenger, NAC could attenuate the activation of all these
Fig. 1 Effects of halofuginone treatment on the cell viability of HUVECs. A. The chemical structure of halofuginone and corresponding molecular formula. B. Cell viability assay of HUVECs treated with different concentrations of halofuginone (0, 50, 100 and 200 nmol/l). The optical density values were detected after 48 hours of treatment. C. Cell viability of HUVECs treatment with 0.5 mmol/l H₂O₂ and 200 nmol/l HF plus H₂O₂ for 24 hours. Cell viability was quantified by MTT assay; ***p < 0.001. D. The expression levels of PCNA upon treatments with H₂O₂ and HF plus H₂O₂ for 24 hours. Three Western blots were quantified by Image J software and were analyzed in the right panel; ***p < 0.001. The results were expressed as the mean ± SEM of three independent experiments.

Fig. 2 Halofuginone protected HUVECs by counteracting H₂O₂-induced apoptosis. A–C. Cells were treated with or without 200 nmol/l Halofuginone for 24 hours, then stimulated with 0.5 mmol/l H₂O₂ for 4 hours. The relative levels of ROS, the concentrations of MDA and SOD of cell lysates were detected; *p < 0.05, **p < 0.01, ***p < 0.001. D. The cells were treated as [A], then the apoptosis rate of HUVECs were detected by flow cytometry; **p < 0.01, ***p < 0.001. E. The cells were treated as [D], then the cleaved-caspase 3, Bcl-2, and Bax were detected by Western blot with β-actin as the loading control. The right panels were the quantitative analysis with three replicates. Data are expressed as means ± SEM from three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001.
MAPKs (Figure 3A). This result showed a persistent activation of JNK and transient activation of ERK and p38, indicating that over-activation of JNK might play a role in the damage caused by \( \text{H}_2\text{O}_2 \). Therefore, using JNK specific inhibitor, SP600125, we found that inhibition of JNK activation could partially reverse the cellular injury by \( \text{H}_2\text{O}_2 \), indicated by cell viability assay and apoptosis counting (Figure 3B, C). Meanwhile, the treatment of SP600125 also partially attenuated the decreased expression levels of PCNA, induced by \( \text{H}_2\text{O}_2 \) (Figure 3B, C). More importantly, we investigated whether halofuginone would inhibit the overactivation of JNK, since it could suppress the ROS levels. It showed in Figure 3E that halofuginone treatment could significantly inhibit the overactivated JNK, while activated ERK1/2 and had little effects on p38 activation. These results indicated that overactivated JNK played dominant roles in initiating apoptosis, induced by \( \text{H}_2\text{O}_2 \), while halofuginone protected endothelial cells by inhibiting JNK phosphorylation.

3.4. Halofuginone-mediated VEGF upregulation exerted protective roles against \( \text{H}_2\text{O}_2 \) in HUVECs

Vascular endothelial growth factor (VEGF) is an important growth factor that confers protective effects on endothelial cells by activating the VEGF receptors-related signaling pathways. In Figure 4A, B, it was shown that halofuginone intervention upregulated both the mRNA and protein levels of VEGF. To test whether halofuginone exerted protective effects by or partially by upregulating VEGF, we used VEGF to test whether it would protect HUVECs from cell injury. It showed in Figure 4C and Figure 4D that addition of VEGF could significantly attenuate \( \text{H}_2\text{O}_2 \)-induced cell viability decrease and apoptosis increase. Moreover, it showed that VEGF could also partially reverse the down-regulation of PCNA and the upregulation of cleaved caspase 3 by \( \text{H}_2\text{O}_2 \) and inhibited the overactivated JNK phosphorylation (Figure 4E). More importantly, to verify whether halofuginone exerted protective effects by or partially by

![Fig. 3 Halofuginone protected HUVECs by inhibiting JNK overactivation-induced apoptosis by \( \text{H}_2\text{O}_2 \). A, HUVECs were treated with or without 10 mmol/l NAC, then treated with 0.5 mmol/l \( \text{H}_2\text{O}_2 \) for different time. The expression levels of p-ERK1/2, ERK1/2, p-p38, p38, p-JNK, and JNK were detected by Western blot. The quantitative analyses were presented in the right panels; ** \( p < 0.01 \), *** \( p < 0.001 \). (B, C), HUVECs were pretreated with 10 mmol/l NAC or 10 mmol/l JNK inhibitor SP600125, then 0.5 mmol/l \( \text{H}_2\text{O}_2 \) was added for 24 hours. After treatment, the harvested cells were analyzed with cell viability assay and flow cytometry for apoptosis. Increased apoptosis in the upper right quadrant indicated enhanced cell injury and death; ** \( p < 0.01 \), *** \( p < 0.001 \). D, The expression profiles of PCNA were detected by Western blot, ** \( p < 0.01 \), *** \( p < 0.001 \). E, The phosphorylation changes of MAPK members were detected by Western blot, with HUVECs treated with 0.5 mmol/l \( \text{H}_2\text{O}_2 \) and \( \text{H}_2\text{O}_2 \) plus 200 nmol/l halofuginone. The bands were quantified and analyzed in the right panels; *** \( p < 0.001 \).]
upregulation of VEGF, we used VEGF specific inhibitor, Cediranib (AZD2171). It showed in Figure 4F, G that addition of VEGF inhibitor significantly decreased the protective effects of halofuginone, indicated by cell viability assay and apoptosis counts. Meanwhile, addition of VEGF inhibitor partially blunted the antiapoptotic effects of halofuginone, indicated by increased expression of cleaved caspase 3, decreased expression of PCNA, compared with halofuginone treatment alone (Figure 4H, I). These results further verified that halofuginone protected endothelial cells by or partially by upregulating VEGF.

4. DISCUSSION

CVD is a group of chronic lipid-driven inflammatory diseases characterized by accumulation of peroxidized lipids in arterial walls, which can lead to a heart attack or stroke. Endothelial cells are the first to be injured during the pathogenesis of atherosclerosis. Therefore, protection of endothelial cells from oxidative stress is very important. In our study, we showed that halofuginone protected HUVECs from H₂O₂-induced apoptosis. Meanwhile, it showed that overactivation of JNK played dominant roles in ROS-mediated apoptosis and halofuginone conferred protective effects by inhibiting JNK activation, while had little effects on ERK and p38 phosphorylation. Moreover,
we found that halofuginone-mediated VEGF upregulation is or partially responsible for the protective effects upon \( \text{H}_2\text{O}_2 \) intervention. Our study elucidated the protective effects and corresponding mechanisms of halofuginone on endothelial cells, suggesting that halofuginone might be a potent antagonist for ROS-induced damage.

Currently, ROS has been shown to play important roles in both physiological and pathophysiological conditions. ROS mainly consists of superoxide (\( \text{O}_2^- \)), peroxides (\( \text{H}_2\text{O}_2 \) and \( \text{ROOH} \)), and free radicals (\( \text{HO}^- \)and \( \text{RO}^- \)). Overproduction of ROS has been shown to play important roles in the pathogenesis of multiple diseases. Therefore, extinguishing ROS has been used widely in the preclinical and clinical settings. In our study, we demonstrated that halofuginone effectively reduced MDA levels, a biomarker of oxidative stress, while simultaneously increasing the activity of SOD, an antioxidant enzyme. MDA levels could reflect the severity of attack in cells by free radicals, and SOD activity levels reflect the capability of scavenging oxygen free radicals. Therefore, these findings suggest that halofuginone protects HUVECs by preventing oxidative stress. In addition to increasing antioxidant activity, halofuginone has been shown to function in antioxidant, antinflammation, and antiapoptosis effects. Recently, halofuginone has been shown to stimulate adaptive remodeling and enhances endothelial-to-mesenchymal transition in balloon-injured carotid arteries. However, the mechanism is not clear. In our study, we clearly demonstrated the effects and mechanisms of halofuginone in protecting endothelial cell from ROS-mediated injuries.

Apoptosis, a form of programmed cell death, is directly or indirectly regulated by complicated pathways in the cells. Apoptosis plays an important role in tissue remodeling, aging, and immune response, while irreversible damage and abnormal apoptosis may be the cause of many diseases. Apoptosis is tightly regulated in the cells. The canonical pathway that triggers apoptosis is the Bcl-2/Bax/Bak pathway. The antiapoptotic protein Bcl-2 is found located at both the cytoplasm and the mitochondria and protects cells from apoptosis by binding to the proapoptotic Bax, Bak, and the BH3-only proteins. If Bcl-2 was downregulated or if it binds with BH3-only proteins, Bax and Bak will experience oligomerization and damage mitochondria, therefore triggering apoptosis. In our study, we showed that \( \text{H}_2\text{O}_2 \) treatment downregulated Bcl-2 protein and upregulated Bax protein, while halofuginone intervention reversed the downregulation of Bcl-2. Furthermore, it has been reported that Bcl-2 was regulated by multiple signaling pathways, and Bcl-2 was considered as the key protein in controlling cell fate, since it is the center of cell death, autophagy, and oxidative stress. The reasons for the changes of Bcl-2 may attribute to the changes of JNK activation, because sustained JNK activation promoted the release of cytochrome C and the cleavage of caspase-3, which results in apoptosis. It was shown in our study that \( \text{H}_2\text{O}_2 \) induced a persistent JNK activation, inhibition of JNK by \( \text{SP600125} \) could significantly attenuate \( \text{H}_2\text{O}_2 \)-mediated apoptosis. Furthermore, halofuginone-mediated VEGF upregulation also inhibited JNK phosphorylation. Therefore, inhibition of JNK by halofuginone might be the key mechanism of protection against \( \text{H}_2\text{O}_2 \) injuries.

VEGFs and their receptors (VEGFRs) have emerged as the principal drivers of angiogenesis and lymph-angiogenesis, and hence the development and maintenance of both of these vascular systems. Now, VEGF/VEGFR signaling have been considered essential in the pathogenesis of CVD. Activation of VEGF-R2 by VEGF leads to stimulation of various intracellular signaling cascades, including activation of the ERK1/2 and p38 MAPK pathways, which mainly confer protective effects on endothelial cells. In our study, we showed that VEGF supplementation decreased \( \text{H}_2\text{O}_2 \)-induced apoptosis and inhibited overactivated JNK. Meanwhile, halofuginone treatment activated ERK1/2, which might correlate with VEGF upregulation and conferred protective effects. However, in our study, we did not further investigate the mechanism of VEGF upregulation upon halofuginone treatment. These results demonstrate that halofuginone protects HUVECs from apoptosis and elucidate a new pathway.

In conclusion, halofuginone has powerful effects in protecting HUVECs from \( \text{H}_2\text{O}_2 \)-induced apoptosis, upregulating VEGF and inhibiting overactivated JNK phosphorylation. These findings suggest that halofuginone might be a potent antioxidant agent and promising preventive drug for CVDs. Further studies will be necessary to determine the exact effects of halofuginone on cardiovascular disease.

**REFERENCES**


