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Protective effects and plausible mechanisms of velvet polypeptide against hydrogen peroxide induced injury in human umbilical vein endothelial cells

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Running title: Velvet polypeptide against HUVECs injury
Abstract:
Velvet antler polypeptide (VAP) is a prominent bioactive component of velvet antlers. Whereas, uncharacterized crude extracts were usually employed in pharmacological studies. In this study, the velvet polypeptide was isolated and purified by acid water extraction, ethanol precipitation, and ammonium sulfate fractionation and precipitation, and chromatography progressively. Human umbilical vein endothelial cells (HUVECs) were induced by H₂O₂ followed purified polypeptide treatment. Cell viability was evaluated by MTT assay. The apoptosis of cells was detected by fluorescence microscopy and flow cytometry. A cell analyzer was used to measure the mitochondrial membrane potential. The intracellular reactive oxygen species (ROS) levels were determined by flow cytometry. The oxidative stress related biochemical parameters were detected, and the expression of apoptosis related proteins was examined by Western blot analysis. Results indicated that a 7.0 kDa polypeptide (VAP II) was isolated from velvet antler. VAP II could enhance cell viability, decrease the cell apoptosis, reverse depolarization of mitochondrial membrane potential, decrease ROS, inhibit oxidative stress, and regulate downstream signaling apoptotic cascade expression caused by H₂O₂. The protective effects of VAP II on HUVEC injury provided a potential strategy for cardiovascular disease treatment.

Key words: Velvet antler polypeptides, Oxidative stress, Antioxidant, Apoptosis
Introduction
Recently, the benefits of natural products have become the limelight in traditional and local medicines. To date, the majority of studies conducted worldwide focused on plant-based medicines, whereas animal-based medicine was neglected (Alves and Alves 2011; Cordell and Colvard 2012). By contrast, various import components are available in the animal-based medicines, such as proteins, peptides, polysaccharides, nucleic acids, and vitamins, which can be used in disease treatment (Blunt et al. 2013). Few investigators have focused on the animal-based medicines to date. As one kind of traditional animal-based medicine, velvet antlers are one of the main products from Sika deer (*Cervus nippon*) widely used as a tonic for the treatment of various diseases traditional Chinese medicine for thousands of years (Sui et al. 2014). Lujiaopan soup has been used to treat mammary hyperplasia and mastitis. Lujiaopan chewable tablets have been used in immune dysfunction, amnesia, and osteoporosis. Pangengushen granules have been used in fracture healing, osteoporosis, and menopausal syndrome (Wu et al. 2013). Accordingly, various pharmacological effects, such as anti-inflammatory, anti-oxidant, anti-lipid peroxidation properties, anti-aging activities, and evaluating bone fracture healing, have been identified to date (Wu et al. 2013). The content of proteins and polypeptides in the velvet antlers is more than 50%, which is considered as the most prominent bioactive components. A study has demonstrated that total velvet antler polypeptides (VAPs) are effective in preventing bone loss by inhibiting IL-1 and IL-6, and by promoting mitosis in anti-osteoporotic experiment (Zhang et al. 2013). Recently, Chen et al. investigated the protective effect of extraction from velvet antlers on acute ischemic myocardial injury induced by isoproterenol in rats and demonstrated that the extraction exerted significant cardio-protective effects on rats. The underlying mechanism might be through the antioxidant and anti-lipid peroxidation properties (Chen et al. 2009). The VAP antioxidant activity was essential for its cardiovascular protective function.

Most pharmacological studies are conducted using uncharacterized crude extracts of deer antler base. In general, reproducing the results of these studies and pinpointing the bioactive compounds are difficult. In a previous report, a 3.2 kDa polypeptide was
purified from the velvet antler of a red deer by ion exchange chromatography, gel filtration, and RP-HPLC, which could stimulate the growth of rat epidermal cells and rabbit costal chondrocytes in a dose-dependent manner (Zhang et al. 2013). Hence, studies on the extraction and isolation of bioactive components of velvet antlers, as well as the physiological mechanisms associated with therapeutic effects, should be given considerable attention to make velvet antler products acceptable as curative medicines. Velvet antlers are rich in amino acids, polypeptides, and proteins, which are considered as the most prominent bioactive components. Isolation and purification of bioactive components from velvet antlers might lead to the discovery of new active molecules that can be investigated for their bioactivities. In the study, we purified the VAP from velvet antlers and described its role in improving resistance of HUVEC to H2O2-induced damage.
Materials and Methods

Materials

Pilose antler (*Cervus nippon*) was purchased from the Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences. Sephadex G-25 Fine were obtained from Amersham Pharmacia (Piscataway, New Jersey, USA). Hoechst 33258 were purchased from Sigma (St. Louis, MO, USA). Muse Annexin V & Dead Cell Kit (Merck Millipore, Germany). Super oxide dismutase (SOD), glutathione peroxidase (GSH), and malondialdehyde (MDA) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Jiancheng Bioengineering Institute, Nanjing, China). Anti-cleaved-caspase-3, cleaved-caspase-9, anti-Bax, anti-Bcl-2, anti-cytochrome c and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit and anti-mouse IgG-HRP were purchased from Beyotime Institute of Biotechnology (Beyotime Institute of Biotechnology, Shanghai, China). All the other reagents were of chemical analytical grade.

Purification of Velvet Antler Polypeptides

Purification of crude VAP was described by Guan (Guan et al. 2006). The pilose antler (2 kg) were cut into small pieces and washed with cold distilled water to remove blood. The pieces were ground with cold acetic acid solution (pH 4.0) using a colloidal mill. The supernatant was obtained by centrifugation at 10,000× g, 4 °C for 30 min, and 95% ethanol was added to the supernatant to make the final ethanol concentration of 65% (v/v). After overnight at 4 °C, the mixture was centrifuged again. The supernatant was evaporated under reduced pressure at 55 °C and the residue was dissolved in distilled water.

The above supernatant was fractionated by precipitation with ammonium sulfate at 30% saturation, followed by 60% saturation. The precipitate obtained from 30% saturation of ammonium sulfate was discarded, and the supernatant was further brought to 60 % saturation of ammonium sulfate. The collected precipitate from 60 % saturation of ammonium sulfate was re-dissolved in 2 ml buffer (20 mM PBS, pH 7.2). Then, the supernatant proteins were collected by centrifugation at 10,000× g for 10 minutes at
4°C. The supernatant was loaded to a 1.6×90 cm Sephadex G 75 column chromatography pre-equilibrated with buffer (20 mM PBS, pH 7.2) to purify the velvet antler polypeptides. Fractions were collected and their absorbance was recorded at a wavelength of 280 nm. The purification of Velvet antler polypeptides was analyzed using Tricine-SDS-PAGE described by Schagger (Schägger 2006). The protein concentrations in the samples were determined with Bradford protein assay.

**Cell Culture**

HUVECs was purchased from the China Center for Type Culture Collection (Wuhan, China) and cultured in DMEM supplemented with 10% FBS, 100 units/ml of penicillin and 100 units/ml of streptomycin (Life Technologies, Carlsbad, CA USA) at 37°C in a humidified atmosphere of 5% CO₂. HUVECs were grown to approximately 80% confluence, maintained with fresh medium as described above, and subcultured every 2 to 3 days. The cells were used within passages 4 to 9 during these experiments.

**Cell Viability Assay**

Cell viability was measured with the MTT cytotoxicity assay as described (Harries et al. 2001). The HUVECs were treated with VAP (25, 50, 100 and 200 µM) for 12 h before testing for the presence of 100 µM H₂O₂ for another 2 h, VE were used as the control. Each treatment condition was tested in 5 replicate wells. At the end of the treatment, cells were incubated with 100 µl of 0.5 mg/ml MTT at 37°C for 4 h. Then 100 µl DMSO was added to each well. Absorbance of each well was detected at 450 nm using a 550 micro plate reader (Bio-Rad, Richmond, CA, USA). The results are expressed as the percentage of MTT reduction relative to the absorbance of control cells. All experiments were performed in triplicate.

**Morphological Examination for Hoechst 33258 Staining**

HUVECs were seeded on slides at a density of 5×10⁴/ml in 6-well plates. After treatment as mentioned above, cells of all five groups were washed twice with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 10 min, and then stained with Hoechst 33258 for 10 min. After the cells were washed twice with PBS, they were observed under a fluorescence microscope. The nuclei of living cells were
homogeneous blue; those of apoptotic cells were compact, condensed, and whitish blue. (Olympus, Shinjuku, Monolith, Tokyo, Japan).

**Flow Cytometric Evaluation of Apoptosis**

The HUVECs was collected after treatment and adjusted to $1 \times 10^6$ cells/ml. A total of 100 µl cell suspension was taken and mixed 100 µl Muse Annexin V & Dead Cell reagents in the dark at room temperature for 20 minutes. Apoptosis was analyzed using Muse Cell Analyzer (Merck Millipore, Germany).

**Mitochondrial Membrane Potential Measurements**

Mitochondria transmembrane potential ($\Delta$ψm) was detected under NucleoCounter NC-3000 Cell Analyzer (ChemoMetec A/S, Denmark). About $1 \times 10^6$ cells were harvested by trypsinization, washed twice with PBS, and resuspended in 1 ml PBS. The cells were incubated with 12.5 µl JC-1 (final JC-1 concentration of 200 µg/ml) for 10 minutes at 37°C. Cells were washed twice with PBS and incubated with 0.25 ml DAPI (final DAPI concentration of 1 µg/ml). Cells were subsequently pelleted by centrifugation (5000 rpm for 5 minutes at 4°C) and re-suspended in 1× phosphate buffered saline (PBS). Mitochondrial membrane potential was detected immediately under Nucleo Counter NC-3000 Cell Analyzer. Prior studies demonstrate a linear relationship between red: green ratio of JC-1 fluorescence and membrane potential over a physiological range. A higher red: green ratio indicates a more polarized, or more negative and hyperpolarized mitochondrial inner membrane (Smiley et al. 1991).

**Determination of Intracellular ROS Production**

Intracellular ROS production was measured by flow cytometry. Following treatment, cells were harvested, washed twice with PBS, and incubated with 10 µM 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) in the dark for 30min. Finally, the cells were analyzed for DCF fluorescence by flow cytometry.

**Measurements of Intracellular SOD, GSH and MDA Contents**

At the end of the treatment, the HUVECs were harvested and sonicated with phosphate buffer (pH 6.8). The homogenates were centrifuged at 4,000 rpm at 4°C for 20 min. MDA, SOD, and GSH were measured in the supernatants with the
commercially available assay kits as the procedures completely described with the manufacturer’s instructions (Jiancheng Bioengineering Institute, Nanjing, China). The activity of the enzymes was expressed as U/mg protein. The MDA content was measured at a wavelength of 532 nm by reaction with thiobarbituric acid (TBA) to form a stable chromophore. The SOD activity assay was based on its ability to inhibit the oxidation of hydroxylamine by the O$_2^-$ produced from the xanthine-xanthine oxidase system. One unit of SOD activity was defined as the quantity that reduced the absorbance at 550 nm by 50%. GSH was determined by a commercial kit based on the reaction with 2, 2'-dinitro-5, 5'-dithio-benzoic acid to yield a chromophore with a maximum absorbance at 412 nm.

**Western Blotting Analysis**

For immunoblot analyses, 40 µg of protein lysates per sample were denatured in 4× SDS-PAGE buffer (Tris-HCl 260 mM, pH 8.0, 40% (v/v) glycerol, 9.2% (w/v) SDS, 0.04% bromophenol blue and 2-mercaptoethanol as reducing agent) and subjected to SDS-PAGE on 12% acrilamide/bisacrilamide gels. Separated proteins were transferred to nitrocellulose membrane (Hybond-P PVDF, Amersham Bioscience). Residual binding sites on the membrane were blocked by incubation in TBST (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) with 5% (w/v) nonfat milk powder overnight at 4 °C. Membranes were then incubated with specific primary antibodies at a dilution of 1:1000 for 16 h at 4°C and with the appropriate peroxidase-linked secondary antibody with a dilution of 1:5000 for 1 h at room temperature. Chemiluminescence signals visualized with an ECL ultra-sensitive light-emitting liquid and quantified by Quantity One software (Bio-Rad, Hercules, CA, USA).

**Statistical Analysis**

All results are presented as mean±SD. Statistical analysis of the data were performed with one-way analysis of variance (ANOVA) followed by Tukey’s test. In all cases differences were considered at $P<0.05$. 

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Result

Isolation and Purification of VAP

The crude VAP was extracted by combination of acid water extraction, ethanol precipitation and ammonium sulfate fractionation precipitation method. Specifically, the crude velvet antler polypeptide was loaded onto a Sephadex G-75 column chromatography. Next, the chromatogram showed three peaks and the peak II (VAP II) were harvested for the following experiments and analyzed by Tricine-SDS-PAGE (Supplemental Fig.1). Notably, the purified VAP II migrated as a single band and the molecular weight was estimated as 7.0 kDa on Tricine-SDS-PAGE (Supplemental Fig. 2). The final yield of VAP II is about 1‰.

VAP II inhibits H$_2$O$_2$-induced Cytotoxicity in HUVECs

Initially, we examined the cytotoxicity of VAP II on HUVECs using the MTT assay. VAP II did not show cytotoxic effects up to 200 mg/l (Fig. 1 A). Then we further evaluated whether VAP II had protection effect. To evaluate whether VAP II has protective effect against oxidative stress, HUVEC cells were pretreated with increasing dose of VAP II for 12 h followed by 2 h of 100 µM H$_2$O$_2$ treatment. After H$_2$O$_2$ treatment, cell viability of each group was measured by MTT assay. The MTT results showed that the H$_2$O$_2$ significantly inhibited the viability of control cells without VAP II treatment. Specifically, H$_2$O$_2$ incubation led to a 57% decrease in cell viability (Fig.1 B). However, as shown in Fig.1 B, pre-treatment of cells with VAP II partially compromised the effects of H$_2$O$_2$-induced cytotoxicity in a dose-dependent manner. But in the same dose, the antioxidant activity of VE is better than VAP II (Supplemental Tab 1). These results suggested that VAP II protected the HUVECs from oxidative stress-induced cell injuries.

VAP II Prevented H$_2$O$_2$-induced Apoptosis

To investigate the preventive effect of VAP II on cell apoptosis, we stained the HUVECs with and without treatment with Hoechst 33258. Hoechst 33258 is an apoptosis marker, which can reflect apoptotic nuclei with condensed and/or fragmented DNA. The uniform morphous of nucleus and well-distributed deep blue
fluorescence are indicated through Hoechst 33258 staining. As shown in Fig. 4, without 
H₂O₂ induction, cell nuclei were uniform blue chromatin with organized 
structure (Fig. 2 A). On the contrary, after incubation with 100 µM H₂O₂ for 2 h, 
HUVECs showed typical features of apoptosis (Fig. 2 B). Moreover, VAP II 
pretreatment reduced H₂O₂-induced apoptosis, demonstrating very few apoptotic 
nuclei (Fig. 2 C, D and E). The data showed that the apoptotic index increased 
dramatically in cells stimulated by H₂O₂, but VAP II could improve the apoptosis 
morphous of HUVECs.

**VAP II Compromised H₂O₂-induced apoptosis in HUVECs**

In order to get quantitatively insight into anti-apoptotic effects of VAP II in 
H₂O₂-induced HUVECs, after treatment with 100 µM H₂O₂ for 2 h, we measured the 
apoptosis rate of HUVECs by Muse Annexin V & Dead Cell reagents. As shown in 
Fig 3, the apoptosis rate grew from 3.26 ±0.4% to 41.15 ± 2.2%. By contrast, 
increased doses of VAP II could evidently attenuate the apoptosis of HUVECs to 
32.75±2.4%, 21.04 ± 2.5% and 9.48 ± 2.8%, respectively (Fig. 3).

**VAP II Reduced Depolarization of Mitochondrial Membrane Potential**

JC-1 fluorescence dye was used to evaluate the permeability of mitochondria 
membrane in HUVECs. As shown in Fig 4, the intensity of green fluorescence 
increased while the intensity of red fluorescence decreased after treatment of H₂O₂ for 
2 h. Pretreatment with the increased doses of VAP II could significantly elevated the 
ratio of JC-1 red to green fluorescence in HUVECs. The result indicated that 
pretreatment with VAP II partially reversed depolarization of mitochondrial 
membrane. These data suggested that VAP II could protect HUVECs from H₂O₂ 
induced oxidative stress injury by restoring the mitochondria function.

**Measurement of ROS Production in HUVECs by Flow Cytometry**

ROS is a mediator of intracellular signals and plays an important role in causing 
apoptotic cell death. The quantitative detection of ROS production in each treatment 
group was performed (Fig. 5). The ROS content in the H₂O₂ group was significantly 
higher than in any other group. The difference between the control group and the 
VAP II protective group was statistically significant. The VAP II group exhibited a
decrease of ROS in HUVECs. The data showed that VAP II can decrease cellular ROS levels induced by H₂O₂, thus reducing damage induced by oxidative stress.

**VAP II Reduced Oxidative Stress in HUVECs**

We measured total SOD, GSH and MDA activities in HUVECs. MDA is regarded as a major marker of lipid peroxidation in tissue, whereas SOD and GSH are two important enzymes in the antioxidant defense system. After treating the cells with H₂O₂ for 2 h, the SOD and GSH levels decreased respectively, (Fig. 6 A and B) ($P<0.001$ versus untreated group). However, incubation with VAP II significantly changes in the content of SOD and GSH (Fig. 6 A and B) ($P<0.01$ versus the H₂O₂ group). In addition, cells with H₂O₂ treatment for 2 h showed increasing intracellular MDA release, ($P<0.001$ versus untreated group), while incubation with VAP II produced a marked decrease in the intracellular level of MDA (Fig. 6 C) ($P<0.01$ versus the H₂O₂ group).

**VAP II Regulated the Apoptosis Associated Proteins Expression in HUVECs**

To investigate whether VAP II has effect on the apoptotic related protein expression in HUVECs. The relative level of Bcl-2, Bax, caspase 9, caspase 3 and Cyto c proteins were detected by western blot. As shown in Fig.7, the expression of Bcl-2 decreased, whereas Bax increased. The ratio of Bax/Bcl-2 increased after treatment with H₂O₂ in the HUVECs, but pretreatment with VAP II could decrease the ratio (Fig. 7). We further investigated the protein expression of caspase-3, caspase-9 and cyto c. Compared with the control group, the expression of caspase-3, caspase-9 and cyto c dramatically increased after treatment with H₂O₂, whereas pretreatment with VAP II inhibited the H₂O₂-induced cleavage of caspase-3, caspase-9 and cyto c. Thus, the results indicated that VAP II might prevent H₂O₂-induced apoptosis in HUVECs by blocking the mitochondria-dependent caspase-3 signaling pathway. However, the underlying mechanism still needs to be further explored.
Discussion

Velvet antler is a precious Chinese medicine material that has been used clinically as a tonic to treat various diseases. VAPs are the main bioactive component of velvet antler; these to regulate immune activities and possess anti-oxidant properties (Kim et al. 2003). Therefore, studies on the extraction and isolation of bioactive polypeptides from velvet antlers and the therapeutic effects should be given considerable attention. In this study, we extracted and isolated a polypeptide from a velvet antler. Functional assay showed the purified polypeptide exerts protective effects on HUVECs undergoing H_2O_2-induced damage in vitro. Moreover, the protective mechanism is that the VAP II regulates downstream signaling apoptotic cascades expression caused by H_2O_2.

VAP is considered as a prominent bioactive component of velvet antler, which has been described as a potential drug; VAP has been used in traditional Chinese medicine (Ba et al. 2016). The extraction and isolation of active components from velvet antlers are critical for studying their bioactivities. The extraction methods used water, salt water, acid water or ethanol in a previous study (Zhao et al. 2011). In this study, we extracted the crude VAP by combination of acid water extraction, ethanol precipitation, and ammonium sulfate fractionation and precipitation. Then, the crude velvet antler polypeptides were isolated by Sephadex G-75 column chromatography. VAP II with a molecular weight of about 7.0 kDa was migrated as a single band in Tricine-SDS-PAGE and harvested for further.

Importantly, endothelial dysfunction is a key precursor to the development of various vascular diseases, such as high blood pressure, high cholesterol, diabetes, atherosclerosis, and cancer (Vanhouette 2009). Meanwhile, oxidative stress is well established as a major contributor to endothelial dysfunction and is one of the main causes of tissue damage in the endothelium (Yu et al. 2010). Therefore, studies on VAP’s therapeutic effect on oxidative stress are particularly valuable. H_2O_2 is a well-known reactive oxygen species formed during normal metabolism and is widely used to mimic oxidative stress-induced injury in a short period (Garcia-Mata 2010). In the present study, HUVEC oxidative injury model was induced by H_2O_2, and the...
protective effects of VAP II were assessed. The viability of HUVECs significantly decreased when the cells were exposed to H$_2$O$_2$. However, pretreatment with different concentrations (50, 100, and 200 µM) of VAP II for 12 h prior to H$_2$O$_2$ administration can improve the cell viability in a concentration-dependent manner. Moreover, the protective effect of VAP II against H$_2$O$_2$ might be regulated by inhibition of cell apoptosis. The results suggested that VAP II protected the HUVECs from oxidative stress-induced cell injuries.

One of the major functions of the mitochondria is linked to the production of oxygen free radicals. Increasing production and decreasing removal of oxygen free radicals caused by abnormal mitochondrial structures and dysfunctional mitochondria are important mechanisms of cellular oxidative stress injury (López-Torres et al. 2000). In this study, mitochondrial membrane potential was measured. The result indicated that pretreatment with VAP II partially reversed depolarization of mitochondrial membrane potential. These findings suggested that mitochondria play a crucial role in VAP II protecting HUVECs from H$_2$O$_2$-induced injury. The imbalance between the production of endogenous reactive oxygen species and the presence of antioxidant molecules is one of the most important mechanisms contributing to endothelial dysfunction (Srivastava et al. 2002). To verify whether the protective effect of VAP II is correlated with oxidative stress, the activities of antioxidant enzymes were determined. Pretreatment with VAP II could reduce MDA content and enhance SOD and GSH activities. Therefore, VAP II could protect HUVECs from relative cellular injuries induced by oxidative stress.

Endothelial cell dysfunction and apoptosis correspond to an important process in the pathogenesis of vascular diseases. Oxidative stress can contribute to endothelial cell dysfunction and induce endothelial cell apoptosis (Li et al. 2015). Therefore, studies on the possible mechanism of the anti-apoptosis effect of VAP II on endothelial cell induced by oxidative stress will facilitate the potential use of VAP II in human vascular diseases. Mitochondrion-related apoptosis is one of the important mechanisms underlying cell apoptosis induced by oxidative stress (Kim et al. 2015). The imbalance of apoptotic regulatory proteins Bcl-2 and Bax located in the
mitochondrial membrane exhibits an important effect on apoptosis. Oxidative stress disturbs the balance between pro-apoptotic Bcl-2 and anti-apoptotic Bax proteins, thereby making the cell susceptible to apoptosis (Lee et al. 2014). Cyto c is an important mitochondrial protein that induces apoptosis when accumulated in the cytosol in response to diverse stress stimuli. Mitochondrial cyto c released from the mitochondria and that from downstream caspase activation are both important in regulating apoptosis (Yang et al. 2014). Our study showed that pretreatment with VAP II could attenuate the increase in Bax/Bcl-2 ratio, prevent the release of cyto c from the mitochondria into the cytosol, and inhibit the activation of caspase-3 and caspase-9. Our data confirmed the anti-apoptosis effect of VAP II against oxidative stress-induced apoptosis and the possible mechanism that might be involved in mitochondrial pathway.
**Conclusion**

In this study, we combined acid water extraction, ethanol precipitation, ammonium sulfate fractionation and precipitation, and Sephadex G-75 column chromatography to isolate and purify a 7.0 kDa polypeptide (VAP II) from a velvet antler. The VAP II protects against \( \text{H}_2\text{O}_2 \)-induced HUVEC injury and apoptosis by inhibiting oxidative stress and regulating downstream signaling apoptotic cascades, including Bax and Bcl-2 expression, cytotoxic release, and caspase-3 and caspase-9 activation. VAP II functions as an efficacious antioxidant, and the underlying mechanism needs to be further investigated.
Acknowledgments

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**Conflict of Interest** The authors declare no conflict of interest.
Reference


Figure legend

Fig. 1. VAP II attenuated H$_2$O$_2$-induced cytotoxicity in human umbilical vein endothelial cells (HUVECs). A. The cytotoxicity of VAP II on HUVECs. B VAP II attenuated H$_2$O$_2$-induced cytotoxicity in HUVECs. The HUVECs were pretreated with VAP II for 12 h and then exposed to 100 µmol/L H$_2$O$_2$ for 2 h. Values are the mean ± SD, $n = 5$. $## P < 0.01$ compared with the untreated group (control); $** P < 0.01$ compared with the HUVECs treated with H$_2$O$_2$ only.

Fig. 2. VAP II prevented H$_2$O$_2$-induced apoptosis in human umbilical vein endothelial cells (HUVECs). (A) Morphological analysis of the HUVECs. (a) HUVECs without H$_2$O$_2$ induction; (b) HUVECs incubated with 100 µmol/L H$_2$O$_2$; (c-e) HUVECs pretreated with 50 mg/L, 100 mg/L and 200 mg/L VAP II before incubation in 100 μmol/L H$_2$O$_2$. Cell nuclei were stained with Hoechst 33258 (fluorescence microscope, 200×). (B) The percentage of nuclear condensation in the cultured cells was counted in response to control. Values are the mean ± SD, $n = 5$. $### P < 0.01$ compared with the untreated group (control); $** P < 0.01$ compared with the HUVECs treated with H$_2$O$_2$ only.

Fig. 3. VAP II suppressed the H$_2$O$_2$ induced apoptosis of HUVEC cells by flow cytometric analysis. (A) Muse Cell Analyzer analysis of apoptosis. (B) Quantitative analysis of data from the lower right quadrant in (A). The HUVEC cells pre-treated with VAP II were exposed to 100 µM H$_2$O$_2$ for 2 h, harvested and then subjected to quantitative analysis of cell apoptosis by Muse Annexin V & Dead Cell reagents and analyzed by Muse Cell Analyzer. Data are representative pictures. (a) Representative flow cytometry results of untreated group. (b) Representative H$_2$O$_2$ alone group. (c-e) Representative cells pretreated with various concentrations of VAP II. Values are the mean ± SD, $n = 5$. $### P < 0.01$ compared with the untreated group (control); $** P < 0.01$ compared with the HUVECs treated with H$_2$O$_2$ only.

Fig. 4. VAP II prevented H$_2$O$_2$-induced loss of mitochondrial membrane potential in HUVECs. (A) NucleoCounter NC-3000 Cell Analyzer analysis of mitochondrial membrane potential. (B) Quantitative analysis of (A). (a-e) Mitochondrial membrane potential analysis of the HUVECs. (a) HUVECs without H$_2$O$_2$ induction; (b)
HUVECs incubated with 100 µmol/L H$_2$O$_2$; (c-e) HUVECs pretreated with 50 mg/L, 100mg/L and 200 mg/L VAP II before incubation in 100µmol/L H$_2$O$_2$. The mitochondrial membrane potential was measured using the fluorescent probe JC-1 and analyzed by NucleoCounter NC-3000 Cell Analyzer. P1, Polarized cells, P2, Depolarized cells.

Fig. 5. The effects of VAP II on the ROS generation in HUVECs. (A) Flow cytometry analysis of the ROS generation. (B) Quantitative analysis of (A). (a-e) The ROS generation of the HUVECs. (a) HUVECs without H$_2$O$_2$ induction; (b) HUVECs incubated with 100 µmol/L H$_2$O$_2$; (c-e) HUVECs pretreated with 50 mg/L, 100mg/L and 200 mg/L VAP II before incubation in 100µmol/L H$_2$O$_2$. Values are the mean ± SD, n = 5. ### P < 0.01 compared with the untreated group (control); ** P < 0.01 compared with the HUVECs treated with H$_2$O$_2$ only.

Fig. 6. The effects of VAP II on the intracellular SOD, GSH, and MDA levels. (A) The intracellular SOD level of HUVECs treated with H$_2$O$_2$. (B) The intracellular GSH level of HUVECs treated with H$_2$O$_2$. (C) The intracellular MDA level of HUVECs treated with H$_2$O$_2$. The HUVECs pretreated with VAP II for 12 h were exposed to 100 µM H$_2$O$_2$ for 2 h. Values are represented as the means ± SEM (n=5). ### p<0.01 versus untreated group (control). # p<0.05 versus untreated group (control). **p<0.01 versus H$_2$O$_2$ alone group, *p<0.05 versus H$_2$O$_2$ alone group.

Fig. 7. The effects of VAP II on apoptotic and antiapoptotic proteins expression. (A) Western blot for Bax, Bcl-2 (B) Quantitation of the effects of VAP II on the ratio of Bax/Bcl-2. (C) Western blot for Cleaved Caspase-3, Cleaved Caspase-9 and Cyto c protein expression. (D) Quantitation of effect of VAP II on Cleaved Caspase-3, Cleaved Caspase-9 and Cyto c protein expression. Values are the mean ± SD, n = 5. ##, P < 0.01 compared with the control cells; **, P < 0.01 compared with the cells treated with H$_2$O$_2$. 
Fig. 1

A

Cell survival (%)

VAP II (mg/l)

B

Cell viability (%)

H₂O₂ (100 µM)  
VAP II (mg/l)  
-  
+  
+  
+  
+  
+  

Fig. 1
Fig. 3
Fig. 4

A

B

| H₂O₂ (100µM) | - | + | + | + | + |
| VAP II (mg/l) | - | - | 50 | 100 | 200 |
Fig. 5

| H\textsubscript{2}O\textsubscript{2} (100 µM) | - | + | + | + | + |
| VAP II (mg/L) | - | - | 50 | 100 | 200 |

** ROS generation

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https://mc06.manuscriptcentral.com/cjpp-pubs
Fig. 6

A

B

C

https://mc06.manuscriptcentral.com/cjpp-pubs
The ratio of protein/β-actin

Fig. 7