Tanshinol protects human umbilical vein endothelial cells against hydrogen peroxide-induced apoptosis

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Abstract. The present study was designed to investigate the effect of tanshinol on hydrogen peroxide (H₂O₂)-induced apoptosis in human umbilical vein endothelial cells (HUVECs), and to determine the underlying mechanisms. HUVECs were pre-incubated with tanshinol (25-200 µM) for 24 h, followed by an incubation with 600 µM H₂O₂ for 6 h. The cell viability was assessed using MTT reagent and the level of cell death was determined by measuring lactate dehydrogenase (LDH) activity. Superoxide dismutase (SOD) activity, levels of malondialdehyde (MDA), reactive oxygen species (ROS) production and NADPH oxidase activity were measured spectrophotometrically using commercially available kits. The apoptotic rate of the HUVECs was detected using Annexin-V/propidium iodide (PI) staining, followed by flow cytometry analysis using a fluorescence microscope. The protein expression of SOD-1, SOD-2, B-cell lymphoma-2 (Bcl-2), cytochrome c and caspase-3 was determined by western blot analysis. Pretreatment with tanshinol resulted in a significant increase in the cellular viability of HUVECs and SOD activity, and a decrease of cell apoptosis, MDA levels and ROS production, induced by H₂O₂. These findings were accompanied by the upregulation of Bcl-2 protein expression, reduction in the release of cytochrome c from the mitochondria to the cytosol and a downregulation of caspase-3 protein expression. This study showed that tanshinol protects against atherosclerosis by preventing H₂O₂-induced apoptosis of HUVECs. These effects appear to be mediated by enhancing the antioxidant defenses and preserving the mitochondrial function of the cells.

Introduction

Atherosclerosis is a chronic, multifactorial disease, in which abnormalities in the structure and function of endothelial cells (ECs) have an initial role in its development (1). Numerous risk factors for atherosclerosis can result in endothelial injury to the lumen of blood vessels, but it has been recently demonstrated that exposure to regional reactive oxygen species (ROS) is one of the major stimuli for EC dysfunction and damage (2,3). ROS production can induce oxidative damage to lipids, proteins and enzymes in ECs, resulting in damage to cellular function and apoptosis of severely damaged ECs (4,5). Hydrogen peroxide (H₂O₂) is one of the most crucial ROS, that can easily penetrate the plasma membrane and cause injury to neighboring cells, as well as H₂O₂-producing cells (6). In vitro models of H₂O₂-induced endothelial cell damage has been extensively applied to mimic oxidative endothelial injury during atherogenesis (7). Previous studies have reported that H₂O₂ could lead to the dysfunction of ECs and apoptosis (8,9). Therefore, the upregulation of the anti-apoptotic pathways in ECs, which are inhibited by H₂O₂, has been considered as an attractive therapeutic strategy in atherosclerosis.

Radix salviae miltiorrhizae, commonly known as 'Danshen', is an herbal supplement that has been used by medical practitioners of traditional Chinese medicine for decades in the therapy of a variety of cardiovascular diseases (10). Tanshinol [3(3,4-dihydroxyphenyl)2-hydroxypropionic acid], is the main effective component of Danshen, which has previously been shown to improve microcirculation, inhibit the production of ROS, restrain platelet adhesion and aggregation, and protect the myocardium from ischemia (11-13). In addition, tanshinol has been shown to have protective effects on ECs suffering from injury caused by hyperhomocysteinemia (14) and inflammation (15).

There have been few studies performed to ascertain the regulatory role of tanshinol in preserving antioxidant defenses and mitochondrial function in ECs. In the present study, the protective effects of tanshinol in ECs were studied in response to H₂O₂-induced oxidative stress. The main focus was to determine whether tanshinol can protect ECs from H₂O₂-induced cytotoxicity by increasing the expression of antioxidant enzymes such as superoxide dismutase (SOD). SOD can detoxify free radicals and preserve mitochondrial function through the upregulation of Bcl-2 expression, which inhibits the cytochrome c-caspase-3 pathway. The presented outcomes
provide further support for a functional role of tanshinol in ECs in the prevention of atherosclerosis.

**Materials and methods**

**Reagents.** H$_2$O$_2$ was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Dimethyl sulfoxide (DMSO), MTT and streptomycin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Penicillin and glutamine were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), trypsin and medium-199 (M199) were purchased from Gibco-BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from PAA Co. (PAA Laboratories GmbH, Piscotaway, NJ, USA), β-endothelial cell growth factor (β-ECGF) was purchased from R&D Systems (Minneapolis, MN, USA), and heparin was purchased from Sinopharm Chemical Reagent Co. Ltd., (Shanghai, China). The SOD Activity Assay kit was purchased from Nanjing Institute of Jiancheng Bioengineering (Nanjing, China), and the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit was obtained from Nanjing KeyGen Biotech Co. (Nanjing, China). Bicinchoninic acid (BCA) Protein Assay and Lipid Peroxidation Malondialdehyde (MDA) Assay kits were purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

**Cell culture.** Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described (16). This study was approved by the Ethics Board of the Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China. Briefly, HUVECs were removed from human umbilical veins (Renji Hospital) by digestion with 0.125% trypsin, and were cultured in M199 containing 20% FBS, penicillin (100 U/ml), streptomycin (100 U/ml), and heparin (50 U/ml) and supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), and β-ECGF (5 ng/ml). The cells were incubated at 37°C in 5% CO$_2$ on 0.1% gelatin-coated culture flasks. HUVECs were identified by their morphology, which appears as a ‘cobblestone’ mosaic appearance after reaching confluence, and the presence of von Willebrand factor (detected by immunofluorescence). HUVECs were used at passages 3-6 for experiments.

**Treatment protocols.** For all experiments, HUVECs were cultured to confluence in 96-well plates or 35 mm dishes. The cells were pre-treated with tanshinol (25, 50, 100, 200 µM) for 24 h before exposure to H$_2$O$_2$ (600 µM). Following exposure to H$_2$O$_2$, the cells were harvested for further analysis.

**Cell viability assay.** The MTT assay was performed to evaluate the viability of the HUVECs. Briefly, HUVECs were seeded onto 96-well plates in M199 media and maintained for 24 h. The cells were subjected to different concentrations of tanshinol (25, 50, 100, 200 µM) for 24 h followed by stimulation with or without 600 µM H$_2$O$_2$ for 6 h. Following the H$_2$O$_2$ incubation, MTT (10 µl/well) reagent was added to the 100 µl medium, and then incubated at 37°C for 4 h prior to the detection of the number of viable cells. The MTT solution was removed and DMSO was added in order to solubilize the formazan crystals. The absorbance of the medium was measured at 570 nm using a BioTek ELx-800 (BioTek Instruments, Inc., Winooksi, VT, USA) plate reader. The extent of cell death was estimated by measuring the activity of lactate dehydrogenase (LDH). The amount of LDH released from the cells in the supernatant was detected using an LDH assay kit (Beyotime Institute of Biotechnology, Shanghai, China), according to the manufacturer's instructions. The absorbance was measured using a microplate reader at 490 nm. The data from each treatment group is expressed as a percentage of the control group.

**Lipid peroxidation assay.** The level of MDA was detected using the Lipid Peroxidation MDA Assay kit, according to the manufacturer's instructions. Briefly, HUVECs were lysed using radio-immunoprecipitation assay buffer, followed by centrifugation at 1600 x g for 10 min to discard the cellular debris. The supernatant was used for the MDA assay and the protein concentration was measured using a BCA assay. The MDA levels were standardized to milligram protein.

**Measurement of intracellular SOD activities.** The culture medium from the HUVECs seeded in the 96-well plates was removed and the cells were washed twice with phosphate-buffered saline (PBS), followed by cell lysis using the freeze-thaw method three times. The SOD activity in the cell lysate was detected using commercial kits according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Shanghai, China).

**Flow cytometric evaluation of apoptosis.** Following tanshinol and H$_2$O$_2$ treatments, the HUVECs were double-stained using an Annexin V-FITC apoptosis detection kit, according to the manufacturer's instructions. Samples stained with Annexin V and propidium iodide (PI) were quantitatively analyzed at 488 nm emission and 570 nm excitation using flow cytometry (BD FACS)calibur; BD Biosciences, San Jose, CA, USA).

**ROS detection.** The 2',7'-dichlorofluorescin diacetate (DCFH-DA) assay was used to detect ROS in the HUVECs as previously described (17). To examine the effects of tanshinol on ROS production, the HUVECs were pretreated with 100 µM tanshinol for 24 h, followed by stimulation with 600 µM H$_2$O$_2$ for 4 h, after which the cells were incubated with DCFH-DA (10 µM) in M199 for 30 min at 37°C in 35-mm dishes or 96-well black-bottomed plates. The fluorescence intensity of the dishes was measured at 485 nm excitation and 538 nm emission by laser-scanning confocal microscopy (FV500, Olympus Optical Co. Ltd., Tokyo, Japan). The fluorescence intensity of the 96-well plates was quantified with an Infinite F500 Microplate Reader (Tecan Group, Ltd., Männedorf, Switzerland) and was standardized to the total milligram protein.

**Western blot analysis.** The HUVECs were lyzed in protein lysis buffer containing a protease inhibitor cocktail. The protein concentrations of the cell lysates was quantified by BCA assay and equal amounts of protein were separated by SDS-PAGE and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked in 5% non-fat dry milk diluted with Tris-buffered saline with Tween® 20 (TBST) (Tris-HCl 20 mM, NaCl 150 mM, pH 7.5, 0.1%
Tween 20) at room temperature for 1 h and probed overnight at 4˚C with either monoclonal rabbit cytochrome c, Bcl-2, SOD-2 or caspase-3 antibodies, or a polyclonal rabbit SOD-1 antibody (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), followed by incubation for 1 h with a goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (Cell Signaling Technology, Inc.). Incubation with either a monoclonal mouse α-tubulin antibody (1:1,000 dilution; Sigma-Aldrich) or a polyclonal mouse β-actin antibody (1:1,000 dilution; Cell Signaling Technology, Inc.) was performed as the loading sample control. The proteins were visualized using Enhanced Chemiluminescence Western Blotting Detection reagents (Amersham Biosciences Corp., Piscataway, NJ, USA). The densitometry of the bands was quantified using Image J 1.38X software (National Institutes of Health, Bethesda, MD, USA).

Cytochrome c release assay. HUVEC cells were harvested by centrifugation at 1,000 x g for 3 min at room temperature. The mitochondrial and cytosol extractions were separated using a Mitochondrial Isolation kit (Pierce Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer’s instructions. The presence of cytochrome c was determined from both the mitochondrial and cytosol extractions by western blotting, using a monoclonal rabbit cytochrome c antibody (1:1,000, Cell Signaling Technology, Inc.).

Statistical analysis. All of the experiments were repeated at least three times. The values are expressed as the means ± standard error of the mean. The data was evaluated for statistical significance using a one-way analysis of variance. A P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using GraphPad Prism® 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

HUVECs exhibit no toxicity to tanshinol administration. Tanshinol was analyzed by MTT assay to determine its toxic effects on HUVECs. Tanshinol, at a concentration range between 25 and 200 µM alone, did not show any toxic effects on HUVECs (Fig. 1A). MTT assay showed that HUVEC viability was significantly decreased following a 600 µM H₂O₂ challenge for 4 h, as compared with the control (P<0.01). Tanshinol significantly attenuated this H₂O₂-induced decrease in cellular viability in a concentration-dependent manner (Fig. 1B). Tanshinol demonstrated the ability to markedly enhance HUVEC cell viability at 100 µM. An LDH release assay supported the results of the MTT assay (Fig. 1C). As compared with the control, the vehicle plus H₂O₂-treated cells exhibited an induced LDH release (244.8±15.57%, P<0.01), while pretreatment with tanshinol significantly decreased LDH release (169.1±7.60%, P<0.01). These results demonstrated the ability of tanshinol to reduce H₂O₂ cytotoxicity.

Tanshinol inhibits H₂O₂-induced HUVEC apoptosis. To quantitatively determine the anti-apoptotic effects of tanshinol in H₂O₂-induced HUVECs, the total apoptotic rate
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Figure 2. Effects of tanshinol on $\text{H}_2\text{O}_2$-induced endothelial apoptosis. (A and B) Annexin V/PI staining detected by flow cytometry. Human umbilical vein endothelial cells were pretreated with vehicle or 100 µM tanshinol for 24 h, followed by exposure to 600 µM $\text{H}_2\text{O}_2$ for a further 6 h. The data are shown as the means ± standard error of the mean (n=6). *$P<0.01$ vs. control; **$P<0.01$, vs. vehicle + $\text{H}_2\text{O}_2$ group. FITC-A, Annexin V-fluorescein isothiocyanate; PI-A, Annexin V-propidium iodide.

Figure 3. Effects of tanshinol on the protein expression of Bcl-2, cytochrome $c$ and Caspase-3 in $\text{H}_2\text{O}_2$-induced HUVECs. (A) Western blot analysis showing the protein expression of Bcl-2 in each group. $\beta$-actin was used as a loading control. The bar chart indicates the levels of protein expression of Bcl-2 between the groups. The mean value in each treatment group was normalized to the control group. (B) Analysis of the release of cytochrome $c$ from the mitochondria. HUVECs were harvested to collect both the mitochondria and cytosol. The protein expression was analyzed by western blotting. $\beta$-actin was used as a loading control. The bar chart showed the ratio of cytochrome $c$ in cytosol to that in mitochondria, indicating the intensity of release of cytochrome $c$. The mean value in each treatment group was normalized to the control group. (C) Western blot analysis showing the intensity of caspase-3 in each group. $\alpha$-tubulin was used as a loading control. The data are shown as the means ± standard error of the mean (n=6). ***$P<0.001$, ****$P<0.001$ vs. control; #P<0.05, ##$P<0.01$, ###$P<0.001$, vs. vehicle plus $\text{H}_2\text{O}_2$ group; Bcl-2, B-cell lymphoma-2.
of the HUVECs was measured by Annexin-V/PI staining, following treatment with 600 µM H₂O₂ for 4 h. As shown in Fig. 2A and B, the rate of apoptosis increased from 4.12±0.56 to 21.40±1.92%, as compared with the control group (P<0.01). By contrast, tanshinol at 100 µM significantly attenuated the apoptotic rate of the HUVECs exposed
Tanshinol inhibits H$_2$O$_2$-induced cytochrome c release from the mitochondria into the cytosol. One mechanism by which oxidative stress may induce cellular toxicity in HUVECs is through the induction of the apoptotic pathway, which is caused by the mitochondrial release of cytochrome c into the cytosol. To demonstrate this hypothesis, the protective action of tanshinol on H$_2$O$_2$-induced toxicity was analyzed by determining the release of cytochrome c from the mitochondria by western blotting (Fig. 3B). As compared with the control group, the cytochrome c levels in the H$_2$O$_2$ group were significantly increased in the cytosol and decreased in the mitochondria (P<0.01). However, pretreatment with tanshinol inhibited H$_2$O$_2$-induced release of cytochrome c, as compared with the vehicle plus H$_2$O$_2$ group (P<0.001).

**Effects of tanshinol on the expression of Bcl-2 and caspase-3 in HUVECs exposed to H$_2$O$_2$.** The activation of caspase-3 is a key step in apoptosis. Furthermore, the Bcl-2 protein family has a critical role in apoptotic cell death, caspase activation and the regulation of apoptosis. Stimulation of HUVECs with H$_2$O$_2$ at 600 µM for 6 h significantly decreased Bcl-2 protein expression to 52.3% of the control protein expression levels (P<0.001), whereas tanshinol at 100 µM attenuated the H$_2$O$_2$-induced downregulation of Bcl-2 protein expression (78.6% of control, P<0.01, Fig. 3A). As compared with the control group, HUVECs exposed to H$_2$O$_2$ alone showed increased protein expression of caspase-3, and tanshinol reduced the ability of H$_2$O$_2$ to increase caspase-3 expression (Fig. 3C).

**Effects of tanshinol on MDA generation and ROS production.** MDA is a product of lipid peroxidation. The levels of MDA were low in the control HUVECs and treatment with H$_2$O$_2$ elevated the cellular MDA levels as compared with the control levels, thus indicating an increase in oxidative stress (P<0.001, Fig. 4A). Pretreatment with tanshinol significantly reduced the generation of MDA induced by H$_2$O$_2$, as compared with the vehicle plus H$_2$O$_2$ group (P<0.01). The redox status was observed using the DCFH-DA assay (Fig. 4B and C). Treatment with H$_2$O$_2$ induced an increase in the fluorescence intensity of DCFH-DA, as compared with the control group (P<0.001). Pre-incubation with tanshinol restrained the production of ROS induced by H$_2$O$_2$ (P<0.01).

**Tanshinol prevents H$_2$O$_2$-induced decrease of SOD activity.** SOD is another indicator of cellular toxicity. To further examine the protective effects of tanshinol, SOD activity was measured in H$_2$O$_2$-treated cells. As shown in Fig. 5A, HUVEC treatment with H$_2$O$_2$ resulted in a significant decrease in SOD activity, as compared with the control group (P<0.01). Conversely, pretreatment with tanshinol increased the SOD activity, as compared with the vehicle plus H$_2$O$_2$ group (P<0.01). To support these results, the protein expression of SOD in HUVECs was measured (Fig 5 B-D). The protein expression levels of SOD-1 and SOD-2 were decreased in the H$_2$O$_2$ group as compared with the control group (P<0.01). When compared with the H$_2$O$_2$ group, higher protein expression levels were observed in the tanshinol group (SOD-1; P<0.01, SOD-2; P<0.05). These results were in accordance with the identified SOD activity of the previous experiment.

**Discussion**

The present study explored the cardiovascular protective function of tanshinol on H$_2$O$_2$-induced injury in HUVECs, as estimated by measures of antioxidative systems and mitochondrial function. Oxidative stress is considered to be a potent atherogenic factor at the initiation of atherosclerotic lesion formation. Oxidized cholesterol particles promote inflammation, the formation of plaques and the migration of smooth muscle cells, which are all conducive to the development of atherosclerotic lesions. Furthermore, mitochondria can also be damaged by these toxic free radicals at the cellular level (18).

In the present study, the effects of tanshinol on H$_2$O$_2$-induced HUVEC apoptosis in vitro, and its possible underlying mechanisms, were estimated. The results indicated that tanshinol could protect HUVECs from apoptosis induced by H$_2$O$_2$, and improve cellular viability. Tanshinol was also shown to significantly inhibit cellular apoptosis and increase SOD activity. An underlying mechanism by which tanshinol prevents HUVECs from apoptosis may be by increasing antioxidant defence systems and preserving mitochondrial function.

It has been previously reported that increased levels of ROS from vessels under pathological conditions is a major process resulting in endothelial injury (17). Following exposure to high levels of H$_2$O$_2$, HUVECs undergo exceptional cytotoxicity. In the present study, it was observed that 600 µM H$_2$O$_2$, a precursor of other ROS, can significantly reduce the cellular viability and increase the apoptotic rate of HUVECs. The present findings are in accordance with previous reports demonstrating increased apoptosis in HUVECs incubated with H$_2$O$_2$ (19-20). However, when HUVECs were pretreated with tanshinol for 24 h, cell viability was significantly enhanced and the apoptotic rate was significantly reduced. These findings suggest that tanshinol may protect HUVECs against H$_2$O$_2$-induced apoptosis.

The mechanisms by which tanshinol prevents EC apoptosis under oxidative stress are currently unknown. Tanshinol was shown to protect HUVECs against oxidative stress which was mainly attributed to the upregulation of cellular antioxidant defenses, including SOD. Thus, it may be assumed that the underlying mechanism behind the anti-apoptotic effects of tanshinol may be due to its upregulation of endogenous cellular antioxidant systems that are capable of scavenging ROS.

It is well known that the antioxidant enzyme SOD has a vital role in EC defenses (21). SOD can dismutate superoxide radicals into hydrogen peroxide, which can then be detoxified into water by other antioxidant enzymes (16). As shown in Figure 4, pretreatment with tanshinol markedly increased SOD activity. These findings suggest that tanshinol may protect ECs by increasing the ability of antioxidant enzymes in the scavenging of H$_2$O$_2$.

Consistent with previous findings (20,23,24), the present study found that H$_2$O$_2$ could markedly decrease Bcl-2 protein expression. In contrast, tanshinol treatment significantly increased H$_2$O$_2$-induced Bcl-2 protein expression. It is well reported that Bcl-2 is an anti-apoptotic protein that can...
inhibit apoptosis through a mitochondria-dependent caspase pathway (25) Bcl-2 has been shown to prevent apoptosis induced by different stimuli, either by restraining the mitochondrial release of cytochrome c, which stimulates caspase activity, or by serving as an antioxidant (26). According to the present study, tanshinol significantly inhibits cytochrome c release from the mitochondria into the cytosol. Therefore, the protective effect of tanshinol on H₂O₂-induced EC injury may be related to the upregulation of Bcl-2 protein expression and therefore reduced caspase activity. In addition, as the interior apoptotic pathway relies on the balance between pro- and anti-apoptotic members of the Bcl-2 family, including Bax, Bcl-XL, Bak and Bad (27-29), the effects of tanshinol on pro-apoptotic proteins requires further study.

In conclusion, the present study showed that tanshinol had a protective effect against H₂O₂-induced cytotoxicity and apoptosis in HUVECs. This anti-apoptotic effect of tanshinol likely contributed to the preservation of mitochondrial function through the upregulation of Bcl-2 protein expression, the inhibition of the cytochrome c caspase-3 pathway, and the stimulation of cellular antioxidant defenses by increased SOD activity. Since oxidative stress-induced EC injury has a vital role in the development of atherosclerosis (30), the findings of the present study may illuminate the pharmacological foundation for the clinical application of tanshinol for the treatment of atherosclerosis.

References