Isolariciresinol-9'-O-α-L-arabinofuranoside protects against hydrogen peroxide-induced apoptosis of human umbilical vein endothelial cells via a PI3K/Akt/Bad-dependent pathway

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Abstract. Isolariciresinol-9'-O-α-L-arabinofuranoside (MWS-19) isolated from *Pinus massoniana* Lamb. Fresh pine needles is the major ingredient of the Songling Xuemaikang capsule therapy used for hypertension. The present study aimed to investigate the effects and underlying mechanisms of MWS-19 on hydrogen peroxide (H_2O_2)-induced apoptosis in human umbilical vein endothelial cells (HUVECs). To investigate the effect of MWS-19 on apoptosis in HUVECs, an oxidative stress-induced apoptosis model was established in HUVECs using H_2O_2, and the present study performed Hoechst 33258 staining and a Cell Counting Kit-8 (CCK-8) assay. Furthermore, western blot analysis was also performed to investigate the underlying mechanism of the effects of MWS-19 on the model. The results demonstrated that MWS-19 reversed the effects of H_2O_2 on cell apoptosis at a concentration range of 15.6-250 μg/ml, with dose-dependent increases in cell growth. Hoechst staining indicated that 500 μM H_2O_2 induced HUVEC apoptosis, and MWS-19 markedly protected HUVECs against apoptosis at 31.3, 62.5 and 125 μg/ml. Furthermore, the protein expression of phosphatidylinositol 3-kinase (PI3K), phosphorylated-Akt and Bcl-2-associated agonist of cell death (Bad) were increased, and reduced caspase-3 activation was observed, following treatment with MWS-19 in H_2O_2-treated HUVECs. Additionally, the PI3K inhibitor wortmannin attenuated PI3K/Akt/Bad signaling induced by MWS-19 treatment and neutralized the effect of MWS-19 on the growth of HUVECs. In conclusion, the results of the present study indicate that MWS-19 may protect against H_2O_2-induced HUVEC apoptosis via the PI3K/Akt/Bad signaling pathway. MWS-19 may serve an important role in the prevention of oxidative damage in vascular endothelial cells in hypertension patients.

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Introduction

Hypertension, a multifactorial disorder, has one of the highest associations with morbidity and mortality, and poses a serious threat to human health in numerous countries (1,2). Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide (O^{2-}), lead to increases in vascular tone and participate in arterial contraction, and have a direct role in the pathogenesis of hypertension (3,4). The vascular endothelium, a thin layer of cells lining the inner surface of the vascular wall, produces and secretes various substances that preserve the integrity and functioning of the vascular system (5,6). Excessive levels of ROS in endothelial cells leads to impaired endothelial nitric oxide (NO) bioactivity (7,8). NO, a type of vasodilator, is secreted by endothelial cells, and 30% of blood NO levels are produced by endothelial nitric oxide synthase (9). Previous studies have reported that increased ROS generation in endothelial cells contributes to their dysfunction in hypertension (10-13). Therefore, compounds that are able to scavenge ROS in endothelial cells may be beneficial in the treatment of hypertension.

The Songling Xuemaikang Caspule (SXC), which has been authorized and recommended by Chinese Pharmacopoeia, and is widely used for treating hypertension, consists of three herbal medicines, including pine needles (*Pinus massoniana* Lamb.), Radix *Puerariae lobata* and powdered nacre (14). Pine needles (*Pinus massoniana* Lamb.) are the major ingredient in a prescription of SXC, and have long been used as a traditional

health-promoting medical food in China. To determine the clinical effect on hypertension of SXC, identification of the mechanisms of each of the major pure compounds is required with regards to hypertension.

Isolariciresinol-9'-O-α-L-arabinofuranoside (also termed MWS-19), a chemical constituent isolated from the fresh pine needles of *Pinus massoniana* Lamb. in the Sichuan province of China, makes up 0.944% (w/w) of each SXC capsule. Characterization was performed by means of spectroscopic analysis and physicochemical properties, and it belongs to the class of lignans (15). Lignans are naturally occurring chemicals that are widespread within the plant and, as they are naturally occurring phenols, they exert cardiovascular protective activity by their functions in oxidation resistance (16-19). However, to the best of our knowledge, the pharmacological effects of MWS-19 have not previously been investigated.

The primary aim of the present study was to investigate the protective effect of MWS-19 of on H_2O_2-induced apoptosis in human umbilical vein endothelial cells (HUVECs) apoptosis and the underlying molecular mechanisms of this effect.

Materials and methods

Materials. MWS-19 (15) was obtained in the authors' lab from the fresh pine needles of *Pinus massoniana* Lamb., in the Sichuan province of China. The chemical structure of MWS-19 is presented in Fig. 1A. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). The Hoechst 33258 staining kit was obtained from Beyotime Institute of Biotechnology (Haimen, China). The E.Z.N.A. DNA/RNA/Protein Isolation kit was purchased from Omega Bio-Tek, Inc. (Norcross, GA, USA). All other reagents were of analytic grade.

Cell culture and reagents. HUVECs (cat. no. 8000; ScienCell Research Laboratories, Inc., San Diego, CA, USA) were cultured in endothelial cell medium (ScienCell Research Laboratories, Inc.) containing 5% fetal bovine serum (ScienCell Research Laboratories, Inc.), 1% endothelial cell growth supplement (ScienCell Research Laboratories, Inc.) and 1% penicillin/streptomycin solution (ScienCell Research Laboratories, Inc.). All cells were maintained at 37˚C in humidified air with 5% CO_2. The medium was changed every other day. The cells were subcultured when they reached >90% confluence.

Cell viability assay. The CCK-8 kit was used to evaluate cell viability. HUVECs were seeded in 96-well plates at $4x10^3$ cells/well with 100 μl culture medium and incubated overnight at 37˚C, subsequently, the medium was replaced with fresh medium for different treatments. To investigate the cytotoxic effect of various concentrations of H_2O_2, cells were treated with 0, 100, 200, 300, 400, 500, 600, 700 and 800 μM H_2O_2 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C for 24 h. To determine the protective effect of MWS-19 on cell viability in H_2O_2-treated HUVECs, cells were randomly divided into the following 11 groups: Negative control group (culture medium treated); model control group (500 μM H_2O_2 only); and cells treated with 500 μM H_2O_2 for 24 h following treatment with 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500 and 1,000 μg/ml MWS-19 for 24 h at 37˚C. In a further experiment, cells were randomly divided into 5 groups: Negative control group (untreated cells); model control group (500 μM H_2O_2 only); 5 nM wortmannin (Cayman Chemical Company, Ann Arbor, MI, USA) treatment only; 500 μM H_2O_2 + 62.5 μg/ml MWS-19 group; and 500 μM H_2O_2 + 62.5 μg/ml MWS-19 + 5 nM wortmannin. Cells were pretreated for 24 h with MWS-19 and/or wortmannin prior to H_2O_2 treatment, and cells were treated with H_2O_2 for 24 h at 37˚C.

Following treatments, 10 μl CCK-8 was added into each well and cells were further incubated for 4 h at 37˚C. The absorbance of samples was measured at a wavelength of 450 nm on a Multiskan FC microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Hoechst 33258 staining. HUVECs were seeded on sterile cover glasses placed in 12-well plates at a density of $1x10^5$ cells/well and cultured at 37˚C for 24 h. Cells were treated with three concentrations of MWS-19 (31.3, 62.5 and 125 μg/ml) for 24 h while the negative control group and model control group were treated with medium for 24 h at 37˚C. The MWS-19 (31.3, 62.5 and 125 μg/ml) groups and model control group were treated with 500 μM H_2O_2 for 24 h at 37˚C, and the negative control group was treated with medium for 24 h at 37˚C. Cells were subsequently fixed by 4% paraformaldehyde at 4˚C overnight, washed twice with PBS and stained with Hoechst 33258 staining solution according to the manufacturer's protocol. Stained nuclei were observed under a confocal microscope (IX73; Olympus Corporation, Tokyo, Japan). A total of five visual fields were randomly selected for each sample, total cell number and apoptotic cell number were counted and calculate the apoptosis ratio. Identical staining procedures were performed with control cells.

Western blot analysis. HUVECs were pretreated with 31.25, 62.5 and 125 μg/ml MWS-19, or 5 nM wortmannin + 62.5 μg/ml MWS-19 for 24 h, which was followed by exposure to 500 μM H_2O_2 for 24 h at 37˚C, and the negative control group (medium treated for 48 h) and the model control group (medium treated for 24 h and 500 μM H_2O_2 treated for another 24 h). Following treatment, HUVECs were washed with ice-cold PBS and cells were lysed in 6-well plates for 5 min with 500 μl guanidine thiocyanate buffer (E.Z.N.A. DNA/RNA/Protein Isolation kit; Omega Bio-Tek, Inc., Norcross, GA, USA) and 20 μl 2-mercaptoethanol, subsequently, 2 ml of ice-cold acetone was added to the cell lysate and incubated at -20˚C for 30 min, which was followed by centrifugation at maximum speed for 10 min at 4˚C. The pellet was washed with 1 ml ice-cold absolute ethanol and centrifuged for 3 min at 4˚C at maximum speed. Protein concentration was detected with a bicinchoninic assay kit.

For western blot analysis, equal amounts of protein extracts (50 μg) were separated by 12% SDS-polyacrylamide gels, and proteins were subsequently transferred on to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked in blocking buffer (Beyotime Institute of Biotechnology) overnight at 4˚C, followed by incubation overnight at 4˚C with the primary antibodies for β-actin (cat. no. sc-8432; 1:1,000; Santa Cruz Biotechnology, Inc. Dallas, TX, USA), phosphatidylinositol 3-kinase (PI3K; cat. no. sc-7175, 1:500, Santa Cruz Biotechnology, Inc.),

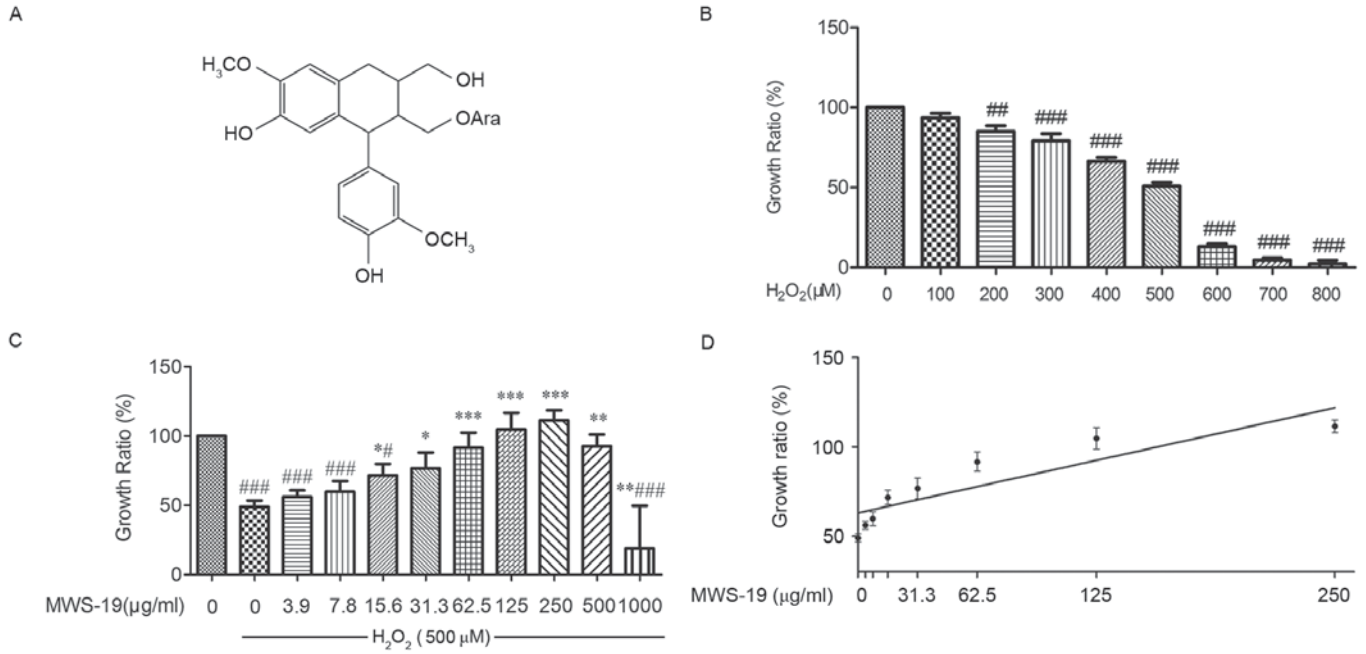

Figure 1. (A) Chemical structure of MWS-19. (B) Cell viability was reduced by 100-800 μM H_2O_2 in HUVECs. $^{##}$P<0.01 and $^{###}$P<0.001 vs. negative control group. (C) Cell viability in HUVECs treated with 3.9-1,000 μg/ml MWS-19 followed by H_2O_2 treatment. *P<0.05, **P<0.01 and ***P<0.001 vs. model control group; $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. negative control group. (D) Correlation analysis was performed to determine the correlation between growth ratio of HUVECs and MWS-19 concentrations between 0 and 250 μg/ml. R^2=0.7842; P=0.0034. MWS-19, isolariciresinol-9'-O-α-L-arabinofuranoside; H_2O_2, hydrogen peroxide; HUVECs, human umbilical vein endothelial cells.

Akt (cat. no. SAB4500799; 1:1,000; Sigma-Aldrich; Merck KGaA), phosphorylated (p)-Akt (cat. no. sc-101629; 1:1,000; Santa Cruz Biotechnology, Inc.), Bcl-2-associated agonist of cell death (Bad; cat. no. sc-8044, 1:500; Santa Cruz Biotechnology, Inc.), p-Bad (cat. no. sc-7998; 1:500; Santa Cruz Biotechnology, Inc.), Bcl-2-associated X (Bax; cat. no. sc-493; 1:500; Santa Cruz Biotechnology, Inc.) and cleaved caspase-3 (cat. no. sc-271759; 1:500; Santa Cruz Biotechnology, Inc.) diluted with primary antibody dilution buffer (Beyotime Institute of Biotechnology). Following three washes with TBS/0.1% Tween-20, the membrane was incubated for 1 h with anti-rabbit, anti-mouse or anti-goat horseradish peroxidase (HRP)-conjugated secondary antibodies (cat. nos. TA130023; TA130003; TA130031, respectively; 1:3,000; OriGene Technologies, Inc., Beijing, China) at room temperature. Following three washes, the proteins were detected using Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore) and visualized using a ChemiDoc Touch imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The relative expression levels of p-Akt and p-Bad were normalized to the total protein expression levels of Akt and Bad, respectively, and all other genes were normalized to the expression of β-actin. The groups were normalized by negative control group, using ImageJ software (version 2x; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are presented as the mean ± standard error of the mean from at least three separate experiments. The differences among groups were analyzed by one-way analysis of variance followed by Fisher's least significant difference test with SPSS statistical software (version 17.0; SPSS, Inc., Chicago, IL, USA). The correlation between growth ratio of HUVECs and MWS-19 concentrations was analyzed by Pearson's correlation analysis with SPSS 17.0 statistical software. P<0.05 was considered to indicate a statistically significant difference.

Results

MWS-19 inhibits 500 μM H_2O_2-induced death of HUVECs. To determine the effects of H_2O_2 in inducing the death of HUVECs, the present study assessed cell viability following exposure various concentrations of H_2O_2 (0-800 μM). The results demonstrated that 200-800 μM H_2O_2 exhibited a cytotoxic effect on HUVEC viability in a dose-dependent manner (Fig. 1B). Based on these results, 500 μM was selected as the model dose (6) to induce cell death for subsequent experiments on apoptotic cell death.

To determine the protective effect of MWS-19 on HUVEC death induced by 500 μM H_2O_2, HUVECs were treated with various concentrations of MWS-19 (0-1,000 μg/ml) for 24 h followed by treatment with 500 μM H_2O_2 for a further 24 h prior to analysis. The results demonstrated that MWS-19 (15.6-500 μg/ml) treatment significantly increased the viability of HUVECs compared with control cells treated with 500 μM H_2O_2 + 0 μg/ml MWS-19 (P<0.05; Fig. 1C), and correlation analysis demonstrated a positive correlation between MWS-19 concentration and the growth ratio of HUVECs, within a dose range of 0-250 μg/ml MWS-19 (P<0.01; R^2=0.7842; Fig. 1D). Furthermore, Fig. 1C and D indicated that the optimal concentration of MWS-19 to prevent H_2O_2-induced cell death was 250 μg/ml. As a dose of 500 μg/ml MWS-19 led to reduced growth compared with lower concentrations, and 1,000 μg/ml led to an increased cytotoxic effect compared with the 500 μM H_2O_2 + 0 μg/ml MWS-19 group (Fig. 1C), doses of 31.3, 62.5

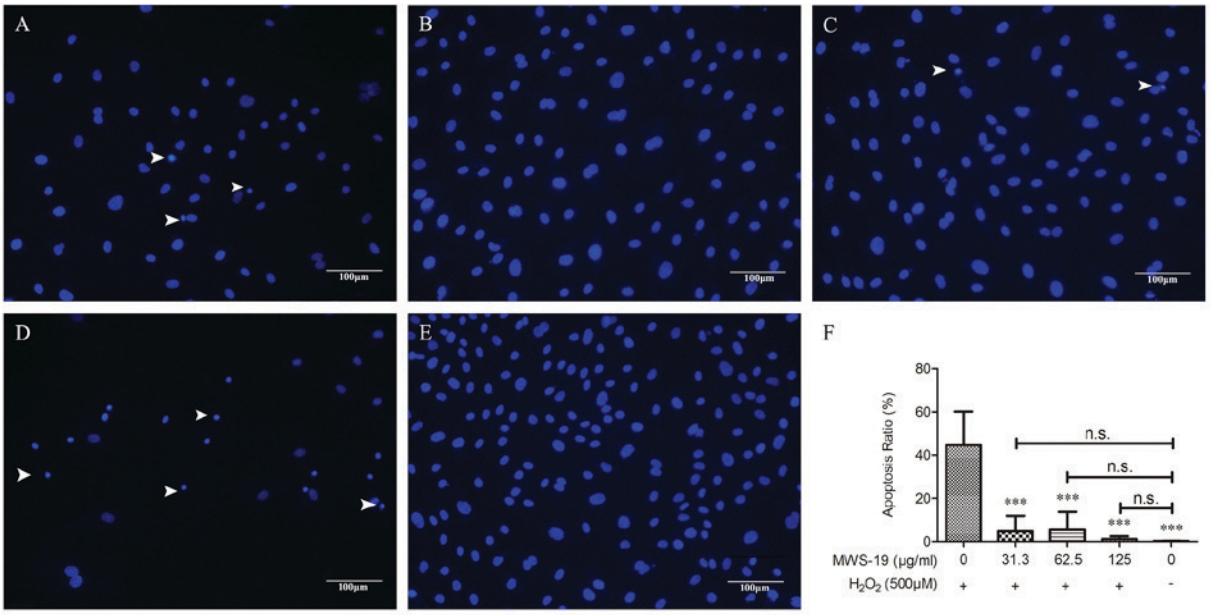

Figure 2. Hoechst 33258 staining of HUVECs. Cells with condensed chromatin (arrowheads) were defined as apoptotic HUVECs. Representative microscopy images for (A) 31.3 μg/ml MWS-19 + 500 μM H_2O_2, (B) 62.5 μg/ml MWS-19 + 500 μM H_2O_2, (C) 125 μg/ml MWS-19 + 500 μM H_2O_2, (D) model control (500 μM H_2O_2) and (E) negative control groups. Scale bars, 100 μm (F) Apoptosis ratios were calculated for each group. ***P<0.001 vs. model control group. n.s., no significant difference; HUVECs, human umbilical vein endothelial cells; MWS-19, isolariciresinol-9'-O-α-L-arabinofuranoside; H_2O_2, hydrogen peroxide.

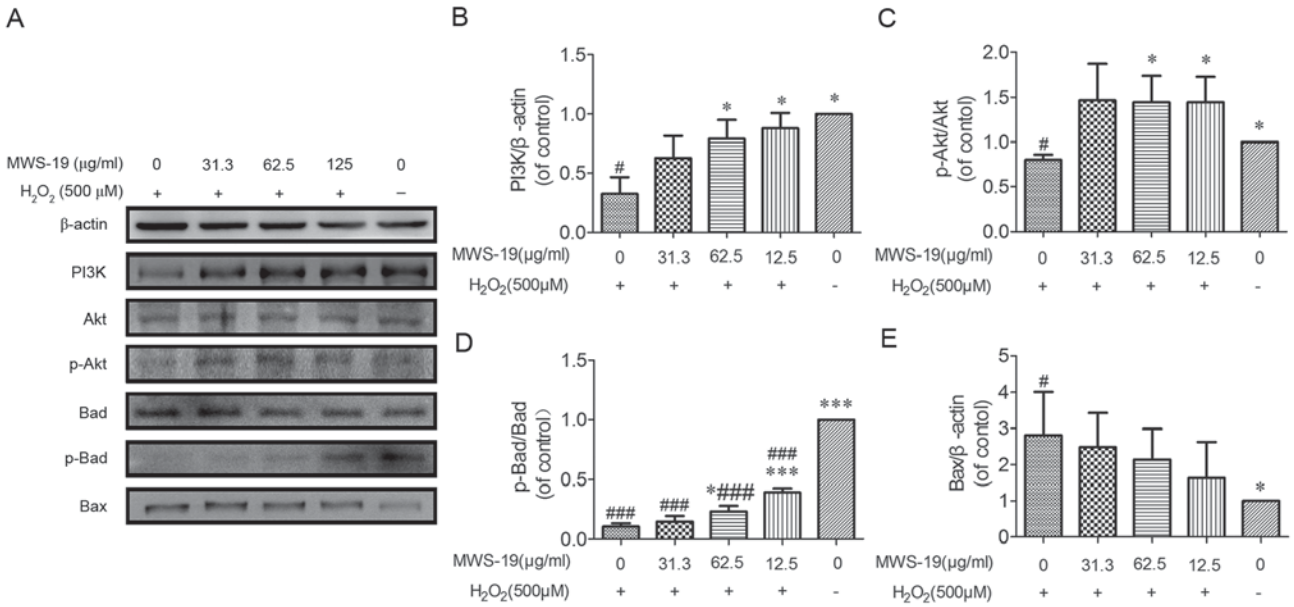

Figure 3. MWS-19 may attenuate the apoptosis of HUVECs via the PI3K/Akt pathway. (A) Cells were pretreated with 31.3, 62.5 and 125 μg/ml MWS-19 for 24 h, followed by treatment with 500 μM H_2O_2 for 24 h and western blotting was performed. The relative protein levels of (B) PI3K, (C) p-Akt, (D) p-Bad and (E) Bax were quantified by densitometric analysis of blots. Data are presented as the mean ± standard error of the mean, n=3. *P<0.05 and ***P<0.001 vs. model control group; $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. negative control group. MWS-19, isolariciresinol-9'-O-α-L-arabinofuranoside; HUVECs, human umbilical vein endothelial cells; PI3K, phosphatidylinositol 3-kinase; H_2O_2, hydrogen peroxide; p-, phosphorylated-; Bad, Bcl-2-associated agonist of cell death; Bax, Bcl-2-associated X.

and 125 μg/ml were selected for subsequent experiments concerning apoptosis and the molecular mechanisms.

Protective effect of MWS-19 against 500 μM H_2O_2-induced apoptosis in HUVECs. Nuclear morphology was evaluated using membrane-permeable blue Hoechst 33258 staining. Hoechst 33258 fluorescence photomicrographs of HUVECs were incubated with MWS-19 (31.3, 62.5 and 125 μg/ml) followed by 500 μM H_2O_2, in addition to control groups, are presented in Fig. 2A-E. In control cultures, the nuclei of cells

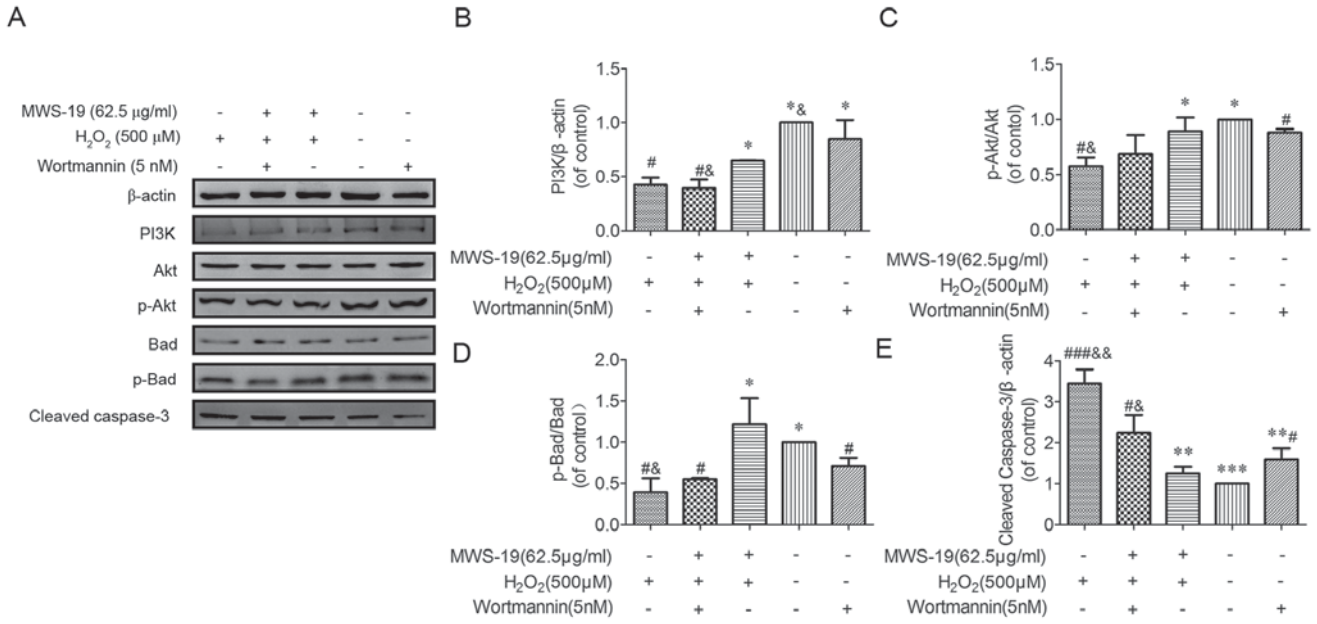

Figure 4. MWS-19 activated the PI3K-Akt pathway. (A) Cells were pretreated with 62.5 μg/ml MWS-19 with or without 5 nM wortmannin for 24 h, followed by treatment with 500 μM H_2O_2 for 24 h, and western blotting was performed. The relative protein levels of (B) PI3K, (C) p-Akt, (D) p-BAD and (E) cleaved caspase-3 were quantified by densitometric analysis. Data are presented as the mean ± standard error of the mean, n=3. *P<0.05, **P<0.01 and ***P<0.001 vs. model control group; $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. negative control group; $^{\&}$P<0.05 and $^{\&\&}$P<0.01 vs. MWS-19 treatment group without wortmannin. MWS-19, isolaricires inol-9'-O-α-L-arabinofuranoside; PI3K, phosphatidylinositol 3-kinase; H_2O_2, hydrogen peroxide; p-, phosphorylated-; Bad, Bcl-2-associated agonist of cell death.

appeared with regular contours and were round and larger in size; cells with smaller nuclei and condensed chromatin were rarely observed (Fig. 2E). By contrast, the majority of nuclei in H_2O_2-treated cells appeared hypercondensed (brightly stained) in the model group (Fig. 2D). Furthermore, the number of cells/field of view were markedly decreased in the model group compared with MWS-19-treated cells (31.3, 62.5 and 125 μg/ml; Fig. 2A-D). As the apoptosis ratios for 31.3, 62.5 and 125 μg/ml MWS-19 treatments were similar to the ratio for the negative control (P>0.05; Fig. 2F), the results indicate that MWS-19 may protect HUVECs against apoptosis induced by 500 μM H_2O_2.

MWS-19 suppresses H_2O_2-induced apoptosis by activating the PI3K/Akt pathway. To gain further insight into the mechanism of MWS-19 on H_2O_2-induced apoptosis, the present study performed western blot analysis to detect alterations in the protein levels of certain genes in HUVECs (Fig. 3A). As demonstrated in Fig. 3A-C, the levels of PI3K and p-Akt in HUVECs were markedly decreased by H_2O_2 in the model group compared with the negative control group, and pretreatment with MWS-19 upregulated the levels of PI3K and p-Akt; PI3K and p-Akt protein expression levels were significantly increased following pretreatment with 62.5 and 125 μg/ml MWS-19, compared with the model group (P<0.05).

Consistent with the increase in p-Akt levels following MWS-19 treatment, the expression levels of p-Bad, an important member of the proapoptotic protein family that is involved in the release of cytochrome C from the mitochondria (3), was increased significantly in 62.5 and 125 μg/ml MWS-19 groups compared with the model group (P<0.05; Fig. 3A and D). However, pretreatment with MWS-19

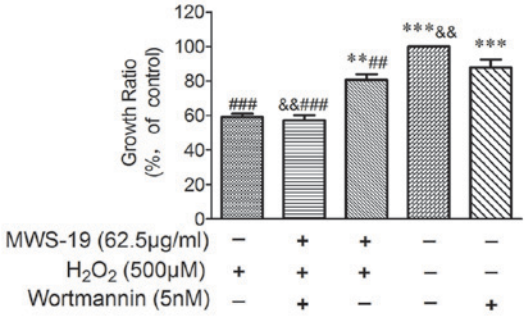

Figure 5. MWS-19 may increase the growth of HUVECs via the phosphatidylinositol 3-kinase/Akt pathway. HUVECs were pretreated with 62.5 μg/ml MWS-19 with or without 5 nM wortmannin for 24 h, followed by treatment with 500 μM H_2O_2 for 24 h, and a Cell Counting Kit-8 assay was performed. Data are presented as the mean ± standard error of the mean, n=3. **P<0.01 and ***P<0.001 vs. model control group; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. negative control group; $^{\&\&}$P<0.01 vs. MWS-19 treatment group without wortmannin. MWS-19, isolariciresinol-9'-O-α-L-arabinofuranoside; HUVECs, human umbilical vein endothelial cells; H_2O_2, hydrogen peroxide.

(31.3, 62.5 and 125 μg/ml) did not affect the protein expression level of Bax compared with the model group (P>0.05; Fig. 3A and E).

Taken together, these results demonstrate that MWS-19 may activate the PI3K/Akt pathway to suppress H_2O_2-induced apoptosis, by upregulating PI3K expression, increasing the phosphorylation of Akt and increasing levels of p-Bad.

MWS-19 promotes HUVECs survival by activating the PI3K/Akt pathway. The present study further investigated whether MWS-19 activated the PI3K/Akt pathway and

suppressed the phosphorylation of Bad to promote HUVEC survival. HUVECs were incubated with a PI3K/Akt signaling inhibitor, wortmannin, in the presence or absence of MWS-19 (62.5 μg/ml) prior to treatment with 500 μM H_2O_2. The results demonstrated that the protein levels of PI3K and p-Akt were markedly increased by combined pretreatment with wortmannin (5 nM) and MWS-19 (62.5 μg/ml), compared with the MWS-19 treatment group without wortmannin ($P<0.05$), and were similar to levels in the model group ($P>0.05$; Fig. 4A-C). Additionally, p-Bad levels were markedly reduced in the MWS-19 + wortmannin treatment group but there was no significant decrease compared with the MWS-19 group without wortmannin ($P<0.05$; Fig. 4D). These results indicate that MWS-19 may activate the PI3K/Akt pathway via upregulation of p-Bad. Additionally, cleaved caspase-3 was upregulated by 500 μM H_2O_2, and pretreatment with wortmannin partially reversed the effect of MWS-19 in downregulating cleaved caspase-3 levels compared with the model group (Fig. 4E), which indicates that MWS-19 may suppress H_2O_2-induced HUVEC apoptosis via activation of the PI3K/Akt/Bad/caspase-3 pathway.

Furthermore, the present study investigated whether downregulation of the PI3K/Akt pathway, and reduced p-Bad levels, may attenuate the MWS-19-induced improvements in cell growth in HUVECs exposed to H_2O_2. As demonstrated in Fig. 5, treatment with wortmannin in the presence of MWS-19 (62.5 μg/ml) reduced the growth ratio to a level that was similar to the model group ($P>0.05$), and was significantly reduced compared with the group treated with MWS-19 in the absence of wortmannin ($P<0.05$). These results indicate that the PI3K/Akt/Bad pathway may be involved in the protective effect of MWS-19 against apoptosis in HUVECs.

The results described above indicate that the protective effects of MWS-19 against H_2O_2-induced HUVEC apoptosis may occur via the PI3K/Akt/Bad/caspase-3 pathway.

Discussion

Oxidative stress is associated with various pathological and degenerative processes, including the development of hypertension and atherosclerosis, and antioxidant supplements may be taken to improve the antioxidant capacity of the body. Naturally occurring antioxidants, such as lignans, have received attention in research (20), with one study reporting that lignans exerted a beneficial effect on cardiovascular disease (17).

The vascular endothelium is involved in maintaining vascular permeability barrier, and endothelial cells function as a barrier to prevent platelet and monocyte adhesion and inhibiting the proliferation of vascular smooth muscle cells. Endothelial dysfunction as a result of oxidative stress is thought to contribute to the pathogenesis and development of hypertension (21-23).

In pathological conditions, ROS may regulate various important biological pathways, including PI3K/Akt and mitogen-activated protein kinases. In particular, H_2O_2 is a major form of ROS and is well established as a method of inducing models of oxidative injury (6,24). Therefore, the present study established a HUVEC oxidative stress model using H_2O_2 to inducing apoptosis of HUVECs, and chose to initially investigate PI3K/Akt as a potential signaling pathway involved in the protective effects of MWS-19 on HUVEC apoptosis. The current study employed a dose of 500 μM H_2O_2 to establish the model group in subsequent experiments based on the results presented in Fig. 1B, as this concentration reduced the growth rate by ~50% compared with the negative control group. Furthermore, a study by Chen *et al* (6) also reported that 500 μM H_2O_2 is an appropriate concentration for inducing optimal HUVEC apoptosis, with minimal amounts of secondary necrosis observed.

CCK-8 assay results in the present study demonstrated that MWS-19 markedly reduced the death of HUVECs induced by H_2O_2 in a dose-dependent manner. These results were verified by results of Hoechst 33258 staining, which indicated that MWS-19 reduced apoptosis of HUVECs at concentrations of 31.3, 62.5 and 125 μg/ml.

To further determine the mechanisms involved in the protection of endothelial cells by MWS-19 against oxidative stress-induced apoptosis, the present study investigated the effects of MWS-19 on the protein expression of components of the PI3K/Akt/Bad pathway. The results demonstrated that treatment with MWS-19 increased the activation of Akt protein in HUVECs exposed to H_2O_2. Akt is an important member of the PI3K pathway that has an important role in the regulation of endothelial cell survival and proliferation, and Akt activation confers protection against H_2O_2-induced apoptosis (1,25). Additionally, the results indicated that MWS-19 (62.5 μg/ml and 125 μg/ml) may protect against apoptosis in HUVECs by upregulation of PI3K and p-Akt, which leads to upregulated levels of p-Bad. Bad, a member of the Bcl-2 family, is a substrate for Akt. In its unphosphorylated form, it combines with the antiapoptotic factors Bcl-2 or Bcl-xl to liberate Bax for permeabilization of the mitochondrial outer membrane (26). Phosphorylation of Bad by Akt suppresses its apoptosis-promoting functions (27). The results of the present study demonstrate that no significant differences were observed between the relative expression of Bax in MWS-19 and model groups (Fig. 3E), which indicates that MWS-19 may prevent the apoptosis of HUVECs by increasing the amount of Bax that is bound to Bcl-2 or Bcl-xl protein rather than decreasing the expression.

Furthermore, the current study also demonstrated that inhibition of PI3K activity with wortmannin significantly suppressed 62.5 μg/ml MWS-19-induced increases in HUVEC growth. Additionally, levels of cleaved caspase-3 protein were increased significantly in the wortmannin + MWS-19 group compared with the group treated by MWS-19 alone (Fig. 4E), while p-Akt and p-Bad levels in control cells treated with wortmannin were decreased significantly compared with the negative control group without wortmannin. These results indicate that MWS-19 may suppress H_2O_2-induced apoptosis via the PI3K/Akt/Bad/caspase-3 pathway.

To summarize, the results of the current study demonstrated that MWS-19 exerted protective effects against H_2O_2-induced apoptosis in HUVECs *in vitro*. The results also indicate that the antiapoptotic effects associated with MWS-19 treatment may occur via the activation of the PI3K/Akt signaling pathway. MWS-19, a type of lignan, was obtained from the fresh pine needles of *Pinus massoniana* Lamb. in the Sichuan province of China. The above findings may provide an important basis

for further investigations to improve the understanding of the actions of SXC in hypertension, and the potential beneficial effects of MWS-19 treatment on the prevention of oxidative damage in vascular endothelial cells in patients with hypertension. In conclusion, MWS exerted protective effects against apoptosis in HUVECs, and this protection may occur via a mechanism involving the PI3K/Akt/Bad signaling pathway. Further studies are required to determine the direct effects of MWS-19 on endothelial cells in spontaneously hypertensive animal models.

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