

Resveratrol attenuates hydrogen peroxide-induced apoptosis, reactive oxygen species generation, and PSGL-1 and VWF activation in human umbilical vein endothelial cells, potentially via MAPK signalling pathways

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Abstract. Reactive oxygen species (ROS) are implicated in the pathogenesis of thrombosis. Studies have reported that resveratrol exhibits antioxidative activities, however, the effect and underlying mechanisms of resveratrol on venous thrombosis remain largely unknown. To investigate the effect of resveratrol on venous thrombosis and the underlying mechanisms, the present study investigated the effects of resveratrol on cell viability, apoptosis, ROS generation and the expression of thrombosis-associated markers in human umbilical vein endothelial cells (HUVECs). HUVECs were pretreated with resveratrol for 2 h and incubated with hydrogen peroxide (H₂O₂) for 24 h prior to the evaluation of cell viability, ROS generation, apoptosis and thrombosis-associated marker expression by performing MTT assays, 2',7'-dichlorofluorescein diacetate reagent, flow cytometry, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis, respectively. Subsequently, to validate whether resveratrol functions via mitogen-activated protein kinase (MAPK) pathways, the expression of thrombosis-associated

markers was detected by western blot analysis and RT-qPCR following treatment of cells with resveratrol and the MAPK pathway activators anisomycin and curcumin. The results demonstrated that cell viability was markedly reduced by H₂O₂, and resveratrol treatment reversed the reductions in cell viability in a dose-dependent manner. In addition, the levels of cell apoptosis and ROS generation were significantly increased by H₂O₂ alone, and resveratrol also reduced these effects in a dose-dependent manner. Furthermore, the mRNA and protein expression of caspase-3, P-selectin glycoprotein ligand-1 and von Willebrand factor was upregulated by H₂O₂ treatment in HUVECs. However, resveratrol decreased the protein expression these proteins in a dose-dependent manner. Resveratrol also significantly inhibited the induction of phosphorylated (p)-p38, P-c-Jun N-terminal kinase and P-extracellular signal-regulated kinase by H₂O₂, and these effects were attenuated by the MAPK pathway activators anisomycin and curcumin. In conclusion, these results indicate that resveratrol protected HUVECs against oxidative stress and apoptosis. Furthermore, to the best of our knowledge, the present study is the first to demonstrate that resveratrol attenuates the expression of thrombosis-associated markers induced by H₂O₂, which may occur through the suppression of the MAPK signalling pathways, indicating a potential novel therapeutic approach to prevent venous thrombosis.

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Abbreviations: DVT, deep venous thrombosis; ROS, reactive oxygen species; HUVECs, human umbilical vein endothelial cells; ECs, endothelial cells; ERK, extracellular signal-regulated kinase; c-Jun, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; VWF, von Willebrand factor; PSGL-1, P-selectin glycoprotein ligand-1

Key words: resveratrol, reactive oxygen species, apoptosis, thrombosis-associated markers, mitogen-activated protein kinase signalling pathway

Introduction

Deep venous thrombosis (DVT) is a global health problem that affects 1 in 1,000 individuals according to epidemiological studies (1). Even with preventive measures, the incidence of DVT ranges from 2.22-3.29% among patients undergoing major orthopaedic surgeries in developed countries (2,3). Pulmonary embolism, the most dangerous complication of DVT, affects between 500,000 and 700,000 patients, which may cause mortality in 10-15% of these patients (4). Structural and functional disorders of venous endothelial cells (ECs) have an initial role in the pathogenesis and progression of DVT (5). Currently, oxidative stress injury, which occurs during hypoxia, sepsis and ischaemia-reperfusion injury, is considered to lead to EC dysfunction and initiation of the

coagulation cascade (6,7). Excessive levels of reactive oxygen species (ROS) cause oxidative damage to ECs and subsequently activate platelets and leukocytes, increasing their adhesion and aggregation ability and triggering procoagulant reactions (8,9). Therefore, the hypothesis of adopting antioxidants to prevent vascular embolism diseases has been proposed and proven effective in several studies (10-12).

Resveratrol (*trans*-3,4,5-trihydroxystilbene), a natural, plant-derived polyphenolic compound that is abundant in grape skin, peanuts, mulberries and red wine, has been proven to exhibit a wide range of pharmacological activities, including antioxidant activity, lipoprotein metabolism modulation, anti-platelet aggregation, and anti-inflammatory and anti-tumour effects (13). In addition, cardioprotective activities of resveratrol have previously been identified, however, studies have primarily focused on cardiac and arterial diseases (14,15). Whether resveratrol may exhibit inhibitory effects on venous thrombosis remains largely unknown. Pathways that have previously been associated with the effects of resveratrol treatment include AMP-activated protein kinase, phosphatidylinositol 3-kinase/Akt and sirtuin 1 (16,17). Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (c-Jun) and p38 mitogen-activated protein kinase (MAPK) are conventional MAPK signalling pathway families that are implicated in various diseases, including tumour growth and haemostasis, among others. Furthermore, MAPK signalling pathways, which promote apoptosis and have roles in venous endothelial oxidative stress injury, have been confirmed as important targets that promote thrombosis by mediating interactions among venous endothelial cells, leukocytes and platelets (18). The present study hypothesises that MAPKs may be involved in mediating the effects of resveratrol on venous thrombosis. In addition, von Willebrand factor (VWF) and P-selectin glycoprotein ligand-1 (PSGL-1) are considered thrombotic risk markers, with increases in these factors indicating a thrombotic phenotype (19,20). Therefore, the present study investigated VWF and PSGL-1 levels in HUVECs following different treatments.

The present study aimed to investigate the effects of resveratrol on apoptosis, ROS generation and the expression of thrombosis-associated markers (VWF and PSGL-1) induced by hydrogen peroxide (H_2O_2) in addition to the underlying mechanisms, *in vitro*. The results of the present study indicated that resveratrol may exert antithrombotic activity *in vitro* by inactivating MAPK signalling pathways.

Materials and methods

Reagents, chemicals and antibodies. Primary human umbilical vein endothelial cells (HUVECs) were purchased from CHI Scientific, Inc. (Maynard, MA, USA). High-glucose Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were both purchased from Hyclone (GE Healthcare Life Sciences, Logan, UT, USA). Resveratrol (purity >99.0%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All primary antibodies were purchased from Proteintech Group, Inc. (Chicago, IL, USA). Phosphorylated antibodies were purchased from ABclonal, Inc. (Cambridge, MA, USA). Anisomycin and curcumin were purchased from Beijing TOP Science Biotechnology Co., Ltd. (Beijing,

China). The MTT kit, dimethyl sulfoxide (DMSO), and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double-staining Apoptosis Detection kit was obtained from Beyotime Institute of Biotechnology (Haimen, China). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

Cell culture and treatments. HUVECs were cultured in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were maintained at 37°C with 5% CO_2 in a humidified atmosphere. Cells were digested with 0.25% trypsin, centrifuged and harvested in a centrifuge (150 x g at room temperature for 5 min) and resuspended in DMEM medium. The cell concentration was adjusted to 1×10^4 cells/ml and cells were inoculated into a culture flask. The resveratrol solution was prepared in DMSO and diluted with culture medium immediately prior to use. DMSO was used as the control group. The cells were pretreated at 37°C with resveratrol (0, 10, 20 and 30 μ M) for 2 h and were then subjected to H_2O_2 (200 μ M) for 24 h. In addition, in the MTT assay, one group received treatment with 30 μ M resveratrol without H_2O_2 treatment. Control cells were treated with medium alone. After the treatments were performed, the cells were harvested for further analysis.

Analysis of cell viability. The viability of HUVECs treated with 0, 10, 20 and 30 μ M resveratrol for 2 h followed by treatment with or without 200 μ M H_2O_2 for 24 h was assessed with an MTT assay. After cells were treated and washed with PBS, 10 μ l MTT solution (5 mg/ml) was added to the cells. The samples were incubated for 4 h at 37°C and 100 μ l dimethyl sulfoxide was added to each well to dissolve the formazan crystals for 4 h at 37°C. The plates were read at 570 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the cell viability was expressed as the optical density (OD) value.

Cellular apoptosis assay. HUVECs that were treated with 0, 10, 20 and 30 μ M resveratrol for 2 h followed by treatment with or without H_2O_2 for 24 h were double stained using an Annexin V-FITC/PI Apoptosis Detection kit, according to the manufacturer's instructions. Briefly, the cells were washed and collected in a 1.5 ml tube, 1×10^5 - 1×10^6 cells were resuspended in 300 μ l 1X binding buffer and cells were transferred to a sterile flow cytometry glass tube. Annexin V-FITC (5 μ l) was added and the tubes were incubated in the dark for 30 min at room temperature. Subsequently, cells were incubated in the dark with 5 μ l PI at room temperature for 15 min and analysed with a flow cytometer (Sysmex Partec GmbH, Gorlitz, Germany) and FloMax[®] software version 2.7 (Sysmex Partec GmbH).

Measurement of intracellular ROS. Intracellular ROS levels were evaluated using the DCFH-DA fluorescent probe reagent and a flow cytometer, according to the manufacturer's instructions. Briefly, after cells (1×10^5) were seeded in 6-well plates and treated with 0, 10, 20 and 30 μ M resveratrol for 2 h followed by treatment with or without H_2O_2 for 24 h, the cells were washed with PBS and incubated with DCFH-DA

(10 μ M) in DMEM medium without FBS at 37°C for 20 min. Following incubation, the cellular 2',7'-dichlorofluorescein (DCF) green fluorescence in each well was subsequently imaged at 488 nm using a fluorescence microscope at x200 magnification (Olympus Corporation, Tokyo, Japan), and DCF fluorescence was quantified using Image-Pro Plus 6 software (Media Cybernetics, Inc., Rockville, MD, USA). Subsequently, flow cytometry (Sysmex Partec GmbH) and FloMax[®] software version 2.7 (Sysmex Partec GmbH) was also performed to quantify DCF fluorescence and measure ROS generation.

Western blot analysis. Cells were treated at 37°C with 0, 10, 20 and 30 μ M resveratrol for 2 h followed by treatment with or without H₂O₂ for 24 h. For experiments including anisomycin/curcumin treatment, cells were treated with resveratrol (0 and 30 μ M) for 2 h followed by treatment with or without anisomycin (0.4 μ g/ml for 6 h) or curcumin (25 μ M for 4 h) prior to treatment with 200 μ M H₂O₂ for 24 h. Cells were homogenized in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology). The lysates were centrifuged (10,625 x g at room temperature for 5 min) and the protein supernatant was collected. Protein concentrations were determined using a BCA Protein assay kit (Beyotime Institute of Biotechnology). The proteins (30 μ g) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and 0.1% Tween-20 (TBS-T, pH 7.6) at room temperature for 2 h and probed with antibodies (at 1:1,000) against phosphorylated (p)-p38 MAPK (cat. no. AP0297), p38 MAPK (cat. no. 14064-1-AP), P-c-Jun (cat. no. AP0048), c-Jun (cat. no. 24909-1-AP), P-ERK (cat. no. AP0472), ERK (cat. no. 16443-1-AP), PSGL-1 (cat. no. 23605-1-AP), VWF (cat. no. 11778-1-AP), caspase-3 (cat. no. 19677-1-AP) and β -actin (cat. no. 60008-1-AP) at 4°C overnight. The membranes were washed with TBS-T and hybridized with secondary goat anti-mouse (cat. no. sc-2039) or goat anti-rabbit (cat. no. sc-2007) horse radish peroxidase antibodies at 1:2,000 dilution (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at room temperature and washed again with TBS-T. The protein bands were developed using an ECL Western Blotting substrate (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and exposed to autoradiography film (Bio-Rad Laboratories, Inc.) for visualization of the bands. β -actin was used as a loading control. The results were quantified using ImageJ software version 1.48 (National Institutes of Health, Bethesda, MD, USA). The results for the control group were defined as 100%.

RT-qPCR assay. Cells were treated at 37°C with resveratrol (0 and 30 μ M) for 2 h followed by treatment with or without 200 μ M H₂O₂ for 24 h. For experiments including anisomycin/curcumin treatment, cells were treated with resveratrol (0 and 30 μ M) for 2 h followed by treatment with or without anisomycin (0.4 μ g/ml for 6 h) or curcumin (25 μ M for 4 h) prior to treatment with 200 μ M H₂O₂ for 24 h. Total RNA was isolated from HUVECs using TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. The integrity of the RNA samples was evaluated using agarose gel electrophoresis. Total RNA was reverse transcribed into

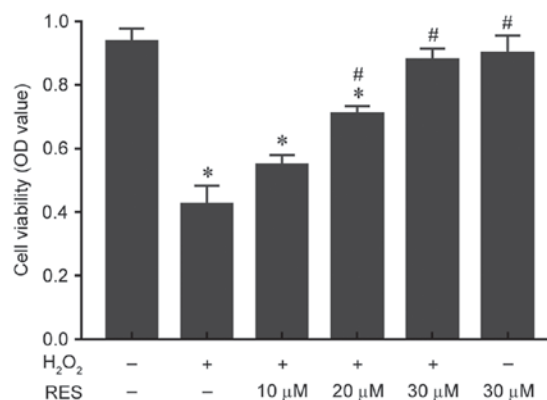


Figure 1. Resveratrol rescues cell viability in H₂O₂-treated HUVECs. HUVECs were exposed to 0, 10, 20 and 30 μ M resveratrol for 2 h prior to treatment with 200 μ M H₂O₂ for 24 h. Cell viability was assessed with an MTT assay. Data are presented as the mean \pm standard error of the mean from six independent experiments, each performed in triplicate. *P<0.05 vs. control; #P<0.05 vs. H₂O₂ only group. H₂O₂, hydrogen peroxide; HUVECs, human umbilical vein endothelial cells; OD, optical density; RES, resveratrol.

cDNA using an PrimeScript[™] RT Master mix kit, followed by SYBR[®] Premix Ex Taq[™] II (both from Takara Biotechnology Co., Ltd.) to detect the expression levels. β -actin was used as an internal control. The thermocycling conditions were as follows: Thirty seconds at 95°C for the activation of Taq DNA polymerase, followed by 40 cycles of amplification at 95°C for 15 sec and 60°C for 1 min. The reactions were performed in triplicate. The specific primers used for qPCR were as follows: Caspase-3, 5'-GGGCTGTGTAAGTGAAGAAA-3' (forward) and 5'-CCGTCTTTGAATTTCTCCA-3' (reverse); PSGL-1, 5'-ATGATTTCTCTGCCAGAAACG-3' (forward) and 5'-GTG GTCAGCTCTGTGACTGC-3' (reverse); VWF, 5'-TCCTCC TACTCTGCCCCC-3' (forward) and 5'-TCCATCCGCTGA ATCACCTC-3' (reverse); β -actin, 5'-ACGGCAAGTTCAA CGGCACAG-3' (forward) and 5'-GACGCCAGTAGACTC CACGACA-3' (reverse); p38, 5'-GGGGCAGATCTGAAC AACAT-3' (forward) and 5'-CAGGAGCCCTGTACCACC TA-3' (reverse); ERK, 5'-TGATCACACAGGGTTCCTGA-3' (forward) and 5'-TGGAAAGATGGGCCTGTTAG-3' (reverse); and c-Jun, 5'-ACAGAGCATGACCCTGAACC-3' (forward) and 5'-CCATTCTGGACTGGATTAT-3' (reverse). Gene expression values were calculated and normalized to β -actin expression using the 2^{- $\Delta\Delta$ C_q} relative expression method (21).

Statistical analysis. Data are presented as the mean \pm standard deviation. Group comparisons were assessed using one-way analysis of variance followed by Tukey's post hoc test using GraphPad Prism 5.0a (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Resveratrol protects HUVECs from H₂O₂-induced injury. HUVECs were pretreated with resveratrol for 2 h and incubated with H₂O₂ for 24 h, and cell viability was measured with an MTT assay. The viability of cells was decreased significantly by treatment with 200 μ M H₂O₂ for 24 h, compared with the control group (P<0.05; Fig. 1), while

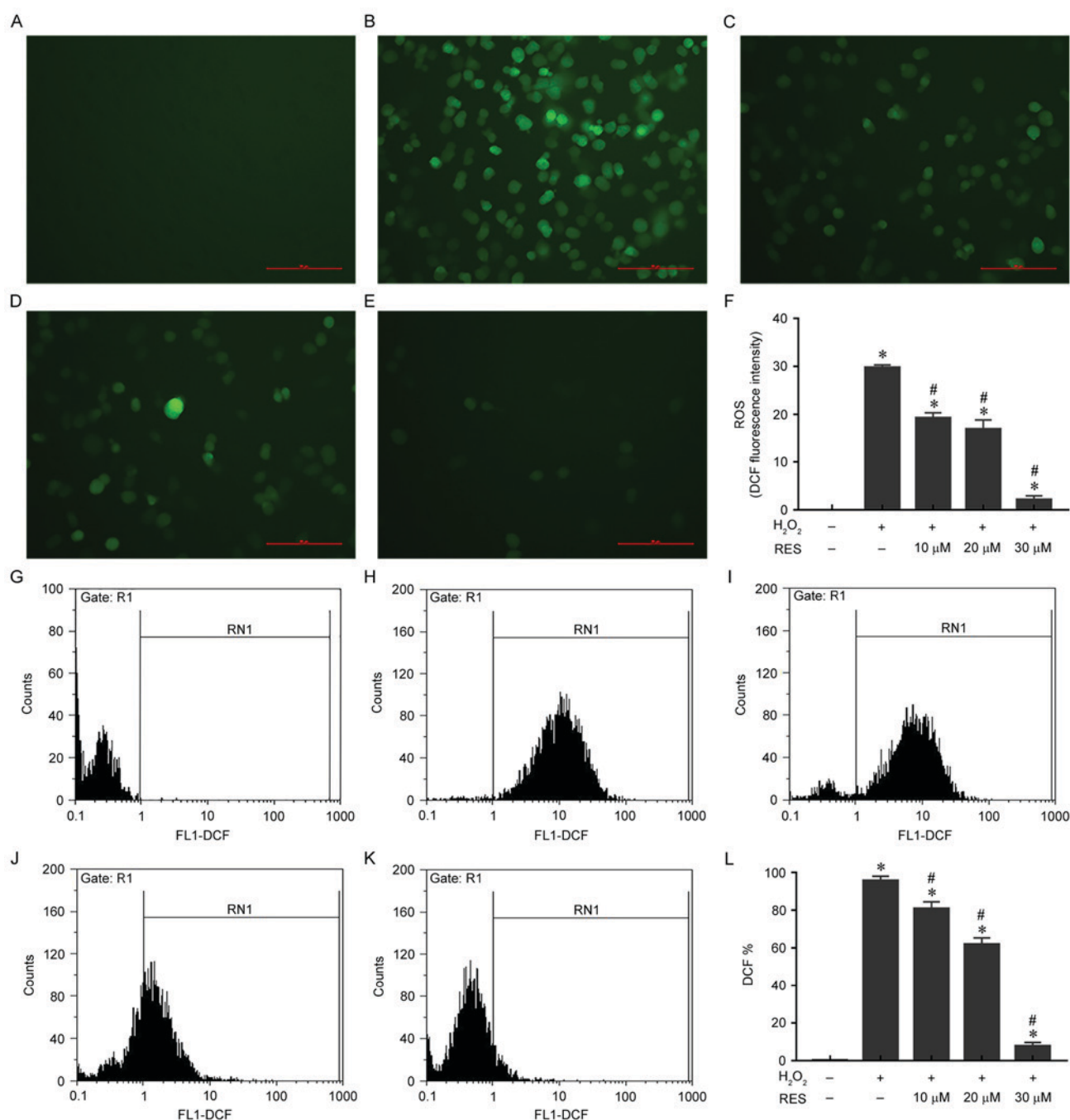


Figure 2. Resveratrol ameliorates H₂O₂-induced ROS generation in HUVECs. Representative micrographs of HUVECs stained with DCF in (A) control, (B) 200 μ M H₂O₂, (C) 200 μ M H₂O₂ + 10 μ M resveratrol, (D) 200 μ M H₂O₂ + 20 μ M resveratrol and (E) 200 μ M H₂O₂ + 30 μ M resveratrol groups, as visualized under a fluorescence microscope at x200 magnification. Scale bar, 100 μ m. (F) Quantification of DCF fluorescence in micrographs using Image-Pro Plus 6.0 software. DCF fluorescence was also quantified by flow cytometry. Representative flow cytometry plots for HUVECs stained with DCF in (G) control, (H) 200 μ M H₂O₂, (I) 200 μ M H₂O₂ + 10 μ M resveratrol, (J) 200 μ M H₂O₂ + 20 μ M resveratrol and (K) 200 μ M H₂O₂ + 30 μ M resveratrol groups. (L) Statistical results of flow cytometry. *P<0.05 vs. control; #P<0.05 vs. H₂O₂ only group. H₂O₂, hydrogen peroxide; ROS, reactive oxygen species; HUVECs, human umbilical vein endothelial cells; DCF, dichlorofluorescein; RES, resveratrol.

resveratrol protected cells from H₂O₂-induced injury in a concentration-dependent manner (10 μ M, 0.55 \pm 0.03 OD; 20 μ M, 0.71 \pm 0.02 OD; and 30 μ M, 0.88 \pm 0.04 OD; Fig. 1). Resveratrol significantly enhanced cell viability at 20 and 30 μ M compared with the H₂O₂ only group (P<0.05), while resveratrol treatment alone exhibited no cytotoxicity compared with the control group (P>0.05). These results indicate that resveratrol may protect HUVECs from H₂O₂-induced injury/death.

Resveratrol attenuates H₂O₂-induced ROS production in HUVECs. ROS are considered indicators of the cellular oxidative stress state. Therefore, intracellular ROS accumulation was determined by measuring DCF-derived fluorescence with the DCFH-DA probe. The results demonstrated that H₂O₂ significantly increased ROS levels compared with the control group (P<0.05) and that resveratrol significantly decreased ROS levels in a concentration-dependent manner compared with the H₂O₂ only group (Fig. 2A-F). The DCF fluorescence

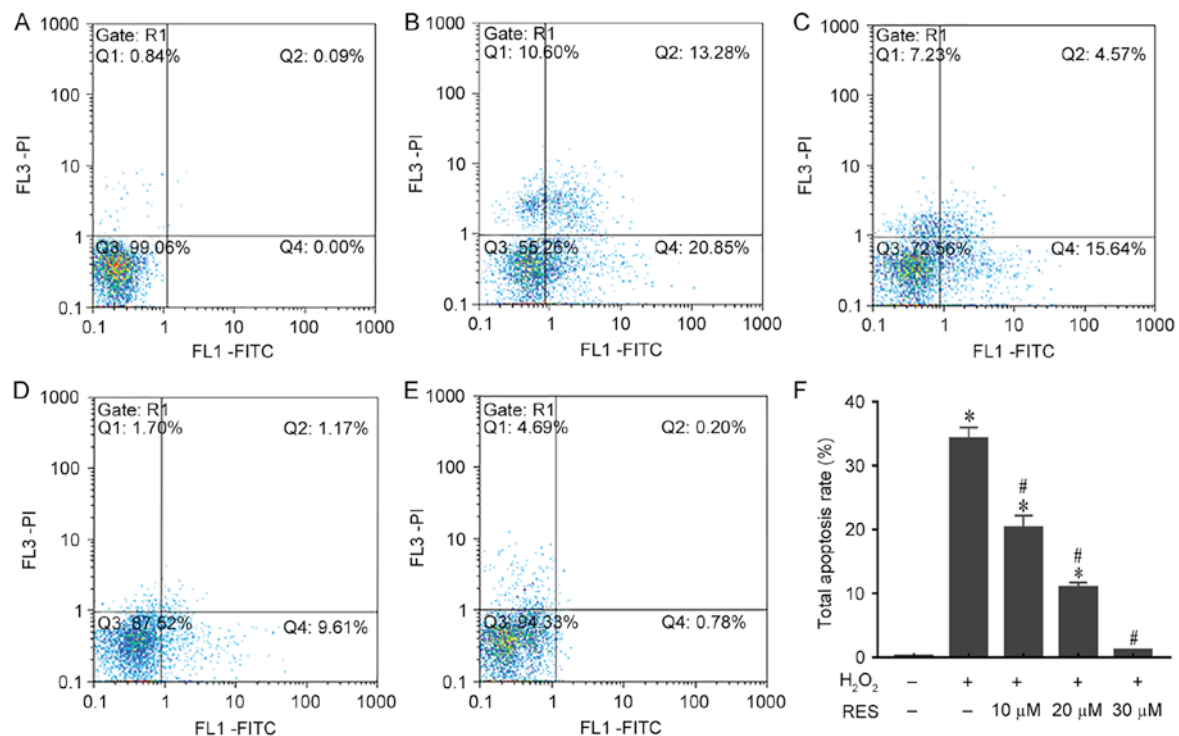


Figure 3. Resveratrol reduces H₂O₂-induced apoptosis of HUVECs. The percentage of apoptotic cells was quantified by Annexin V-PI labelling and flow cytometry analysis. Representative flow cytometry plots indicating apoptosis levels in HUVECs in (A) control, (B) 200 μ M H₂O₂, (C) 200 μ M H₂O₂ + 10 μ M resveratrol, (D) 200 μ M H₂O₂ + 20 μ M resveratrol and (E) 200 μ M H₂O₂ + 30 μ M resveratrol groups. Quadrants 2 and 4 were considered to indicate apoptotic cells. (F) The apoptosis ratio was statistically analysed and presented in a histogram. *P<0.05 vs. control; #P<0.05 vs. H₂O₂ only group. H₂O₂, hydrogen peroxide; HUVECs, human umbilical vein endothelial cells; PI, propidium iodide; RES, resveratrol; FITC, fluorescein isothiocyanate; Q, quadrant.

quantification results obtained by flow cytometry and presented in Fig. 2G-L were consistent with the micrographs. These results indicate that resveratrol may attenuate H₂O₂-induced ROS production and oxidative stress in HUVECs.

Resveratrol reduces H₂O₂-induced apoptosis in HUVECs. To quantitatively investigate the anti-apoptotic effects of resveratrol in H₂O₂-treated HUVECs, cell apoptosis was measured by Annexin V/PI staining. Quadrants 2 and 4 were considered to indicate apoptotic cells. As demonstrated in Fig. 3, HUVEC apoptosis increased to 34.19 \pm 1.8% (P<0.05 vs. control group) when exposed to H₂O₂. By contrast, increasing doses of resveratrol clearly reduced the apoptosis of HUVECs to 20.24 \pm 1.95, 10.93 \pm 0.77 and 1.03 \pm 0.05% (P<0.05 vs. H₂O₂ only group). These results indicate that resveratrol may prevent apoptosis induced by H₂O₂.

Resveratrol inhibits the activation of caspase-3, PSGL-1 and VWF induced by H₂O₂. Caspase-3 is a key mediator of cell death and an executioner for the death programme in cells in response to H₂O₂. PSGL-1 and VWF are markers of a prothrombotic status and predict the occurrence of thrombosis. As demonstrated in Fig. 4A and B by western blotting results, the protein expression of caspase-3 was significantly increased by H₂O₂ treatment compared with the control group (P<0.05). However, when cells were pretreated with resveratrol for 2 h prior to incubation with H₂O₂, caspase-3 protein expression was significantly decreased in a concentration-dependent manner compared with the H₂O₂ only group (P<0.05). The protein levels of PSGL-1 and VWF were also

significantly increased by H₂O₂ compared with the control group (P<0.05), while PSGL-1 and VWF protein expression was significantly decreased by resveratrol pretreatment in a concentration-dependent manner compared with the H₂O₂ only group (P<0.05; Fig. 4A and B). The results for the mRNA levels of caspase-3, PSGL-1 and VWF were similar to those observed for protein levels, although only a resveratrol concentration of 30 μ M was used (Fig. 4C).

Resveratrol suppresses the activation of PSGL-1 and VWF via MAPK signalling pathways. To investigate the underlying mechanisms of the effects of resveratrol on thrombosis-associated markers, the present study also detected the expression of MAPK signalling pathway-associated proteins and mRNA by western blotting and RT-qPCR, respectively. The mRNA levels of p38, ERK and c-Jun, and the protein levels of p-p38, P-ERK and P-c-Jun, were significantly increased by H₂O₂ compared with the control group (P<0.05; Figs. 5 and 6). However, these increases were significantly decreased by pretreatment with 30 μ M resveratrol (P<0.05; Figs. 5 and 6). When HUVECs were exposed to anisomycin (0.4 μ g/ml for 6 h), an activator of p38 and c-Jun, or curcumin (25 μ M for 4 h), an activator of ERK, following treatment with resveratrol, the suppressive effects of resveratrol on PSGL-1 and VWF protein and mRNA expression were attenuated (P<0.05 vs. the H₂O₂ + resveratrol group; Figs. 5 and 6). In addition, anisomycin or curcumin treatment also significantly attenuated the effects of resveratrol treatment on the mRNA expression of p38, ERK and c-Jun, and the protein levels of p-p38, p-ERK and p-c-Jun (P<0.05 vs. the H₂O₂ + resveratrol group; Figs. 5 and 6). However, no

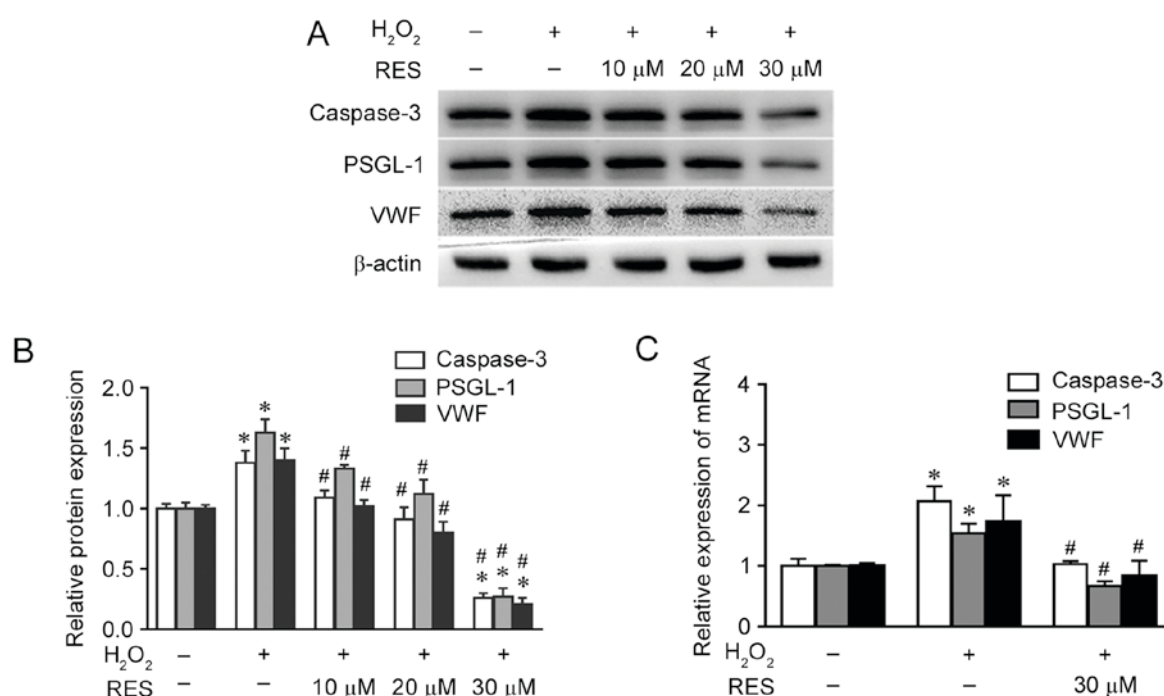


Figure 4. Resveratrol inhibits the protein and mRNA expression of caspase-3, VWF and PSGL-1 induced by H₂O₂ in HUVECs. (A) Protein expression levels of caspase-3, VWF and PSGL-1 in HUVECs exposed to 0, 10, 20 and 30 μM resveratrol for 2 h prior to treatment with or without 200 μM H₂O₂ for 24 h, as measured by western blot analysis. β-actin was used as the loading control. (B) Relative expression ratios of caspase-3, VWF and PSGL-1 were determined by densitometric analysis. (C) mRNA expression of caspase-3, PSGL-1 and VWF in the different groups was investigated by reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. control; #P<0.05 vs. H₂O₂ only group. VWF, von Willebrand factor; PSGL-1, P-selectin glycoprotein ligand-1; H₂O₂, hydrogen peroxide; HUVECs, human umbilical vein endothelial cells; RES, resveratrol.

significant differences were observed in the expression of total p38, ERK and c-Jun protein expression among the different groups (Fig. 5). These results indicate that resveratrol may suppress the activation of PSGL-1 and VWF induced by H₂O₂ via MAPK signalling pathways.

Discussion

DVT is a common postoperative complication following high-energy trauma that leads to localized clot (thrombus) formation within a femoral or popliteal vein. Venous ECs, platelets and leukocytes have crucial roles in regulating the local microenvironmental balance between coagulation and anticoagulation. Under normal physiological conditions, ECs maintain a vasodilatory and local fibrinolytic state, in which the coagulation, adhesion and activation of platelets and leukocytes is suppressed (22). Oxidative stress, defined as the excessive production of ROS and reactive nitrogen species, has been implicated in numerous diseases, including venous thrombosis. Under pathological conditions, excess ROS production is induced by venous stasis and hypoxia, which destroys endothelial structure and function, promotes procoagulation reactions by upregulating cell adhesion molecules (VWF and PSGL-1), inflammatory cytokines and antifibrinolytic substances (plasminogen activator inhibitor-1), and downregulates anticoagulants and fibrinolytic substances (23,24).

Resveratrol, a potent antioxidant, was recently determined to suppress oxidative stress and decrease ROS generation;

the study by Pan *et al* (25) demonstrated that resveratrol suppressed tumour necrosis factor-α-triggered ROS generation in HUVECs. Additionally, Zhou *et al* (26) reported that resveratrol pretreatment significantly attenuated the detrimental effect of tert-butyl hydroperoxide on cell viability and apoptosis by inhibiting mitochondrial ROS generation in HUVECs. In addition, Zhang *et al* (27) reported that the effects of vinorelbine on cell apoptosis, intracellular ROS generation and intracellular superoxide dismutase activity were attenuated when ECV-304 human vascular endothelial cells were pretreated with resveratrol. To confirm these studies, in the present study, HUVECs were pretreated with resveratrol for 2 h and incubated with H₂O₂ for 24 h. HUVEC viability was decreased significantly by H₂O₂, one of the major components of ROS. In addition, resveratrol protected cells from H₂O₂-induced cytotoxicity in a concentration-dependent manner, as demonstrated in Fig. 1. Furthermore, resveratrol significantly decreased ROS levels and clearly attenuated H₂O₂-induced apoptosis, as presented in Figs. 2 and 3. As sufficient evidence indicates that resveratrol may suppress oxidative stress and decrease ROS generation, resveratrol may have potential in the prevention of DVT.

VWF, which is produced by megakaryocytes and ECs, is a multimeric glycoprotein that is expressed in the subendothelial matrix, blood plasma, platelets and endothelium (28). VWF binds to exposed subendothelial collagen rapidly upon damage to the vascular surface and triggers a complex pathway, including platelet activation, adhesion and aggregation (29).

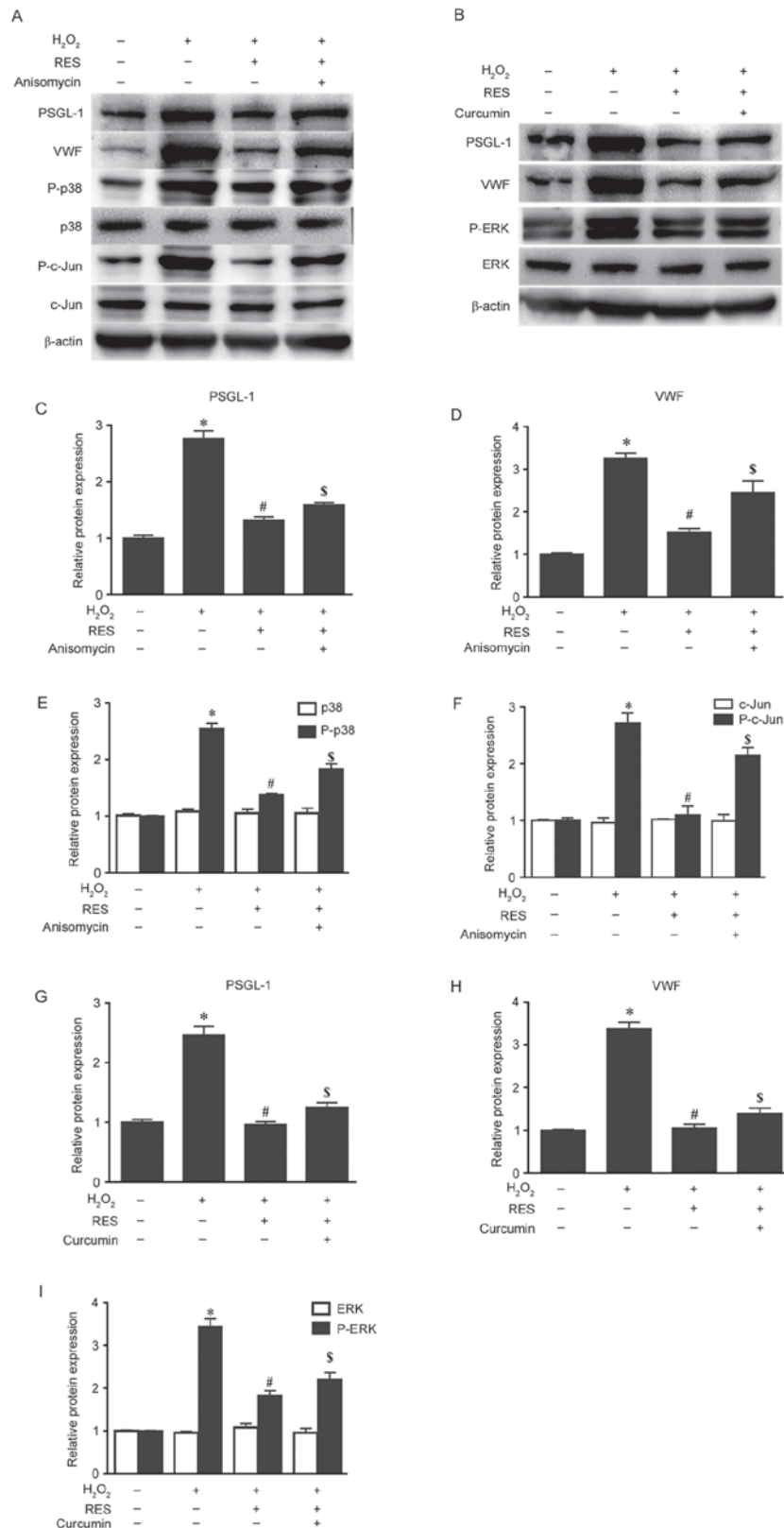


Figure 5. Resveratrol suppresses the protein expression of PSGL-1 and VWF induced by H₂O₂ via the mitogen-activated protein kinase signalling pathway. (A) Protein expression levels of PSGL-1, VWF, p-p38, p38, p-c-Jun and c-Jun in HUVECs exposed to 30 μ M resveratrol for 2 h and 0.4 μ g/ml anisomycin for 6 h prior to treatment with 200 μ M H₂O₂ for 24 h were measured by western blot analysis. (B) Protein expression levels of PSGL-1, VWF, p-ERK and ERK in HUVECs exposed to 30 μ M resveratrol for 2 h and 25 μ M curcumin for 4 h prior to treatment with 200 μ M H₂O₂ for 24 h were measured by western blot analysis. β -actin was used as the loading control. The relative expression ratios of (C) PSGL-1, (D) VWF, (E) p-p38 and p38, and (F) p-c-Jun and c-Jun, in cells treated with H₂O₂ with or without pretreatment with resveratrol and anisomycin were determined by densitometric analysis. The relative expression ratios of (G) PSGL-1, (H) VWF, and (I) P-ERK and ERK, in cells treated with H₂O₂ with or without pretreatment with resveratrol and curcumin were determined by densitometric analysis. *P<0.05 vs. control; #P<0.05 vs. H₂O₂ only group; \$P<0.05 vs. H₂O₂ + RES group. PSGL-1, P-selectin glycoprotein ligand-1; VWF, von Willebrand factor; H₂O₂, hydrogen peroxide; p, phosphorylated; c-Jun, c-Jun N-terminal kinase; HUVECs, human umbilical vein endothelial cells; ERK, extracellular signal-regulated kinase; RES, resveratrol.

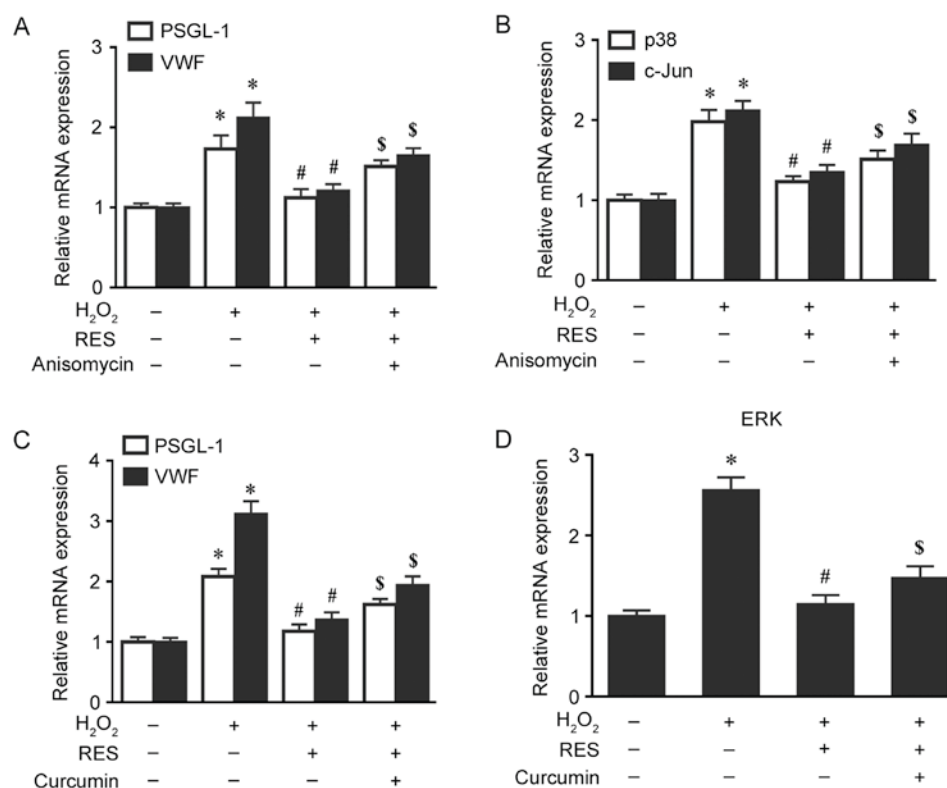


Figure 6. Resveratrol suppresses the mRNA upregulation of PSGL-1 and VWF induced by H₂O₂ via the mitogen-activated protein kinase signalling pathway. The relative mRNA expression levels of (A) PSGL-1 and VWF, and (B) p38 and c-Jun, in HUVECs exposed to 30 μ M resveratrol for 2 h and 0.4 μ g/ml anisomycin for 6 h prior to treatment with 200 μ M H₂O₂ for 24 h were measured by RT-qPCR. The relative mRNA expression levels of (C) PSGL-1 and VWF, and (D) ERK, in HUVECs exposed to 30 μ M resveratrol for 2 h and 25 μ M curcumin for 4 h prior to treatment with 200 μ M H₂O₂ for 24 h were measured by RT-qPCR. *P<0.05 vs. control; #P<0.05 vs. H₂O₂ only group; \$P<0.05 vs. H₂O₂ + RES group. PSGL-1, P-selectin glycoprotein ligand-1; VWF, von Willebrand factor; H₂O₂, hydrogen peroxide; c-Jun, c-Jun N-terminal kinase; HUVECs, human umbilical vein endothelial cells; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ERK, extracellular signal-regulated kinase; RES, resveratrol.

PSGL-1 is a glycoprotein that was originally identified in leukocytes as the ligand of P-selectin and has an important role in the selective recruitment of leukocytes and activated platelets into the venous wall, which amplifies the coagulation cascade and results in increased thrombin generation (30,31). Consequently, VWF and PSGL-1 are referred to as thrombosis-associated markers, and increased levels of these markers in the plasma predicts the occurrence of thrombosis (20,32). To validate the inhibitory effect of resveratrol on DVT *in vitro*, the present study investigated the effect of resveratrol on VWF and PSGL-1 in HUVECs. The results demonstrated that VWF and PSGL-1 secretion from the HUVECs was stimulated by H₂O₂, while resveratrol markedly decreased VWF and PSGL-1 elevation in a concentration-dependent manner, as demonstrated in Fig. 4. To the best of our knowledge, the present study is the first to investigate the effect of resveratrol on thrombosis-associated markers, and the results indicate that resveratrol may inhibit venous thrombosis by suppressing VWF and PSGL-1. However, it should be noted that this conclusion is only based on *in vitro* experiments and at the molecular level. Follow-up animal studies are required to confirm this hypothesis.

Various studies have reported that MAPK pathways contribute to vascular endothelial oxidative stress and venous thrombosis. The expression and secretion of VWF via the activation of ERK was blocked by inhibitors of ERK in HUVECs (33). Venous thrombosis triggered by PSGL-1

and P-selectin was reported to depend on MAPK signalling pathways (34). In the present study, the results demonstrated that H₂O₂ (200 μ M) induced upregulation of mRNA and protein phosphorylation levels of p38, ERK and c-Jun in HUVECs, and resveratrol downregulated these effects at the mRNA and protein phosphorylation levels. When HUVECs were pretreated with the MAPK activators anisomycin and curcumin, the suppressive effects of resveratrol on PSGL-1 and VWF were attenuated, as demonstrated in Figs. 5 and 6. A studies reported that MAPKs cascades were maximally activated within 10-20 min after treatment with numerous stimuli (35). To investigate the simultaneous alterations in MAPKs with PSGL-1 and VWF, levels of p-p38, P-ERK and p-c-Jun were assayed after treatment with H₂O₂ for 24 h. Due to this discrepancy and limitation of the present study, MAPK pathways should be assayed within 20 min to validate the results of the present study.

The results of the present study indicated that resveratrol may inhibit the activation of VWF and PSGL-1 via MAPK pathways. However, regarding the development of venous thrombosis, the underlying molecular mechanisms regulating the expression of VWF and PSGL-1 is complex and requires further investigation. In conclusion, these findings demonstrate that resveratrol may protect HUVECs from oxidative stress and apoptosis, and suppress the expression of thrombosis-associated markers, by inactivating MAPK signalling pathways.

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