Monotropein alleviates H$_2$O$_2$-induced inflammation, oxidative stress and apoptosis via NF-κB/AP-1 signaling

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Abstract. Aging is a major risk factor in cardiovascular disease (CVD). Oxidative stress and inflammation are involved in the pathogenesis of CVD, and are closely associated with senescent vascular endothelial cells. Monotropein (Mtp) exerts various bioactive roles, including anti-inflammatory and antioxidative effects. The aim of the present study was to investigate the function of Mtp in senescent endothelial cells. An MTT assay was performed to evaluate the influence of Mtp on H$_2$O$_2$-stimulated human umbilical vein endothelial cells (HUVECs). Senescent cells were assessed by determining the expression of senescence-associated β-galactosidase, high mobility group AT-hook 1 and DNA damage marker γ-H2A.X variant histone. Malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and proinflammatory cytokine concentrations were estimated using assay kits to evaluate the levels of oxidative stress and inflammation in HUVECs. The TUNEL assay was performed to identify apoptotic cells. Furthermore, the expression levels of endothelial cell adhesion factors, NF-κB, activator protein-1 (AP-1) and apoptotic proteins were determined via western blotting. Mtp enhanced HUVEC viability following H$_2$O$_2$ stimulation. H$_2$O$_2$-mediated increases in MDA, proinflammatory cytokine and endothelial cell adhesion factor levels were decreased by Mtp treatment, whereas Mtp reversed H$_2$O$_2$-mediated downregulation of SOD and GSH-Px activity. Furthermore, Mtp inhibited cell apoptosis, NF-κB activation and AP-1 expression in H$_2$O$_2$-stimulated HUVECs; however, NF-κB activator counteracted the anti-inflammatory, anti-oxidative and antiapoptotic effects of Mtp. The present study indicated that Mtp ameliorated H$_2$O$_2$-induced inflammation and oxidative stress potentially by regulating NF-κB/AP-1.

Introduction

Cardiovascular disease (CVD) is a primary cause of death worldwide (1), resulting in a public health burden for society and patients. Age is considered as a major contributor to CVD, the incidence of which increases significantly with age (2,3). Vascular endothelial cells are a single layer of squamous cells covering the surface of the vascular intima, which forms the biological barrier of the vascular wall (4). Dysfunction of vascular endothelial cells is closely associated with senescence, increasing the risk of CVD in the elderly population. Senescent endothelial cells impair the function of vessels, which involves oxidative stress and a proinflammatory phenotype (5). Inflammation and oxidative stress are the primary factors of cell senescence. Inflammatory cytokines secreted by senescent cells further trigger inflammation and senescence in the surrounding tissue (6). Previous studies have demonstrated that proinflammatory cytokines are increased, and superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities are decreased in the process of cell senescence (6-8). Therapeutic strategies for inflammatory disorders and normalizing oxidative stress have been demonstrated to be effective in various types of CVD (9).

Monotropein (Mtp) is an iridoid glycoside isolated from the roots of Morinda officinalis (10). A previous study demonstrated that Mtp protected osteoblasts from H$_2$O$_2$-induced oxidative stress via regulating autophagy (11). Mtp induced the differentiation of bone marrow-derived endothelial progenitor cells and prevented cell apoptosis by decreasing the release of reactive oxygen species (ROS) (12). NF-κB is a classical transcription factor that is activated in response to extracellular stimulus, and serves a crucial role in oxidative stress and inflammatory responses (13,14). He et al (15) demonstrated that Mtp significantly inhibited lipopolysaccharide (LPS)-induced secretion of inflammatory cytokines by suppressing activation of the NF-κB signaling pathway. However, to the best of our knowledge, there is limited research available regarding the role of Mtp in the pathogenesis of CVD. H$_2$O$_2$-stimulated human umbilical vein endothelial cells (HUVECs) are a well-established senescent cell model (16). The aim of the present study was to simulate an oxidative environment with
H$_2$O$_2$-stimulated vascular endothelial cells, and to determine the effects and mechanisms underlying Mtp in H$_2$O$_2$-stimulated endothelial cells.

**Materials and methods**

**Cell culture.** HUVECs (Sigma-Aldrich; Merck KGaA) were maintained in endothelial growth medium (Sigma-Aldrich; Merck KGaA) at 37°C with 5% CO$_2$. HUVECs were pretreated with Mtp (0.1, 1, 10, 100 or 1,000 µM; dissolved in deionized water; purity >98%; Chengdu Herbpurify Co., Ltd.) for 4 h at 37°C. H$_2$O$_2$ has been widely used to induce an oxidative environment in vascular endothelial cell models in vitro (16). To induce cell senescence, HUVECs were treated with 100 µM H$_2$O$_2$ for 12 h at 37°C. HUVECs were incubated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich; Merck KGaA) for 12 h at 37°C to activate NF-κB, as previously described (17).

**MTT assay.** HUVECs were seeded (5x10$^5$ cells/well) into a 96-well plate. Following treatment with H$_2$O$_2$ and Mtp, 20 µL MTT reagent (5 mg/ml; Beijing Solarbio Science & Technology Co., Ltd.) was added to each well for 4 h at 37°C. Methylthiazolyl tetrazolium (MTT) formazan was dissolved in dimethyl sulfoxide (dMSo) at 37°C. The absorbance was measured at a wavelength of 570 nm using a microplate reader (Bio-rad laboratories, inc.).

**Senescence-associated β-galactosidase (β-Gal) activity.** HUVECs were collected into a centrifuge tube containing the extracting reagent of the β-Gal assay kit (Beijing Solarbio Science & Technology Co., Ltd.). HUVECs (5x10$^5$) were centrifuged at 15,000 x g for 10 min at 4°C. The corresponding reagents in the kit were added into the tube and incubated for 30 min at 37°C. The absorbance value was immediately determined at a wavelength of 405 nm according to manufacturer's protocol.

**ELISA.** Cell culture medium was centrifuged at 1,000 x g at 4°C for 10 min. The levels of secreted IL-6, TNF-α and monocyte chemoattractant protein-1 (MCP-1) in cell culture medium were measured using human IL-6 (cat. no. EH004-48), TNF-α (cat. no. EH009-48) and MCP-1 (cat. no. EH019-48) ELISA kits (Shanghai ExCell Biology, Inc.) according to the manufacturer's protocol. Optical density values were recorded at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.). Each group was assessed in triplicate.

**Western blotting.** HUVECs were seeded (5x10$^6$) into a 100-mm petri dish. Total protein was extracted using RIPA buffer containing phosphatase inhibitors (Beijing Solarbio Science & Technology Co., Ltd.). Proteins (30 µg) were separated via 10% SDS-PAGE and transferred onto PVDF membranes (EMD Millipore). After blocking with 5% skimmed milk for 2 h at room temperature, the membranes were incubated at 4°C overnight with primary antibodies targeted against: Phosphorylated (p)-NF-κB p65 (phosphor S276; cat. no. ab194726; 1:1,000; Abcam), NF-κB p65 (cat. no. ab16502; 1:1,000; Abcam), high mobility group AT-hook 1 (Hmg1; cat. no. ab129153; 1:20,000; Abcam), vascular cell adhesion molecule-1 (VCAM-1; cat. no. bs-0920R; 1:500; BIOSS), intercellular cell adhesion molecule-1 (ICAM-1; cat. no. bs-4618R; 1:500; BIOSS), Bcl-2 (cat. no. bs-4563R; 1:500; BIOSS), Bax (cat. no. bs-0127R; 1:500; BIOSS), GAPDH (cat. no. bs-33033M; 1:500; BIOSS), cleaved caspase-3 (cat. no. 9661; 1:1,000; Cell Signaling Technology, Inc.), caspase-3 (cat. no. 9662; 1:1,000; Cell Signaling Technology, Inc.); γ-H2A.X variant histone (H2AX; cat. no. 2577; 1:1,000; Cell Signaling Technology, Inc.), H2AX (cat. no. 2595; 1:1,000; Cell Signaling Technology, Inc.), p-activator protein-1 (AP-1; phospho Ser63; cat. no. ABP50621; 1:1,000; Abbkine Scientific Co., Ltd.) and AP-1 (cat. no. ABP50668; 1:1,000; Abbkine Scientific Co., Ltd.). Subsequently, the membranes were incubated with HRP-linked anti-mouse IgG (cat. no. 7076; 1:3,000; Cell Signaling Technology, Inc.) or HRP-linked anti-rabbit IgG (cat. no. 7074; 1:3,000; Cell Signaling Technology, Inc.) secondary antibodies. Protein bands were visualized using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.). Protein expression levels were semi-quantified using Image Lab software (version 4.0; Bio-Rad Laboratories, Inc.).

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from cultured cells using the TRIzol® Purification kit (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Promega Corporation) in the presence of oligo(dT) primers and dNTP. The following temperature protocol was used for reverse transcription: Denaturation at 70°C for 5 min; annealing at 25°C for 10 min; and extension at 42°C for 50 min. Subsequently, qPCR was performed using the Power SYBR Green PCR Master mix (Thermo Fisher Scientific, Inc.) and a 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions used for qPCR were as follows: 95°C for 10 min; followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The following primers were used for qPCR: VCAM-1 forward, 5’-CAGGCT GTGAAGTCCCATTT-3’ and reverse, 5’-TTCAGCTGTGATC GGCTTCC-3’; ICAM-1 forward, 5’-ACCATCTACAGCTT CGCG-3’; and reverse, 5’-TTCTTGCCACGTCCAGTT TT-3’; GAPDH forward, 5’-GCACCGTGCAAGGCTGAGA AC-3’ and reverse, 5’-TGTTGAAGACGCCTAGTAGGAA-3’. GAPDH was used as an internal control for quantification using the 2$^{-\Delta\Delta Ct}$ method (18).

**Measurement of malondialdehyde (MDA).** The MDA assay kit (Beijing Solarbio Science & Technology Co., Ltd.) was used to evaluate the content of MDA. HUVECs (4x10$^5$) were collected and centrifuged at 8,000 x g for 10 min at 4°C after adding the extracting reagent of the MDA assay kit. Subsequently, the MDA detection reagent was added and fully mixed at 100°C for 60 min, cooled and then centrifuged at 10,000 x g for 10 min at room temperature. The supernatant (200 µl) was plated into a 96-well plate and the absorbance value was determined at wavelengths of 450, 532 and 600 nm according to the manufacturer's protocol.

**Measurement of SOD.** A SOD assay kit (Beijing Solarbio Science & Technology Co., Ltd.) was used to evaluate SOD activity. HUVECs (5x10$^5$) were collected and centrifuged at
8,000 x g for 10 min at 4°C after adding the extracting reagent of the SOD assay kit. Subsequently, corresponding reagents were added into the sample and fully mixed at 37°C for 30 min. The absorbance value was measured at a wavelength of 560 nm according to the manufacturer’s protocol.

Measurement of GSH-Px. The GSH-Px assay kit (Beijing Solarbio Science & Technology co., ltd.) was used to evaluate GSH-Px activity. HUVECs were collected and centrifuged at 8,000 x g for 10 min at 4°C after adding the extracting reagent of the GSH-Px assay kit. Subsequently, the corresponding reagents were added into the sample and fully mixed. The absorbance value was immediately determined at a wavelength of 412 nm according to the manufacturer’s protocol.

TUNEL apoptosis assay kit. Adherent cell slides were prepared to locate apoptotic cells using the Tunel apoptosis assay kit (Nanjing KeyGen Biotech Co., Ltd.). Biotin-labeled dUTP could connect to the 3'-OH terminal of apoptotic cells via TdT Enzyme and combine specifically with streptavidin-HRP. Briefly, treated cells were fixed with fresh 4% paraformaldehyde for 15 min at room temperature, gently rinsed with PBS and incubated with 0.1% Triton X-100 for 2 min at 4°C. The TdT enzyme was added and incubated for 60 min at 37°C in the dark, and then with streptavidin-HRP solution for 30 min in the dark at 37°C. Finally, diaminobenzidine solution was used to assess the color-reaction for 10 min at room temperature. Apoptotic cells were visualized in six randomly selected fields of view using a light microscope (magnification, x200).

Statistical analysis. Data are presented as the mean ± SD. Each experiment was performed in triplicate. Statistical analyses were performed using GraphPad Prism software (version 6.0; GraphPad Software, Inc.). Comparisons between two groups were analyzed using the unpaired Student’s t-test. Comparisons among multiple groups were analyzed using one-way ANOVA followed by Tukey’s post hoc test. *P<0.05, **P<0.01 and ***P<0.001 vs. control; #P<0.05, ##P<0.01 and ###P<0.001 vs. H2O2. Mtp, monotropein; HuVEC, human umbilical vein endothelial cell; β-Gal, β-galactosidase; Hmga1, high mobility group AT-hook 1; H2AX, H2a.X variant histone.

Results

Mtp regulates HUVEC viability and senescence. Initially, varying concentrations of Mtp were prepared for the pretreatment of HUVECs for 24 h. The results indicated that, compared with the control group, 0.1-100.0 µM Mtp did not significantly affect HUVEC viability, but 1,000 µM Mtp significantly reduced cell viability (Fig. 1A). For subsequent experiments, 0.1-100.0 µM Mtp were used for pretreating HUVECs, which were subsequently incubated with 100 µM H2O2 for 12 h. The results suggested that H2O2 significantly inhibited cell viability in the absence of Mtp pretreatment compared with the control group. By contrast, 10 and 100 µM Mtp significantly increased cell viability in H2O2-stimulated HUVECs compared with the H2O2 group,
and 100 µM Mtp exhibited an improved efficacy compared with 10 µM Mtp (Fig. 1B). Subsequently, HUVECs were pretreated with 100 µM Mtp or vehicle, and then stimulated with H2O2 for 12 h. Compared with the control group, H2O2 significantly enhanced β-Gal activity, but Mtp pretreatment significantly decreased H2O2-induced β-Gal activity (Fig. 1C). Additionally, by measuring the expression levels of the senescence marker Hmgal1 and the DNA damage marker γ-H2AX, the results also indicated that H2O2 increased HUVEC senescence compared with the control group, whereas Mtp pretreatment significantly inhibited H2O2-induced senescence (Fig. 1D). The results suggested that Mtp reversed H2O2-mediated downregulation of cell viability and induction of senescence.

**Mtp alleviates the inflammatory response of HUVECs.** To investigate the effect of Mtp on the inflammatory response in H2O2-stimulated HUVECs, cell culture medium was collected to estimate the release of proinflammatory cytokines, such as IL-6, TNF-α and MCP-1. The results indicated that H2O2 significantly upregulated the release of proinflammatory cytokines compared with the control group, and Mtp pretreatment significantly reduced H2O2-induced proinflammatory cytokine release, suggesting a potent anti-inflammatory effect of Mtp (Fig. 2A-C). TNF-α can cause vascular endothelial cell dysfunction, resulting in the production of a variety of cytokines, such as ICAM-1 and VCAM-1, and triggering vascular inflammation (19,20). The results indicated that ICAM-1 and VCAM-1 expression levels were significantly increased in the H2O2 group compared with the control group (Fig. 2D). Similarly, the mRNA levels of ICAM-1 and VCAM-1 were upregulated in the H2O2 group compared with the control group (Fig. 2E). Collectively, the results indicated that Mtp protected HUVECs against H2O2-induced inflammation.
HUVEC oxidative stress and apoptosis are suppressed by Mtp. It has previously been reported that Mtp is capable of inhibiting H$_2$O$_2$-induced ROS generation in osteoblasts (21). In the present study, MDA content was estimated to evaluate membrane lipid peroxidation. The results indicated that H$_2$O$_2$ significantly increased MDA content compared with the control group, whereas pretreatment with Mtp significantly decreased MDA levels compared with the H$_2$O$_2$ group (Fig. 3A). In addition, significantly decreased SOD and GSH-Px activities were observed in the H$_2$O$_2$ group compared with the control group, but Mtp pretreatment inhibited H$_2$O$_2$-mediated downregulation of SOD and GSH-Px activities (Fig. 3B and C), which indicated that Mtp protected HuVecs against H$_2$O$_2$-induced oxidative injury. Subsequently, whether there was an association between Mtp and cell apoptosis was investigated. The TUNEL assay indicated that apoptotic cells (brown-stained) were observed in the H$_2$O$_2$ group and Mtp pretreatment significantly decreased H$_2$O$_2$-induced cell apoptosis (Fig. 3D). Collectively, the results indicated that Mtp ameliorated H$_2$O$_2$-induced oxidative stress and apoptosis.

Mtp anti-inflammatory effects are reversed by PMA. NF-κB is a key transcription factor associated with oxidative stress and the inflammatory response (13,14). AP-1 has been demonstrated to interact with NF-κB, and NF-κB/AP-1 signaling cascades serve an important role in inflammation (22,23). The present study suggested that the phosphorylation of NF-κB and AP-1 was significantly increased following H$_2$O$_2$ treatment compared with the control group. By contrast, pretreatment with Mtp significantly decreased the phosphorylation of NF-κB and AP-1 compared with the H$_2$O$_2$ group (Fig. 4A). PMA (100 ng/ml) was prepared and incubated with HuVecs for 12 h to activate NF-κB as previously described (17). The phosphorylation levels of NF-κB and AP-1 were significantly increased by PMA incubation compared with the control group (Fig. 4B). Furthermore, the results indicated that the anti-inflammatory effects of Mtp were significantly reversed by PMA (Fig. 4C-E), which was further indicated by significantly elevated protein expression levels of ICAM-1 and VCAM-1 in the PMA + Mtp + H$_2$O$_2$ group compared with the Mtp + H$_2$O$_2$ group.
Figure 4. Mtp anti-inflammatory effects are reversed by PMA. (A) NF-κB and AP-1 protein expression levels were measured via western blotting. (B) HUVECs were treated with PMA for 12 h, and NF-κB and AP-1 protein expression levels were determined via western blotting. The levels of secreted proinflammatory cytokines (C) IL-6, (D) TNF-α and (E) MCP-1 were determined by performing ELISAs. (F) The protein expression levels of VCAM-1 and ICAM-1 were measured via western blotting. **P<0.01 and ***P<0.001 vs. control; #P<0.05, ##P<0.01 and ###P<0.001 vs. H2O2. Mtp, monotropein; PMa, phorbol 12-myristate 13-acetate; AP-1, activator protein-1; HUVEC, human umbilical vein endothelial cell; MCP-1, monocyte chemoattractant protein-1; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular cell adhesion molecule-1.
In summary, the results suggested that Mtp exerted an anti-inflammatory effect potentially via regulating the activation of NF-kB/αP-1 signaling cascades. NF-kB/αP-1 signaling may be associated with the inhibitory effects of Mtp on oxidative stress and apoptosis. To further evaluate the molecular mechanism underlying HUVEC oxidative stress and apoptosis, cells were incubated with Mtp and PMA. The results suggested that Mtp pretreatment-mediated decreases in MDA content were counteracted by PMA (Fig. 5A). PMA also significantly decreased Mtp-mediated upregulation of SOD and GSH-Px in H2O2-stimulated HUVECs (Fig. 5B and C). In addition, Mtp pretreatment decreased H2O2-induced cell apoptosis, which was weakened by PMA treatment (Fig. 5D). Accordingly, Mtp-mediated downregulation of the expression levels of proapoptotic proteins Bax and cleaved-caspase 3 was reversed by PMA, whereas the protein expression levels of Bcl-2 displayed the opposite effect (Fig. 5E). The results suggested that the inhibitory effects of Mtp on HUVEC oxidative stress and apoptosis were partially counteracted by PMA.

Discussion

Previous studies have demonstrated that Mtp exerts anti-apoptotic and anti-inflammatory effects in osteoarthritis chondrocytes (21,24,25). Nevertheless, the potential functions of Mtp in the progression of CVD are not completely understood. CVD is closely associated with senescent vascular endothelial cells, which secrete proinflammatory mediators and further exacerbate the progression of CVD (26). In the present study, HUVECs were cultured in vitro and stimulated with H2O2 to mimic a senescent cell model. An appropriate concentration of Mtp was selected to treat HUVECs and was assessed using an MTT assay. Subsequently, by measuring the proinflammatory mediators secreted by HUVECs exposed to different stimuli, the results indicated that Mtp pretreatment ameliorated the inflammatory response...
triggered by H₂O₂. In addition, the markers of oxidative stress and apoptosis were also decreased in H₂O₂-stimulated HuVECs in the presence of Mtp; however, a potential limitation of the present study may be the lack of ROS determination.

A previous study indicated that Mtp decreased the DNA binding activity of NF-κB in LPS-induced RAW 264.7 macrophages, and inhibited the phosphorylation and degradation of inhibitory κB-α, thereby inhibiting the translocation of NF-κB (27). Furthermore, Mtp inhibits the phosphorylation of NF-κB in MC3T3-E1 murine embryonic osteoblastic precursor cells (15). AP-1 is capable of interacting with NF-κB, which triggers inflammatory cytokines, including TNF-α and IL-1β, via regulating their corresponding mediator genes (28). In the present study, the phosphorylation of NF-κB was increased in H₂O₂-stimulated HuVECs compared with the control group. Pretreatment with Mtp decreased the phosphorylation of NF-κB and AP-1 in H₂O₂-stimulated HuVECs. Moreover, the results indicated that elevating the activation of NF-κB by PMA counteracted the ameliorative effects of Mtp on H₂O₂-stimulated HuVECs, suggesting that Mtp exerted its protective role by modulating the NF-κB/AP-1 signaling pathway. When cells are stimulated with PMA, the phosphorylation of p38MAPK is increased (29, 30). The signaling pathway activates a variety of transcription factors, including NF-κB (p50/p65) and AP-1 (c-Fos/c-Jun), that coordinate the induction of numerous genes encoding inflammatory mediators (31), such as IL-6, TNF-α, and MCP-1 (32). To date, studies on Mtp have primarily focused on osteoarthritis. He et al (15) demonstrated that Mtp attenuates inflammatory impairment on osteoblasts via inactivation of the NF-κB signaling pathway. Moreover, Mtp suppresses IL-1β-induced apoptosis and catabolic responses on osteoarthritis chondrocytes (24), and in Mtp-treated osteoblasts, oxidative stress was alleviated via Akt/mTOR-mediated autophagy (11). However, the role of Mtp on endothelial cells in CVD has not been previously reported.

In summary, the present study indicated that Mtp protected HuVECs against H₂O₂-induced inflammation, oxidative stress and apoptosis, potentially via mediating the NF-κB/AP-1 signaling pathway. Therefore, Mtp may serve as a candidate therapeutic for protecting HuVECs in patients with CVD via monitoring NF-κB/AP-1 signaling cascades or inhibiting NF-κB activation. The present study suggested the protective effect of Mtp on H₂O₂-induced vascular endothelial cells, indicating a potential therapeutic effect for patients with CVD via targeting endothelial functions. Nevertheless, the effects of Mtp on vascular endothelial cells were only investigated at the cellular level in the present study. Therefore, how Mtp activates NF-κB and whether Mtp affects other signaling pathways in endothelial cells requires further investigation. Moreover, animal experiments and clinical trials are required to further validate the curative effect of Mtp.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

FJ and XRX made substantial contribution to data acquisition. WML and KX contributed to data analysis. LFW and XCY designed the present study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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