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Triptolide Alleviates Oxidized LDL-Induced Endothelial Inflammation by Attenuating the Oxidative Stress-Mediated Nuclear Factor-Kappa B Pathway

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A B S T R A C T

Background: Endothelial inflammation triggered by oxidized LDL (ox-LDL) is a crucial mechanism involved in atherosclerosis. Triptolide (TP), a primary active ingredient of the traditional Chinese medicine *Tripterygium wilfordii* Hook F, possesses antioxidant and anti-inflammatory properties in vivo. However, limited information is available regarding these effects on endothelial inflammation occurring in atherosclerosis.
Objectives: This study investigated the effects and possible mechanisms of action of TP on ox–LDL-induced inflammatory responses in human umbilical vein endothelial cells.
Methods: Human umbilical vein endothelial cells were preincubated with TP at the indicated concentrations for 1 hour and then incubated with ox-LDL (50 μg/mL) for the indicated times.
Results: Preincubation of cultured human umbilical vein endothelial cells with TP inhibited ox–LDL-induced cytokine and chemokine production, adhesion molecule expression, and monocyte adhesion in a concentration-dependent manner. The concentrations of 8-isoprostane, malondialdehyde, and superoxide increased after human umbilical vein endothelial cells were exposed to ox-LDL, which were associated with decreased activities of total superoxide dismutase and its isoenzyme (ie, CuZn- superoxide dismutase). Preincubation with TP reversed ox–LDL-induced effects in all events. Moreover, preincubation with TP also attenuated ox–LDL-induced nuclear factor-kappa B transcriptional activation in a concentration-dependent manner, via the suppression of inhibitor of kappa Balpha (IκBα) phosphorylation and subsequent nuclear factor-kappa B DNA binding.
Conclusions: These data indicate that TP inhibits ox–LDL-induced endothelial inflammation, possibly via suppression of the oxidative stress-dependent activation of the nuclear factor-kappa B signaling pathway.
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Introduction

Atherosclerosis is among the primary causes of several cardiovascular diseases such as cardiac ischemia and stroke. Endothelial cells, especially through their production of endothelium-derived nitric oxide, are critically involved in cardiovascular homeostasis by modulating inflammatory, atherogenic, and thrombotic conditions.[1–3] In addition to hemodynamic dysregulation, endothelial dysfunction, defined as a reduction in nitric oxide availability, is characterized by an impaired response to an endothelium-dependent vasodilator, such as acetylcholine. Endothelial dysfunction is also accompanied by high levels of reactive oxygen species (ROS) and proinflammatory mediators (eg, cytokines and adhesion molecules) along with enhanced permeability of the vascular endothelium.[2,3] Mounting evidence shows that endothelial dysfunction is an early hallmark of atherosclerosis that contributes to the development of pathological conditions.[3,4]

Increased blood levels of oxidized LDL (ox-LDL) are the most common characteristics of numerous diseases, including atherosclerosis, hypertension, and diabetes.[5,6] Ox-LDL is recognized as a crucial factor in the development of atherosclerosis. It has been demonstrated that ox-LDL leads to endothelial dysfunction at the onset of atherosclerosis.[7,8] A growing body of evidence has shown that ox-LDL causes enhanced levels of intracellular ROS, leading to transcription-dependent inflammation-related factor expression and synthesis via the activation of nuclear factor kappa B

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(NF-κB).[9–11] These are considered the central mechanisms for the development of atherosclerosis.

Triptolide (TP) is a major biologically active compound extracted from the traditional Chinese medicinal herb *Tripterygium wilfordii* Hook F (TWHF) (family: Celastraceae).[12,13] In China, TWHF and its preparations are widely used in the treatment of autoimmune and inflammatory diseases, including systemic lupus, rheumatoid arthritis, nephritis, and psoriatic arthritis.[12] Recently, increasing evidence has shown that TP attenuates inflammatory responses in animal models of autoimmune and inflammatory diseases, such as nephritis, asthma, and arthritis.[14–16] However, the precise mechanisms by which TP attenuates ox-LDL-induced inflammatory responses in endothelial cells remain to be fully determined.

Because TP has broad-spectrum therapeutic potential due to its immunosuppressive, anti-inflammatory, and antioxidant properties, it was hypothesized that it could have potential implications in the modulation of atherosclerosis. Therefore, this study was designed to determine the in vitro effect of TP on oxidative stress and inflammatory responses triggered by ox-LDL in human umbilical vein endothelial cells (HUVECs) and to clarify the potential molecular mechanisms involved in TP-mediated actions.

Materials and methods

Reagents and chemicals

TP (molecular weight = 360.4 and purity ≥98% by HPLC, as described in the product sheet supplied by Sigma-Aldrich (St Louis, Missouri), catalog No. T3652), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), proteinase inhibitor solution, dimethyl sulfoxide (DMSO), Tiron, and Triton X-100 were purchased from Sigma-Aldrich. Ox-LDL was purchased from the Beijing Solarbio Life Science Company (Beijing, China). RPMI-1640 medium, carboxyfluorescein diacetate succinimidyl ester, fetal bovine serum, and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, California). Cytokine and chemokine ELISA kits were obtained from R&D Systems (Minneapolis, Minnesota). An 8-isoprostane assay kit was purchased from Cayman Chemical (Ann Arbor, Michigan). Malondialdehyde (MDA) and superoxide dismutase (SOD) activity assay kits were obtained from Beyotime Biotechnology Institute (Shanghai, China). A protein assay kit and RIPA lysis buffer were supplied by Bio-Rad Laboratories (Hercules, California) and Thermo Scientific (Waltham, Massachusetts), respectively.

Cell culture and experimental protocols

HUVECs, obtained from Lonza (Allendale, New Jersey), were cultured in an EGM-2 Bullet kit medium supplemented with growth supplements (Lonza) and used at passage 3-7. HUVECs were cultured to confluence (80%–90%) and starved in endothelial basal medium-2 without growth supplements for 4 hours before the start of experiments. THP-1, a human monocytic cell line, was purchased from ATCC (Manassas, Virginia) and cultured in RPMI-1640 medium containing 10% fetal bovine serum at a density of 2 through 5×10^6 cells/mL, as suggested in the product specification sheet supplied by the vendor. The cells were cultured in a humidified 37°C incubator at 5% carbon dioxide.

TP was dissolved in DMSO and stock solutions (1 mg/mL) were stored at –20°C. The reagents were freshly diluted to the indicated concentrations in the culture medium before use. For the experiments, the cells were cultured in a medium containing growth supplements. At confluence, cells were exposed to TP at concentrations of 25, 50, or 100 nM for 1 hour and then incubated with ox-LDL (50 µg/mL) for the indicated times. The cells were used for subsequent experiments. Cells exposed to equal dilutions of DMSO alone were used as controls. The DMSO concentration in the culture medium was adjusted to a final concentration of 0.1% v/v.

Cell viability assay

The viability of HUVECs was determined using an MTT colorimetric assay. Briefly, HUVECs were seeded in 96-well plates (1×10^4 cells/well) and allowed to attach overnight. The MTT reagent was then added to a final concentration of 0.5 mg/mL at the indicated times after the corresponding exposure and incubated for an additional 4 hours. After MTT removal, 100 µL DMSO was added to solubilize the crystals. Absorbance was measured at 570 nm using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, California). The viability of HUVECs was expressed as the change relative to the control with no TP exposure.

Cytokine/chemokine assay

Cell-free supernatant fractions from HUVECs exposed to ox-LDL in the presence or absence of TP in 24-well plates (1×10^5 cells/well) were collected to determine the concentrations of cytokines and chemokines. The concentrations of cytokines and chemokines were assayed using the corresponding ELISA kits following the manufacturer's instructions. The concentrations of cytokines and chemokines were recorded as picogram per milliliter of the culture media.

Cell-based ELISA assay

The effects of TP on the levels of adhesion molecules (intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1)) and phosphorylated inhibitor of kappa Balpha (IκBα) (Ser32) were determined using a cell-based ELISA assay as described previously.[17] Briefly, HUVECs in a 96-well plate were fixed with 4% paraformaldehyde for 5 minutes after incubation with ox-LDL and washed 3 times with phosphate-buffered saline. The fixed cells were permeabilized with prechilled methanol for 10 minutes at 4°C, followed by blocking with phosphate-buffered saline containing 1% bovine serum albumin (BSA) and 0.2% Triton X-100 for 1 hour. Next, the cells were incubated with mouse anti-ICAM-1 monoclonal antibody, mouse anti-VCAM-1 monoclonal antibody (dilutions, 1:100; Santa Cruz Biotechnology, Santa Cruz, California), or rabbit antiphosphorylated IκBα (Ser32) polyclonal antibody (dilution, 1:200; Cell Signaling Technology Inc, Danvers, Massachusetts) overnight at 4°C. After washing with phosphate-buffered saline, the cells were incubated with fluorescein isothiocyanate (FITC) conjugated anti-mouse immunoglobulin G or FITC-conjugated anti-rabbit immunoglobulin G (dilutions, 1:200; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) at room temperature for 1 hour. Negative control experiments were performed by omitting the primary antibody during incubation. The optical density of each well was quantified using a SpectraMax microplate reader an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Optical density of the negative control was subtracted from that of each well. Data are presented as changes relative to the control.

Monocyte adhesion assay

A monocyte adhesion assay was performed as previously reported.[17] Briefly, HUVECs were cultured in 96-well plates at a density of 1×10^4 cells/well for 12 hours, exposed to TP at the indicated concentrations for 1 hour, and incubated with ox-LDL (50 µg/mL) for an additional 6 hours. THP-1 cells prelabeled with 1 µM carboxyfluorescein diacetate succinimidyl ester at 37°C for 30

minutes were added to HUVECs exposed to ox-LDL. The labeled monocytes were then co-incubated with HUVECs and allowed to adhere at 37°C for 30 minutes in a 5% carbon dioxide incubator. The nonadherent cells were gently aspirated. Subsequently, the cells were washed 3 to 5 times with phosphate-buffered saline and incubated in phosphate-buffered saline containing 2% Triton X-100 to lyse the monocytes. The total fluorescence intensity of the samples was quantified at a 485 nm excitation wavelength and 520 nm emission wavelength using a SpectraMax microplate reader. Based on the fluorescence intensity per well of adherent THP-1 cells, the data were recorded as the change relative to the control. In another set of experiments, HUVECs were grown in 24-well plates (0.5×10^5 cells/well). Fluorescence staining of monocyte attachment to HUVECs was performed as described above. Finally, after the removal of nonadherent pre-labeled THP-1 cells, residual cells were washed 3 times with phosphate-buffered saline and observed under a fluorescence microscope (DMI 3000 B, Leica, Wetzlar, Germany) at 485 nm excitation and 535 nm emission wavelengths.

Oxidative stress-related parameter assay

HUVECs in 6-well plates (3×10^5 cells/well) were exposed to TP at the indicated concentrations for 1 hour, followed by incubation with ox-LDL (50 µg/mL) for 6 hours. After incubation, the supernatants and cells were collected and cell extracts were prepared in ice-cold RIPA buffer with a proteinase inhibitor or 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.2) containing 1 mM ethylene glycol-bis-(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 210 mM mannitol, and 70 mM sucrose. Measurements of 8-isoprostane, MDA, and total SOD and its isoenzyme activities were conducted using the corresponding assay kits. Protein levels of the cell extracts were measured using a protein assay kit and used to normalize the levels of MDA and activities of total SOD and its isoenzymes. MDA levels were determined using a lipid peroxidation MDA assay kit: The cell extract was mixed with the assay solution and incubated at 100°C for 15 minutes in the dark. After cooling to room temperature, the mixtures were centrifuged at $1000 \times g$ for 10 minutes. The supernatants (200 μL) were transferred to a 96-well plate, and the absorbance was immediately measured at 532 nm using a SpectraMax microplate reader. A standard curve was constructed according to the manufacturer's recommended protocol. The activity of total SOD and its isoenzymes was determined using a copper and zinc/manganese-SOD (CuZn/Mn-SOD) assay kit (water-soluble tetrazolium salt-8,WST-8). The activity of Mn-SOD was assayed by adding an inhibitory solution to inactivate CuZn-SOD activity. The difference between the activities of total SOD and Mn-SOD was considered the activity of CuZn-SOD. Absorbance was measured at 450 nm using a SpectraMax microplate reader. SOD activity was expressed as units per milligram of protein (1 unit of SOD activity was defined as the amount of enzyme that attenuated WST-8 reduction by approximately 50%).

Superoxide ($O_2^{-}\cdot$) assay

Superoxide production in HUVECs was determined by a lucigenin-enhanced chemiluminescence assay as reported previously.[18] In brief, HUVECs in 6-well plates (3×10^5 cells/well) were exposed to TP at the indicated concentrations for 1 hour, followed by incubation with ox-LDL (50 µg/mL) for 6 hours. Cells were then washed with a modified Krebs-HEPES buffer containing 119 mM sodium chloride, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4.6 mM potassium chloride, 1.0 mM magnesium sulfate, 0.15 mM sodium phosphate, 0.4 mM potassium dihydrogen phosphate, 5 mM sodium bicarbonate, 1.2 mM calcium dichloride, and 5.5 mM glucose (pH 7.4), and scraped off using a rubber policeman. To prepare cell lysates, the cells were homogenized in ice-cold modified Krebs-HEPES buffer and centrifuged at $5000 \times$ gram for 10 minutes. The protein contents of the samples were measured using a protein assay kit. Cell lysates (10–30 μg protein) were incubated at 37°C for 30 minutes in the modified Krebs-HEPES buffer containing diethyldithiocarbamate (10 mM) and β-nicotinamide adenine dinucleotide phosphate (NADPH) (0.1 mM). Lucigenin (5 µM final concentration) was gently added to the tube and incubated at 37°C for an additional 30 minutes in the dark. After incubation, repeated measurements were made over 10 minutes at 1-minute intervals using a TD-20/20 luminometer (Turner BioSystems, Sunnyvale, California) and averaged for each tissue sample. The cell-permeant superoxide scavenger Tiron (10 mM final concentration) was added to the tube, and 10 additional measurements were recorded and averaged. The remaining samples were incubated with 0.1 mM apocynin, a selective NADPH oxidase inhibitor, to determine the potential role of NADPH oxidase in superoxide production. Data were recorded as the difference in average counts per minute per milligram of protein before and after Tiron supplementation.

NF-κB p65 DNA-binding activity assay

HUVECs in 6-well plates (3×10^5/well) were exposed to TP at the indicated concentrations for 1 hour, followed by incubation with ox-LDL (50 µg/mL) for an additional 30 minutes. After incubation, nuclear protein was extracted from the cells using a nuclear extraction kit (Active Motif, Carlsbad, California), as described by the manufacturer. A specific TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif) was used to quantify the DNA-binding activity of NF-κB p65 following the manufacturer's instructions. Briefly, extracted nuclear proteins were added to each well coated with an unlabeled oligonucleotide containing the consensus binding site for NF-κB (5′-GGGACTTTCC-3′) and incubated for 1 hour. After washing, a primary antibody directed against the NF-κB p65 subunit was applied and incubated for 1 hour. Subsequently, a secondary antibody conjugated to horseradish peroxidase was added and incubated for 1 hour. A colorimetric reaction was initiated by adding a developing solution. After termination with a stop solution, the plate was read at 450 nm with an absorbance microplate reader (Molecular Devices, Downingtown, Pennsylvania). The protein levels of the nuclear extract were assayed using a protein assay kit and used to normalize the DNA-binding activity of NF-κB p65.

Dual-luciferase reporter assay for NF-κB

For the NF-κB transcriptional activity assays, HUVECs were transfected with a cis-reporter plasmid containing 5 copies of consensus NF-κB sequences linked to a minimal E1B promoter-luciferase gene (pNF-κB Luc; Stratagene, La Jolla, California) using Lipofectamine 2000, following the manufacturer's instructions. The pRL-SV40 *Renilla* luciferase control reporter vector (Promega Corporation, Madison, Wisconsin) was used as an internal control. Twenty-four hours after transfection, the cells were exposed to TP at the indicated concentrations for 1 hour, followed by incubation with ox-LDL (50 µg/mL) for an additional 1 hour. Afterward, the cells were harvested and lysed with a cell lysis buffer (Cell Signaling Technology Inc, Danvers, Massachusetts). Firefly luciferase activity was assayed using a dual-luciferase reporter assay system (Promega Corporation) with a TD-20/20 luminometer (Turner BioSystems). To express relative luciferase activity, *Renilla* luciferase activity was used to normalize firefly luciferase activity.

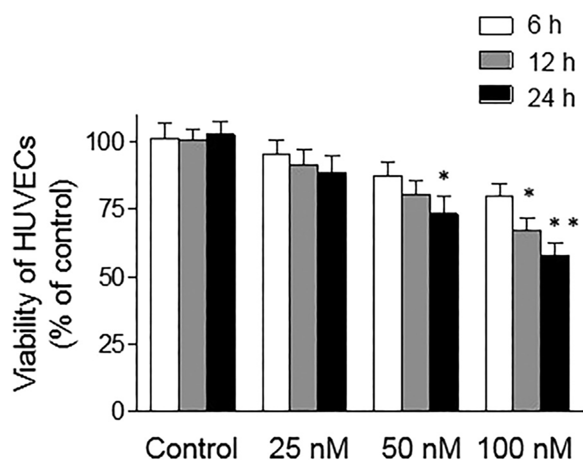

Figure 1. Triptolide (TP) reduced the viability of human umbilical vein endothelial cells (HUVECs) in a concentration- and time-dependent manner. HUVECs were incubated with TP at the indicated concentrations for the indicated times. Cell viability was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Values are presented as the mean (SE) of 4 independent analyses. $^*P < 0.05$ and $^{**}P < 0.01$ compared with the corresponding control group.

Statistical analysis

All data from at least 3 independent experiments were expressed as mean (SE). Comparisons between more than 2 groups were performed using 1-way analysis of variance, followed by Bonferroni's adjustment for multiple comparisons. A value of $P < 0.05$ indicated a statistically significant difference. GraphPad Prism version 6.0 (GraphPad Software Inc, La Jolla, California) was used to perform all the analyses.

Results

Effects of TP on the viability of HUVECs

As shown in **Figure 1**, TP reduced the viability of HUVECs in a concentration- and time-dependent manner. Compared with the control, the viability of HUVECs was reduced 12 or 24 hours after exposure to 50 or 100 nM TP, respectively. However, no changes in the viability of HUVECs were observed when HUVECs were exposed to 25 to 100 nM TP for 6 hours. In addition, a pilot study showed that cell viability did not change when HUVECs were exposed to ox-LDL (50 µg/mL) for 6 hours (data not shown). Based on the results, 50 µg/mL ox-LDL, 25-100 nM TP, and an incubation duration of 6 hours were chosen for the multiplex assay in the following experiments.

Effects of TP on ox–LDL-induced production of proinflammatory cytokines/chemokines and adhesion molecules in HUVECs

To investigate the possible effects of TP on endothelial inflammation, the inhibitory effect of TP on ox–LDL-induced production of inflammatory mediators in endothelial cells was first determined. As illustrated in **Figure 2**, incubation with ox-LDL (50 µg/mL) for 6 hours enhanced the production of proinflammatory cytokines and chemokines, including tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1), in HUVECs. Preincubation with TP attenuated the ox–LDL-induced effects on proinflammatory cytokines and chemokines in a concentration-dependent manner. Taken together, these results show that TP can attenuate inflammatory responses in endothelial cells at the tested exposure conditions.

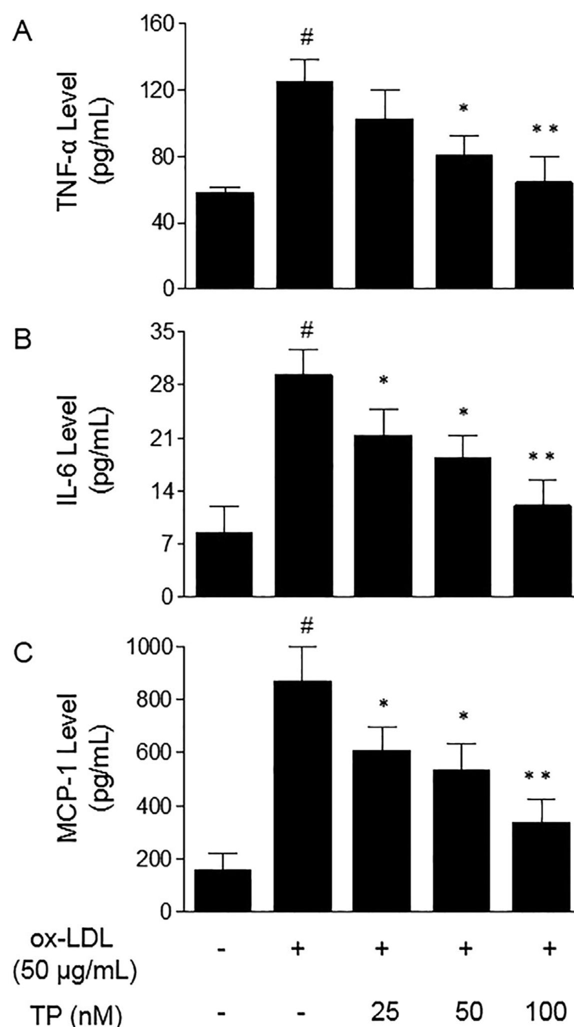

Figure 2. Triptolide (TP) inhibited ox-LDL-induced production of proinflammatory cytokines and chemokines, including tumor necrosis factor alpha (TNF-α) (A), interleukin (IL) 6 (B), and chemokines (MCP-1) (C) in human umbilical vein endothelial cells (HUVECs). After preincubation with TP at the indicated concentrations for 1 hour, HUVECs were incubated with oxidized LDL (ox-LDL) (50 µg/mL) for 6 hours. ELISA was used to determine the concentrations of proinflammatory cytokines and chemokines in the culture supernatant. Values are presented as the mean (SE) of 3 to 4 independent analyses. $^*P < 0.05$ and $^{**}P < 0.01$ compared with HUVECs exposed to ox-LDL alone; #$P < 0.05$ compared with the control group.

It has been demonstrated that adhesion molecules are involved in the pathological process of leukocyte recruitment. Thus, the effect of TP in the attenuation of ox–LDL-induced expression of adhesion molecules in endothelial cells was determined. As shown in **Figure 3**, incubation with ox-LDL (50 µg/mL) for 6 hours increased the expression of ICAM-1 and VCAM-1 in HUVECs, and the responses were inhibited in a concentration-dependent manner by preincubation with TP 1 hour before ox-LDL incubation. Together, the decreased expression of adhesion molecules induced by TP may lead to inhibition of leukocyte adhesion to endothelial cells.

Effects of TP on ox–LDL-induced monocyte adhesion to HUVECs

Because the adhesion of leukocytes to endothelial cells via adhesion molecules is a crucial step in the process of inflammation, the effect of TP in the attenuation of ox–LDL-induced leukocyte attachment to endothelial cells was determined. As shown in **Figure 4**, incubation with ox-LDL increased the number of monocytes that

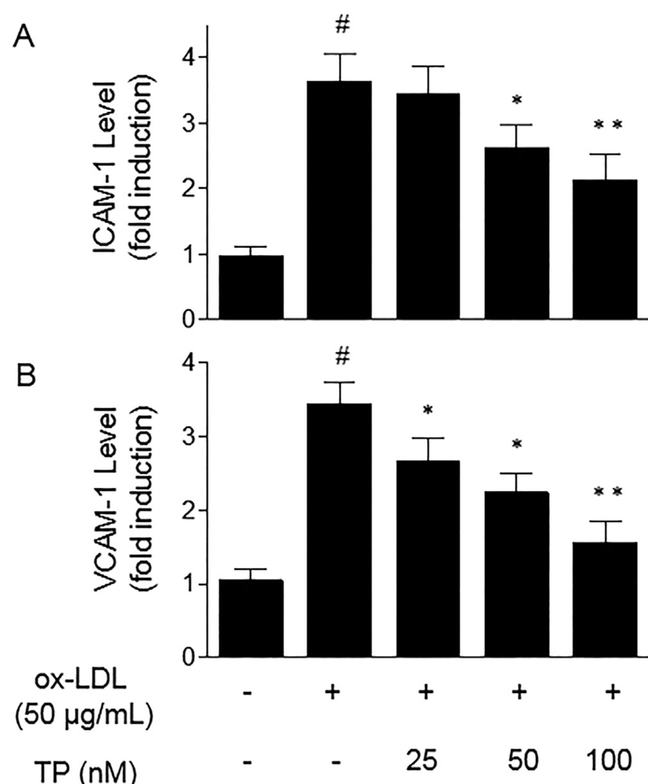

Figure 3. Triptolide (TP) inhibited oxidized LDL (ox-LDL)-induced intercellular adhesion molecule 1 (ICAM-1) (A) and vascular cell adhesion molecule 1 (VCAM-1) (B) expression in human umbilical vein endothelial cells (HUVECs). After preincubation with TP at the indicated concentrations for 1 hour, HUVECs were incubated with ox-LDL (50 μg/mL) for 6 hours. The cell-based ELISA was used to determine the expression of ICAM-1 and VCAM-1 in HUVECs. Values are presented as the mean (SE) of 4 independent analyses. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with HUVECs exposed to ox-LDL alone; $\#P < 0.05$ compared with the control group.

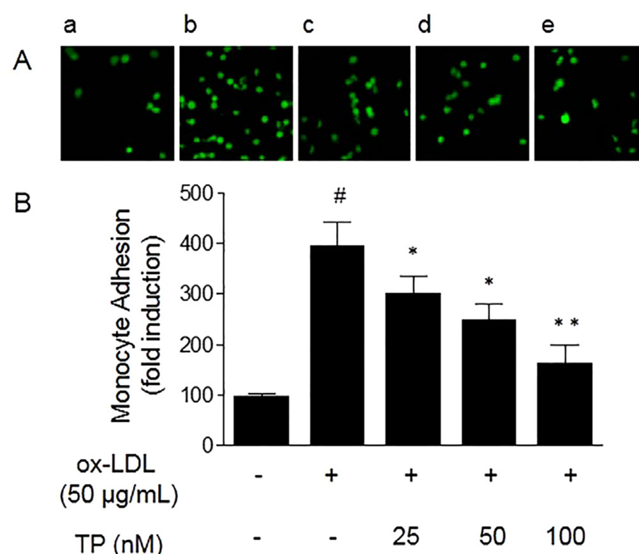

Figure 4. Triptolide (TP) inhibited oxidized LDL (ox-LDL)-induced monocyte adhesion to human umbilical vein endothelial cells (HUVECs). After preincubation with TP at the indicated concentrations for 1 hour, HUVECs were incubated with ox-LDL (50 μg/mL) for 6 hours, and followed by coincubation with fluorescence-labeled THP-1 cells for 30 minutes. (A) Representative images of fluorescence-stained monocyte attachment to HUVECs. Magnification, × 200. HUVECs were incubated with (a) a vehicle (control) or (b) ox-LDL alone and in the presence of TP at the concentration of (c) 25, (d) 50, or (e) 100 nM before coincubation with fluorescence-labeled THP-1 cells. (B) Fluorescence intensity of THP-1 cells was measured using a spectrofluorometer. Values are presented as the mean (SE) of 4 independent analyses. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with HUVECs exposed to ox-LDL alone; $\#P < 0.05$ compared with the control group.

adhered to HUVECs. Preincubation with TP attenuated ox–LDL-mediated monocyte adhesion in a concentration-dependent manner. Overall, the data suggest that TP, at the tested exposure conditions, can attenuate ox-LDL-induced leukocyte attachment to endothelial cells via the inhibition of adhesion molecule expression.

Effects of TP on ox–LDL-induced changes in oxidative stress-related parameters in HUVECs

To confirm the potential antioxidative action of TP in ox–LDL-treated HUVECs, 8-isoprostane, MDA, total SOD and its isoenzyme activities, and superoxide production were assayed in the supernatants and cell extracts of HUVECs exposed to ox-LDL. As shown in Figure 5 and Figure 6, compared with the control group, incubation with ox-LDL for 6 hours increased the production of 8-isoprostane and MDA in HUVECs. In contrast, incubation with ox-LDL lowered the total SOD and CuZn-SOD activities in HUVECs, but the activity of Mn-SOD did not change following incubation with ox-LDL in HUVECs. All ox–LDL-induced effects, except for those on Mn-SOD activity, were reversed by pre-incubation with TP.

In addition, as illustrated in Figure 7, incubation with ox-LDL for 6 hours increased superoxide production in HUVECs compared with that in the control group. These responses were inhibited in a concentration-dependent manner by preincubation with TP. Ex vivo incubation of samples with apocynin (0.1 mm), a selective NADPH oxidase inhibitor, prevented ox–LDL-induced superoxide production (data not shown). These results suggest that NADPH oxidase is the major source of ox–LDL-induced superoxide production in HUVECs.

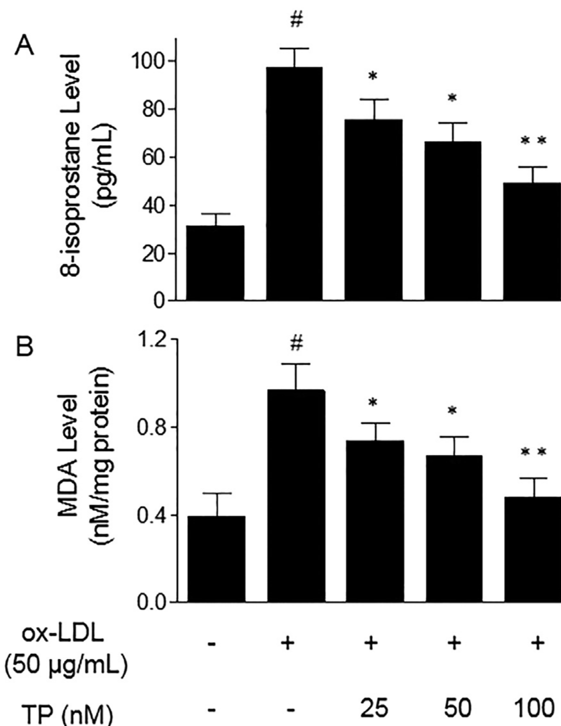

Figure 5. Triptolide (TP) inhibited oxidized LDL (ox-LDL)-induced production of 8-isoprostane (A) and malondialdehyde (MDA) (B) in human umbilical vein endothelial cells (HUVECs). After preincubation with TP at the indicated concentrations for 1 hour, HUVECs were incubated with ox-LDL (50 μg/mL) for 6 hours. The levels of 8-isoprostane in the culture supernatant and MDA in HUVECs were assayed using the corresponding kit, respectively. Values are presented as the mean (SE) of 4 independent analyses. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with HUVECs exposed to ox-LDL alone; $\#P < 0.05$ compared with the control group.

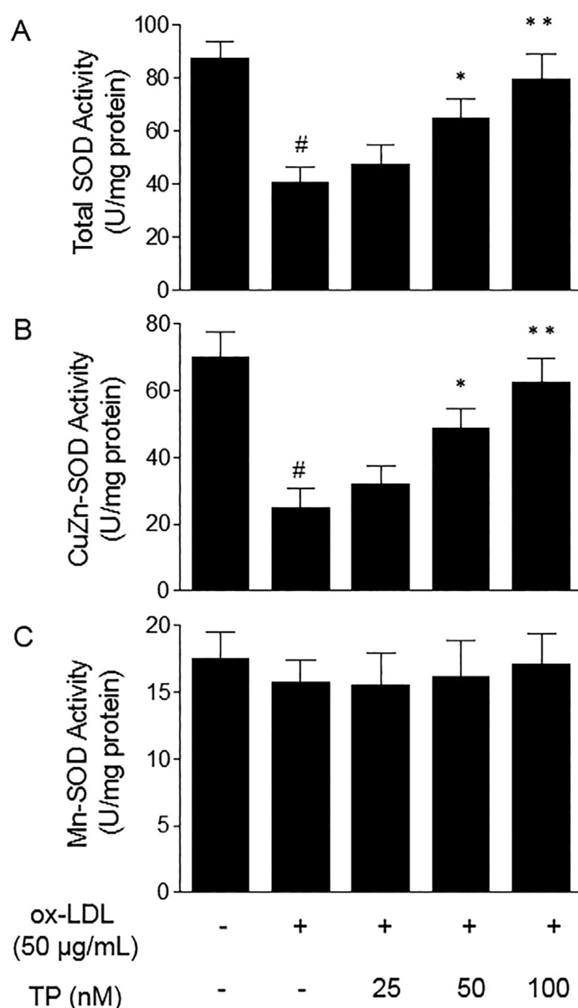

Figure 6. Triptolide (TP) reversed oxidized LDL (ox-LDL)-induced changes in the activities of total superoxide dismutase (SOD) (A) and its isoenzymes, including copper- and zinc-SOD (CuZn-SOD) (B) and manganese-SOD (Mn-SOD) (C) in human umbilical vein endothelial cells (HUVECs). After preincubation with TP at the indicated concentrations for 1 hour, HUVECs were incubated with ox-LDL (50 µg/mL) for 6 hours. The activities of total SOD and its isoenzymes, including CuZn-SOD and Mn-SOD, in HUVECs were assayed using a commercial assay kit. Values are presented as the mean (SE) of 4 independent analyses. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with HUVECs exposed to ox-LDL alone; #$P < 0.05$ compared with the control group.

Collectively, these results suggest that TP reduces ox–LDL-induced ROS production, possibly by attenuating NADPH oxidase activity and enhancing antioxidative enzyme activity in endothelial cells.

Effects of TP on ox–LDL-induced activation of the NF-κB signaling pathway in HUVECs

The transcription factor NF-κB plays a critical role in the expression of inflammatory factors. Phosphorylation of IκBα (Ser32) is necessary for the activation and subsequent nuclear translocation of NF-κB. As shown in **Figure 8**, the phosphorylation of IκBα increased 30 minutes after incubation with ox-LDL in HUVECs, and this effect was suppressed by preincubation with TP in a concentration-dependent manner. Because phosphorylation of IκBα is followed by the binding of NF-κB to DNA and transcriptional activation, the effects of TP on ox-LDL-mediated NF-κB activation were also determined by measuring the DNA-binding activity of NF-κB p65 and NF-κB transcriptional activity. Incubation with ox-LDL increased the DNA-binding activity of NF-κB p65 in HUVECs, and this effect was inhibited in a concentration-dependent manner by preincubation with TP. Moreover, incubation with ox-LDL enhanced NF-κB reporter activity in HUVECs, which was attenuated by preincubation with TP in a concentration-dependent manner. Taken together, these data demonstrate that preincubation with TP, at the tested exposure conditions, attenuated ox-LDL-mediated NF-κB transcriptional activation by suppressing IκBα phosphorylation and subsequent NF-κB DNA binding.

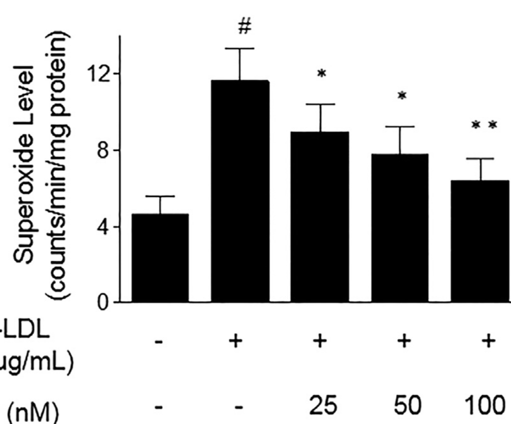

Figure 7. Triptolide (TP) inhibited oxidized LDL (ox-LDL)-induced increases in superoxide production in human umbilical vein endothelial cells (HUVECs). After preincubation with TP at the indicated concentrations for 1 hour, HUVECs were incubated with ox-LDL (50 µg/mL) for 6 hours. The levels of superoxide were measured using a lucigenin-enhanced chemiluminescence assay. Values are presented as the mean (SE) of 4 independent analyses. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with HUVECs exposed to ox-LDL alone; #$P < 0.05$ compared with the control group.

Discussion

The studies described were performed to investigate the effects of TP, a major bioactive ingredient of TWHF, on ox–LDL-induced inflammatory responses in endothelial cells. First, the results showed that TP suppressed the release of inflammation-related factors and inflammatory cell adhesion to endothelial cells (ie, HUVECs) exposed to ox-LDL. Second, the data suggest that the anti-inflammatory actions of TP are mediated via the attenuation of the signaling cascade that results from oxidative stress-induced NF-κB activation. These results improve our understanding of the potential role of TP in the suppression of ox-LDL triggered endothelial inflammation. Moreover, the results highlight the therapeutic potential of TP in atherosclerosis treatment.

Natural products derived from plants, animals, marine organisms, and bacteria have been used by human beings for many centuries to treat various illnesses. Natural products and their derivatives have been investigated for their potential pharmacological applications.[12,19–21] TP was first extracted from TWHF in 1972.[12] Commonly referred to as thunder god vine, TWHF has been widely used as a therapeutic agent to treat autoimmune and inflammatory diseases in China for hundreds of years. Increasing evidence has shown that TP attenuates inflammatory responses in vivo.[14–16] Although considerable effort has been made to determine the effects of TP on inflammatory responses, the specific mechanisms of TP in the regulation of ox–LDL-induced inflammatory responses have not yet been fully elucidated. In this study, the results showed that TP significantly suppressed inflammatory responses triggered by ox-LDL in endothelial cells, suggesting that TP is involved in the regulation of ox–LDL-induced inflammatory responses in endothelial cells. Moreover, the studies described suggest that the potential mechanisms for TP-mediated inhibition of inflammatory responses

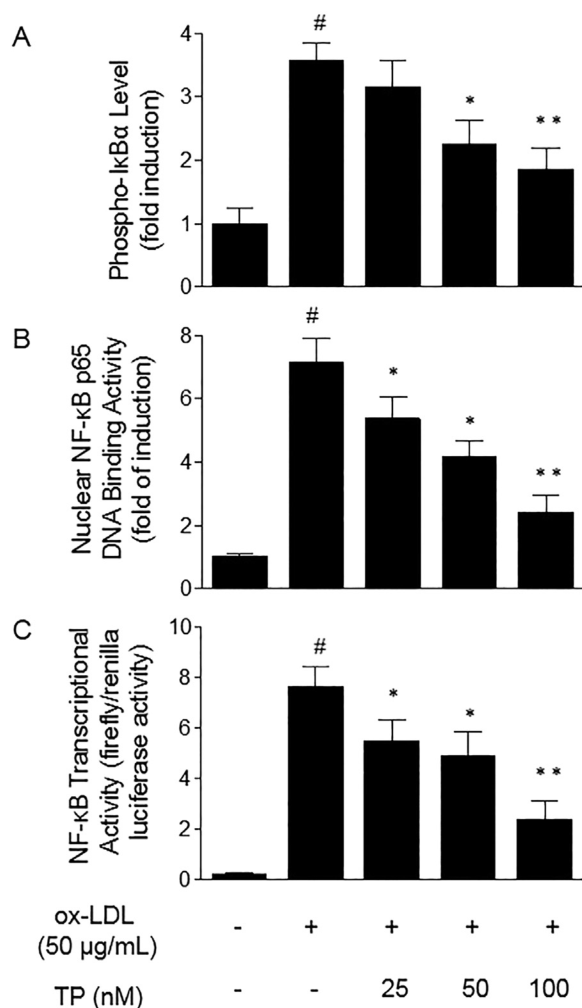

Figure 8. Triptolide (TP) inhibited oxidized LDL (ox-LDL)-induced increases in the protein levels of phosphorylated IκBα (phospho-IκBα) (A) and activities of nuclear factor kappa B (NF-κB) p65 DNA binding (B) and its transcription (C) in human umbilical vein endothelial cells (HUVECs). After preincubation with TP at the indicated concentrations for 1 hour, HUVECs were incubated with ox-LDL (50 μg/mL) for 30 minutes or 1 hour. The protein levels of phospho-IκBα and activities of NF-κB p65 DNA binding and its transcription were assayed using a cell-based ELISA, a nonradioactive ELISA-based assay kit, and a dual luciferase reporter assay system, respectively. Values are presented as the mean (SE) of 3 to 4 independent analyses. $^*P < 0.05$ and $^{**}P < 0.01$ compared with HUVECs exposed to ox-LDL alone; $\#P < 0.05$ compared with the control group.

induced by ox-LDL in endothelial cells may be related to the suppression of the oxidative stress and NF-κB signaling pathway.

Atherosclerosis is recognized to involve a chronic inflammatory process.[8] In addition to the overproduction of inflammatory mediators, inflammation is characterized by a massive infiltration of leukocytes from the blood into the sites of inflammation. The recruitment of leukocytes involves the interaction between adhesion molecules on endothelial cell surfaces and their counter receptors on the surface of leukocytes.[22,23] Ox–LDL-bound MCP-1 is also involved in facilitating the recruitment of monocytes to endothelial cells.[24] The results of this study showed that TP, at some of the exposures tested, clearly inhibited the ox–LDL-induced production of proinflammatory factors, including cytokines (TNF-α and IL-6)(tumor necrosis factor alpha and interleukin 6), chemokines (eg, MCP-1), and adhesion molecules (eg, ICAM-1 and VCAM-1). Moreover, the co-culture cellular adhesion experiments described demonstrated the inhibitory effects of TP on monocyte recruitment to endothelial cells. Collectively, these results suggest that TP has the potential to act as a protective agent against vascular inflammation.

ROS have been shown to be involved in the development of atherosclerosis.[8,25] Atherosclerosis has been found to be associated with an oxidative stress imbalance, including enhanced ROS and reduced net antioxidant activity. It has been demonstrated that oxidative stress produced by ROS induces an initial inflammatory response through positive feedback, resulting in additional ROS generation and further tissue injury.[8,26] ROS generation and elimination are involved in many cellular processes, including inflammatory responses. Increasing evidence has demonstrated that oxidative stress, reflected by the excessive generation of ROS, contributes to the pathogenesis of atherosclerosis.[8,25] Therefore, targeting the site of inflammation and eliminating ROS may be an effective strategy to treat atherosclerosis.

Ox-LDL is a critical factor contributing to the development of atherosclerosis.[27,28] It increases vascular oxidative stress, thereby leading to endothelial dysfunction.[7,8] As a result, inflammatory factor production is increased, which in turn induces leukocyte adherence to the endothelium and infiltration.[8] ROS derived from NADPH oxidase is strongly associated with atherosclerosis.[29] Accumulating evidence has shown the link between ox-LDL and NADPH oxidase, which in turn gives rise to ROS generation, including superoxide and hydrogen peroxide.[8,29] Previous studies have demonstrated that ox–LDL-mediated endothelial inflammation is prevented by radical-scavenging agents.[7] In this study, TP inhibited the ox-LDL-induced increase in intracellular ROS, which was associated with decreased NADPH oxidase activity as determined using a lucigenin-enhanced chemiluminescence assay. These results suggest that NADPH oxidase is involved in the ox–LDL-mediated increases in intracellular ROS levels. Normalization of ROS also appears to inhibit endothelial inflammation. These data imply that the decrease in ROS levels in endothelial cells is a critical mechanism involved in TP-mediated effects on endothelial inflammation. The results are congruent with previous studies demonstrating that the suppression of endothelial dysfunction in atherosclerotic lesions in ApoE$^{-/-}$ mice is linked to a reduction in oxidative stress derived from NADPH oxidase activation.[30]

Cellular ROS levels are regulated by the balance between the oxidant and antioxidant defense systems. SOD protects against superoxide-mediated cytotoxicity by catalyzing superoxide to form hydrogen peroxide. Thus, intracellular levels of superoxide are very low under normal physiological conditions. However, CuZn-SOD (SOD-1), not Mn-SOD (SOD-2), is inactivated by hydrogen peroxide derived from dismutation of the superoxide anion. Consistent with previous studies,[31] these data suggest that TP significantly reduced NADPH oxidase-mediated superoxide production in endothelial cells exposed to ox-LDL, which subsequently improved the CuZn-SOD activity. Based on these results, it was speculated that the inhibitory effects of TP on oxidative stress might be due to the attenuation of NADPH oxidase activity and enhancement of antioxidative enzyme activity.

NF-κB is an oxidant-sensitive transcription factor, and its activation has been linked with endothelial dysfunction.[8,32,33] In the pathogenesis of atherosclerosis, ox-LDL induces enhanced levels of intracellular ROS, which results in transcription-dependent proinflammatory factor synthesis via IκBα phosphorylation and subsequent NF-κB activation.[8,34] In addition to attenuating NF-κB transcriptional activation, as determined by an NF-κB luciferase reporter gene assay, the studies described also showed that TP inhibited events upstream of NF-κB transcriptional activation, including IκBα phosphorylation and translocation of NF-κBp65 into the nucleus from the cytoplasm. Based on the finding that TP attenuated the activity of NADPH oxidase associated with a concomitant decrease in ROS generation, we assumed that TP attenuates endothelial inflammation induced by ox-LDL by blocking the ox-

idative stress and NF-κB signaling cascade, and reducing the generation of proinflammatory factors. Thus, in addition to inhibiting NF-κB activation at the transcriptional level, this study suggests that the protective effects of TP are a result of the suppression of oxidative stress and the ROS/NF-κB signaling pathway in ox-LDL-mediated endothelial inflammation. Previous studies demonstrated that TP attenuated LPS-induced endothelial inflammation by inhibiting NF-κB activation at the transcriptional level, but did not investigate the effect of TP on oxidative stress.[35]

The concentrations of TP (25–100 nM) used in this study were similar to those that have been reported to inhibit inflammatory responses in human lung epithelial cells, and induce apoptotic cell death and cell cycle arrest in leukemia cell lines.[36,37] The concentrations were much lower than the plasma concentrations of TP in rats after oral administration of TP at a dose of 1 mg/kg, or similar to those detected in human beings when the extracts of TWHF were orally administered (200 mg, 3 times daily) as anti-systemic lupus agents.[38,39] Thus, the concentrations of TP used in this study are comparable to those achieved pharmacologically. The current study initially examined the effects of TP on the viability of endothelial cells. These data showed that the viability of HUVECs noticeably decreased when higher concentrations of TP ($\geq$50 nM) were present in the growth media for 12 or 24 hours. Therefore, the cytotoxicity caused by TP may restrict its clinical application. The use of appropriate structural modifications and combination pharmacotherapy as possible solutions to alleviate the cytotoxicity of TP and broaden its therapeutic window and clinical applications are proposed. An additional limitation of these studies is that the experiments described, including the intervention allocation and outcome assessment, were not conducted in a blinded manner. To diminish the possibility of a subjective effect in data collection and analysis, blinding procedures should be considered in future studies.

Overall, TP attenuated ox–LDL-induced inflammatory responses in endothelial cells. The effects of TP appear to be attributable to its suppression of ROS generation triggered by ox-LDL, thereby leading to the inhibition of NF-κB activation.

Conclusions

Inflammation is involved in the pathogenesis of a number of cardiovascular diseases, including atherosclerosis and hypertension. This suggests that the prevention or control of these diseases will require the development of effective therapeutic regimens for the treatment of inflammation. The data generated by this study provide insights into the mechanisms responsible for the suppression of ox–LDL-induced inflammatory responses of endothelial cells by TP, a major bioactive ingredient of the Chinese herb TWHF. The ability of TP to attenuate endothelial cell-associated inflammatory responses triggered by ox-LDL suggests that TP may be useful for the prevention of atherosclerosis.

Authors' Contributions

Y. Wang was responsible for conception and design of research; S. Zhang, S. Xie, and Y. Gao performed experiments; Y. Wang and S. Zhang analyzed data and interpreted results of experiments; and S. Zhang and Y. Wang drafted, edited, and revised manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors have indicated that they have no conflicts of interest regarding the content of this article.

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References

1. Cyr AR, Huckaby LV, Shiva SS, Zuckerbraun BS. Nitric oxide and endothelial dysfunction. *Crit Care Clin*. 2020;36(2):307–321.
2. Salvatore T, Pafundi PC, Galiero R, Rinaldi L, Caturano A, Vetrano E, Aprea C, Albanese G, Di Martino A, Ricozzi C, Imbriani S, Sasso FC. Can metformin exert as an active drug on endothelial dysfunction in diabetic subjects? *Biomedicines*. 2020;9(1):3.
3. Salvatore T, Caturano A, Galiero R, Di Martino A, Albanese G, Vetrano E, Sardu C, Marfella R, Rinaldi L, Sasso FC. Cardiovascular benefits from gliflozins: effects on endothelial function. *Biomedicines*. 2021;9(10):1356.
4. La Sala L, Prattichizzo F, Ceriello A. The link between diabetes and atherosclerosis. *Eur J Prev Cardiol*. 2019;26(Suppl 2):15–24.
5. Trpkovic A, Resanovic I, Stanimirovic J, Radak D, Mousa SA, Cenic-Milosevic D, Jevremovic D, Isenovic ER. Oxidized low-density lipoprotein as a biomarker of cardiovascular diseases. *Crit Rev Clin Lab Sci*. 2015;52(2):70–85.
6. Zheng J, Liu B, Lun Q, Gu X, Pan B, Zhao Y, Xiao W, Li J, Tu P. Longxuetongluo capsule inhibits atherosclerosis progression in high-fat diet-induced $ApoE^{-/-}$ mice by improving endothelial dysfunction. *Atherosclerosis*. 2016;255:156–163.
7. Luchetti F, Crinelli R, Nasoni MG, Benedetti S, Palma F, Fraternale A, Iuliano L. LDL receptors, caveolae and cholesterol in endothelial dysfunction: ox-LDLs accomplices or victims? *Br J Pharmacol*. 2021;178(16):3104–3114.
8. Marchio P, Guerra-Ojeda S, Vila JM, Aldasoro M, Victor VM, Mauricio MD. Targeting early atherosclerosis: a focus on oxidative stress and inflammation. *Oxid Med Cell Longev*. 2019;2019:8563845.
9. Zhao W, Wu C, Chen X. Cryptotanshinone inhibits oxidized LDL-induced adhesion molecule expression via ROS dependent NF-kB pathways. *Cell Adh Migr*. 2016;10(3):248–258.
10. Ou HC, Chou WC, Hung CH, Chu PM, Hsieh PL, Chan SH, Tsai KL. Galectin-3 aggravates ox-LDL-induced endothelial dysfunction through LOX-1 mediated signaling pathway. *Environ Toxicol*. 2019;34(7):825–835.
11. Ahotupa M. Oxidized lipoprotein lipids and atherosclerosis. *Free Radic Res*. 2017;51(4):439–447.
12. Chen SR, Dai Y, Zhao J, Lin L, Wang Y, Wang Y. A mechanistic overview of triptolide and celastrol, natural products from *Tripterygium wilfordii* Hook F. *Front Pharmacol*. 2018;9:104.
13. Cheng Y, Zhao Y, Zheng Y. Therapeutic potential of triptolide in autoimmune diseases and strategies to reduce its toxicity. *Chin Med*. 2021;16(1):114.
14. Tang B, Zhu J, Zhang B, Wu F, Wang Y, Weng Q, Fang S, Zheng L, Yang Y, Qiu R, Chen M, Xu M, Zhao Z, Ji J. Therapeutic potential of triptolide as an anti-inflammatory agent in dextran sulfate sodium-induced murine experimental colitis. *Front Immunol*. 2020;11:592084.
15. Yuan K, Li X, Lu Q, Zhu Q, Jiang H, Wang T, Huang G, Xu A. Application and mechanisms of triptolide in the treatment of inflammatory diseases-A review. *Front Pharmacol*. 2019;10:1469.
16. Zhang J, Zhu M, Zhang S, Xie S, Gao Y, Wang Y. Triptolide attenuates renal damage by limiting inflammatory responses in DOCA-salt hypertension. *Int Immunopharmacol*. 2020;89(Pt A):107035.
17. Wang Y, Cui L, Xu H, Liu S, Zhu F, Yan F, Shen S, Zhu M. TRPV1 agonism inhibits endothelial cell inflammation via activation of eNOS/NO pathway. *Atherosclerosis*. 2017;260:13–19.
18. Wang Y, Chen AF, Wang DH. Enhanced oxidative stress in kidneys of salt-sensitive hypertension: role of sensory nerves. *Am J Physiol Heart Circ Physiol*. 2006;291(6):H3136–H3143.
19. Lotfy BMM, Mousa MR, EI-Shehry MSFES, Ahmed SHAF, Ali SB, AI Shawoush AM, Mohamed AS. Therapeutic potency of Gallium verum extract on ethanol-induced gastric ulcer in rats. *Biointerface Res Appl Chem*. 2022;12(5):6010–6020.
20. Mohamed AS, Mahmoud SA, Soliman AM, Fahmy SR. Antitumor activity of saponin isolated from the sea cucumber, *holothurian arenicola* against ehrlich ascites carcinoma cells in swiss albino mice. *Nat Prod Res*. 2021;35(11) 1928-1923.
21. Sadek SA, Hassanein SS, Mohamed AS, Soliman AM, Fahmy SR. Echinochrome pigment extracted from sea urchin suppress the bacterial activity, inflammation, nociception, and oxidative stress resulted in the inhibition of renal injury in septic rats. *J Food Biochem*. 2022;46(3):e13729.
22. Gerhardt T, Ley K. Monocyte trafficking across the vessel wall. *Cardiovasc Res*. 2015;107(3):321–330.
23. Schnoor M, Alcaide P, Voisin MB, van Buul JD. Crossing the vascular wall: common and unique mechanisms exploited by different leukocyte subsets during extravasation. *Mediat Inflamm*. 2015;2015:946509.
24. Wiesner P, Tafelmeier M, Chittka D, Choi SH, Zhang L, Byun YS, Almazan F, Yang X, Iqbal N, Chowdhury P, Maisel A, Witztum JL, Handel TM, Tsimikas S,

Miller YI. MCP-1 binds to oxidized LDL and is carried by lipoprotein (a) in human plasma. *J Lipid Res*. 2013;54(7):1877–1883.
25. Förstermann U, Xia N, Li H. Roles of vascular oxidative stress and nitric oxide in the pathogenesis of atherosclerosis. *Circ Res*. 2017;120(4):713–735.
26. Papaconstantinou J. The role of signaling pathways of inflammation and oxidative stress in development of senescence and aging phenotypes in cardiovascular disease. *Cells*. 2019;8(11):1383.
27. Herrington W, Lacey B, Sherliker P, Armitage J, Lewington S. Epidemiology of atherosclerosis and the potential to reduce the global burden of atherothrombotic disease. *Circ Res*. 2016;118(4):535–546.
28. Matsuzawa Y, Guddeti RR, Kwon TG, Lerman LO, Lerman A. Treating coronary disease and the impact of endothelial dysfunction. *Prog Cardiovasc Dis*. 2015;57(5):431–442.
29. Yuan T, Yang T, Chen H, Fu D, Hu Y, Wang J, Yuan Q, Yu H, Xu W, Xie X. New insights into oxidative stress and inflammation during diabetes mellitus-accelerated atherosclerosis. *Redox Biol*. 2019;20:247–260.
30. Aguilar EC, Dos Santos LC, Leonel AJ, de Oliveira JS, Santos EA, Navia-Pelaez JM, da Silva JF, Mendes BP, Capettini LSA, Teixeira LG, Lemos VS, Alvarez-Leite JI. Oral butyrate reduces oxidative stress in atherosclerotic lesion sites by a mechanism involving NADPH oxidase down-regulation in endothelial cells. *J Nutr Biochem*. 2016;34:99–105.
31. Lee WJ, Ou HC, Hsu WC, Chou MM, Tseng JJ, Hsu SL, Tsai KL, Sheu WHH. Ellagic acid inhibits oxidized LDL-mediated LOX-1 expression, ROS generation, and inflammation in human endothelial cells. *J Vasc Surg*. 2010;52(5):1290–1300.
32. Zhang Q, Lenardo MJ, Baltimore D. 30 years of NF-κB: A blossoming of relevance to human pathobiology. *Cell*. 2017;168(1-2):37–57.
33. Leonard A, Rahman A, Fazal F. Importins α and β signaling mediates endothelial cell inflammation and barrier disruption. *Cell Signal*. 2018;44:103–117.
34. Yan S, Zhang X, Zheng H, Hu D, Zhang Y, Guan Q, Liu L, Ding Q, Li Y. Clematichinenoside inhibits VCAM-1 and ICAM-1 expression in TNF-α-treated endothelial cells via NADPH oxidase-dependent IκB kinase/NF-κB pathway. *Free Radic Biol Med*. 2015;78:190–201.
35. Song C, Wang Y, Cui L, Yan F, Shen S. Triptolide attenuates lipopolysaccharide-induced inflammatory responses in human endothelial cells: involvement of NF-kB pathway. *BMC Complement Altern Med*. 2019;19(1):198.
36. Hoyle GW, Hoyle CI, Chen J, Chang W, Williams RW. Identification of triptolide, a natural diterpenoid compound, as an inhibitor of lung inflammation. *Am J Physiol Lung Cell Mol Physiol*. 2010;298(6):L830–L836.
37. Kang D, Liu Y, Song Y, Fang B, Zhang Q, Hu L. Triptolide shows high sensitivity and low toxicity against acute myeloid leukemia cell lines through inhibiting WSTF-RNAPII complex. *Front Oncol*. 2022;12:811850.
38. Gong X, Chen Y, Wu Y. Absorption and metabolism characteristics of triptolide as determined by a sensitive and reliable LC-MS/MS method. *Molecules*. 2015;20(5):8928–8940.
39. Yao J, Zhang L, Zhao X, Hu L, Jiang Z. Simultaneous determination of triptolide, *wilforlide A* and triptonide in human plasma by high-performance liquid chromatography-electrospray ionization mass spectrometry. *Biol Pharm Bull*. 2006;29(7):1483–1486.