Original Research

Ethanol extract of propolis protects endothelial cells from oxidized low density lipoprotein-induced injury by inhibiting lectin-like oxidized low density lipoprotein receptor-1-mediated oxidative stress

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Abstract

Lectin-like oxidized low density lipoprotein receptor-1 (LOX-1), as the primary oxidized low-density lipoprotein (ox-LDL) receptor on endothelial cells, plays a crucial role in endothelial injury, which is a driving force in the initiation and development of atherosclerosis. Our previous studies have shown that ethanol extract of propolis (EEP) promotes reverse cholesterol transport and inhibits atherosclerotic lesion development. However, the protective effects of EEP against ox-LDL-induced injury in endothelial cells and the underlying mechanisms are still unknown. This study was designed to test the hypothesis that EEP attenuates ox-LDL-induced endothelial oxidative injury via modulation of LOX-1-mediated oxidative stress. Our results showed that exposure of human umbilical vein endothelial cells (HUVECs) to ox-LDL (100 mg/L) led to the decrease in cell viability and increase in lactate dehydrogenase (LDH) release, caspase-3 activation, and apoptosis, whereas pretreatment with EEP (7.5, 15 and 30 mg/L) protected against such damages in a dose-dependent manner. In addition, EEP mitigated ox-LDL uptake by HUVECs and attenuated ox-LDL-upregulated LOX-1 expression both at the mRNA and protein levels. Moreover, EEP suppressed the ox-LDL-induced oxidative stress as assessed by decreased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation, reactive oxygen species (ROS), and malondialdehyde (MDA) generation as well as increased antioxidant enzyme activities. Similar results were observed in the anti-LOX-1 antibody or diphenyleneiodonium (DPI)-pretreated HUVECs. These data indicate that EEP may protect HUVECs from ox-LDL-induced injury and that the mechanism at least partially involves its ability to inhibit endothelial LOX-1 upregulation and subsequent oxidative stress.

Keywords: Ethanol extract of propolis, oxidized low-density lipoprotein, lectin-like oxidized low-density lipoprotein receptor-1, oxidative stress, vascular endothelial cell

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Introduction

Atherosclerosis is a chronic, progressive cardiovascular disease that results from complex interactions of circulating factors and various cell types in the vessel wall, including endothelial cells, monocytes/macrophages, and smooth muscle cells.[1] Endothelial injury, associated to dysfunction, is a driving force in the initiation and development of atherosclerosis.[2] In the oxidation hypothesis of atherosclerosis, oxidized low-density lipoprotein (ox-LDL) plays a pivotal role in initiating the pathological process that leads to endothelial injury prerequisite for macrophage uptake and accumulation of cholesterol, inflammation, and proliferation of vascular smooth muscle cells in the subintimal space.[3] Ox-LDL uptake is mediated by several receptors such as CD36, CD68, and scavenger receptor A (SR-A) on macrophages,[4] whereas its uptake depends mainly on lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) on endothelial cells.[3] LOX-1, a class E scavenger receptor induced by multiple metabolic factors such as ox-LDL,

tumor necrosis factor-α (TNF-α), angiotensin II, and shear stress,[5] is upregulated in atherosclerotic plaque and injured endothelial cells,[6] and its genetic deletion is associated with reduced atherosclerotic plaque formation in hypercholesterolemic mice.[7,8] Moreover, ox-LDL-induced activation of LOX-1 triggers the expression and release of inflammatory factors involved in endothelial injury and atherogenesis such as intercellular adhesion molecule-1 (ICAM-1), E-selectin and monocyte chemoattractant protein-1 (MCP-1),[9] and reduces nitric oxide (NO) availability through increased reactive oxygen species (ROS) production.[10] Therefore, LOX-1 may be an attractive therapeutic target for ox-LDL-mediated endothelial injury and atherosclerosis.[11]

Propolis, a natural resinous bee-hive product collected by honeybees from various plant sources, has been used in folk medicines and complementary therapies since ancient times and has become one of the most popular functional foods all around the world, because of its broad spectrum of biological and pharmacological properties such as antioxidant, antimicrobial, antiviral, antitumoral, antiinflammatory, immunomodulatory, and cardioprotective functions.[12] These activities are mainly attributed to the active compounds in propolis such as flavonoids, phenolic acids, and their esters.[12,13] Flavonoids and related compounds are able to inhibit lipid peroxidation, platelet aggregation, capillary permeability and fragility, and the activity of enzyme systems including cyclo-oxygenase and lipoxygenase.[14] It has been reported that ethanol extract of propolis (EEP) and its flavones exert inhibitory effects on inflammatory responses and inflammation-related transcription factors in macrophages.[15,16] Our previous studies have shown that EEP promotes reverse cholesterol transport and inhibits atherosclerotic lesion development.[17,18] However, whether EEP protects endothelial cells from ox-LDL-induced injury by inhibiting the LOX-1 expression has not yet been determined.

In the present study, we explored the potential protective effect of EEP on ox-LDL-induced insults in human umbilical vein endothelial cells (HUVECs) and investigated whether the cytoprotective effect of EEP would be related to regulation of LOX-1 expression and subsequent oxidative stress.

Materials and methods

Reagents

RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco (Rockville, MD). RIPA lysis buffer and bicinchoninic acid (BCA) protein quantitation kits were purchased from Solarbio (Beijing, China) and DiI-ox-LDL was from Xiesheng Biotech (Beijing, China). 2′,7′-Dichlorofluorescin diacetate (DCHF-DA) and anti-LOX-1 monoclonal antibody (anti-LOX-1 mAb) were purchased from Molecular Probes (Eugene, OR) and R&D Systems (Minneapolis, MN), respectively. Anti-β-actin antibody and diphenyleneiodonium (DPI) were purchased from Sigma (St Louis, MO). 3-(4,5-Dimethylthiazol-2-y-l)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and Annexin V-FITC apoptosis detection kits were purchased from Genview (Houston, TX) and KeyGEN Biotech (Nanjing, China), respectively. Polyvinylidene fluoride (PVDF) membranes and enhanced chemiluminescence (ECL) kits were purchased from Millipore (Bedford, USA) and Thermo Scientific Pierce (Rockford, IL), respectively. Real-time PCR reagent kits were purchased from Tiangen Biotech (Beijing, China) and caspase-3 activity assay kits from Calbiochem (San Diego, CA). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase assay kits were purchased from Genmed Scientifics Inc. (Shanghai, China). Assay kits of lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) were obtained from Jiancheng Biotech (Nanjing, China).

Preparation of EEP and total flavonoids measurement[15]

Propolis was obtained from Shandong province (China) and kept desiccated pending its processing. The propolis powder (100 g) was extracted in 95% (v/v) ethanol (1 L) under the condition of sonication at 40°C for 3 h. The supernatant was then filtered with Whatman No. 4 filter papers. The residues were collected and extracted with 95% ethanol for another two times. Thereafter, all of the supernatants were collected together and evaporated in a rotary evaporator under a reduced pressure at 50°C. Finally, the EEP was dried in the oven and stored at −20°C. The colorimetric method of Chinese Standard (GB/T 20574-2006) was used to determine the total flavonoids content of EEP, which was 213.46 ± 2.93 mg rutin equivalent per gram. Immediately prior to use, EEP sample was weighed, dissolved in dimethylsulfoxide (DMSO), and diluted with cell culture medium into appropriate concentrations. The final concentration of DMSO did not exceed than 0.1% (v/v). The control cells received the same amount of DMSO.

Isolation and oxidation of LDL

Human LDL was isolated from plasma of normolipidemic donors by sequential ultracentrifugation, and incubated with 10 μmol/L $CuSO_4$ for 18 h at 37°C as described recently.[19]

Cell culture

HUVECs were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 100 U/mL penicillin/streptomycin in a 37°C humidified incubator containing 5% CO_2 until subconfluent, and then the medium was replaced with 1% FBS medium.

Cell viability and LDH assay

HUVECs were seeded in 96-well plates, pretreated with/without different concentrations (7.5, 15 and 30 mg/L) of EEP, anti-LOX-1 mAb (4 mg/L) or DPI (5 μmol/L) for 1 h and then stimulated with ox-LDL (100 mg/L) for 24 h. Cell viability was determined by MTT assay as described previously[20] and expressed as the percentage of the optical

density of treated cells relative to that of the untreated control cells (100%).

To further measure the level of cell injury, LDH leakage in the media was determined according to the protocols of a LDH activity assay kit.

Detection of apoptosis by flow cytometry analysis

The Annexin V-FITC/PI double-staining assay was used to quantify apoptosis according to the manufacturer's instructions. Following treatment, cells were centrifuged, washed with phosphate-buffered saline (PBS) and resuspended in 500 μL binding buffer, and incubated with 5 μL Annexin V-FITC and 5 μL of propidium iodide (PI) solution for 10 min in the dark at room temperature. Cellular fluorescence was measured using flow cytometry analysis with a FAC Scan flow cytometer (Becton-Dickinson, San Jose, CA).

Measurement of caspase-3 activity

Caspase-3 activity was determined with an assay kit following the manufacturers' instructions. Briefly, after treatment, HUVECs were harvested, rinsed with PBS, and lysed with iced lysis buffer supplied in the kit. The lysate was centrifuged at 12,000 *g* at 4°C for 15 min. The mixture of 10 μL lysate supernatant, 80 μL reaction buffer, and 10 μL caspase-3 substrate together in 96-well microtiter plates were incubated at 37°C for 2 h. Caspase-3 activity was read in an Infinite F200 microplate reader (Tecan, Switzerland) at 405 nm and expressed as a percentage of the control.

Uptake of Dil-ox-LDL

HUVECs were pretreated with EEP (7.5, 15 and 30 mg/L) or anti-LOX-1 mAb (4 mg/L) for 1 h and then incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil)-ox-LDL (25 mg/L) for 6 h. Cells were washed three times with PBS and lysed in 200 μL lysis buffer. Protein concentration of each sample was determined using BCA protein assay. Fluorescence was detected with excitation and emission wavelengths of 530 and 590 nm, respectively, using an Infinite F200 microplate reader (Tecan, Switzerland). These data were normalized to the protein concentration of each sample, as described previously.[21]

Dil-ox-LDL uptake was further assessed by fluorescence microscopy after washing the cells with ice-cold PBS and fixing in 4% paraformaldehyde for 20 min. For counterstaining, cell nuclei were stained with 4′,6-diamidino-2′-phenylindole dihydrochloride (DAPI) for 5 min at room temperature.

Measurement of ROS levels

Intracellular ROS levels were determined using redox-sensitive dye DCHF-DA, which could be oxidized into 2′,7′-dichlorofluorescein (DCF) by intracellular ROS when entering into cells. Briefly, culture medium was removed and the treated HUVECs in six-well plates were washed with PBS twice. Subsequently, DCHF-DA, diluted to a final concentration of 10 μmol/L with RPMI 1640, was added and incubated in the dark at 37°C for 20 min. Then HUVECs were harvested, suspended in PBS, and the mean fluorescence intensity of cells was analyzed using a FAC Scan flow cytometer.

Determination of lipid peroxidation level and antioxidant enzyme activities

The treated HUVECs were washed with ice-cold PBS, collected, and centrifuged at 1000 r/min for 5 min. The cells were resuspended in 0.5 mL of lysis buffer and lysed with an electromotive cell crusher. The MDA content, activities of SOD, and CAT were assayed using commercial kits according to the manufacturer's instructions. Protein content was determined using BCA assay. MDA content and enzyme activities were converted to nmol/mg protein and units/mg protein, respectively.

Measurement of NADPH oxidase activity

The activity of NADPH oxidase in the treated HUVECs grown in six-well plates was determined by lucigenin-enhanced chemiluminiscence with a commercial kit according to manufacturer's instructions.

Western blot analysis

Cells were lysed in RIPA buffer and the protein concentration was determined by BCA protein assay. Equivalent amounts of protein from each lysate sample were separated by 8% SDS-PAGE and then transferred onto PVDF membranes by electroblotting. After blocking in 10% nonfat dry milk diluted with Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 2 h at room temperature, the membranes were incubated with primary antibodies overnight at 4°C. After four washes with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. At last, protein bands were visualized by ECL reaction. The Image-Pro Plus software (version 6.0, Media Cybernetics, LP, USA) was used to measure the band intensities, which were normalized to β-actin levels.

Quantitative real-time PCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). First strand cDNA was synthesized using MuLV reverse transcriptase from 2 μg of total RNA. Real-time PCR was performed with a Rotor-Gene Q real-time PCR cycler (Qiagen, Shanghai, China) using SYBR-green PCR master mix kits. The data were analyzed using the Rotor-Gene Q software (version 1.7, Qiagen), and relative mRNA levels were calculated using the $2^{-\triangle\triangle Ct}$ method and normalized against GAPDH. The primers were synthesized by Sangon Biotech (Shanghai, China) and the oligonucleotide sequences were 5′-CTATTTTCCTCGGGCTCATT-3′ (forward) and 5′-AAGTCCAGATCAGCTGTGCTA-3′ (reverse) for LOX-1, 5′-CCTCCCGCT TCGCTCTCT-3′ (forward) and 5′-GCTGGCGACGCAAAAGA-3′ (reverse) for GAPDH.

Statistical analysis

Results are shown as the mean ± standard error of the mean (SEM) for at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Student–Newmann–Keuls multiple comparison tests with the SPSS13.0 software for Windows. *P*-values less than 0.05 were considered statistically significant.

Results

EEP attenuates ox-LDL-induced cytotoxicity in HUVECs

Cytotoxicity of EEP on HUVECs was evaluated by MTT assay. As shown in Figure 1a, except for the concentration of 60 mg/L, treatment with EEP at concentrations up to 30 mg/L for 24 h had no significant effects on cell viability. To evaluate the cytoprotective function of EEP, HUVECs were preincubated with EEP (7.5, 15 and 30 mg/L) for 1 h, followed by 100 mg/L of ox-LDL treatment for 24 h. The results of MTT assay (Figure 1b) showed that EEP protected HUVECs from ox-LDL-induced cytotoxicity. EEP treatment at various concentrations increased the cell viability by 16.0%, 38.6% and 50.7%, respectively, compared to the ox-LDL-treated cells.

LDH, which leaks from cells after plasma membrane disruption, was measured to further confirm the protective effect of EEP on HUVECs. As seen in Figure 1c, LDH release increased significantly in the media after HUVECs were stimulated with ox-LDL, whereas pretreatment with EEP significantly attenuated the LDH release in a dose-dependent manner.

In addition, preincubation with anti-LOX-1 mAb and DPI (a ROS inhibitor) also prevented the reduced cell viability and the LDH release induced by ox-LDL (Figure 1b and c).

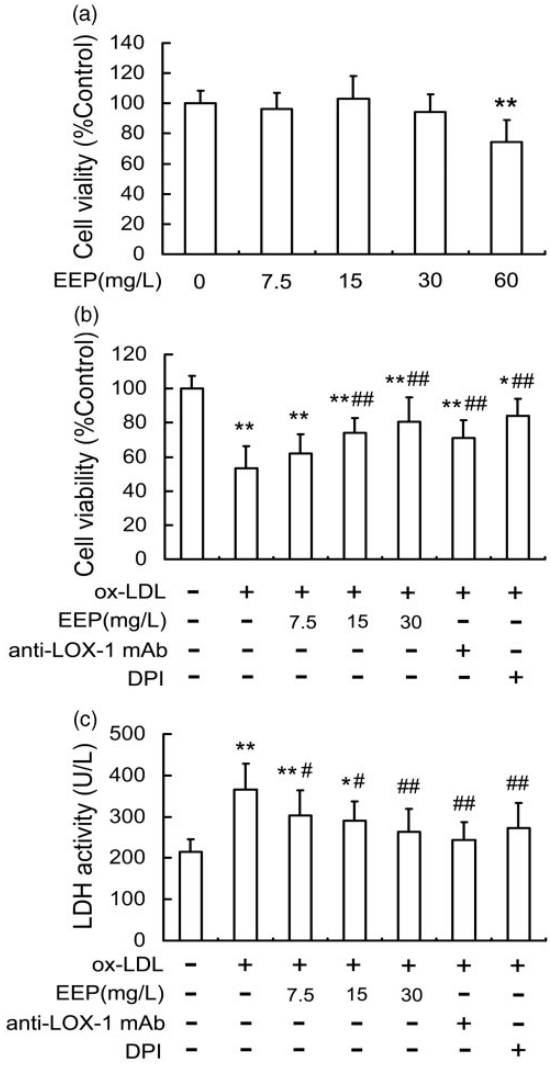

Figure 1 Effects of EEP on ox-LDL-induced cytotoxicity in HUVECs. HUVECs were incubated with the indicated concentrations of EEP for 24 h and cell viability was measured by the MTT assay and expressed as the percentage of control (a). HUVECs were pretreated with or without EEP (7.5, 15 and 30 mg/L), anti-LOX-1 mAb (4 mg/L) or DPI (5 μmol/L.) for 1 h, followed by incubation with ox-LDL (100 mg/L) for 24 h, and then cell viability (b) and LDH activity in media (c) were determined by MTT assay and a kit, respectively. Data are expressed as the mean ± SEM of six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle-treated control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus ox-LDL treatment

EEP inhibits ox-LDL-induced HUVEC apoptosis

Annexin V-PI double staining assay through flow cytometry (Figure 2a and b) showed that treating HUVECs with 100 mg/L ox-LDL for 24 h increased the apoptotic cells by 183.9%, compared to the vehicle-treated control cells. However, pretreating the cells with EEP (7.5, 15 and 30 mg/L) decreased the cell apoptotic rate in a dose-dependent manner.

The protective effect of EEP on ox-LDL-induced cell apoptosis was further confirmed through determining the caspase-3 activity, a marker of apoptosis. As shown in Figure 2c, the ox-LDL-induced increase in caspase-3 activity was significantly suppressed by EEP in a concentration-dependent manner.

Moreover, the ox-LDL-induced cell apoptosis and activation of caspase-3 were also inhibited by anti-LOX-1 mAb and DPI (Figure 2a, b and c).

EEP attenuates ox-LDL uptake by HUVECs

As seen in Figure 3a, clear uptake of Dil-ox-LDL was detected in control cells, whereas the uptake of Dil-ox-LDL was significantly attenuated by EEP in a dose-dependent manner. To further obtain the experimental evidence, we visualized the uptake of Dil-ox-LDL by using fluorescence microscopy. The increase in intracellular fluorescence signals of Dil-ox-LDL was remarkably blocked by treatment with EEP (Figure 3b). Similar results were obtained using cells treated with anti-LOX-1 mAb, suggesting that EEP may inhibit the cellular uptake of ox-LDL via suppression of LOX-1 pathway.

EEP attenuates ox-LDL-induced LOX-1 expression

Since the endothelial uptake of ox-LDL is mainly mediated by LOX-1, we investigate the effects of EEP on the ox-LDL-induced LOX-1 expression in HUVECs. LOX-1 protein (Figure 4a) and mRNA (Figure 4b) expression were increased after exposure to ox-LDL (100 mg/L) in

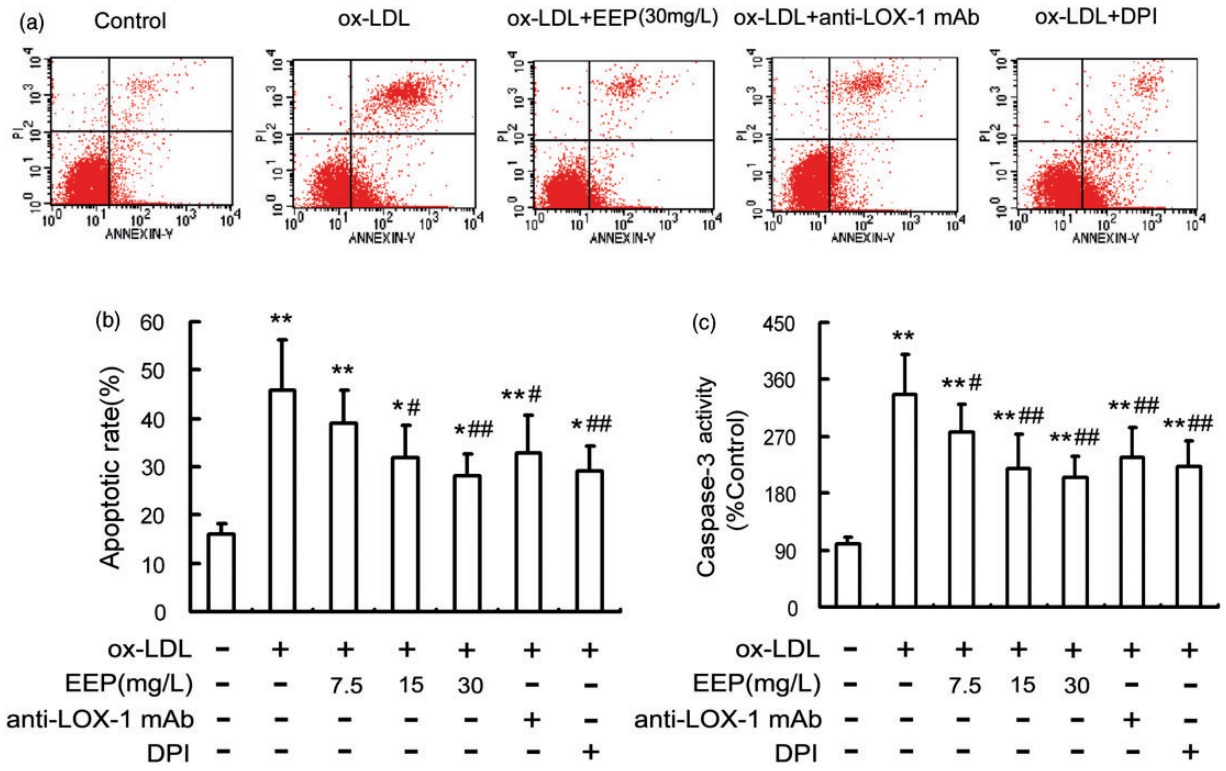

Figure 2 Effect of EEP on ox-LDL-induced apoptosis in HUVECs. Cells were treated as described in Figure 1b. (a) HUVEC apoptosis was detected using flow cytometry. (b) The total apoptotic cells (early and late-stage apoptosis) were represented by the right side of the panel (Annexin V staining alone or together with PI). (c) Caspase-3 activity was determined by colorimetric assay. Data are expressed as the mean ± SEM of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle-treated control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus ox-LDL treatment. (A color version of this figure is available in the online journal)

HUVECs. However, pretreatment with EEP before exposure to ox-LDL resulted in the reduction of LOX-1 expression in a dose-dependent manner. Furthermore, pretreatment with the ROS inhibitor DPI also markedly inhibited ox-LDL-upregulated LOX-1 expression, indicating that ROS plays a key role in the increased expression of LOX-1.

EEP reduces ox-LDL-induced oxidative stress in HUVECs

To investigate whether the protective effects of EEP on HUVECs could be attributed to reduced oxidative stress, DCHF was used as a fluorescent probe to determine intracellular ROS levels. As shown in Figure 5a, exposure of HUVECs to ox-LDL for 4 h led to a 2.4-fold increase in ROS, whereas EEP pretreatment significantly reduced the increased fluorescence induced by ox-LDL in a dose-dependent manner. Additionally, preincubation with anti-LOX-1 mAb and DPI (a NADPH oxidase inhibitor) also inhibited ox-LDL-generated ROS, suggesting that ROS production was largely dependent on the binding of ox-LDL to LOX-1 and the subsequent activation of NADPH oxidase.

Since the reduced activities of antioxidant enzymes under the condition of oxidative stress additionally increase ROS generation, we explored the effects of EEP on the activities of SOD and CAT in HUVECs in response to ox-LDL. As seen in Figure 5b and c, similar to anti-LOX-1 mAb and DPI, EEP pretreatment significantly decreased the suppression of SOD and CAT activities caused by ox-LDL.

Membrane lipid oxidation is one of the primary events in oxidative damage, which can be assessed by its degradation product MDA. Treating HUVECs with ox-LDL markedly increased the intracellular MDA levels. However, preincubation with EEP, anti-LOX-1 mAb, and DPI significantly decreased MDA levels (Figure 5d).

EEP suppresses ox-LDL-stimulated NADPH oxidase activation in HUVECs

ROS production in vascular endothelial cells was largely dependent on the binding of ox-LDL to LOX-1 and the subsequent activation of NADPH oxidase.[22] We therefore measured NADPH oxidase activity in HUVECs. The results showed that incubation of HUVECs with 100 mg/L ox-LDL for 4 h led to a significant increase in NADPH oxidase activity. However, EEP, anti-LOX-1 mAb, and DPI significantly attenuated the ox-LDL-stimulated NADPH oxidase activation (Figure 6).

Discussion

LOX-1, as the primary ox-LDL receptor on endothelial cells, plays a crucial role in the endothelial injury, which is one of the earliest hallmarks of atherosclerosis. In the present study, we first demonstrated that EEP attenuated ox-LDL-induced endothelial injury as assessed by the increased cell viability and the suppressed LDH release, caspase-3 activation, and apoptosis. Second, we showed that EEP mitigated ox-LDL uptake by HUVECs and inhibited LOX-1 mRNA

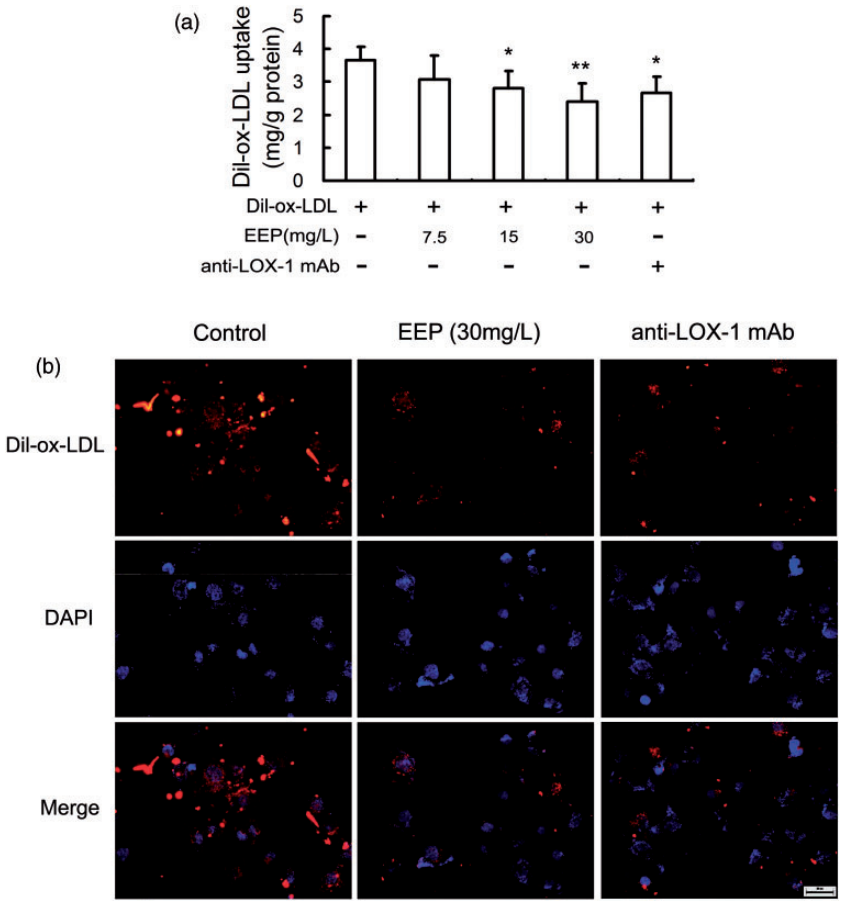

Figure 3 Effect of EEP on ox-LDL uptake by HUVECs. (a) Dil-ox-LDL fluorescence intensity in cells incubated with EEP at the indicated concentrations or anti-LOX-1 mAb (4 mg/L) for 1 h and then treated with Dil-ox-LDL (25 mg/L) for 6 h. Data are presented as the mean ± SEM of at least four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle-treated control. (b) Visualization of Dil-ox-LDL uptake in HUVECs using fluorescence microscopy. Red staining denotes Dil-ox-LDL fluorescence, and the blue staining denotes nuclear staining by DAPI (scale bar = 20 μm). (A color version of this figure is available in the online journal)

and protein upregulation induced by ox-LDL. In addition, our results indicated that EEP prevented ox-LDL-induced oxidative stress as reflected by reduced NADPH oxidase activation, ROS and MDA generation as well as increased antioxidant enzyme activities. Similar results were observed in the anti-LOX-1 mAb or DPI-pretreated HUVECs. These data indicate that EEP inhibits ox-LDL-induced endothelial cell injury and proatherogenic effects by suppressing endothelial LOX-1 upregulation and subsequent oxidative stress.

Endothelial cells regulate cardiovascular health and maintain a delicate balance in the vasculature between vasodilation and vasoconstriction, anti-inflammation and pro-inflammation, and also antioxidation and pro-oxidation. Many major risk factors for atherosclerosis such as ox-LDL, ROS, and angiotensin II, can promote endothelial cell injury and apoptosis, and subsequently may contribute to the initiation of atherosclerosis. Thus, endothelial injury has been considered to be an initial event in the pathogenesis of cardiovascular diseases and the key point in the prevention and control of atherosclerosis is inhibiting the excessive injury of vascular endothelial cells.[23] Recently, people are interesting in natural products as a daily supplement to protect against cardiovascular disease and growing evidence has confirmed that the dietary supplement has positive outcomes in preventing atherogenesis through protection of endothelial cells, inhibition of oxidative stress, and inflammatory responses.[24–26] Several observations including our previous studies have revealed that EEP and its flavones can inhibit the inflammatory responses mediated by macrophages and mitigate atherosclerotic development.[15–18] In addition, it is reported that propolis also exhibit inhibitory effects on the oxidative modification of LDL.[27] However, there are no studies exploring the inhibitory effects of EEP on ox-LDL-induced endothelial damage. In the present study, MTT assay and LDH measurement showed that pretreatment with EEP could effectively inhibit the reduced cell viability and the LDH release induced by ox-LDL in a dose-dependent manner. Furthermore, the increased apoptotic rate and caspase-3 activity in ox-LDL-treated cells were remarkably suppressed by EEP pretreatment. These data indicate that EEP is able to inhibit ox-LDL-induced endothelial cell injury.

To elucidate why EEP could inhibit endothelial cell injury so effectively, we next investigated the underlying mechanisms. LOX-1 is a type II membrane glycoprotein that has a C-terminal extracellular C-type lectin-like

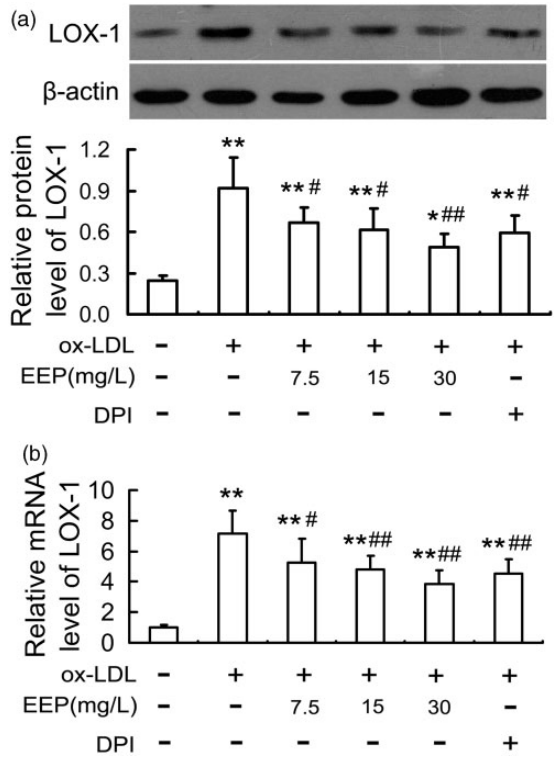

Figure 4 Inhibitory effect of EEP on ox-LDL-induced endothelial LOX-1 expression. HUVECs were pretreated with/without the indicated concentrations of EEP or DPI (5 μmol/L) for 1 h and then stimulated with ox-LDL (100 mg/L) for 24 h. At the end of the incubation, LOX-1 protein (a) and mRNA (b) levels were analyzed by Western blotting and quantitative real-time PCR, respectively. Data are presented as the mean ± SEM of at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle-treated control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus ox-LDL treatment

domain, which is essential for binding to and uptaking ox-LDL by endothelial cells.[28] Accumulating evidence has confirmed that LOX-1 expression is increased in atherosclerotic plaques and its overexpression in endothelium increases plaque formation and promotes atherosclerosis, whereas LOX-1 knockout mice fed a high-cholesterol diet have been reported to show reduced binding of ox-LDL to the aortic endothelium, which preserves endothelial function.[6–8,29,30] In addition, the LOX-1 expressed on the cell surface can be proteolytically cleaved and released in a soluble form (sLOX-1) in the circulation under pathological conditions, and serum levels of sLOX-1 are elevated at the early stages of acute coronary syndrome and are associated with coronary plaque vulnerability. Thus, sLOX-1 might be a diagnostic and prognostic marker for atherosclerotic-related events.[31] LOX-1 activation by ox-LDL has been shown to stimulate endothelial proinflammatory gene expression and production of superoxide radicals,[9,10] promote formation of foam cells, and trigger apoptosis via activation of the proapoptotic agents caspase-9/caspase-3 pathway.[5,32] Many anti-atherosclerotic drugs may exert their atheroprotective effects via direct or indirect down-regulation of LOX-1 expression in vascular lesions.[11,33–35] Therefore, LOX-1 may be a potential therapeutic target for treating atherogenesis leading to beneficial outcome in vasculature. Our current study showed that EEP alleviated the Dil-ox-LDL uptake, which was mimetic to anti-LOX-1 antibody. We also found that ox-LDL induced LOX-1 expression as was reported previously.[34] However, EEP significantly reduced such induction both at the mRNA and protein levels. It suggests EEP can suppress the uptake of ox-LDL by diminishing LOX-1 expression. Previous investigations have demonstrated that LOX-1 activation by ox-LDL induces ROS production and in turn, stimulates LOX-1 expression, suggesting a positive feedback cycle between ROS and LOX-1 expression.[36] In our study, the ROS inhibitor DPI also inhibited ox-LDL-upregulated LOX-1 expression, which indicates that EEP, like DPI, may inhibit LOX-1 expression by reducing intracellular oxidative stress.

Oxidative stress is regarded as a critical mechanism in endothelial cell injury and pathogenesis of atherosclerosis.[37] Extensive reports have demonstrated that oxidative stress induced by ox-LDL is mainly resulted from excessive increase in NADPH oxidase-derived ROS generation and decrease in activities of antioxidative enzymes.[38] NADPH oxidase is the major source of ROS in endothelial cells and the increased NADPH activity has been detected in atherosclerotic arterie,[39] while quercetin, one of the flavonoid in propolis, inhibits vascular superoxide production through suppressing NADPH oxidase activation[40] and resveratrol protected endothelial cells from ox-LDL-induced oxidative damages via the inhibition of NADPH oxidase activity.[41] In addition, SOD and CAT are two major endogenous antioxidant enzymes that protects endothelial cells against the oxidative injury. SOD is able to catalyze O_2^- to form H_2O_2, and then H_2O_2 is eliminated by CAT to water. In the present study, ox-LDL led to a significant increase in NADPH oxidase activity and a decrease in the activities of SOD and CAT with concomitant elevated production of ROS, which are consistent with previous studies.[33,34,41] However, EEP and anti-LOX-1 antibody blocked these effects. Membrane peroxidation leads to changes in membrane fluidity and permeability and promotes protein degradation, which leads to cell injury.[37] Our results showed that the levels of MDA, a lipid peroxidation marker, were markedly elevated in response to ox-LDL stimulation. Similar to anti-LOX-1 antibody and DPI, EEP pretreatment protected endothelial cells from ox-LDL-induced lipid peroxidation. These data suggest that the protective effect of EEP on HUVECs may be involved in blocking LOX-1-mediated oxidative stress response.

In conclusion, this study showed for the first time that EEP protected HUVECs from ox-LDL-induced injury through the inhibition of LOX-1 upregulation and subsequent oxidative stress *in vitro*. These results might explain the diverse physiological activities of EEP and add to a growing body of evidence that EEP has beneficial effects on atherosclerosis-related diseases.

Author contributions: YF carried out the study design, data collection and analysis, and drafted the manuscript. JL helped to carry out the caspase-3 activity and cell viability assay. MD and JZ helped to prepare ox-LDL and determine LDH, MDA, and antioxidant enzyme activities. XX and PH

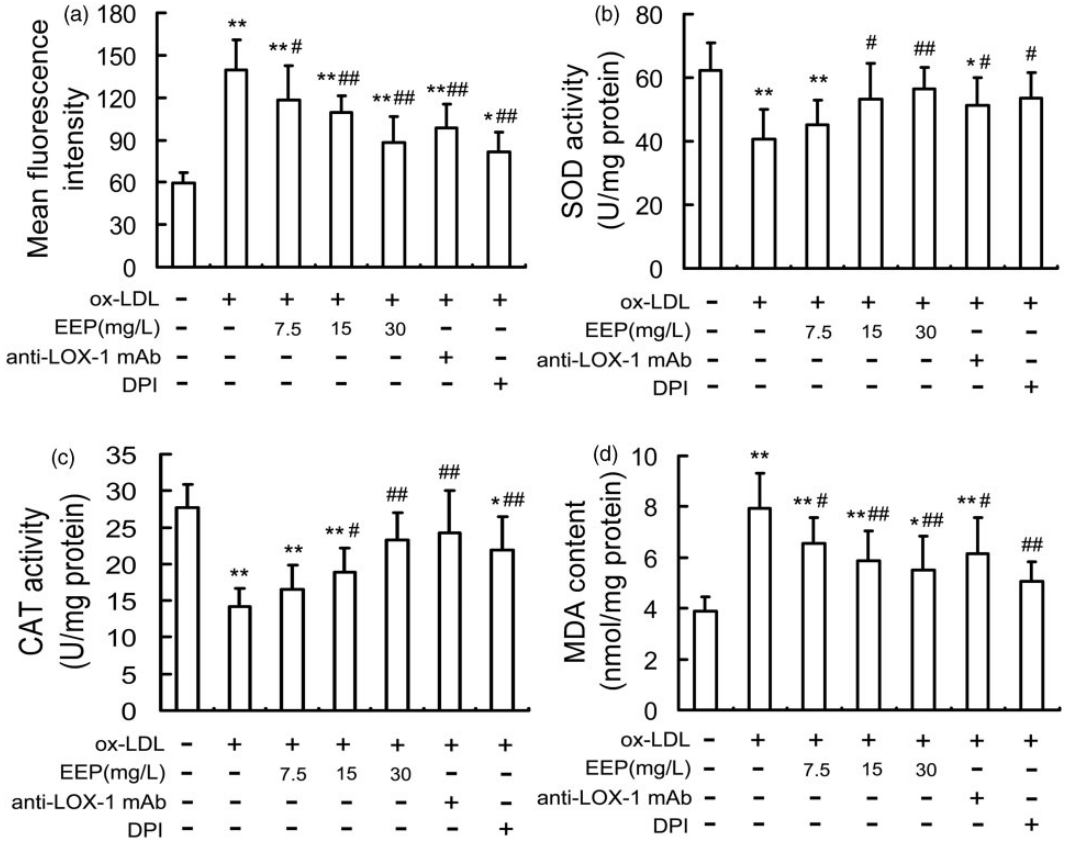

Figure 5 Inhibitory effects of EEP on ox-LDL-induced oxidative stress in HUVECs. (a) Cells were pretreated with or without the indicated concentrations of EEP, anti-LOX-1 mAb (4 mg/L,) or DPI (5 μmol/L) for 1 h, followed by stimulation with ox-LDL (100 mg/L) for 4 h, and then intracellular ROS levels were measured by DCF analysis using a flow cytometer. (b–d) Effects of EEP on the activities of SOD and CAT and the production of MDA in HUVECs pretreated with or without the indicated concentrations of EEP, anti-LOX-1 mAb (4 mg/L), or DPI (5 μmol/L) for 1 h and then stimulated with ox-LDL (100 mg/L) for 24 h. Data are presented as the mean ± SEM of at least four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle-treated control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus ox-LDL treatment

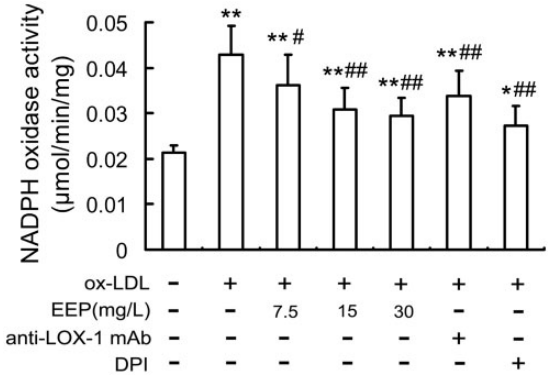

Figure 6 Effect of EEP on NADPH oxidase activity in the treated HUVECs. Cells were pretreated with or without the indicated concentrations of EEP, anti-LOX-1 mAb (4 mg/L) or DPI (5 μmol/L) for 1 h, followed by stimulation with ox-LDL (100 mg/L) for 4 h, and then NADPH oxidative activity was determined by lucigenin chemiluminescence. Data are presented as the mean ± SEM of six independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle-treated control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus ox-LDL treatment

helped to carry out cell culture, Western blot, and quantitative real-time PCR analysis. PJ helped to perform flow cytometry analysis. JW participated in the study design. SY was responsible for the study design, the funding, the data analysis, and the manuscript draft. All authors read and approved the final manuscript.

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