Inhibitory effects of oleoylethanolamide (OEA) on \( \text{H}_2\text{O}_2 \)-induced human umbilical vein endothelial cell (HUVEC) injury and apolipoprotein E knockout (ApoE-/-) atherosclerotic mice

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Abstract: Atherosclerosis (AS) is initiated by vascular endothelial cell injury, which is induced by lipid and protein oxidation. Oleoylethanolamide (OEA), a dietary fat-derived lipid, has shown atheroprotective effect. In vitro studies demonstrated that OEA showed cytoprotective effects on \( \text{H}_2\text{O}_2 \)-induced primary cultured human umbilical vein endothelial cell (HUVEC) injury model. Further investigation of the cytoprotective effects of OEA demonstrated that OEA exerted its function by scavenging for reactive oxygen species, as well as increasing anti-oxidative enzymes, reducing lipid peroxidation, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)-positive cells and apoptosis-related proteins expression. The in vivo study using an ApoE-/- mouse model fed with high-fat diet for 8 weeks showed that OEA (10 mg/kg/day, i.g.) administration reduced blood lipid levels, prevented endothelial cell damage and inhibited early AS plaque formation. In conclusion, our results suggested that OEA exerted a pharmacological effect on ameliorating atherosclerotic plaque formation through the inhibition of oxidative stress-induced endothelial cell injury and therefore OEA can be a potential candidate drug for anti-atherosclerosis.

Keywords: Atherosclerosis, endothelial cell, oxidative stress, apoptosis, OEA

Introduction

Atherosclerosis (AS) is the leading cause of coronary heart disease for its high morbidity and mortality in western developed countries [1]. Vascular endothelial cell injury is the initial event in the development of AS, which is frequently linked with endothelial dysfunction [2]. Endothelial dysfunction would trigger numbers of pathophysiologic processes, such as macrophage aggregation, vascular smooth muscle cell proliferation and ultimate atheromatous plaque formation [3, 4]. Thus, how to protect endothelial cell injury is an effective method to prevent AS.

Oxidative stress, because of a broken scale between antioxidants and pro-oxidants, is the key incentive in the development of AS [5]. The excessive reactive oxygen species (ROS) increases endothelial cell apoptosis, which is the main cause of endothelial injury [6]. The integrity of the endothelial monolayer was destroyed by endothelial cell apoptosis, leading to the degeneration of vascular structures and increasing endothelial permeability [7]. Activation of caspase-mediated mitochondrial pathway has been notably involved in the pathogenesis of most ROS-induced endothelial cell injury [8]. Therefore, antioxidants that preferentially remove ROS may have therapeutic applications in ROS-induced endothelial injury.

Oleoylethanolamide (OEA), a natural occurring lipid and agonist of peroxisome proliferator-activated receptor alpha (PPAR-α), antiobesity and has atheroprotective effects through the inhibition of low density lipoprotein (LDL) modification in vascular system [9-12]. In spite of many papers have devoted to the exploration of OEA on AS, however, the protect role of OEA on endothelial cell injury induced by oxidative stress still remain largely unknown.

In present study, we investigated the effect of OEA on \( \text{H}_2\text{O}_2 \)-induced HUVEC injury and atherosclerosis development in high fat diet (HFD)-induced ApoE-/- atherosclerosis mice. Our data
showed that OEA could prevent H$_2$O$_2$-induced HUVEC injury and ameliorate atherosclerotic plaque formation. Thus, OEA may be a potential candidate for anti-atherosclerosis drug.

**Materials and methods**

**Reagents**

Oleylethanolamide (≥ 98%), H$_2$O$_2$, dimethylsulfoxide (DMSO), oil red O were obtained from Sigma-Aldrich (St. Louis, MO, USA). The cell counting kit-8 was obtained from Dojindo Laboratory (Japan). M-200 medium and low-serum growth supplements were obtained from Invitrogen Corporation (New York, NY, USA). The kits for determining superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-px) activity were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Triacylglycerol (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) commercial kits were purchased from Zhongsheng Bio-tech Co., Ltd. (Beijing, China). The enzyme-linked immunosorbent assay (ELISA) kits of human 4-hydroxynonenal (HNE), mouse-soluble intercellular adhesion molecule-1 (sICAM-1), mouse-monocyte chemotactic protein 1 (MCP-1), mouse C-reactive protein (CRP) were obtained from R&D Systems Inc. (Minneapolis, MN, USA). The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Caspase-3 activity and ROS fluorometric assay kits were purchased from Biovision Inc. (Palo Alto, CA, USA). All primary and secondary antibodies labeled with horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

**Cell culture and treatment**

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh human umbilical veins using 0.1% collagenase I. The neonate cords were donated by the Maternal and Child Care Service Centre in our hospital. The M-200 medium with low-serum growth supplements, penicillin (100 U/ml), and streptomycin (100 μg/ml) were used for the HUVEC culture. The HUVECs were treated with OEA (100 μM) for 8 h before testing for the presence of 100 μM H$_2$O$_2$ (24 h).

**Cell proliferation assay**

Cell viability analyzed by a Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Shanghai, China). The HUVECs were incubated on 96-well plates at a density of 8,000 cells per well and cultured for 24 hours. Cell media were removed; the cells were incubated with tetrazolium reagent for 1 hour and then washed with PBS. Colorimetric dye was tested at wavelength 450 nm in spectrophotometer. The data were obtained from three independent experiments.

**Measurement of CAT, 4-HNE and GSH-px levels and SOD activity**

The HUVEC cells were cultured in six-well plates at 3 × 10$^5$ cells/well. The supernatant and cells were collected respectively after the indicated
treatments for detecting CAT, 4-HNE and MDA levels as well as SOD activity using the corresponding detection kits according to the manufacturer's brochures.

**Detection of intracellular ROS production**

The effect of OEA on intracellular ROS levels was tested using the total ROS detection kit according to the manufacturer's instructions (Enzo Life Sciences Inc., Farmingdale, NY, USA) as previously described [13]. Briefly, following drug treatment, the HUVEC cells were harvested and washed with 1 × washing buffer, and then the cells were incubated with 100 μl of 5-(and-6)-carboxy-2, 7-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) (25 μM final concentration) in darkness at 37°C for 30 min. Cellular DCF fluorescence intensity was determined through microplate reader with excitation wavelength of 495 nm and emission wavelength of 529 nm. The ROS level was expressed as a percentage of the control.

**TUNEL assay**

The DNA fragmentation was tested by the TUNEL kit. Briefly, HUVEC cells were cultured on cover slips overnight. After exposure to 100 μM H₂O₂ for 24 h, cells were fixed by incubation in 4% neutral buffered formalin solution for 30 min at room temperature. HUVECs were incubated with a 0.3% H₂O₂ methanol solution for 15 min at room temperature to inactivate endogenous peroxidase activity. Then, cells were treated with a permeabilizing solution (0.1% sodium citrate and 0.1% Triton X-100) at 4°C for 1 min. Subsequently, cells were incubated in the TUNEL reaction mixture at 37°C for 60 min and visualized by fluorescence microscopy (DM4000B, Leica, Wetzlar, Germany). The apoptotic cells were counted in at least 100 cells from five randomly selected fields in each sample, and counts were expressed as a percentage of the total number of cells.

**Analysis of caspase-3 activation**

Caspase-3 activation was detected using a fluorescein active caspase-3 staining kit (BioVision). HUVECs (1 × 10⁶ cells/mL) were resuspended in 50 μL of chilled lysis buffer and incubated on ice for 10 min. Approximately 50 μL of 2 × reaction buffer (containing 10 mM dithiothreitol, DTT) and 5 μL of 1 mM DEVD-AFC substrate was added to each sample, which was then incubated at 37°C for 2 h. Fluorescence was tested on a microplate reader (Spectrafluor, TECAN, Sunrise, Austria) with an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

**Western blot**

Protein levels of Bax, Bcl-2, caspase-3 were determined in the HUVECs. Cytoplasmic extracts were obtained by lysing the cells in lysis buffer containing 1% protease inhibitors. The cell lysates were centrifuged at 13,000 rpm for 15 min at 4°C, and the supernatant was obtained. Protein content was detected using a bicinchoninic acid assay. Equal amounts of the protein (30 μg) were separated on 10% sodium dodecyl sulfate polyacrylamide gel and
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A

B

Control

H₂O₂

H₂O₂+OEA(100μM)

OEA(100μM)

PI

TUNEL

C

D

E

Bcl-2

Bax

Caspase-3

β-actin

TUNEL Apoptotic Index (%)

control

H₂O₂

H₂O₂+OEA

OEA

control

H₂O₂

H₂O₂+OEA

OEA

Protein level (% of control)

Bax

caspase-3

Bcl-2

transferred onto nitrocellulose membranes. These membranes were blocked with 3% bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 2 h at room temperature. The membranes were incubated overnight with primary antibodies at 4°C, washed with TBST, incubated with HRP-conjugated IgG at room temperature for 1 h, washed in TBST, and the membranes were visualized by enhanced chemiluminescence. Band intensity was measured and quantified.

**Animals**

Male C57BL/6N and ApoE-/- mice (6 weeks old) were provided by the Experimental Animal Center of Medical Department of Peking University. All animal care procedures were executed in accordance with the Guidelines and Policies for Animal Surgery provided by our institute (Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China) and were permitted by the Institutional Animal Use and Care Committee as previously reported [14]. The mice were maintained in a temperature-controlled facility (temperature: 22°C ± 1°C, humidity: 60%) with a 14 h light/10 h dark photoperiod and free access to food and water. Ten male C57BL/6N mice comprised the control group (n = 10). Twenty male ApoE-/- mice were divided randomly into a model group (n = 10), OEA group (n = 10). All mice were fed a high fat diet (containing 0.3% cholesterol and 20% fat) for 8 weeks. The animals in the OEA group were orally administered OEA (10 mg/kg/day, i.g.) constantly for 8 weeks, whereas the other groups were given the same volume of vehicle. The mice were fasted for 4 h at the start and end period of the experiment and blood samples were collected from their inner canthus for serum preparation. The serum was stored at -80°C prior to analysis.

**Levels of lipids and inflammatory factors in the serum of mice**

At the end of the experiment, the serum LDL-C, TC, TG and HDL-C were detected by a Hitachi 7600 Automatic Biochemistry Analyzer (Tokyo, Japan) and serum inflammatory factors CRP, sICAM-1, MCP-1 were measured by ELISA assays according to the brochures.

**Oil red O staining**

The atherosclerotic plaque area in each aortic section was assessed under oil red O staining using Image-Pro Plus (Media Cybernetics Inc., Silver Spring, MD). Briefly, hearts were embedded in Tissue-Tek OCT compound at -20°C, and sections were obtained through the aorta root. Subsequently, frozen sections were immersed in 60% isopropanol and stained in oil red O for 55 min at 60°C. Then, the sections were washed by deionized water and cell nucleus was stained by hematoxylin. To evaluate the seriousness of the lesions, the ratio of the oil red O-positive area was compared with the whole vessel area, including the lumen, intima, media, and adventitia, as previously described [15]. Every five section from each animal was examined, and the mean fraction area was calculated and expressed as a percentage.

**Assessment of EC apoptosis in mice aortae**

DNA fragmentation in the ECs was measured using the TUNEL staining kit according to the manufacturer’s instructions. Frozen sections of the aortae from the ApoE-/- mice were maintained for 15 min at room temperature and washed with PBS to remove the OCT compound. The TUNEL reaction mixture was added to the sections, which were subsequently incubated for 1 h at 37°C in darkness. After incubation, the cells were washed twice for 5 min in PBS.

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**Figure 3.** OEA treatment reverses H_2O_2-induced lipid peroxidation and cellular antioxidant potential descent and inhibits H_2O_2-induced HUVEC apoptosis. A. Effects of OEA on the production of SOD, GSH-px, CAT, 4-HNE activity. B. Effect of OEA on H_2O_2-induced HUVEC apoptosis by TUNEL staining. C. Statistic data of TUNEL-positive cells. D. Apoptosis related proteins expression detected by western blot. E. Statistic data of Bax, Bcl-2, caspase-3 expression compared with control group. Values (n = 3 per group) are expressed as means ± S.E. *P < 0.05 compared with control group; **P < 0.01 compared with H_2O_2-treated group.
Figure 4. OEA reduces the atherosclerotic plaque in the aorta of ApoE-/- mice. A. Levels of CRP, MCP-1, sICAM-1 expression measured by ELISA in mice at 8 weeks of the administration. B. Atherosclerotic lesion formation under Oil Red O staining in mice at 8 weeks of the administration. C. Statistical data of lipid accumulation in the aortic root. D. Blood lipid levels of serum in mice at 8 weeks of the administration. Values (n = 10 per group) are expressed as means ± S.E. **P < 0.01 compared with C57 control group; *P < 0.05, ***P < 0.01, compared with vehicle treated ApoE-/- model group.
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and stained with DAPI solution. After rinsing twice for 5 min in PBS, the samples were observed under a fluorescence microscope (Leica DM4000, Germany). The ratio of TUNEL-positive cells was calculated according to the previous method [14].

Immunofluorescence

Endothelial cell caspase-3 deposition in the aortic arch was measured through immunofluorescence. Briefly, the aorta sections from the ApoE-/- mice were incubated overnight at 4°C with anti-caspase-3 rabbit polyclonal antibodies (1:100). After rinsing, the sections were incubated with anti-rabbit FITC antibodies (1:100) for 45 min at room temperature and then incubated with DAPI for 5 min. Then the sections were mounted with the fluorescence quenching agent and the samples were observed under a fluorescence microscope (Leica DM4000, Germany). Caspase-3 expression was quantified using Image-Pro Plus 6.0.

Statistical analysis

Results are expressed as mean ± S.E. Differences between the groups were analyzed using one-way ANOVA, followed by a t test. Differences with \( P < 0.05 \) were considered statistically significant.

Results

**OEA protects against \( H_2O_2 \)-induced cytotoxicity in HUVEC**

Firstly, in order to obtain the optimized oxidative stress conditions, we investigate HUVECs treated for 6, 12, 24, and 48 h with different \( H_2O_2 \) concentrations. Cell viabilities were tested using CCK-8 assay. As shown in **Figure 1A**, a dose and time-dependent increase of cytotoxicity on HUVECs was observed in response to \( H_2O_2 \). Cell viability was reduced to 50.2% ± 2.5% at 100 μM \( H_2O_2 \) treated for 24 h, which was the concentration selected for the next experiments. To assess the cytoprotective effect of OEA, HUVECs were pretreated with different concentrations (25, 50, 100 μM) of OEA for 8 h, followed by 24 h of 100 μM \( H_2O_2 \) treatment. As shown in **Figure 1B**, to our excitement, OEA pretreatment groups showed a significant increase in cell viability, the cell viability from 49.9% ± 2.2% up to 70.9% ± 7.3% at 100 μM, which indicates that OEA pretreatment protects against \( H_2O_2 \)-induced cell damage. To exclude the proliferative and toxic effects of OEA, we also evaluated the OEA treatment alone on the cell viability level. As shown in **Figure 1C**, treatment with OEA 8 h did not significantly influence cell proliferation and survival, therefore, OEA treatment alone does not significantly influence cell viability.

**OEA decreases intracellular ROS level and caspase-3 activation**

Oxidative stress may lead to the up-regulation of ROS in cardiovascular diseases [3]. As **Figure 4** shown, OEA pretreatment can significantly decrease ROS level compared with \( H_2O_2 \) treated group. The activation of the caspase-3 is crucial in the initiation of apoptosis in diverse biological processes. Our results show that 100 μM \( H_2O_2 \) significantly increased the caspase-3 activity, whereas the addition of OEA to the culture system significantly suppressed \( H_2O_2 \)-induced caspase-3 activation in **Figure 4**.

**OEA treatment reverses \( H_2O_2 \)-induced lipid peroxidation and cellular antioxidant potential descent**

One of the primary events in oxidative damage is the membrane lipid oxidation, which can be determined by its degradation products 4-HNE [16]. As shown in **Figure 2A**, treatment with 100 μM \( H_2O_2 \) notably increased the intracellular 4-HNE levels, whereas significantly decreased the antioxidant enzymes CAT, SOD, and GSH-Px activity. However, pretreatment with different concentrations of OEA markedly decreased the 4-HNE levels and increased the antioxidant enzymes activity. These results were coincident with the serum level of inflammatory factors, CRP, MCP-1, sICAM-1 in vivo as shown in **Figure 3A**. The aforementioned results showed that OEA can increase the antioxidative ability of the damaged endothelial cells.

**OEA inhibits \( H_2O_2 \)-induced HUVEC apoptosis**

Apoptosis in HUVEC was detected using TUNEL staining. As shown in **Figure 2B** and **2C**, treatment with 100 μM \( H_2O_2 \) for 24 h obviously increased the number of apoptotic cells. However, pretreatment with 100 μM OEA for 8 h reduced the TUNEL-positive cells. Next, we
investigated the effects of OEA on apoptosis-related proteins expression. As shown in Figure 2, H$_2$O$_2$ significantly increased the pro-apoptosis proteins Bax and caspase-3 expression as well as decreased the anti-apoptosis protein Bcl-2 expression. However, treatment with OEA significantly reversed the H$_2$O$_2$ injury.

**OEA reduces the atherosclerotic plaque in the aorta of ApoE/- mice**

To assess the cytoprotective function of OEA in vivo, we established an experimental ApoE/-AS mice model following the method previously described [14, 15]. After 8 weeks of OEA (10
mg/kg) administration, we initially measured the effect of OEA on serum lipids LDL-C, HDL-C, TC and TG levels. As shown in Figure 3D, the serum lipid levels in ApoE-/- model group were remarkably higher than those in C57BL/6N control group and OEA administration group could reduce the lipid levels. We also evaluated the AS plaque formation in the experimental ApoE-/- mice. As shown in Figure 3B and 3C, OEA (10 mg/kg) remarkably reduced the oil red O positive plaque area in the experimental ApoE-/- mice compared with the C57 group. These results showed that OEA treatment may inhibit atherosclerotic plaque formation in the aorta of ApoE-/- mice.

OEA protects against endothelial damage in the aorta of ApoE-/- mice

(Figure 5) Excessive ROS levels causing oxidative injury play a crucial role in the pathogenesis of AS [17]. To assess the cytoprotective function of OEA in vivo, we established an experimental AS mice model as the method described previously [18]. As shown in Figure 4A and 4C, the ratio of apoptotic EC in the aortic arch vessel was significantly decreased in the OEA treatment group compared with the model group. We also evaluated caspase-3 activation. As shown in Figure 4B and 4D, OEA treatment remarkably decreased the activated caspase-3 expression compared with the ApoE-/- model mice. These results suggest that OEA treatment in vivo inhibits AS development.

Discussion

Endothelial dysfunction and cellular injury that caused by oxidative stress play crucial roles in the development of AS [19]. In the normal conditions, ROS levels in the plasma are maintained in low levels by reacting with heme proteins [20]. However, under pathologic conditions, such as hypertension, hyperlipidemia, obesity, and diabetes could cause an increase of ROS levels [21]. Also, the increased ROS levels may induce endothelial cell apoptosis from oxidative stress [22]. Therefore, protecting endothelial cells from ROS-induced damage is an effective treatment for the AS.

OEA, a naturally occurring lipid, has many pharmacological activities, anti-obesity [12, 23], neuroprotection [24], and anti-atherosclerosis [25]. Fan and his colleagues referred that OEA played a key role in AD lesion development via targeting oxidized LDL [9]. In this study, we investigated the effects of OEA on H$_2$O$_2$-induced endothelial cell injury in vitro and high-fat diet-induced ApoE-/- AS mice in vivo. After exposed to H$_2$O$_2$, HUVEC exhibited high oxidative stress injury, such as increased ROS level, caspase-3 activation, up-regulation of oxidative enzymes and down-regulation of anti-oxidative enzymes. Furthermore, H$_2$O$_2$ group showed a more TUNEL-positive cells. However, pretreatment with OEA may reverse the H$_2$O$_2$-induced aforementioned effects. AS is considered as a chronic inflammatory disease and inflammation is also characterized by excessive ROS production [26]. So, these results suggested that the protective effect of OEA against oxidative stress induced endothelial cell injury may be due to its ability to inhibit ROS production. Excessive ROS may cause cell apoptosis. So, we also evaluated the effects of OEA on apoptosis-related proteins expression in H$_2$O$_2$-induced HUVEC damage. In accordance with the result of cytoprotective effects, OEA treatment can increase the anti-apoptosis protein Bcl-2 expression, whereas decrease the pro-apoptosis proteins expression, such as Bax and caspase-3. We also confirmed the protective effects of OEA against endothelial cell damage in vivo. OEA administration can significantly decrease endothelial cell caspase-3 activation and apoptosis in the aorta of ApoE-/- mice.

Previous studies revealed that the increasing of blood lipid levels is the characteristic of AS [27, 28]. Lopez and his colleagues have demonstrated that blood lipid levels was associated with atrial fibrillation, which was a risk factor for AS [29]. In this study, to assess the effect of OEA on HFD-induced ApoE-/- AS mice, we measured the lipid accumulation in the aortic root by oil red O. Our results showed that the blood lipid level of mice in OEA treatment group could be remarkably decreased by OEA compared with that in the model group, indicating that OEA may performed an important inhibition role on AS. Meanwhile, the cytokines level in the plasma were also measured, our results displayed that OEA administration group could decrease the MCP-1, CRP, sICAM-1 levels, which was consistent with the in vitro experiment.
In conclusion, our study attempts to investigate the potential role of OEA on H₂O₂-induced HUVEC injury and apolipoprotein E knockout (Apoe⁻/⁻) AS mice. OEA may play an important role in protecting HUVECs against H₂O₂-induced apoptosis and ameliorates AS plaque formation. The underlying mechanism is related to its antioxidant activity. AS, a chronic inflammatory procession, is initiated by EC dysfunction [30]. Our study may provide evidence that OEA treatment may be a potential drug for treating AS. However, further experimental studies are still needed to explore the molecular mechanism of OEA on protecting AS induced by oxidase stress.

Disclosure of conflict of interest

None.

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