RESEARCH PAPER

Aspirin eugenol ester attenuates oxidative injury of vascular endothelial cells by regulating NOS and Nrf2 signalling pathways

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Background and Purpose: Aspirin eugenol ester (AEE) is a new drug compound synthesized by combining aspirin with eugenol. It was reported to possess anti-thrombotic, anti-atherosclerotic, and anti-oxidative effects. However, its molecular mechanism against oxidative injury is unclear. This study investigated how AEE affected the oxidative injury of vascular endothelial cells in vivo and in vitro.

Experimental Approach: A hamster model of atherosclerosis induced by a high fat diet (HFD) and an in vitro model of oxidative stress, H₂O₂-induced apoptosis of HUVECs, were used to investigate the anti-oxidative effects of AEE.

Key Results: AEE significantly reduced the stimulatory effect of HFD on malondialdehyde, the inhibitory effect of HFD on SOD activity and GSH/GSSG ratio, and the overexpression of inducible NOS (iNOS) in the aorta. In vitro, incubation of HUVECs with H₂O₂ led their apoptosis, dysfunctions of the NO systems (including increased iNOS activity, decreased endothelial NOS activity, and increased production of NO), an imbalance in calcium homeostasis and energy metabolism with an increase in intracellular free calcium and decrease in ATP, and a down-regulation of Nrf2. In contrast, in the HUVECs pretreated with 1 µM AEE for 24 hr, the above adverse effects induced by H₂O₂ were significantly ameliorated. Moreover, the decrease in NO production and activity of iNOS induced by AEE was significantly attenuated in Nrf2-inhibited HUVECs.

Conclusion and Implication: AEE protects vascular endothelial cells from oxidative injury by regulating NOS and Nrf2 signalling pathways. This suggests that AEE is a novel potential agent for the prevention of atherosclerosis.

1 | INTRODUCTION

Aspirin eugenol ester (AEE) is synthesized by combining aspirin with eugenol based on the prodrug principal (Li et al., 2012). Pharmacological and pharmacodynamic studies showed that AEE has significantly reduced side effects and enhanced pharmacological activity as an anti-thrombus,

anti-atherosclerosis, and anti-oxidant, compared with either [aspirin](#) or [eugenol](#) alone (Karam et al., 2015; Karam et al., 2016; Li et al., 2011; Ma et al., 2015; Ma et al., 2016; Ma, Yang, Liu, Yang, et al., 2017; Ye et al., 2011). However, the molecular mechanisms through which AEE inhibits atherosclerosis, thrombus, and oxidative stress are unclear. A metabolomic analysis in high fat diet (HFD)-induced atherosclerotic hamsters and AEE-treated hamsters suggested that AEE protects the aorta from injury, and this suggests it has an effect on oxidative stress (Ma, Yang, Liu, Kong, et al., 2017). Oxidative stress is a well-known cause of

Abbreviations: AEE, aspirin eugenol ester; eNOS, endothelial NOS; HFD, high fat diet; iNOS, inducible NOS; MDA, malondialdehyde; Nrf2, nuclear factor (erythroid-derived 2)-like 2; SERCA, sarco/endoplasmic reticulum Ca²⁺-ATPase

cardiovascular diseases. It well-documented that oxidative stress is responsible for cardiovascular endothelial dysfunction, the development of thrombus, and atherosclerosis (Heitzer, Schlinzig, Krohn, Meinertz, & Munzel, 2001; Incalza et al., 2018; Rocha, Apostolova, Hernandez-Mijares, Herance, & Victor, 2010). Moreover, many studies have shown that the effects of many drugs and active ingredients on cardiovascular diseases are related to their antioxidant activity.

Recently, many oxidative stress models were established in vitro and in vivo to elucidate the process of cardiovascular disease. The atherosclerosis model in Syrian golden hamsters induced by HFD is widely used to study the mechanism of thrombosis development and treatment, which is beneficial for the development, design, and screening of anti-atherosclerotic drugs (Dillard, Matthan, & Lichtenstein, 2010; Romain et al., 2012; Yamanouchi et al., 2000). Due to the complex regulation of systems in this organism, it is difficult to clarify the antioxidant mechanism of AEE. Therefore, cellular models are needed to further elucidate the mechanism of AEE in vitro.

Since the oxidative stress induced in HUVECs by H_2O_2 is a useful and sensitive model of this condition, it has been widely used to assess cardiovascular oxidative damage in vitro (Chen et al., 2016; Kaczara, Sarna, & Burke, 2010; Sohel et al., 2016; Wijeratne, Cuppett, & Schlegel, 2005). The development of oxidative stress in an organism and cell involves complex molecular mechanisms. NO plays an important role in oxidative stress based on its concentration and biological micro-environment (Bredt, 1999; Palmer, Ferrige, & Moncada, 1987; Zhang et al., 2017). NO can protect against oxidative stress at a physiological concentration, while excessive NO and NO derivatives generated by **inducible NOS (iNOS)** and **endothelial NOS (eNOS)** cause oxidative stress of cells, manifest as energy metabolism imbalance, dysfunction of calcium homeostasis, and apoptosis (Adachi, 2010; Beckman & Koppenol, 1996; Chen, Zhao, Zhang, Wu, & Qi, 2012). Many reports suggest that some drugs reduce the oxidative stress by affecting the activity or levels of eNOS and iNOS (Chen et al., 2012; Xu et al., 2010). It is still unclear whether the effect of AEE on oxidative stress is mediated by an effect on iNOS and eNOS.

In this study, the antioxidant effect of AEE was investigated in the HFD-induced hamster model of atherosclerosis and the H_2O_2 -induced HUVEC model of oxidative stress. The mechanism through which AEE protects cells from oxidative stress was investigated on the basis of NOS. This study provides important information for the identification of novel potential candidates for treating cardiovascular diseases.

2 | METHODS

2.1 | Sample collection

The atherosclerosis model of Syrian golden hamsters was developed, and the treatments were prepared following the methods described in our previous studies (Ma, Yang, Liu, Kong, et al., 2017). In brief, All hamsters were housed in facilities by group at a controlled relative humidity (45–65%), 12 h light/12 h dark cycle and temperature ($22 \pm 2^\circ\text{C}$). Hamsters were assigned to three groups ($n = 10$): (a) normal

What is already known

- The oxidative injury of vascular endothelial cells could cause atherosclerosis.

What this study adds

- In the study, it was proved that AEE protected vascular endothelial cells from oxidative injury by regulating NOS and Nrf2 signalling pathways.

What is the clinical significance

- This suggests that AEE is a novel potential agent for the prevention of atherosclerosis.

group (the hamsters were fed a normal diet); (b) HFD group (the hamsters were fed a HFD); (c) AEE-treated HFD-fed group (the hamsters were simultaneously fed a HFD with AEE $27 \text{ mg}\cdot\text{kg}^{-1}$ body weight). Blood samples were collected from the heart into heparin-treated vacuum tubes. All hamsters were sacrificed under anesthesia induced with phenobarbital (intraperitoneal injection, $30 \text{ mg}\cdot\text{kg}^{-1}$). Plasma was obtained after centrifugation of blood at $1,000\times g$ and 4°C for 10 min and stored at -80°C until analysis. The aortas were carefully isolated from the hamster and fixed in 4% formalin for pathological observations. All experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee of Lanzhou Institute of Husbandry and Pharmaceutical Science of Chinese Academy of Agricultural Sciences (approval no. NKMYD201601). Animal welfare regulation was complied with, and experimental procedures were performed strictly in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by the U.S. National Institutes of Health. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010) and with the recommendations made by the *British Journal of Pharmacology*.

2.2 | Oxidation and antioxidant markers in plasma

The concentrations of malondialdehyde (MDA), SOD activity, and GSH/GSSG ratio were assessed using commercial kits according to the manufacturer's protocols.

2.3 | Cell culture and treatments

HUVECs (ATCC® Cat# CRL-4053™, RRID:CVCL_9Q53) purchased from ATCC (Rockville, MD) were cultured on cell culture flasks in DMEM/F12 (1:1) medium with 10% FBS. The media were refreshed once every 2 days. Subcultures were performed with trypsin-EDTA. Experiments were subsequently conducted on passage 6–7 cells.

HUVECs were randomly divided into three groups ($n = 6$): normal group, model group, and AEE pretreatment group. Cells in the normal group were incubated with the growth conditions. Dose-response studies with H_2O_2 were carried out to optimize the model. The model

group was incubated for 22 hr with the medium containing 200 μM H_2O_2 . In the AEE pretreatment groups, the cells were pre-incubated for 24 hr with culture medium containing 1 μM AEE, and then incubated with the medium containing 200 μM H_2O_2 for 22 hr.

2.4 | Cell viability measurement

Cells were seeded at a density of 5×10^4 cells ml^{-1} in 96-well plates, and the cell viability was tested using CCK-8. Briefly, CCK-8 was added in culture medium at 37°C for 1 hr. The absorption values were measured at 450 nm using an Enspire Microplate Reader (PerkinElmer, Germany). The viability of HUVECs in each well was presented as percentage of control cells.

2.5 | Transduction of Nrf2 to HUVECs

The GFP lentivirus containing Nrf2 cDNA or control lentivirus was used to transduce HUVECs for overexpression tests. In brief, the cells were plated in 6-well cell culture plates. After 24 hr, transfections were carried out using a lentivirus expressing Nrf2 gene labelled with green fluorescent proteins (GFPs). Transduction efficiency was determined by the measurement of GFP positive cells using fluorescence microscopy. Then, the transfected cells were incubated for 22 hr in the medium containing 200 μM H_2O_2 .

The lentivirus labelled with ubiquitin IRES-puromycin containing short interference (si) RNA oligonucleotides against Nrf2 (siNrf2) or control siRNA was used to transfect HUVECs for knock-down experiments.

2.6 | Measurement of intracellular NO

DAX-J2 Red probe was used to examine intracellular NO. Briefly, the HUVECs or Nrf2 overexpressing HUVECs were cultured on 24-well glass bottom cell culture plates and treated with AEE and H_2O_2 . To verify the role of iNOS in the NO generation induced by H_2O_2 , the HUVECs were cultured on the 24-well glass bottom cell culture plates and treated with the iNOS inhibitor S-Methylisothiourea Sulfate (SMT) and H_2O_2 . Then, the cells were incubated with DAX-J2 Red probe work solutions (6 μM) at 37°C for 30 min in the dark. The relative fluorescence intensity of the cells was measured by a laser scanning confocal microscope (ZEISS LSM-800, Jena, German).

2.7 | NOS activity assay

A NOS detection kit was used to assay the total intracellular activity of NOS and activity of iNOS. In brief, the HUVECs were cultured in 96-well black cell culture plates. Then, the cells were incubated with the NOS reaction mixture containing SMT or NOS reaction mixture with L-NAME (eNOS inhibitor) at 37°C for 40 min in the dark to detect total NOS, iNOS, and eNOS activity. The relative fluorescence intensity of the cells was measured by an Enspire Microplate Reader (PerkinElmer), and the blank controls were set up to deduct background fluorescence.

2.8 | Measurement of intracellular tetrahydrobiopterin (BH4)

The intracellular BH4 was detected by the BH4 ELISA kits according to the manufacturer's protocols.

2.9 | Protein expression analysis

The expression of eNOS and p-eNOS was evaluated by Western blot analysis. In brief, the total protein of the HUVECs was isolated using RIPA and quantified using bicinchoninic acid method. SDS-PAGE (10%) and transfer of the separated proteins onto PVDF membrane (Merck Millipore, Billerica, Massachusetts, USA) were performed using standard procedures. The blots were incubated with primary antibodies against eNOS, p-eNOS, and internal control β -actin and then incubated with HRP-conjugated secondary antibody. The results were detected using a BIO-RAD ChemiDoc™ MP imaging system (Alfred Nobel Drive Hercules, California, USA) and were normalized to the corresponding internal control β -actin for eliminating the variations in total protein.

The expression of Nrf2 and iNOS was evaluated by immunofluorescence. Briefly, the cells were cultured on glass coverslips with 4% paraformaldehyde and fixed; 0.1% Triton X-100 (Cayman, USA) permeabilized the cells and they were labelled with a primary antibody against Nrf2 and iNOS. After this, they were incubated with an Alexa Fluor® 488-conjugated secondary antibody (Abcam Cat# ab150077, RRID:AB_2630356) or Alexa Fluor 647-conjugated secondary antibody (Abcam Cat# ab150079, RRID:AB_2722623). The relative fluorescence intensity of the cells was measured by laser scanning confocal microscope (ZEISS LSM-800).

In order to investigate the expression of iNOS in the aorta, the aortas were formalin-fixed and paraffin embedded, sectioned, dewaxed, and antigen-repaired. They were incubated with iNOS primary antibody, HRP-labelled goat anti-rabbit IgG (H + L), and 3,3'-diaminobenzidine tetrahydrochloride using the standard protocol. The results were analysed by ImageJ 1.8.0 software (ImageJ, ver 1.8.0, RRID:SCR_003070; National Institutes of Health, Bethesda, Maryland).

2.10 | Measurement of $[\text{Ca}^{2+}]_i$

The $[\text{Ca}^{2+}]_i$ levels in the HUVECs were determined with Fura-2/AM fluorescence staining. Briefly, the cells were suspended in HBSS (pH 7.2) and incubated at 37°C for 5 min. Then, the samples were treated with Fura-2/AM and incubated at 37°C for 45 min. The suspension was centrifuged at 1,200 $\times$ g and 4°C for 10 min and washed twice using HBSS (pH 7.2). Then, fluorescence value (F) was measured using an Enspire Microplate Reader (PerkinElmer) at an excitation wave length of 340 nm and an emission wave length of 500 nm. The final concentration of 0.1% Triton X-100 was added to the suspension to measure the F_{max} . EDTA (5 mM, pH 7.6; Solarbio, China) was added to the cell suspension containing Triton X-100 to measure the F_{min} . $[\text{Ca}^{2+}]_i$ is calculated according to the following formula (K_d : 224 nM): $[\text{Ca}^{2+}]_i$ (nM) = $K_d \times ((F - F_{\text{min}})/(F_{\text{max}} - F))$.

2.11 | SERCA function assay

Cellular **sarco/endoplasmic reticulum Ca^{2+} -ATPase** (SERCA) function was assayed by a SERCA function detection kit. Briefly, the cells were collected and stained with a Mag-Fluo-AM probe. Then, the stained cells were permeated, and the mitochondrial calcium pump inhibitor (FCCP), $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibitor (**benzamil**), and Mg-ATP were added to the permeated cells. Then, the relative fluorescence value was measured using an Enspire Microplate Reader (PerkinElmer) at an excitation wave length of 490 nm and an emission wave length of 525 nm.

2.12 | ATP production

Cellular **ATP** levels were determined using an enhanced ATP assay kit. The treated HUVECs were lysed with ATP assay lysis buffer. The lysed cells were centrifuged at 4°C and 12,000× g for 5 min, and the supernatant was collected. Before ATP detection, detecting solution was added to a 96-well white plate and incubated at room temperature for 5 min. The supernatant was then added to the plate, mixed quickly, and read within 30 min. Total ATP levels were calculated from the luminescence signals and were normalized to the protein concentrations.

2.13 | Apoptosis detection

2.13.1 | Apoptosis detection by flow cytometry

The apoptosis in the HUVECs or Nrf2 overexpressing HUVECs induced by H_2O_2 was quantified with Annexin V/PE apoptosis detection kit using flow cytometry. Briefly, the cells were collected and washed three times with cold PBS. Next, the cells were incubated with PE-Annexin V and stained with 7-ADD for 20 min at room temperature in the dark. The double stained cells were analysed by flow cytometry, and the following controls were used to set up compensation and quadrants: unstained cells, cells stained with PE-Annexin V, and cells stained with 7-ADD.

2.14 | Hoechst 33342 staining

The cells were cultured on glass coverslips in medium. After being washed twice with ice cold PBS, the cells were fixed with cold 4% paraformaldehyde for 10 min. They were incubated with Hoechst 33342 work solutions at room temperature for 10 min in the dark. The stained cells were examined with a laser scanning confocal microscope (ZEISS LSM-800).

2.15 | Statistical analysis

All experiments and data analysis were performed according the blinding principles. Statistical analysis was carried out using the SAS 9.2 (Statistical Analysis System, ver 9.2, RRID:SCR_008567; SAS Institute Inc., NC, USA). All data are presented as means $\pm$ SD. The differences among groups were analysed with one-way ANOVA. Where significance was achieved, Duncan's multiple comparisons were carried out to identify the sources of differences. Statistical significance was considered at $P < 0.05$. The data and statistical analysis comply

with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology.

2.16 | Chemicals

AEE a transparent crystal with a purity of 99.5% by RE-HPLC was prepared by our group. The lipid peroxidation MDA assay kit, enhanced ATP assay kit, SOD activity assay kit, and GSH/GSSG assay kit were purchased from Beyotime (Shanghai, China). DMEM/F12 (1:1) and FBS were supplied by Gibco (NY, USA). DMSO, NOS detection kit and trypsin-EDTA were supplied by Sigma (St. Louis, MO). The cell counting kit-8 (CKK-8) was bought from MedChemExpress (NJ, USA). Annexin V/PE apoptosis detection kit was from BD Biosciences (NY, USA) and DAX-J2™ Red and Fura-2/AM probe from AAT Bioquest (CA, USA). Sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) function detection kit was from Genmed Scientifics Inc. (Shanghai, China). BH4 (tetrahydrobiopterin) ELISA kit was purchased from Elabscience (Wuhan, China). Anti-eNOS (Abcam Cat# ab5589, RRID:AB_304967) anti-eNOS (phospho S1177; Abcam Cat# ab184154, RRID:AB_2768154), anti-iNOS (Abcam Cat# ab115819, RRID:AB_10898933), and anti-nuclear factor (erythroid-derived 2)-like 2 (Nrf2; Abcam Cat# ab62352, RRID:AB_944418/RRID:AB_944418) were from Abcam (Cambridge, USA). The lentivirus expressing Nrf-2 gene and inhibiting Nrf-2 gene was constructed respectively by Genechem (Shanghai, China). The normal diet (12.3% lipids, 63.3 carbohydrates and 24.4% proteins) was purchased from Keao Xieli Feed Co., Ltd (Beijing, China) and the HFD (40% lipids, 43% carbohydrates and 17% proteins) was supplied by Research Diet, Inc. (product D12079B, New Brunswick, NJ).

2.17 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Fabbro et al., 2017; Alexander, Kelly et al., 2017).

3 | RESULTS

3.1 | AEE enhanced the body's antioxidant capacity

The results for MDA and SOD levels, and GSH/GSSG ratio are shown in Table 1. AEE significantly attenuated the increase in MDA ($P < 0.05$) and prevented the decrease in SOD activity and GSH/GSSG ratio ($P < 0.05$) caused by HFD in Syrian golden hamsters.

3.2 | Effects of AEE on the apoptosis of HUVECs

To verify the effects of H_2O_2 on apoptosis in HUVECs, cells were incubated with H_2O_2 at 10, 20, 40, 80, 120, 200, and 300 μM for 22 hr. As shown in Figure 1a, apoptosis of HUVECs showed a dose-dependent

TABLE 1 Effects of AEE on oxidation/antioxidant markers in Syrian golden hamsters' plasma

Group	SOD (u ml^{-1})	MDA (μM)	GSH (μM)	GSSG (μM)	GSH/GSSG
Normal	285.15 $\pm$ 25.36	2.10 $\pm$ 0.15	4.21 $\pm$ 0.12	1.25 $\pm$ 0.19	3.39 $\pm$ 0.27
Model	149.30 $\pm$ 11.40*	8.48 $\pm$ 0.80*	2.65 $\pm$ 0.10	1.58 $\pm$ 0.12	1.69 $\pm$ 0.16*
AEE	208.9 $\pm$ 7.37**	5.60 $\pm$ 0.60**	3.19 $\pm$ 0.45	1.34 $\pm$ 0.29	2.42 $\pm$ 0.22**

Note. AEE, aspirin eugenol ester; MDA, malondialdehyde.

Normal: The hamsters were fed a normal diet; Model: The hamsters received high fat diet; AEE: The hamsters were simultaneously fed a HDF and AEE at 27 $\text{mg}\cdot\text{kg}^{-1}$ body weight.

* $P < 0.05$, compared with the normal group.

** $P < 0.05$, compared with the model group.

FIGURE 1 The effect of aspirin eugenol ester (AEE) and H_2O_2 on HUVECs. (a) H_2O_2 caused apoptosis of HUVECs. (b) AEE decreased apoptosis of HUVECs induced by H_2O_2 . (c) The changes in endothelial NOS (eNOS) activity with the different concentrations of H_2O_2 . (d) AEE reduced the decrease in eNOS activity induced by H_2O_2 . (e) The changes in inducible NOS (iNOS) activity with the different concentrations of H_2O_2 . (f) AEE reduced the increase in eNOS activity induced by H_2O_2 . Values are expressed as means $\pm$ SD where applicable. $n = 6$. * $P < 0.05$, compared with the normal group; # $P < 0.05$, compared with the H_2O_2 group. r.u.: relative units

relationship with hydrogen peroxide. After exposure to 200 μM of H_2O_2 for 22 hr, the early apoptosis of HUVECs increased significantly. However, pre-incubation of HUVECs with different concentrations of AEE for 24 hr markedly decreased the early cell apoptosis (Figure 1b).

3.3 | The dose-response effect of H_2O_2 on the activity of iNOS and eNOS

The activity of iNOS and eNOS induced by different concentrations of H_2O_2 (10, 20, 40, 80, 120, 200, and 300 μM) was detected in the HUVECs. The results are shown in Figure 1c,e. Low-dose H_2O_2

(20–40 μM) significantly increased the activity of eNOS, while high-dose H_2O_2 (>120 μM) significantly decreased the activity of eNOS. There was no significant effect on the activity of iNOS induced by low-dose H_2O_2 . High-dose H_2O_2 significantly increased the activity of iNOS.

3.4 | The dose-response for the effect of AEE on the activity of iNOS and eNOS

To verify the effects of AEE on the activity of iNOS and eNOS in HUVECs, cells were incubated with AEE at 0.5, 1.0, 2.0, and 4.0 μM for 24 hr in the absence of H_2O_2 . As shown in Figure 1d, different

concentrations of AEE did not cause a significant change in eNOS and iNOS activity in HUVECs. Treatment of HUVECs with 200 μM H_2O_2 for 22 hr significantly increased the activity of iNOS and decreased the activity of eNOS. Moreover, there was a dose-response relationship for the effect of AEE on the activity of iNOS and eNOS induced by H_2O_2 (Figure 1d,f).

3.5 | AEE ameliorated H_2O_2 -induced NO dysregulation

The DAX-J2 Red fluorescent dye was used to detect the generation of NO by the HUVECs. The DAX-J2 Red fluorescence intensity increased significantly after the HUVECs were treated with 200 μM H_2O_2 for 22 hr. Pretreatment with 1 μM AEE for 24 hr significantly inhibited the increase in DAX-J2 Red fluorescence intensity induced by H_2O_2 ($P < 0.05$; Figure 2a,b). In addition, the results of NOS activity assay showed that in the HUVECs treated with H_2O_2 for 22 hr, their eNOS activity was decreased (Figure 2c) and the activity of iNOS was increased (Figure 3c), while pretreatment with 1 μM AEE for 24 hr significantly ameliorated the decrease in eNOS activity (Figure 2c) and

the increase in iNOS activity (Figure 3c). Furthermore, after treatment with SMT, the H_2O_2 -enhanced NO production in the HUVECs was significantly inhibited (Figure 2a,b).

The protein expression of eNOS and iNOS was detected by Western blotting and immunofluorescence. There were no significant differences in the expression and phosphorylation of eNOS between treatment groups (Figure 2d,e). After treatment with 200 μM H_2O_2 , the expression of iNOS was significantly increased (Figure 3a,b). Pretreatment with 1 μM AEE for 24 hr significantly attenuated the H_2O_2 -induced expression of iNOS (Figure 3a,b). In the cells treated with H_2O_2 , the intracellular production of BH4 was significantly decreased. In the cells receiving AEE and H_2O_2 treatments, BH4 concentration was higher than the cells treated with H_2O_2 alone, although it did not reach the levels in the normal group (Figure 2f).

To verify whether AEE can affect the expression of iNOS in vivo, the expression of iNOS was examined in the aorta of HFD-induced atherosclerotic hamsters in the presence and absence of AEE. The results showed that the expression of iNOS in HFD-fed group was significant higher than those in the control group ($P < 0.05$).

FIGURE 2 The effect of aspirin eugenol ester (AEE) on H_2O_2 -induced production of NO and endothelial NOS (eNOS). Values are expressed as means $\pm$ SD where applicable. (a–b) AEE reduced H_2O_2 -enhanced NO formation. “+”: with the treatments in the HUVECs; “–”: without the treatments in the HUVECs. $n = 6$. * $P < 0.05$, compared with the normal group; # $P < 0.05$, compared with the H_2O_2 treated group. (c) AEE prevented the decrease in eNOS activity induced by H_2O_2 . $n = 6$. * $P < 0.05$, compared with the normal group; # $P < 0.05$, compared with the animals receiving H_2O_2 alone. (d–e) The expression and phosphorylation of eNOS by HUVECs were not significantly different between the treatment groups. “+”: with the treatments in the HUVECs; “–”: without the treatments in the HUVECs. (f) The overexpression of Nrf2 attenuated the decrease in intracellular BH4 production induced by H_2O_2 . “+”: with the treatments in the HUVECs; “–”: without the treatments in the HUVECs. $n = 6$. * $P < 0.05$, compared with the normal group; # $P < 0.05$, compared with the H_2O_2 group

FIGURE 3 Aspirin eugenol ester (AEE) down-regulated the expression and activity of inducible NOS (iNOS) in vitro and in vivo. Values are expressed as means $\pm$ SD where applicable. (a–b) AEE ameliorated the H_2O_2 -induced expression of iNOS in the HUVECs. iNOS: targeted protein; Hoechst 33342: the nuclei were stained with Hoechst 33342. “+”: with the treatments in the HUVECs. “-”: without the treatments in the HUVECs. $n = 6$. * $P < 0.05$, compared with the normal group; # $P < 0.05$, compared with the H_2O_2 group. (c) AEE attenuated the effect on iNOS production induced by H_2O_2 . “+”: with the treatment on the HUVECs; “-”: without the treatments on the HUVECs. $n = 6$. * $P < 0.05$, compared with the normal group; # $P < 0.05$, compared with the H_2O_2 group. (d–e) AEE attenuated the high fat diet (HFD)-induced expression of iNOS in the aorta from Syrian golden hamsters. Normal group: The hamsters were fed a normal diet; HFD group: The hamsters were fed a HFD; AEE group: The hamsters were simultaneously fed a HFD and AEE. $n = 10$. * $P < 0.05$, compared with the normal group; # $P < 0.05$, compared with the HFD group. All data were normalized to the corresponding control and expressed in relative units (r.u.)

In the presence of AEE, the expression of iNOS was significantly reduced compared with those in HFD-fed groups ($P < 0.05$; Figure 3d,e).

3.6 | AEE reduced the impairment of intracellular calcium homeostasis induced by H_2O_2

The basal $[Ca^{2+}]_i$ was increased in H_2O_2 -treated HUVECs after 22 hr, whereas pretreating HUVECs with 1 μ M AEE for 24 hr significantly reduced this rise in intracellular calcium (Figure 4a). The SERCA function assay showed that in the H_2O_2 -treated group, the transfer efficiency of SERCA was significantly decreased ($P < 0.05$).

Pretreatment of the cells with 1 μ M AEE for 24 hr significantly enhanced the transfer efficiency of SERCA compared with that of the H_2O_2 alone-treated group (Figure 4b).

3.7 | AEE reduced the energy imbalance induced by H_2O_2

The ATP concentration in the HUVECs treated with 200 μ M H_2O_2 for 22 hr was decreased by about fourfold compared with the normal group ($P < 0.05$). In the cells pretreated with 1 μ M AEE for 24 hr, the ATP concentrations were significantly higher than in those receiving H_2O_2 treatment alone ($P < 0.05$; Figure 4c).

FIGURE 4 Aspirin eugenol ester (AEE) attenuated the H_2O_2 -induced imbalance in calcium homeostasis and energy. Values are expressed as means $\pm$ SD where applicable. (a) AEE reduced intracellular calcium accumulation induced by H_2O_2 . $n = 6$. * $P < 0.05$, compared with the normal group; # $P < 0.05$, compared with the H_2O_2 group. (b) AEE attenuated the decreased transportation efficiency of sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) induced by H_2O_2 . $n = 6$. * $P < 0.05$, compared with the normal group; # $P < 0.05$, compared with the H_2O_2 group. (c) AEE attenuated the decreased ATP concentration caused by H_2O_2 . $n = 6$. * $P < 0.05$, compared with the normal group; # $P < 0.05$, compared with the H_2O_2 group. All data were normalized to the corresponding control and expressed as relative units (r.u.)

3.8 | Effect of AEE on Nrf2 expression by HUVECs treated with H_2O_2

Multiple immunofluorescence staining was used to analyse the protein expression of Nrf2. The nuclear and microtubule were stained with Hoechst 33342 and Tubulin Tracker Red respectively. The protein expression of Nrf2 in the HUVECs treated with H_2O_2 was significantly decreased, and the protein was mainly located in the nucleus.

Pretreatment with 1 μM AEE for 24 hr significantly enhanced the expression of Nrf2 compared with the H_2O_2 alone-treated group, and the protein was predominantly located in the nucleus (Figure 5a,b).

To detect the effect of Nrf2 on NO production induced by H_2O_2 , the HUVECs were transduced with lentivirus to overexpress and inhibit Nrf2 respectively (Figure 6a). After the HUVECs with down-regulated Nrf2 were given 200 μM H_2O_2 for 6 hr, the NO production, apoptosis, and activity of iNOS were significantly increased, while the

FIGURE 5 The effect of aspirin eugenol ester (AEE) on the expression of Nrf2 induced by H_2O_2 . (a–b) AEE enhanced the expression of Nrf2 in the H_2O_2 group. Nrf2: targeted protein; Hoechst 33342: The nuclei were stained with Hoechst 33342; Tubulin Tracker: Cellular tubulins were stained with tubulin tracker probe; “+”: with the treatments in the HUVECs; “-”: without the treatments in the HUVECs. Values are expressed as means $\pm$ SD. $n = 6$. * $P < 0.05$, compared with the normal group; # $P < 0.05$, compared with the H_2O_2 group. All data were normalized to the corresponding control and expressed as relative units (r.u.)

FIGURE 6 Genetic inhibition of Nrf2 reduced the effect of aspirin eugenol ester (AEE) on H₂O₂-induced apoptosis of HUVECs and dysfunctions of NOS. (a) Transfection of HUVEC with lentivirus to overexpress Nrf2 and inhibit Nrf2. (b) The changes in the apoptosis between different treatment groups. (c) The changes in the inducible NOS (iNOS) expression and activity, as well as endothelial NOS (eNOS) activity and the levels of BH4 and NO in different treatment groups. "+": with the treatment in the HUVECs; "-": without the treatments in the HUVECs. Values are expressed as means $\pm$ SD. $n = 6$. * $P < 0.05$, compared with the normal group; # $P < 0.05$, compared with the H₂O₂ group and & $P < 0.05$ between H₂O₂-induced, normal, and Nrf2 inhibited HUVECs. All data were normalized to the corresponding control and expressed as relative units (r.u.). si: short interference; wt: wild type

BH4 concentrations and the activity of eNOS were significantly decreased compared with those in the normal HUVECs stimulated with H₂O₂. Pre-incubating the Nrf2-inhibited HUVECs with AEE did not significantly reverse the changes induced by H₂O₂ (Figure 6b,c).

Overexpression of Nrf2 in the HUVECs could significantly attenuate the increase in both the expression and activity of iNOS induced by H₂O₂ (Figure 7a-c). Overexpression of Nrf2 in the HUVECs did not affect the expression and phosphorylation of eNOS but restored the intracellular BH4 concentrations decreased by H₂O₂ (Figures 3d, e and 7d). The H₂O₂-induced NO production was significantly decreased by overexpressing Nrf2 in the HUVECs (Figure 7e,f). Overexpression of Nrf2 in the HUVECs significantly reduced the cell apoptosis induced by H₂O₂.

4 | DISCUSSION

The oxidative injury of vascular endothelial cells could cause various cardiovascular diseases such as thrombus, atherosclerosis, and hypertension (Higashi, Noma, Yoshizumi, & Kihara, 2009; Incalza et al.,

2018; Mei, Thompson, Cohen, & Tong, 2015). All kinds of stimuli, including physical, chemical, and biological factors, trigger oxidative injury in vascular endothelial cells (Hogg et al., 1999; Kojima et al., 2011; Toborek, Blanc, Kaiser, Mattson, & Hennig, 1997). HFD is a major risk factor for oxidative injury in vascular endothelial cells. The HFD-fed hamster is thought to be a convenient and sensitive model for rapid establishment of atherosclerosis (Singhal, Finver-Sadowsky, McSherry, & Mosbach, 1983; Zhao, Chen, Mao, Min, & Cao, 2018). It had been confirmed that AEE can prevent the severe atherosclerotic lesions in the aorta of HFD-fed Syrian golden (Ma, Yang, Liu, Kong, et al., 2017), while the mechanism by which AEE protects against the injury of aorta induced by HFD is still unknown. The metabolomic analysis of HFD-fed hamsters and AEE-treated HFD-fed hamsters suggested that protective effect of AEE against the injury of aorta might be related to a reduction in oxidative stress (Ma, Yang, Liu, Kong, et al., 2017). In the present study, the oxidative balance was disturbed in plasma of HFD-fed atherosclerotic hamsters, and AEE treatment significantly attenuated the oxidative imbalance caused by HFD ($P < 0.05$). The results suggest that AEE treatment may reduce the

FIGURE 7 Protective effect of aspirin eugenol ester (AEE) on NO dysregulation induced by H_2O_2 mediated by enhancing the expression of Nrf2. Values are expressed as means $\pm$ SD where applicable. (a–b) The overexpression of Nrf2 ameliorated the H_2O_2 -enhanced expression of inducible NOS (iNOS) in the HUVECs. (c) Overexpression of Nrf2 attenuated the disorder of iNOS induced by H_2O_2 . (d) Overexpression of Nrf2 attenuated the decrease in intracellular BH4 induced by H_2O_2 . (e, f) Overexpression of Nrf2 reduced H_2O_2 -enhanced NO formation. $n = 6$. * $P < 0.05$, compared with the normal group; # $P < 0.05$, compared with the H_2O_2 group. (c–d) The expression and phosphorylation of endothelial NOS were not different between treatment groups in the HUVECs. “+”: with the treatments in the HUVECs. “-”: without the treatments in the HUVECs. $n = 6$. All data were normalized to the corresponding control and expressed as relative units (r.u.). wt: wild type

oxidative injury. In order to simulate oxidative injury in vascular endothelial cells in vivo, many oxidative stress models of endothelial cells have been established. H_2O_2 -induced oxidative stress is well established as a common model for oxidative injury (Kaczara et al., 2010; Li, Zhao, Ge, Yu, & Ma, 2018). In the present study, apoptosis of HUVECs showed a dose-dependent relationship with H_2O_2 . Approximately 60% apoptosis was induced in the HUVECs treated with 200 μM H_2O_2 for 22 hr.

The previous studies and the present study suggest that AEE exhibits pharmacological activity against oxidative stress in vivo. In our in vitro experiments, 1 μM AEE pretreatment of HUVECs for

24 hr reduced the H_2O_2 -induced apoptosis in the HUVECs. This suggests that AEE inhibits oxidative stress in vitro. To further investigate the mechanism of the effect of AEE on oxidative stress, NO as a key molecular in the regulation of oxidative stress was examined. NO plays a key role in cell signal transduction at physiological concentrations, while excessive NO production would form nitrogen free radicals causing oxidative stress (Beckman & Koppenol, 1996). NO production was significantly increased in the H_2O_2 -treated HUVECs, and pretreating the HUVECs with AEE significantly reduced the H_2O_2 -induced NO production via inhibiting the expression and activity of iNOS. Moreover, after treatment with SMT, the H_2O_2 -induced NO

production was significantly inhibited. These findings suggest that iNOS is a key regulatory target of AEE in reducing H₂O₂-induced dysregulation of the NO pathway. Notably, AEE treatment also significantly attenuated the increased expression of iNOS in aorta from the HFD-fed atherosclerotic hamsters, which suggests that AEE possesses an inhibitory effect on iNOS *in vivo*. In the present study, there were no significant differences in the expression and phosphorylation of eNOS between the treatment groups, while after treatment with H₂O₂, the intracellular BH4 was significantly decreased, and the changes were restored by pretreating HUVECs with AEE. This suggests that the activity of eNOS is influenced by regulating the eNOS uncoupling. When the essential cofactor BH4 is oxidized or decreased, eNOS is uncoupled, which would generate radicals leading to further exacerbation of the redox imbalance. It was interesting that low-dose H₂O₂ (20–40 µM) significantly increased the activity of eNOS in the HUVECs, which was consistent with the results of previous studies (Ballinger et al., 2000; Drummond, Cai, Davis, Ramasamy, & Harrison, 2000).

Excess NO production might cause cell apoptosis by disrupting Ca²⁺ homeostasis and the respiratory chain (Xu, Eu, Meissner, & Stamler, 1998; Xu, Liu, Charles, & Moncada, 2004). In a previous study it was shown that excessive NO could inhibit the activity of SERCA by tyrosine nitration within the channel-like domain causing an imbalance of Ca²⁺ homeostasis (Xu et al., 1998). In the present study, we found that the cellular level of Ca²⁺ was significantly increased, and the transfer efficiency of SERCA was significantly decreased in the HFD group compared with the control animals. The above phenomenon was significantly ameliorated by AEE treatment. An increased concentration of NO can prevent cytochrome C oxidase from using any available oxygen, and it can also inhibit the electron transport chain complexes I–III to reduce energy production to cause the disruption of the respiratory chain (Brookes et al., 2000; Pearce, Kanai, Epperly, & Peterson, 2005). In the present study, the ATP concentration was significantly decreased in the H₂O₂ group. Pretreating the HUVECs with AEE significantly increased the ATP concentration. Our present study illustrates that excessive NO production is a key factor in causing cell apoptosis through disrupting the cellular energy metabolism and Ca²⁺ homeostasis in the H₂O₂ group. Moreover, we observed that 1 µM AEE pretreatment of the HUVECs for 24 hr could significantly reverse the down-regulation of Nrf2 in the HUVECs induced by H₂O₂.

Nrf2 is a key component in cellular redox homeostasis for attenuating oxidative stress-associated pathological processes (Ding et al., 2017; Satta, Mahmoud, Wilkinson, Yvonne Alexander, & White, 2017; Vomund, Schafer, Parnham, Brune, & von Knethen, 2017). To explore whether the overexpression of Nrf2 can ameliorate the increase in NO production in the HUVECs treated with H₂O₂, we constructed Nrf2-overexpressed HUVECs and Nrf2-inhibited HUVECs with a lentivirus vector. The results showed that the overexpression of Nrf2 significantly inhibited the increase in NO production in the HUVECs induced by H₂O₂. After the HUVECs with down-regulated Nrf2 were given 200 µM H₂O₂ for 6 hr, the NO production, apoptosis, and activity of iNOS were significantly increased, while the BH4 concentrations and the activity of eNOS were significantly decreased

compared with those in the normal HUVECs treated with H₂O₂. Pre-incubating the Nrf2-inhibited HUVECs with AEE did not significantly reverse the changes induced by H₂O₂. These findings suggest that the effect of AEE on Nrf2 is an important mechanism through which it prevents the increase in NO production induced by H₂O₂.

The principal findings of this study show that the protective effect of AEE on oxidative injury of vascular endothelial cells is associated with its ability to prevent NO dysregulation via decreasing the expression of iNOS, reducing the eNOS uncoupling, and increasing the expression of Nrf2. However, the interdependent relationship between iNOS and Nrf2 in the protective effect of AEE on oxidative injury in vascular endothelial cells needs to be further investigated.

5 | CONCLUSION

AEE effectively protected vascular endothelial cells from oxidative injury by down-regulating iNOS, reducing eNOS coupling, and up-regulating Nrf2 to ameliorate the dysregulated NO pathway induced by H₂O₂.

AUTHOR CONTRIBUTIONS

M.H. designed and performed experiments and wrote the manuscript. J.L. and Y.Y. performed the synthesis and purification of AEE. X.L. and Z.Q. helped with the animal experiments. M.H. and J.L. supervised the study and revised the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for [Design & Analysis](#), [Immunoblotting and Immunochemistry](#), and [Animal Experimentation](#), and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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