

Original Paper

Allicin Decreases Lipopolysaccharide-Induced Oxidative Stress and Inflammation in Human Umbilical Vein Endothelial Cells through Suppression of Mitochondrial Dysfunction and Activation of Nrf2

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Key Words

Allicin • HUVEC • Atherosclerosis • Mitochondrial dysfunction • Inflammation • Nrf2

Abstract

Background: Allicin, a major component of garlic, is regarded as a cardioprotective agent and is associated with increased endothelial function. **Methods:** The effects of allicin on lipopolysaccharide (LPS)-induced vascular oxidative stress and inflammation in cultured human umbilical vein endothelial cells (HUVECs) and the mechanisms underlying these effects were studied. The protective effects were measured using cell viability, a lactate dehydrogenase (LDH) assay and cell apoptosis as indicators, and the anti-oxidative activity was determined by measuring reactive oxygen species (ROS) generation, oxidative products and endogenous antioxidant enzyme activities. HUVEC mitochondrial function was assessed by determining mitochondrial membrane potential (MMP) collapse, cytochrome c production and mitochondrial ATP release. To investigate the potential underlying mechanisms, we also measured the expression of dynamic mitochondrial proteins using western blotting. Furthermore, we evaluated the Nrf2 antioxidant signaling pathway using an enzyme-linked immunosorbent assay (ELISA). **Results:** Our results demonstrated that allicin enhanced HUVEC proliferation, which was suppressed by LPS exposure, and LDH release. Allicin ameliorated LPS-induced apoptosis, suppressed ROS overproduction, reduced lipid peroxidation and decreased the endogenous antioxidant enzyme activities in HUVECs. These protective effects were associated with the inhibition of mitochondrial dysfunction as indicated by decreases in the MMP collapse, cytochrome c synthesis and mitochondrial ATP release. In addition, allicin attenuated the LPS-induced inflammatory responses, including endothelial cell adhesion and TNF- α and IL-8 production. Furthermore, allicin increased the expression of LXR α in a dose-dependent manner. Allicin-induced attenuation of inflammation was inhibited by LXR α siRNA treatment. Finally, allicin activated NF-E2-related factor 2 (Nrf2), which controls the defense against oxidative stress and inflammation. **Conclusions:** Taken together, the present data

suggest that allicin attenuated the LPS-induced vascular injury process, which may be closely related to the oxidative stress and inflammatory response in HUVECs. Allicin modulated Nrf2 activation and protected the cells against LPS-induced vascular injury. Our findings suggest that allicin attenuated the LPS-induced inflammatory response in blood vessels.

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Introduction

Cardiovascular diseases (CVDs) have high mortality rates worldwide and have become a serious burden in many countries [1, 2]. Atherosclerosis (AS) is a progressive condition involving accumulation of lipids and fibrous elements in the large arteries, which is a key trigger for CVD [3]. Endothelial cell dysfunction is an important characteristic of AS [4, 5]. Both oxidative stress and mitochondrial dysfunction are responsible for the development of endothelial dysfunction [6, 7]. Given that endothelial dysfunction plays an important role in AS, protection of the vascular system is important. Many pharmacological agents that target oxidative stress and mitochondrial dysfunction have been considered for AS treatment [8].

For reasons of both safety and economy, garlic has been used as an anti-oxidative and anti-inflammatory agent [9]. Allicin (diallyl thiosulfinate), a key constituent of garlic [10], has potential health benefits, including anti-inflammatory, anti-oxidative stress and anti-hypertensive activities [11-13]. Previous studies have indicated that allicin protects cells against oxidative stress by inducing the generation of antioxidant products [13]. Allicin altered the fatty acid levels of rats fed high-fat diets [14]. Recently, several studies have demonstrated that allicin decreases cholesterol and blood pressure [10, 15]. Taken together, these findings indicate that allicin suppresses oxidative stress and inflammatory responses *in vitro* and *in vivo*.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key regulator of oxidative stress and the inflammatory response in endothelial cells [16, 17]. Nrf2 binds to the antioxidant response element (ARE) and triggers the expression of ARE-regulated genes, including heme oxygenase-1 (HO-1). HO-1 is an important inducible antioxidant enzyme in vascular tissue. Although recent studies have demonstrated that Nrf2 has a role in protecting against hyperoxic damage [18] and in angiogenesis [19, 20] in human endothelial cells, the underlying molecular mechanism by which allicin induces Nrf2 activation has not been fully elucidated.

Lipopolysaccharide (LPS) promotes AS by enhancing the apoptosis of vascular endothelial cells [21], smooth muscle cells [22] and macrophages [23]. Primary human umbilical vein endothelial cells (HUVECs) have been widely used in studies of endothelial cell function *in vitro*. In this study, we investigated the mechanisms underlying the potential protective effects of allicin on LPS-induced cytotoxicity in HUVECs with a focus on oxidative stress and mitochondrial dysfunction. We also found that allicin protected HUVECs against oxidative stress-induced cytotoxicity in a Nrf2 activation-dependent manner. Collectively, our studies demonstrated that allicin exhibits anti-oxidative and anti-inflammatory activities in LPS-treated HUVECs by activating Nrf2 signaling and inhibiting the NF- κ B pathway. Our findings showed that allicin has the potential to be used as an anti-oxidative stress and anti-inflammatory mediator.

Materials and Methods

Materials

Allicin (purity > 98%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and was stored at -80°C until use. Allicin was dissolved in dimethyl sulfoxide (DMSO) (<0.1%, which had no toxicity), and 0.1 % DMSO was used as a vehicle control. LPS was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Commercial HUVECs were purchased from Allcells (Shanghai Biological Technology Co., Ltd., China). The cells were seeded in RPMI 1640 medium with 10 % fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 60 µg/mL endothelial cell growth supplement (BD, San Diego, CA) at 37°C in a 5% CO₂ incubator. HUVECs in passages 3–5 were used. The HUVECs were treated with various concentrations of allicin (0–40 µg/mL) and LPS (1 µg/mL) for 24 h.

Neutrophils (HL-60 cells: No. CCL-240) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The cells were seeded in RPMI 1640 medium with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 1% (w/v) penicillin/streptomycin at 37°C in a 5% CO₂ incubator.

MTT assay

Cell viability was determined using MTT assays [24]. Briefly, HUVECs (2 × 10⁵ cells/well) were seeded in RPMI 1640 medium with 10% FBS for 24 h. After incubation in serum-free RPMI 1640 medium overnight, the HUVECs were treated with various concentrations of allicin (0–40 µg/mL) and LPS (1 µg/mL) for 24 h. After addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, MO, USA) to the samples, the absorbance at 490 nm was measured using a microplate reader (Bio-Rad, CA, USA), and the value of the untreated HUVECs was set as 100%. All experiments were repeated three times.

Lactate dehydrogenase (LDH) assay

Cytotoxicity was measured using a LDH assay kit according to the manufacturer's protocols. Briefly, 50 µl of culture supernatant from each sample was collected for the following investigations. The samples were combined with nicotinamide-adenine dinucleotide (NADH) and pyruvate for 15 min at 37°C. The LDH activity was measured at 440 nm using a microplate reader (Bio-Rad, CA, USA).

Apoptosis analysis

HUVEC apoptosis was assessed using a commercial apoptosis assay kit (BD Biosciences, USA) [24]. HUVECs (2 × 10⁵ cells/well) were seeded into 6-well plates, and the cells were treated with various concentrations of allicin (0–40 µg/mL) and LPS (1 µg/mL) for 24 h. Then, the cells were trypsinized and washed with PBS. The suspensions were treated with 5 µL of Annexin V and 10 µL of a propidium iodide (PI) solution in the dark at room temperature for 20 min. Apoptotic cells were quantified using flow cytometry. The data were analyzed using FlowJo software (FlowJo, Ashland, OR, USA). Q1 indicates FITC-, PI-; Q2 indicates FITC+, PI-; Q3 indicates FITC+, PI+; and Q4 indicates PI+. Q1 indicated viable cells, Q2 was cells in early-stage apoptosis, cells in late-stage apoptosis were in Q3, and dead cells were Q4. The cells in Q2 and Q3 were defined as apoptotic cells.

Measurement of intracellular ROS

The ROS levels were evaluated using the Reactive Oxygen Species Assay Kit (Beyotime, Shanghai, China). The samples were incubated with DCFH-DA at 37°C for 45 min in the dark. Then, the samples were washed three times with PBS. The fluorescence was analyzed at 485 nm (excitation) and 535 nm (emission) using a microplate reader (Bio-Rad, CA, USA).

Measurement of oxidative products

The MDA (malondialdehyde) content was measured using a commercial kit (Ann Arbor, MI, USA). The protein carbonyl content was assayed using a commercial ELISA kit (Cell Biolabs, USA) according to the manufacturer's instructions.

Measurement of antioxidant enzyme activity

The catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione S-transferase (GST) activities were assessed according to the protocols provided with the assay kits (Cayman Chemical, USA). The enzyme activities were normalized to those of the control.

Measurement of mitochondrial membrane potential (MMP)

The MMP was analyzed using Rh123 staining. Rh123 (10 µM) was added to the HUVECs for 30 min at 37°C. The fluorescence was measured at 480 nm (excitation) and 530 nm (emission) using a microscope, and the value of the untreated HUVECs was set as 100%.

Quantification of cytochrome c release

Cytochrome c levels in the cytosolic and mitochondrial fractions were measured in the supernatants from the cytosolic extracts and in isolated mitochondrial suspensions, respectively, using the Quantikine M Cytochrome C Immunoassay kit obtained from R&D Systems (Minneapolis, MN, USA). The HUVECs were lysed with lysis buffer containing protease inhibitors. The lysates were centrifuged for 10 min at 750 g, and the supernatants were collected. Then, the supernatants were centrifuged at 15,000 g for 15 min at 4°C. The subsequent supernatants were used as the cytosolic fractions. The pellet containing the mitochondria was combined with PBS plus 0.5% Triton X-100 for 10 min. Then, the pellet was centrifuged at 15,000 g for 10 min, and the supernatant was used as the mitochondrial fraction. The absorbance was determined at 450 and 540 nm using a microplate reader (Thermo Scientific, CA, USA).

Quantification of ATP release

ATP release was detected using a conventional luciferin-luciferase assay according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). HUVECs (5.0×10^5) were trypsinized and re-suspended in 200 µl of buffer (100 mM Tris, 4 mM EDTA, pH 7.7). The lysates were collected, and ATP release was detected using a microplate reader (Bio-Rad, CA, USA). For evaluation of ATP release, the quantities detected in the samples were corrected for total volume and time.

Quantification of TNF-α and IL-8 by ELISA

HUVECs (2.0×10^4 cells/well) were seeded into a 24-well plate, and the culture supernatants were harvested. TNF-α and IL-8 concentrations were detected using ELISA kits (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocols.

Cell adhesion assay

Measurement of the neutrophils adhering to the HUVECs was as performed as previously described [25]. Briefly, HUVECs that had been seeded in 96-well plates were treated with allicin (0–40 µg/mL) and LPS (1 µg/mL). The neutrophils were labeled with 5 µM Vybrant DiD for 30 min. The cells were then re-suspended in an adhesion medium and added to confluent monolayers of HUVECs for 1 h. The cells that did not adhere to the HUVECs were removed by washing with PBS. The fluorescent signals of the adherent cells were measured using a microplate reader (Bio-Rad, CA, USA).

NF-κB p65 transcription factor assay

For evaluation of the NF-κB activation, the nuclear fractions of the HUVECs were harvested, and the bound NF-κB was measured using an NF-κB p65 Transcription Factor Assay Kit (ab133112, Abcam, UK) according to the protocol.

LXRα siRNA transfections

HUVECs (2.0×10^5 cells/well) were cultured in 6-well plates and transfected with LXRα siRNA (5 nM) or control siRNA (5 nM) (Santa Cruz Biotechnology Inc., CA, USA, Cat: sc-38828) using the DharmaFECT transfection reagent (Thermo Scientific Dharmacon, USA) for 36 h. Then, the cells were treated with allicin (0–40 µg/mL) and LPS (1 µg/mL) for 24 h. The production of TNF-α and IL-8 was determined using ELISA.

Western blot analysis

The total proteins from cells were extracted, and protein concentrations were determined using the BCA assay. Equal amounts of protein (40 µg) were resolved on 12% SDS-PAGE gels and transferred to a 0.45 µm nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk at room temperature for 1 h. The membrane was probed with the primary antibodies overnight at 4°C. Then, the membrane was incubated with the horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The signals were visualized using enhanced chemiluminescence reagents (ECL), and a densitometric analysis was performed.

Measurement of Nrf2 activity by ELISA

Nrf2 activity was detected using a TransAM NRF2 assay Kit (Active Motif, Carlsbad, CA) according to the protocol. First, 5 µg aliquots of the nuclear extracts of the HUVECs were incubated in a 96-well plate that

had been precoated with an oligonucleotide containing a binding site for Nrf2. The absorbance was detected at 450 nm using a microplate reader (Thermo Scientific, CA, USA).

Statistical analysis

The experiments were performed at least three times. The results are expressed as the mean \pm SD. They were statistically analyzed using two-tailed, unpaired Student's t-tests, and scores were considered significant at $p < 0.05$.

Results

Allicin inhibits LPS-decreased HUVEC viability

The potential cytotoxicity of allicin to the HUVECs was assessed using MTT assays, and HUVEC viability was not affected by allicin at the doses used (from 0–40 $\mu\text{g/mL}$) (Fig. 1a). Moreover, allicin prevented the LPS-induced decrease in HUVEC viability (Fig. 1b) and the increase in LDH release (Fig. 1c) at 24 h.

Allicin inhibits LPS-induced HUVEC apoptosis

HUVEC apoptosis was evaluated using the Annexin-V/PI staining assay. Our data showed that allicin (at concentrations of 0–40 $\mu\text{g/mL}$) did not affect HUVEC apoptosis, whereas LPS induced apoptosis in the HUVECs (Fig. 1d) ($p < 0.01$) compared to that of the control group. Moreover, allicin prevented LPS-induced HUVEC apoptosis at 24 h (Fig. 1d).

Allicin reduces LPS-enhanced oxidative stress in HUVECs

To evaluate the effects of allicin on the LPS-induced ROS levels, we used the DCDHF-DA assay. LPS clearly increased ROS production in the HUVECs compared to that of the

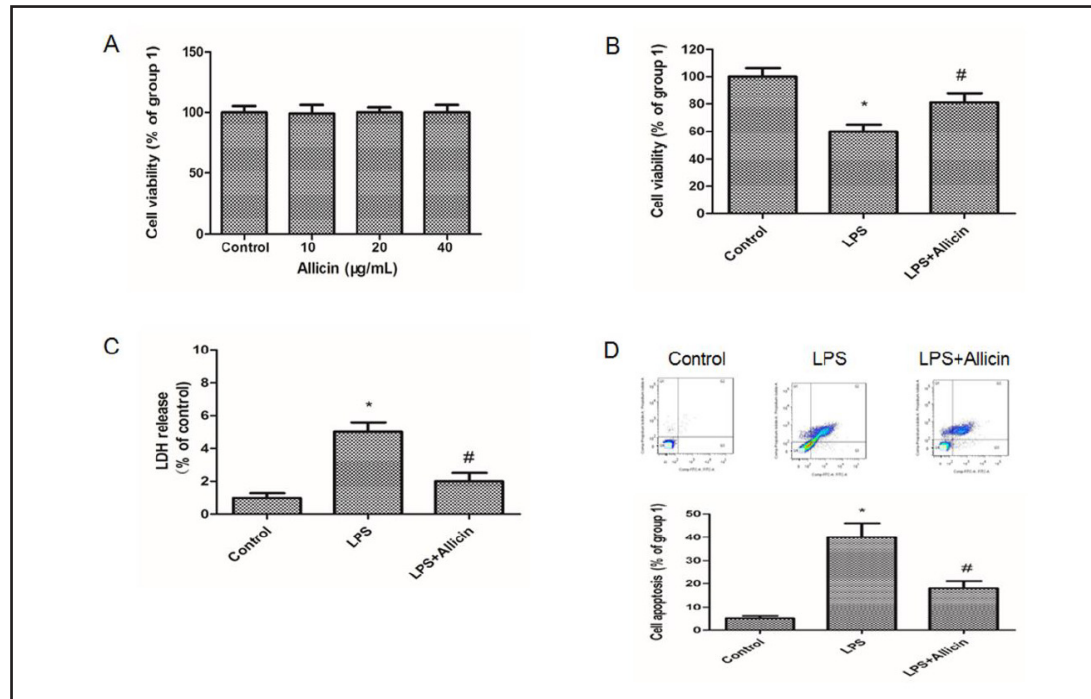


Fig. 1. Allicin prevents LPS-induced cytotoxicity to HUVECs. (A). HUVECs were treated with 0–40 $\mu\text{g/mL}$ allicin, and cell viability was evaluated. (B) HUVECs were treated with 40 $\mu\text{g/mL}$ allicin and LPS, and cell viability was measured. (C) HUVECs were treated with 40 $\mu\text{g/mL}$ allicin and LPS, and the LDH release was measured. (D) HUVECs were treated with 40 $\mu\text{g/mL}$ allicin and LPS, and apoptotic cell death was determined by flow cytometry. * $p < 0.05$ vs. the Control group. # $p < 0.05$ vs. the LPS group. (n = 3 independent experiments).

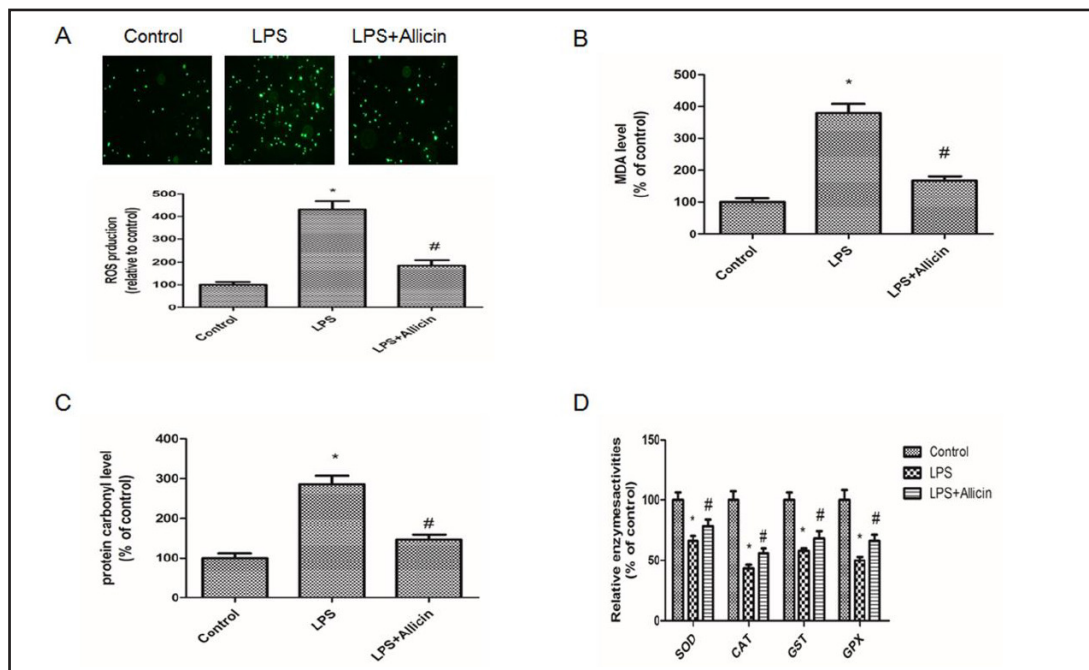


Fig. 2. Allicin reduces the LPS-induced oxidative stress. HUVECs were treated with allicin and LPS for 24 h. (A) HUVECs were treated with 40 μ g/mL allicin and LPS, and the intracellular ROS production was determined. (B) HUVECs were treated with 40 μ g/mL allicin and LPS, and the MDA levels were determined. (C) HUVECs were treated with 40 μ g/mL allicin and LPS, and the protein carbonyl levels were determined. (D) HUVECs were treated with 40 μ g/mL allicin and LPS, and the antioxidant enzyme activities were determined. * $p < 0.05$ vs. the Control group. # $p < 0.05$ vs. the LPS group. (n = 3 independent experiments).

control group ($p < 0.01$) (Fig. 2a). Moreover, allicin reversed the LPS-induced increase in ROS production in the HUVECs (Fig. 2a).

Lipid peroxidation was assessed by measuring MDA production, and the data showed that allicin significantly alleviated the MDA production in the HUVECs (Fig. 2b).

Furthermore, we assessed oxidative stress by measuring protein carbonyl production. As shown in Fig. 2c, LPS increased the protein carbonyl levels compared to those in the control cells, whereas allicin significantly prevented the increase in protein carbonyl levels.

To investigate the effects of allicin on the intracellular antioxidant system, we measured the SOD, CAT, GST and GPx activities. The results showed that LPS-induced decreases in the enzymatic activities were alleviated in the allicin-treated cells (Fig. 2d).

Allicin reduces LPS-induced mitochondrial dysfunction in HUVECs

To analyze the potential anti-mitochondrial dysfunction effects of allicin, we analyzed the changes in MMP. The data showed that the LPS-induced decreases in the MMP were partially reversed by allicin treatment (Fig. 3a).

Cytochrome c release was measured using an immunoassay kit, and increased mitochondrial cytochrome c levels (Fig. 3b) and decreased cytoplasmic cytochrome c levels (Fig. 3c) were found in the allicin-treated HUVECs compared to those of HUVECs treated with LPS.

Moreover, allicin partly rescued the mitochondrial ATP production that was decreased by LPS in the HUVECs (Fig. 3d).

Allicin regulates the expression of dynamic mitochondrial proteins

Mitochondrial fusion is regulated by Mfn-1 and Opa-1, whereas mitochondrial fission is regulated by the production of Drp-1 and Fis-1. To determine whether mitochondrial fission and fusion were affected by LPS and allicin treatment, we measured the Mfn-1, Opa-1, Drp-

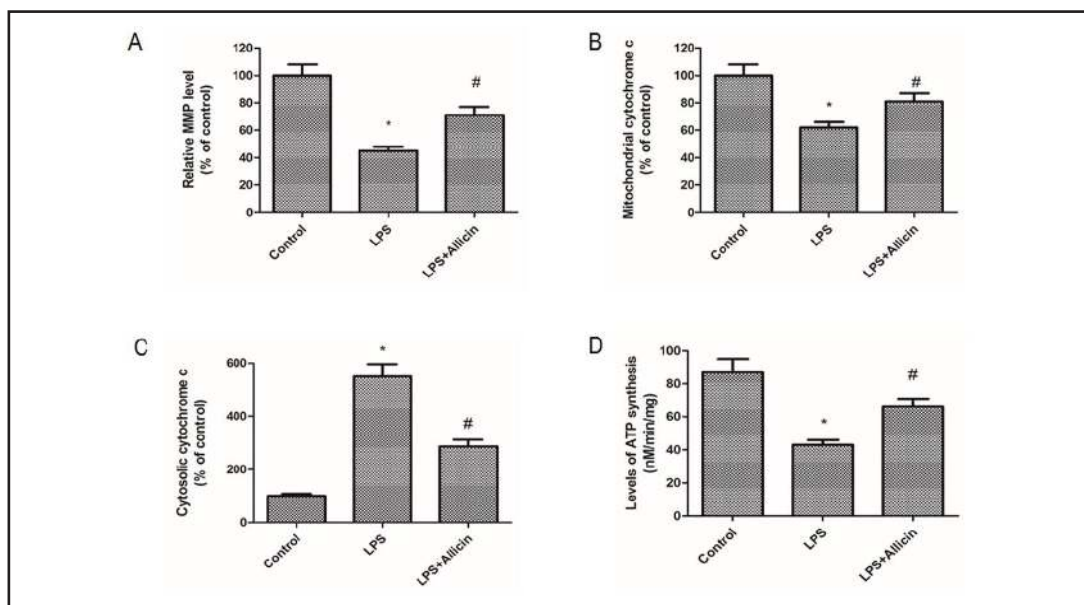


Fig. 3. Allicin reduces the LPS-induced mitochondrial dysfunction. HUVECs were treated with allicin and LPS for 24 h. (A) HUVECs were treated with 40 μ M allicin and LPS, and the MMP was measured. (B) HUVECs were treated with 40 μ M allicin and LPS, and the mitochondrial cytochrome c levels were measured. (C) HUVECs were treated with 40 μ M allicin and LPS, and the cytosolic cytochrome c levels were measured. (D) HUVECs were treated with 40 μ M allicin and LPS, and ATP synthesis was measured. * p < 0.05 vs. the Control group. # p < 0.05 vs. the LPS group. (n = 3 independent experiments).

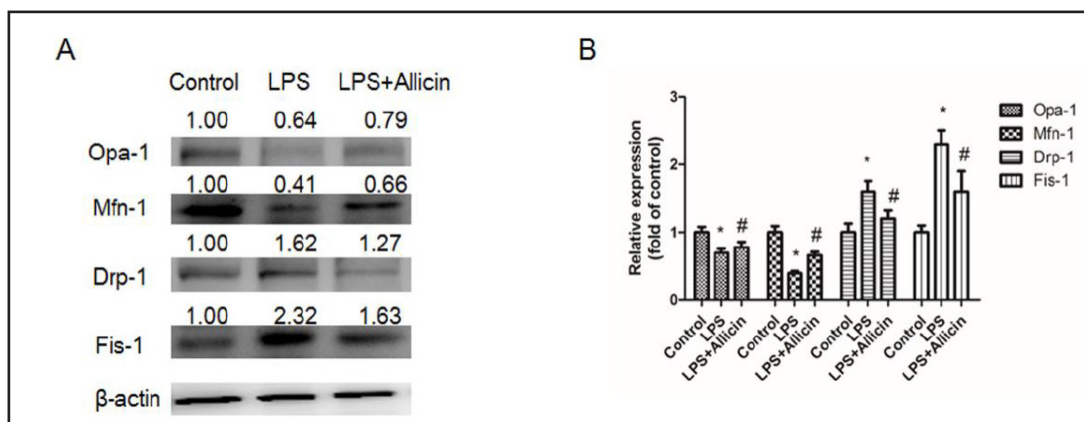


Fig. 4. Effects of allicin on the levels of the dynamic mitochondrial proteins. HUVECs were treated with allicin and LPS for 24 h. The expression levels of the dynamic mitochondrial proteins Opa-1, Mfn-1, Drp-1 and Fis-1 were determined by western blotting (A), and the relative levels were calculated (B). The results of the western blot analyses are presented as a fold of the control value (the optical densities). * p < 0.05 vs. the Control group. # p < 0.05 vs. the LPS group. (n = 3 independent experiments).

1 and Fis-1 levels using western blotting (Fig. 4). We found that LPS exposure significantly decreased the Opa-1 and Mfn-1 expression, and allicin partially prevented this effect. With respect to the fission proteins, both Drp-1 and Fis-1 were increased by LPS exposure, whereas the expression of Drp-1 and Fis-1 in the allicin-treated HUVEC was down-regulated.

Allicin inhibits LPS-promoted inflammatory mediators in HUVECs

To assess inflammatory response in HUVECs after LPS treatment, we measured the TNF- α and IL-8 expression levels. Our studies showed that LPS strongly enhanced the

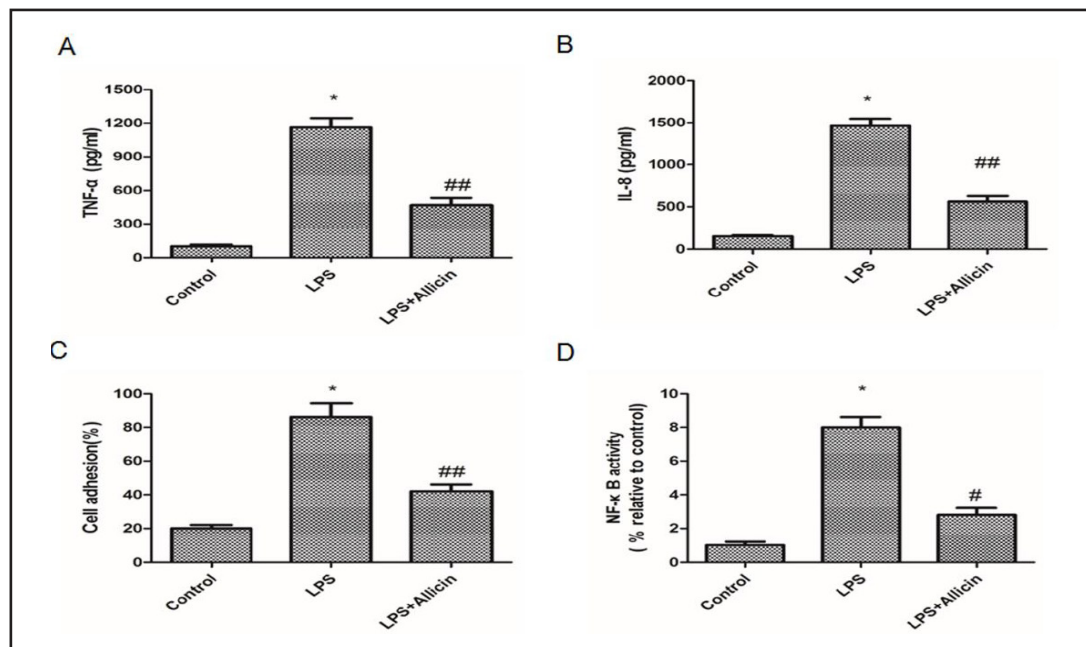


Fig. 5. Allicin inhibits the LPS-induced inflammatory response. HUVECs were treated with allicin and LPS for 24 h. The levels of TNF- α (A), IL-8 (B), cell adhesion (C) and NF- κ B activation (D) were measured. * p < 0.05 vs. the Control group. # p < 0.05 vs. the LPS group. (n = 3 independent experiments).

secretion of TNF- α (Fig. 5a) and IL-8 (Fig. 5b). Notably, the IL-8 levels were substantially increased compared to those of the other inflammatory cytokines. Moreover, the results demonstrated that allicin treatment inhibited the LPS-promoted inflammatory response that resulted in the secretion of TNF- α and IL-8, particularly IL-8, which subsequently led to HUVEC apoptosis.

Allicin inhibits adhesion of neutrophils to LPS-exposed HUVECs

Neutrophil adhesion to the endothelium is a key step in the inflammatory response. The effects of allicin on neutrophil adhesion to LPS-exposed HUVECs were evaluated. As shown in Fig. 5c, allicin inhibited the adhesion of the neutrophils to the LPS-exposed HUVECs.

Allicin suppresses the LPS-promoted NF- κ B binding activity in HUVECs

To evaluate the effects of allicin on NF- κ B signaling, we evaluated the NF- κ B binding activity in the endothelial cells. As shown in Fig. 5d, the HUVEC NF- κ B binding activity was substantially increased after LPS treatment. Moreover, allicin (at levels of 0–40 μ g/mL) reversed the LPS-induced increases in NF- κ B binding activity in the HUVECs.

Allicin regulates anti-inflammatory responses by activating LXR α

The effects of allicin on LXR α expression were evaluated. Our data indicated that allicin increased LXR α expression (Fig. 6a). To investigate whether LXR α participated in the allicin-induced anti-inflammatory response, we used LXR α siRNA. As shown in Fig. 6b, LXR α knockdown significantly reversed the decreased TNF- α and IL-8 levels induced by allicin (Fig. 6c, d), as well as the allicin-induced decreases in the NF- κ B binding activity (data not shown).

Allicin increases the LPS-suppressed Nrf2 activity

We explored the role of the transcription factor Nrf2 in the allicin-mediated anti-oxidative effects in HUVECs. Nrf2 expression in the HUVECs was determined by western blotting. As expected, treatment with allicin increased the Nrf2 levels that had been reduced

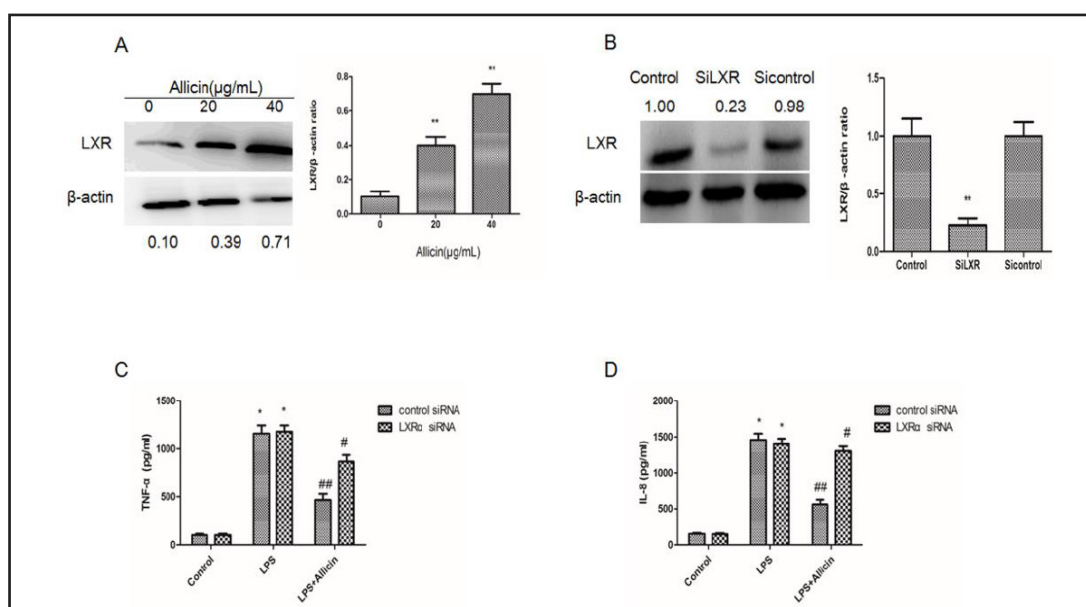


Fig. 6. LXRα knockdown prevents the allicin-induced decrease in the inflammatory response. (A) Effects of allicin on LXRα expression. (B) The siRNA-induced reduction in LXRα expression was determined by western blot analysis. (C) siRNA LXRα reversed the allicin-induced decrease in the TNF-α level. (D) siRNA LXRα reversed the allicin-induced decrease in the IL-8 level. * $p < 0.05$ vs. the Control group. # $p < 0.05$ vs. the LPS group. ## $p < 0.05$ vs. the LPS group. (n = 3 independent experiments).

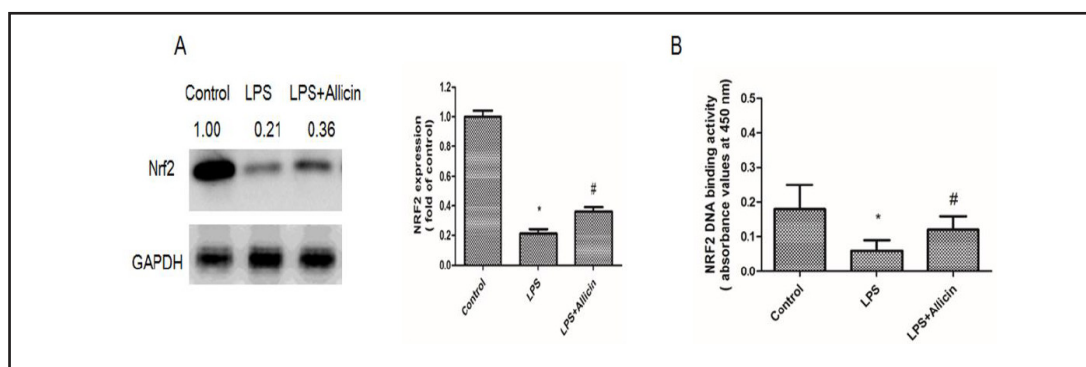


Fig. 7. Allicin increases the LPS-inhibited Nrf2 activation in HUVECs. (A) The effects of allicin on Nrf2 levels in HUVECs were determined using western blotting. (B) The effects of allicin on Nrf2 activation in HUVECs was determined using the ELISA-based TransAM Nrf2 assay. * $p < 0.05$ vs. the Control group. # $p < 0.05$ vs. the LPS group. (n = 3 independent experiments).

by LPS (Fig. 7A). Nrf2 activation in the HUVECs was evaluated using ELISA and was reduced by LPS. Allicin also prevented the LPS-mediated reduction of Nrf2 activation (Fig. 7B).

Discussion

CVD is the leading cause of death worldwide, and endothelial cell injury has been confirmed as a key factor in the development of CVDs [26]. Allicin has been reported to have anti-oxidative and anti-inflammatory effects [27, 28]. In this study, the effects of allicin on LPS-induced vascular injury were investigated. Our results showed that allicin reduced the LPS-induced oxidative stress and inflammation in HUVECs. Allicin may thus have beneficial effects on CVD.

Vascular inflammation plays a key role in the development of CVDs. LPS-induced endothelial injury has been used as a model to evaluate potentially protective compounds and to explore the underlying mechanisms [29, 30]. Our study assessed the protective effect of allicin against LPS-induced HUVEC cytotoxicity *in vitro*. LPS-induced oxidative stress, which was indicated by the increased levels of oxidative products (MDA and protein carbonyls) and the decreased activities of the antioxidant enzymes, was attenuated by allicin. Furthermore, allicin notably regulated LPS-induced mitochondrial dysfunction by controlling mitochondrial biogenesis.

In this study, LPS increased the oxidative stress as shown by ROS, MDA and protein carbonyl overproduction in HUVECs. Oxidative stress induced cell apoptosis when the endogenous antioxidant factors were decreased [31]. Our data demonstrated that LPS induced apoptosis in HUVECs by decreasing the SOD, CAT, GST and GPx activities, and preservation of these endogenous antioxidant activities may protect the HUVECs against oxidative stress. Previous experiments have demonstrated that allicin reduces intracellular ROS expression [32] and also attenuates oxidative stress by directly increasing glutathione (GSH) expression [33]. Consistent with these previous results, our data confirmed that allicin attenuates the LPS-induced endothelial injury by decreasing the ROS level and inducing the endogenous antioxidant activities.

Mitochondria are the key sources of ROS production. However, these organelles are extremely susceptible to oxidative stress, which triggers mutations in the mtDNA and regulates the membrane permeability [34]. Furthermore, past studies have also supported the involvement of mitochondrial dysfunction in AS development [35-37]. In the present study, collapse of the MMP, release of cytochrome c and decreased mitochondrial ATP production were observed following LPS treatment in HUVECs.

Additional mitochondrial biogenesis enhances mitochondrial ATP generation, which is the key indicator of mitochondrial function [38]. Mitochondrial biogenesis encompasses mitochondrial growth and division [39]. When stress exceeds tolerable levels, the mitochondria promote cellular apoptosis by inducing several mitochondrial cascades, including MMP, caspase family proteins, and Bcl-2 family proteins [40-42]. In this study, allicin attenuated the negative effects of LPS on the mtDNA and proteins, which suggested that the maintenance of mitochondrial biogenesis may be responsible for the allicin-mediated attenuation of LPS-induced oxidative stress and mitochondrial dysfunction.

Mitochondrial functions adapt to various stress conditions by continuously remodeling mitochondrial biogenesis [43]. High-molecular-weight GTPases, including Mfn-1, Opa-1, Drp-1 and Fis-1, are key constituents that participate in these processes, and these proteins have been associated with cellular apoptosis [44]. In this study, allicin attenuated the LPS-induced increases in Drp-1 and Fis-1 as well as the decreases in Opa-1 and Mfn-1, which indicated that allicin inhibited LPS-induced impairment of the endothelial cells via regulation of mitochondrial biogenesis.

LPS stimulation of endothelial cells can induce TNF- α and IL-8 production. These cytokines have been shown to play vital roles in vascular inflammation, which can cause cardiovascular complications. IL-8 plays an important role in recruiting monocytes to the site of inflammation. TNF- α can activate endothelial cells to express other inflammatory cytokines, which may irreversibly damage the vascular integrity. In this study, we found that allicin inhibited the LPS-induced production of TNF- α and IL-8. Furthermore, LXR α knockdown significantly prevented the allicin-mediated decreases in the TNF- α and IL-8 levels in HUVECs. LPS has been shown to induce the production of ROS, which can subsequently activate the transcription factor NF- κ B. NF- κ B has an important role in the inflammatory response, and NF- κ B activation was shown to regulate the IL-8 level [45]. Several studies have reported that LPS modulates the expression of cytokines and adhesion molecules, and these alterations play a crucial role in the pathogenesis of systemic inflammatory reactions [46]. LPS-treated HUVECs release ROS, and a mechanism that involves ROS and NF- κ B activation could at least partially explain the cytokine induction in HUVECs. Therefore, the effects of allicin on LPS-induced NF- κ B activation were evaluated. The results demonstrated

that allicin inhibited LPS-induced NF- κ B activation. Furthermore, LXR α activation inhibit the LPS-mediated NF- κ B induction. In this study, the effects of allicin on LXR α expression were also investigated. Our results demonstrated that allicin up-regulated LXR α expression. Additionally, we found that LXR α knockdown significantly reversed the anti-inflammatory effects of allicin.

Nrf2 has been reported to suppress oxidative stress and the inflammatory response [47, 48]. Furthermore, allicin attenuates oxidative stress responses through the Nrf2 antioxidant signaling pathway as previously suggested in other models [49-53]. However, whether Nrf2 activation mediates the allicin-induced protection from LPS-induced vascular injury is currently unknown. In this study, we demonstrated that allicin exerts cytoprotective effects via the Nrf2 activation pathway. Interestingly, our experiments showed that the LPS-induced reduction in Nrf2 expression and activation was prevented by allicin, indicating that allicin ameliorated the LPS-mediated reduction in Nrf2 transcriptional activation and expression. Recent evidence has identified cross-talk between the Nrf2 and NF- κ B pathways. However, there is little data to show that the activation of Nrf2 suppresses NF- κ B signaling. Our findings strongly implied that activation of Nrf2 inhibited the LPS-induced NF- κ B signaling. However, further studies are required to elucidate the underlying molecular mechanism.

In summary, all the findings from the present study indicated that allicin is be an anti-oxidative and anti-inflammatory agent that protects HUVECs from LPS-associated injury by regulating the endogenous antioxidant system and mitochondrial function. The protective effects elicited by allicin were accompanied by the preservation of mitochondrial biogenesis and inhibition of inflammatory responses via suppression of mitochondrial dysfunction and activation of Nrf2. In conclusion, allicin has the potential to ameliorate injury to the blood vessels as demonstrated here in LPS-treated HUVECs, suggesting that this molecule has clinical applications in the prevention of AS.

Acknowledgments

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Disclosure Statement

The authors report no conflicts of interest.

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