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Research Article

Myricetin Inhibits Mitochondrial Dysfunction and Oxidative Stress in Trimethylamine N-Oxide-Induced Human Umbilical Vein Endothelial Cells

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Abstract

Background: Myricetin (MYR) has attracted significant attention for its broad range of biological activities, including anti-inflammatory, anti-cancer, and anti-obesity effects. MYR also exerts notable protective effects against cardiovascular diseases and osteoporosis. Thus, this study aimed to investigate the therapeutic potential and underlying mechanisms of MYR in alleviating trimethylamine N-oxide (TMAO)-induced mitochondrial dysfunction and oxidative stress in human umbilical vein endothelial cells (HUVECs), thereby providing insights into potential strategies for treating atherosclerosis. **Methods:** Mitochondrial dysfunction and oxidative stress *in vitro* were modeled by treating HUVECs with TMAO. The protective effects of MYR were evaluated by assessing the levels of PTEN-induced putative kinase 1 (PINK1) and Parkin RBR E3 ubiquitin-protein ligase (Parkin), measuring reactive oxygen species (ROS) production, and determining the expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Furthermore, the mechanisms underlying the protective effects of MYR were explored. **Results:** MYR treatment reduced the expression of PINK1 and Parkin in TMAO-treated HUVECs, thereby attenuating mitochondrial damage and lowering ROS levels, underscoring the role of MYR in preserving mitochondrial integrity. Moreover, MYR suppressed COX-2 and iNOS expression in TMAO-treated HUVECs, reduced ROS and malondialdehyde (MDA) levels, and increased reduced glutathione (GSH) levels, indicating its ability to alleviate oxidative stress. Additionally, MYR facilitated the nuclear translocation of the nuclear factor erythroid 2–related factor 2 (NRF2) protein and upregulated the expression of heme oxygenase-1 (HO-1) and NAD(P)H quinone dehydrogenase 1 (NQO-1). Notably, when NRF2 signaling was inhibited by ML385, an NRF2 signaling inhibitor, MYR no longer mitigated oxidative stress in TMAO-treated HUVECs, suggesting that its antioxidant effects are mediated by the NRF2 signaling pathway. **Conclusion:** These findings demonstrate that MYR protects against mitochondrial dysfunction and oxidative stress in HUVECs, likely through associated antioxidant properties and activation of the NRF2 signaling pathway.

Keywords: atherosclerosis; mitochondrial dysfunction; oxidative stress; MYR; HUVECs

1. Introduction

Atherosclerosis, a chronic inflammatory condition affecting medium and large arteries, is characterized by plaque formation in the vascular intima, composed of various cells, lipids, and debris [1]. As the leading cause of cardiovascular disease (CVD) worldwide, it underlies severe clinical outcomes, including myocardial infarction, stroke, heart failure, and disabling peripheral artery disease [2,3]. The disease significantly contributes to morbidity and mortality and remains one of the primary causes of death in the elderly. Although the precise etiology of atherosclerosis is multifactorial and not yet fully understood, both high-density lipoprotein (HDL) and low-density lipoprotein (LDL) are critical in cholesterol transport and central to the disease's progression [4].

Both animal models and atherosclerotic patients exhibit elevated levels of free-radical peroxidation products, accompanied by reduced antioxidant enzyme activity [5,6]. Reactive oxygen species (ROS) play pivotal roles in regulating cellular functions and biological processes; however, their overproduction can induce oxidative stress and promote vascular injury [7]. Oxidative stress is characterized by an imbalance between oxidants and the antioxidant defense systems [8], and is considered a central pathogenic mechanism in atherosclerosis [7]. Consequently, it represents a potential therapeutic target for patients with atherosclerosis. Mitochondrial dysfunction has been implicated in both the pathogenesis and progression of atherosclerosis [9]. Beyond their well-established role in ATP generation, mitochondria are significant sources of ROS in various cell types [10]. Upon stimulation, mitochondria trigger inflammatory responses through ROS production and the release of mitochondrial DNA into the cytosol, initiating immune signaling. Mitochondrial impairment not only disrupts cellular energy metabolism but also increases ROS release, exacerbating oxidative stress and promoting cellular damage and death. Both primary and secondary mitochondrial dysfunction contribute to the onset and progression of atherosclerosis by enhancing ROS

generation [11]. In conclusion, oxidative stress and mitochondrial dysfunction play critical roles in the development and progression of atherosclerosis, and targeting these mechanisms may offer promising therapeutic strategies for the disease.

Myricetin (MYR, 3, 5, 7, 3′, 4′, 5′-hexahydroxy flavonol, $C_{15}H_{10}O_8$), a polyhydroxyflavonol compound characterized by light-yellow crystals [12], is widely distributed in plant families such as Vitaceae, Leguminosae, Rosaceae, and Fagaceae. It is also found in common dietary sources, including berries, vegetables, fruits, red wine, and tea [13,14]. MYR exhibits a range of pharmacological properties, including anticancer [15], antidiabetic [16], anti-inflammatory [17], antioxidant [18], and cardiovascular protective effects [19]. MYR protects against H_2O_2-induced injury by inhibiting ROS generation [20], and restoring the activity and protein expression of key antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [21]. These findings highlight the potent antioxidant capacity of MYR in various conditions. However, despite substantial research on MYR's antioxidant properties, its protective role in TMAO-induced HUVECs, particularly regarding the attenuation of oxidative stress and mitochondrial dysfunction, remains unexplored. Therefore, this study aims to investigate the protective effects of MYR against oxidative stress and mitochondrial dysfunction in TMAO-induced HUVECs.

2. Materials and Methods

2.1 Materials

MYR (purity ≥99.6%) was obtained from Shanghai Yuanye Bio-Technology (Cat # B21458, Shanghai, China). The yellow powder was dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions. Based on prior research by Bertin et al. [22], a working concentration of 1 μM was selected for MYR. TMAO was purchased from TargetMol (Cat # T41245, Shanghai, China), and its usage concentration was referenced from a previous study [23].

2.2 Cell Culture and Treatment

HUVECs were sourced from BeNa Culture Collection (BNCC342387, Beijing, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Suzhou, China) supplemented with 10% fetal bovine serum (FBS, SORFA, Beijing, China) and incubated at 37 °C in a 5% CO_2 atmosphere. Cells at passages 3–6 were used for the experiments. HUVECs were seeded at a density of 5×10^4 cells/cm^2. All cell lines were validated by STR profiling and tested negative for mycoplasma.

The experimental design comprised distinct sets of treatment groups. In the first experiment, four groups were established: Control, MYR, TMAO, and TMAO+MYR. Following a 2 h starvation period, cells were pretreated with 1 μM MYR for 1 h and subsequently exposed to 0.6 mM TMAO for 12 h. The second experiment consisted of two groups: Control and ML385. Following 2 h of starvation, cells were pretreated with 5 μM ML385 for 1 h. A third experiment involved five groups: Control, ML385, TMAO, TMAO+MYR, and TMAO+ML385+MYR. After 2 h of starvation, HUVECs were first treated with 5 μM ML385 for 1 h, followed by 1 μM MYR for 1 h, and then exposed to 0.6 mM TMAO for 12 h.

2.3 Western Blot

Following treatment with MYR and TMAO, total proteins were extracted from HUVECs using RIPA lysis buffer (Solarbio, Beijing, China) containing 0.1% phenylmethylsulfonyl fluoride (PMSF, Solarbio, Beijing, China). Protein concentration was determined using a bicinchoninic acid (BCA) Protein Assay Kit (Solarbio, Beijing, China) and then boiled at 100 °C for 5–10 minutes. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels, initially at 80 V for 30 minutes, followed by 110 V for 90 minutes. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane at 110 V for 1 h. The membranes were blocked with 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST) for 2 h at room temperature. After washing with TBST, the membranes were incubated overnight at 4 °C with the following primary antibodies: cyclooxygenase-2 (COX-2) (1:2000, R23971), inducible nitric oxide synthase (iNOS) (1:2000, R381545), nuclear factor erythroid 2–related factor 2 (NRF2) (1:2000, R380773), heme oxygenase-1 (HO-1) (1:2000, R24541), NAD(P)H quinone dehydrogenase 1 (NQO-1) (1:2000, R381695), Parkin (1:2000, R381626), and PTEN-induced putative kinase 1 (PINK 1) (1:2000, 507131) purchased from Zenbio (Chengdu, Sichuan, China), and β-actin (1:5000, AB0011) purchased from Abways Technology (Shanghai, China). After five 10-minute washes with TBST, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000, AF594, Abways Technology, Shanghai, China). After washing with TBST, protein bands were visualized using an enhanced chemiluminescence (ECL) western blot substrate (Solarbio, Beijing, China).

2.4 ROS Measurement

Intracellular ROS levels in HUVECs were measured using a commercial ROS Assay Kit (BTK023, Bioswamp, Wuhan, China). After treatment with MYR and TMAO, the culture medium was replaced with DMEM containing 10 μM of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (CA1410, Solarbio, Beijing, China) and 10 μg/mL Hoechst (C00212, Solarbio, Beijing, China). The cells were then incubated at 37 °C for 20 minutes and washed three times with ice-cold PBS to remove residual probes and Hoechst. Fluorescence microscopy was used for observation and image acquisition.

2.5 MDA Measurement

Malondialdehyde (MDA) levels in HUVECs were quantified using the MDA Assay Kit (BTK022, Bioswamp, Wuhan, China). After treatment with MYR and TMAO, cells were collected in PBS and lysed using a tissue homogenizer. The lysate was centrifuged at 10,000 ×g for 10 minutes to obtain the supernatant for MDA measurement. To prepare the MDA working solution, 50 µL of 0.37% TBA stock solution, 150 µL of TBA diluent, and 3 µL of antioxidant were mixed. For analysis, 100 µL of supernatant was combined with 200 µL of the MDA working solution and heated at 100 °C for 15 minutes. After centrifugation at 1000 ×g for 10 minutes, the samples were transferred to a 96-well plate. MDA levels were measured using a microplate reader at a wavelength of 532 nm.

2.6 GSH Measurement

Glutathione (GSH) levels in HUVECs were measured using the GSH Assay Kit (BTK034, Bioswamp, Wuhan, China). After treatment with MYR and TMAO, cells were collected using ice-cold PBS and centrifuged to remove the supernatant. A triple volume of protein reagent S was added to the cell pellet. The samples underwent two rapid freeze-thaw cycles using liquid nitrogen and a 37 °C water bath. After centrifugation at 10,000 ×g for 10 minutes, the supernatant was collected for GSH quantification. To prepare the GSH working solution, 6.6 µL of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) stock solution was mixed with 150 µL of GSH detection buffer. For measurement, 50 µL of sample and 150 µL of the GSH working solution were added to a 96-well plate, incubated at room temperature for 5 minutes, and GSH levels were determined using a microplate reader.

2.7 MitoSOX Red Staining

Mitochondrial ROS levels in HUVECs were assessed with the MitoSOX Red kit (S0061S, Beyotime, Beijing, China) following the manufacturer's protocol. Briefly, after treatment with MYR and TMAO, cells were incubated with MitoSOX Red solution and Hoechst (C00212, Solarbio, Beijing, China) at 37 °C for 20 minutes. After incubation, residual staining solutions were removed by washing with ice-cold PBS. Images were then acquired using fluorescence microscopy.

2.8 Mito-Tracker Staining

Mitochondrial dysfunction in HUVECs was assessed using the Mito-Tracker Green kit (C1048, Beyotime, Beijing, China) according to the manufacturer's protocol. A working solution of Mito-Tracker Green was prepared at a concentration of 100 nM in Hank's balanced salt solution. The cell culture medium was replaced with the Mito-Tracker Green working solution and Hoechst (C00212, Solarbio, Beijing, China), followed by incubation at 37 °C for 30 minutes. After incubation, residual staining reagents were removed by washing with ice-cold PBS. Fluorescence microscopy was used to observe and record the results.

2.9 ATP Assay

ATP production was measured using an ATP Assay Kit (S0026, Beyotime, Beijing, China) following the manufacturer's instructions. Briefly, 200 µL of lysis buffer was added to each well of a 6-well plate. After complete cell lysis, samples were centrifuged at 12,000 ×g for 5 minutes at 4 °C. The supernatant was carefully collected for analysis. Next, 100 µL of ATP detection working solution was added to each well of a 96-well plate, followed by 20 µL of the sample. Relative light unit (RLU) values were recorded using a chemiluminescence analyzer, and ATP levels were determined based on a standard curve.

2.10 Cell Immunofluorescence

HUVECs were seeded into a 24-well plate. After reaching 60% confluency, the cells were treated with MYR and TMAO, then washed with ice-cold PBS. Fixation was performed using 4% paraformaldehyde (P1110, Solarbio, Beijing, China) for 10 minutes, followed by washing with ice-cold PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes and washed again with ice-cold PBS. Blocking was carried out with 5% BSA (Solarbio, Beijing, China) at room temperature for 1 h. The cells were then incubated overnight at 4 °C with an anti-NRF2 antibody (1:200) diluted in 5% BSA. After an additional wash with ice-cold PBS, the cells were incubated for 1 h at room temperature with Goat Anti-Rabbit IgG (H+L) FITC (1:5000) diluted in 5% BSA, followed by another ice-cold PBS wash. The cells were then stained with DAPI for 5 minutes at room temperature. Finally, the cells were observed and imaged using fluorescence microscopy.

2.11 Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). One-way ANOVA followed by Tukey's multiple comparisons test (three groups) was used, while an unpaired *t*-test was employed to compare differences between two groups. Significance levels are denoted as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. All results represent data from three or more independent biological experiments. Technical replicates within each experiment were averaged before statistical analysis.

3. Results

3.1 MYR Suppresses Mitochondrial Dysfunction in TMAO-Induced HUVECs

Dysfunctional mitochondria not only impair energy production but also act as a primary source of ROS, contributing to oxidative stress and promoting the development of atherosclerosis through enhanced ROS generation. To investigate the effects of MYR on TMAO-induced mito-

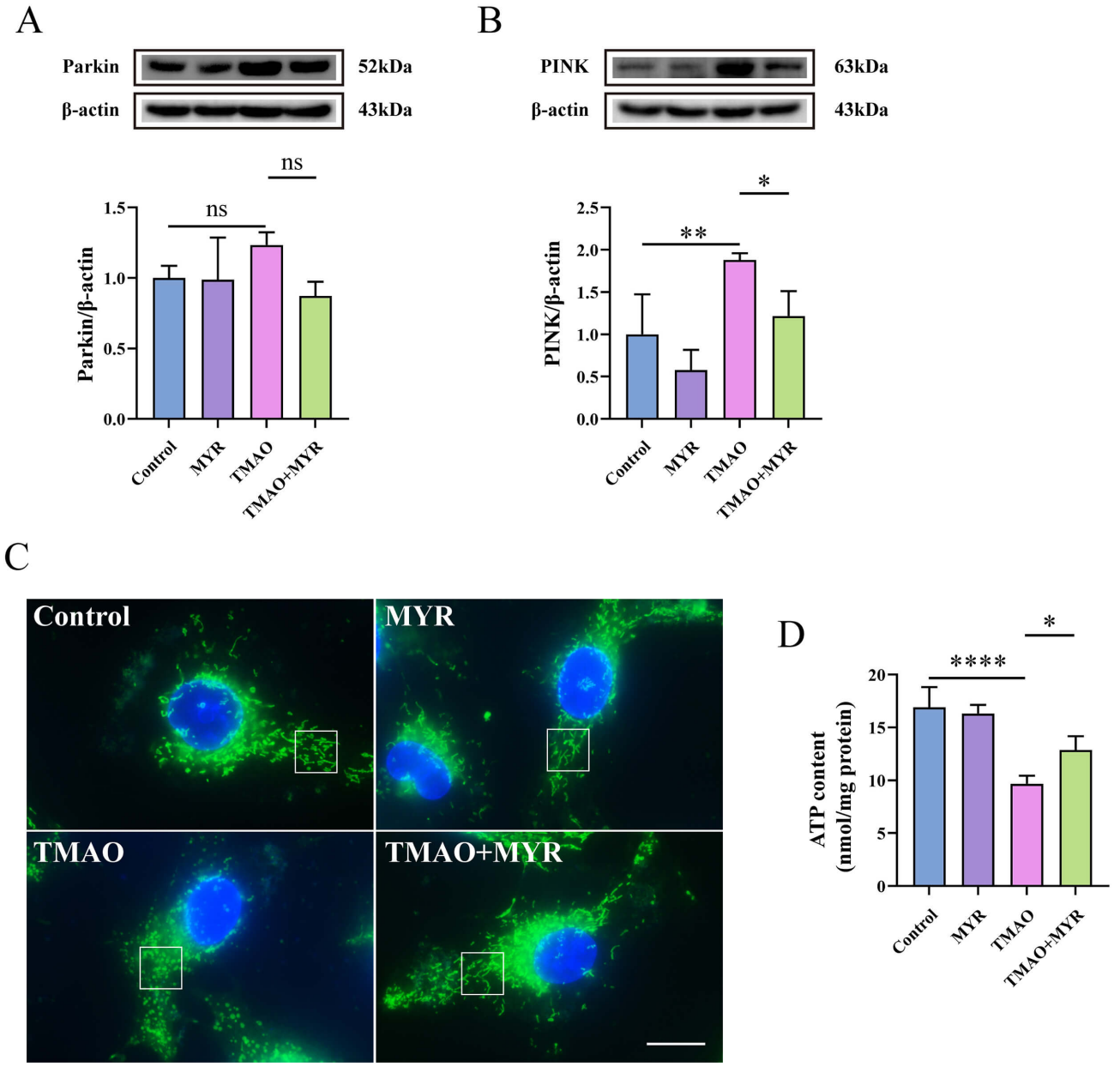

Fig. 1. Effects of MYR on mitochondrial dysfunction in TMAO-induced HUVECs. After a 2 h starvation, cells were pretreated with 1 μM of MYR for 1 h, followed by exposure to 0.6 mM of TMAO for 12 h. (A,B) Western blot (WB) analysis was performed to assess the expression of PINK1 and Parkin in HUVECs. (C) Mitochondrial morphology was visualized using Mito-Tracker staining according to the manufacturer's protocol. Images were magnified at 150×, with a scale bar representing 20 μm. (D) ATP levels were measured in HUVECs using a commercial kit. Statistical significance is denoted as $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$ and ns, not significant. The white box indicates the characteristics of mitochondria. MYR, Myricetin; TMAO, trimethylamine N-oxide; HUVECs, human umbilical vein endothelial cells; PINK1, PTEN-induced putative kinase 1.

chondrial dysfunction in HUVECs, the expression of Parkin and PINK was evaluated. TMAO treatment moderately upregulated PINK expression (Fig. 1A,B), with a particularly significant increase in PINK levels. However, MYR supplementation effectively decreased the expression of PINK (Fig. 1B). Additionally, Mito-Tracker staining revealed that TMAO treatment induced a punctate and fragmented mitochondrial morphology in HUVECs; however, this fragmentation was ameliorated by MYR supplementation (Fig. 1C). To further assess the protective effects of MYR on mitochondrial dysfunction, ATP production in HUVECs was measured. The results showed that TMAO significantly reduced ATP production, while MYR supplementation effectively restored ATP levels (Fig. 1D). These results suggest that MYR mitigates TMAO-induced mitochondrial dysfunction in HUVECs.

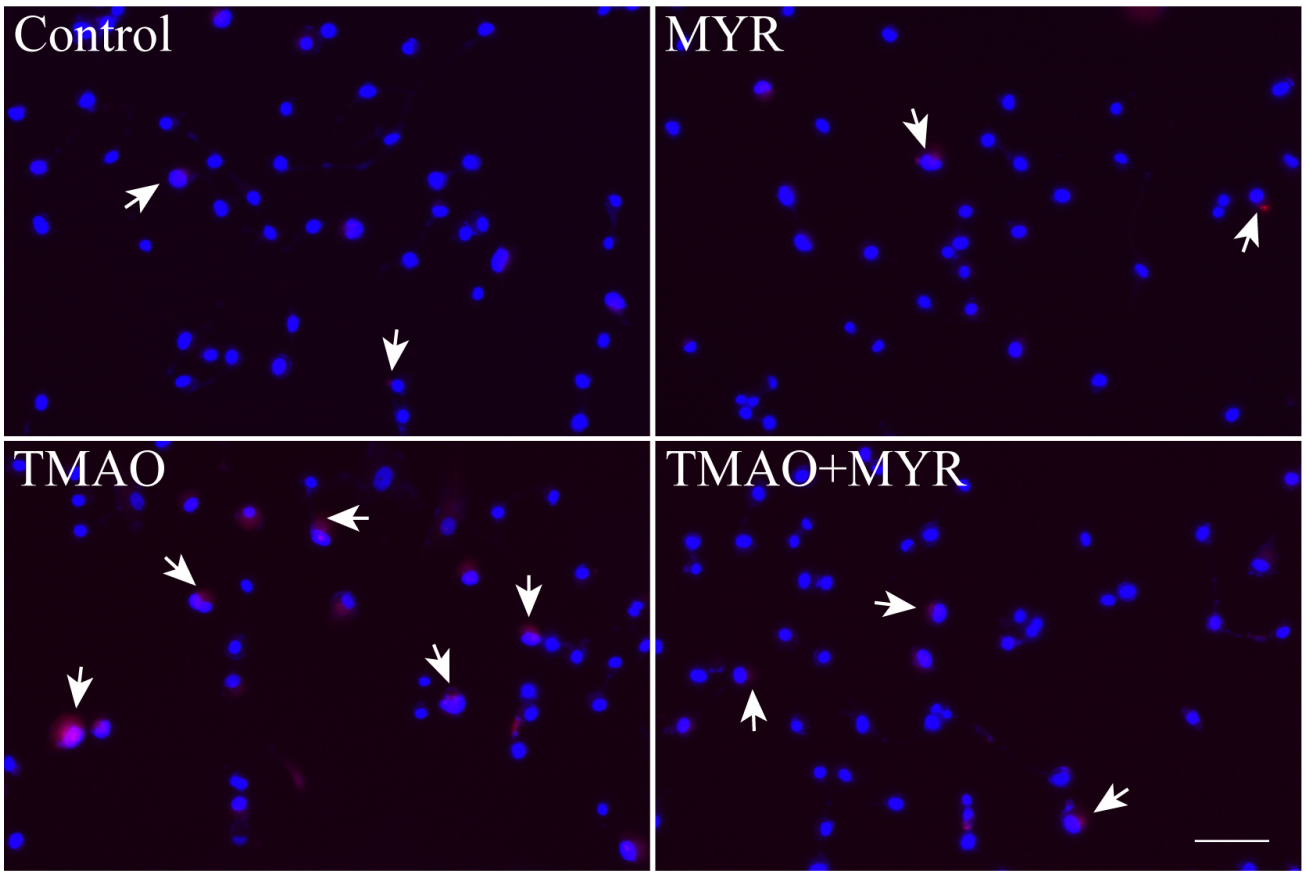

Fig. 2. MYR attenuates mitochondrial ROS production in TMAO-induced HUVECs. After a 2 h starvation period, cells were pretreated with 1 μM of MYR for 1 h, followed by exposure to 0.6 mM of TMAO for 12 h. Mitochondrial ROS levels were assessed using MitoSox staining according to the manufacturer's protocol. Images were magnified at 10×, with a scale bar representing 200 μm. The white arrows denote ROS-positive cells. ROS, reactive oxygen species.

3.2 MYR Inhibits the Production of Mitochondrial ROS in TMAO-Induced HUVECs

In addition to compromising energy production, dysfunctional mitochondria are a major source of ROS. To assess the effect of MYR on mitochondrial ROS levels in TMAO-treated HUVECs, MitoSOX staining was performed. The results showed that TMAO treatment increased mitochondrial ROS production, whereas MYR supplementation suppressed ROS generation (Fig. 2). These results suggest that MYR may exert antioxidant effects by inhibiting mitochondrial ROS production in TMAO-induced HUVECs.

3.3 MYR Inhibits Oxidative Stress in TMAO-Induced HUVECs

To further explore the antioxidant activity of MYR, the levels of iNOS, COX-2, ROS, MDA, and GSH in TMAO-treated HUVECs were quantified. Our results demonstrated that TMAO upregulated the expression of iNOS and COX-2, while pretreatment with MYR significantly reduced their levels (Fig. 3A,B). Moreover, TMAO increased ROS generation in HUVECs, and MYR pretreatment effectively suppressed ROS production (Fig. 3C). TMAO decreased GSH levels and increased MDA content, whereas MYR supplementation elevated GSH levels and inhibited MDA accumulation (Fig. 3D,E). These results highlight the potential antioxidant role of MYR in mitigating oxidative stress in TMAO-induced HUVECs.

3.4 MYR Activates NRF2 Signaling in TMAO-Induced HUVECs

The transcription factor NRF2 signaling pathway plays a critical role in cellular defense against oxidative stress, making it essential for maintaining cellular homeostasis. In contrast, TMAO promotes atherosclerosis development and induces oxidative stress, negatively impacting vascular health. To explore whether MYR can mitigate TMAO-induced oxidative stress, a series of experiments were conducted. MYR treatment stimulated the nuclear translocation of NRF2 protein in HUVECs (Fig. 4A). Additionally, MYR upregulated the expression of downstream targets NQO-1 in TMAO-treated HUVECs (Fig. 4B,C). Additionally, our findings revealed that MYR moderately upregulates the expression of HO-1 and NQO-1; however, under TMAO treatment, MYR produced an additional enhancement of HO-1 and NQO-1 expression.

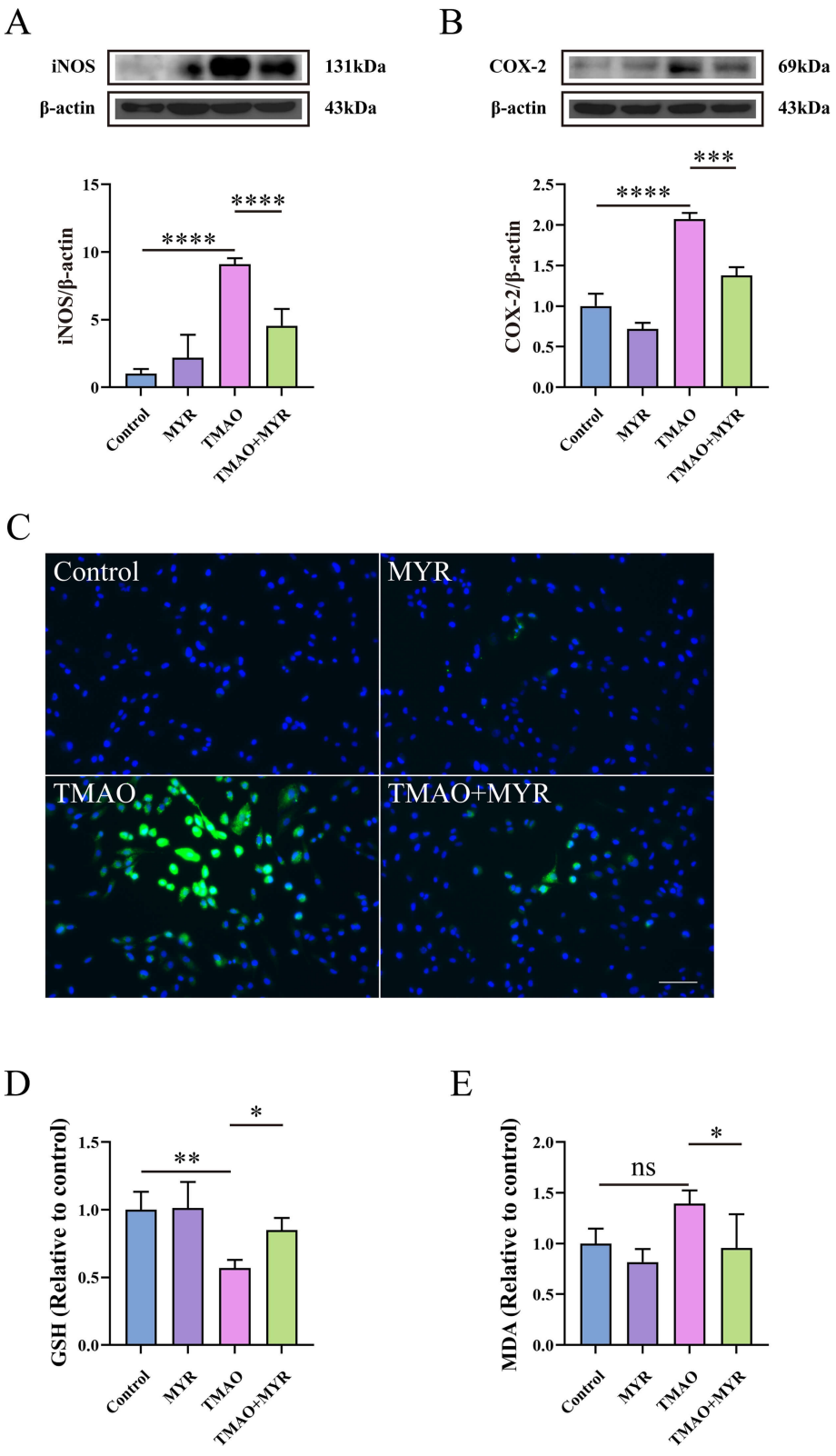

Fig. 3. MYR attenuates oxidative stress in TMAO-induced HUVECs. (A,B) WB analysis was performed to evaluate the expression of iNOS and COX-2 in HUVECs. (C) Intracellular ROS levels were evaluated using Hoechst/ROS staining. Images were magnified at 10×, with a scale bar representing 200 μm. (D,E) The levels of GSH and MDA in HUVECs were quantified using relevant kits. Statistical significance is indicated as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ and ns, not significant. iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; GSH, glutathione; MDA, malondialdehyde.

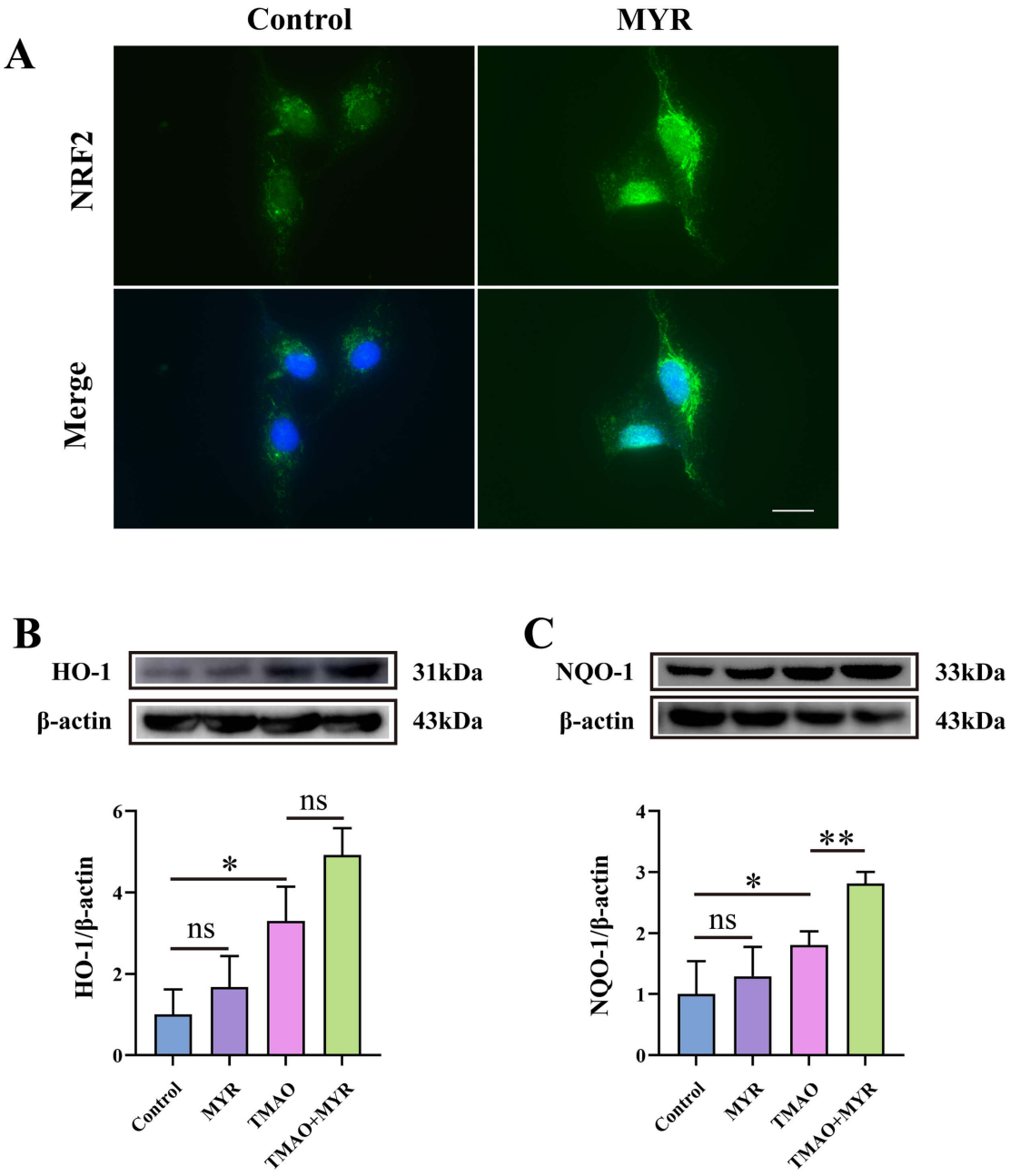

Fig. 4. MYR activates NRF2 signaling in TMAO-induced HUVECs. (A) After a 2-h starvation period, cells were pretreated with 1 μM of MYR for 6 h. Cellular immunofluorescence analysis was conducted to evaluate the nuclear localization of NRF2 protein, with images magnified at 100× and a scale bar indicating 20 μm. (B,C) After a 2 h starvation period, cells were pretreated with MYR for 1 h and then exposed to TMAO for 6 h. WB analysis was employed to measure the expression of heme oxygenase-1 (HO-1) (B) and NAD(P)H quinone dehydrogenase 1 (NQO-1) (C). Statistical significance is indicated as $*p < 0.05$, $**p < 0.01$ and ns, not significant.

3.5 The Antioxidant Properties of MYR Depend on the Activation of NRF2 Signaling Pathway

To further validate the dependence of MYR's antioxidant properties on NRF2 signaling activation, ML385, a specific NRF2 signaling inhibitor, was used. The results showed that ML385 treatment reduced the expression of NRF2, HO-1, and NQO-1 in HUVECs (Fig. 5A–C), confirming the effective inhibition of the NRF2 pathway. Moreover, in TMAO-treated HUVECs, MYR no longer suppressed the production of iNOS and COX-2 following ML385 blockade (Fig. 5D,E). Additionally, ML385-mediated inhibition abolished MYR's ability to reduce MDA levels and elevate GSH levels in TMAO-treated HUVECs (Fig. 5F,G). These results suggest that MYR exerts its antioxidant properties through the NRF2 signaling pathway. To further confirm these results, intracellular ROS levels were measured in TMAO-treated HUVECs. While TMAO increased ROS production, MYR failed to reduce

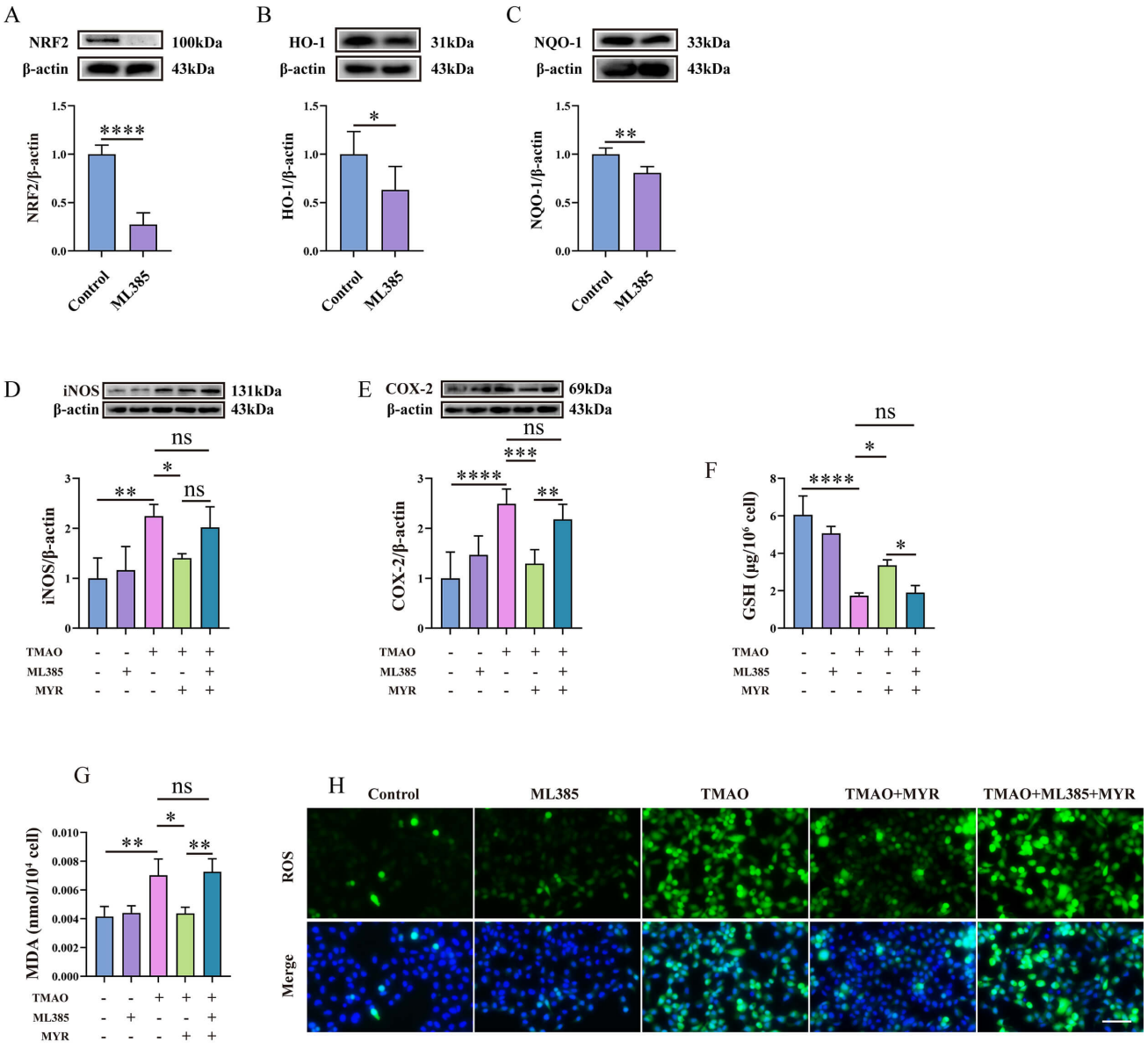

Fig. 5. The antioxidant properties of MYR depend on the activation of the NRF2 signaling pathway. (A–C) HUVECs were treated with ML385 for 1 h, followed by WB analysis to assess the levels of NRF2 protein (A), HO-1 (B), and NQO-1 (C) proteins. Following 2 h serum-free starvation, HUVECs were pretreated with ML385 for 1 h, followed by a 1 h incubation with MYR, and subsequently exposed to TMAO for 12 h. (D,E) WB analysis was performed to evaluate the protein expression of iNOS (D) and COX-2 (E). (F,G) GSH and MDA levels in HUVECs were measured using relevant kits. (H) Intracellular ROS levels in HUVECs were assessed by Hoechst/ROS staining. Images were magnified at 10×, with a scale bar representing 200 μm. Statistical significance is indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ and ns, not significant.

ROS levels after NRF2 inhibition by ML385 (Fig. 5H). Overall, our findings demonstrate that the antioxidant properties of MYR depend on the activation of the NRF2 signaling pathway in TMAO-treated HUVECs.

4. Discussion

Atherosclerosis is a chronic inflammatory disorder primarily characterized by vascular endothelial injury, inflammation of the vascular wall, and the accumulation of cholesterol and other lipids within the arterial wall. It is closely linked to various CVDs, including coronary heart disease, hypertension, and stroke [2,3]. Atherosclerosis can lead to vessel narrowing or complete occlusion, thereby compromising blood and oxygen delivery to vital organs [24,25], potentially resulting in severe outcomes such as myocardial infarction and stroke. Therefore, the prevention and management of atherosclerosis are critical for reducing the risk of cardiovascular events. This study aims to investigate whether MYR can alleviate mitochondrial dysfunction and oxidative stress in a TMAO-induced cellular model of

atherosclerosis, with the goal of providing insights for the development of new anti-atherosclerotic drugs.

Mitochondrial dysfunction affects various arterial wall cell types, including macrophages, smooth muscle cells, lymphocytes, and endothelial cells. This dysfunction leads to increased ROS levels, inducing oxidative stress, chronic inflammation, and intracellular lipid accumulation [26]. In addition to impairing energy production, dysfunctional mitochondria generate excessive ROS, which promotes oxidative stress and contributes to cellular damage and death. Mitochondrial dysfunction is currently considered a key factor in the onset and progression of atherosclerosis [27]. Thus, maintaining mitochondrial integrity is vital for the prevention and management of atherosclerosis. PINK1 accumulates on the outer membrane of damaged mitochondria, where it recruits Parkin and activates its E3 ubiquitin ligase activity. Parkin-mediated ubiquitination of proteins on the outer mitochondrial membrane induces selective autophagy, a hallmark of mitochondrial dysfunction, with PINK1 and Parkin serving as key biomarkers [28]. The present study demonstrates that MYR supplementation downregulates the expression of both Parkin and PINK1, while reducing mitochondrial ROS levels in TMAO-induced HUVECs. Consistent with these findings, Mito-Tracker staining revealed that MYR decreased mitochondrial spot aggregation under TMAO induction. These results are in line with previous studies reporting MYR's protective effects against mitochondrial dysfunction, including its ability to ameliorate Aβ-induced deficits in N2a-SW cells [29] and counteract aluminum phosphide-induced damage in cardiomyocytes [30]. Our data support the role of MYR as a potent agent against TMAO-induced mitochondrial impairment in HUVECs.

Mitochondrial damage represents a critical cellular event that, upon induction by external stressors, promotes the release of ROS and subsequent oxidative stress [31]. Oxidative stress arises from an imbalance between excessive free radical production and impaired antioxidant defenses, ultimately leading to macromolecular damage (e.g., lipids, DNA, proteins) [32] and contributing to the pathogenesis of various diseases. Notably, both animal models of atherosclerosis and human patients exhibit increased levels of lipid peroxidation markers (e.g., MDA) and reduced activity of antioxidant enzymes (e.g., GSH) [5,6], supporting the view that oxidative stress serves as a unifying mechanistic pathway in atherosclerosis progression [7] and represents a potential therapeutic target. In this study, MYR supplementation suppressed the expression of iNOS and COX-2, attenuated ROS generation, reduced MDA levels, and restored GSH content in TMAO-stimulated HUVECs. These findings demonstrate that MYR provides antioxidant efficacy by mitigating oxidative injury in TMAO-induced HUVECs.

The NRF2/HO-1 pathway, a critical cascade in the cellular response to oxidative stress, plays an essential role in regulating antioxidant defense, anti-inflammatory processes, and apoptosis. This pathway has thus been identified as a promising therapeutic target for atherosclerosis [33]. Under conditions of oxidative stress or pathogen stimulation, NRF2 protein dissociates from Keap1 and translocates to the nucleus, where it forms a heterodimer with the musculoaponeurotic fibrosarcoma oncogene homolog (Maf) protein to initiate the expression of cytoprotective genes. Due to its capacity to alleviate oxidative injury in vascular endothelial cells, NRF2 signaling has become a key focus for herbal medicine–based interventions in atherosclerosis [34]. The present study observed that MYR enhances the nuclear translocation of NRF2, suggesting that MYR may exert antioxidant effects on TMAO-induced HUVECs *via* activation of the NRF2 signaling pathway. Moreover, while the effect was not statistically significant, MYR led to a moderate upregulation in HO-1 and NQO-1 expression. This observation aligns with earlier studies showing that MYR enhances the expression of these antioxidant enzymes [35,36]. The absence of statistical significance in the present study may be attributed to variations in cell types or animal models, which could affect the sensitivity to MYR. To confirm these results, ML385, an NRF2 signaling inhibitor, was applied. The results showed that inhibition of NRF2 signaling prevented MYR from regulating iNOS, COX-2, and ROS production, as well as MDA and GSH levels in TMAO-induced HUVECs. This suggests that the antioxidant properties of MYR depend on NRF2 signaling activation.

This study has several limitations. First, the findings were obtained only from an in vitro TMAO-induced HUVEC model, which cannot fully reflect the complex pathological environment of atherosclerosis in vivo. Second, MYR was tested mainly at a single concentration and treatment duration; therefore, its optimal dose, effective time window, and potential cytotoxicity require further investigation. Third, although NRF2 involvement was supported using the inhibitor ML385, genetic approaches such as NRF2 knockdown would provide stronger mechanistic evidence. Finally, the bioavailability and metabolism of MYR were not evaluated, limiting direct extrapolation of these findings to clinical application. Future in vivo and clinical studies are needed to confirm the protective effects of MYR against TMAO-related endothelial dysfunction and atherosclerosis.

5. Conclusions

Atherosclerosis, a major contributor to CVDs, is closely linked to mitochondrial dysfunction and oxidative stress. Therefore, targeting these pathological processes may represent a key strategy for the prevention and treatment of atherosclerosis and related cardiovascular conditions. This study demonstrates that MYR effectively alleviates TMAO-induced mitochondrial damage and oxidative stress in HUVECs, with its antioxidant properties poten-

tially mediated through activation of the NRF2 signaling pathway. These findings not only reveal a novel protective function of MYR but also provide a molecular basis for its potential application in the prevention and therapy of atherosclerosis.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

DZ, conceived the study and designed the experiments. HT, performed the experiments. HT, analyzed the data, HL and HB carried out the statistical analysis, LS prepared figures. HT, wrote the original draft. DZ, reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

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