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Research Article

Ginsenoside Rg1 ameliorates chronic intermittent hypoxia-induced vascular endothelial dysfunction by suppressing the formation of mitochondrial reactive oxygen species through the calpain-1 pathway

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ABSTRACT

Background: As the major pathophysiological feature of obstructive sleep apnea (OSA), chronic intermittent hypoxia (CIH) is vital for the occurrence of cardiovascular complications. The activation of calpain-1 mediates the production of endothelial reactive oxygen species (ROS) and impairs nitric oxide (NO) bioavailability, resulting in vascular endothelial dysfunction (VED). Ginsenoside Rg1 is thought to against endothelial cell dysfunction, but the potential mechanism of CIH-induced VED remains unclear.
Methods: C57BL/6 mice and human coronary artery endothelial cells (HCAECs) were exposed to CIH following knockout or overexpression of calpain-1. The effect of ginsenoside Rg1 on VED, oxidative stress, mitochondrial dysfunction, and the expression levels of calpain-1, PP2A and p-eNOS were detected both in vivo and in vitro.
Results: CIH promoted VED, oxidative stress and mitochondrial dysfunction accompanied by enhanced levels of calpain-1 and PP2A and reduced levels of p-eNOS in mice and cellular levels. Ginsenoside Rg1, calpain-1 knockout, OKA, NAC and TEMPOL treatment protected against CIH-induced VED, oxidative stress and mitochondrial dysfunction, which is likely concomitant with the downregulated protein expression of calpain-1 and PP2A and the upregulation of p-eNOS in mice and cellular levels. Calpain-1 overexpression increased the expression of PP2A, reduced the level of p-eNOS, and accelerated the occurrence and development of VED, oxidative stress and mitochondrial dysfunction in HCAECs exposed to CIH. Moreover, scavengers of $O_2^{\bullet -}$, H_2O_2, complex I or mitoK_{ATP} abolished CIH-induced impairment in endothelial-dependent relaxation.
Conclusion: Ginsenoside Rg1 may alleviate CIH-induced vascular endothelial dysfunction by suppressing the formation of mitochondrial reactive oxygen species through the calpain-1 pathway.

1. Introduction

Chronic intermittent hypoxia (CIH) is the crucial pathophysiological feature of obstructive sleep apnea (OSA), which is a major and underrecognized concern for public health. OSA occurs in 14% of males and 5% of females, and the rate tends to increase with age, especially after middle age [1]. OSA is increasingly considered a primary independent risk factor for the presence of cerebrovascular and cardiovascular diseases (CVDs), including stroke, congestive heart failure, hypertension and atherosclerosis [2]. Endothelial dysfunction and the formation of mitochondrial reactive oxygen species (ROS) play vital and initiating roles in the pathogenesis and progression of the vascular complications induced by CIH [3]. Calpain-1, as the most studied gene product of the calpain family, is a calcium-dependent protease. The activation of calpain-1 augments high glucose-induced vascular endothelial dysfunction (VED) through the eNOS/NO signaling pathway in RAOECs and HAECs [4]. Moreover, inhibition of calpain activity upregulates the eNOS/NO signaling pathway in endothelial cells via suppression of PP2A phosphatase [5]. However, the contribution of the calpain-1/

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PP2A/eNOS signaling pathway to the development of CIH-induced VED is unknown.

Mitochondrial dysfunction has been implicated as a crucial result of increased calpain-1 activity in several disease states [6]. Considering the effect of calpain-1, ROS and the involvement of mitochondrial dysfunction and oxidative stress in CIH-induced endothelial dysfunction [7,8], we hypothesized that calpain-induced mitochondrial ROS (mitoROS) signaling contributes to the development of VED after CIH. A possible target of calpain-1 in the regulation of mitoROS in VED is the mitochondrial ATP-sensitive K (mitoK$_{ATP}$) channel [9]. When activated, this channel facilitates the entry of K^+ into the matrix and reduces the mitochondrial membrane potential, which increases $O_2^{\bullet -}$ production by the mitochondrial electron transport chain (ETC) [10]. Thus, we further hypothesized that calpain-1 mediates VED via a new mechanism involving the activation of mitoK$_{ATP}$ channels, and consequently ROS generation.

Ginseng is a traditional Chinese medicinal herb, and its purified and main active ingredient ginsenoside Rg1 has wide potential therapeutic effects on cardiovascular diseases, including anti-vascular remodeling, antiatherosclerosis, antihypertension, and antimyocardial hypertrophy [11–14]. Recent studies indicated that ginsenoside Rg1 could ameliorate hypoxia-induced vasoconstriction in rats with pulmonary arterial hypertension, protect cerebral mitochondrial function in hypoxia-exposed rats and reduce ischemia/reperfusion injury-induced oxidative stress [15–18]. In addition, ginsenoside Rg1 has protective potential against high glucose-induced endothelial barrier dysfunction in HUVECs and low-shear stress-induced vascular endothelial cell dysfunction in a model of atherosclerosis [12,19]. However, the effect of ginsenoside Rg1 on VED induced by CIH is still unclear. In the present study, we aimed to investigate the effect of ginsenoside Rg1 on CIH-induced VED and its potential mechanisms.

2. Materials and methods

2.1. Reagents

Ginsenoside Rg1 was obtained from Nanjin Jingzhu Biotechnology Company. Assay kits for Cell Counting Kit-8, NO nitrate reductase, dihydroethidium (DHE), MDA, JC-1, and MitoSOX were obtained from Beyotime Biotechnology (Shanghai, China). Antibodies against calpain-1 and β-actin were obtained from Proteintech (Wuhan, China). Antibodies against Drp1, Mfn2, PP2A, Ser^{1177} eNOS phosphorylation and endothelial nitric oxide (eNOS) were obtained from ABclonal (Wuhan, China). Phenylephrine (PE), acetylcholine (Ach), polyethylene glycol-catalase (PEG-Cat), 5-hydroxydecanoate (5-HD) and okadaic acid (OKA) were obtained from Sigma Aldrich (Shanghai, China). MDL-28170, L-NAME, acetylcysteine (N-acetyl-l-cysteine, NAC), TEMPOL and rotenone were obtained from Selleck (Houston, TX, USA).

2.2. Animal experiments

All animal procedures of this study were performed under the Animal Experimentation Ethics Committee of Jinzhou Medical University. Forty male wild-type C57BL/6 mice (obtained from Liaoning Changsheng Biotechnology Co. Ltd) were randomly assigned to four groups: the control group (Con), chronic intermittent hypoxia group (CIH), CIH + ginsenoside Rg1 low-dose group (Rg1 L, 10 mg/kg/d) and ginsenoside Rg1 high-dose group (Rg1 H, 20 mg/kg/d). Twenty male mice deficient in μ-calpain (Capn1 EK684$^{-/-}$; Cyagen Biosciences Inc.) were randomly divided into two groups: the control group (CK) and the chronic intermittent hypoxia group (MK). Mice subjected to CIH were placed into the cages of automated, computer-controlled O_2 concentration exchange systems. The mice were exposed to 20 hypoxic events/h for 8 h/day during the light period (9 a.m.–5 p.m.). During each hypoxic event, the FiO_2 was decreased from 21% to 10% over a 90-s period and then restored to 21% during the following 90 s [20]. Mice from the control group were placed into cages with normoxic gas under the same conditions. During the CIH interval, ginsenoside Rg1 groups received ginsenoside Rg1 (suspended in 5% sodium carboxymethyl cellulose, 10 or 20 mg/kg per day) gavage for 4 weeks. After 4 weeks, mice were anesthetized with 20% urethane by intraperitoneal injection, serum was collected from eye blood, and the thoracic aortas were used for vascular reactivity study, western blotting, immunohistochemistry and immunofluorescence staining.

2.3. Human coronary artery endothelial cell culture and treatment

Human coronary artery endothelial cells (HCAECs) were obtained from BLUEFBIO and cultured in DMEM supplemented with 10% fetal bovine serum (HyClone, Logan, Utah, USA) in an incubator at 37 °C with 5% CO_2. The CIH exposure method was performed as previously described [21]. HCAECs were exposed to 1% O_2 for 5 min followed by 20% O_2 for 5 min at 37 °C for 24 h. Cells were incubated with ginsenoside Rg1 (20 μM), MDL-28170 (calpain-1 inhibitor, 20 μM), okadaic acid (OKA, PP2A inhibitor, 2 μM), NAC (ROS inhibitor, 100 μM) and TEMPOL (mitoROS inhibitor, 1 μM) for 48 h before CIH exposure. To further study the potential mechanism of calpain-1 in the accommodation of VED, the CAPN1-lentiviral vector (pLV-CAPN1, GV367, obtained from Genechem Co., LTD. Shanghai, China) was added to HCAECs for 12 h according to the manufacturer's instructions. Then, the transfected cells were exposed to CIH for another 48 hours. The efficacy of overexpression was verified by western blot analysis.

2.4. Immunohistochemistry and immunofluorescence staining

Immunohistochemical staining of thoracic aortas was conducted to measure the levels of calpain-1, PP2A and eNOS Ser^{1177} phosphorylation (p-eNOS). Briefly, the collected aortic rings were fixed in 4% paraformaldehyde overnight for paraffin embedding, and subsequently cut into slices. Slices were dewaxed, treated with antigen retrieval, and separately incubated with primary antibodies against calpain-1, PP2A and p-eNOS at 1:100 dilutions overnight at 4 °C. After washing with PBS, slices were incubated with horseradish peroxidase-conjugated secondary antibodies for 20 min at 37 °C. Next, 50 μl of DAB solution was added to each aortic section and stained for 1-5 min. After washing with distilled water, slices were counterstained with hematoxylin for 2 min at room temperature. Slices were mounted, observed and captured under a Leica DMI 3000B microscope. Semiquantitative analysis of the tissue staining was implemented with ImageJ software by counting the percentage of positive cells.

Immunofluorescence staining was performed to measure the level of p-eNOS in aortas and HACECs. Following treatments, the slices and cells were fixed in 4% paraformaldehyde for 30 min, soaked in 0.5% Triton for 20 min and blocked with 5% BSA for 30 min. Then, the slices and cells were incubated with primary antibodies against p-eNOS (1:1000) overnight at 4 °C. Thereafter, the slices and cells were incubated with anti-rabbit FITC for 1 hour at 37 °C in the dark. Nuclear counterstaining was labeled with Hoechst 33342 for 5 min. The images were collected by fluorescence microscopy and analyzed by ImageJ software.

2.5. Measurement of reactive oxygen species production

Intracellular ROS were detected by dihydroethidium (DHE) staining, and mitoROS were assessed by MitoSOX staining in HACECs and aortas according to the manufacturer's instructions. The images were captured using fluorescence microscopy and analyzed using ImageJ software.

2.6. Assessment of MDA and NO

The levels of MDA and NO in the serum and cultured supernatant were detected by the MDA assay kit and the nitrate reductase method according to the manufacturer's instructions.

2.7. Vascular reactivity

Freshly isolated thoracic aortas were rapidly removed and placed in oxygenated ice-cold PBS buffer, gently cleaned of fat and adherent connective tissue, and cut into 2 mm-3 mm lengths. Isolated aortic rings were mounted on a separate wire myograph chamber to detect isometric tension. Each chamber was filled with warmed (37 °C) physiologic salt solution (PSS, pH 7.4, 130 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 1.16 mM $CaCl_2$, 14.9 mM $NaHCO_3$, 0.026 mM EDTA, and 11.1 mM glucose). The aortic rings from all groups were preconstricted with PE (10^{-5} M) before detecting Ach (10^{-10} M-10^{-4} M)-induced vasodilation and precontracted by K-PSS (60 mM KCl in PSS solution) before measuring PE (10^{-10} M-10^{-4} M)-induced vasoconstriction. To determine the role of NO in vasodilation, the aortic rings of all groups were incubated with L-NAME (100 μM) for 30 min before relaxation. To verify the role of PP2A, $O_2^{\bullet -}$, H_2O_2, ROS, mitoROS, complex I and mitoK_{ATP} on CIH-induced VED, aortic rings from the Con group and CIH group were incubated with OKA, tiron, PEG-Cat, NAC, TEMPOL, rotenone and 5-HD for 24 hours before PE-induced vasoconstriction.

2.8. Cell viability assay

A Cell Counting Kit-8 (CCK-8 kit) was used to evaluate the cell viability of HCAECs under different conditions. Cell viability was calculated based on the percentage of the optical density relative to that of untreated controls.

2.9. Detection of mitochondrial transmembrane potential ($\Delta\Psi m$)

The mitochondrial membrane potential of HACECs was detected by JC-1 fluorescence staining according to the manufacturer's instructions. Briefly, the cells were incubated with JC-1 at 10 μg/ml for 15 min at 37 °C, and then the images were observed by fluorescence microscopy. Increased monomer (green) fluorescence levels and decreased J-aggregate (red) fluorescence levels indicated ΔΨm depolarization. All images were analyzed by ImageJ software.

2.10. Western blot

The collected aortic tissue samples and cultured HACECs were homogenized with RIPA lysis buffer. The lysate was centrifuged at 12 000 rpm and 4 °C for 20 min. The supernatant was collected to quantify the total protein concentrations by the BCA method. Protein samples (20 μg) from different groups were separated by 10% SDS–PAGE electrophoresis and then transferred to PVDF membranes. After blocking with 5% nonfat milk for 1.5 h, the membranes were incubated with the following primary antibodies: rabbit anti-calpain 1 (1:1000), rabbit anti-PP2A (1:1000), rabbit anti-Ser^{1177} eNOS (1:1000), rabbit anti-eNOS (1:1000), rabbit anti-Drp1 (1:1000), rabbit anti-Mfn2 (1:1000) and β-actin (1:10000). After overnight incubation at 4 °C, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h. The density of protein bands was quantified by ImageJ software, and the results were normalized to β-actin.

2.11. Statistical analyses

All results are presented as the means ± S.E.M. One-way ANOVA followed by Bonferroni's test was applied to examine differences between the various groups. SPSS 26.0 software was used for all statistical analyses. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Ginsenoside Rg1 and Calpain-1 Knockout Protected Against CIH-induced vascular endothelial dysfunction

Decreased nitric oxide (NO) production, impaired endothelial-dependent relaxation and an enhanced vascular contraction response are linked with endothelial dysfunction. A vascular reactivity study revealed that compared with the control group, Ach-induced vasodilation and PE-induced vasoconstriction were damaged in the aortas of mice exposed to CIH, and all of these effects were restored by ginsenoside Rg1 treatment. The effect of vasodilation in all groups was abolished after the aortas were incubated with L-NAME for 30 min (Fig. 1 (A-D)). Immunofluorescence staining and the nitrate reductase method showed that the levels of p-eNOS and NO were reduced in the aortas of mice exposed to CIH, and all of these changes were reversed by ginsenoside Rg1 treatment (Fig. 1 (E-F)). To further determine whether calpain-1 is involved in the regulation of CIH-induced VED, the above experiments were performed in $Capn1^{-/-}$ mice exposed to CIH. Our results suggested that compared with CIH treatment, knockout of calpain-1 protected against the decreased level of p-eNOS in aortas and NO in the serum of mice exposed to CIH (Fig. 1(G-H)). Furthermore, calpain-1 knockout can ameliorate the impaired Ach-induced vasodilation and PE-induced vasoconstriction in the aortas of mice exposed to CIH, while the effect of vasodilation was eliminated after the aortas were incubated with L-NAME in all groups (Fig. 1(I)).

3.2. Ginsenoside Rg1 and Calpain-1 knockout improved CIH-induced oxidative stress and mitochondrial dysfunction n

To investigate the roles of ginsenoside Rg1 and calpain-1 knockout in the regulation of CIH-induced oxidative stress and mitochondrial dysfunction, DHE staining, MDA assay kits, western blotting and MitoSOX staining were used. DHE staining and MDA assay kits showed that the levels of intercellular ROS and MDA were augmented in the thoracic aortas of mice exposed to CIH, and all of these changes were restored by ginsenoside Rg1 treatment and calpain-1 knockout (Fig. 2(A-B)). Mitochondrial fission and fusion maintain a balance to maintain mitochondrial function in cells. Western blot analysis and MitoSOX staining demonstrated that the levels of Drp1 and mitoROS were augmented, while the level of Mfn2 was decreased in the thoracic aortas of mice subjected to CIH,

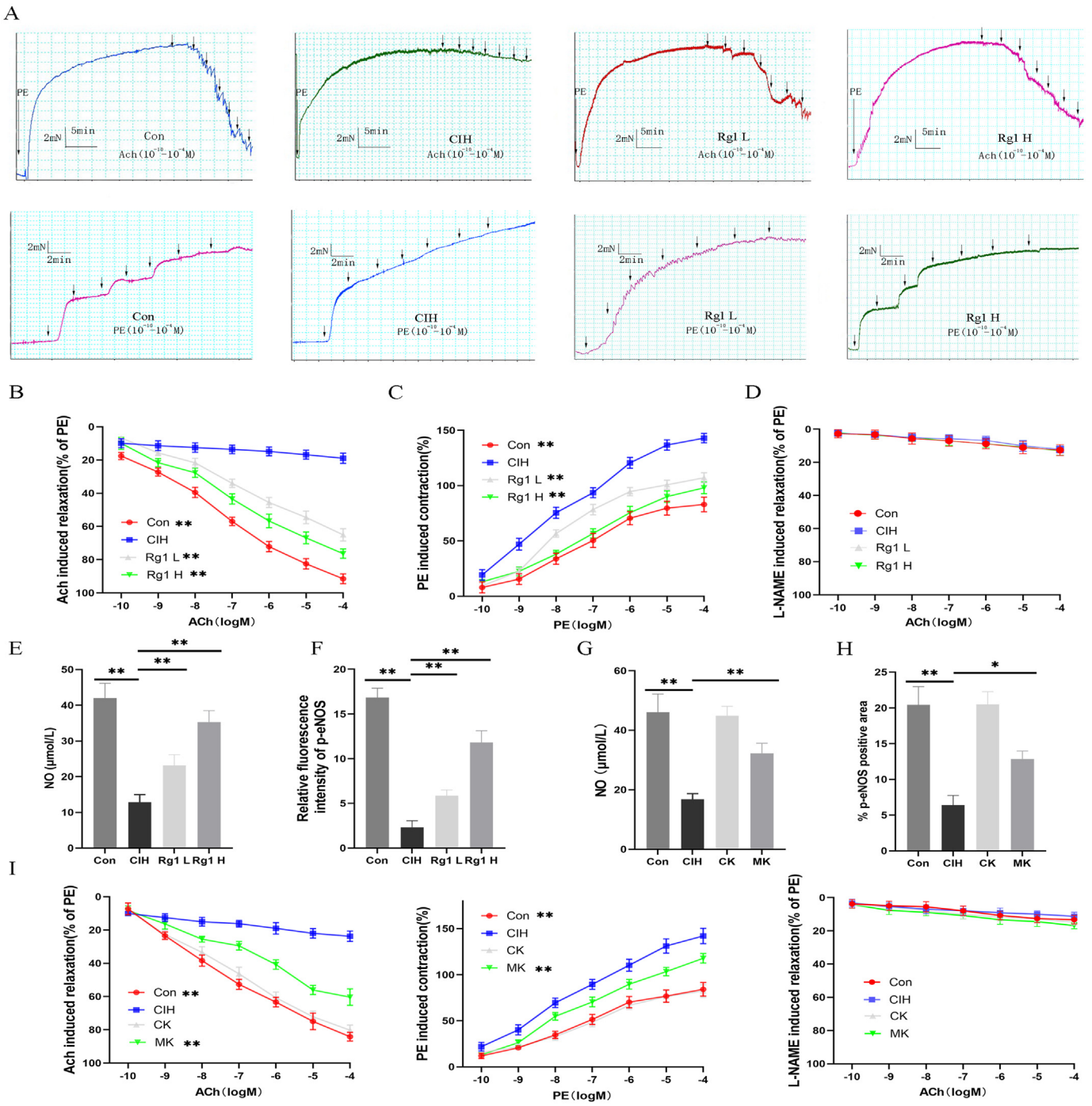

Fig. 1. Ginsenoside Rg1 and Calpain-1 Knockout Protected Against CIH-induced Vascular Endothelial Dysfunction. (A) Representative images of the impaired endothelial-dependent relaxation and vascular contraction response. (B) The effect of ginsenoside Rg1 treatment on Ach-induced vasodilation in aortas of mice exposed to CIH (n = 5). (C) The effect of ginsenoside Rg1 treatment on the vascular contraction response to PE in aortas of mice exposed to CIH (n = 5). (D) The effect of L-NAME treatment on the vasodilation responses of all groups (n = 5). (E, G) Serum NO levels detected by the nitrate reductase method (n = 8). (F) Fluorescence intensity of p-eNOS in aortic sections (n = 3). (H) The percentage of p-eNOS-positive cells was determined by immunohistochemistry staining (n = 3). (I) The effects of calpain-1 knockout on Ach-induced vasodilation, the PE-induced vascular contraction response and the vasodilation responses after incubation with L-NAME in the aortas of mice exposed to CIH (n = 5). Data are presented as the mean ± SD (**P < 0.01 compared with the CIH group).

and these changes were reversed by ginsenoside Rg1 treatment and calpain-1 knockout (Fig. 2(A, C)).

3.3. Ginsenoside Rg1 and Calpain-1 knockout suppressed CIH-induced activation of the calpain-1/PP2A/eNOS signaling pathway in the thoracic aorta of mice

To determine the effects of ginsenoside Rg1 and calpain-1 knockout on CIH-induced activation of the calpain-1/PP2A/eNOS

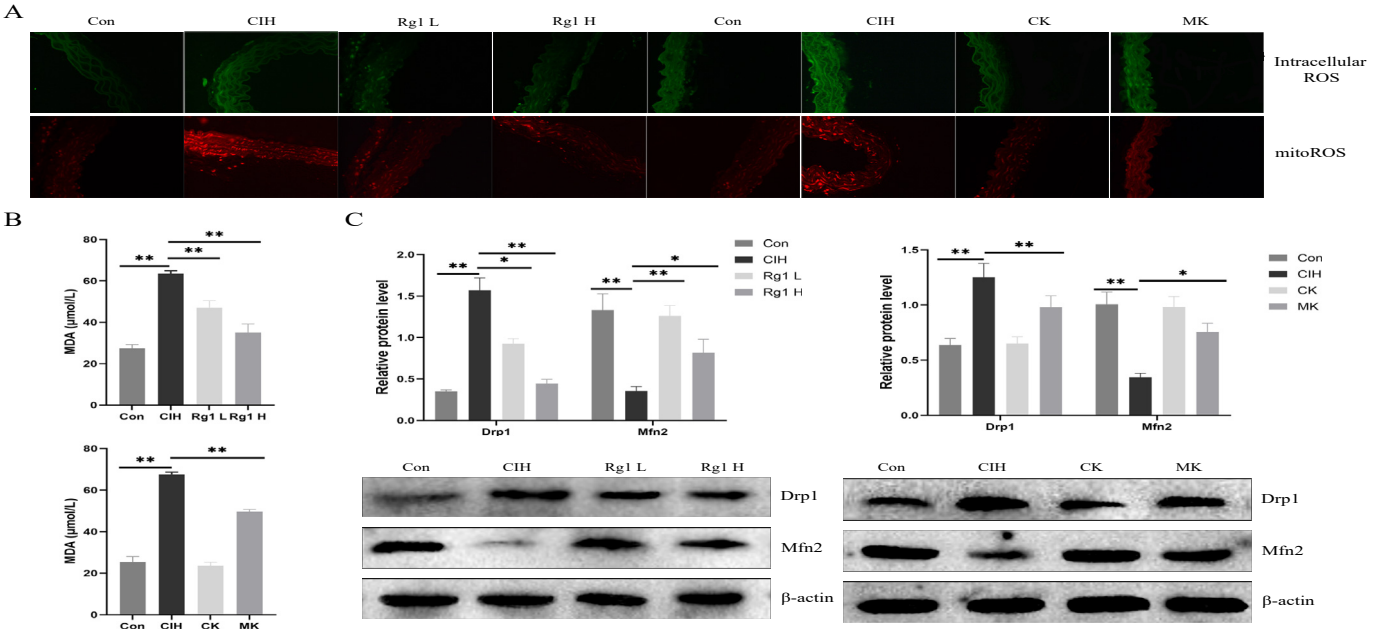

Fig. 2. Ginsenoside Rg1 and Calpain-1 Knockout improved CIH-induced oxidative stress and mitochondrial dysfunction. (A) Representative fluorescence images intercellular ROS and mitoROS in aortas (n = 3). (B) MDA levels in the serum of mice subjected to CIH were determined by an MDA assay kit (n = 8). (C) Drp1 and Mfn2 protein expression in the aortas of mice exposed to CIH was examined by western blotting (n = 3). Data are presented as the mean ± SD (**P < 0.01, *P < 0.05 compared with the CIH group).

signaling pathway, immunohistochemistry staining and western blot experiments were conducted. Our data indicated that compared with the control group, calpain-1 knockout only reduced the calpain-1 expression level in aortas; other indices were not statistically significant. Compared with CIH, calpain-1 knockout reduced the protein levels of calpain-1 and PP2A and increased the level of p-eNOS in the aortas of mice exposed to CIH (Fig. 3(A-B)). Additionally, ginsenoside Rg1 treatment enhanced the protein expression levels and the percentage of cells positive for calpain-1 and PP2A and downregulated the level of p-eNOS in the aortas of mice subjected to CIH (Fig. 3(C-E)).

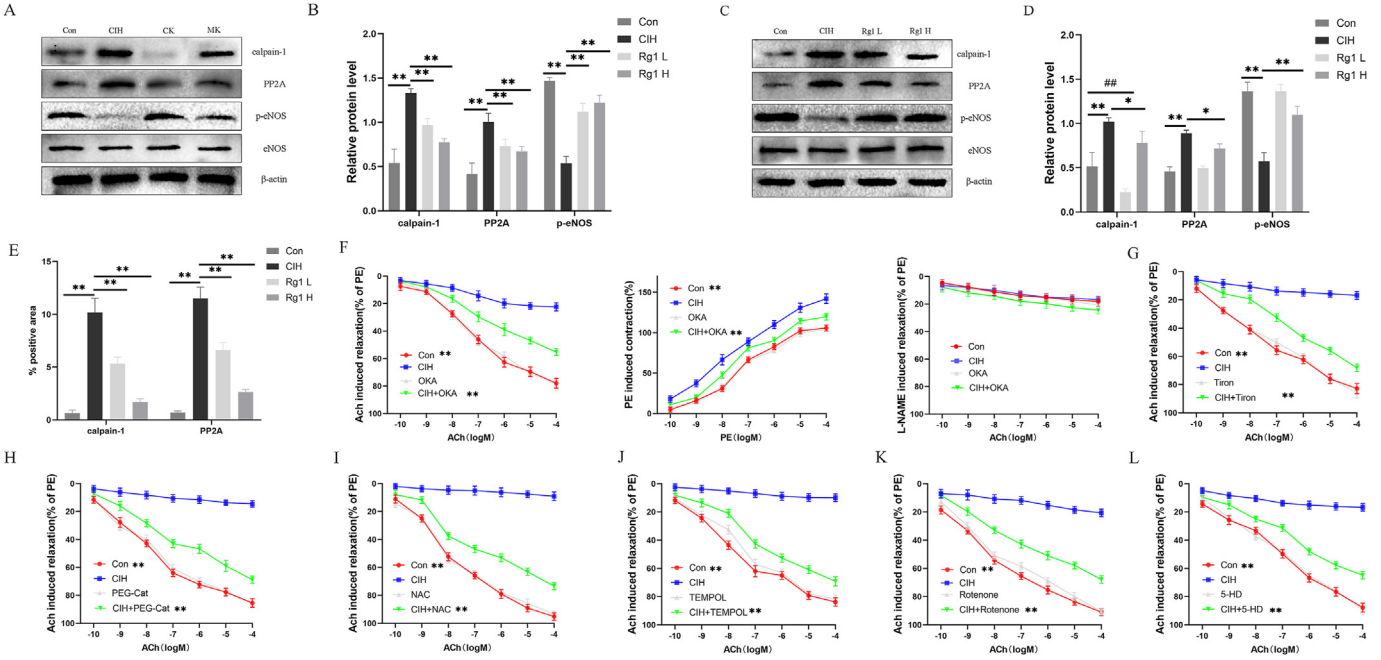

Fig. 3. The activation of the calpain-1/PP2A/eNOS signaling pathway and vascular reactivity studies in aortas of mice exposed to CIH. (A-D) The levels of calpain-1, PP2A and p-eNOS were examined by western blot analysis (n = 3). (E) The percentage of cells positive for calpain-1 and PP2A was determined by immunohistochemistry staining (n = 3). (F) The effects of PP2A treatment on Ach-induced vasodilation, the PE-induced vascular contraction response and the vasodilation responses after incubation with L-NAME in the aortas of mice exposed to CIH (n = 5). (G-L) The effects of tiron, PEG-Cat, NAC, TEMPOL, rotenone and 5-HD treatment on the impaired endothelium-dependent relaxation in aortas of mice with CIH exposure (n = 5). Data are presented as the mean ± SD (**P < 0.01, *P < 0.05 compared with the CIH group; $^{\#\#}P$ < 0.01 compared with the control group).

3.4. Effects of PP2A, $O_2^{\bullet -}$, H_2O_2, ROS, MitoROS, complex I and $MitoK_{ATP}$ on CIH-induced vascular endothelial dysfunction

The roles of PP2A, $O_2^{\bullet -}$, H_2O_2, ROS, mitoROS, complex I and $mitoK_{ATP}$ in CIH-induced VED were examined in the presence of OKA, tiron, PEG-Cat, NAC, TEMPOL, rotenone and 5-HD. The results indicated that compared with the CIH group, OKA treatment improved the damaged Ach-induced vasodilation and PE-induced vasoconstriction in aortas of mice with CIH exposure, while the effect of vasodilation in all groups was removed after the aortas were incubated with L-NAME for 30 min (Fig. 3 (F)). Additionally, treatment with tiron, PEG-Cat, NAC, TEMPOL, rotenone and 5-HD protected against the impaired endothelium-dependent relaxation in the aortas of mice subjected to CIH (Fig. 3 (G-L)).

3.5. Ginsenoside Rg1 improved CIH-induced vascular endothelial dysfunction and oxidative stress through the calpain-1/PP2A/eNOS signaling pathway in HACECs

To test whether ginsenoside Rg1 can improve VED and the activation of oxidative stress induced by CIH through the calpain-1/PP2A/eNOS signaling pathway in HACECs, DHE staining, immunofluorescence staining and western blot analysis were performed. The results suggested that compared with the CIH group, treatment with MDL-28170, OKA, NAC, TEMPOL and ginsenoside Rg1 increased the cell viability, the levels of NO, and the expression level of p-eNOS and degraded the levels of MDA and intracellular ROS in HCAECs subjected to CIH (Fig. 4(A-G)). Meanwhile, MDL-28170, OKA, NAC, TEMPOL and ginsenoside Rg1 treatment degraded the expression of calpain-1 and PP2A protein while upregulating the level of p-eNOS in HCAECs exposed to CIH (Fig. 4(H–K)).

3.6. Ginsenoside Rg1 alleviated CIH-induced mitochondrial dysfunction via the calpain-1/PP2A/eNOS signaling pathway in HACECs

To further confirm whether ginsenoside Rg1 can inhibit CIH-induced mitochondrial dysfunction via the calpain-1/PP2A/eNOS signaling pathway in HACECs, western blot, MitoROS and JC-1 staining experiments were performed. The results showed that compared with CIH, treatment with MDL-28170, OKA, NAC, TEMPOL and ginsenoside Rg1 ameliorated the upregulated mitoROS and protein expression of Drp1 and reversed the downregulated mitochondrial membrane potential and protein expression of Mfn2 in HACECs subjected to CIH (Fig. 5).

3.7. Effect of Calpain-1 overexpression on CIH-induced vascular endothelial dysfunction, oxidative stress and mitochondrial dysfunction in HACECs

To further determine the role of calpain-1 in CIH-induced VED, oxidative stress and mitochondrial dysfunction in HACECs, we introduced pLV-CAPN1 into HCAECs to overexpress calpain-1. After stable transfection, the expression of calpain-1 in the pLV-CAPN1 control group was higher than that in the pLV-NC control group (Fig. 6(A-B)). Compared with the pLV-NC CIH group, pLV-CAPN1 decreased the cell viability, the levels of NO, Mfn2 and enhanced the levels of MDA, intracellular ROS and mitoROS in HCAECs exposed to CIH (Fig. 6(C–K)). Meanwhile, pLV-CAPN1 also upregulated the expression levels of calpain-1 and PP2A protein, while decreasing the level of p-eNOS in HCAECs after CIH exposure (Fig. 6(A-B)).

4. Discussion

CIH, as the major feature of OSA, which is a common sleep breathing disorder, can induce vascular endothelial dysfunction (VED) in vivo and in vitro. VED is an early precipitant of CVD [22]. This impairment mainly manifested as an unusual nitric oxide (NO) endothelium-dependent vasomotion and an augmented response to vasoconstrictors in the vascular bed [23]. In the present study, we found that ginsenoside Rg1 treatment restored the impaired endothelium-dependent vasomotion and degraded the contraction response to PE in the thoracic aorta of mice after 4 weeks of CIH exposure. The promotional effect of ginsenoside Rg1 on impaired endothelium-dependent relaxation induced by CIH was eliminated by L-NAME. Meanwhile, ginsenoside Rg1 treatment increased the cell viability and the levels of p-eNOS and NO in aortas and HCAECs subjected to CIH. These results demonstrated that ginsenoside Rg1 ameliorated CIH-induced VED via the eNOS/NO signaling pathway. In the CIH environment, mitochondrial dysfunction causes the activation of oxidative stress, sets to become a crucial factor in the pathogenesis and progression of VED [24–26]. Mitochondrial fusion and fission regulate the mitochondrial dynamics-related proteins Drp1 and Mfn2 and maintain a balance to maintain mitochondrial function [27,28]. Previous studies have reported that CIH reduces the mitochondrial membrane potential and disrupts the balance of mitochondrial dynamics by decreasing Mfn2 protein and increasing Drp1 protein expression [29,30]. Mitochondrial ROS (mitoROS) can exacerbate ROS production by directly regulating the functionality of mitochondrial electron transport chain (ETC) complexes [31]. The imbalance between oxidizing and antioxidant defense systems contributes to the activation of oxidative stress, which is manifested as enhanced levels of ROS and MDA, causing endothelial activation and eventually leading to VED [32,33]. The occurrence of VED facilitates increased vascular resistance, leading to vascular diseases, including coronary artery disease, cerebrovascular disease and peripheral artery disease [3,34,35]. In our study, ginsenoside Rg1 treatment reversed the downregulated mitochondrial membrane potential in HCAECs and the upregulated level of mitoROS in aortas and HCAECs exposed to CIH. Additionally, ginsenoside Rg1 treatment reversed the reduced expression of Mfn2, the increased expression of Drp1, and the enhanced levels of intracellular ROS and MDA in vivo and in vitro. These data revealed that ginsenoside Rg1 ameliorated CIH-induced oxidative stress and mitochondrial dysfunction.

PP2A is a complex heterotrimeric serine/threonine phosphatase that implicates in many cellular processes, including mitochondrial dysfunction and apoptosis [36–38]. Studies have shown that blocking the expression of PP2A attenuates high glucose-induced ROS accumulation and inflammation [30]. The activation of PP2A directly degrades eNOS phosphorylation at Ser^{1177} and then damages NO bioavailability, which contributes to VED [39]. Our results revealed that CIH accelerates the expression level of PP2A, while ginsenoside Rg1 treatment downregulates the expression of PP2A. Moreover, OKA treatment enhanced cell viability and the levels of NO, Mfn2 and p-eNOS while downregulating the levels of Drp1, intracellular ROS, mitoROS and MDA in HCAECs exposed to CIH. OKA treatment improved the damaged endothelium-dependent relaxation and vascular contraction response, while L-NAME treatment abolished the vasodilation response in all groups. These results suggested that PP2A could be involved in CIH-induced mitochondrial dysfunction, oxidative stress and VED through the eNOS/NO signaling pathway. Calpain-1, as the major member of the calpain family, is a calcium-dependent cysteine protease [40]. Our lab and others have shown that the activation of calpain-1 not only participates in the regulation of diabetes-induced and arsenic-induced VED but also plays a crucial role in mitochondrial

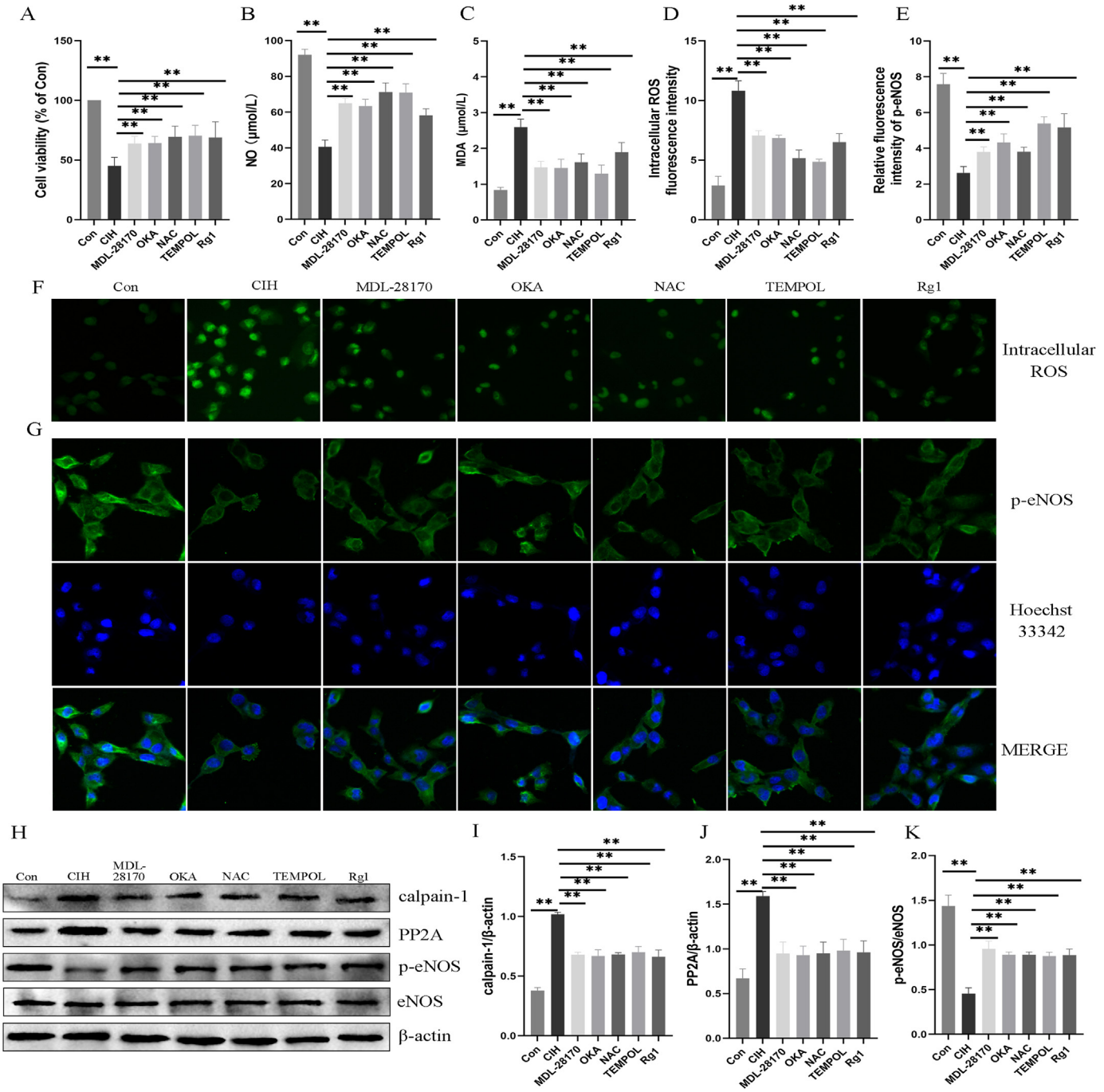

Fig. 4. Ginsenoside Rg1 improved CIH-induced vascular endothelial dysfunction and oxidative stress through the calpain-1/PP2A/eNOS signaling pathway in HACECs. (A) Cell viability of HCAECs in different groups (n = 8). (B) Level of NO in HCAEC supernatant in different groups (n = 8). (C) Level of MDA in HCAEC supernatant in different groups (n = 8). (D-G) Representative fluorescence images and fluorescence intensity of intercellular ROS and p-eNOS in HCAECs exposed to CIH (n = 3). (H–K) Western blotting was conducted to examine the levels of calpain-1, PP2A and p-eNOS in HCAECs exposed to CIH (n = 3). Data are presented as the mean ± SD (**P < 0.01 compared with the CIH group).

dysfunction and oxidative stress [8,41,42]. Meanwhile, the inhibition of calpain upregulates the eNOS/NO signaling pathway through suppression of PP2A phosphatase in endothelial cells stimulated with MPO [5]. These findings indicated that CIH facilitates the protein expression of calpain-1, while ginsenoside Rg1 treatment downregulates the expression of calpain-1. To test whether calpain-1 contributes to VED induced by CIH, calpain-1 knockout mice and HCAECs transfected with a lentiviral vector were used. The results showed that calpain-1 knockout could ameliorate VED, mitochondrial dysfunction, oxidative stress and the augmented level of PP2A and increase the levels of p-eNOS and NO in the aortas of mice exposed to CIH. In addition, the effects of calpain-1 knockout and OKA treatment were similar to those of ginsenoside Rg1 treatment. Overexpression of calpain-1 can augment the expression level of PP2A, degrade the levels of p-eNOS and NO and contribute to the activation of mitochondrial dysfunction, oxidative stress and VED in HCAECs exposed to CIH. These results suggested that ginsenoside Rg1 ameliorated CIH-

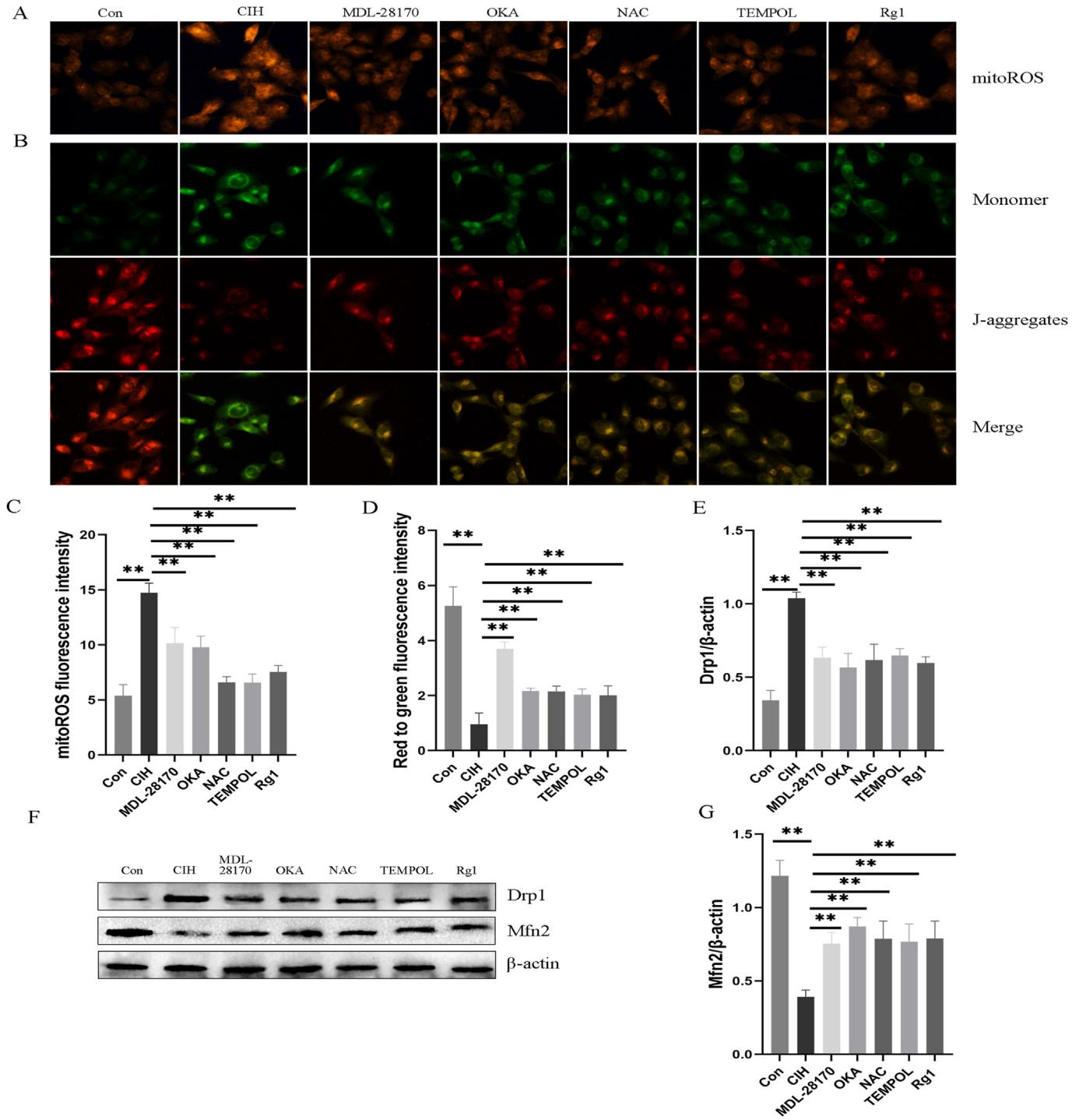

Fig. 5. Ginsenoside Rg1 alleviated CIH-induced mitochondrial dysfunction via the calpain-1/PP2A/eNOS signaling pathway in HACECs exposed to CIH. (A-D) Representative fluorescence images and fluorescence intensity of mitoROS and ΔΨm in HCAECs subjected to CIH (n = 3). (E-G) Western blotting was conducted to examine the levels of Drp1 and Mfn2 in HCAECs exposed to CIH (n = 3). Data are presented as the mean ± SD (**P < 0.01 compared with the CIH group).

induced VED by inhibiting the calpain-1/PP2A/eNOS signaling pathway.

As the starting point of ROS formation, O_2 is reduced to $O_2^{\bullet -}$ through the mitochondrial ETC. Complex I, resided in the mitochondrial inner membrane, as the major member of the mitochondrial ETC, plays a crucial part in the source of ROS [9,43]. $O_2^{\bullet -}$ is also responsible for the formation of other types of ROS in the vascular endothelium, including hydrogen peroxide (H_2O_2) and hydroxyl radicals [44,45]. Mitochondria themselves are the alternative source of ROS that support a primary contribution to VED [46]. The increased mitoROS can exacerbate the production of ROS by directly mediating the function of the ETC. Previous studies suggested that the blocking of mitochondrial K_{ATP} (mitoK_{ATP}) channels prevents angiotensin II-induced endothelial dysfunction [47]. When activated, the channels directly contribute to the entry of K^+ and depolarize the mitochondrial membrane potential, which

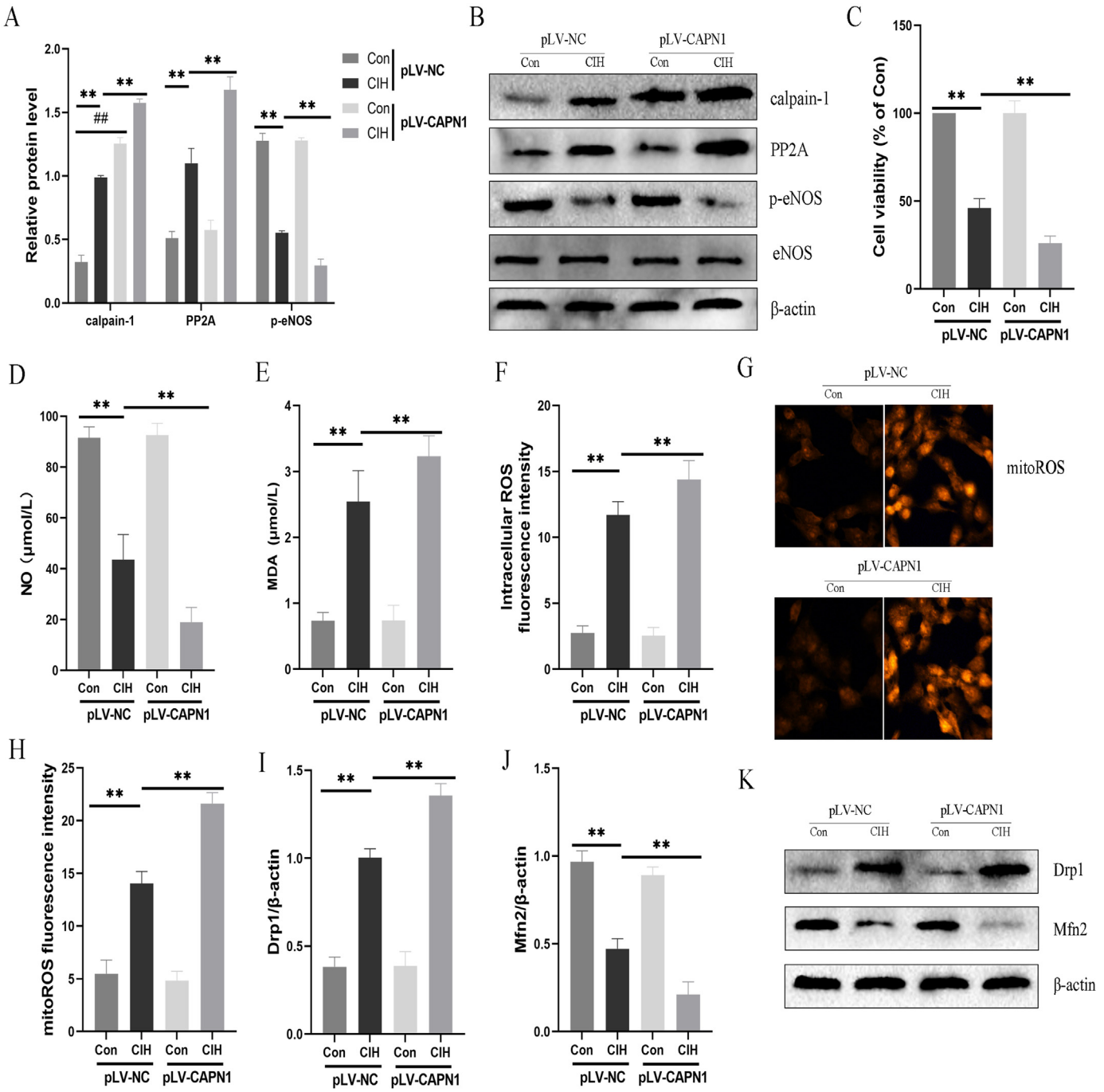

Fig. 6. Effect of calpain-1 overexpression on CIH-induced vascular endothelial dysfunction, oxidative stress and mitochondrial dysfunction in HACECs. (A-B) Western blotting was conducted to examine the levels of calpain-1, PP2A and p-eNOS in HCAECs exposed to CIH (n = 3). (C) Cell viability of HCAECs in different groups (n = 8). (D) Level of NO in HCAEC supernatant in different groups (n = 8). (E) Level of MDA in HCAEC supernatant in different groups (n = 8). (F) Level of intracellular ROS in HCAECs exposed to CIH (n = 3). (G-H) Representative fluorescence images and fluorescence intensity of mitoROS in HCAECs subjected to CIH (n = 3). (I–K) Western blotting was conducted to examine the levels of Drp1 and Mfn2 in HCAECs exposed to CIH (n = 3). Data are presented as the mean ± SD (**$P < 0.01$, *$P < 0.05$ compared with the CIH group; $^{\#\#}P < 0.01$ compared with the control group).

causes mitochondrial matrix swelling and uncoupling and increases $O_2^{\bullet -}$ production by the ETC, subsequently leading to ROS generation [48,49]. Our current findings demonstrated that treatment with tiron, PEG-Cat, NAC, TEMPOL, rotenone and 5-HD protected against the impaired endothelium-dependent relaxation in the aortas of mice subjected to CIH. Furthermore, NAC and TEMPOL treatment increased the cell viability, the levels of p-eNOS and NO, the mitochondrial membrane potential, and the level of Mfn2 and decreased the levels of mitoROS, ROS, MDA and the protein expression of Drp1 in HCAECs exposed to CIH. These effects were similar to those of ginsenoside Rg1 treatment. Taken together, our

data further supported that ginsenoside Rg1 may alleviate CIH-induced VED by suppressing the formation of mitoROS.

In conclusion, although the detailed mechanisms still need to be determined, our work reveals the possible mechanism of action of ginsenoside Rg1 for CIH-induced vascular endothelial dysfunction, at least in part, by suppressing the formation of mitochondrial reactive oxygen species via the calpain-1 pathway. These studies shed a deeper light on the application of ginsenoside Rg1 in the treatment of vascular complications in OSA patients.

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