

RESEARCH ARTICLE

Propofol prevents human umbilical vein endothelial cell injury from Ang II-induced apoptosis by activating the ACE2-(1-7)-Mas axis and eNOS phosphorylation

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Abstract

Angiotensin II (AngII), a vasoactive peptide that elevates arterial blood pressure and results in hypertension, has been reported to directly induce vascular endothelial cell apoptosis. Recent work has demonstrated that propofol pre-treatment attenuates angiotensin II-induced apoptosis in human coronary artery endothelial cells. However, the underlying mechanism remains largely unknown. Here, we investigated human umbilical vein endothelial cells (HUVECs) subjected to angiotensin II-induced apoptosis in the presence or absence of propofol treatment and found that angiotensin II-induced apoptosis was attenuated by propofol in a dose-dependent manner. Furthermore, ELISA assays demonstrated that the ratio of angiotensin (1–7) (Ang (1–7)) to Ang II was increased after propofol treatment. We examined the expression of ACE2, Ang (1–7) and Mas and found that the ACE2-Ang (1–7)-Mas axis was up-regulated by propofol, while ACE2 overexpression increased phosphorylated endothelial nitric oxide synthase (phosphorylated eNOS) expression and siACE2 resulted in the repression of endothelial nitric oxide synthase (eNOS) phosphorylation. In conclusion, our study revealed that propofol can inhibit endothelial cell apoptosis induced by Ang II by activating the ACE2-Ang (1–7)-Mas axis and further up-regulating the expression and phosphorylation of eNOS.

Introduction

The vascular endothelium, a barrier between the vascular wall and the bloodstream, plays an important role in maintaining cardiovascular homeostasis [1]. Endothelial apoptosis is a pivotal risk factor for cardiovascular disease, and participates in the pathological development of

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vascular injury through endothelial dysfunction, directly resulting in cardiovascular dysfunction [2].

The renin-angiotensin-aldosterone system (RAAS) [3, 4] is thought to play a vital role in regulating vascular function and pathological responses to vascular injury. Ang II, a major active peptide of the RAAS, functions in modulating vascular tone and structure [5, 6]. However, AngII has been reported to directly induce vascular endothelial cell apoptosis [7], which aggravates vessel injury by binding the Ang II type 1 receptor (AGTR1) [8, 9]. Recent studies showed that Ang II-induced endothelial apoptosis is related to the stimulation of excessive reactive oxygen species (ROS) production and oxidative stress reactions to reduce the expression of endothelial nitric oxide synthase (eNOS) [10, 11]. eNOS is key to the generation of NO. Known as an 'endothelium-derived relaxing factor', NO produced by eNOS in the vascular endothelium not only maintains constant vascular tension but also has antioxidant, anti-inflammatory and anti-proliferative effects on vascular smooth muscle [12].

Recently, new members of the RAAS such as angiotensin converting enzyme 2 (ACE2) [7, 10, 13, 14], angiotensin (1–7) (Ang (1–7)) and Mas were identified. ACE2 is mainly expressed in endothelial cells and catalyses the conversion of AngII to Ang (1–7), which mediates effects that are opposite to those of AngII, with the consequent binding of Mas [2]. Ang (1–7) functions in vasodilation, as an anti-inflammatory and anti-proliferative agent, and in protecting against ischemia-reperfusion injury [2]. Additionally, Ang (1–7) activates protein-tyrosine phosphatases to suppress the MAPK activation and inhibits the generation of reactive oxygen species (ROS) [11, 15].

Propofol is widely used in clinical anaesthesia and in other fields as a commonly used intravenous anaesthetic. Propofol is structurally similar to the endogenous antioxidant α -Tocopherol (vitamin E) and possesses anti-inflammatory [11, 15–18] properties. Recent research showed that propofol suppresses oxidative stress-mediated endothelial cell dysfunction induced by hydrogen peroxide [19], tumour necrosis factor- α (TNF- α) [5] and ischemia-reperfusion injury [6]. In addition, propofol has been demonstrated to prevent oxidative stress and TNF- α -mediated endothelial cell activation and dysfunction induced by increased generation of endogenous NO [8]. However, it is still unclear whether propofol exerts a protective effect in Ang II-induced endothelial dysfunction. In this study, we prepared HUVEC model with Ang II treatment and investigated the potential mechanisms of propofol against Ang II-induced vascular endothelial cells injury and oxidative stress.

Materials and methods

Reagents and antibodies

Reagents include propofol (Sigma-Aldrich, St Louis, MO, USA), dulbecco's modified Eagle's medium (DMEM), new-born calf serum (NCS), penicillin, streptomycin, trypsin-EDTA (GIBCO Laboratories, Grand Island, New York, USA), dimethylsulfoxide (DMSO), annexin V-FITC apoptosis detection kit, TUNEL detection kit, ELISA kit, and lipofectamine 3000 transfection reagent. Propofol was dissolved in DMSO and further diluted in phosphate buffered saline (PBS). The final DMSO concentration was 0.1%, which did not affect either cell function or the assay system (S1 Fig). The following primary antibodies were used in this study: beta-tubulin polyclonal antibody (Bioworld Technology, AP0064), Bcl-2 antibody (Abcam, ab32124), cleaved-caspase-9 antibody (CST, 7237), caspase-9 antibody (PTG, 66169-1-Ig), eNOS antibody (Bioworld Technology, BS6837), phosphorylated eNOS antibody (Bioworld Technology, BS4848), anti-cytochrome C antibody (Abcam, ab13575), anti-angiotensin converting enzyme 2 (Abcam, ab15348), anti-MAS1 antibody (GeneTex, GTX45868), and AGTR1 antibody (PTG, 66415-1-Ig). The above-mentioned primary antibodies are diluted to

the appropriate concentration ratio (1:500~1:1000) with a proprietary anti-dilution solution (Bio Sharp, BL506A) according to the instructions.

HUVEC culture and sample preparation

HUVECs were obtained from the type culture collection of the Chinese Academy of Sciences, Shanghai, China. Briefly, HUVECs were cultured at 37°C, in a 95% O₂ and 5% CO₂ humidified atmosphere in DMEM supplemented with 20% foetal bovine serum, 100 µg/mL streptomycin, and 100 IU/mL penicillin. For sample preparation, the culture media was replaced with glucose- and serum-free DMEM, with AngII added to the medium at different concentrations (10⁻⁷, 10⁻⁶, 10⁻⁵, 10⁻⁴M), and incubated in a normal incubator with 5% CO₂ and 95% N₂ for 24 h, after which the HUVECs were then returned to the maintenance medium (NCS-DMEM). At the same time, prepared propofol, together with the Mas receptor antagonist A779 and eNOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME), was added to the medium at different concentrations (50 µmol/L-150 µmol/L) for 4 h. We used H₂O₂ as another inducer for apoptosis [17, 19], the proper concentration of which was set to 400 µM (S2 Fig).

Flow cytometry

Cells were treated with angiotensin II for 24 hr, followed by propofol for 4 hr, and on the day of the experiment, they were trypsinized, washed once with ice-cold PBS and stained with annexin V and propidium iodide using a specific kit (BD Biosciences, Franklin Lakes, NJ, USA). Flow cytometry analysis of 20 000 cells per sample was performed on a COULTER EPICS XL-MCLTM flow cytometer (Beckman Coulter, Fullerton, CA, USA) and quantified using EXPO32 ADC analysis software (Beckman Coulter).

TUNEL assay

The presence of death-associated DNA fragmentation was assessed *in situ* by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL). On day two after plating, 50% confluent cells grown on coverslips were incubated with Ang II as indicated. After 24 h, cells were treated with different concentrations of propofol for another 4 h, after which cells were fixed with Triformol for 10 min, and equilibration buffer was directly applied on the cells for 10 min at room temperature. TdT enzyme was mixed in reaction buffer and applied to the cells for 1 h at 37°C. Next, 2×SSC (saline-sodium citrate buffer) was added to the coverslips and incubated at room temperature for 15 min. Following PBS washes, diaminobenzidine (DAB) peroxidase substrate was applied, and cells were counterstained with 6-diamidino-2-phenylindole (DAPI). Cells were photographed on a Leica TCS SP5II microscope.

Measurement of ROS and NO

The level of ROS was determined as oxidative stress. HUVECs were stimulated by Ang II and propofol as described, incubated with DCFH-DA for 40 min at 37°C for measurement. ROS production in HUVECs was detected using a reactive oxygen species assay kit to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). The fluorescence intensity was detected using a fluorescence microplate reader with the wavelength at 525 nm.

HUVECs were treated with Ang II in the absence or presence of propofol. NO production in medium was detected using a nitric oxide assay kit (Nitrate reductase method) according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). The fluorescence intensity was detected with the wavelength at 520-560 nm.

Western blotting

The expression of apoptosis- and ACE2-Ang (1–7)-Mas axis-related proteins were determined by Western blotting. Whole-cell lysates were prepared in RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 1% Triton X-100 plus proteinase inhibitors; Sigma). Protein concentrations were determined using the Bradford assay, and samples containing 30 µg were separated via 12% SDS-PAGE. Proteins were transferred to nitrocellulose membranes, which were then blocked in 5% non-fat milk in 20mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20 for 1 h at room temperature, followed by overnight incubation with different primary monoclonal antibodies at 4°C; β-Tubulin antibody was used as a loading control. The membranes were then incubated with secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature and then exposed to enhanced chemiluminescence reagents. Densitometric analysis was performed to quantify signal intensity.

Measurement of the concentration of Ang II and Ang (1–7)

ELISA assay kit for Ang (1–7) /Ang II was purchased from CUSABIO Company (cat. no. CSB-E04493h). The detection range of human angiotensin 1–7 (Ang1-7) ELISA kit is 7.8pg/ml–500pg/ml, and the minimum detectable dose of human Ang1-7 is typically less than 1.95pg/ml. The detection range of human Ang II ELISA kit is 31.25pg/ml–2000pg/ml, and the minimum detectable dose of human Ang II is typically less than 7.8pg/ml. Cell culture supernates were centrifuged for 15 minutes at 1000 x g at 2–8°C to remove particulates and assay immediately. Standards and samples are pipetted into the antibody pre-coated microplate and incubated for 2 hours at 37°C, after washing, avidin conjugated horseradish peroxidase (HRP) and biotin-antibody are added into the wells and incubated for 1 hour at 37°C. The TMB substrate is added and colour develops in proportion to the amount of Ang (1–7) /Ang II, the absorbance of each sample was measured by enzyme-linked immunosorbent analyser with the readings at 450 nm. The content of Ang II or Ang (1–7) was calculated in accordance with the standard curve.

Transfections and siRNA interference for ACE2

Human ACE2 siRNA (5′ – GAGGAGACUAUGAAGUAAATT–3′), (5′ – UUUACUUCUAUGUCUCCUCTT –3′) and negative control siRNA (GenePharma, China) were transfected using lipofectamine 3000 (Invitrogen, Carlsbad, USA) following the manufacturer's protocol. siRNAs and plasmids were evaluated with western blot (S3 Fig). For ACE2 overexpression, HUVECs were transfected with plasmid DNA carrying the transcriptional target sequence of ACE2 promoter sequences together with a GFP reporter vector used to normalize experimental variations, including transfection efficiency. Cells were harvested 12 h later and lysed for western blotting. All transfections were performed in triplicate, and three independent experiments were similarly performed.

Statistical analysis

Statistical comparisons were made using the Kruskal Wallis test in SPSS V11.0 software. $P < 0.05$ was considered significant.

Results

Protective effects of propofol in AngII-induced apoptosis

To confirm the inhibitory effect of propofol on AngII-induced cell apoptosis, HUVECs were stained with annexin V-FITC and propidium iodide, then analysed by flow cytometry. The

percentage of apoptosis in the control groups was $5.31 \pm 1.15\%$. The fraction of apoptotic cells increased to $34.15 \pm 3.4\%$, $52.9 \pm 13.9\%$, $46.05 \pm 6.3\%$, and $67.5 \pm 3.9\%$ when cells were treated with 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} M AngII, respectively, compared with the control group ($P < 0.05$ versus control group). Propofol treatment resulted in reduced AngII-induced cell apoptosis (Fig 1A–1J). The percentage of apoptotic cells decreased to $11.4 \pm 3.25\%$, $11.7 \pm 2.0\%$, $13.18 \pm 3.0\%$, and $43.4 \pm 2.54\%$ at 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-4} M AngII, respectively ($P < 0.01$ versus AngII group) (Fig 1A–1J). According to these results, we choose 10^{-6} M AngII for subsequent studies. TUNEL assays were performed to find the most effective concentration of propofol against AngII-induced cell apoptosis. (Fig 1K–1Q) The percentage of apoptotic cells was $9 \pm 0.12\%$ and $9 \pm 0.39\%$ in the control and DMSO groups (to exclude the possibility of solvent effects, 0.1% DMSO was added to the medium). Addition of AngII to cells resulted in accelerated cell apoptosis ($91 \pm 0.12\%$). Cell apoptosis decreased in all of the propofol-treated groups compared with the AngII group ($53 \pm 0.12\%$ and $16 \pm 0.05\%$ at 50 and 100 $\mu\text{mol/L}$ propofol, respectively) ($P < 0.01$), except for the 150 $\mu\text{mol/L}$ group ($40 \pm 0.06\%$), indicating that administration of excessive propofol has no extra protective effects.

Propofol inhibits AngII-induced apoptosis by up-regulating the ACE2-Ang (1–7)-Mas axis

AngII can directly lead to vascular endothelial cell apoptosis; however, Ang (1–7) play an opposing role to that of AngII [11]. To determine whether Ang (1–7) is involved in the suppression of AngII induced apoptosis by propofol, we used the Mas receptor antagonist A779 to inhibit Ang (1–7) binding to Mas. We then examined the apoptosis-related proteins by western blotting and found increased levels of cleaved caspase-9, the active form of caspase-9, whereas the anti-apoptotic protein Bcl-2 was down-regulated upon AngII treatment (Fig 2A). However, caspase-9 activation was blocked, and Bcl-2 expression increased in response to increasing concentrations of propofol. Notably, in the presence of A779, the reduction on Bcl-2 and cleaved caspase 3 by propofol were reversed. These data demonstrate that propofol protected AngII-induced apoptosis involves Mas receptor.

The ACE2-Ang (1–7)-Mas axis was recently described as a novel component of the RAAS, in which ACE2 hydrolyses AngII to form Ang (1–7) [2]. To clarify whether propofol up-regulates ACE2 and Ang (1–7) to prevent AngII-induced apoptosis, we monitored the expression of ACE2 and Mas (Fig 2B) after propofol treatment. As expected, AngII treatment resulted in the down-regulation of ACE2, slight decrease in Mas expression and up-regulation of AGTR1, indicating the suppression of Ang (1–7) expression and the activation of AngII-AGTR1 pathway. After propofol treatment, ACE2 levels were up-regulated and the expression of Mas receptor and AGTR1 recovered to a similar level as the control, this effect was blocked by the Mas receptor antagonist A779.

Propofol increases Ang (1–7) levels

The above data suggest that propofol treatment results in ACE2 up-regulation to hydrolyse AngII to form Ang (1–7). To confirm that propofol directly contributes to the generation of Ang (1–7), we performed ELISA to evaluate the AngII/Ang (1–7) ratio after propofol treatment. The AngII group was used to exclude solvent on against apoptosis. As shown in Fig 3, Ang (1–7) was up-regulated after propofol treatment; however, no significant difference was found in AngII expression compared with the control. The ratio of Ang (1–7) to AngII expression was calculated, and the data distinctly reflects a dose-dependent increase in Ang (1–7)/AngII with propofol treatment.

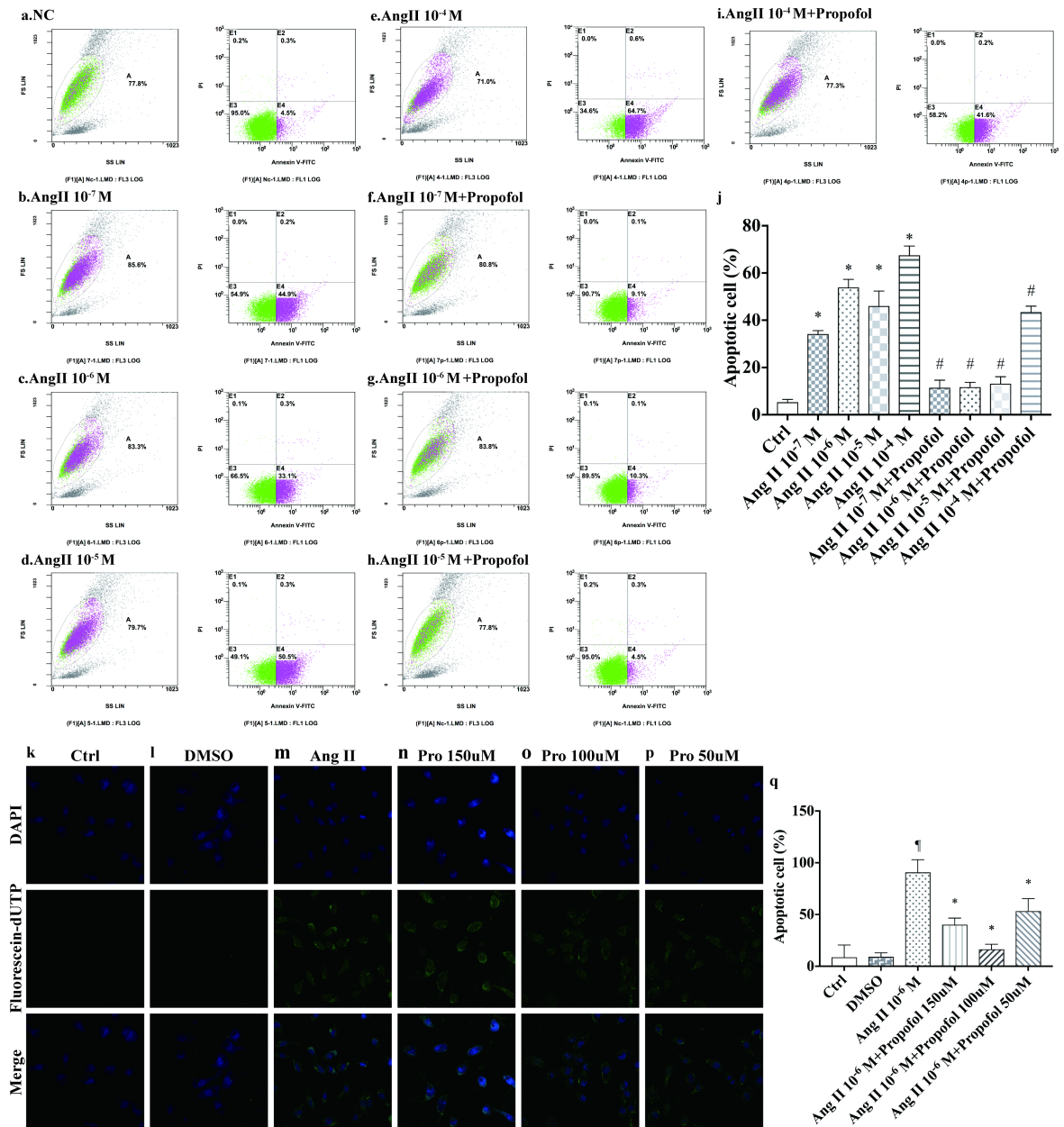


Fig 1. Propofol inhibits AngII-induced apoptosis in HUVECs. (a-j) Detection of apoptosis with Annexin V-FITC and propidium iodide staining. Cells were preconditioned with increasing concentrations of AngII (10⁻⁷–10⁻⁴M) for 24 h and then treated with or without propofol for 4 h. Each group of Annexin V- and propidium iodide-stained cells was measured by flow cytometry. The histogram represents the percentage of apoptotic cells, and the data represent the mean±s.d. of three independent experiments. (n = 3) *P<0.01 versus the control group and # P<0.05 versus corresponding concentration AngII injury groups (k-q). AngII-pre-treated HUVECs were treated with different concentrations (0–150µmol/L) of propofol for 4 h. Cell apoptosis was evaluated via TUNEL assay. Data are from n = 3 independent experiments. ¶versus the DMSO group (P<0.01) and *versus the AngII injury group (P<0.01).

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Propofol inhibited Ang II-mediated oxidative damage

Oxidative stress is accepted as a critical pathogenic factor in endothelial cell dysfunction. In order to elucidate whether propofol contribute to inhibiting Ang II -mediated oxidative damage, we investigated some oxidative stress related indicators such as ROS and NO.

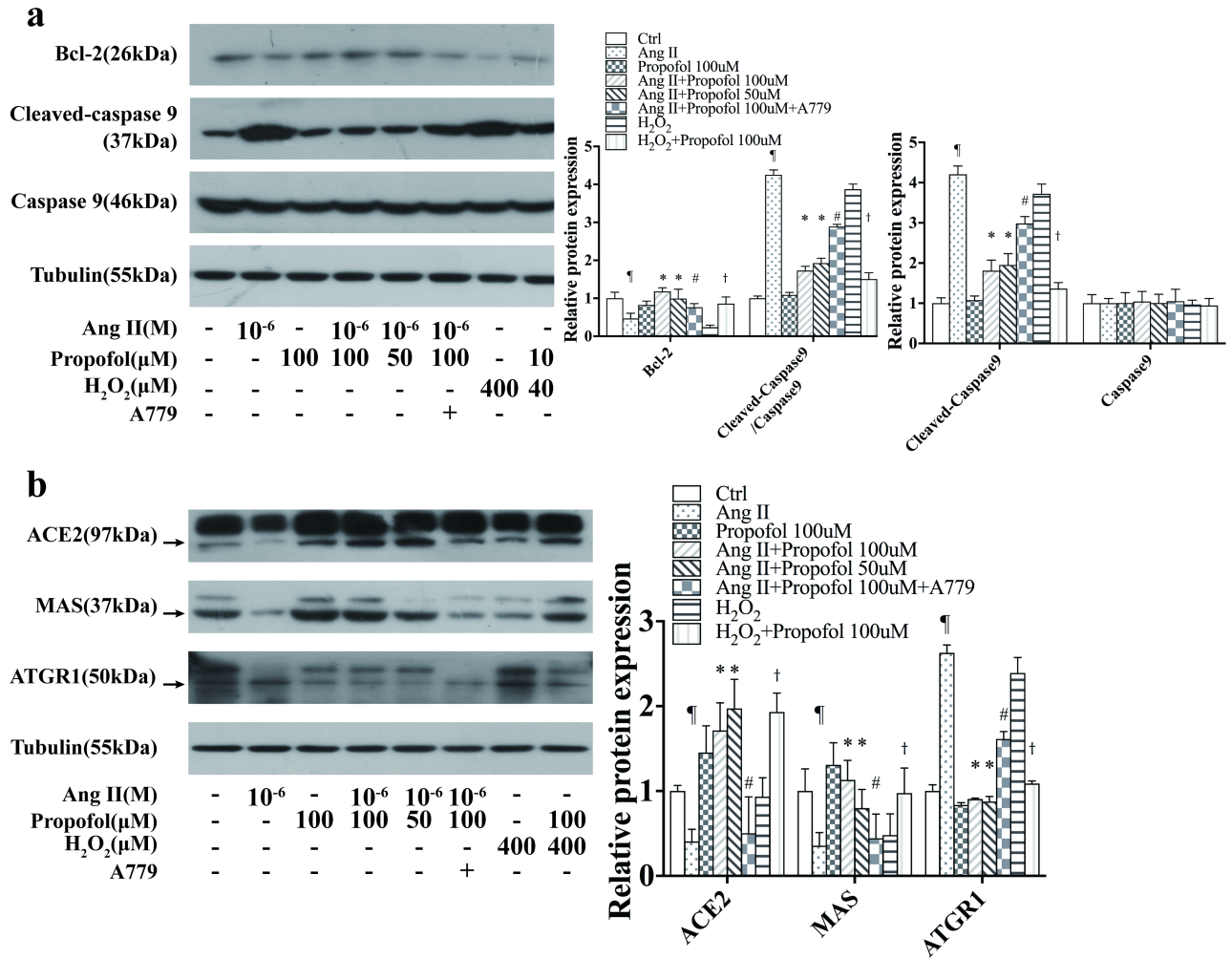


Fig 2. Propofol inhibits AngII-induced apoptosis by up-regulating the ACE2-Ang (1-7)-Mas axis. (a) Western blotting was performed to evaluate the expression of the apoptosis-related proteins caspase-9 and Bcl-2. The expression of cleaved caspase-9 decreased and that of Bcl-2 was significantly increased after propofol treatment for 4 h compared with the control group. After A779 administration, the expression of cleaved caspase-9 was down-regulated and Bcl-2 levels increased. (b) ACE2 and Mas protein levels were up-regulated; however, ATGR1 protein level was decreased in the propofol group compared with the control group. A779 decreased the protein levels of ACE2 and Mas while up-regulated ATGR1 after A779 treatment. The data are representative of six independent experiments, *P<0.01 versus the AngII -treated group, # P<0.01 versus the AngII+propofol 100μM group, ¶P<0.01 versus the control group, and †P<0.01 versus the H₂O₂ -treated group.

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Treatment with Ang II led to a sharp augment in ROS generation (Fig 4A-4I), which was reduced by propofol, whereas the effect was counteracted when A779 is present. By contrast, Ang II treatment resulted in a decrease in NO production compared with control. The addition of propofol reversed the inhibitory effects of Ang II on NO production (Fig 4J). A779 and eNOS inhibitor L-NAME slightly decreased the level of NO. These data suggested that propofol inhibited Ang II -mediated oxidative damage in HUVECs, which might be benefit in attenuating endothelial dysfunction.

Effect of propofol on AngII-induced apoptosis through eNOS phosphorylation

Studies have shown that AngII-induced endothelial apoptosis is mediated by the stimulation of ROS generation to reduce eNOS expression [9, 10]. We examined eNOS and

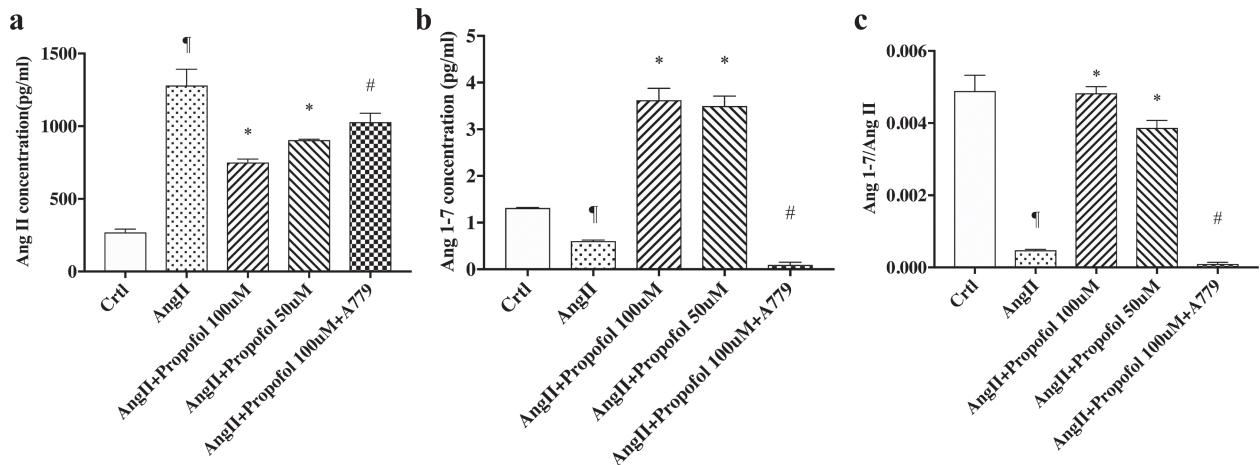


Fig 3. Propofol up-regulates the expression of Ang (1–7), as quantified by ELISA. HUVECs cultured in 96-well plates were treated with AngII for 24 h, followed by treatment with propofol with or without A779 for 4 h. Data are shown as the mean±s.d. from n = 3 independent experiments. (a) The level of Ang (1–7) was quantified by ELISA; *P<0.01 versus the AngII group, and #P<0.05 versus the control group. (b) The level of AngII was quantified by ELISA using a commercial kit. # P<0.05 versus the AngII group. (c) The ratio of Ang (1–7) to AngII expression was calculated and shown as (c). *P<0.01 versus the AngII -treated group, # P<0.05 versus the AngII+propofol 100µM group, and ¶P<0.01 versus the control group.

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phosphorylated eNOS by western blotting and found that the expression of phosphorylated eNOS was up-regulated following propofol treatment, whereas this effect was counteracted by L-NAME or A779 (Fig 5A). This result, therefore, indicated that eNOS phosphorylation may be an important step in the prevention of Ang II-induced apoptosis by propofol and that the ACE2-Ang (1–7)-Mas axis may also play a role in this process.

To clarify the effect of the ACE2-Ang (1–7)-Mas axis on eNOS phosphorylation, we knocked down and overexpressed ACE2 in HUVECs. The construction of the cellular transfection model was confirmed by the expression of GFP in ACE2-GFP via western blotting, and ACE2 expression was decreased in the siRNA-ACE2 group (scrambled- siRNA was used as a negative control) (Fig 5B) and increased in ACE2-GFP cells (Fig 5C). We then examined the expression of caspase-9 and found that cleaved caspase-9 levels increased in the siRNA-ACE2 group compared with the scrambled-siRNA group but decreased in the ACE2-GFP group compared with the GFP group, whereas the effect was counteracted when L-NAME is present. (P<0.05), indicating that ACE2 expression plays an important role in AngII-induced endothelial apoptosis. However, phosphorylated eNOS levels decrease in the siRNA-ACE2 group compared with the scrambled-siRNA group and increased in the ACE2-GFP group compared with the GFP group (P<0.05). The addition of L-NAME inhibited the Promoting effects of propofol on eNOS phosphorylation.

Discussion

Endothelial cell dysfunction could be caused by numerous risk factors and is intimately linked to progression of acute respiratory distress syndrome [20], systemic inflammatory response syndrome, and multi-organ dysfunction syndrome. Hydrogen peroxide (H₂O₂) has been shown to cause severe cell dysfunction [21] in human umbilical vein endothelial cells (HUVECs). Alvarez E and colleagues reported that Ang II induces endothelial cell apoptosis by triggering NADPH oxidase to produce a large amount of ROS [22].

Propofol (2, 6-diisopropylphenol) is an intravenous sedative-hypnotic agent that provides an enhanced antioxidant effect and protection against oxidative damage both in vitro and in

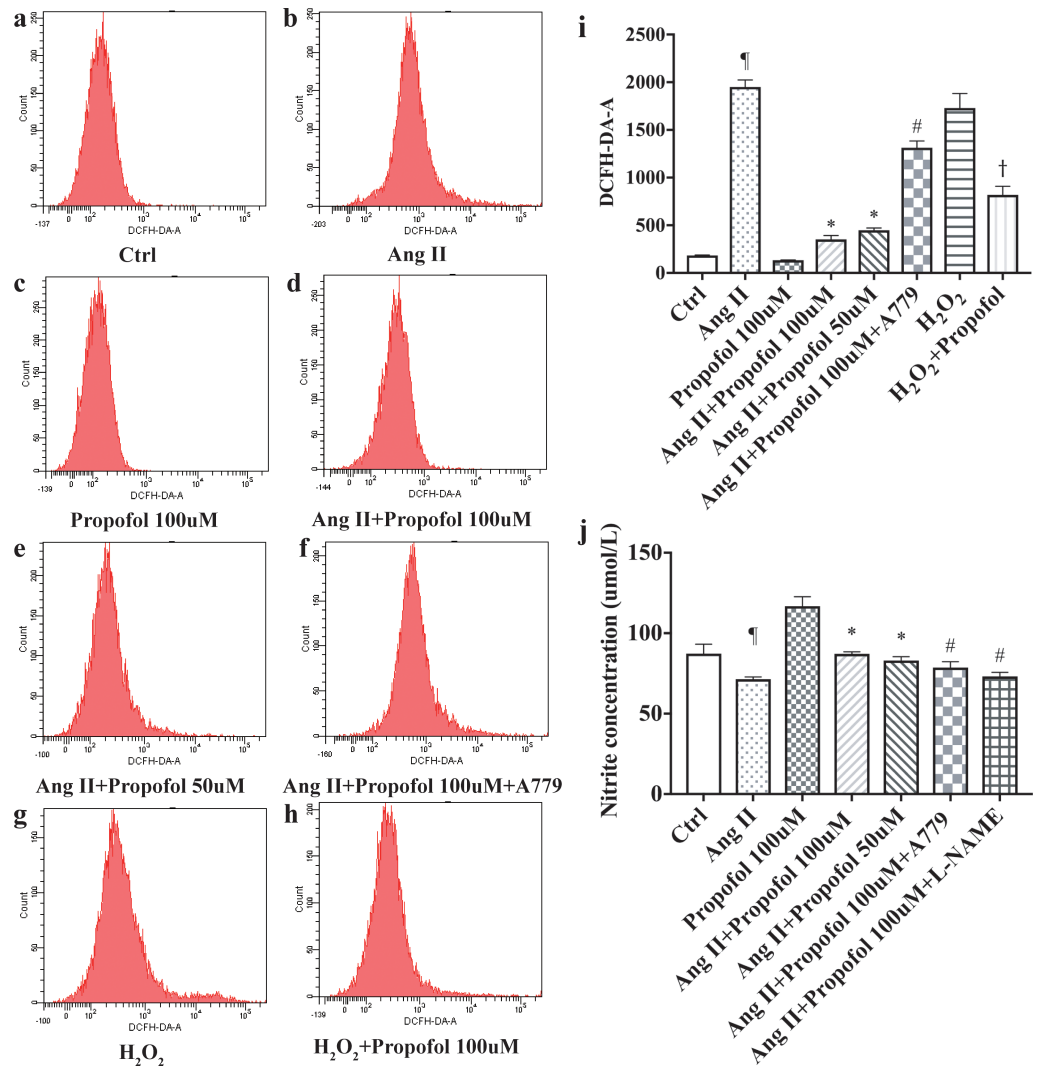


Fig 4. Propofol inhibited Ang II-mediated oxidative damage. (a–h) ROS assay showed that treatment with propofol reduced Ang II-stimulated ROS formation. (i–j) NO assay showed that Addition of propofol exhibited higher NO production. The results are shown as the mean ± SD (n = 3). † p<0.05 compared to the Ctrl group; * p<0.05 compared to the Ang II group; # p<0.05 compared to the Propofol 100uM group; and ‡ p<0.05 compared to H₂O₂ group.

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vivo [19, 23–25]. Chen et al. reported that propofol pre-treatment can attenuate angiotensin II-induced apoptosis in human coronary artery endothelial cells. Therefore, we hypothesized that propofol improves endothelial function and inhibit HUVEC apoptosis subjected to Ang II treatment. Our data demonstrate that propofol significantly inhibited HUVEC apoptosis, which is enhanced by Ang II. These results suggested the mechanism of the protective effect of propofol may be involved in suppressed apoptosis. We further examined the expressions of apoptosis related proteins bcl-2, caspase 9 and cleaved caspase 9 in order to explore the signaling pathway responsible for the protective effect of propofol and found propofol markedly up-regulated the bcl-2 expression and decrease the level of cleaved caspase-9. Thus, propofol may exert its suppressive effects on HUVEC apoptosis via regulation of bcl-2 and caspase-9.

Ang II, the biologically active key effector in the RAAS, is a generally accepted biomarker for predicting the progression of cardiovascular diseases [12]. The newly defined ACE2- Ang

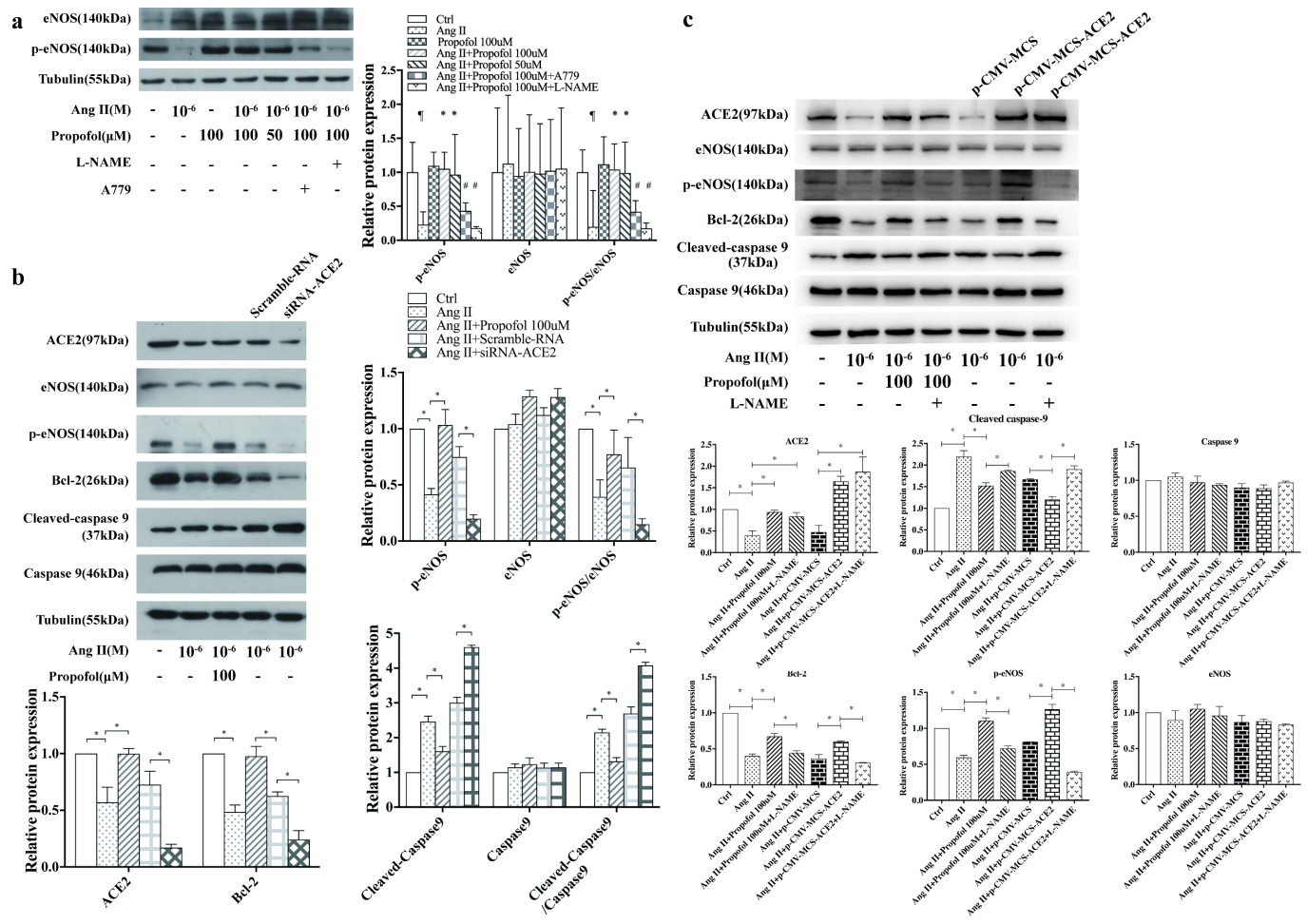


Fig 5. Propofol prevents AngII-induced apoptosis by regulating ACE2 and phosphorylated eNOS. (a) A representative Western blot of eNOS activation in AngII-pre-treated HUVECs among normal (Control), propofol-treated and with or without A779 and L-NAME addition after propofol treatment for 4 h. Following the process described in the Materials and Methods, equal amounts of total protein from each cell sample were subjected to Western blotting using specific antibodies against eNOS and phosphorylated eNOS. β -Tubulin was used to confirm equal protein loading. The results are shown as the mean \pm SD ($n = 3$). ¶ $p < 0.05$ compared to the Ctrl group; $*$ $p < 0.05$ compared to the Ang II group; $\#$ $p < 0.05$ compared to the Propofol 100 μ M group. (b) Expression of phosphorylated eNOS in HUVECs in AngII-treated medium, with or without propofol-treatment. HUVECs transfected with siRNA-ACE2 and scramble-RNA were harvested 48h after transfection. Data are representative of $n = 3$ independent WB experiments; $*$ $P < 0.01$. (c) Expression of phosphorylated eNOS in HUVECs in AngII-treated medium, propofol-treated and with or without L-NAME addition after propofol treatment for 4 h. HUVECs transfected with an ACE2-overexpression plasmid were harvested 48h after transfection. Data are representative of $n = 3$ independent WB experiments; $*$ $P < 0.01$.

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(1-7)-Mas axis was shown to contribute to causing vasodilation, anti-hypertrophy and improving vascular function, exerting the opposite actions of Ang II [26]. We hypothesized that endothelial dysfunction could be attenuated by propofol by modulating the balance of Ang II and Ang (1-7). ELISA assay demonstrated that propofol led to prominent conversion of Ang II to Ang-(1-7), with a reduction in the level of Ang II and an increase in Ang-(1-7), which indicated the recovery of endothelial function. ACE2, highly expressed in the vascular endothelium, efficiently metabolizes Ang II into a putatively protective peptide Ang-(1-7) [27, 28]. Hence, ACE2 has been accepted as a promising therapeutic target for Hypertension. Propofol has been shown to increase ACE2 in earlier study via a PI3K-dependent mechanism [29], so we postulated propofol can increase ACE2 to protect endothelial from dysfunction through catalysing the conversion of Ang II to Ang-(1-7). In the study, propofol improved ACE2 expression and ACE2 activity which were inhibited by Ang II. Besides, Mas expression

was also increased, and AGTR1 was suppressed. Ang II can be activated when interacted with AGTR1 to play a central role in the up-regulation of blood pressure [30, 31]. However, Excessive activation of the ACE-AngII-ATIR pathway can directly cause endothelial injury [22]. Our data suggested propofol protect endothelial from apoptosis by increasing ACE2 to catalyze Ang II to Ang-(1-7), resulting in suppression of the activation of the ACE-AngII-ATIR.

Evidence has shown that oxidative stress is considered to be a critical pathogenic factor responsible for endothelial cell dysfunction and the development of cardiovascular diseases [32, 33]. To inhibit or interfere with ROS generation and reducing ROS accumulation might be the potential ways to protect endothelial cells from injury. Propofol was reported to demonstrate antioxidant effects and shown to exhibit potent H₂O₂ [34] and hydroxyl radical [35] scavenging activity. Since Ang II is a potent activator of oxidative stress, we speculated that propofol treatment may lead to a reduction in oxidative stress to improve endothelial function. Our data demonstrated that propofol effectively attenuates oxidative stress by inducing lots of variation in some oxidative stress indicators in the Ang II-damaged HUVECs. Propofol decreased ROS generation and further stimulated NO production.

Endothelial cells play a role in the regulation of the vascular tone, by ways of generation of vasodilator such as nitric oxide (NO). Propofol was reported to regulate nitric oxide production derived from endothelium [36, 37], but the exact mechanism has not been fully elucidated. Rabelo LA and Alenina N found that Ang II reduces NO activity in endothelial cells by enhancing NADPH oxidase phosphorylation [26]. However, angiotensin-(1-7), by interacting with its receptor Mas, induces the release of NO from endothelial cells and thereby counteracts the effects of angiotensin II. Thus, we hypothesized that propofol up-regulates ACE2/Ang-(1-7)/Mas axis to protect against endothelial injury by modulating the release of NO. In ECs, NO is largely synthesized enzymatically by endothelial nitric oxide synthase (eNOS) [38]. Propofol was reported to increase phosphorylation of eNOS at Ser¹¹⁷⁷ for the NO production, which was involved in PKC activation and intracellular translocation [39, 40]. Thus, we explored the expression of eNOS and P-eNOS, indeed, propofol efficiently increased the expression of P-eNOS and the effect severely blocked by addition of Mas receptor antagonist A779 and L-NAME, suggesting that the NO generation was induced by propofol through eNOS phosphorylation and activation of MAS. To further explore the signalling pathway responsible for MAS activation, plasmids were transfected to knockout or overexpress ACE2. The result revealed that the eNOS phosphorylation was increased in an ACE2 dependent manner, and the up-regulation of ACE2 is responsible for the protective effect of propofol against epithelial apoptosis.

In conclusion, our study revealed that propofol significantly attenuates Ang II-induced endothelial dysfunction. The mechanism involved the up-regulation of ACE2-Ang (1-7)-Mas axis, subsequent phosphorylation of eNOS to generate NO, and regulation of apoptosis-related protein such as bcl-2, caspase9. The present study provides new insights into propofol's protective effect against endothelial dysfunction as well as its therapeutic potential for hypertension.

Supporting information

S1 Fig. The effects of DMSO on HUVECs apoptosis. Cells were treated with or without 0.1% DMSO for 4h, and then cleaved-caspase 9, caspase 9, Bcl-2 were detected by Western blot analysis. The results are shown as the mean \pm SD (n = 3). (TIF)

S2 Fig. Propofol promoted HUVECs proliferation. The cell viability was measured by CCK8 assay. HUVECs were incubated with different concentration of H₂O₂ for 4 h. The results are

shown as means \pm SD (n = 3). * P < 0.01, compared with control.
(TIFF)

S3 Fig. siRNAs and plasmids were evaluated with western blot. Western blot showed ACE2 protein expression which was knockout or overexpressed. The results are shown as means \pm SD (n = 3). * P < 0.01, compared with control.

(TIF)

S4 Fig. Propofol increased ACE2 expression in a time-dependent manner. Western blot showed ACE2 protein expression which was treated with propofol in AngII injured HUVECs. The results are shown as means \pm SD (n = 3). # P < 0.01, compared with control; * p < 0.05 compared to the Ang II group.

(TIF)

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