

# Cyclosporin-A reduced the cytotoxicity of propranolol in HUVECs via p38 MAPK signaling

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## Abstract

Propranolol (PROP) is a nonselective  $\beta$ -adrenergic receptor antagonist used to treat hypertension and cardiac arrhythmias. Oral administration of PROP has recently emerged as a new treatment modality for hemangiomas. However, the side effects of PROP at the cellular level have not been adequately described.

The present study investigates and highlights the mechanisms of coupling of the drugs cyclosporin-A (CyA) and PROP on cell proliferation and the occurrence of apoptosis. It also relays the antioxidant effect of PROP on human umbilical vein endothelial cells (HUVECs).

HUVECs were treated with CyA and PROP. At 24 hours after treatment, the levels of reactive oxygen species (ROS), cell proliferation, and apoptosis were determined using the ROS kit, MTT assay, and Annexin V staining. In addition, the related proteins of phospho-p38 mitogen-activated protein kinase were determined by western blotting. Subsequently, HUVECs pretreated with CyA or PROP were treated with the p38 inhibitor (SB203580). Finally, the ROS level, cell proliferation, and apoptosis were measured again in both active HUVECs and HUVECs, in which the p38 proteins were inhibited.

The combination of CyA and PROP reversed the effect of CyA on cell viability, reduced the ROS level and the cell apoptosis induced by PROP. Moreover, inhibition of p38 protein catalase activity immediately stopped the effect of CyA–propranolol in HUVECs.

The effect of the CyA–propranolol combination on HUVECs is associated with the p38 pathway changes, which is proven to be a potential chemotherapeutic agent that minimizes the side effects of PROP in hemangioma therapy.

**Abbreviations:** CyA = Cyclosporin-A, HUVECs = human umbilical vein endothelial cells, MAPKs = mitogen-activated protein kinases, PROP = propranolol, PROP–CyA = propranolol supplemented with 10  $\mu$ g/mL CyA, PROP–CyA–SB = propranolol supplemented with 10  $\mu$ g/mL CyA and p38MAPK specific inhibitor, ROS = reactive oxygen species.

**Keywords:** cyclosporin-A, hemangioma, human umbilical vein endothelial cells, p38, propranolol

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

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## 1. Introduction

Propranolol (PROP) is a nonselective  $\beta$ -blocker (beta-blocker) used to treat many cardiovascular diseases like hypertension and arrhythmias. Recently, it was suggested that beta-blockers could also be used in hemangioma patients.<sup>[1,2]</sup> However, PROP has a reputation for inducing apoptosis of vascular endothelial cells. Its use could cause some adverse effects in patients<sup>[3]</sup>; although the side effects of PROP in patients remain insomnia, fatigue, and general weakness.

Cyclosporine (cyclosporin-A, CyA) is a cornerstone of immunosuppression and a potent immunosuppressant<sup>[4]</sup>; it inhibits T-cell proliferation by deflecting signal transduction of calcium-dependent signaling pathways in leukocytes, thereby knocking down calcineurin.<sup>[5]</sup> On the other hand, a study on isolated mouse liver mitochondria showed that CyA also slows down the exit of cytochrome C from mitochondria and blocks the reorganization of their cristae structure.<sup>[6]</sup> Although most studies focused on the effect of CyA on T cells, few reports have claimed that CyA may have different biological effects in different cell types, including endothelial cells,<sup>[7]</sup> vascular smooth muscle cells,<sup>[8]</sup> cardiac myocytes,<sup>[9]</sup> and tumor cells.<sup>[10]</sup> The functional role of CyA in cell proliferation, survival, and apoptosis depends largely on its concentration. The signal transduction pathway by which CyA might stimulate apoptosis of HUVECs has not been fully elucidated. It is known that CyA normally blocks the enzyme activities of phosphatases and calcineurins by preventing nuclear translocation of nuclear factor of activated T cells. A recent study suggested that CyA plays a protective role for

microvascular endothelial cells; yet, the understanding of its mechanism also remains unclear.<sup>[11]</sup>

Mitogen-activated protein kinases (MAPKs) regulate various cellular reactions by relaying signals from the extracellular to the intracellular medium, leading to cellular responses to external stresses. In mammals, the MAPK3/MAPK1 (ERK1/ERK2) pathway regulates cell proliferation, differentiation, angiogenesis, and embryonic development.<sup>[12]</sup> However, a recent study suggested that CyA may activate the MAPK3/MAPK1 pathway in different types of cells.<sup>[13]</sup> Based on these data, we hypothesize that combined treatment of CyA and PROP might reduce the cytotoxicity of PROP on vascular endothelial cells. This study investigated the combined effect of CyA and PROP on the proliferation and apoptosis of HUVECs, and its underlying mechanism was discussed. The results showed that the combined treatment of CyA and PROP increased cell proliferation and inhibited cell apoptosis in HUVECs. At the same time, phospho-p38 mitogen-activated protein kinase was involved in the protective effect induced by CyA in HUVECs. Moreover, we demonstrated that CyA promoted the proliferation of HUVECs in vitro by activating the MAPK3/MAPK1 signaling pathway and inhibiting the apoptotic side effects induced by PROP.

## 2. Methods

### 2.1. Chemicals and antibodies

PROP and CyA were purchased from Sigma Aldrich (NY). SB203580 was purchased from Selleck (Houston, TX). MTT and reactive oxygen species (ROS) detection kits were purchased from Beyotime (Shanghai, China). An apoptosis detection kit was purchased from Sigma Aldrich (NY). Anti-c-caspase3, anti-p-MKK3, anti-p-MKK6, anti-P38MAPK, anti-NQO1, anti-Bax, and anti-GAPDH antibodies were purchased from Abcam (Cambridge, UK). This study does not involve more than minimal risk. All cells and chemicals were obtained from approved researchers and therefore do not require ethical review.

### 2.2. Culture of HUVECs

HUVECs were purchased from ATCC (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, USA). The culture flasks were incubated at 37°C in a humidified atmosphere with 10% CO<sub>2</sub> in the air.

### 2.3. Western blot analysis

After incubation, cellular proteins were recovered by radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Haimen, China) for western blot analysis. The BCA method (BCA Protein Assay Kit, Beyotime Company, China) was used to determine the protein concentration of the supernatant. The supernatants were then resolved on polyacrylamide gels with SDS and transferred to a nitrocellulose membrane. The PVDF membrane was incubated in blocking buffer (TBS containing 5% skim milk and 0.1% Tween20) for 2 hours, then incubated with the primary antibody diluted in the same buffer. The specific secondary antibody was detected with peroxidase-conjugated anti-IgG at 1:2000. Relative proteins were detected using the

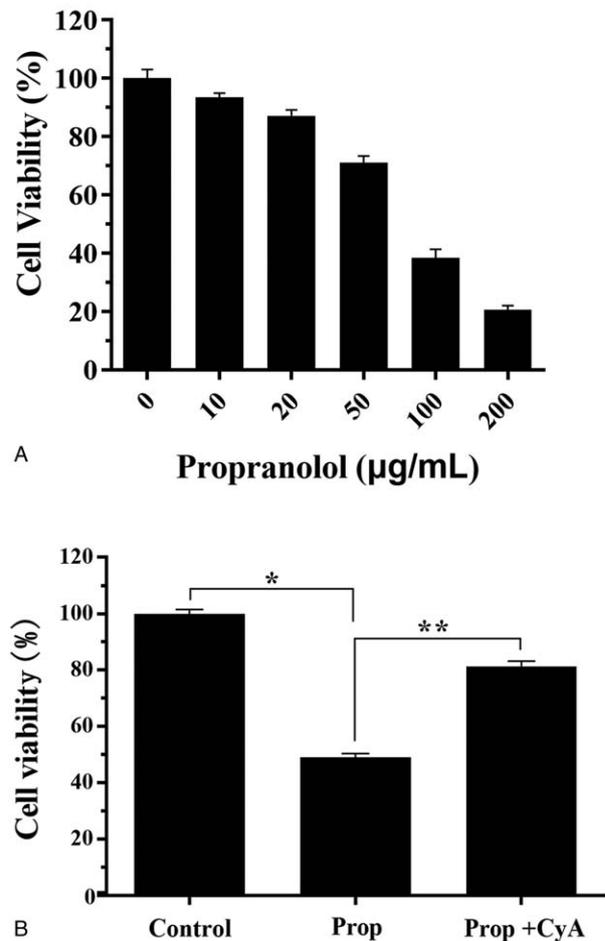
super signal chemiluminescence system (ECL, Pierce) and then exposed to autoradiography film.

### 2.4. Apoptosis assay

We used an Annexin V Apoptosis Detection Kit (BD Biosciences, Rockville, MD) to monitor the apoptosis assay. HUVECs were grown to 70% confluence, treated with PROP and CyA with or without SB203580 for 24 hours, and then collected. According to the manufacturer's instructions, cells were stained with Annexin V-FITC and propidium iodide (PI), using untreated cells as a double staining control. Finally, cell analyzes were performed immediately using a FACS flow cytometer. At least 20,000 cells were counted for each measurement.

### 2.5. Measurement of reactive oxygen species

According to the manufacturer's protocol, ROS content was measured using dichloro-dihydrofluorescein diacetate. In brief, cells were incubated with dichloro-dihydrofluorescein diacetate



**Figure 1.** CyA reduced propranolol-induced growth inhibition. (A) HUVECs were treated with DMSO (control) or propranolol (10–200 µg/mL) for 24 hours. Cell viability was measured by MTT assay, and results were expressed in the percentage of viable cells. (B) HUVECs were treated with propranolol or CyA (10 µg/mL) combined with propranolol (82.277 µg/mL) for 24 hours. Cell viability was measured by MTT assay. \* $P < .05$  and \*\* $P < .01$ . DMSO = dimethyl sulfoxide, HUVECs = human umbilical vein endothelial cells.

(final concentration of 10  $\mu$ M) at room temperature in the dark for 30 minutes, washed with cold Hank balanced salt solution (pH 7.2), and immediately analyzed using a CLARIO star multifunction fluorescent enzyme labeling instrument.

### 2.6. MTT assay

Cells (3000 per well) were cultured in DMEM medium (10% FCS, 1% Pen/Strep) in 96-well plates and grown overnight at 37° C. The next day, the indicated concentrations of the drugs were added to the wells. After 24 hours of treatment, cell proliferation was determined by MTT assay according to the manufacturer's instructions. In brief, MTT solution (5 mg/mL in PBS) was added to each well and incubated for an additional 30 minutes. The formazan product was analyzed spectrophotometrically (570 nm) after dissolved in 100  $\mu$ L dimethyl sulfoxide (DMSO). Cell counts were calculated using a standard curve constructed between the cell count and the OD570 value.

### 2.7. Statistical analysis

Results are reported as mean  $\pm$  SE. Statistical analysis was performed using Student *t* test or one-way ANOVA. *P* < .05 was considered statistically significant.

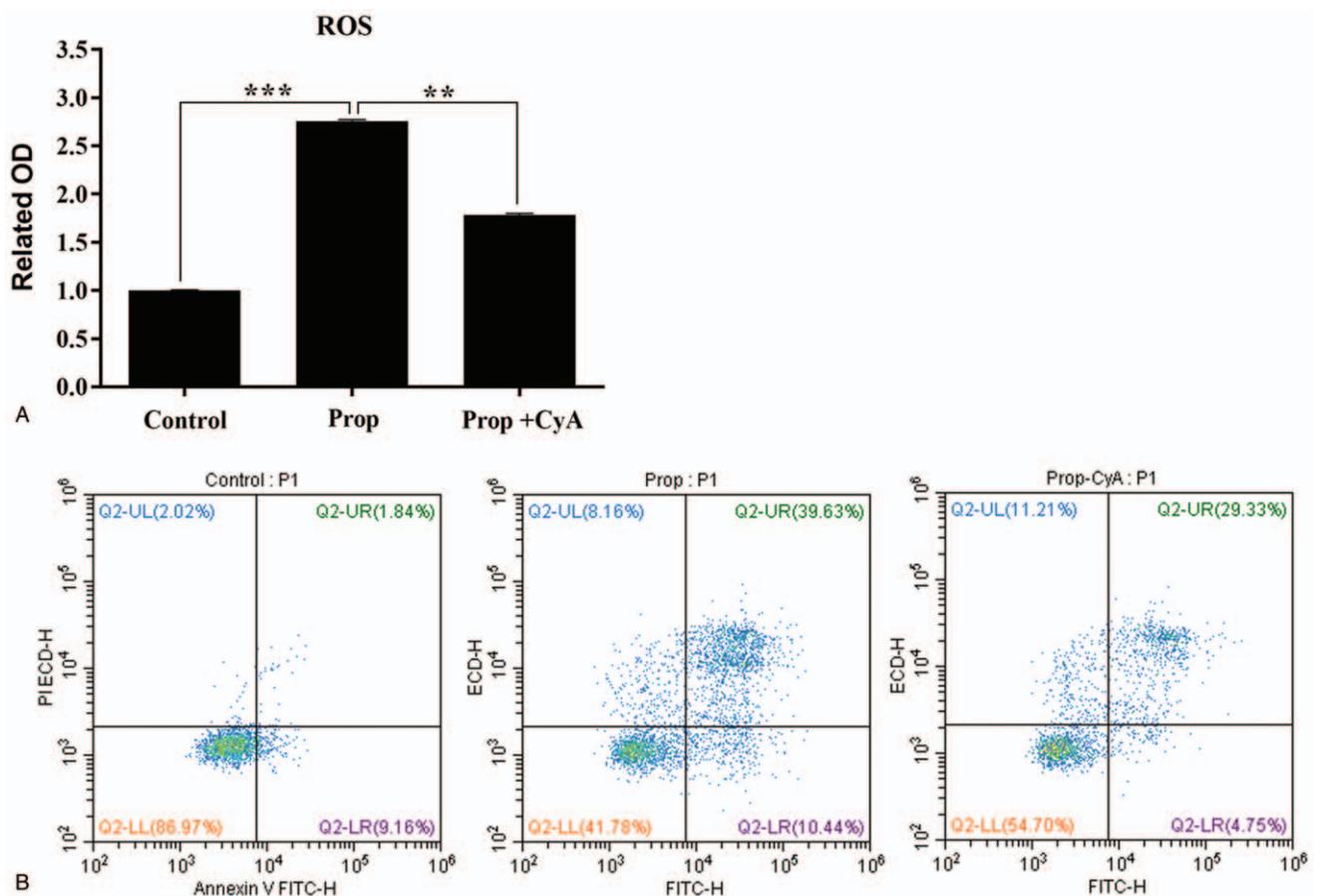
## 3. Results

### 3.1. CyA reduced PROP-induced growth inhibition

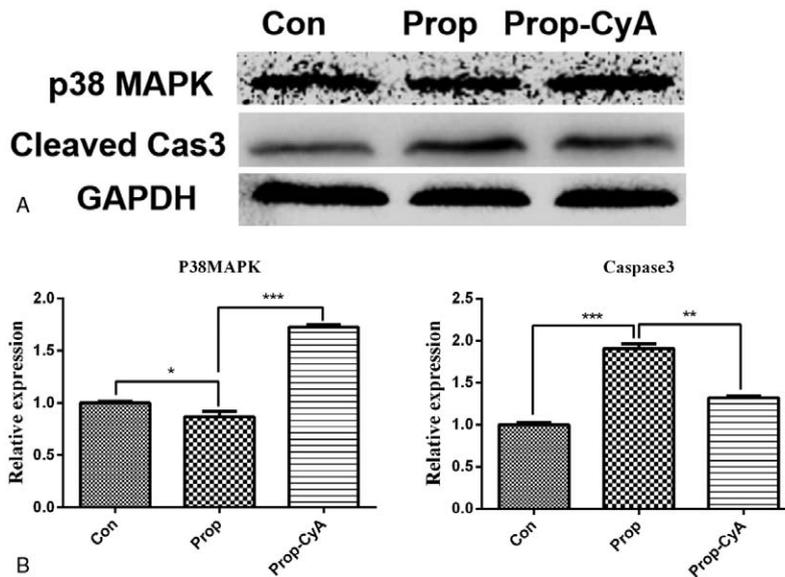
We first examined the effects of PROP on endothelial cell proliferation using MTT analysis. HUVECs were treated with various concentrations of PROP (10–200  $\mu$ g/mL) for 24 hours, and the inhibition rate of the cells was calculated. As shown in Fig. 1A, the cell viability of HUVECs treated with PROP decreased sharply with increasing doses, with the maximum reduction index at 24 hours and the IC<sub>50</sub> value at 82.277  $\mu$ g/mL, as shown by the MTT results. To further elucidate the inhibition rate of cells, HUVECs were treated with 82.277  $\mu$ g/mL free PROP and 82.277  $\mu$ g/mL PROP supplemented with 10  $\mu$ g/mL CyA (propranolol supplemented with 10  $\mu$ g/mL CyA [PROP–CyA]) for 24 hours. Then, the untreated cells were used as control, and the inhibition rate of the cells was calculated. Our results showed that HUVECs treated with PROP–CyA had higher cell viability than PROP (Fig. 1B).

### 3.2. Propranolol–CyA combination decreased propranolol-induced mitochondrial oxidative stress in resistant cells

We examined intracellular ROS production to investigate the existing relationship between CyA and PROP on HUVECs. The



**Figure 2.** CyA decreases propranolol-induced mitochondrial oxidative stress in HUVECs. (A) The level of ROS of HUVECs treated with propranolol (82.277  $\mu$ g/mL) and CyA (10  $\mu$ g/mL) for 24 hours was detected. (B) Cells were treated with DMSO (control), propranolol, and CyA (10  $\mu$ M) for 24 hours. Apoptosis assay using flow cytometry after staining with annexin V-FITC/propidium iodide (PI). Representative scatter plots of PI (Y-axis) versus annexin V (X-axis). \*\**P* < .01 and \*\*\**P* < .001. HUVECs = human umbilical vein endothelial cells, ROS = reactive oxygen species.



**Figure 3.** Effect of CyA and propranolol combination on p38 MAPK pathway. (A) Cells were treated with DMSO (control), propranolol (82.277  $\mu\text{g}/\text{mL}$ ), and/or CyA (10  $\mu\text{g}/\text{mL}$ ) for 24 hours. After treatment, cell extracts were prepared and subjected for Western blot analysis. Expression of p38, c-caspase3 was measured in HUVECs. GAPDH was used as an internal loading control. (B) Intensities of protein signals were normalized to GAPDH. \*\* $P < .01$  and \*\*\* $P < .001$ . DMSO = dimethyl sulfoxide, HUVECs = human umbilical vein endothelial cells, MAPKs = mitogen-activated protein kinases.

results showed that the intracellular production of ROS increased in the groups treated with both PROP and PROP-CyA compared with the untreated cells (control); notably, the intracellular ROS production PROP-CyA was lower than that produced by PROP treatment (Fig. 2A). Therefore, cells were stained with fluorescein isothiocyanate (Annexin-V FITC) and propidium iodide (PI) and analyzed by flow cytometry. The results showed that treatment with PROP could induce apoptosis of cells in a dose-dependent manner. As shown in Fig. 2B, the increased percentage of Annexin V-negative/PI-positive staining in the cells treated with PROP and PROP-CyA (39.63% and 29.33%, respectively) suggested that PROP could induce cell death by a different mechanism or occur more rapidly than PROP-CyA-induced necrosis. Thus, CyA attenuated the PROP apoptotic effect in HUVECs cells. The average of dead cells (Annexin V-negative/PI-positive) was not significantly increased and remained below 40% throughout the experiment.

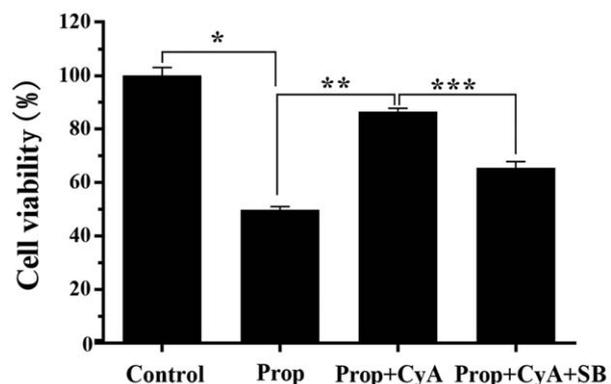
### 3.3. Effect of propranolol-CyA combination on MAPKs

To elucidate the mechanism underlying the effect of CyA on the resistance of HUVECs to the side effects of PROP, we examined by Western blot the effects of PROP and the propranolol-CyA combination on the major proteins p38 MAPKs, since p38 is known to be strongly involved in the regulation of cell survival and apoptosis. As shown in Fig. 3A and B, the application of PROP significantly decreased the relative protein expression of p38 MAPK but increased the expression of cleaved caspase3. In contrast, PROP-CyA increased the protein expression of p38 MAPK but decreased that of cleaved caspase3 when both groups were compared with the control groups.

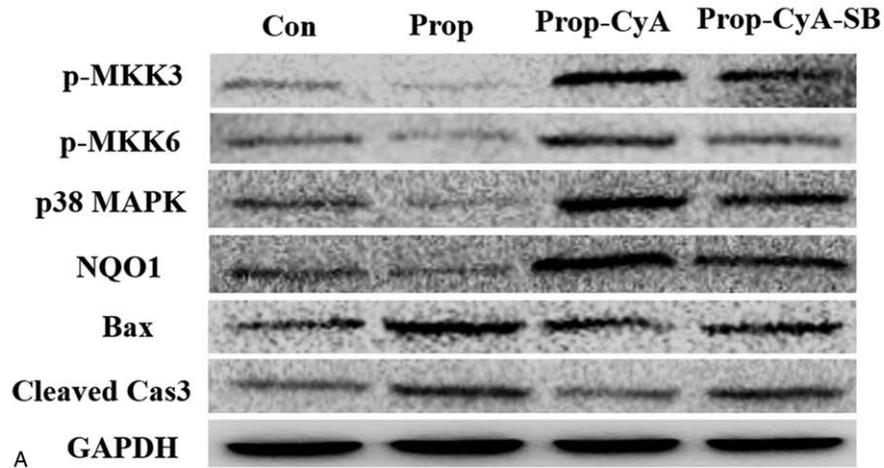
### 3.4. CyA affects PROP reactions on HUVECs via the p38 MAPK-dependent mechanism

To investigate the possible involvement of the p38 MAPK pathway in the PROP-CyA effects on HUVECs, cells were

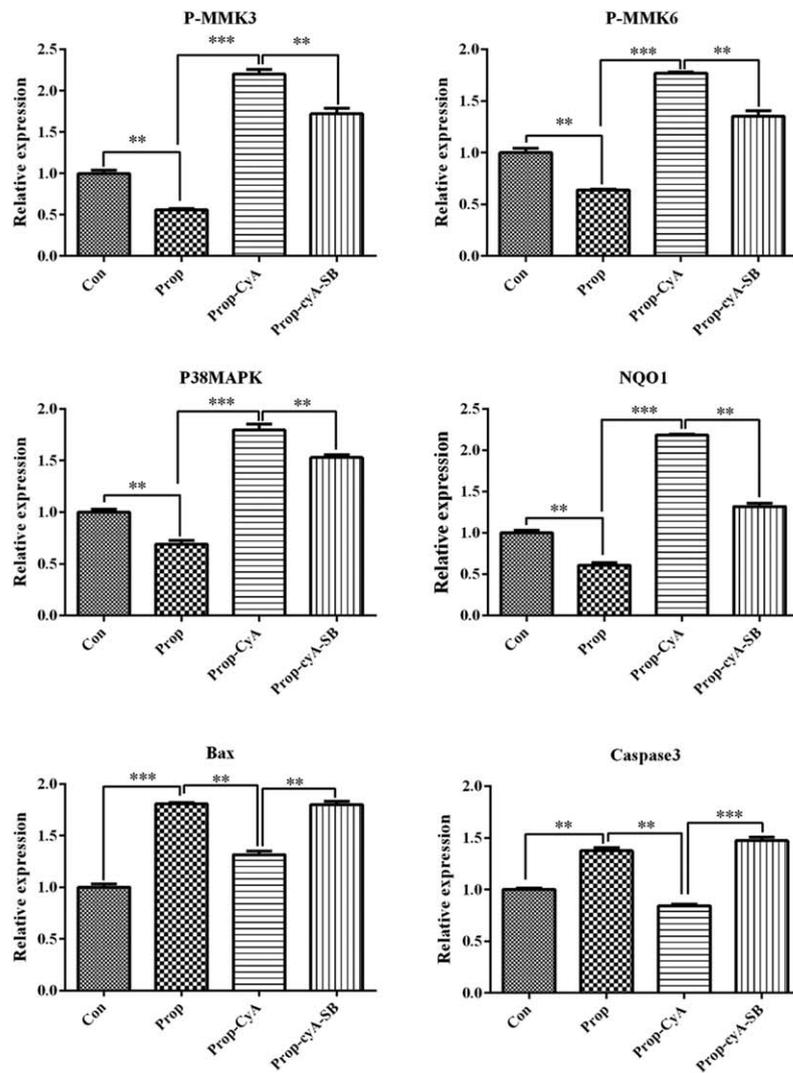
pretreated with the p38MAPK-specific inhibitor SB203580 (10  $\mu\text{M}$ ) and then treated with PROP and CyA for 24 hours. The levels of p-MKK3, p-MKK6, p38MAPK, and NQO1 were detected by Western blot analysis. As shown in Fig. 4A and B, PROP decreased the expression of p-MKK3, p-MKK6, P38MAPK, and NQO1 compared with the control. In contrast, the propranolol-CyA combination increased the expression of p-MKK3, p-MKK6, P38MAPK, and NQO1 compared with the control and PROP groups. Furthermore, we tested whether the p38MAPK-specific inhibitor p38 could reverse this effect. We found that the inhibitor p38 (propranolol supplemented with 10  $\mu\text{g}/\text{mL}$  CyA and p38MAPK specific inhibitor [PROP-CyA-SB]) significantly decreased the expression of p-MKK3, p-MKK6, P38MAPK, and



**Figure 4.** CyA affects HUVECs to propranolol via activating the p38 signaling pathway. (A) HUVECs were pretreated with p38 inhibitor sb203580 (10  $\mu\text{M}$ ), then treated with 82.277  $\mu\text{g}/\text{mL}$  propranolol and/or CyA (10  $\mu\text{g}/\text{mL}$ ) for 24 hours. The level of p-MKK3, p-MKK6, P38MAPK, NQO1, Bax, and cleaved caspase3 was detected by Western blot analysis (WB). Intensities of proteins signals were normalized to GAPDH. Results are representative of three different experiments. Values are mean  $\pm$  SE. \*\* $P < .01$  and \*\*\* $P < .001$ . HUVECs = human umbilical vein endothelial cells.

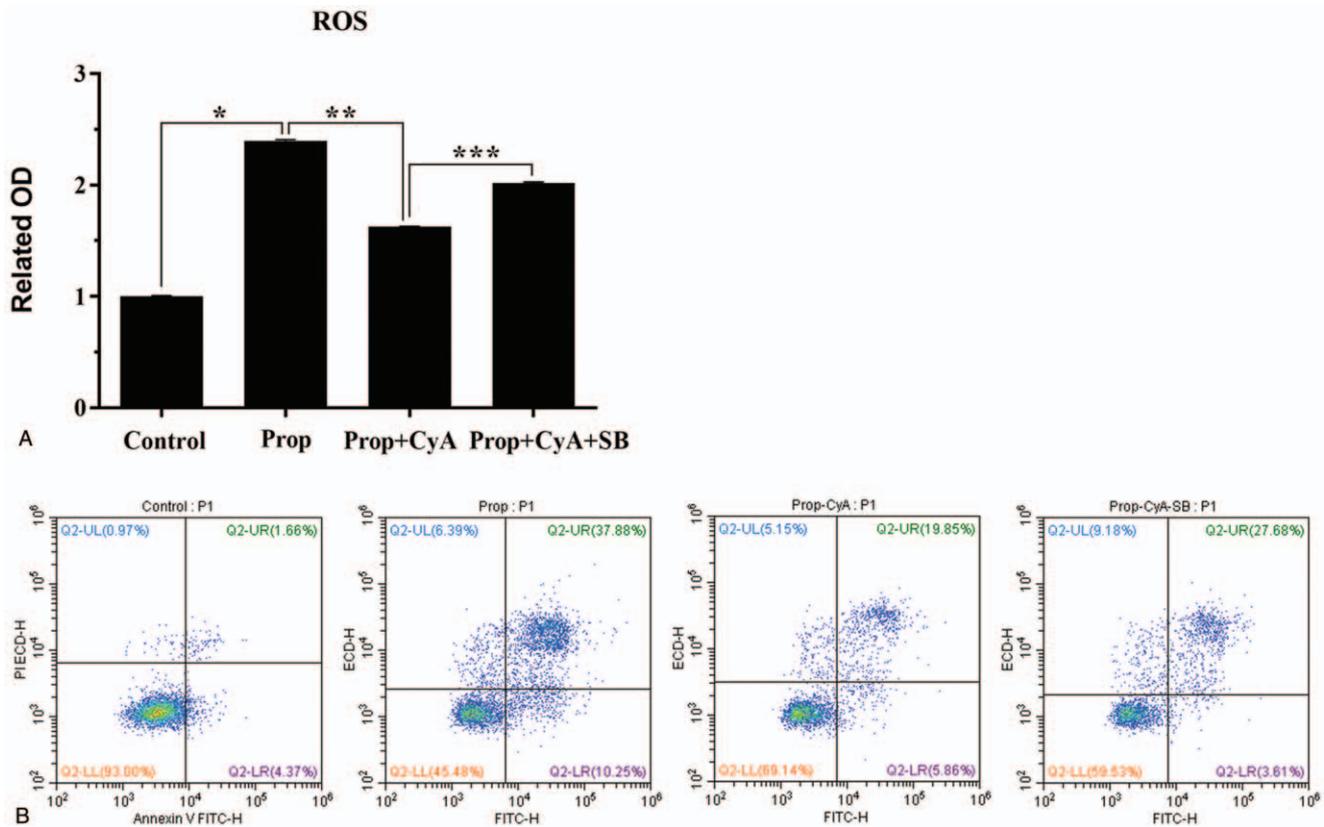


A



B

**Figure 5.** HUVECs were pretreated with p38 inhibitor SB203580 (10  $\mu$ M), then treated with propranolol (82,277  $\mu$ g/mL) and/or CyA (10  $\mu$ g/mL) for 24 hours. MTT detected the inhibition rate of cell. Values are mean  $\pm$  SE of three measurements.  $P < .05$ ,  $^{**}P < .01$ , and  $^{***}P < .001$ . HUVECs=human umbilical vein endothelial cells.



**Figure 6.** CyA affects HUVECs apoptosis and ROS level to propranolol via activating the p38 signaling pathway. (A) HUVECs were pretreated with p38 inhibitor sb203580 (10  $\mu$ M), then treated with propranolol (82.277  $\mu$ g/mL) and/or CyA (10  $\mu$ g/mL) for 24 hours. The level of ROS was detected. (B) HUVECs were pretreated with p38 inhibitor sb203580, then treated with propranolol (82.277  $\mu$ g/mL) and/or CyA (10  $\mu$ g/mL) for 24 hours. The apoptosis was detected using flow cytometry after staining with annexin V-FITC/propidium iodide. Representative scatter plots of PI (Y-axis) versus annexin V (X-axis) were shown. \* $P < .05$ , \*\* $P < .01$ , and \*\*\* $P < .001$ . HUVECs=human umbilical vein endothelial cells.

NQO1 compared with the propranolol–CyA groups. We also examined the apoptosis-related proteins Bax and cleaved caspase-3. PROP treatment increased the expression of Bax and cleaved caspase-3 compared with control. In contrast, PROP–CyA subsequently inhibited the expression of Bax and cleaved caspase-3. Therefore, inhibition of p38MAPK may increase the expression of Bax and cleaved caspase-3.

To confirm that the p38-MAPK pathway is involved in the effects of PROP–CyA on the proliferation of HUVECs, cell survival rates were determined by MTT assay. As shown in Fig. 5, the proliferation of HUVECs was significantly increased by treatment with PROP–CyA after 24 hours of incubation compared with the PROP groups. Moreover, the application of PROP–CyA-SB significantly decreased the cell survival rate compared to the PROP–CyA combination groups. Then, the apoptosis rate was determined by flow cytometry after staining with annexin V-FITC/propidium iodide. We checked whether knocking down p38 MAPK could modulate the effect of PROP–CyA on apoptosis of cultured HUVECs. A second cell staining with annexin V-FITC/propidium iodide was examined by flow cytometry. The increased percentage of annexin V-negative/PI-positive staining in cells treated with PROP and PROP–CyA (37.88% and 19.85%, respectively) confirmed that the PROP-induced cell death mechanism occurs more rapidly than PROP–CyA-induced necrosis. However, the addition of the selective p38 kinase inhibitor SB -203580 (PROP–CyA-SB) increased the

apoptosis rate of PROP–CyA-SB. It restored the percentage of PI-positive staining to 27.68%, suggesting that CyA targets the p38 kinase inhibitors reduce the cytotoxicity of PROP in HUVECs (Fig. 6A and B). Interestingly, the PROP–CyA-SB and PROP–CyA treatments significantly increased the apoptosis rate compared with PROP and the control groups.

#### 4. Discussion

PROP has recently been introduced as an important treatment for certain heart diseases, minimization of essential tremor, and hemangiomas.<sup>[14–17]</sup> PROP inhibits proliferation and induces apoptosis of endothelial cells derived from hemangiomas.<sup>[3]</sup> However, a high concentration of PROP can produce various cytotoxicity events, including cell death and apoptosis as side effects. Therefore, it is important to find out a potential drug or supplement that can decrease the side effect of PROP and optimize its utilization for several diseases.

Besides its role in inhibiting calcineurin enzymes, CyA is a potent immunosuppressive drug widely used to reduce the activity of the patient's immune system after solid organ transplantation. Previous studies have shown that CyA selectively blocks T-cell receptor-induced proliferation, differentiation, and cytokine production.<sup>[18]</sup> Here, by focusing on HUVECs development, our study revealed that a combined treatment by PROP and CyA induced cell proliferation and decreased

apoptosis. Importantly, the inhibition of p38 protein expression resulted in increased apoptosis, indicating that p38 protein is actively involved in the propranolol–CyA mechanism of action on apoptosis and proliferation of HUVECs.

Furthermore, we examined the molecular mechanism for propranolol–CyA combined effects, although wide evidence suggests that p38 signaling could be strongly activated by environmental stresses and growth factor responsiveness notably.<sup>[19,20]</sup> Many chemotherapeutic agents require p38 activity for the inhibition of apoptosis.<sup>[21]</sup> Accordingly, propranolol–CyA treatment of HUVECs induced cell proliferation and prevented apoptosis, largely prevented by the selective p38 kinase inhibitor SB-203580, thus establishing an essential role for p38 kinase in propranolol–CyA combine effect.

Previous studies have demonstrated that stress-induced cell death is linked to ROS activities and that oxidative stress measurement can be used to assess drugs and toxins. Although some studies have shown antioxidant properties for Cyclosporine A and PROP independently,<sup>[22,23]</sup> some other studies have demonstrated their toxic effects.<sup>[24]</sup> Recent research has suggested that ROS can trigger PROP-induced cell death; therefore, ROS inhibition is responsible for reducing PROP-induced cell apoptosis.

The present study indicated that the simultaneous treatment of PROP and CyA significantly decreased the level of ROS in HUVECs; revealed that the inhibition of p38 protein expression could inhibit cell's proliferation and induce apoptosis consequently; finally, it suggested that p38 protein serves as a stimulator of CyA while PROP inhibits HUVECs proliferation. The primary MAPKs for p38 modules are MKK3 and MKK6,<sup>[25]</sup> and once these p38s are activated, they phosphorylate regulatory molecules and play essential roles in cell proliferation, migration, and differentiation. p38 can also regulate BCL-2 family members (e.g., BCL-xL, BCL-2) and the IAP family members (e.g., XIAP and cFLIP), important pro-apoptotic and inhibitors of apoptosis, respectively.<sup>[26]</sup> In addition, p38 is considered an indicator of tissue homeostasis and several vascular pathologies, heart, and neurodegenerative diseases.<sup>[19,27]</sup> Induction of apoptosis by CyA may also relate to the NF- $\kappa$ B pathway.<sup>[28]</sup> Recent reports have demonstrated that p38 activates the NF- $\kappa$ B pathway, and NF- $\kappa$ B promotes apoptosis induced by tumor necrosis factor and some other agents, but these need to be deeply explored in the future.

Importantly, our study revealed a clear anti-apoptotic and induced proliferation pathway through the CyA and PROP treatments. To sum up, these results demonstrate that p38 plays a crucial role in the mechanism in HUVECs under CyA and PROP medications. Endothelial cell apoptosis and proliferation have been suggested to contribute to pathology lesions of atherosclerosis, vascular remodeling and angiogenesis. These findings are of potential pathophysiological importance for understanding the signal transduction pathways that regulate endothelial cell proliferation and apoptosis. These might contribute to the development of important therapeutic strategies to understand the role of endothelial cell proliferation and apoptosis in diseases.

## Author contributions

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**Formal analysis:** Zhi Yao.

**Funding acquisition:** Bailing Chen.

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**Methodology:** Zhong Lv, Guanhao Xie.

**Project administration:** Bailing Chen.

**Resources:** Guanhao Xie, Haowen Cui.

**Software:** Congxiang Shao.

**Supervision:** Haowen Cui, WeiQuan Yuan.

**Validation:** Congxiang Shao.

**Visualization:** Zhi Yao, Congxiang Shao.

**Writing – original draft:** WeiQuan Yuan.

**Writing – review & editing:** Bailing Chen.

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