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Cyclophilin A–FoxO1 signaling pathway in endothelial cell apoptosis

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ABSTRACT

Cyclophilin A (CyPA), which is encoded by *PPIA*, is a ubiquitously expressed intracellular protein and is secreted in response to inflammatory stimuli. CyPA stimulates proinflammatory signaling pathways in vascular smooth muscle cells (VSMCs) and endothelial cells (ECs), promotes VSMC migration and proliferation, EC adhesion molecules expression, and inflammatory cell chemotaxis and apoptosis. Moreover, CyPA activates mitogen-activated protein kinases, including ERK1/2, JNK, and p38, and stimulates IkB-α phosphorylation, NF-kB activation, and vascular cell adhesion molecule-1 and E-selectin expression in human umbilical vein ECs. Therefore, we hypothesized that CyPA regulated transcription factor FoxO1 phosphorylation and transcriptional activity and the expression of downstream genes involved in vascular EC activation, thus activated vascular ECs *in vitro*. We found that intracellular CyPA promoted FoxO1 dephosphorylation at Ser256, nuclear accumulation, and transcriptional activity by interacting with it. Moreover, CyPA induced FoxO1-dependent expression of downstream genes involved in EC chemotaxis and apoptosis, including monocyte chemoattractant protein-1 and BCL-2-interacting mediator of cell death, and stimulated the apoptosis of human umbilical vein ECs *in vitro*.

1. Introduction

Endothelial cell (EC) migration, permeability, and apoptosis play significant roles in the pathogenesis of several vascular inflammatory and cardiovascular diseases, including atherosclerosis and vascular remodeling. Atherosclerosis is a vascular disease characterized by chronic inflammation of arterial walls [1] and involves complex interactions among modified lipoproteins, monocyte-derived macrophages or foam cells, T lymphocytes, ECs, and smooth muscle cells (SMCs) [2,3]. Atherosclerosis development is initiated by EC activation. Oxidized LDL and other stimuli induce EC dysfunction, which increases the adhesion of ECs to leukocytes and production of adhesion molecules and proinflammatory cytokines, including E-selectin, vascular cell adhesion molecule-1 (VCAM-1) [4], and monocyte chemoattractant protein-1 (MCP-1), resulting in the recruitment of inflammatory cells into the intima [5]. Moreover, activated ECs facilitate the passage of lipids (such as LDL) in the plasma and promote arterial lesion development, hemodynamic changes, and vascular remodeling [6–9].

Cyclophilin A (CyPA) is a ubiquitously expressed protein belonging to the immunophilin family and is an intracellular receptor of cyclosporine A (CsA), a potent immunosuppressive drug [10]. CyPA has peptidyl-prolyl isomerase (PPIase) activity [11] and regulates the *cis–trans* isomerization of the Xaa-Pro peptide bond. Moreover, CyPA regulates diverse cellular functions, including protein modification, folding, and trafficking [12,13], intracellular trafficking [14], signal transduction [15,16], and transcription regulation [17], through its enzymatic activity and non-enzymatic scaffold function. CyPA is present in both the cytoplasm and nucleus [18,19–21]. Although CyPA was

Abbreviations: CyPA, Cyclophilin A; FoxO1, Forkhead box protein O1; P-FoxO1, Phosphorylated Forkhead box protein O1; FoxO1 A3, FoxO1 (two Ser and one Thr) three phosphation sites mutated to Ala; FoxO1 ΔDB, FoxO1 deletion of the DNA binding domain; ECs, Endothelial cells; HUVEC, Human umbilical vein endothelial cell; MCP-1, Monocyte chemotactic protein 1; Bim, Bcl-2 interacting mediator of cell death; VCAM-1, Vascular cell adhesion molecule 1; ICAM-1, Intercellular cell adhesion molecule-1; Bax, Bcl-2 associated X protein; siNeg, siNegative; DMSO, Dimethyl sulfoxide; Endo, Endogenous; CsA, Cyclosporin A; CHX, Cycloheximide; IP, Immunoprecipitation; IgG, Immunoglobulin G; FSK, Forskolin; RT-qPCR, Real time-quantitative polymerase chain reaction; RT-sqPCR, Reverse transcription-semi-quantitative polymerase chain reaction

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initially believed to primarily function as an intracellular protein, recent studies have shown that it is secreted by cells in response to inflammatory stimuli, especially ROS generation [22–27]. Recent studies have also reported that both intracellular and extracellular CyPA plays a key role in inflammation and cardiovascular disease pathogenesis. Oxidative stress (ROS generation) and inflammatory stimuli, including cardiovascular diseases, promote CyPA expression and secretion from vascular SMCs (VSMCs) [24–27]. Extracellular CyPA stimulates proinflammatory signals in ECs, induces the expression of adhesion molecules, including E-selectin and VCAM-1, activates mitogen-activated protein kinases, including ERK1/2, JNK, and p38, and promotes IkB-α phosphorylation, NF-kB activation, and EC apoptosis *in vitro* [4,25]. In addition, CyPA functions as a potent leukocyte chemoattractant for inflammatory cells (human monocytes, neutrophils, eosinophils, and T cells) [21,23,28–33] and stimulates inflammatory responses *in vivo* [23]. CyPA also promotes the activation of matrix metalloproteinases (MMPs), especially MMP-1 and MMP-9 [33,34]. Vascular CyPA plays a crucial role in intima formation, vascular remodeling, angiotensin II-induced abdominal aortic aneurysm development, and atherosclerosis progression [5,35–37]. Therefore, determination of the mechanism of action of CyPA is crucial for preventing cardiovascular disease progression.

Forkhead box O1 (FoxO1) is a member of the FoxO transcription factor family that plays important roles in regulation of cell cycle arrest, apoptosis, stress resistance, and fat and glucose homeostasis, energy metabolism, endothelial cells morphology and vascular development and homeostasis in response to growth factors [38–40]. FoxO1 function is regulated, in part, by post-translational modifications including phosphorylation, acetylation, and ubiquitination [41–43]. Phosphorylation of FoxO1 at a number of specific regulatory sites results in translocation of FoxO1 from the nucleus to the cytosol that impairs its transcriptional activity [39], such as Akt, a serine/threonine kinase downstream from PI3K in insulin signaling pathways, phosphorylate FoxO1 at Thr24, Ser256, and Ser319 to promote nuclear exclusion of FoxO1 [44,45]. In addition to Akt, other kinases including SGK phosphorylate FoxO1 at Thr24 and Ser319 [45,46] and PKA at Thr24, Ser256, and Ser319 to regulate its function in a similar manner. And dephosphorylation of Foxo factors in turn stimulates nuclear entry, leading to the activation or repression of apoptosis and cell cycle-related genes such as Bim, p27, MnSOD, or GADD45 [47–50].

In the present study, we hypothesized that CyPA regulated transcription factor FoxO1 phosphorylation and transcriptional activity and the expression of downstream genes involved in vascular EC activation that activated vascular ECs *in vitro*. We found that CyPA interacted with FoxO1 to promote its dephosphorylation at Ser256, nuclear accumulation, and transcriptional activity and FoxO1-dependent MCP-1 and Bim expression that stimulated the apoptosis of human umbilical vein endothelial cells (HUVECs) *in vitro*.

2. Materials and methods

2.1. Cell culture, expression constructs, and reagents

HUVECs were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and were cultured in ECM (ScienCell Research Laboratories). pcDNA3.1-FoxO1 WT, pcDNA3.1-FoxO1 A3 (expressing constitutively active gain-of-function FoxO1 mutant), and pcDNA3.1-FoxO1 ΔDB (expressing loss-of-function FoxO1 mutant) plasmids were kindly gifted by Dr. Weiguo Zhu, Peking University Health Science Center, and pcDNA3.1-Flag-CyPA was constructed.

Cyclosporin A (CsA), and cycloheximide (CHX) were obtained from Sigma, and 4',6-diamidino-2-phenylindole (DAPI) was obtained from Invitrogen, and DEB025 (Alisporivir) was obtained from MedchemExpress. The following antibodies were used in this study: anti-β-actin antibody (Sigma), anti-CyPA antibody (Abcam), anti-FoxO1 antibody (Cell Signaling), anti-phosphorylated FoxO1 (Ser256) antibody (Cell Signaling), anti-Flag antibody (Cell Signaling) and anti-GFP antibody (Cell Signaling).

Immunoprecipitation was performed using Pierce™ Protein A/G Agarose beads (Thermo Fisher). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Reverse transcription-semi-quantitative polymerase chain reaction (RT-sqPCR) were performed using a real-time PCR kit (TAKARA) and *Ex Taq* PCR kit (TAKARA) respectively, and luciferase assay was performed using a luciferase assay system (Promega). The cell viability assay was performed using CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega).

2.2. Immunoblotting, immunoprecipitation, immunostaining, RT-qPCR, and RT-sqPCR

Immunoblotting, immunoprecipitation, immunostaining, RT-qPCR, and RT-sqPCR were performed as described previously [51,52].

2.3. siRNA and plasmid transfection

CyPA, FoxO1 and negative siRNAs (siCyPA,siFoxO1 and siNeg, respectively) were synthesized by GenePharma (Shanghai). The target sequences of these siRNAs are as follows: siCyPA: 5′-AACACAAATGG TTCCCAGT-3′ and siFoxO1: 5′-GAGCGTGCCCTACTTCAAG-3′. All cells in this study were transfected with the siRNAs by using Lipofectamine™ RNAiMAX (Invitrogen) and with the respective plasmids by using Lipofectamine™ LTX reagent with PLUS™ reagent (Invitrogen), according to the manufacturer's instructions.

2.4. Luciferase assay

A FoxO reporter gene system containing a FoxO-responsive luciferase construct encoding a firefly luciferase reporter gene and tandem repeats of a FoxO transcriptional response element was obtained from Yeasen (Shanghai, China) and was used to evaluate FoxO1 transcriptional activity. HUVECs were transfected with the FoxO-responsive luciferase construct after pretreatment with the CyPA inhibitor CsA or pre-transfected with siCyPA to block CyPA function. Next, the cells were collected and washed once with cold PBS, followed by lysis in a cell lysis buffer (Promega). After one freezing and thawing cycle, whole-cell lysates were centrifuged in a cold room (4°C) at 12,000 rpm for 15 min, and the supernatant obtained was collected in a fresh tube. Next, 20 μl supernatant was added to equal amounts of luciferase assay substrate, and luminescence was detected as relative light units by using the LUMIstar OPTIMA reader (BMG Labtech). Each assay was repeated three times. Fold change in values is represented as a mean of three experiments.

2.5. Cell viability assay

HUVECs were grown in a six-well plate, transfected with the specific siRNAs for indicated time periods, and dissociated. Next, the dissociated transfected cells were seeded at the same density into a 96-well plate, cultured for 4 h, and treated with CHX for 24 h at 37°C. Cell viability assay was performed using the CellTiter-Glo® Luminescent Cell Viability Assay Kit, according to the manufacturer's instructions.

2.6. Apoptosis assay

Apoptotic cells were identified by performing the morphological staining of nuclei. HUVECs were fixed in 3.7% formaldehyde, stained with DAPI for 10 min, and photographed using a fluorescence microscope (Lecia). Apoptotic cells were identified based on their typical morphological appearance, including chromatin condensation and nuclear fragmentation.

2.7. Data collection and statistical analysis

The intensity of the Western blot results was analyzed by densitometry using Image J software for quantification. All experiments were repeated at least three independent batchs and each batch was done at least three biological replicates, the averages and standard errors of all data shown were calculated from three biological replicates of one batch experiment. Data are expressed as mean ± SD. For experiments concerning multiple groups, one-way ANOVA with post-hoc Tukey test were performed to evaluate the differences. The differences between two (control and experimental) groups were determined by two-tailed Student's *t*-test. A p value of < 0.05 was considered to be statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

3. Results

3.1. CyPA promotes FoxO1 dephosphorylation at Ser256 in HUVECs

To determine whether human intracellular CyPA promoted FoxO1 dephosphorylation at Ser256 in ECs, we knocked down the intracellular CyPA gene or overexpressed CyPA gene by transfecting HUVECs with different doses of siCyPA or Flag-CyPA plasmid and determined the effect of CyPA gene knockdown or overexpression on FoxO1 phosphorylation. CyPA gene knockdown dramatically increased FoxO1 phosphorylation at Ser256 in a dose-dependent manner (Fig. 1A), and CyPA gene overexpression significantly decreased FoxO1 phosphorylation at Ser256 in a dose-dependent manner (Fig. 1B), indicating that CyPA promoted FoxO1 dephosphorylation at Ser256 in HUVECs. We also used the CyPA inhibitor CsA to block CyPA activity. For this, HUVECs were treated with 10 μM CsA for different time periods and with different concentrations of CsA for 30 min. CsA treatment promoted FoxO1 phosphorylation at Ser256 in a time- and concentration-dependent manner by blocking CyPA activity (Fig. 1C and D). FoxO1 phosphorylation at Ser256 was observed after CsA treatment for 5 min, and maximal FoxO1 phosphorylation was observed after CsA treatment for 30 min (Fig. 1C). Moreover, treatment with 1 μM CsA induced FoxO1 phosphorylation at Ser256, and maximal FoxO1 phosphorylation at Ser256 was observed after treatment with 100 μM CsA (Fig. 1D). To ensure the specificity of CyPA inhibitor regulation of FoxO1 phosphorylation, we used another non-immunosuppressive inhibitor of CyPA, DEB025 (Alisporivir), in different concentrations, to verifty the results of CsA treatment. Similar results (Fig. 1E) were observed in DEB025 (Alisporivir) treatment. FoxO1 phosphorylation was also analyzed by performing immunoblotting. Results of normalized FoxO1 phosphorylation are shown in the corresponding quantitation bar graphs (Fig. 1F, G, H, I and J) from three biological replicates of one batch experiment.

3.2. CyPA associates with FoxO1 in HUVECs

To determine whether human intracellular CyPA promoted FoxO1 dephosphorylation by directly associating with it in ECs, co-immunoprecipitation analysis was performed using HUVECs. CyPA was immunoprecipitated from HUVEC lysates (Fig. 2A), and associated FoxO1 was detected by performing western blotting and reciprocal immunoprecipitation (Fig. 2B). Results of the immunoprecipitation analysis showed a dramatic association between CyPA and FoxO1 (Fig. 2A and B). Then, to determine whether this association is mediated by the CyPA active site, we performed the co-immunoprecipitation using HUVECs in the presence of CsA compared with the DMSO control group. CsA treatment significantly attenuated the association between CyPA and FoxO1 (Fig. 2C and D), indicating that CyPA associated with FoxO1, partly depended on its PPIase active site. Moreover, the association of CyPA with FoxO1 was specific because no immunoprecipitation of CyPA and FoxO1 was observed when total cell lysate was used as a positive control and IgG was used as a negative control.

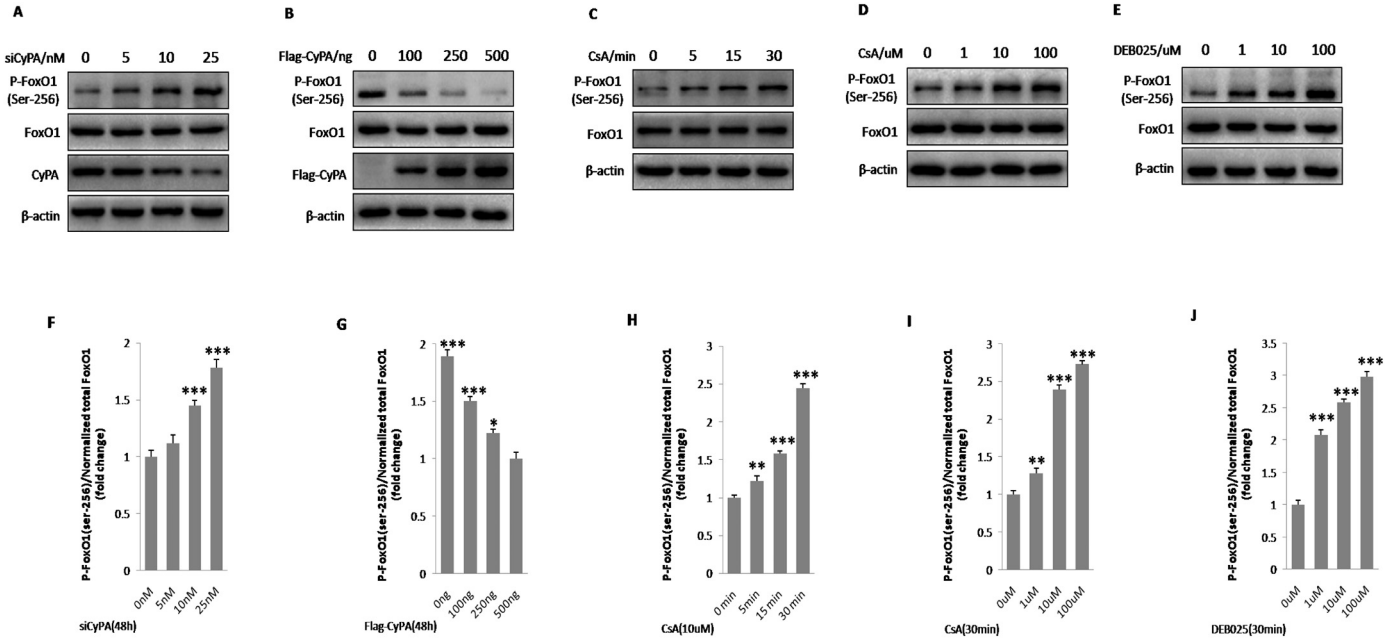

Fig. 1. CyPA promotes FoxO1 dephosphorylation at Ser256 in HUVECs. (A) CyPA knockdown or (B) overexpression by using different dose of siCyPA or Flag-CyPA, promoted or inhibited FoxO1 phosphorylation at Ser256 in a dose-dependent manner, respectively. HUVECs were transiently transfected with siNeg/siCyPA or vector/Flag-CyPA plasmid for 48 h. CyPA inhibition promoted FoxO1 phosphorylation at Ser256 in a (C) time- and (D and E) concentration-dependent manner. HUVECs were treated with 10 μM CyPA inhibitor CsA for the indicated time periods and with the indicated concentrations of CsA or another inhibitor DEB025 for 30 min. (F, G, H, I and J) The results are shown in the corresponding quantitation bar graphs. All experiments were repeated three times. Error bars represent standard deviation. $^{**}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (two-tailed Student's *t*-test).

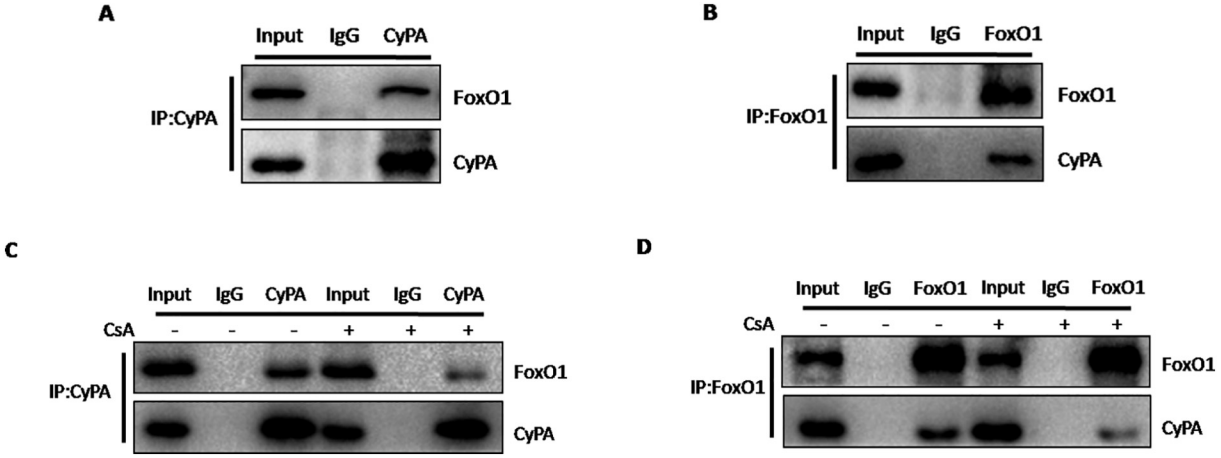

Fig. 2. CyPA associates with FoxO1 in HUVECs. (A) CyPA or (B) FoxO1 was immunoprecipitated from HUVEC lysates. (C) CyPA or (D) FoxO1 was immunoprecipitated from HUVEC lysates in the presence of CsA compared with the DMSO control group. The immunoprecipitates (IP) were examined by performing SDS-PAGE and western blotting analysis with the corresponding antibodies. Our results showed that CyPA co-immunoprecipitated with FoxO1 and vice versa, and the association partly depended on CyPA PPIase active site. All experiments were repeated three times.

3.3. CyPA stimulates the nuclear accumulation of FoxO1 in HUVECs

FoxO1 regulates its nuclear and cytoplasmic translocation as a post-translational modification [53]. To determine whether CyPA regulated the subcellular localization of FoxO1, we performed immunofluorescence analysis by using HUVECs. We observed that FoxO1 was mainly located in the nucleus of HUVECs treated with a negative control siRNA (siNeg) (Fig. 3A) and DMSO (Fig. 3B). However, transfection of HUVECs with siCyPA (Fig. 3A) or treatment of HUVECs with the CyPA inhibitor CsA (Fig. 3B) resulted in the cytoplasmic localization of FoxO1, indicating that CyPA is a key regulator of the subcellular localization of FoxO1. The nuclear and cytoplasmic localization of FoxO1 was determined by manually counting 400 FoxO1-positive HUVECs. The percentage of HUVECs showing the nuclear and cytoplasmic localization of FoxO1 per total number of cells was determined and shown in the corresponding quantitation bar graphs (Fig. 3C) from three biological replicates of one batch experiment. CyPA-knockdown cells was evaluated by performing immunoblotting (Fig. S1).

3.4. CyPA promotes the transcriptional activity of FoxO1 in HUVECs

To determine whether CyPA regulated FoxO1 transcriptional activity associated with its subcellular localization, we performed the luciferase assay by using HUVECs. FoxO reporter luciferase activity was significantly inhibited in HUVECs pre-transfected with siCyPA (Fig. 4A) or pretreated with 10 μM CyPA inhibitor CsA (Fig. 4B) to knockdown or

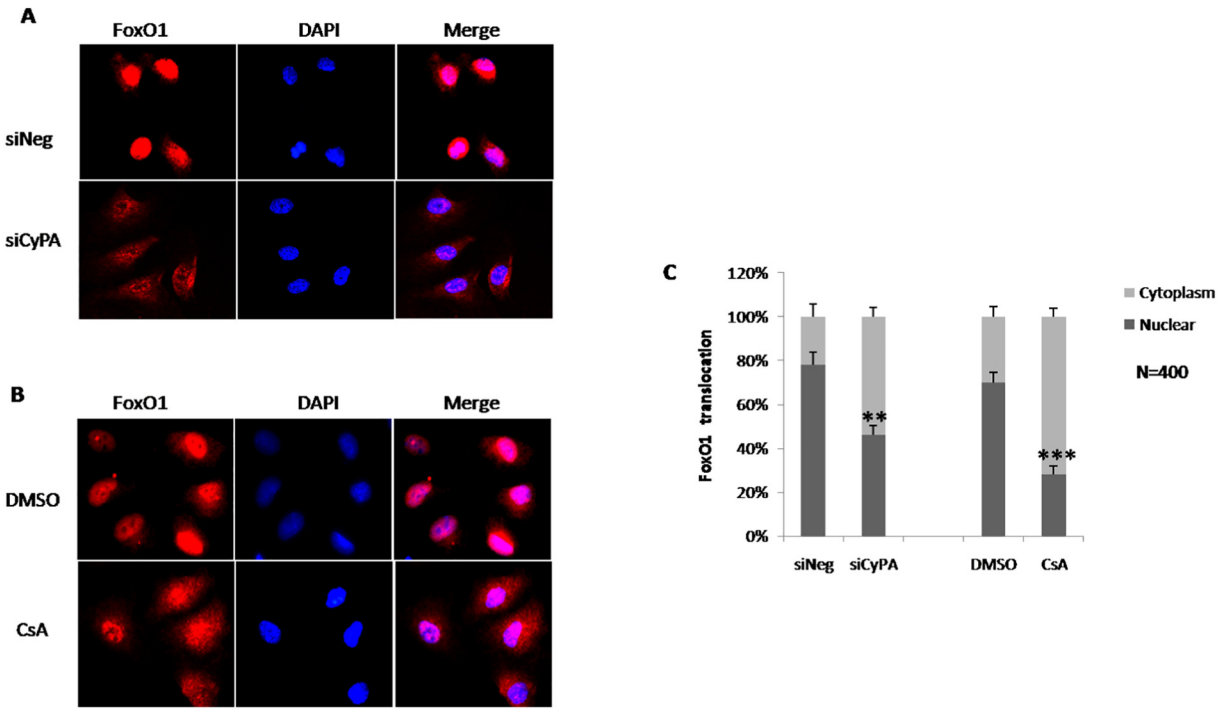

Fig. 3. CyPA stimulates FoxO1 nuclear accumulation in HUVECs. (A) CyPA knockdown by using siCyPA or (B) CyPA inhibition by using CsA increased FoxO1 nuclear translocation. HUVECs were transiently pre-transfected with siNeg or siCyPA for 48 h or were pretreated with DMSO or 10 μM CsA for 30 min. (C) The results are shown in corresponding quantitation bar graphs. All experiments were repeated three times. Error bars represent standard deviation. $^{**}p < 0.01$, $^{***}p < 0.001$ (two-tailed Student's *t*-test).

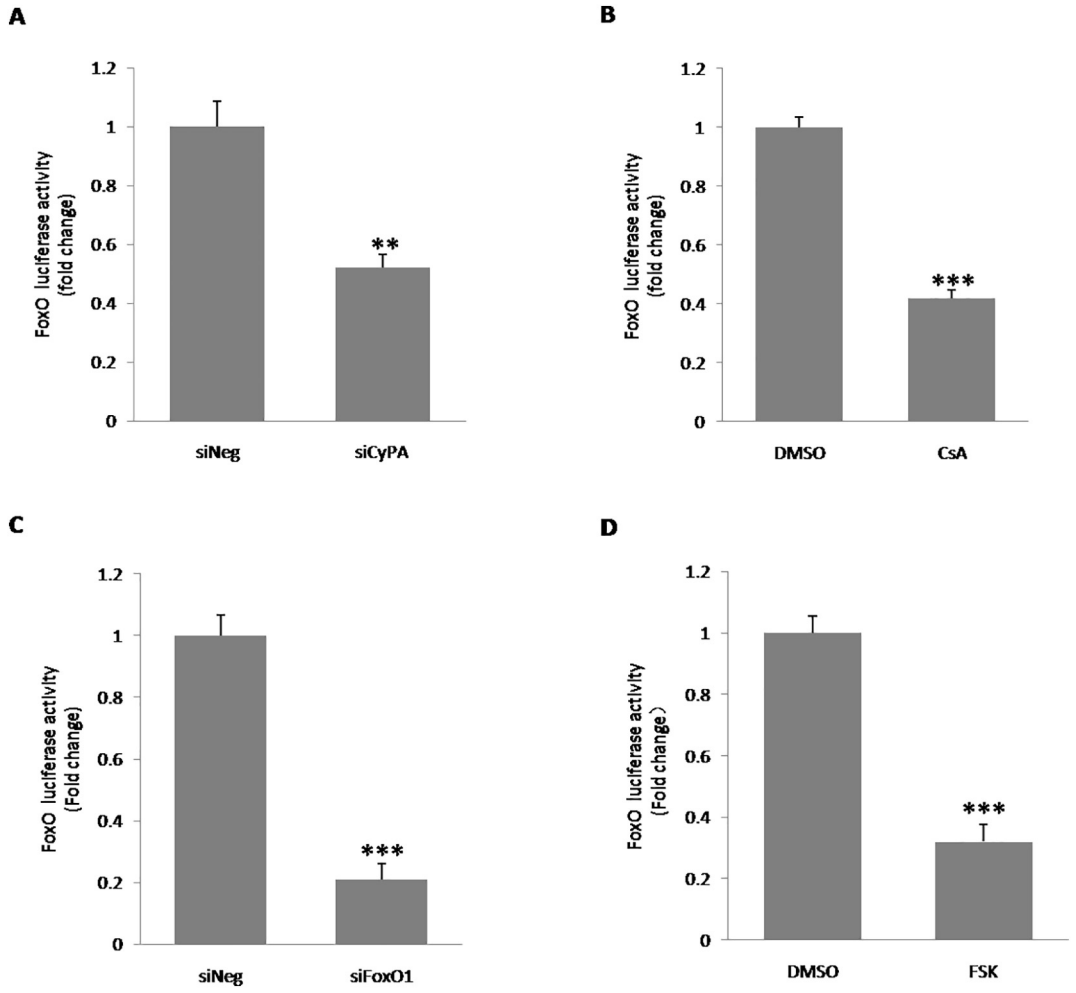

Fig. 4. CyPA promotes FoxO1 transcriptional activity in HUVECs. (A) CyPA knockdown by using siCyPA or (B) CyPA inhibition by using CsA decreased FoxO1 transcriptional activity. HUVECs were transiently pre-transfected with siNeg or siCyPA for 24 h or were pretreated with DMSO or 10 μM CsA for 6 h. (C) siFoxO1 or (D) FSK, which decreased FoxO1 luciferase activity, was used as the positive control. All experiments were repeated three times. Error bars represent standard deviation. $^{**}p < 0.01$, $^{***}p < 0.001$ (two-tailed Student's *t*-test).

block CyPA activity, respectively, indicating that CyPA is the key regulator of FoxO1 transcriptional activity. siFoxO1 (Fig. 4C) and FoxO1 inhibitor FSK (Fig. 4D) were used as positive controls to decrease FoxO reporter luciferase activity. CyPA or FoxO1-knockdown cells was evaluated by performing immunoblotting (Fig. S1).

3.5. CyPA induces FoxO1-dependent expression of MCP-1 and Bim genes in HUVECs

Because CyPA promoted FoxO1 dephosphorylation, nuclear accumulation, and transcriptional activity, we examined whether human intracellular CyPA regulated the expression of downstream EC-related functional responsive genes through FoxO1. Results of RT-qPCR showed that there was a robust decrease in the expression of FoxO1 downstream genes MCP-1 and Bim, which are involved in EC-related chemotaxis and apoptosis, in cells transfected with siCyPA (Fig. 5A, B, C and D) compared with that in control cells. However, this decrease in the expression of MCP-1 and Bim genes was not observed in cells tansfected with siFoxO1(Fig. 5A and B), and in cells expressing WT FoxO1 and constitutively active FoxO1 (FoxO1 A3), and the FoxO1 A3 group rescued more compared to the WT group, but not in cells expressing loss-of-function FoxO1 (FoxO1 ΔDB) (Fig. 5C and D). Consistently, MCP-1 and Bim genes expression was significantly downregulated in cells treated with CyPA inhibitor CsA (Fig. 5E, F, G and H) compared with that in control cells. However, this decrease in the expression of MCP-1 and Bim genes was not observed in cells transfected with siFoxO1(Fig. 5E and F), and in cells expressing WT FoxO1 and constitutively active FoxO1 (FoxO1 A3), and the FoxO1 A3 group rescued more compared to the WT group, but not in cells expressing loss-of-function FoxO1 (FoxO1 ΔDB) (Fig. 5G and H), indicating that CyPA-mediated regulation of MCP-1 and Bim gene transcription partly requires FoxO1 transcriptional activity. We also examined the effect of FoxO1 on the expression of other chemotaxis- and apoptosis-related downstream genes, including the genes encoding VCAM-1, ICAM-1, P21, P27, and Bax. However, no difference was observed in the expression of these genes in cells transfected with siCyPA and those transfected with siFoxO1, indicating that the CyPA–FoxO1 axis did not regulate the expression of these genes (Fig. S2). Primers used in RT-qPCR and RT-sqPCR are listed in Table S1. CyPA and FoxO1-knockdown, WT FoxO1-, FoxO1 A3- and FoxO1 ΔDB-overexpressing cells were evaluated by performing immunoblotting or RT-sqPCR and agarose gel electrophoresis Fig. S3).

3.6. CyPA–FoxO1 signaling pathway regulates HUVEC viability and triggers EC apoptosis

Based on the observation that CyPA induced FoxO1-dependent expression of MCP-1, Bim genes, which play a key role in EC chemotaxis and viability [4,54], we assessed the ability of the CyPA–FoxO1 signaling pathway to regulate EC death by examining the viability of

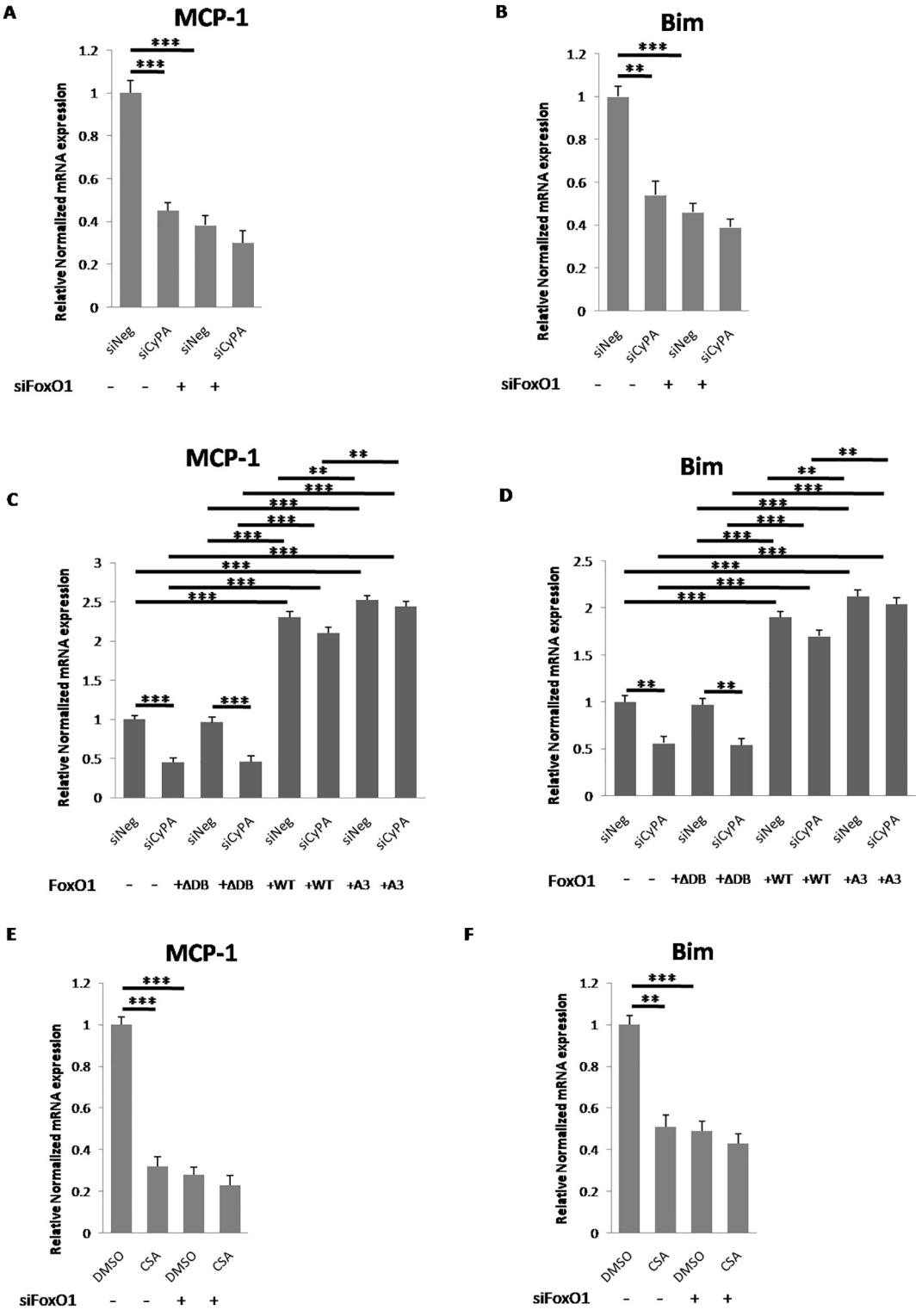

Fig. 5. CyPA induces FoxO1-dependent expression of MCP-1 and Bim genes in HUVECs. HUVECs were transiently pre-transfected with (A and B) siNeg or siFoxO1, or (C and D) WT FoxO1,constitutively active FoxO1 (FoxO1 A3) or loss-of-function FoxO1 (FoxO1 ΔDB) for 24 h, followed by transfection with siNeg or siCyPA for 24 h, or (E, F, G and H) treated with DMSO or 10 μM CyPA inhibitor CsA for 6 h. Next, the cells were analyzed by performing RT-qPCR. MCP-1 and Bim gene expression decreased robustly in cells transfected with siCyPA (Fig. 5A, B, C and D) compared with that in control cells. However, this decrease in the expression of MCP-1 and Bim genes was not observed in cells transfected with siFoxO1 (Fig. 5A and B), and in cells expressing WT FoxO1 and constitutively active FoxO1 (FoxO1 A3), and the FoxO1 A3 group rescued more compared to the WT group, but not in cells expressing loss-of-function FoxO1 (FoxO1 ΔDB) (Fig. 5C and D). Consistently, MCP-1 and Bim genes expression was significantly downregulated in cells treated with CyPA inhibitor CsA (Fig. 5E, F, G and H) compared with that in control cells. However, this decrease in the expression of MCP-1 and Bim genes was not observed in cells transfected with siFoxO1 (Fig. 5E and F), and in cells expressing WT FoxO1 and constitutively active FoxO1 (FoxO1 A3), and the FoxO1 A3 group rescued more compared to the WT group, but not in cells expressing loss-of-function FoxO1 (FoxO1 ΔDB) (Fig. 5G and H), indicating that CyPA promotes MCP-1 and Bim genes transcription partly requires FoxO1 transcriptional activity. All experiments were repeated three times. Error bars represent standard deviation. $^{**}p < 0.01$, $^{***}p < 0.001$ (one-way ANOVA with post hoc Tukey test).

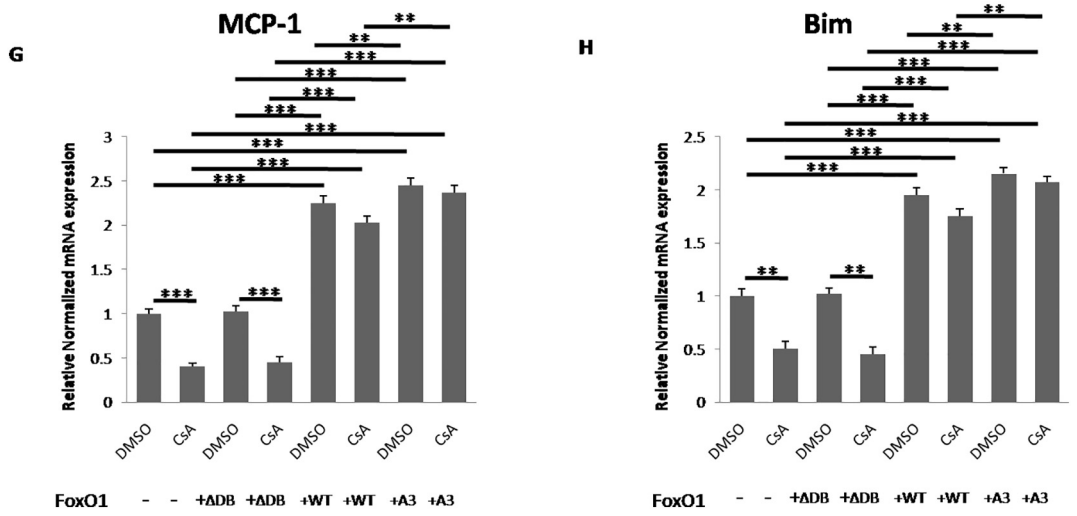

Fig. 5. (*continued*)

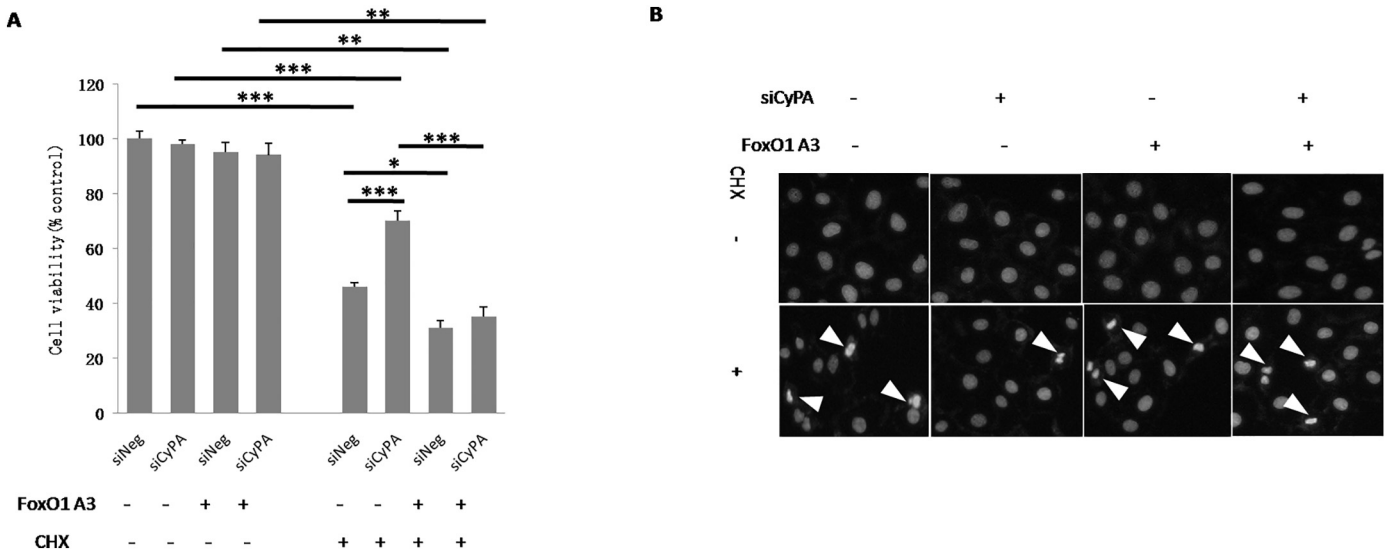

Fig. 6. The CyPA–FoxO1 signaling pathway regulates HUVEC viability and triggers EC apoptosis. The cell viability and apoptosis assays were performed using HUVECs transiently pre-transfected with vector or constitutively active FoxO1 (FoxO1 A3) plasmid for 24 h, followed by transfection with siNeg or siCyPA for 24 h and treatment with CHX for 24 h.The CyPA–FoxO1 signaling pathway promotes EC death. (A) Cell death decreased robustly in cells transfected with siCyPA and treated with CHX. However, this decrease in cell death was not observed in cells overexpressed constitutively active FoxO1 (FoxO1 A3). (B) EC death was confirmed to be caused by apoptosis (arrowhead) based on the analysis of nuclear morphology by performing fluorescence microscopy. For this, HUVECs were fixed, and apoptotic cells were identified based on the morphological staining of nuclei, as described in the Materials and Methods. All experiments were repeated three times. Error bars represent standard deviation. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (one-way ANOVA with post hoc Tukey test).

HUVECs in the presence of the protein synthesis inhibitor CHX (Fig. 6A). No significant reduction was observed in the viability of HUVECs in the absence of CHX. However, CHX treatment and siCyPA transfection significantly increased the viability of HUVECs compared with siNeg. However, the enhanced effect of CyPA knockdown on cell viability decreased in HUVECs overexpressed constitutively active FoxO1 (FoxO1 A3). To determine whether HUVEC death was caused by apoptosis, we analyzed cell nuclei by performing fluorescence microscopy (Fig. 6B). No significant apoptosis induction was observed in the absence of CHX. However, CHX treatment and siNeg transfection significantly induced the apoptosis of HUVECs (arrowhead indicated) compared with siCyPA transfection. However, the overexpression of constitutively active FoxO1 (FoxO1 A3) diminished the apoptosis of HUVECs that CyPA derived, indicating that the CyPA–FoxO1 signaling pathway regulated EC viability and apoptosis. CyPA-knockdown and FoxO1 A3-overexpressing cells were evaluated by performing immunoblotting (Fig. S4).

4. Discussion

The results of the present study highlight the essential role of the intracellular CyPA–FoxO1 signaling pathway in EC apoptosis. CyPA promoted FoxO1 dephosphorylation at Ser256, nuclear accumulation, and transcriptional activity by directly associating with it. Moreover, CyPA induced FoxO1-dependent expression of downstream genes associated with EC chemotaxis and apoptosis, including MCP-1 and Bim, to form a signaling pathway for stimulating the apoptosis of HUVECs *in vitro*. The possible mechanism of this pathway is shown in Fig. 7.

CyPA associates with FoxO1 and dephosphorylates it directly, partly via its PPIase active site. However, detailed mechanisms underlying this are unclear. CyPA may form a complex with other upstream proteins such as the classical PKA, AKT, and SGK or with other kinases and phosphatases to play role in FoxO1 phosphorylation regulation. However, additional data are also needed to confirm the specific binding sites and/or mediators between CyPA and FoxO1. Moreover,

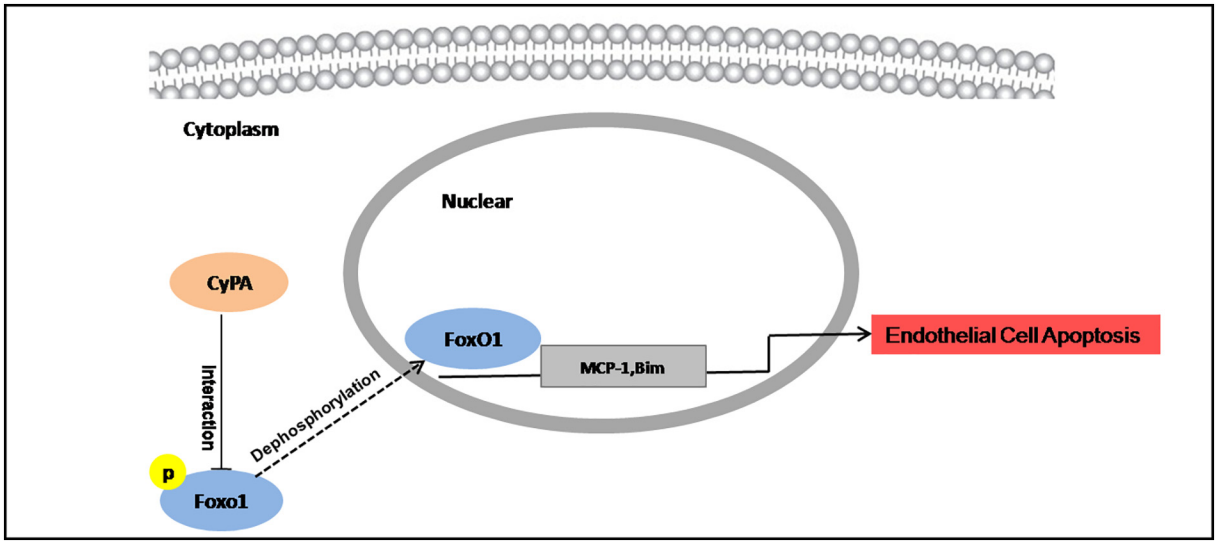

Fig. 7. Possible mechanism of the CyPA–FoxO1 signaling pathway underlying EC apoptosis. CyPA interacted with FoxO1 to promote its dephosphorylation, nuclear accumulation, and transcriptional activity. Moreover, CyPA induced FoxO1-dependent expression of MCP-1 and Bim genes to form a signaling pathway for stimulating EC apoptosis.

whether CyPA regulates the post-translational modification of FoxO3a or FoxO4, which are FoxO family proteins with functions similar to FoxO1 in vascular remodeling. The results of these studies may provide more evidence and insight on the functions and mechanisms of action CyPA and FoxO1 in vascular remodeling and atherosclerosis. In the present study, we could not determine whether CyPA modified FoxO1 *in vivo* or *ex* vivo. Therefore, *in vivo* or *ex vivo* studies involving CyPA- and FoxO1-KO mouse models or human atherosclerosis samples, respectively, should be performed to validate the results of our *in vitro* study. The results of these studies could be used to determine the implications and key biological roles of the CyPA–FoxO1 signaling pathway in cardiovascular diseases.

However, the present study is the first to show that CyPA mediated the post-translational modification and transcriptional activity of FoxO1 in vascular ECs to form a signaling pathway for promoting vascular EC apoptosis *in vitro*. The results of the present study broaden our understanding of the role of FoxO1 post-translational modification and downstream target gene transcription, which are important for EC homeostasis, and of the function and regulatory role of CyPA in vascular remodeling. An understanding of the functions of CyPA and FoxO1 through the CyPA–FoxO1 signaling pathway may help in developing clinical therapies targeting CyPA and FoxO1 for treating cardiovascular diseases.

5. Conclusions

- CyPA promoted FoxO1 dephosphorylation at Ser256 by interacting with it in human umbilical vein ECs.
- CyPA increased FoxO1 nuclear accumulation and transcriptional activity in human umbilical vein ECs.
- CyPA induced FoxO1-dependent expression of downstream genes associated with EC chemotaxis and apoptosis, including MCP-1 and Bim, to form a signaling pathway in human umbilical vein ECs.
- CyPA–FoxO1 signaling pathway stimulated the apoptosis of human umbilical vein ECs *in vitro*.

Author contributions

Y.X. designed, performed, and discussed the experiments and wrote the manuscript. X.L.conceived the project, supervised the experiments, and wrote the manuscript. J.G. conceived the project, wrote the manuscript, and provided funding.

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Appendix A. Supplementary data

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