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Roles of I_2^{PP2A} in the downregulation of eNOS Ser1177 phosphorylation by angiotensin II-activated PP2A

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ABSTRACT

The chronic elevation of angiotensin II (Ang II) is an important cause of endothelial dysfunction (ED). The Ang II/type 1 receptor (AT_1R) signaling pathway can cause endothelial nitric oxide synthase (eNOS)/nitric oxide (NO) dysfunction through various mechanisms leading to ED. The modulation of eNOS phosphorylated at Ser1177 is an important mechanism upregulating eNOS activity. Protein phosphatase 2 A (PP2A) has been reported to dephosphorylate eNOS at Ser1177. The PP2A inhibitor 2 protein (I_2^{PP2A}) is a specific endogenous inhibitor that binds the catalytic subunit of PP2A and directly inhibits PP2A activity. Therefore, we hypothesized that Ang II might attenuate I_2^{PP2A} expression to activate PP2A, which downregulates eNOS Ser 1177 phosphorylation, leading to eNOS dysfunction. In our study, we used Ang II-treated human umbilical vein endothelial cells (HUVECs) and, found that the eNOS Ser1177 phosphorylation levels were downregulated, the activity of PP2A was increased, and I_2^{PP2A} expression was decreased. Furthermore, these effects were blocked by candesartan (CAN). The phosphorylation levels of eNOS Ser1177 were decreased after I_2^{PP2A} was knocked down by specific siRNA but increased after I_2^{PP2A} overexpression. We also found that the Ang II treatment decreased the association of I_2^{PP2A} with PP2A but increased the association between PP2A and eNOS. Taken together, our results suggest that Ang II activates PP2A by downregulating the I_2^{PP2A} expression through the AT_1R signaling pathway leading to the loss of eNOS Ser1177 phosphorylation and ED.

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1. Introduction

Vascular endothelial cells (VECs) constitute a layer of flat epithelial cells that cover the inner surface of the vascular lumen and play critical roles in maintaining circulatory homeostasis [1]. Endothelial cells produce and release endothelium-derived contractile factors (EDCFs) and endothelium-derived relaxing factors (EDRFs); these factors exist in a dynamic equilibrium, and their imbalances can result in endothelial dysfunction (ED) [2]. Nitric oxide (NO) is an important EDRF produced along with L-citrulline from L-arginine in a reaction catalyzed by endothelial nitric oxide synthase (eNOS). eNOS/NO dysfunction is among the important manifestations of ED, and numerous studies have shown that the mechanism underlying ED may be related to a decrease in eNOS activity accompanied by reductions in NO production [3,4].

Angiotensin II (Ang II) is a key component in the renin-angiotensin system (RAS) that participates in a variety of cardiovascular diseases through its specific Ang II type 1 receptor (AT_1R) [5,6]. Previous studies have reported that the chronic elevation of Ang II is associated with decreased eNOS activity and the loss of NO production, resulting in ED and hypertension [5,7]. The post-

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translational phosphorylation modulation of eNOS is an important mechanism to regulate its activity. Studies have shown that eNOS can be dephosphorylated through interactions with protein phosphatase 2 A (PP2A) [8–10].

PP2A, which is a serine/threonine phosphatase, is a heterotrimeric complex that consists of a scaffolding subunit, a regulatory subunit and a catalytic subunit, and its activity regulatory mechanisms include the binding of its inhibitors [11,12]. There are two specific endogenous inhibitors of PP2A, i.e. inhibitor 1 (I_1^{PP2A}) and inhibitor 2 (I_2^{PP2A}) [13]. Both inhibitors can bind the PP2A catalytic subunit (PP2Ac) and directly inhibit PP2A activity [11,14]. Thus far, knowledge regarding the roles of the PP2A protein inhibitors in eNOS phosphorylation and functional regulation upon Ang II stimulation is limited.

Although studies have reported the roles of eNOS in Ang II-related cardiovascular disease, the mechanism by which how Ang II triggers eNOS dysfunction remains not fully understood. In this study, we sought to explore the roles of PP2A endogenous inhibitor in the Ang II-induced PP2A activation and in the eNOS functional dysregulation.

2. Materials and methods

2.1. Materials and reagents

Fetal bovine serum (FBS) (Biological Industries, Connecticut, USA); 0.25% Trypsin EDTA, Pen-Strep Solution, and high-glucose Dulbecco's modified Eagle's medium (DMEM; HyClone, UT, USA); Ang II (Sigma-Aldrich, St. Louis, MO, USA); candesartan (CAN; Selleck, Houston, Texas, USA); a primary antibody against eNOS Ser1177 (Millipore, Billerica, MA, USA); primary antibodies against I_2^{PP2A} and PP2Ac (Santa Cruz, CA); and a primary antibody against β-tubulin and secondary antibodies conjugated with horseradish peroxidase (PMK Biotechnology, Wuhan, China) were purchased. Lipofectamine™ 3000 (Invitrogen, Waltham, MA, USA) and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) (Beyotime, Shanghai, China) were also purchased.

2.2. Cell culture

The human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords within 30 min of delivery of normal term pregnancies through elective cesarean section and provided by the Affiliated Hospital of Guizhou Medical University. The whole study was approved by the ethics committee of Guizhou Medical University. The HUVECs were isolated as previously described [15]. The isolated cells were cultured in 20% FBS in high-glucose DMEM. Then, the expression of factor VIII related antigen (vWF) and CD34 was detected by an immunochistochemical assay for cell characterization. The purity of the HUVECs was 100%. The HUVECs were grown in 10% FBS in high-glucose DMEM in a humidified incubator at 37 °C with 5% CO_2. The cells received the subsequent treatments once confluence reached 70%–80%. The cells used in the experiments were from the 4th to the 8th passage.

2.3. Western blot analysis

The total proteins were collected from the HUVECs by using RIPA lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.25 mM sodium pyrophosphate, 1 mM EDTA, 1% Na_3VO_4, and 5 mM phenylmethanesulfonyl). Then the proteins were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to PVDF membranes (Millipore), which were blocked with 5% nonfat milk for 1 h at room temperature. Then, the membranes were incubated with the primary antibodies overnight at 4 °C. After three 10-min washes, the membranes were incubated with the secondary antibodies. The protein bands were visualized by enhanced chemiluminescence (ECL, Bio-Rad). The densitometric analysis was conducted with Bio-Rad software.

2.4. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted with TRIzol reagent; the RNA integrity was verified by agarose gel electrophoresis. Reverse transcription was performed by the PrimeScript RT Reagent Kit (Takara, Dalian, China), and the Tli RNaseH Plus system (Takara) was utilized for qPCR assay. The experiment was repeated three times. The differences in expression between the groups were determined by calculating $2^{-\Delta\Delta Ct}$. The expression levels were normalized to the expression of β-actin. The following PCR primers were used in the study: I_2^{PP2A} F: ATCTGGAAAGGATTTGACGAAA, I_2^{PP2A} R: CTAACTCATCAGCACCTGCA C; β-Actin F: CTTAGTTGCGTTACACCCTTTCTTG, and β-Actin R: CTGTCACCTTC ACCGTTCCAGTTT. All primers were synthesized by Sangon Biotech (Shanghai, China).

2.5. NO measurement

The NO-sensitive fluorescence probe DAF-FM DA was used to measure the content of intracellular NO as described [16]. After washed with phosphate-buffered saline (PBS), the cells were incubated with a 10-μM solution of the NO-sensitive fluorescent dye DAF-FM DA without phenol red medium at 37 °C for 30 min in dark. The measurement of NO production was carried out under an Olympus microscope (IX71, Japan). The mean values of the fluorescence intensity were analyzed by ImageJ software.

2.6. PP2A activity assay

PP2A activity was measured with a V2460 kit from Promega (Madison, WI, USA) using a previously described method [17]. The HUVECs were collected using precooled phosphatase storage buffer, lysed on ice for 30 min, and then centrifuged at 4 °C at 12,000×*g* for 25 min to remove the supernatant. According to the instructions of the PP2A test kit, 250 μl of supernatant were added to the column, and the filtrate was collected as the sample to be tested. Finally, the phosphate content of the sample after the reaction was measured. The absorbance (OD) value was determined at 600 nm, and the enzyme activity of PP2A in the sample was calculated according to a standard curve.

2.7. Transient transfection

The pCIGPS01-I_2^{PP2A} plasmid was constructed by Era Biotech (Shanghai, China). The specific I_2^{PP2A} siRNA was constructed by GenePharma (Shanghai, China). The cells were seeded in six-well plates. When the confluence reached to 40%–50%, the cells were transfected with the plasmids or siRNA by Lipofectamine™ 3000 regent according to manufacturer's instructions. Transfected cells were collected 24 h later.

2.8. Co-immunoprecipitation (Co-IP)

The cell lysates were collected and incubated with the PP2A antibody overnight at 4 °C. Then, IP of PP2A was performed. Protein A/G agarose (Santa Cruz, CA) was added to the PP2A immune complex and incubated overnight at 4 °C. The immune complexes were washed three times with PBS, and an immunoblot analysis was performed using primary antibodies against eNOS Ser1177, eNOS and I_2^{PP2A} as appropriate in each experiment.

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2.9. Statistical analysis

SPSS 17. 0. statistical software was used to analyze the data, and the data are expressed as the mean ± standard deviation (mean ± SD). The homogeneity of variance was tested for comparisons of multiple groups of samples. A one-way ANOVA was used for comparisons between groups, and the SNK-q method was used for comparisons of variances. $P < 0.05$ indicated a statistically significant difference.

3. Results

3.1. Ang II/AT$_1$R pathway activation downregulates eNOS Ser1177 phosphorylation leading to a reduction in NO production

We carried out experiments with cultured human endothelial cells. We incubated the HUVECs with different concentrations of Ang II for different durations and observed the expression of total eNOS protein and eNOS Ser1177 in each group. After the treatment with Ang II at concentrations of 10^{-8} M, 10^{-7} M, 10^{-6} M, and 10^{-5} M for 12 h, the phosphorylation levels of eNOS Ser1177 were significantly lower than those after control treatment (Fig. 1-A). The phosphorylation levels of eNOS Ser1177 were also lower than those in the control group after treatment with Ang II at a concentration of 10^{-7} M for 6 h, 12 h, 24 h and 36 h (Fig. 1-B). There were no statistically significant differences in the expression levels of the total eNOS protein among the groups. Based on the above results, the concentration of Ang II chosen for the subsequent experiments was 10^{-7} M, and the selected treatment duration was 12 h.

Compared with the control treatment, the treatment with Ang II at a concentration of 10^{-7} M for 12 h substantially downregulated the eNOS Ser1177 phosphorylation levels. The CAN pretreatment prevented the downregulation effect of AngII on eNOS Ser1177 phosphorylation. There were no statistically significant differences in the eNOS protein expression levels among the groups (Fig. 1-C).

To further clarify the effects of Ang II on NO production in the HUVECs, we used DAF-FM DA fluorescent probes to detect the production of NO in each group. After the treatment with Ang II, the fluorescence intensity was lower than that in the control group. As expected, the loss of NO production in the Ang II-treated cells was reversed by CAN (Fig. 1-D, 1-E). Altogether, the above data indicate that the Ang II treatment reduced eNOS Ser1177 phosphorylation, leading to the loss of NO formation.

3.2. Ang II/AT$_1$R pathway downregulates I_2^{PP2A} protein and mRNA expression and activates PP2A

I_2^{PP2A} is an endogenous inhibitor of PP2A that binds subunit C of PP2A to inhibit PP2A activity [11,14]. Subsequently, we examined the protein expression of I_2^{PP2A}. Compared with that in the control group, the protein expression of I_2^{PP2A} was significantly decreased after the treatment with Ang II. This effect on I_2^{PP2A} expression could be prevented by the CAN pretreatment (Fig. 2-A). Furthermore, the mRNA levels of I_2^{PP2A} were determined by real-time PCR. Similarly, after the Ang II treatment, the mRNA expression of I_2^{PP2A} was markedly reduced, and the CAN pretreatment reversed this result (Fig. 2-B). These data demonstrate that AngII suppressed I_2^{PP2A} expression.

To confirm that Ang II/AT$_1$R downregulated the phosphorylation of eNOS Ser1177 through PP2A, we measured the activity of PP2A.

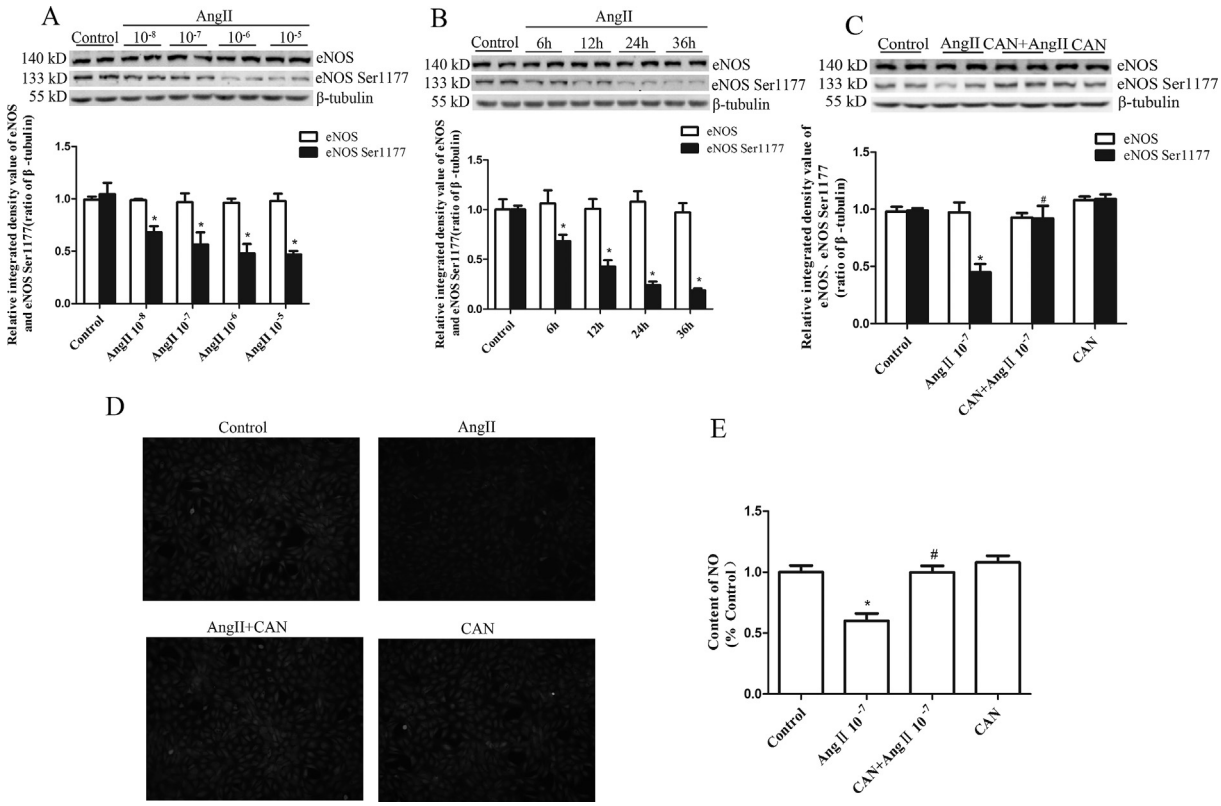

Fig. 1. Ang II/AT$_1$R signaling pathway downregulates eNOS Ser1177 phosphorylation and reduces NO production. (A) In HUVECs, after treatment with Ang II, the phosphorylation of eNOS Ser1177 was downregulated in a concentration-dependent and (B)time-dependent manner ($n = 6$). (C) CAN pretreatment (10^{-6} M, 3 h) blocked the effect of Ang II (10^{-7} M, 12 h) ($n = 9$). (D) HUVECs were incubated with DAF-FM DA (10 μmol/L) for 30 min to determine the NO content ($n = 6$). Representative images were captured under a fluorescence microscope (100 ×). (E) Semiquantitative evaluation of DAF-FM fluorescence intensity. $^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the Ang II group.

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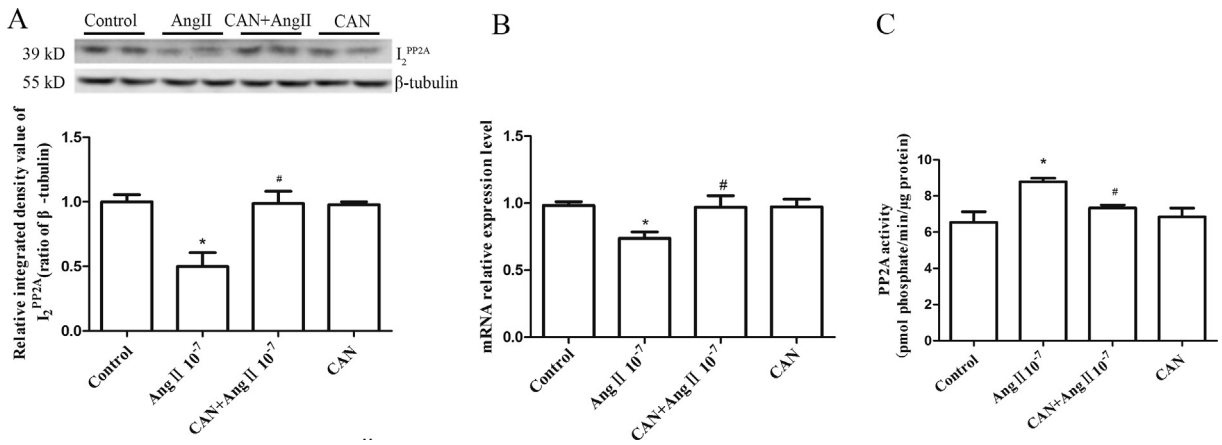

Fig. 2. Ang II/AT_1R pathway activates PP2A and downregulates the expression of $I_2^{PP2A.}$ (A) In HUVECs, PP2A activity was increased after the Ang II (10^{-7} M, 12 h) stimulation, and the CAN pretreatment (10^{-6} M, 3 h) had a blocking effect ($n = 4$). (B) Ang II downregulated I_2^{PP2A} protein expression and (C) mRNA expression through the AT_1R pathway ($n = 9$). * $P < 0.05$ compared with the control group; # $P < 0.05$ compared with the Ang II group.

The activity of PP2A was significantly increased after the treatment with Ang II at a concentration of 10^{-7} M for 12 h. After the pretreatment with CAN, the activity of PP2A was downregulated (Fig. 2-C).

3.3. Effect of I_2^{PP2A} on eNOS Ser1177 phosphorylation

To clarify the role of I_2^{PP2A} in the effects of AngII on eNOS Ser1177 phosphorylation, we transferred I_2^{PP2A} siRNA into HUVECs. The results showed that the expression of the I_2^{PP2A} protein was significantly reduced after the I_2^{PP2A} gene was knocked down by siRNA (Fig. 3-A). Meanwhile, regarding the levels of eNOS Ser1177 phosphorylation, the results showed that the phosphorylation levels were notably downregulated after the knockdown of I_2^{PP2A} (Fig. 3-A). Then, we increased the I_2^{PP2A} protein levels by transfecting the pCIGPS01-SET overexpression plasmid into HUVECs. Western blotting was performed to detect the protein expression. The expression of I_2^{PP2A} in the overexpressed I_2^{PP2A} group was markedly higher than that in the Vector group (Fig. 3-B). In addition, the phosphorylation levels of eNOS Ser1177 were dramatically increased in the I_2^{PP2A} overexpression group compared to those in the Vector group. There were no statistically significant differences in eNOS protein expression among the groups (Fig. 3-B). These results reveal the important role of I_2^{PP2A} in controlling eNOS Ser1177 phosphorylation.

3.4. Ang II inhibits the interaction between I_2^{PP2A} and PP2Ac

I_2^{PP2A} has been reported to inhibit the activity of PP2A by binding PP2Ac [10]. We used a co-immunoprecipitation (Co-IP) assay to observe the effects of AngII on I_2^{PP2A} and PP2A interactions and their effect on eNOS Ser1177 phosphorylation. The Co-IP studies showed that Ang II treatment attenuated the binding between I_2^{PP2A} and PP2A. In contrast, the association between eNOS and PP2A was enhanced. Consequently, the phosphorylation levels of eNOS Ser1177 were downregulated. The above results were reversed after the pretreatment with CAN (Fig. 4).

4. Discussion

According to previous studies, impaired eNOS/NO function is among the most important manifestations of ED [3,4,18]. Moreover, the regulation of eNOS activity is a complex process involving gene transcription, translation and posttranslational modifications. Phosphorylation and dephosphorylation are the most versatile posttranslational regulatory mechanisms of eNOS proteins. The phosphorylation of Ser1177, which is an important phosphorylation site of eNOS, can upregulate eNOS activity [19]. Previous studies have shown that Ang II is involved in the pathophysiological process of ED [20], and some studies have shown that the chronic elevation of Ang II can impair the activity of eNOS and the production of NO, leading to ED and hypertension [5–7,21]. Ang II has four receptors; however, Ang II exerts its biological effects mainly through AT_1 receptors [5,6]. eNOS Ser1177 phosphorylation has been shown to be downregulated, while NO production has been shown to be decreased in HUVECs by the action of Ang II, and these effects are mainly mediated by the AT_1R pathway [22]. In this study, we also observed the phosphorylation levels of eNOS Ser1177 in HUVECs. We further measured the NO content in each group and found that the NO content was significantly decreased after the Ang II treatment, and that this could be reversed by the CAN pretreatment. These results confirm that Ang II downregulates eNOS Ser1177 phosphorylation, eNOS activity and NO production through the AT_1R-mediated signaling pathway in HUVECs.

PP2A has been implicated in the process of eNOS Ser1177 dephosphorylation [10,23,24]. Thus, we measured the activity of PP2A. We found that the Ang II treatment could increase PP2A activity, which could be prevented by the CAN pretreatment. This finding suggests that the Ang II/AT_1R pathway can activate PP2A. PP2A is a heterotrimeric complex. The complicated mechanism regulating its activity mainly includes the assembly of the PP2A holoenzyme, posttranslational modification of related subunits and inhibitors [11,12,25,26]. Due to the complexity of the structure and physiological function of PP2A, there are many types of PP2A inhibitors, which can be divided into endogenous inhibitors and exogenous inhibitors. The endogenous specific inhibitors include I_1^{PP2A} and I_2^{PP2A} [13,14[13,14,27], while the exogenous inhibitors include okadaic acid, cantharidin, etc [28]. Among the inhibitors, I_1^{PP2A} and I_2^{PP2A} can directly inhibit PP2A activity by binding the PP2Ac subunit; these are noncompetitive inhibitors of PP2A, but they do not inhibit the activity of other protein phosphatases, such as PP1, PP2B and PP2C [13]. Then, we determined the protein expression level of I_2^{PP2A}. We found that the protein level of I_2^{PP2A} was downregulated after the Ang II treatment. In addition, we measured the mRNA level of I_2^{PP2A}. The I_2^{PP2A} mRNA level was also downregulated after the Ang II treatment, which was consistent with the results of the protein expression. These results indicate that the Ang II/AT_1R pathway activates PP2A by downregulating

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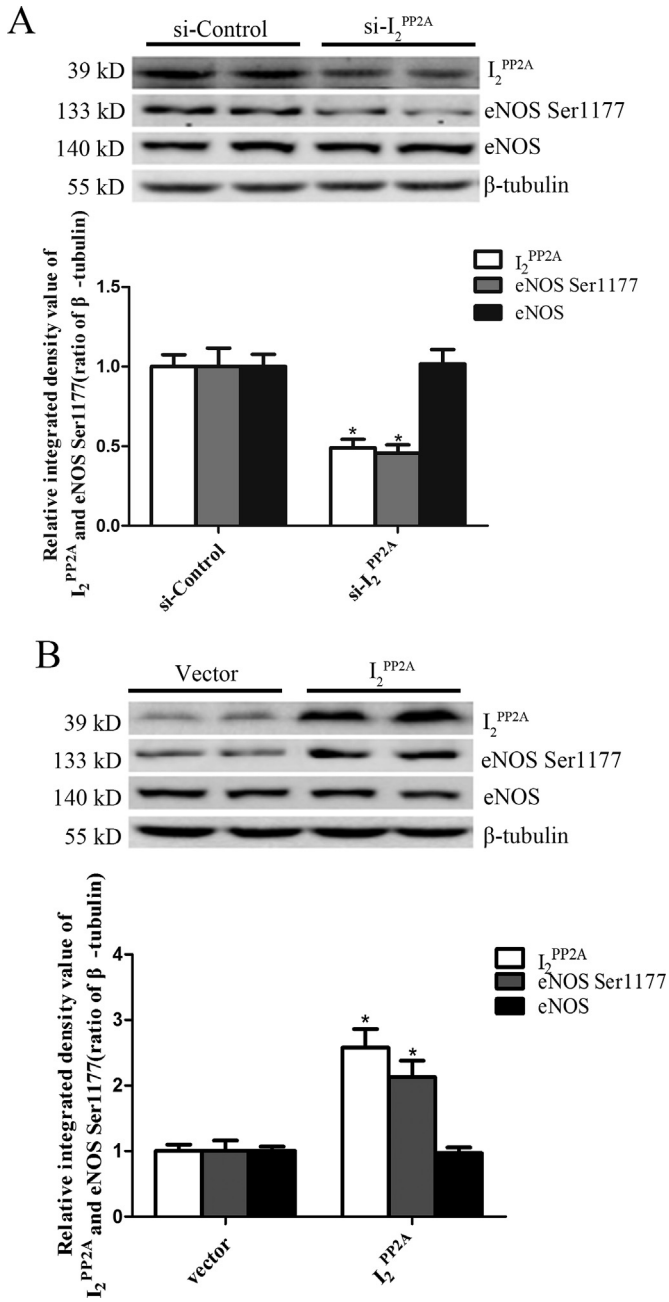

Fig. 3. I_2^{PP2A} participates in eNOS Ser1177 dephosphorylation. (A) In HUVECs, after transfection with I_2^{PP2A}-specific siRNA for 24 h, the expression of I_2^{PP2A} and the phosphorylation level of eNOS Ser1177 were downregulated ($n = 6$). $^*P < 0.05$ compared with the si-control group. (B) Then, the I_2^{PP2A}-overexpression plasmid was transfected into the HUVECs, the expression of the I_2^{PP2A} protein and the phosphorylation level of eNOS Ser1177 were dramatically increased ($n = 6$). $^*P < 0.05$ compared with the Vector group.

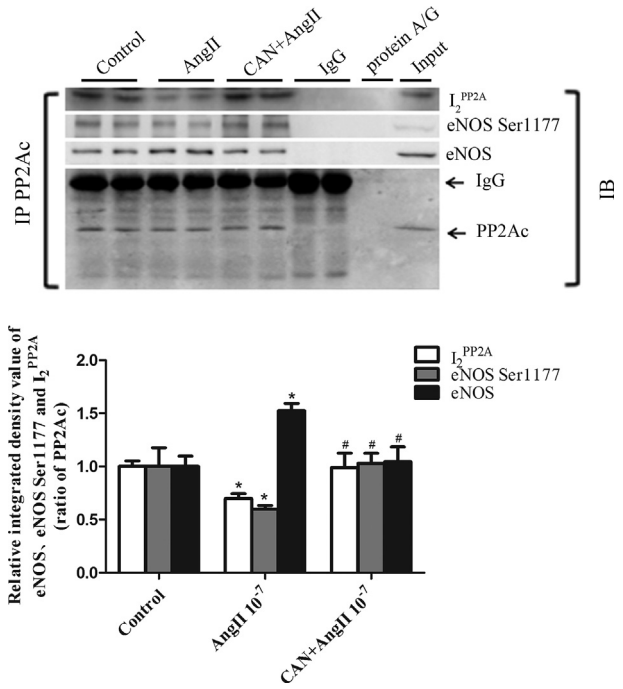

Fig. 4. Ang II inhibited the interaction between PP2Ac and I_2^{PP2A}. After pretreatment with CAN (10^{-6} M) for 3 h, HUVECs were treated with Ang II (10^{-7} M) for another 12 h. Cell lysates were incubated with PP2Ac antibody for an immunoprecipitation (IP) assay, and then, the PP2Ac, I_2^{PP2A}, eNOS and eNOS Ser1177 expression levels were determined by immunoblotting(IB). In the PP2Ac immunoprecipitates, the expression of I_2^{PP2A} was decreased, while the expression of eNOS was increased ($n = 6$). $^*P < 0.05$ compared with the control group; $^\#P < 0.05$ compared with the Ang II group.

I_2^{PP2A} expression. Actually, we tried to assay the I_1^{PP2A} protein expression simultaneously. However, the I_1^{PP2A} protein was not detected in HUVECs (data not shown). This suggests that the protein expression of I_1^{PP2A} and I_2^{PP2A} might have tissue or cell differences, as reported in a previous study [30].

To further demonstrate the roles of I_2^{PP2A} in the effects of Ang II on eNOS Ser1177 phosphorylation, we knocked down intracellular I_2^{PP2A} using siRNA. We found that the phosphorylation level of eNOS Ser1177 was downregulated after the I_2^{PP2A} knockdown, indicating that the expression of I_2^{PP2A} could affect the activity of eNOS. Then, we transfected an I_2^{PP2A} overexpression plasmid into HUVECs to observe whether the high expression of I_2^{PP2A} had the same effect on the phosphorylation level of eNOS Ser1177. In this study, our results showed that when I_2^{PP2A} was highly expressed, the phosphorylation levels of eNOS Ser1177 were dramatically increased. These results demonstrate that the expression of the I_2^{PP2A} protein has further effects on the phosphorylation levels of eNOS Ser1177.

Earlier studies have shown that ceramide can reduce the activity of PP2A by reducing the effect of I_2^{PP2A} on PP2Ac, ultimately downregulating the phosphorylation levels of eNOS Ser1177 and reducing the activity of eNOS [29,31]. Therefore, we speculated that Ang II may affect the activity of eNOS by reducing the interaction between PP2Ac and I_2^{PP2A}, and examined the effects of AngII on PP2Ac–I_2^{PP2A} interactions. We found that Ang II decreased the binding of I_2^{PP2A} and PP2Ac; but on the other hand, the binding between PP2Ac and eNOS was augmented. As a result, eNOS Ser1177 phosphorylation was markedly reduced. These results indicate that Ang II/AT_1R increases the activity of PP2A by reducing the binding of I_2^{PP2A} to PP2A, leading to a decrease in eNOS activity. The decreased binding of I_2^{PP2A} to PP2Ac may be due to the decrease in I_2^{PP2A} protein expression induced by Ang II or other effects of Ang II. Further studies are needed to elucidate these potential mechanisms.

In conclusion, Ang II can activate PP2A through the AT_1R signaling pathway leading to downregulation of eNOS Ser1177 levels, which may be related to the decreased I_2^{PP2A} expression and the interaction between PP2Ac and I_2^{PP2A}. These identified mechanisms may suggest novel targets to protect eNOS and improve endothelial function.

Conflicts of interest

The authors have no conflicts of interest to declare.

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