



## Research Paper

# Critical role of vascular peroxidase 1 in regulating endothelial nitric oxide synthase



Zhaoya Liu<sup>a</sup>, Yanbo Liu<sup>a</sup>, Qian Xu<sup>a</sup>, Haiyang Peng<sup>a</sup>, Yixin Tang<sup>a</sup>, Tianlun Yang<sup>a</sup>, Zaixin Yu<sup>a</sup>, Guangjie Cheng<sup>b</sup>, Guogang Zhang<sup>a,\*</sup>, Ruizheng Shi<sup>a,\*</sup>

<sup>a</sup> Department of Cardiovascular Medicine, Xiangya Hospital, Central South University, Changsha 410008, China

<sup>b</sup> Division of Pulmonary, Allergy & Critical Care Medicine, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294, USA

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## ABSTRACT

Vascular peroxidase 1 (VPO1) is a member of the peroxidase family which aggravates oxidative stress by producing hypochlorous acid (HOCl). Our previous study demonstrated that VPO1 plays a critical role in endothelial dysfunction through dimethylarginine dimethylaminohydrolase2 (DDAH2)/asymmetric dimethylarginine (ADMA) pathway. Hereby we describe the regulatory role of VPO1 on endothelial nitric oxide synthase (eNOS) expression and activity in human umbilical vein endothelial cells (HUVECs). In HUVECs AngiotensinII (100 nM) treatment reduced Nitric Oxide (NO) production, decreased eNOS expression and activity, which were reversed by VPO1 siRNA. Knockdown of VPO1 also attenuated ADMA production and eNOS uncoupling while enhancing phosphorylated ser1177 eNOS expression level. Furthermore, HOCl stimulation was shown to directly induce ADMA production and eNOS uncoupling, decrease phosphorylated ser1177 eNOS expression. It also significantly suppressed eNOS expression and activity together with NO production. Therefore, VPO1 plays a vital role in regulating eNOS expression and activity via hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-VPO1-HOCl pathway.

## 1. Introduction

Vascular peroxidase (VPO1) is a heme-containing peroxidase that is primarily found in the cardiovascular system [1]. As a member of peroxidase family, VPO1 aggravates oxidative stress utilizing hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and producing hypochlorous acid (HOCl) [2]. Recent study suggested that VPO1 decreases eNOS expression by increasing Asymmetric dimethylarginine (ADMA) level [3]. VPO1 also was found to decrease dimethylarginine dimethylaminohydrolase2 (DDAH2) expression and activity in HUVECs, which contributes to endothelial dysfunction [3].

ADMA is the L-arginine analogue that inhibits eNOS expression [4]. In the murine model of diabetic nephropathy with angiotensin II (Ang II) infusion, it was shown to have higher level of reactive oxygen species (ROS) and ADMA, as well as decreased eNOS expression [5]. This suggests that oxidative stress induced by Ang II correlates with ADMA production and eNOS expression. Protein arginine methyltransferase1 (PRMT1) is the predominant enzyme catalyzing the formation of ADMA. However, the exact relationship between ROS

and PRMT1 regulation has not been fully determined.

eNOS has been shown to be a source of superoxide. Under pathological conditions, the homodimer of eNOS could be uncoupled, which result in dissociation of eNOS dimers into monomer. The uncoupling prompts eNOS to produce superoxide instead of NO [6], which may induce oxidative stress. It has been demonstrated that HOCl treatment induced eNOS uncoupling in endothelial cells and with recombinant eNOS protein [7]. In addition, phosphorylation on serine (Ser1177) and threonine (Thr495) residues [8] have been discussed extensively to regulate eNOS. The phosphorylation of Ser1177 is involved in multiple signaling pathways in the cardiovascular conditions [9].

Overall, we hypothesize that under oxidative stress, eNOS expression and activity are regulated through a VPO1 mediated signaling pathway.

*Abbreviation:* DDAH2, dimethylarginine dimethylaminohydrolase2; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; ADMA, asymmetric Dimethylarginine; HOCl, hypochlorous acid; eNOS, endothelial nitric oxide synthase; PRMT1, Protein arginine methyltransferase1; ROS, reactive oxygen species; NO, nitric oxide

\* Corresponding authors.

E-mail addresses: [xyzgg2006@sina.com](mailto:xyzgg2006@sina.com) (G. Zhang), [xyshirui Zheng@csu.edu.cn](mailto:xyshirui Zheng@csu.edu.cn) (R. Shi).

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## 2. Material and methods

### 2.1. Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were obtained from ATCC. HUVECs were cultured in Dulbecco's modified Eagle's medium (DMEM, 1 g/L glucose, 10 mmol/L sodium pyruvate) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin at 37 °C in 5% CO<sub>2</sub>. HUVECs from passage 3 to passage 10 were used.

HUVECs were incubated with DMEM containing AngII (100 nmmol/l) for 24 h which was shown to be the optimal treatment for VPO1 expression by our previous work. The protein and mRNA expression of VPO1, PRMT-1, eNOS, the levels of ADMA in the supernatant, the ratio of eNOS dimer and monomer, the concentration of H<sub>2</sub>O<sub>2</sub>, HOCl, cGMP in the cell lysate were determined. In addition, the cells were also exposed to Hank's buffered saline solution (HBSS) with 100 μmol/L HOCl at 37 °C for 2 h. HOCl was removed by extensive wash with PBS, the cells were then cultured in DMEM for another 24 h [10].

### 2.2. RNA interference and cell transfection

The small interference RNA (siRNA) including negative controls were synthesized and purchased from RiboBio Co Ltd (Guangzhou, China). HUVECs were seeded in six-well plates at a density of 5×10<sup>5</sup> per well and cultured in 10% FBS DMEM for 12–24 h until 40–50% confluent. The cells were transfected with VPO1 siRNA or negative control siRNA at a final concentration of 50 nM using the ribo FECT™ CP Transfection Kit (RiboBio Co Ltd, Guangzhou, China), following manufacturer's protocol. 24 h after transfection, cells were washed with PBS and treated with AngII(100 nM) for 24 h.

### 2.3. Western blot analysis

The cells were lysed with RIPA(Beyotime, China) containing 1 mM phenylmethanesulfonyl fluoride (PMSF) on ice for 30 min to 1h. Cell lysate was sonicated at 4 °C and centrifuged at 12000 rpm for 10 min. Cell lysate containing 50–60 μg protein that was solubilized in 5×loading buffer (Beyotime, China) and resolved by 10% SDS-PAGE gels and transferred onto 0.22 μm polyvinylidene difluoride membranes (PVDF, Millipore). Specifically, eNOS dimers and monomers were assayed by low-temperature SDS-PAGE as described previously [11]. Briefly, after blocking the residual protein sites on the membranes with 5% nonfat dry milk, the membrane was incubated with anti-VPO1 (from Dr. Guangjie Cheng, University of Alabama at Birmingham), anti-PRMT-1 (Sigma-Aldrich, USA), eNOS, eNOS ser 1177 (BD transduction laboratories), 3-chlorotyrosine antibody (Cell Science), anti-GAPDH (Sigma-Aldrich, USA) overnight at 4 °C. And then the membrane was washed by TBST for 10 min 3 times and further incubated with a second horseradish peroxidase-conjugated antibody against with mouse or rabbit IgG for 1 h at room temperature. Lastly, the membrane was visualized using SuperSignal West Pico Chemiluminescent Substrates (PIERCE) with a gel documentation system (Bio-Rad).

### 2.4. RNA isolation, reverse transcription, real-time PCR analysis

Total RNA isolation from HUVECs was carried out by trizol (Invitrogen, USA) at a constant temperature of 4 °C. The RNA was quantified by ultraviolet absorbance spectrophotometry. cDNA was synthesized from RNA (500 ng) using the PrimeScript™ RT Reagent Kit (TakaRa, Japan) according to the manufacture's instruction. Real-time PCR was performed by ABI 7500 Real-time PCR system (Foster City, CA, USA). Results were analyzed as the ratio of VPO1 to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expres-

sion.

### 2.5. ADMA concentration

ADMA concentration was detected by using High Performance Liquid Chromatography (HPLC) as described previously [12].

### 2.6. Hydrogen peroxide assay

Changes in intracellular H<sub>2</sub>O<sub>2</sub> levels were measured by the hydrogen peroxide assay kit (Beyotime, China) according to the manufacturer's instruction. In this kit, ferric ions (Fe<sup>3+</sup>) that oxidated by H<sub>2</sub>O<sub>2</sub> can form a purple complex, xylenol orange (3,3'-bis[N,N-di(carboxymethyl)-aminomethyl]-o-cresolsulfonephthalein, disodium salt and xylenol orange), which can be detected at a wavelength of 560 nm [13].

### 2.7. Hypochlorous acid assessment

HOCl can be combined with tyrosine to produce 3-chlorotyrosine (3-Cl-Tyr) [14]. Western blot and immunohistochemistry were used for detecting 3-Cl-Tyr expression. Cells were seeded to six-well glass slide. The slides of cells fixed by 4% paraformaldehyde for 20 min, permeabilized by 0.1% Triton×100 for 10 min, blotted by 1% BSA for 1 h, and then incubated anti-3-Cl-Tyr (Hycult Biotech, The Netherlands) overnight 4 °C. Secondary antibody was incubated for 1 h at room temperature. Lastly, 3,3'-diaminobenzidine was used as color developing reagent. The results were determined by the amount of granular brown substance in the cytoplasm.

### 2.8. Assay of cGMP

The concentration of cGMP was determined using ELISA from R & D system.

### 2.9. eNOS activity

Cellular NOS activity was measured by the conversion of L-arginine to NO using a nitric-oxide synthase assay kit (Beyotime Institute of Biotechnology). After treated AngII (100 nM) for 24 h, the culture supernatant was removed and 100 uL NOS assay buffer (1×) with or without eNOS inhibitor (L-NAME) were added to each well. Then 100 uL of NOS assay reaction solution(50%NOS assay buffer, 38.8% MilliQ water, 5% L-Arginine solution, 5% 0.1 mM NADPH, 0.2% DAF-FMDA) was added to each well and incubated for 2 h at 37 °C. Cells were collected and washed twice by PBS. Fluorescence was measured by flow cytometry. eNOS activity was calculated by the decrease in NOS activity when applying eNOS inhibitor to HUVECs.

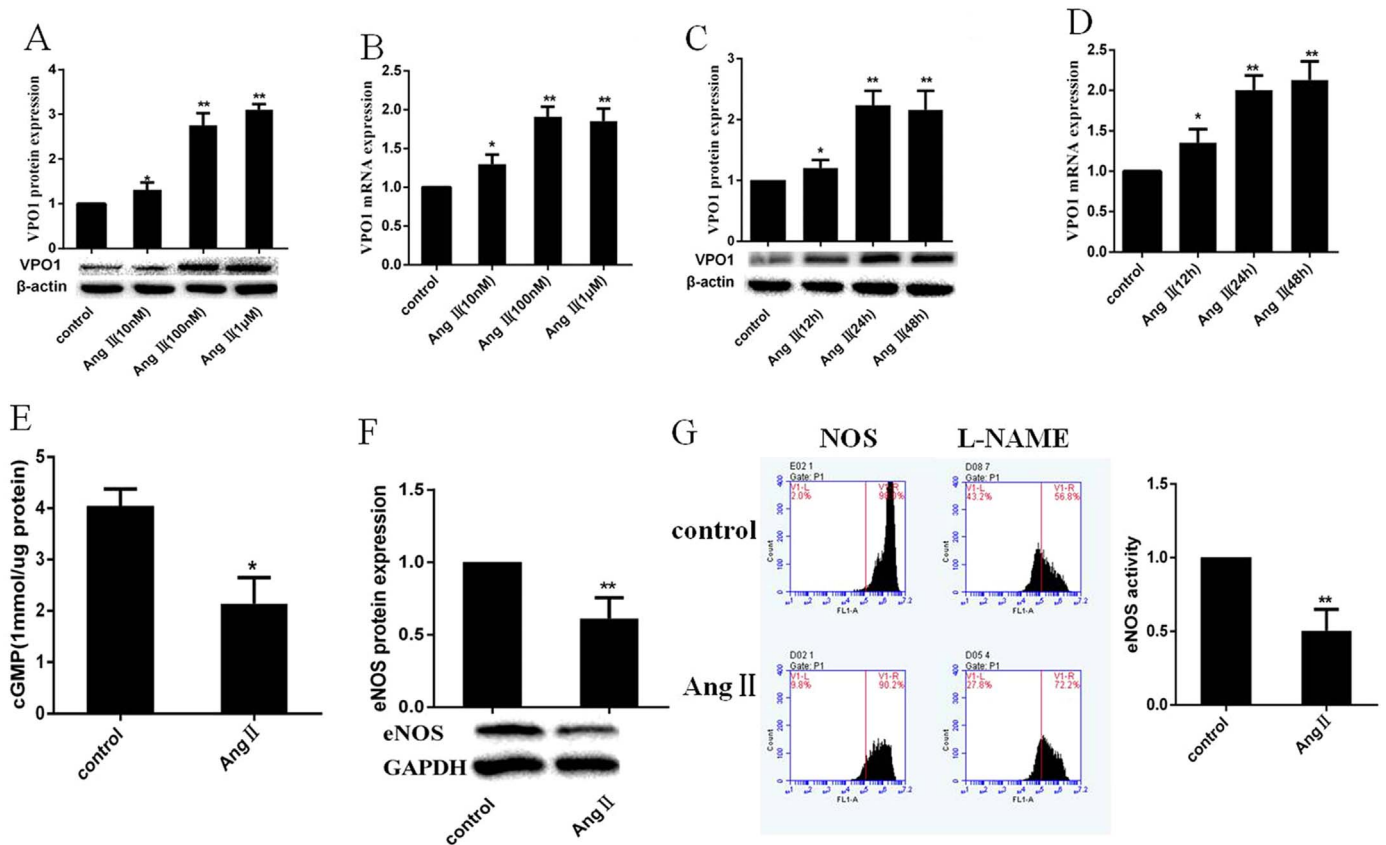
## 3. Results

### 3.1. Angiotensin II enhances VPO1 expression, decreases eNOS expression and activity as well as NO production

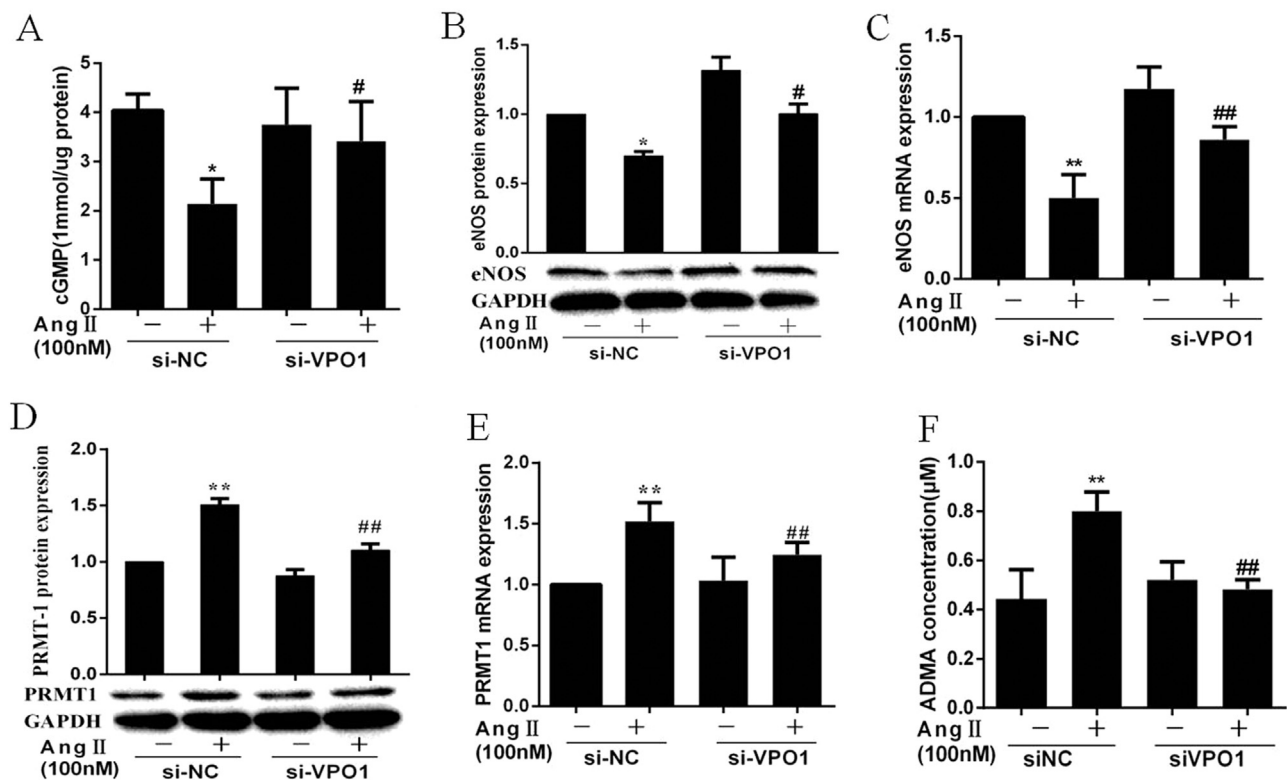
As described by our groups previously, AngII induced VPO1 protein and mRNA expression in a concentration and time-dependent manner in VASMCs [10]. In this study, AngII also up-regulated VPO1 protein and mRNA expression in the same manner in HUVECs. The induction effect peaked at 24 h of treatment with 100 nM AngII (Fig. 1A–D). Also, AngII (100 nM) significantly decreased eNOS expression and activity as well as cGMP level (Fig. 1E–G).

### 3.2. VPO1 regulates eNOS expression through PRMT1/ADMA pathway

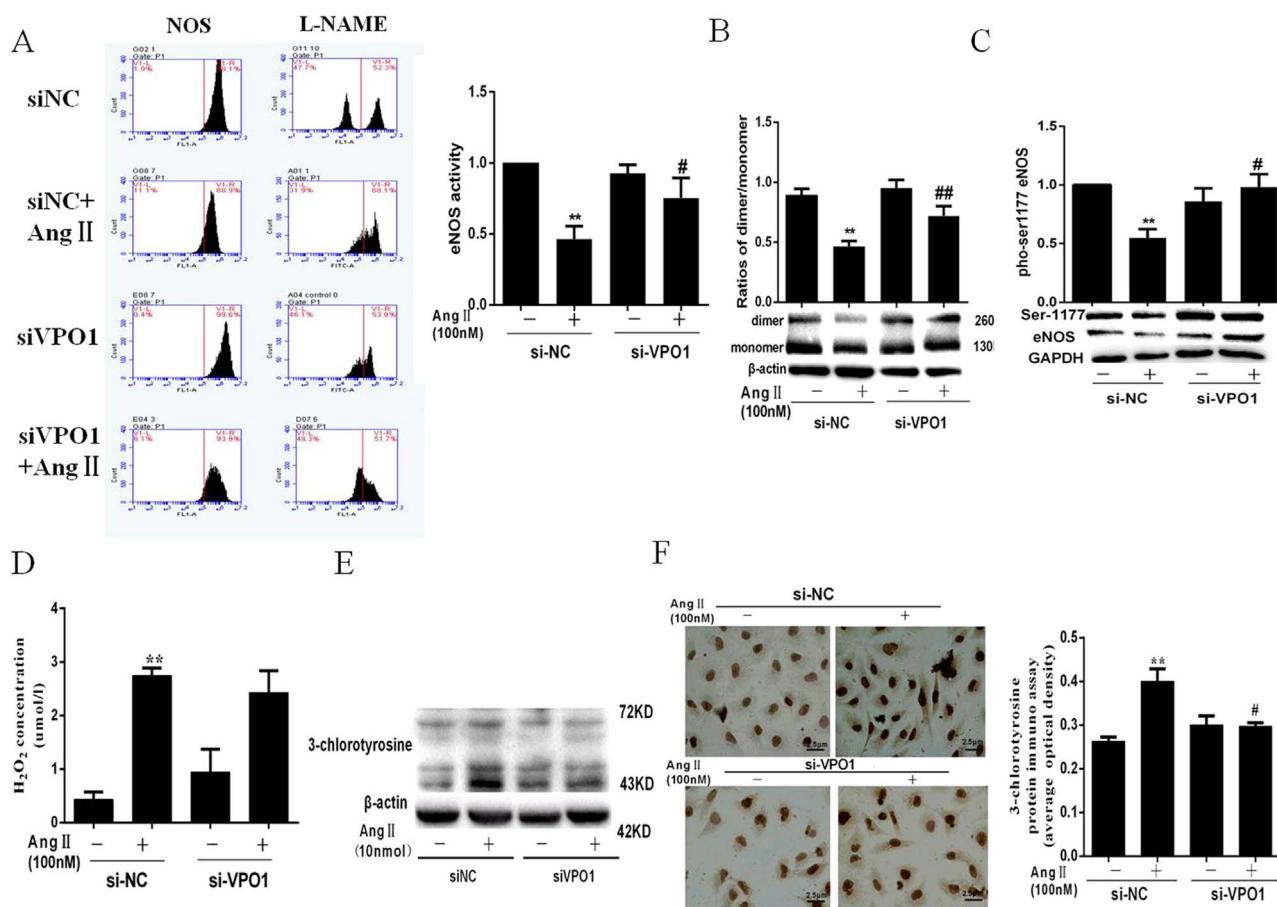
siRNA transfection was used to knockdown gene and protein expression of VPO1 in HUVECs. VPO1 expression (both protein and



**Fig. 1.** Effects of AngII on VPO1 and eNOS in HUVECs. (A–D): AngII treatment up-regulated protein and mRNA of expression of VPO1 in a time and dose dependent manner (\*\* $P < 0.01$ ) vs control group. (E) AngII (100 nM) treatment inhibited cGMP production. (\* $P < 0.05$ ) vs control group. (F–G) incubation of HUVECs with Ang (100 nM) for 24 h decreased eNOS expression and activity. (\*\* $P < 0.01$ ) vs control group. (n=3 per group).



**Fig. 2.** VPO1 regulates eNOS expression through PRMT1/ADMA pathway. HUVECs were treated with AngII for 24 h after transfected with siVPO1. (A) VPO1 gene knockdown reversed AngII induced decrease of cGMP generation. (B–C) the eNOS protein and mRNA expression. (D–E) the PRMT1 protein and mRNA expression. (F) AMDA was measured by HPLC(n=3 per group) (\*\* $P < 0.01$ ) vs control group. (## $P < 0.01$ ) vs AngII group, (n=3 per group).



**Fig. 3.** The effect of VPO1 on eNOS activity in AngII treated HUVECs. (A) eNOS activity was measured by Flow Cytometry. VPO1-siRNA attenuated the decreased eNOS activity induced by AngII. (B) The eNOS dimer and monomer were measured by low-temperature SDS-PAGE. (C) The level of p-eNOS. (D) The level of intracellular H<sub>2</sub>O<sub>2</sub>. (E–F) the 3-chlorotyrosine expression assessed by western blot and immunohistochemistry. (\*\*P < 0.01) vs control group. (##P < 0.01) vs AngIIgroup. (n=3 per group).

mRNA) was significantly inhibited compared with the negative control (Supplementary Figs. A–B). we treated HUVECs with AngII (100 nM) for 24 h after siRNA transfection. The decrease in cGMP concentration induced by AngII was reversed by VPO1siRNA transfection (Fig. 2A). VPO1 siRNA also alleviated the inhibiting effect of AngII on eNOS gene and protein expression (Fig. 2B–C). ADMA is a competitive inhibitor of eNOS and synthesized by protein arginine methyltransferase1 (PRMT1) [15]. As shown in Fig. 2D–F, AngIIup-regulated PRMT1 protein and mRNA expression and ADMA concentration, which were inhibited by VPO1 siRNA.

### 3.3. The effect of VPO1 on eNOS activity in AngII treated HUVECs

As shown in Fig. 3A, VPO1 siRNA successfully attenuated the decreased eNOS activity which induced by AngII. eNOS activity can be regulated by several mechanisms. Studies have shown the dimeric structure is crucial to eNOS activity [16]. We assessed the ratio of eNOS dimer and monomer after transfection with VPO1 siRNA. The results showed that VPO1 siRNA reversed the eNOS uncoupling induced by AngII (Fig. 3B). In addition, eNOS phosphorylation is a critical way of regulating its activity [17,18]. We subsequently examine the ser1177 phosphorylation of eNOS. As depicted in Fig. 3C, AngIIimpaired ser1177 phosphorylation of eNOS, which reversed by VPO1 siRNA.

### 3.4. Influence of VPO1 on H<sub>2</sub>O<sub>2</sub> and HOCl production

Our previous study has been demonstrated that VPO1 is responsible for the production of HOCl in the cardiovascular diseases [1]. As shown in Fig. 3D–F, AngIIincubation significantly increased the levels

of H<sub>2</sub>O<sub>2</sub> and HOCl. Also, VPO1-siRNA evidently inhibited the HOCl generation compared with the controls (Fig. 3E–F). However, in the AngII treated state, VPO1-siRNA increased H<sub>2</sub>O<sub>2</sub> production without statistical significance (Fig. 3D).

### 3.5. HOCl directly decreases eNOS expression and activity in HUVECs

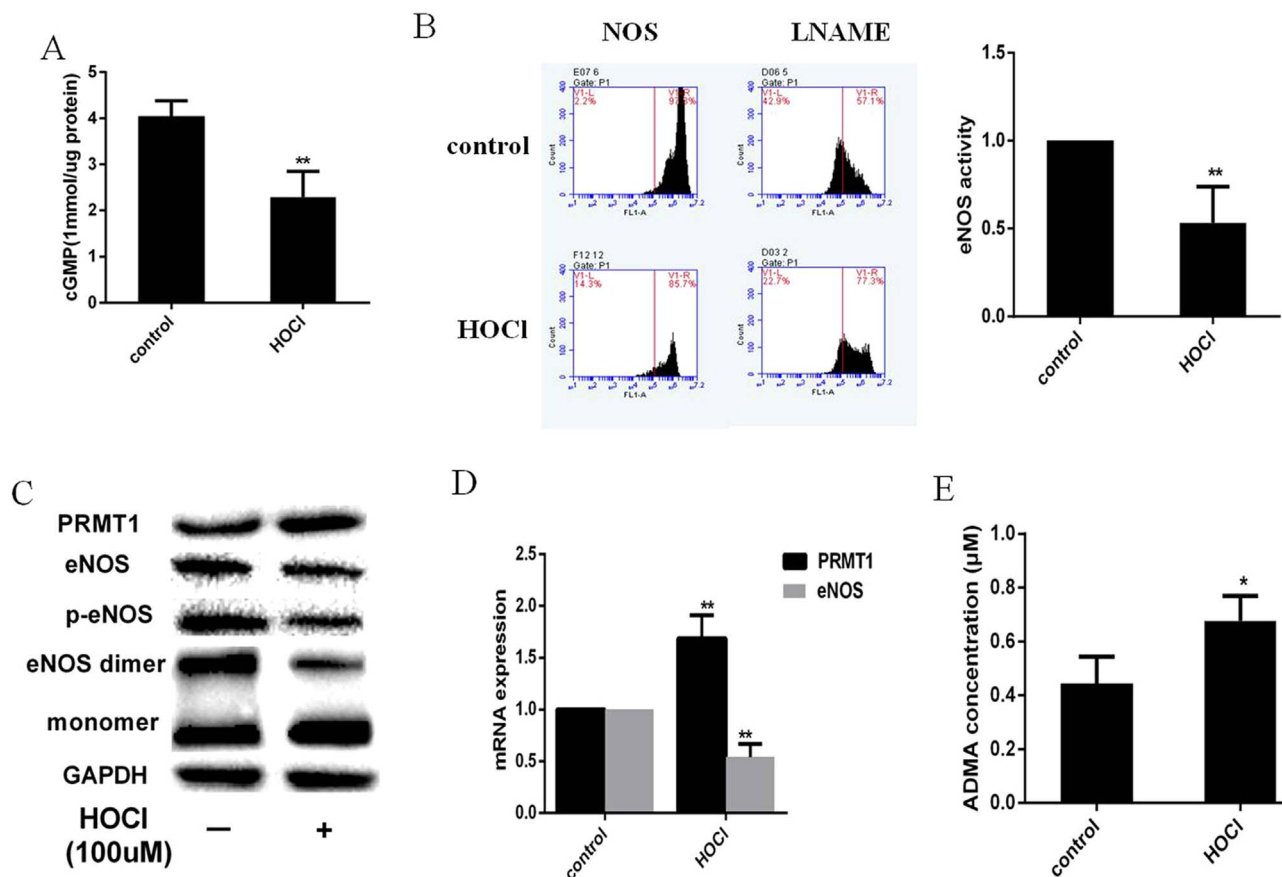
It has been demonstrated that VPO1-generated HOCl aggravates vascular oxidative stress that induced vascular smooth cell proliferation and endothelial dysfunction [19]. HUVECs were treated with HOCl (100 μmol/L) for 2 h and replaced with normal medium for 24 h. As shown in Fig. 4A–D, HOCl significantly decreased eNOS expression and activity, cGMP production. Moreover, PRMT-1 expression and ADMA production were increased while the dimer and phosphorylation of eNOS were decreased compared with the controls (Fig. 4C–E).

## 4. Discussion

The data presented above indicates that VPO1 plays a critical role in regulating eNOS expression and activity. VPO1 expression was up-regulated in AngII-treated HUVECs. Knockdown of VPO1 was sufficient to attenuate the AngII induced decrease in eNOS expression and activity. Moreover, the effect of VPO1 on eNOS was mainly achieved by HOCl formation. This experiment implied that VPO1 may be a key regulator on eNOS, which is a potential therapeutic target in cardiovascular diseases.

Vascular peroxidase 1 (VPO1) is a newly identified family member of peroxidases expressed in cardiovascular system. VPO1-mediated





**Fig. 4.** HOCl directly decreases eNOS expression and activity in HUVECs. HUVECs were incubated with HOCl (100  $\mu\text{mol/L}$ ) for 2 h, and then cultured with DMEM for 24 h. (A) cGMP production measured by ELISA. (B) eNOS activity. (C) PRMT1, total eNOS, eNOS dimer and monomer were analyzed by western blot. (D) The eNOS and PRMT1 mRNA expression. (E) ADMA production.  $n=3$  per group (\*\*  $P < 0.01$ , \*  $P < 0.05$ ) vs control group. ( $n=3$  per group).

oxidative stress plays an essential role in endothelial cell apoptosis, smooth muscle cell proliferation, cardiac hypertrophy and vascular calcification [10,20–22]. Recently our laboratory demonstrated that activation of NOX/VPO1/HOCl pathway played an important role in endothelial dysfunction via DDAH/ADMA pathway in SHR and HUVECs treated with AngII [3].

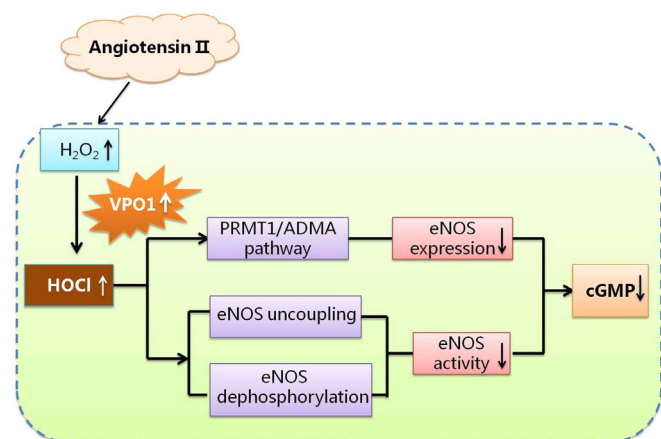
NO is one of the factors secreted by endothelium to maintain the vascular tone. These factors modulate the relaxatory behavior of vascular smooth muscle, platelet aggregation and inflammation [23]. NO synthesized enzymatically from L-arginine by eNOS [24,25] is the most important signaling molecule that modulates physiological endothelium function. Previous studies have shown that increased oxidative stress is often associated with decreased eNOS expression and activity [23,26,27]. This is consistent with our data that eNOS expression and activity were decreased in AngII treated HUVECs.

ADMA is the most important upstream regulator for eNOS expression [28]. It is potentially an indicator for cardiovascular diseases, as decrease in NO production leads to adverse vascular effects including dysregulation of blood pressure, loss of antithrombotic activity, disorder in homeostasis and fibrinolysis, and inhibition of platelet aggregation [29,30]. Protein arginine methyltransferase1 (PRMT1) is the predominant enzyme catalyzing the formation of ADMA by transferring methyl groups from S-adenosyl-methionine to guanidino groups of arginine residues in a variety of proteins; DDAH metabolize ADMA to produce citrulline [31]. Our group has demonstrated that VPO1 was correlated with ADMA metabolism through regulating DDAH expression and activity [12]. In this study, we found that AngII-induced PRMT1 expression was also inhibited by VPO1 siRNA, which significantly suppressed ADMA production and enhanced eNOS expression. These results implicated that VPO1 is a key enzyme

regulating ADMA generation and eNOS expression.

The activity of eNOS is subjected to a discreet and complexed mechanism of regulation. The active form of eNOS enzyme consists of two identical subunits that form a head to tail homodimer [27]. Intriguingly, it has been demonstrated that the eNOS could be a source of superoxidase when uncoupled [32]. Recent studies in vivo and in vitro demonstrated increased eNOS uncoupling in many cardiovascular disorders including hypertension [33], ischemia reperfusion [34] and diabetes [35]. In this study, we observed that the VPO1 siRNA attenuated AngII-induced eNOS uncoupling. In addition, protein phosphorylation is a posttranslational modification and a key mediator of eNOS activity. The primary sites where eNOS gets phosphorylated are serine residues and, to a lesser extent, on tyrosine (Tyr) and threonine residues [36]. Studies showed that Resveratrol restored endothelial function by preserving eNOS phosphorylation (Ser1177). In our study, we observed the knockdown of VPO1 expression significantly increased serine1177 phosphorylation of eNOS. These data suggest that VPO1 may act as a novel regulator of eNOS activity via structure change and phosphorylation.

The predominant role of VPO1 entails utilization of  $\text{H}_2\text{O}_2$  to yield HOCl, which aggravates oxidative stress in the pathological conditions [19]. In this study, we demonstrated that AngII increased  $\text{H}_2\text{O}_2$  and HOCl production in HUVECs. Interestingly, VPO1 expression inhibition had no effect on  $\text{H}_2\text{O}_2$  production, but significantly decreased the level of HOCl. HOCl was shown to inactivate eNOS by inducing eNOS uncoupling and inhibiting eNOS phosphorylation [37,38]. To further test the hypothesis that VPO1 regulates eNOS via HOCl formation, HUVECs were incubated with HOCl for an hour. HOCl is the strong oxidant substance that participates in redox reaction [37]. We found that HOCl treatment decreased eNOS expression and activity by



**Fig. 5.** The proposed pathway of VPO1 regulation of eNOS. AngII up-regulates H<sub>2</sub>O<sub>2</sub> production and VPO1 expression. VPO1-derived HOCl decreases eNOS expression through PRMT1/ADMA pathway and down-regulates eNOS activity via inducing eNOS uncoupling and eNOS ser1177 dephosphorylation.

inducing ADMA production, eNOS uncoupling and inhibiting p-eNOS. These results indicate that HOCl-derived from VPO1 directly induced a decreased eNOS expression and activity which inhibited NO generation.

However, the mechanism of how HOCl regulates PRMT-1 and eNOS uncoupling remains unexplained. Recently, a novel redox-based PRMT regulatory mechanism was reported. It is revealed that two cysteine residues (C101 and C208) which can influence PRMT1 activity can be regulated by the redox environment [39]. Additionally, S-glutathionylation of eNOS is a new and important mechanism providing redox regulation of cellular signaling [40]. It has been demonstrated that HOCl contributed to the formation of s-glutathionylation [41]. These are interesting directions in which further investigation is necessary in uncovering the mechanism behind.

In conclusion, our work suggested that VPO1 inhibited eNOS expression by decreasing ADMA production. It also decreases eNOS activity by structural change and de-phosphorylation. The proposed pathway of VPO1 regulation of eNOS is summarized in Fig. 5. Overall, VPO1 is a pivotal regulator of eNOS.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2017.02.022.

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