VASCULAR PEROXIDASE 1 UP-REGULATION BY ANGIOTENSIN II ATTENUATES NITRIC OXIDE PRODUCTION THROUGH INCREASING ASYMMETRICAL DIMETHYLARGININE IN HUVECS

Short title: Vascular peroxidase1 attenuates nitric oxide in HUVECs.

Haiyang Peng, MD¹, Luyao Chen, MD¹, Xiao Huang, MD, PhD¹, Tianlun Yang, MD,
PhD¹, Zaixin Yu, MD, PhD¹, Guangjie Cheng, PhD², Guogang Zhang, MD, PhD¹,
Ruizheng Shi, MD, PhD¹

¹Department of Cardiovascular Medicine, Xiangya Hospital, Central South
University, Changsha 410008, China

²Division of Pulmonary, Allergy & Critical Care Medicine, Department of Medicine,
University of Alabama at Birmingham, Birmingham, AL 35294, USA

*Corresponding author:

Dr. Ruizheng Shi, Department of Cardiovascular Medicine, Xiangya Hospital, Central
South University, Changsha 410008, China. E-mail: xysrz2010@sina.com; Phone:
086-731-84327695; Fax: 086-731-84327695.

Abstract : Asymmetric dimethylarginine (ADMA), the endogenous inhibitor of nitric oxide synthase (NOS), contributes to endothelial dysfunction and subsequent cardiovascular events including hypertension. Vascular peroxidase 1 (VPO1) is a novel heme-containing peroxidase that utilizes hydrogen peroxide (H_2O_2) generated from co-expressed nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to catalyze peroxidative reactions. Our previous study revealed a clear connection between VPO1 gene expression and endothelial dysfunction in spontaneously hypertensive rats. In the present study, we explored whether VPO1 participates in endothelial dysfunction during hypertension by increasing ADMA production. Spontaneously hypertensive rats displayed impaired endothelium-dependent relaxation, decreased eNOS expression and nitric oxide production, significantly increased VPO1 expression in both plasma and aorta tissue, and an increased ADMA level in plasma. In cultured endothelial cells, angiotensin II increased the ADMA level by inhibiting dimethylarginine dimethylaminohydrolase activity, which was inhibited by knockdown of VPO1 using small hairpin RNA. Moreover, the NADPH oxidase inhibitor, and the hydrogen peroxide scavenger attenuated angiotensin II-mediated up-regulation of VPO1 and generation of hypochlorous acid. Furthermore, VPO1-derived hypochlorous acid suppressed recombinant dimethylarginine dimethylaminohydrolase activity and increased ADMA production. VPO1 plays a critical role in ADMA production via H_2O_2 -VPO1-hypochlorous acid pathways, which may contribute to endothelial dysfunction in hypertension.

Keyword: vascular peroxidase 1; nicotinamide adenine dinucleotide phosphate oxidase; asymmetrical dimethylarginine; endothelial dysfunction; hypertension

Introduction

Asymmetric dimethylarginine (ADMA), the endogenous inhibitor of nitric oxide (NO) synthase (NOS), contributes to endothelial dysfunction and subsequent cardiovascular events.¹⁻³ Numerous clinical and animal studies have shown a relationship between high blood pressure and high plasma ADMA concentrations.⁴⁻⁸ Increased bioavailability of reactive oxygen species (ROS) and altered redox signaling (oxidative stress) have been implicated in the onset and/or progression of chronic diseases, including hypertension.⁹⁻¹⁰ Although patients with early hypertension display elevated plasma levels of ADMA and markers of ROS,¹¹ it is unclear how ROS increase the ADMA levels during hypertension.

Vascular peroxidase 1 (VPO1) is a newly identified heme-containing peroxidase that primarily localizes to the cardiovascular system.¹² We have previously demonstrated that VPO1 is able to utilize hydrogen peroxide (H₂O₂) generated from co-expressed nicotinamide adenine dinucleotide phosphate oxidases (NOXs) to catalyze peroxidative reactions.¹² Several studies from our group suggested that NOX/VPO1 pathway-mediated oxidative stress plays an important role in myocardial ischemia-reperfusion injury, endothelial cell apoptosis and smooth muscle cell proliferation.¹³⁻¹⁵ Our more recent work revealed the up-regulation of VPO1, accompanied by endothelial dysfunction, decreased NO levels, and elevated NOX and ROS activities during the development of hypertension. These data suggest a connection between VPO1 gene expression, endothelial dysfunction and hypertension.

The present study was designed to test the hypothesis that VPO1 is a source of ROS in the cardiovascular system, enhances ADMA production, and subsequently induces endothelial dysfunction under conditions of hypertension. First, in the *in vivo* portion of this study, we assessed the relationship between VPO1, endothelial dysfunction and ADMA in spontaneously hypertensive rats (SHR). Then, in the *in vitro* study, we investigated the role of VPO1 in angiotensin II-induced changes in endothelial NOS (eNOS) expression, ADMA and NO production in human umbilical vein endothelial cells (HUVECs). Furthermore, as cellular ADMA is metabolized by dimethylargininedimethylaminohydrolases (DDAHs),³ and DDAH activity could be attenuated by ROS,¹⁶ we tested whether the role of VPO1 in enhancing ADMA was fulfilled by the inhibition of DDAH activity.

Methods

Animals

This study was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the 8-week experimental protocol was approved by the Medicine Animal Welfare Committee of Xiangya Medical School, Central South University (Changsha, China). Male SHR were obtained from SLRC Laboratory Animal Inc. (Shanghai, China). SHR (n = 10) at 12 weeks of age were randomly assigned to groups. Age- and sex-matched normotensive Wistar-Kyoto (WKY) rats (n = 10) were used as control animals.

The systolic blood pressure of the rats was measured using the tail-cuff method before

the experiment. The rats were anesthetized with pentobarbital sodium (60mg/kg) intraperitoneally. Paw & eye flex tests were performed to confirm that animals were under complete anaesthesia. The left carotid artery was catheterized to collect blood sample. Then, the rats were sacrificed. The thoracic aorta was isolated and divided into segments. A segment of aorta was immediately incubated in Krebs solution for the assay of vascular function. Other segments were frozen in liquid nitrogen for the extraction of total RNA or protein. Blood samples were collected in 5 mL tubes containing EDTA and anticoagulant and were immediately centrifuged at -4 °C (20 min, 4000 rpm); plasma samples were obtained and stored at -80 °C until analysis.

Vascular tone assay

Endothelium-dependent and endothelium-independent relaxation of the rat aorta in response to acetylcholine (ACh) and S-nitroso-N-acetylpenicillamine (SNAP), respectively, were assessed as previously described.¹⁷ Briefly, thoracic aortas were dissected free of adventitia and cut into 2-3 mm ring segments. Following contraction induced using phenylephrine (PE, 10 µmol/L) (Sigma-Aldrich), relaxation in response to increasing concentrations of ACh (0.01-10 µmol/L) or SNAP (0.01-10 µmol/L) was evaluated to obtain dose-response curves. The degree of precontraction in response to PE was chosen to approximate 80% of the maximal response to KCl (60 mmol/L). The dose-response curves were analyzed, and the maximal relaxation value (E_{max}), expressed as the percentage of PE-induced contraction, was calculated for each ring.

ADMA concentration assay

The HPLC system consisted of a Shimadzu LC-10Adv pump, an SCL-10Adv system

controller, a CTO-10Avp column oven, an FCV-10ALvp low-pressure gradient unit, and a DGU-14A degasser (Shimadzu, Kyoto, Japan). Chromatographic separation was performed using a Thermo HypersilHyPURITY C18 analytical column (150×2.1 mm, 5 μm). The oven temperature was set at 35 °C. The mobile phase, containing 20 mmol/L ammonium acetate (pH 3.9)/methanol (95:5, v/v), was applied at a flow rate of 0.2 mL/min. The mass spectrometer was a single quadrupole LCMS-2010 equipped with an atmospheric pressure chemical ionization (APCI) interface (Shimadzu, Kyoto, Japan). The flow rate of the nebulizer gas and the dried gas were set at 1.5 and 10 L/min, respectively. The ions selected for monitoring were determined based on positive scanning from 100 to 1000 m/z. For the quantification of ADMA, the positive protonated molecular ions of ADMA ($[M+H]^+$) were detected at $m/z = 203$.

DDAH activity assay

ADMA was added to the cell lysates at a final concentration of 500 μmol/L in an ice bath. 5-sulfosalicylic acid (30%) was immediately added to the cell lysates as a negative control. The cell lysates were incubated at 37 °C for 2 h, followed by the addition of 30% 5-sulfo-salicylic acid. The ADMA levels were measured via LC-MS. DDAH activity was reflected by the difference in the ADMA concentration between each group.

Cell culture and treatment

HUVECs were purchased from ATCC. The cells were seeded at a density of 10^5 per 60-mm dish in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cultures were maintained at

37 °C in a gas mixture of 5% CO₂/95% air. Cells between passages 4 and 15 were used for the experiments. The cells were cultured in DMEM containing 1% FBS for 24 h before the treatments described below.

To investigate the effects of angiotensin II on VPO1 expression in HUVECs, the cells were treated with angiotensin II (100 nmol/L) for 12, 24, or 48 h or with angiotensin II (10, 100, or 1000 nmol/L) for 24 h. Subsequent experiments were performed as follows. First, the cells were incubated in 10 μM diphenyliodonium (DPI; a NOX inhibitor) or 10 μmol/L catalase (a H₂O₂ hydrolase) and then exposed to Ang II (100 nmol/L) for 24 h. The expression levels of Nox4, VPO1, eNOS, and DDAH-2; the DDAH activity level; and the concentrations of H₂O₂, hypochlorous acid (HOCl), ADMA and NO in the supernatants were determined. Second, the cells were treated with HOCl. As described previously¹⁴, the cells were incubated for 1 h in Hank's buffered saline solution (HBSS), which consisted of HOCl stock solution at a final HOCl concentration of 20 μmol/L. Then, the cells were washed, followed by incubation in DMEM for an additional 24 h.

RNA interference and cell transfection

RNA interference and cell transfection were performed as described previously. The VPO1 siRNA sequences were designed and synthesized by Sangon (Shanghai, China). HUVECs were transfected with VPO1 siRNA using LipofectamineRNAiMAX (Roche) according to the manufacturer's instructions (see supporting information to gain more information of RNAi and primer sequences). Forty-eight hours after transfection, the cells were exposed to Ang II (100 nmol/L) for 24 h.

Effects of VPO1 and HOCl on recombinant human DDAH2 (hDDAH2) activity

The effects of VPO1 and its enzymatic product HOCl on the activity of the recombinant hDDAH2 enzyme were measured as described previously.¹⁸ Briefly, 1 µg of hDDAH2 was incubated for 15 min at 37 °C in the presence of VPO1 (10, 30, 100 and 300 nmol/L) with or without substrate (10 µmol/L H₂O₂ and NaCl 140 mmol/L) or, in a different experiment, in the presence of HOCl (0.1, 1, 10, and 100 µmol/L). After the addition of the DDAH substrate ADMA at 1 mmol/L in a final reaction volume of 100 µL, the samples were incubated for 1 h at 37 °C. After incubation, 200 µL of freshly prepared color-developing reagent was added to each sample. After 15 minutes of incubation at 95 °C in an oven, the plate was cooled to room temperature, and citrulline formation was measured at 540 nm.

NO assay

The level of NO in plasma and cell supernatants was indirectly determined using an NO assay kit according to the protocol provided by the manufacturer (Nanjing Jiancheng Bioengineering Institute, China)¹⁶. Nitrate was reduced to nitrite, which can be detected by Griess reagent to reflect the concentration of NO.

Real-time PCR analysis, Western blot analysis, and H₂O₂ and HOCl assays

Real-time PCR analysis, Western blot analysis, and H₂O₂ and HOCl assays were performed as described previously.¹⁶

Materials

DMEM, penicillin, streptomycin, and FBS were obtained from Hyclone. Angiotensin II, ADMA, DPI, catalase, 4-aminobenzoic hydrazide (ABAH), and HOCl stock solution

were purchased from Sigma-Aldrich (St. Louis, MO, USA). The rabbit anti-eNOS antibody and the rabbit anti-DDAH-2 antibody were obtained from Abcam (Cambridge, UK). The H₂O₂ assay kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Other chemical reagents were obtained from Beijing Chemical Industry (Beijing, China), unless otherwise noted.

Statistical analysis

The results are expressed as the means±SEM. All statistical evaluation was performed with the statistics program IBM SPSS 19.0 for windows. Quantitative variables were compared using Student's paired t-test for two groups or ANOVA followed by the Newman-Student-Keuls test for multiple groups. The significance level was set at $P<0.05$.

Results

As shown in Figure 1, compared to WKY rats, systolic blood pressure was significantly higher in SHR at 20 weeks of age. The endothelium-dependent relaxation of the thoracic aorta ring in SHR was significantly impaired, accompanied by increased VPO1 expression in aorta tissue and plasma and an elevated ADMA level in plasma. Alternatively, eNOS expression in aorta tissue and NO production in plasma were significantly decreased in SHR compared to WKY rats (Figure 1).

The angiotensin II level in SHR was significantly increased compared to that in WKY rats (see Supplementary material online, Figure S1). We therefore utilized angiotensin II as a stimulus to mimic the pathological state of endothelial dysfunction under

conditions of hypertension in *in vitro* experiments. As shown in Figure 2, treatment with angiotensin II up-regulated VPO1 expression (at both the mRNA and protein levels) in HUVECs in a concentration- and time-dependent manner. The induction effect peaked at 24 h of treatment with 100 nmol/L Ang II. To determine the role of VPO1 in angiotensin II-mediated eNOS expression and NO production, we developed VPO1-specific siRNA. As shown in Figure S2, VPO1-siRNA successfully knocked down VPO1 expression by 80% in HUVECs. Angiotensin II treatment significantly decreased eNOS expression and NO production in HUVECs, but VPO1-siRNA transfection significantly attenuated the inhibitory effects of angiotensin II on eNOS expression and NO production (Figure 3).

It is well known that an elevated ADMA level is responsible for decreasing NO production by inhibiting eNOS expression or activity.¹⁻³ As shown in Figure 3E, in line with the increased ADMA level observed in SHR from our *in vivo* study, angiotensin II treatment significantly increased ADMA production and up-regulated VPO1 expression, but VPO1-siRNA transfection attenuated the effect of angiotensin II on ADMA production. It has been found that more than 70% of ADMA is metabolized by DDAH into citrulline and dimethylamine. In humans, there are two major isoforms of DDAH (DDAH-1 and DDAH-2), which display partially overlapping tissue distributions.¹⁹ DDAH-1 expression tends to predominate in tissues with high expression of the neuronal isoform of NOS (nNOS), whereas DDAH-2 expression appears to follow the expression pattern of eNOS.¹⁹ In this study, we found that the activity, but not the expression, of DDAH-2, the major enzyme responsible for ADMA

metabolism in endothelial cells, was significantly inhibited by angiotensin II treatment but that VPO1-siRNA transfection attenuated the effect of angiotensin II. These results indicated that VPO1 might play a regulatory role in ADMA production by regulating DDAH-2 activity in endothelial cells.

We have previously shown that the pathophysiological activity of VPO1 depends on the generation of HOCl.¹⁴⁻¹⁶ In the present study, we found that angiotensin II treatment significantly increased HOCl production but that VPO1-siRNA transfection attenuated the effect of angiotensin II (Figure 3G). Next, to clarify the role of VPO1 in NO production, we observed the effect of HOCl treatment on ADMA and NO production and eNOS expression in HUVECs. As shown in Figure 4, incubation of these cells in HOCl significantly increased the ADMA levels by decreasing DDAH activity, and this alteration was accompanied by decreased eNOS expression and NO production. To further elucidate how VPO1 and its derived product HOCl enhanced ADMA production, we treated recombinant hDDAH2 with VPO1 or HOCl. As shown in Figure 5, treatment with H₂O₂ and VPO1 resulted in a significant reduction in recombinant hDDAH2 activity, whereas treatment with VPO1 alone exerted no effect (Figure 5A). The VPO1 product HOCl also dose-dependently impaired hDDAH1 activity (Figure 5B). Taken together, these data suggest that VPO1 impairs DDAH-2 activity by generating HOCl.

Several lines of evidence from our group suggested that NOX/H₂O₂/VPO1 pathway-mediated oxidative stress plays an important role in myocardial ischemia-reperfusion injury, endothelial cell apoptosis and smooth muscle cell

proliferation.¹⁴⁻¹⁶ Recently, Luo *et al* found that NOX increased the cellular ADMA levels; however, the specific mechanism underlying this activity is not well understood.²⁰ In the present study, to define the mechanism upstream of VPO1 that is involved in the angiotensin II-induced changes in the ADMA, eNOS and NO levels, we examined the effects of pharmacologic inhibitors of NOX and H₂O₂. Both DPI (a NOX oxidase inhibitor) and catalase (a specific H₂O₂ scavenger) attenuated the induction of VPO1 expression and HOCl and ADMA production by angiotensin II, and these effects were accompanied by the suppression of DDAH-2 activity, eNOS expression and NO production in HUVECs (Figure 6).

Discussion

In the present study, we found that the regulatory effect of VPO1 on angiotensin II-induced ADMA production was fulfilled by the inhibition of DDAH-2 activity via the VPO1 producing HOCl in HUVECs, indicating that VPO1 might play a role in endothelial dysfunction in SHR.

Endothelial dysfunction is thought to contribute to the pathogenesis and development of hypertension.^{4-8, 12,21} The risk of developing hypertension is increased in normotensive individuals with impaired flow-mediated vasodilation,²² whereas antihypertensive treatments have a beneficial effect on endothelial dysfunction.²³ The major factor leading to endothelial dysfunction is the insufficient production and bioavailability of NO, which is generated from the hydrolysis of L-arginine by NOS.²⁴ The down-regulated expression or enzymatic activity of eNOS in endothelial

cells is closely associated with endothelial dysfunction during hypertension.^{25,26} The blood pressure of eNOS^{-/-} mice was significantly elevated compared to that of normal control mice.²⁷ In the present study, in line with our previous study,¹⁷ we found that systolic blood pressure was significantly increased, eNOS expression and NO production were decreased, and aortic endothelium-dependent relaxation was impaired in SHR.

ROS, such as superoxide and H₂O₂, have emerged as important molecules in the pathogenesis of endothelial dysfunction in hypertension.^{9,10} Peroxidases are an important source of ROS, and myeloperoxidase (MPO), the peroxidase derived from leukocytes, plays a role in endothelial dysfunction by reducing the bioavailability of NO under conditions of diabetic vascular disease or lipopolysaccharide-induced acute inflammation.²⁸⁻³¹ However, MPO is primarily localized to leukocytes and must be released by leukocytes to then bind to and infiltrate the vascular wall.²⁸⁻³¹ In the present study, we found no significant elevation in the plasma MPO level in SHR, and this result was in line with our previous finding of no significant MPO binding to the vascular tissues of SHR.¹⁴ Similarly, we found that the expression of VPO1, a secreted peroxidase that is primarily expressed in the cardiovascular system and is much more highly concentrated in human plasma than MPO,³² was elevated in both plasma and in aorta tissue in SHR. This increase in VPO1 expression was accompanied by impaired endothelium-dependent relaxation of the thoracic aorta ring and decreased eNOS expression and NO levels. These results indicate that VPO1 plays another important role potentially in endothelial dysfunction in SHR.

ADMA, the endogenous inhibitor of eNOS, is another marker of endothelial dysfunction.¹⁻³ There is a strong association between high blood pressure and high plasma ADMA concentrations.⁴⁻⁶ In the present study, we confirmed that the ADMA content in plasma was significantly elevated in SHR, and this alteration was accompanied by impaired aortic endothelium-dependent relaxation and decreased eNOS expression and NO production. Increased ADMA levels have been observed in several conditions associated with ROS. However, the mechanism by which ROS increase the ADMA level is unclear.⁶ Recently, Eike-Christinet *al* performed an elegant study showing that MPO regulates ADMA production under inflammatory conditions.¹⁸ In the present study, we found that the ADMA level was elevated and that VPO1 expression was significantly increased in plasma and vascular tissues of SHR. In cultured endothelial cells, angiotensin II treatment significantly increased ADMA production and VPO1 expression, but VPO1-siRNA transfection attenuated the effect of angiotensin II on ADMA production. Furthermore, the activity of DDAH-2, the major enzyme responsible for ADMA metabolism in endothelial cells,³ was significantly inhibited by VPO1-siRNA transfection, although DDAH-2 expression was not significantly altered by this treatment. Taken together, these data support the notion that VPO1 might regulate ADMA production by suppressing DDAH-2 activity in endothelial cells.

It has been demonstrated that the role of peroxidases in ROS-induced injury is at least partially dependent on the production of HOCl.^{31,32} MPO impaired endothelium-dependent relaxation induced by derived HOCl and chlorinating

species.²⁹⁻³² Our previous studies revealed that the pathophysiological role of VPO1 also depends on the generation of HOCl.¹⁴⁻¹⁶ In the present study, angiotensin II treatment significantly increased HOCl production, but VPO1-siRNA transfection attenuated this effect of angiotensin II. Therefore, to further investigate the role of VPO1-derived HOCl in nitric oxide production, we treated endothelial cells with HOCl and found that HOCl treatment significantly enhanced ADMA production by inhibiting DDAH-2 activity, which led to decreases in eNOS expression and NO level. It has been revealed that the crystal structure of microbial DDAH contains a Cys-His-Glu catalytic triad (Cys-249, His-162, and Glu-114).³³ The presence of a reactive cysteine residue in the active site of DDAH raised the possibility that DDAH activity might be altered under conditions of oxidative or nitrosative stress.³⁴ In the present study, to further define how VPO1 enhances ADMA production, we employed recombinant hDDAH2 and found that treatment with H₂O₂ and VPO1 or with HOCl alone resulted in a significant reduction in recombinant hDDAH2 activity but that treatment with VPO1 alone exerted no effect on recombinant hDDAH2 activity. These data indicated that the inhibitory effect of VPO1 on DDAH-2 activity might be fulfilled by the generation of HOCl, as the cysteine residue of DDAH is a well-established target of HOCl.³⁵ Although it has been reported that HOCl induces the uncoupling of eNOS to ONOO⁻ generated from NOX³⁶ and that HOCl plays a role in inhibiting NOS in endothelial cells via the intracellular mislocalization of eNOS by modified low density lipoprotein,³⁷ our data suggested that HOCl inhibited eNOS expression by enhancing ADMA production, and this effect might be related to the reaction of HOCl with the cysteine residue of

DDAH-2.

NOX, the major source of ROS in vascular cells, plays an important role in the development of cardiovascular diseases, including hypertension.^{38, 39} However, the NOX-mediated or NOX-activated oxidative stress pathway is not fully understood. Based on several lines of evidence from our group, as a novel pathway that acts downstream of NOX, the NOX/VPO1 pathway has been shown to mediate oxidative stress, thereby playing an important role in myocardial ischemia-reperfusion injury, endothelial cell apoptosis and smooth muscle cell proliferation.¹⁴⁻¹⁶ Recently, Luo *et al* found that NOX increased the cellular ADMA levels; however, the specific mechanism underlying this effect is not well understood.²⁰ In the present study, both DPI (a NOX inhibitor) and catalase (a specific H₂O₂ scavenger) attenuated the angiotensin II-induced increase in ADMA production by inhibiting VPO1 expression. These results indicated that the activation of the NOX/H₂O₂/VPO1/HOCl pathway plays an important role in endothelial dysfunction during hypertension by modulating the DDAH/ADMA pathway. Because there is no VPO1 transgenic or knock-out animal model right now, this mechanism in vivo cannot be explained more clearly, which is one of the limitations of this study.

Perspectives

ADMA, the endogenous inhibitor of NOS, contributes to endothelial dysfunction and subsequent cardiovascular events.¹⁻³ MPO has been identified as a regulatory switch of

ADMA bioavailability under inflammatory conditions.¹⁸ However, whether other peroxidases are responsible for HOCl-mediated endothelial dysfunction under noninflammatory conditions such as hypertension has remained unknown. The present study supports the notion that VPO1 plays a critical role in endothelial dysfunction by regulating ADMA bioavailability in hypertension. Considering its specific tissue distribution, the involvement of VPO1 in endothelial dysfunction warrants further investigation. The findings of the present study suggest that understanding the mechanisms underlying the VPO1-ADMA interaction may open a new avenue for the treatment of cardiovascular diseases.

Acknowledgements

International Postdoctoral Exchange Fellowship Program for supporting RuizhengShi.

Sources of funding

This project was supported by grants from the National Science Foundation of China (No. 81570453 and No.81102440 to S.R.Z.)and the National Nature Science Fund of China(NO.81170261 TO Z.G.G). This project was also supported by National Basic Research Program of China (973 Program) (No. 2014CB542402).as well as grant from the Postdoctoral Fund of China (No. 2013M542143).

Disclosure of conflict of interest: None.

Reference

1. Rochette L, Lorin J, Zeller M, Guillard JC, Lorgis L, Cottin Y, Vergely C. Nitric

oxide synthase inhibition and oxidative stress in cardiovascular diseases: possible therapeutic targets? *Pharmacol Ther* 2013;140:239-257.

2. Bouras G, Deftereos S, Tousoulis D, Giannopoulos G, Chatzis G, Tsounis D, Cleman MW, Stefanadis C. Asymmetric Dimethylarginine (ADMA): a promising biomarker for cardiovascular disease? *Curr Top Med Chem* 2013;13:180-200.

3. Palm F, Onozato ML, Luo Z, Wilcox CS. Dimethylargininedimethylaminohydrolase (DDAH): expression, regulation, and function in the cardiovascular and renal systems. *Am J Physiol Heart Circ Physiol* 2007;293:H3227-H3245.

4. Perticone F, Sciacqua A, Maio R, Perticone M, Maas R, Boger RH, Tripepi G, Sesti G, Zoccali C. Asymmetric dimethylarginine, L-arginine, and endothelial dysfunction in essential hypertension. *J Am Coll Cardiol* 2005; 46:518-523.

5. Surdacki A, Nowicki M, Sandmann J, Tsikas D, Boeger RH, Bode-Boeger SM, Kruszelnicka-Kwiatkowska O, Kokot F, Dubiel JS, Froelich JC. Reduced urinary excretion of nitric oxide metabolites and increased plasma levels of asymmetric dimethylarginine in men with essential hypertension. *J Cardiovasc Pharmacol* 1999;33:652-658.

6. Wang D, Strandgaard S, Iversen JS, Wilcox CS. Asymmetric dimethylarginine, oxidative stress, and vascular nitric oxide synthase in essential hypertension. *Am J Physiol Regul Integr Comp Physiol* 2009;296:R195-R200.

7. Li D, Xia K, Li NS, Luo D, Wang S, Jiang DJ, Deng HW, Li YJ. Reduction of asymmetric dimethylarginine involved in the cardioprotective effect of losartan in spontaneously hypertensive rats. *Can J Physiol Pharmacol* 2007;85:783-789.

8. Matsuoka H, Itoh S, Kimoto M, Kohno K, Tamai O, Wada Y, Yasukawa H, Iwami G, Okuda S, Imaizumi T. Asymmetrical dimethylarginine, an endogenous nitric oxide synthase inhibitor, in experimental hypertension. *Hypertension* 1997;29:242-247.
9. Münzel T, Gori T, Bruno RM, Taddei S. Is oxidative stress a therapeutic target in cardiovascular disease? *Eur Heart J* 2010;31:2741-2748.
10. Stuhlinger MC, Oka RK, Graf EE, Schmolzer I, Upson BM, Kapoor O, Szuba A, Malinow MR, Wascher TC, Pachinger O, Cooke JP. Endothelial dysfunction induced by hyperhomocyst(e)inemia: role of asymmetric dimethylarginine. *Circulation* 2003;108:933-938.
11. Wang D, Strandgaard S, Iversen J, Wilcox CS. Asymmetric dimethylarginine, oxidative stress, and vascular nitric oxide synthase in essential hypertension. *Am J PhysiolRegulIntegr Comp Physiol* 2009;296: R195-R200.
12. Cheng G, Salerno JC, Cao Z, Pagano PJ, Lambeth JD. Identification and characterization of VPO1, a new animal heme-containing peroxidase. *Free RadicBiol Med* 2008;45:1682-1694.
13. Shi R, Hu C, Yuan Q, Yang T, Peng J, Li Y, Bai Y, Cao Z, Cheng G, Zhang G. Involvement of vascular peroxidase 1 in angiotensin II-induced vascular smooth muscle cell proliferation. *Cardiovasc Res* 2011;91:27-36.
14. Bai YP, Hu CP, Yuan Q, Peng J, Shi RZ, Yang TL, Cao ZH, Li YJ, Cheng G, Zhang GG. Role of VPO1, a newly identified heme-containing peroxidase, in ox-LDL induced endothelial cell apoptosis. *Free RadicBiol Med* 2011;51:1492-1500.
15. Zhang YS, He L, Liu B, Li NS, Luo XJ, Hu CP, Ma QL, Zhang GG, Li YJ, Peng J.

A novel pathway of NADPH oxidase/vascular peroxidase 1 in mediating oxidative injury following ischemia-reperfusion. *Basic Res Cardiol* 2012;107:266.

16. Hong L, Fast W. Inhibition of human dimethylarginine dimethylaminohydrolase-1 by S-nitroso-L-homocysteine and hydrogen peroxide. Analysis, quantification, and implications for hyperhomocysteinemia. *J Biol Chem* 2007; 282(48):34684-34692..

17. Yang L, Bai Y, Li N, Hu C, Peng J, Cheng G, Zhang G, Shi R. Vascular VPO1 expression is related to the endothelial dysfunction in spontaneously hypertensive rats. *Biochem Biophys Res Commun* 2013;439:511-516.

18. Von Leitner EC, Klinker A, Atzler D, Slocum JL, Lund N, Kielstein JT, Maas R, Schmidt-Haupt R, Pekarova M, Hellwinkel O, Tsikas D, D'Alecy LG, Lau D, Willems S, Kubala L, Ehmke H, Meinertz T, Blankenberg S, Schwedhelm E, Gadegebeku CA, Böger RH, Baldus S, Sydow K. Pathogenic cycle between the endogenous nitric oxide synthase inhibitor asymmetrical dimethylarginine and the leukocyte-derived hemoprotein myeloperoxidase. *Circulation* 2011;124:2735-2745.

19. Hu X, Atzler D, Xu X, Zhang P, Guo H, Lu Z, Fassett J, Schwedhelm E, Böger RH, Bache RJ, Chen Y. Dimethylarginine dimethylaminohydrolase-1 is the critical enzyme for degrading the cardiovascular risk factor asymmetrical dimethylarginine. *Arterioscler Thromb Vasc Biol* 2011;31:1540-1546.

20. Zaiming Luo, Tom Teerlink, Kathy Griendling, Shakil Aslam, William J. Welch and Christopher S. Wilcox. Angiotensin II and NADPH oxidase increase ADMA in vascular smooth muscle cells. *Hypertension* 2010;56:498-504.

21. Li LJ, Geng SR, Yu CM. Endothelial dysfunction in normotensive Chinese with a

- family history of essential hypertension. *ClinExpHypertens* 2005;27:1-8.
22. Rossi R, Chiurlia E, Nuzzo A, Cioni E, Origliani G, Modena MG. Flow-mediated vasodilation and the risk of developing hypertension in healthy postmenopausal women. *J Am CollCardiol* 2004;44:1636-1640.
23. Muiesan ML, Salvetti M, Monteduro C, Rizzoni D, Zulli R, Corbellini C, Brun C, Agabiti-Rosei E. Effect of treatment on flow-dependent vasodilation of the brachial artery in essential hypertension. *Hypertension* 1999;33:575-580.
24. Perticone F, Ceravolo R, Pujia A, Ventura G, Iacopino S, Scozzafava A, Ferraro A, Chello M, Mastroroberto P, Verdecchia P, Schillaci G. Prognostic significance of endothelial dysfunction in hypertensive patients. *Circulation* 2001;104:191-196.
25. Schiffrin EL. Vascular endothelin in hypertension. *VasculPharmacol* 2005;43:19-29.
26. Forstermann U, Closs EI, Pollock JS, Nakane M, Schwarz P, Gath I, Kleinert H. Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertension* 1994;23:1121-1131.
27. Van Vliet BN, Chafe LL, Montani JP. Characteristics of 24 h telemetered blood pressure in eNOS-knockout and C57Bl/6J control mice. *J Physiol* 2003;549:313-325.
28. Eiserich JP, Baldus S, Brennan ML, Ma W, Zhang C, Tousson A, Castro L, Lusis AJ, Nauseef WM, White CR, Freeman BA: Myeloperoxidase, a leukocyte-derived vascular NO oxidase. *Science* 2002;296:2391-2394.
29. Zhang C, Yang J, Jacobs JD, Jennings LK: Interaction of myeloperoxidase with

vascular NAD(P)H oxidases derived reactive oxygen species in the vasculature: implications for vascular diseases. *Am J Physiol* 2003;285:H2563-H2572.

30. Abu-Soud HM, Hazen SL: Nitric oxide is a physiological substrate for mammalian peroxidases. *J BiolChem* 2000;275:37524-37532.

31. Chunxiang Zhang, Jian Yang, and Lisa K. Jennings. Leukocyte-Derived Myeloperoxidase Amplifies High-Glucose-Induced Endothelial Dysfunction Through Interaction With High-Glucose-Stimulated, Vascular Non-Leukocyte-Derived Reactive Oxygen Species. *Diabetes* 2004;53:2950-2959.

32. Li H, Cao Z, Zhang G, Thannickal VJ, Cheng G. Vascular peroxidase 1 catalyzes the formation of hypohalous acids: characterization of its substrate specificity and enzymatic properties. *Free RadicBiol Med* 2012;53:1954-1959.

33. Murray-Rust J, Leiper J M, McAlister M, Phelan J, Tilley S, Santa Maria J, Vallance P, McDonald N. Structural insights into the hydrolysis of cellular nitric oxide synthase inhibitors by dimethylargininedimethylaminohydrolase. *Nat StructBiol* 2001;8:679-683.

34. Leiper J, Murray-Rust J, McDonald N, Vallance P. S-nitrosylation of dimethylargininedimethylaminohydrolase regulates enzyme activity: further interactions between nitric oxide synthase and dimethylargininedimethylaminohydrolase. *ProcNatlAcadSci U S A* 2002;99:13527-13532.

35. Fu X, Kassim SY, Parks WC, Heinecke JW. Hypochlorous acid oxygenates the cysteine switch domain of pro-matrilysin (MMP-7). A mechanism for matrix

metalloproteinase activation and atherosclerotic plaque rupture by myeloperoxidase. *J Biol Chem* 2001;276(44):41279-87.

36. Xu J, Xie Z, Reece R, Pimental D, Zou MH. Uncoupling of endothelial nitric oxidase synthase by hypochlorous acid: role of NAD(P)H oxidase-derived superoxide and peroxynitrite. *ArteriosclerThrombVascBiol* 2006;26:2688-2695.

37. Nuzkowski A, Grabner R, Marsche G, Unbehauen A, Malle E, Heller R: Hypochlorite-modified low density lipoprotein inhibits nitric oxide synthesis in endothelial cells via an intracellular dislocalization of endothelial nitric oxide synthase. *J BiolChem* 2001;276:14212–14221.

38. Montezano AC, Touyz RM. Reactive oxygen species, vascular Noxs, and hypertension: focus on translational and clinical research. *Antioxid Redox Signal* 2014;20:164-182.

39. Touyz RM. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance? *Hypertension* 2004; 44:248-252.

Novelty and Significance

What is new?

We demonstrated for the first time that VPO1, the novel peroxidase localized to the cardiovascular system, plays a role in endothelial dysfunction under conditions of hypertension. We also revealed for the first time that the enhancing effect of NOX on ADMA production was regulated, at least in part, by VPO1.

What is relevant?

ADMA contributes to endothelial dysfunction and subsequent cardiovascular events. NOX, the major source of ROS in vascular cells, plays an important role in the development of cardiovascular diseases. The findings of the present study suggest that understanding the mechanisms underlying the NOX-ADMA interaction may open a new avenue for the treatment of cardiovascular diseases.

Summary

The present study revealed that VPO1 plays a role in endothelial dysfunction in SHR by enhancing ADMA production and that this effect of VPO1 was fulfilled by inhibiting DDAH-2 activity via the VPO1 product HOCl. The present study also elucidated that VPO1 acts downstream of NOX and plays an important role in NOX-mediated oxidative stress in cardiovascular diseases such as hypertension.

Figure Legends

Figure 1. Mean $\pm$ SEM values for rats (n=10 per group). In 20-week-old SHR, the changes in systolic blood pressure (A), NO production in plasma (B), the ADMA level in plasma (C), the endothelium-dependent and endothelium-independent relaxation of

the thoracic aorta ring (D, E), the eNOS expression level in aorta tissue (G), and significantly increased VPO1 expression in both aorta tissue and plasma (F, G, H) were observed compared to WKY rats. All the data are reported with three independent experiments. * $P < 0.05$, ** $P < 0.01$.

Figure 2. Dose- and time-dependent effect of angiotensin II on VPO1 expression in HUVECs.

Angiotensin II up-regulated the expression of VPO1 (at the mRNA and protein levels as analyzed by real-time PCR and Western blot, respectively) in a dose- and time-dependent manner. * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control, All the data are reported as the mean $\pm$ SD of three independent experiments.

Figure 3. Effect of VPO1 knockdown on angiotensin II-induced eNOS and DDAH-2 mRNA (A) and protein expression (B, C), NO production (D), ADMA production (E), DDAH-2 activity (F) and HOCl production (G) in HUVECs. HUVECs were incubated in angiotensin II (100 μ mol/L) for 24 h after VPO1-siRNA transfection. * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control, ### $P < 0.01$ vs. Ang II. All the data are reported as the mean $\pm$ SD of three independent experiments.

Figure 4. HUVECs were incubated in HOCl (100 μ mol/L) for 1 h, and the changes in ADMA production (A), DDAH-2 activity (B), eNOS mRNA expression (C), eNOS and DDAH-2 protein expression (D,E) and NO production (F) were measured. * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control. All the data are reported as the mean $\pm$ SD of three independent experiments.

Figure 5. Effect of VPO1 on recombinant human DDAH-2 enzyme activity. The addition of VPO1 and its substrate H_2O_2 , but not VPO1 or H_2O_2 alone, resulted in a significant decrease in hDDAH-2 activity (A). In addition, HOCl dose-dependently impaired hDDAH2 activity (B). * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control. All the data are reported as the mean $\pm$ SD of three independent experiments.

Figure 6. Effect of pharmacologic inhibitors of NOX and H_2O_2 on angiotensin II-induced eNOS, DDAH-2 and VPO1 mRNA (A) and protein expression (B, C), DDAH-2 activity, NO production (D,E) and ADMA production (F) in HUVECs. AngII,

the cells were incubated in angiotensin II(100 μ mol/L) for 24 h; +DPI, the cells were incubated in DPI (10 μ mol/L) for 30 min and then exposed to angiotensin II(100 μ mol/L) for 24 h; +CAT, the cells were incubated in catalase (200 U/mL) for 30 min and then exposed to angiotensin II(100 μ mol/L) for 24 h. * P <0.05 vs. control, ** P <0.01 vs. control, # P <0.05 vs. Ang II, ** P <0.01 vs. control. All the data are reported as the mean $\pm$ SD of three independent experiments.

Supporting Information

S1 Text. Including primers and RNAi information involved in this research.

S2 supplemental figures.

Figure-1

Figure-2

Figure-3

Figure-4

Figure-5

A

B

Figure-6