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Vascular VPO1 expression is related to the endothelial dysfunction in spontaneously hypertensive rats

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ABSTRACT

Reactive oxygen species (ROS) contributes to endothelial dysfunction that is involved in the pathogeneses of hypertension. Vascular peroxidase 1 (VPO1) can utilize ROS to catalyze peroxidative reactions, possibly enhancing endothelial dysfunction. This study is to identify VPO1's involvement in endothelial dysfunction and hypertension. Sixty-four spontaneously hypertensive rats (SHRs) and 64 age-matched, body-weight controlled normotensive Wistar–Kyoto rats (WKYs) were randomly grouped and studied at the age of 5, 8, 13 and 20 weeks (16 animals, each). Blood pressure and vasodilator responses to acetylcholine in aortic rings were observed. The expressions of VPO1 and endothelial NO synthase (eNOS) in aortas were assessed by quantitative reverse transcription-PCR and western blotting analysis. Plasma concentrations of hydrogen peroxide (H_2O_2) and NO, NOX activity, hypochlorous acid (HOCl) production, and 3-nitrotyrosine content in aortic homogenates were also determined in this study. Along with the development of hypertension in SHR rats, VPO1 expression was up-regulated together with a significant increase in NOX activity, HOCl production, 3-nitrotyrosine content, and plasma H_2O_2 level compared with WKYs at 8, 13 and 20 weeks of age. In contrast, blood NO levels were decreased and aortic relaxation to acetylcholine was deteriorated in SHRs. The over-expression of VPO1 during the development of hypertension, accompanied by the endothelial dysfunction, the decreased NO levels, the elevated NOX and ROS activities, indicates a clear connection between VPO1 gene and hypertension. VPO1 may pathogenetically contribute to hypertension via signal pathways involving NOX–H_2O_2–VPO1–HOCl or JNK/p38 MAPK although further studies are needed to determine the precise mechanisms.

1. Introduction

Hypertension is the major risk factor for cardiovascular diseases and the pathogenesis of essential hypertension has been under intensive investigations. Accumulated evidence suggests that oxidative stress caused by ROS is a strong underlying factor in hypertension [1]. Enzymatic sources of ROS in hypertension include xanthine oxidoreductase, uncoupled NO synthase (NOS), mitochondrial respiratory enzymes, and NOX found in many cell types in vessels, the heart, kidney, and central nervous system [2–4]. NOX is the major source of ROS in the cardiovascular systems, accompanying hypertension, it generates superoxide and other downstream ROS [1,5]. Each member of the NOX family contains a catalytic unit termed NOX and at least five NOX isoforms (NOX1-5) have been identified so far in the mammalian, NOX1, NOX2, and NOX4 (and NOX5 in humans) mainly influence the cardiovascular system [6–9].

Furthermore, heme-containing peroxidase is widely distributed in the body and has highlyz distinct cell/tissue distributions. It participates in host defense, biosynthesis of thyroid hormone, extracellular matrix, as well as in pathological conditions by the generation of hypohalous acids which have the capacity to oxidize proteins, lipids, and DNA, resulting in damage to cells and tissues [10,11]. Among the peroxidase studied so far, myeloperoxidase (MPO) is capable of catalyzing H_2O_2 and chlorine ion to generate strong oxidizer HOCl [12], and it plays an important role in innate immunity, inflammatory responses and cardiovascular disease [13,14]. On the other hand, NO that is generated by endothelial nitric oxide synthase (eNOS) plays an important protective role in the regulation of vascular relaxation [15]. It is well known that impairment of endothelial nitric oxide synthesis and/or bioavailability causes endothelial dysfunction, and endothelial dysfunction is a hallmark of hypertension.

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Vascular peroxidase 1 (VPO1), a newly discovered member of the peroxidase family, expresses in a variety of tissues including cardiovascular system, lung and liver and is secreted into blood. In cardiovascular system, it is expressed mainly in the cells including vascular endothelial cells, smooth muscle cells and myocardial cells [16]. Two VPO isoforms namely VPO1 and VPO2 have been identified; while VPO1 is found both in rats and humans, VPO2 is found only in humans [16]. The enzymatic properties and substrate specificity of VPO1 are similar to MPO; it utilizes chloride in the presence of H_2O_2 to generate HOCl [17,18], which possesses a powerful oxidizing capacity and will further contribute to oxidative stress within vascular tissue.

There are some studies on the role of NOX/VPO1 pathway: In an AngII-induced vascular smooth muscle cell proliferation model, the cell proliferation is found to be significantly reduced by either the NOX inhibitor or VPO1 expression silence [19]; in the ox-LDL-induced vascular endothelial cell apoptosis model, NOX inhibitor or VPO1 expression silence significantly reduces the quantity of the apoptosis cell [20]; and in a myocardial ischemia–reperfusion injury rat model, VPO1 synergistically works with NOX to enlarge and promote the myocardial oxidative damage induced by ischemia–reperfusion [21]. Nevertheless, little is known whether NOX/VPO1 pathway is involved in endothelial dysfunction and the development of hypertension.

To explore the possible involvement of VPO1 in the development of hypertension in the present study, VPO1 expression together with the status of NOX activity, ROS production, oxidative stress, and endothelial dysfunction were observed in the genetic hypertensive rat model. The tissue samples from aorta were used for this study; as aorta is the common site for vascular diseases, aortic endothelium and smooth muscle host a number of mediators involved in a delicate homeostatic balance [19,22,23].

2. Materials and methods

2.1. Animals and experimental design

A total of 64 male spontaneously hypertensive rats (SHRs) aged at 5 weeks and another 64 male Wistar–Kyoto rats (WKYs), as their normotensive genetic controls, were purchased from Vital River Laboratory (Beijing, China). The animals were randomly divided into following groups: (1) Groups WKY_5 and SHR_5(n = 16, each); the rats at age of 5 weeks were used to perform the studies as control; (2) Groups WKY_8 and SHR_8(n = 16, each); animals were killed at 8 weeks of age to study in the initial stage of hypertension; (3) Group WKY_{13} and SHR_{13}(n = 16, each); animals were killed at 13 weeks of age to study in the aggressive phase of hypertension; (4) Group WKY_{20} and SHR_{20} (n = 16, each); animals were killed at 20 weeks of age to study in the stationary phase of hypertension. In each group, 8 rats were used to measure endothelial function in intact thoracic aorta, NOX activity, HOCl production and 3-nitrotyrosine content, and the other 8 rats were used to determine related vascular protein messenger RNA (mRNA) and western-blot expression.

The study was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the experimental protocol was approved by the medicine animal welfare committee of Xiangya Medical School, Central South University (Changsha, China).

2.2. Measurement of blood pressure and postmortem

At the designed age of weeks, the rats were anesthetized with pentobarbital sodium (Sigma–Aldrich) (60 mg/kg) intraperitoneally. The left carotid artery was catheterized with PE-50 tubing for monitoring blood pressure and heart rate using a Statham P23AC pressure transducer and a data acquisition system (PolyView, Grass Ins, West Warwick, RI). Then, 2 ml of blood from each rat was collected and centrifuged at 1000g for 10 min at 4 °C to separate plasma. Then, the rats were killed by decapitation. The thoracic aorta was carefully excised, dissected, and placed in chilled Krebs–Henseleit buffer (pH 7.4). Aortic tissues for immunoblotting or mRNA expression analyses and so on were cleaned with ice-cold phosphate-buffered saline (PBS), snap-frozen in liquid nitrogen immediately, and stored at −80 °C until processing.

2.3. Vascular tone

Endothelium-dependent/independent relaxation of rat aorta to acetylcholine (ACh), and to S-nitroso-*N*-acetylpenicillamine (SNAP) (Sigma–Aldrich) were performed as previously described respectively [24]. Briefly, thoracic aortas were dissected free of adventitia, cut into 2–3 mm ring segments. Following contraction by phenylephrine (PE, 10 μmol/L) (Sigma–Aldrich), relaxations to cumulative concentrations of Ach (0.01–3 μmol/L) or SNAP (0.01–10 μmol/L) were tested to obtain cumulative concentration–response curves. The degree of precontraction to PE was chosen to approximate 80% of the maximal response to KCl (60 mmol/L). Dose–response curves were analyzed (see Fig. 1B and C), and the maximal relaxation value (Emax) in percent of the PE induced contraction for each ring was calculated.

2.4. RNA preparation and quantitative reverse transcription-PCR (qRT-PCR) analysis

Total RNA was extracted by TRIzol (Invitrogen). For detection of mRNAs, RNA (0.5 μg) was subjected to reverse transcription reaction using the PrimeScript reverse transcription reagent Kit (TaKaRa, China) according to the manufacturer's instructions. Quantitative analysis of the change in expression levels was performed using SYBR Premix Ex Taq (TaKaRa, China) at ABI 7300 (Applied Biosystems, Foster City, CA). PCR cycling conditions were an initial incubation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, and annealing at 60 °C for 31 s. The method was used to examine eNOS, an endothelial nitric oxide synthase, and VPO1 mRNA in aorta tissues. GAPDH was used to normalize the mRNA expression. Data analysis was performed by comparative Ct method using the ABI software. The primers for eNOS, VPO1 and GAPDH are shown in Table 1.

2.5. Western blot (WB) analysis

Western blots were performed on aortic homogenates with RIPA buffer (contain 0.1% PMSF), and equal amounts of protein from each sample (40 μg) using the BCA protein assay kit (Beyotime, China) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidine fluoride membranes. The membranes were then incubated with primary antibodies against eNOS (Abcam), VPO1 (a generous gift from Dr. Guangjie Cheng, University of Alabama at Birmingham) or GAPDH (Sigma–Aldrich), and finally, horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Promega). The chemiluminescence signals were detected with the ECL plus Western blotting reagents (GE Healthcare). The densitometric analysis was conducted with Image J 1.43 (National Institutes of Health).

2.6. Markers of oxidative stress

NOX activity was measured by a commercially available kit (GENMED, China) following the manufacturer's instructions. Briefly, the supernatant of aortal tissue homogenates was

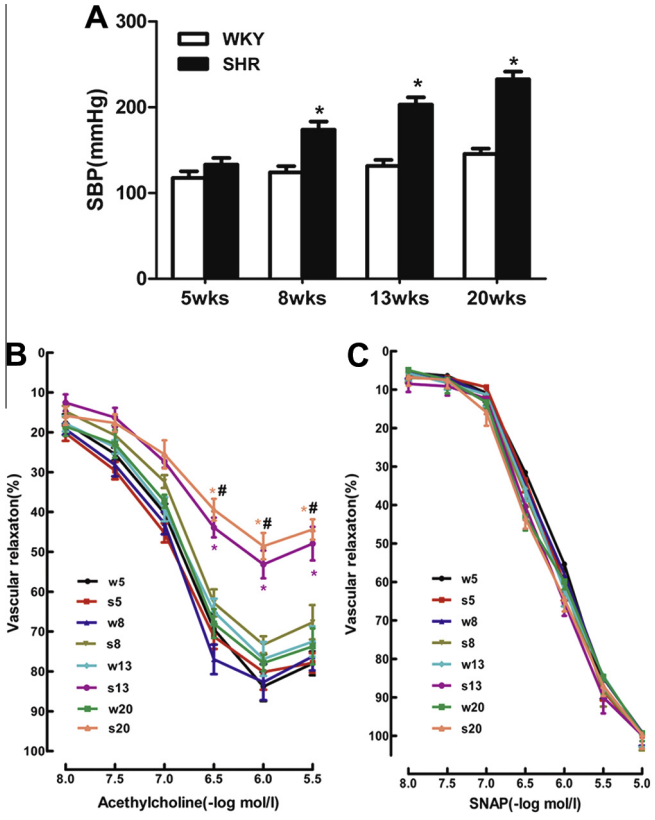

Fig. 1. The development of hypertension and vascular endothelium-dependent relaxation from different groups. (A) Systolic blood pressure (SBP) of SHR and age-matched WKY rats at 5, 8, 13, 20 weeks (wks) of age. (B) ACh-induced relaxation is determined by isometric tension studies: As pink triangles and purple circles show that vascular relaxation in SHR is significantly impaired in comparison with the week-matched WKY group at 13, 20 week-age; and pink triangles also show that vascular relaxation in SHR at 20 week-age is further impaired; red squares and inverted gold triangles show that both strains at 5, 8 week-age possess unchanged vascular relaxation. (C) SNAP – induced relaxation: vascular endothelium-independent relaxation by SNAP is similar in SHR and WKY rats less than 20 week-age. $^{*}P < 0.05$ vs the same week-age WKY group; $^{\#}P < 0.05$ vs the 8 week-age SHRs. Values are expressed as means ± s.e.m. ($n = 8$ each group).

incubated with oxidized cytochrome c in a quartz cuvette at 30 °C for 3 min, then, NOX substrate (NADPH) was added to the reaction and incubated for 15 min. The absorbance at 550 nm was monitored by a spectrophotometer. NOX activity was determined by calculating cytochrome c reduction per min.

Plasma H_2O_2 measurement was performed using 100 μl of plasma following the instructions of Hydrogen peroxide assay kit (Beyotime, China). The ferrous oxidation–xylenol orange method was adopted.

Hypochlorous acid (HOCl) production was determined by a 3,3′,5,5′-tetramethylbenzidine (TMB) assay as previously reported [19,25]. HOCl production was calculated by the standard curve of the TMB method shown in Supplementary methods.

3-Nitrotyrosine, a product by protein tyrosine nitration [5] that represents NO scavenging/deactivation by reactive oxygen species that react rapidly with NO, is an indicator of protein modification mediated primarily by peroxynitrite [26]. It was measured in aortic homogenate with an ELISA kit (Millipore), using a method of specific immunological reaction.

2.7. The plasma NO levels

NO levels were determined by a nitrite/nitrate assay kit (Beyotime, China) using the Griess assay as previously described [27]. In the Griess assay, nitrate is reduced to nitrite by the nitrate reductase, and then detected through the classic Griess reagent, which determines the total nitric oxide.

2.8. Statistical analysis

Results were presented as mean ± s.e.m. (standard errors). Statistical analysis was performed by Student's *t*-test for two groups or analysis of variance followed by Newman–Student–Keuls test for multiple groups. *P* values <0.05 were considered to be significant. All statistical tests were carried out using the SPSS 13.0.

3. Results

3.1. Increased SBP and deteriorated endothelium-dependent vasorelaxation in SHRs

As shown in Fig. 1A, there was no difference in SBP between SHR and WKY rats in young rats at 5 weeks of age. However, SBP was increased ($P < 0.05$) along the subsequent 12-week period from 8 week-age onward in SHR but remained unchanged in WKY rats. No significant differences were found in SBP between SHR_{13} and SHR_{20}. The findings agreed well with the previous studies [28,29].

Vascular tone observation showed that the maximal relaxation in response to Ach was significantly decreased in SHR than in WKY rats from the age of 13 weeks onward (Fig. 1B, $P < 0.05$). This parameter became much lower in SHR_{20} than in SHR_8 ($P < 0.05$), whereas no differences were observed between WKY_8 and SHR_8, indicating that vascular endothelium-dependent relaxation to Ach was impaired in SHR_{13} from the aggressive stage of hypertension. Fig. 1C showed that vascular endothelium-independent relaxation by SNAP was similar in SHR and WKY rats less than 20 week-age. This indicates the presence of an intact and functional vascular smooth muscle cell (VSMC) layer in both strains. An endothelial dysfunction in SHR is clearly seen here even though it is hard to rule out the possibility of a functional difference in VSMC between WKY and SHR as shown by other investigators [30].

3.2. Increased VPO1 expression in SHRs

qRT-PCR and WB analysis showed that VPO1 expression at both mRNA and protein levels in SHR aorta was significantly upregulated from 8 week-age, the initial stage of hypertension (Fig. 2A and B). VPO1 expression was in concurrence with BP development in SHRs, indicating a possible involvement of the gene in the pathogenesis of hypertension.

Table 1
Primers for real-time for PCR.

Gene	Forward primer	Reverse primer	Product size (bp)
eNOS	ACTTTTTATTGGGCGTCCTC	AATGTGAGTCCGAAAATGTCCT	161
VPO1	CTGCTACCGGCTGGATGAGAACT	GTGCCATGGAGAAAAGCCTCTCTGT	205
GAPDH	AACTCCCTCAAGATTGTCAGC	GGGAGTTGCTGTTGAAGTCACA	448

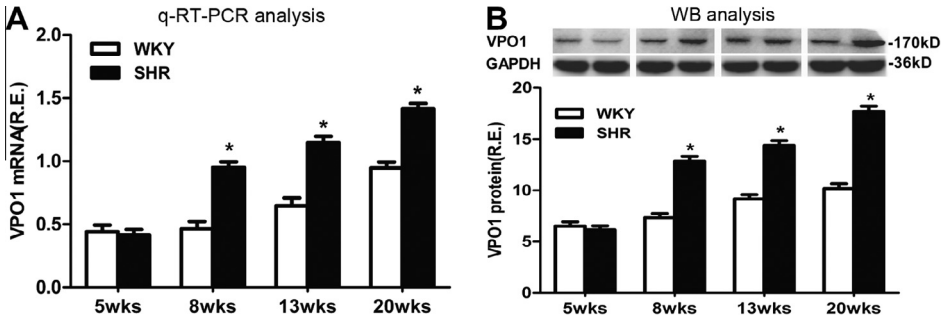

Fig. 2. VPO1 expression at both mRNA and protein levels in Aorta tissue from different groups. (A) qRT-PCR shows that VPO1 mRNA expression in SHR thoracic aorta is significantly increased from 8 week-age onwards, the initial stage of hypertension to the stationary phase, when compared with the age-matched WKY group. (B) WB analysis shows an over-expression of VPO1 protein in SHR thoracic aorta from 8 week-age onwards when compared with the age-matched WKY group. Bars represent means ± s.e.m. of 8 animals in each group. $^{*}P < 0.05$ compared with the same week-age WKY group.

3.3. Elevated NOX activity, H_2O_2, HOCl, and 3-nitrotyrosine content in SHRs

The NOX activity was significantly increased in SHR at 8–20 week-age compared with the age-matched WKY rats (Fig. 3A). Parallel to the changes in NOX activity, plasma H_2O_2 level and aortic HOCl production at 8, 13, 20 weeks of age in SHRs were significantly up-regulated in comparison with WKY rats (Fig. 3B and C). Aortic 3-nitrotyrosine content was significantly higher in SHRs than in WKY rats (Fig. 3D) as well. These results indicated that the NOX pathway was significantly enhanced in SHRs alongside the development of hypertension.

3.4. Decreased NO production and eNOS expression in SHRs

NO production as determined by measuring plasma nitrite/nitrate levels was significantly reduced in SHR compared with WKY rats at 8, 13, 20 weeks (Fig. 4A). The expression of eNOS was significantly down-regulated at both mRNA and protein levels from the age of 8 weeks in SHRs compared with WKY rats (Fig. 4B and C). It indicated that eNOS expression was down-regulated during hypertension, resulting in the decreased NO production that might contribute to the impaired endothelium-dependent vasorelaxation in SHRs.

4. Discussion

In this study, we have confirmed that there is up-regulated VPO1 expression during the development of hypertension in the SHR rats. Accompanying with VPO1 overexpression, the NOX pathway is activated and the parameters for oxidative stress are increased, which is marked as increased H_2O_2, and HOCl, 3-nitrotyrosine content. On the other hand, both NO levels in plasma and aortic eNOS expression are decreased, which could directly contribute to the endothelial dysfunction marked by deteriorated endothelium-dependent vasorelaxation in the SHR rats.

During the process of ROS production by NOX, the immediate product of NOX is superoxide anion, which rapidly dismutates to H_2O_2 either catalyzed by SOD or spontaneously [31]. H_2O_2 is further catalyzed by peroxidases to form hypochlorous acid (HOCl), which has a powerful oxidizing capacity and further provokes

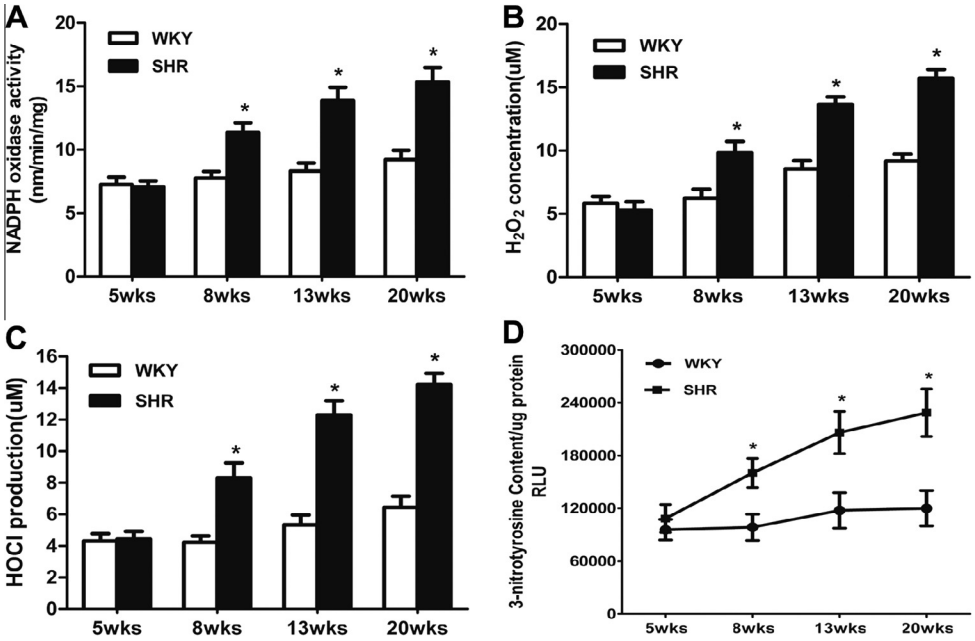

Fig. 3. NOX activity and ROS makers' levels in the rats of different groups. (A) NOX activity in aorta tissue is significantly increased from 8 week-age onwards in SHR, when compared with the age-matched WKY group. (B) Plasma H_2O_2 concentration is significantly increased from 8 week-age onwards in SHR in comparison with the age-matched WKY group. (C) A significantly increased HOCl production in aorta tissue is seen from 8 week-age onwards in SHR, when compared with the age-matched WKY group. (D) A significantly increased 3-nitrotyriosine level in aorta tissue is seen from 8 week-age onwards in SHR, when compared with the age-matched WKY group. Data represent means ± s.e.m. of 8 animals in each group. $^{*}P < 0.05$ compared with the same week-age WKY rats group.

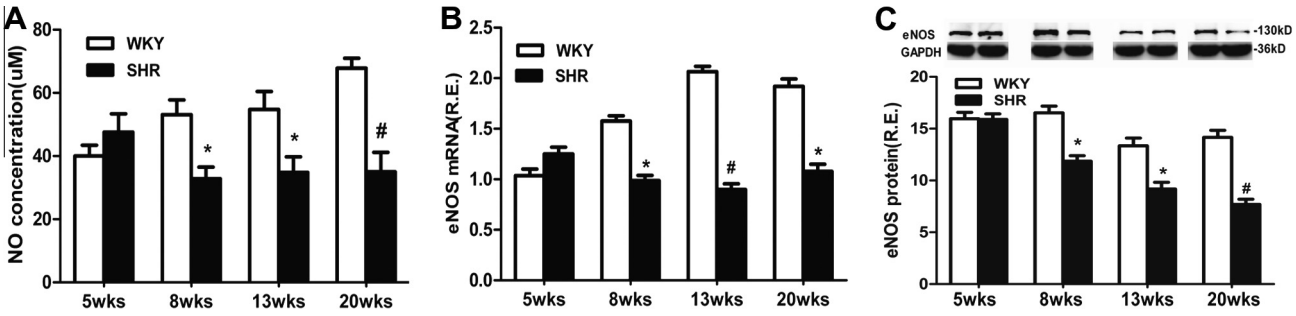

Fig. 4. Plasma NO levels and endothelial eNOS expression in different groups. (A) Plasma NO concentration is significantly decreased in SHR alongside the development of hypertension from 8 week-age onwards. (B) The expression of eNOS mRNA, as determined by qRT-PCR, is obviously decreased in SHR from 8 week-age onwards. (C) A decrease in eNOS protein expression, as determined by Western blot analysis, is seen in SHR from 8 week-age onwards. Bars represent means ± s.e.m. of 8 animals in each group. $^{\#}P < 0.001$, $^{*}P < 0.05$ compared with the same week-age WKY group.

and aggravates oxidative damage within vascular tissue [32]. On the other hand, superoxide avidly reacts with NO to form the highly reactive intermediate peroxynitrite, leading to NO degradation and impaired NO bioactivity in vasculature. In this study, 3-nitrotyrosine was significantly increased in aortic homogenate from SHRs while plasma NO content was decreased. The decreased NO could be resulted from increased ROS activity mediated by NOX–H_2O_2–HOCl pathway, which down-regulated eNOS expression and enhanced the scavenging/deactivation of NO. And the decreased NO level could contribute to the development of hypertension in SHR rats.

Except from utilizing chloride in the presence of H_2O_2 to generate HOCl [17,18], leading to increased HOCl production, VPO1 can also oxidase both Apolipoprotein E in very low density lipoprotein and recombinant ApoE3 via VPO1/H_2O_2/Cl system, playing a role in the genesis and development of atherosclerosis [33]. An increased H_2O_2 concentration can cause an upregulated VPO1 expression which in turn increases HOCl production [21]. In the present study, it was confirmed that an upregulated VPO1 expression was increased along with the development of hypertension and the increased H_2O_2 concentration.

Study has also shown that HOCl can decrease NO bioavailability in the vasculature [34]. And HOCl is able to oxidize the zinc-thiolate center of endothelial nitric oxide synthase and uncouples the enzyme [35]. Moreover, HOCl can provoke endothelial cell desquamation and death by either apoptotic or oncotic cell-death pathways. Sublethal concentrations of HOCl can increase endothelial tissue factor in a concentration-dependent manner [32]. Therefore, we infer that these effects of H_2O_2 and HOCl on endothelial cell NO production may contribute to impaired vascular tone. That may explain its role underlying the findings of decreased NO levels, impaired endothelium-dependent vasorelaxation to Ach alongside the development of hypertension in SHRs. Since JNK and p38 MAPK are the best studied signaling molecules that respond to numerous stress stimuli including oxidative stress [36,37], and HOCl can activate JNK/p38 MAPK dependent signaling pathways [38,39], whether HOCl-induced endothelial dysfunction is related to the activation of the JNK/p38 MAPK pathways and pathogenetically contributes to hypertension is unknown. Further studies are needed to explore the importance of the interaction between NOX–H_2O_2–VPO1–HOCl signaling pathway and JNK/p38 MAPK pathway.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.012.

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