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Assessment of Renal Functional Phenotype in Mice Lacking gp91\textsuperscript{PHOX} Subunit of NAD(P)H Oxidase

Mohammed Z. Haque, Dewan S.A. Majid

Abstract—To determine the role of endogenous superoxide (O2\textsuperscript{-}) in the kidney, we assessed renal hemodynamics and excretory function in gp91\textsuperscript{PHOX} (a NAD(P)H oxidase subunit) gene knockout (KO) mice and compared these findings with those of wild-type (WT) strain C57BL/6 mice. Renal blood flow (RBF) and glomerular filtration rate (GFR) were determined by PAH and inulin clearances respectively in anesthetized mice (n=8 in each group). There were higher baseline RBF (4.3±0.4 versus 2.5±0.2 mL/min per gram; P<0.002) and lower renal vascular resistance (RVR) (16±1.4 versus 29±2.3 mm Hg/mL/min per gram; P<0.0001) in KO compared with WT without a significant difference in mean arterial pressure (MAP) (67±2 versus 71±2 mm Hg) and GFR (0.66±0.09 versus 0.73±0.05 mL/min per gram) between the strains. Intravenous infusion of angiotensin II (Ang II) (2 ng/min per gram of body weight) for 30 minutes caused a lesser degree of decreases in RBF (−8% versus −33%) and of increases in RVR (+73% versus +173%) in KO compared with WT. GFR was increased (43%) in KO but not in WT during Ang II infusion. Urinary excretion of nitrate/nitrite was higher in conscious KO (n=5) than in WT (n=5), indicating an increase in nitric oxide bioavailability that could be the cause of high RBF and low RVR in KO. These data indicate that gp91\textsuperscript{PHOX}, a subunit of NAD(P)H oxidase, plays a regulatory role in the maintenance of renal vascular tone. These results also suggest that the mechanism of Ang II-mediated renal vascular action involves concomitant generation of O2\textsuperscript{-}.

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Key Words: angiotensin II • superoxide • renal circulation

Considerable evidence supports a critical role for reactive oxygen species, particularly superoxide (O2\textsuperscript{-}), in the pathogenesis of various cardiovascular disorders, including hypertension.\textsuperscript{1-4} Recent studies have suggested that an inappropriate generation of O2\textsuperscript{-} contributes to the pathophysiology of hypertension, primarily by modulating vascular smooth muscle tone and potentially affecting the vascular growth leading to endothelial dysfunction.\textsuperscript{2,3,5-7} Among various enzymes involved in generation of reactive oxygen species, NAD(P)H oxidase seem to be the major source of O2\textsuperscript{-} production in the vessel walls\textsuperscript{8-10} and in the kidney.\textsuperscript{11-13} The NAD(P)H oxidase is a membrane-associated enzyme that catalyzes the 1-electron reduction of oxygen using NADH or NADPH as the electron donor:

\[ \text{NADP(H)} + 2O_2 = \text{NADP(H)} + H^+ + 2O_2^{-} \]

Activity of NAD(P)H oxidase can be markedly upregulated by various agonists, such as angiotensin II (Ang II),\textsuperscript{13,15,16} It has been reported that Ang II can induce O2\textsuperscript{-} production in vascular tissue,\textsuperscript{9,15,16} in fibroblasts\textsuperscript{17} and in renal mesangial cells\textsuperscript{18} by activating NAD(P)H enzymes, and this action was shown to be mediated via AT\textsubscript{1} receptors.\textsuperscript{12,15}

NAD(P)H oxidase is composed of five subunits. Two of the subunits, gp91\textsuperscript{PHOX} and p22\textsuperscript{PHOX}, are located in the cellular membrane and others, p45\textsuperscript{PHOX}, p67\textsuperscript{PHOX}, and p40\textsuperscript{PHOX}, are located in the cytosolic compartment.\textsuperscript{14,15}

Among these, the catalytic subunit gp91\textsuperscript{PHOX} was shown to be essential for Ang II-induced vascular hypertrophy and oxidant stress.\textsuperscript{19,20} It has been reported that incubation with Ang II increases gp91\textsuperscript{PHOX} expression and O2\textsuperscript{-} production in the mice aorta.\textsuperscript{17,21} The aorta, collected from gp91\textsuperscript{PHOX} gene knockout (KO) mice, was also shown to have enhanced endothelium-dependent relaxation, indicating that the absence of O2\textsuperscript{-} generation caused by lack of this subunit may enhance the nitric oxide (NO) activity in that vascular tissue.\textsuperscript{22} Thus, these findings indicate an essential functional role of gp91\textsuperscript{PHOX} in the regulation of normal vascular tone as well as in various vascular disorders.

Several experimental studies have also indicated that O2\textsuperscript{-} plays an important role in the control of renal function.\textsuperscript{11,23,24} We have demonstrated that an enhancement of O2\textsuperscript{-} generation by inhibiting superoxide dismutase enzyme in the canine kidney leads to renal vasoconstriction and decreases in urine flow and sodium excretion.\textsuperscript{23} Similar observation was also reported in renal medullary circulation in rats.\textsuperscript{11} Given the potential implications of NAD(P)H oxidase-induced O2\textsuperscript{-} generation in the regulation of body function, we designed this study to assess the renal hemodynamics and excretory function in mice lacking the gene for gp91\textsuperscript{PHOX} subunit of NAD(P)H oxidase to

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understand more of the role of endogenous $O_2^-$ in the renal vascular and tubular function. In these experiments, renal effects of acute Ang II infusion were also evaluated in the gp91PHOX KO mice and compared these effects with those in their genetic background wild-type (WT) strain C57BL/6 mice to examine the possible involvement of $O_2^-$ in Ang II induced responses in the kidney.

Methods

The studies described in this article were performed in accordance with the guidelines and practices established by the Tulane University Animal Care and Use Committee. Male KO and WT mice (Jackson Laboratories, Bar-Harbor, Me) were housed in a temperature- and light-controlled room and allowed free access to standard diet (Ralston-Purina, St. Louis, Mo) and tap water. Because the C57BL/6 mice are commonly used as the genetic background for gene-targeted mutations, this strain was used as control group for KO mice. Two sets of animals were tested. One set of KO (n=8) and WT (n=8) mice were used for acute experiments under anesthesia to measure renal blood flow (RBF), glomerular filtration rate (GFR), and renal excretory function as well as to evaluate the systemic and renal effects of Ang II infusion. To assess the normal renal excretory function at conscious state, another set of KO (n=5) and WT (n=5) mice were used for 24-hour collection of urine to measure excretion rate of sodium/potassium, nitrate/nitrite, 8-isoprostane, and creatinine. Systolic arterial pressure was also measured in these conscious mice by tail-cuff method. Then mice were trained in the metabolic cages for 3 days before 24-hour urine collection and blood pressure measurement. All the mice used in this study were given the same food and water in daily ration. At the end of experiments, these mice were sacrificed with a high dose of anesthesia to remove and weigh the kidneys.

On the day of the acute experiments, mice with $\approx$25 g of body weight (BW) were anesthetized with a combination of Inactin (thiobutabarbital sodium, 100 mg/kg per BW) and Ketalar (a mixture of ketamine 10 mg/kg per BW, xylazine 8 mg/kg per BW intraperitoneally (IP)). Supplemental doses of Ketalar (5 mg/kg ketamine and 4 mg/kg xylazine IP) were administrated as required. The mice were placed on a servo-controlled surgical table that maintained body temperature at 37°C, and a tracheostomy was performed. The animals were allowed to breathe air enriched with oxygen (O2) by placing the exterior end of the tracheal cannula inside a small plastic chamber into which humidified 95% O2/5% CO2 was supplied. The animals were allowed to breathe air enriched with O2. The animals were allowed to breathe air enriched with O2 by placing the exterior end of the tracheal cannula inside a small plastic chamber into which humidified 95% O2/5% CO2 was supplied. The animals were allowed to breathe air enriched with O2. The animals were allowed to breathe air enriched with O2 by placing the exterior end of the tracheal cannula inside a small plastic chamber into which humidified 95% O2/5% CO2 was supplied. The animals were allowed to breathe air enriched with O2. The animals were allowed to breathe air enriched with O2 by placing the exterior end of the tracheal cannula inside a small plastic chamber into which humidified 95% O2/5% CO2 was supplied. The animals were allowed to breathe air enriched with O2. The animals were allowed to breathe air enriched with O2 by placing the exterior end of the tracheal cannula inside a small plastic chamber into which humidified 95% O2/5% CO2 was supplied. The animals were allowed to breathe air enriched with O2.

Analytical Procedures

Blood and urine samples collected during acute experiments were analyzed for inulin, PAH, and sodium/potassium concentrations. Inulin and PAH concentrations were determined by spectrophotometry and sodium/potassium concentrations were determined by flame photometry. The value for inulin clearance was considered as GFR and the value for PAH clearance was considered as renal plasma flow. RBF was calculated from renal plasma flow and hematocrit value. Renal vascular resistance (RVR) was calculated as systemic blood pressure divided by the RBF. The 24-hour urine samples, collected from conscious mice, were analyzed for sodium/potassium (flame photometry), creatinine (Jaffe reaction), nitrate/nitrite, and 8-isoprostane (Enzyme Immunoassay; Assay Design). The 8-isoprostane excretion values were expressed per milligram of creatinine excretion in urine. All values were normalized per gram of kidney weight. Results were expressed as mean±SEM. Statistical analyses were performed using Student t test. Significance was deemed as $P<0.05$.

Results

Baseline Values of Renal Parameters and Arterial Pressure in KO and WT Mice

Baseline value for RBF was higher in KO than in WT mice. Figure 1A illustrates these baseline values in both strains. RBF in KO was 4.3±0.4 mL/min per gram, which was significantly ($P<0.002$) different than that in WT (2.5±0.2 mL/min per gram). Baseline GFR was not statistically different between KO and WT (0.66±0.09 and 0.73±0.05 mL/min per gram, respectively; Figure 1B). Baseline value for RVR was lower in KO compared with WT (16±1.3 versus 29±2.3 mm Hg/mL/min per gram, respectively; $P<0.0001$; Figure 2A). The average baseline mean arterial pressure in KO was 66±2 mm Hg, which was not significantly different from the average value of 71±2 mm Hg in WT (Figure 2B). Baseline urine flow (V) (11±1.4 versus 11±1.7 $\mu$L/min per gram), sodium excretion (U NV) (0.44±0.12 versus 0.92±0.23 µmol/min per gram), fractional excretion of sodium (FE Na) (0.40%±0.09% versus 0.86%±0.25%), and potassium excretion (U K) (1.4±0.2 versus 1.2±0.2 µmol/min per gram) were not significantly different between the groups in anesthetized animals.

When 24-hour urine collections were performed in conscious animals (n=5 in each group), it was also observed that the basal level of V was not different in both strains (KO: 4.2±0.5 mL/day per gram; WT: 3.9±0.8 mL/day per gram;
much smaller (8% in KO mice, Ang II-induced decreases in RBF was mice decreased from the baseline value of 4.3 to 3.9 KO compared with WT mice (807 vs. 132 mm Hg/mL/min per gram) in anesthetized KO and WT mice. *P<0.001 versus WT mice.

Figure 3A). However, the basal level of UNaV was higher in KO mice compared with WT mice (807 ± 69 versus 545 ± 94 µmol/day per gram; P=0.058; Figure 3B). Urinary excretion rates of nitrate/nitrite were also significantly higher in KO than in WT (23.7 ± 3.0 versus 13.3 ± 2.5 µmol/day per gram; P<0.001; Figure 3A). The excretion rate of creatinine was different between KO (1.4 ± 0.4 mg/day per gram) and WT (1.0 ± 0.2 mg/day per gram) mice. Although not statistically different, the average excretion of 8-isoprostane was slightly higher in KO compared with WT (43.1 ± 4.9 versus 37.1 ± 5.5 µg/mg of creatinine excretion; Figure 4B). Systolic arterial pressure measured by tail-cuff method in these conscious animals showed no difference between the strains. The mean systolic arterial pressure was 133 ± 3.7 and 132 ± 2.1 mm Hg in KO and WT mice, respectively.

Responses to Ang II in KO and WT Mice

Figure 1A shows the RBF responses to Ang II infusion in anesthetized animals. In WT mice, Ang II decreased 33 ± 9.5% (P<0.05) in RBF from the baseline value of 2.5 ± 0.2 to 1.64 ± 0.2 mL/min per gram (P<0.008). However in KO mice, Ang II-induced decreases in RBF was much smaller (8 ± 8%) than that in WT mice. RBF in KO mice decreased from the baseline value of 4.3 ± 0.4 to 3.9 ± 0.4 mL/min per gram (P=NS) during Ang II infusion. The percent changes in RBF in response to Ang II in KO was significantly lower than in WT (P<0.001).

Figure 1B illustrates the GFR responses to Ang II in these mice. Ang II did not cause significant change in GFR (from 0.73 ± 0.05 to 0.77 ± 0.07 mL/min per gram) in WT mice. Interestingly, Ang II caused an increase in GFR from 0.66 ± 0.09 to 0.86 ± 0.08 mL/min per gram (P<0.02) in KO mice (mean percent increase was 43% ± 21%; P=0.06).

The increment in RVR in response to Ang II was less in KO compared with WT mice (Figure 2A). RVR increased to 28.5 ± 3 and 76 ± 9 mm Hg/mL/min per gram from their respective baseline values in KO and WT mice. These responses to Ang II were significantly different between the groups of mice (KO 73% ± 10% versus WT 173% ± 39%; P<0.01). Intravenous administration of Ang II caused similar increments in mean arterial pressure in both groups of mice (Figure 2B). Mean arterial pressure increased to 103 ± 6 mm Hg in KO mice (55% ± 8%) and 111 ± 3 mm Hg in WT mice (58% ± 5%) from their respective baseline values.

Ang II increased V, UNaV, and FE Na in both strains of mice because of associated increases in arterial pressure. V increased to 45 ± 9 and 32 ± 3 µL/min per gram. UNaV increased to 6.9 ± 1.5 and 6.2 ± 0.8 µmol/min per gram, and FeNa increased to 5.2% ± 0.8% and 5.8% ± 0.8% in KO and WT mice, respectively, from their baseline values without statistical differences in the mean responses between the groups. Acute Ang II infusion did not cause any significant changes in UNaV in KO or WT mice (1.7 ± 0.1 and 1.2 ± 0.1 µmol/min per gram, respectively).

In time-control experiments (n=3), no significant difference was observed between the values obtained from the first and the second collection periods, which were as follows: RBF, 3.4 ± 0.3 to 3.2 ± 0.1 mL/min per gram; GFR, 0.64 ± 0.13 to 0.62 ± 0.13 mL/min per gram; V, 14.7 ± 0.4 to 15.1 ± 1.3 µL/min per gram; and UNaV, 1.4 ± 0.2 to 1.5 ± 0.4 µmol/min per gram.

Discussion

In the present investigation, we have demonstrated that in KO mice lacking the gene for gp91PHOX, the baseline blood flow to the kidney is significantly higher than that in WT mice.
that lack of $O_2^-$ generation by vascular NAD(P)H oxidase in response to Ang II in KO mice increased NO bioavailability in the renal vessels and thus reduces the vasoconstrictor action of Ang II. It may be argued that endogenous levels of Ang II may be at a higher level or could have lower AT$_1$ receptor population in KO compared with WT mice, which caused such diminished responses to exogenous Ang II in KO mice. At present, we have no data regarding endogenous Ang II or AT$_1$ receptor levels in KO and WT mice, because future experiments are planned to examine such an issue. However, this possibility seems most unlikely because of the observation that Ang II infusion resulted in similar increases in arterial pressure in both strains of mice, indicating that there were no differences in endogenous levels of Ang II or AT1 receptor population. In preliminary experiments conducted previously in our laboratory, Ang II-induced vasoconstrictor response was seen attenuated in dogs treated with the superoxide dismutase enzyme mimetic, tempol (D.S.A. Majid & A. Nishiyama, unpublished data 2002). Collectively, these findings suggest that $O_2^-$ generation by activation of vascular NAD(P)H oxidase during Ang II infusion partially contributes to the vasoconstrictor response to Ang II infusion. Although baseline GFR was not different in both strains of mice, Ang II infusion increased GFR in KO mice but not in WT mice (Figure 1B). Ang II usually elicits less or no change in GFR than in RBF, as reported in many studies involving rats and dogs. $^{31}$ We are not aware of any other report in the literature that has shown the effects of acute Ang II infusion on GFR in mice. Ang II elicits no changes in GFR in WT mice observed in the present study, indicating proportionate increases in the resistances of both preglomerular and postglomerular arterioles and thus causing a minimal change in glomerular filtration pressure. $^{31}$ The finding that Ang II increased GFR in KO mice indicates that exogenous Ang II infusion has exerted proportionately less constrictor action on the afferent than on the efferent arterioles in KO mice, which resulted in an increase in glomerular pressure. $^{31}$ Because experimental evidence suggests that the afferent arterioles exhibit a greater interaction between NO and Ang II actions than do efferent arterioles, $^{32}$ it is possible that increased NO bioavailability in KO mice may have buffered the Ang II-induced vasoconstrictor action to a greater extent in afferent than in efferent arterioles, resulting in an increase in GFR during Ang II infusion. An interactive role of NO and $O_2^-$ in the control of afferent arteriolar tone has also been suggested in recent studies. $^{24,33}$ Ang II was shown to stimulate mesangial cells to synthesize $O_2^-$, $^{18}$ It is also recognized that an interaction of NO and Ang II plays an important role in the mesangial regulation of glomerular microcirculation. $^{32}$ Although the relative distribution of the presence of NAD(P)H oxidase enzymes between preglomerular and postglomerular vessels has not yet been clearly defined, it could be possible that Ang II-induced $O_2^-$ production is normally greater in renal afferent than that in efferent arterioles. Thus, the results of the present investigation suggest that local generation of $O_2^-$ plays an important regulatory role in modulating glomerular dy-
namics during the altered state of the renin–angiotensin system.

It has also been noted that the urinary excretion rate of sodium is higher in conscious KO than that in WT mice (Figure 3B), indicating that an increase in NO activity and a decrease in NAD(P)H oxidase-induced generation of O$_2^-$ influences sodium reabsorption$^{23,34–36}$ in KO mice. However, further comprehensive experiments using this model of KO mice are needed to examine the NAD(P)H oxidase-induced O$_2^-$ formation in the regulation of tubular reabsorptive function.

In conclusion, the present investigation demonstrates a regulatory role of gp91$^\text{phox}$, a subunit of NAD(P)H oxidase enzyme, in the maintenance of normal renal vascular tone and suggests that the renal vasoconstrictor action of Ang II is mediated, at least in part, by a mechanism that involves concomitant generation of O$_2^-$ by this enzyme.

**Perspectives**

In the past, O$_2^-$ was considered as a molecule of pathological interest$^{1–3}$. However, the physiological role of O$_2^-$ is now increasingly appreciated as the findings from recent investigations implicate that this reactive oxygen species is an important part of vascular biology because of its effects on various cellular function and its potential interaction with vascular NO-signaling mechanisms.$^{4,10}$ Although the NAD(P)H oxidase activity has been implicated in various renal diseases, its potential role in the regulation of renal vascular and tubular function is yet to be clarified. The catalytic subunit of NAD(P)H oxidase, gp91$^\text{phox}$, is normally expressed in the smooth muscle layer of the resistance arteries and in the adventitia and endothelium.$^{16,17,19,20}$ Current interest in elucidating the renal functional role of gp91$^\text{phox}$ and other members of the Nox (NADPH oxidase) family stems from the fact that this enzyme is abundantly present in the kidney.$^{11–14}$ The findings in mice lacking the gp91$^\text{phox}$ gene in the present study provide the evidence that the NAD(P)H oxidase activity exerts a regulatory influence on the basal tone and on the responses to Ang II in the renal vascular system. Further comprehensive studies using such KO mice are required to examine the potential importance of the Nox family in regulating glomerular microcirculation and renal tubular function during chronic alterations in the renin–angiotensin system.

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