p47phox Is Required for Afferent Arteriolar Contractile Responses to Angiotensin II and Perfusion Pressure in Mice

En Yin Lai, Glenn Solis, Zaiming Luo, Mattias Carlstrom, Kathryn Sandberg, Steven Holland, Anton Wellstein, William J. Welch, Christopher S. Wilcox

Abstract—Myogenic and angiotensin contractions of afferent arterioles generate reactive oxygen species. Resistance vessels express neutrophil oxidase-2 and -4. Angiotensin II activates p47phox/neutrophil oxidase-2, whereas it downregulates NOX-4. Therefore, we tested the hypothesis that p47phox enhances afferent arteriolar angiotensin contractions. Angiotensin II infusion in p47phox+/+ mice did not change afferent arteriolar angiotensin contractions. However, p47phox−/− mice increased renal cortical NADPH oxidase activity (7 ± 1–12 ± 1 [P < 0.01] versus 5 ± 1–7 ± 1 103 · RLU · min−1 · μg protein−1 [P value not significant]), mean arterial pressure (77 ± 2–91 ± 2 [P < 0.005] versus 74 ± 2–77 ± 1 mm Hg [P value not significant]), and renal vascular resistance (7.5 ± 0.4–10.1 ± 0.7 [P < 0.01] versus 7.9 ± 0.4–8.3 ± 0.4 mm Hg/mL · min−1 · gram kidney weight−1 [P value not significant]). Afferent arterioles from p47phox−/− mice had a lesser myogenic response (3.1 ± 0.4 versus 1.4 ± 0.2 dynes · cm−1 · mm Hg−1; P < 0.02) and a lesser (P < 0.05) contraction to 10−6 M angiotensin II (diameter change +/+: 9.3 ± 0.2–3.4 ± 0.6 μm versus −/−: 9.9 ± 0.6–7.5 ± 0.4 μm). Angiotensin and increased perfusion pressure generated significantly (P < 0.05) more reactive oxygen species in p47phox+/+ than −/− arterioles. Angiotensin II infusion increased the maximum responsiveness of afferent arterioles from p47phox+/+ mice to 10−6 M angiotensin II yet decreased the response in p47phox−/− mice. The angiotensin infusion increased the sensitivity to angiotensin II only in p47phox+/+ mice. We conclude that p47phox is required to enhance renal NADPH oxidase activity and basal afferent arteriolar myogenic and angiotensin II contractions and to switch afferent arteriolar tachyphylaxis to sensitization to angiotensin during a prolonged angiotensin infusion. These effects likely contribute to hypertension and renal vasoconstriction during infusion of angiotensin II. (Hypertension. 2012;59[part 2]:415-420.)

Key Words: NADPH oxidase ■ hypertension ■ oxidative stress ■ reactive oxygen species

Blockade of angiotensin II (Ang II) reduced the blood pressure (BP) and renal vascular resistance (RVR) in many models of hypertension1 and in humans with essential hypertension.2 Afferent arteriolar contractions with Ang II increase the RVR and reduce the transmission of arterial pressure into the kidney, which may protect the glomeruli from potential barotrauma.3 Ang II increases reactive oxygen species (ROS) in the afferent arteriole4–6 and the kidney,7,8 which increase the vascular contractility and the RVR.4–6,9–18 ROS are implicated in Ang II responses, because Tempol10 reduced the hypertension and the renal vasoconstriction of mice infused with Ang II at a slow pressor rate,7 which is considered a model of human essential hypertension.19

NADPH oxidase has been implicated in Ang II–induced increases in ROS.12,15,16,20,21 However, NADPH oxidase is a complex enzyme, and ≥2 neutrophil oxidases (NOX-2 and -4) are expressed in rodent microvessels with different regulation8,9,12,20 and activation by cytosolic subunits.20 NOX-2 is a prominent oxidase in small blood vessels and glomeruli where it interacts with p22phox in the membrane and p47phox, p67phox, p40phox, and Rac2 from the cytosol.12 Ang II reduced the expression of NOX-4 but increased the afferent arteriolar mRNA expression for p22phox and increased the vascular membrane association12,22–24 and c-Src–dependent phosphorylation and activation of p47phox,13 which assembled with p22phox, NOX-2, and other cytosolic subunits to form a functional membrane oxidase.12 Although knockout of p47phox−/− attenuated the increase in ROS9–11 and rate of rise of BP14,25 with pressor infusions of Ang II and attenuated large vessel myogenic responses,14 prolonged Ang II infusion at a slow pressor rate did not change p47phox expression in the kidney,5,24 and p47phox−/− mice had a maintained,25 or even increased,9,10,14,27 basal level of superoxide (O2•−) generation in blood vessels, kidneys, and vascular smooth muscle cells (VSMCs). Moreover, p47phox−/− mice had a normal basal BP,11,14,25,27 Thus, the role of p47phox in basal and Ang II–stimulated regulation of BP and RVR is not completely understood.

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From the Hypertension, Kidney, and Vascular Research Center and Division of Nephrology and Hypertension (E.Y.L., G.S., Z.L., M.C., K.S., W.J.W., C.S.W.) and Lombardi Cancer Center (A.W.), Georgetown University Medical Center, Washington, DC; Clinical Infectious Diseases (S.H.), National Institute of Allergy and Infectious Diseases, Bethesda, MD.
Correspondence to Christopher S. Wilcox, Division of Nephrology and Hypertension, Georgetown University Medical Center, 3800 Reservoir Rd, NW, PHC Building, F6003, Washington, DC 20007. E-mail wilcoxch@georgetown.edu
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understood. We tested the hypothesis that p47phox is required for full afferent arteriolar contractions to Ang II and perfusion pressure (myogenic responses) and for increases in mean arterial pressure (MAP) and RVR in mice receiving a slow pressor (low-dose) infusion of Ang II by contrasting responses in p47phox +/+ versus −/− mice.

Methods

Experimental Design
Male p47phox +/+ and −/− littermate mice aged 10 to 14 weeks were bred from +/+ founders and backcrossed ≥8 times to the C57BL/6 background.14 Because these mice are prone to infection, we followed the advice of our veterinarians that they be individually housed with precautions to minimize infection and with trimethoprim/sulfamethoxazole added to the drinking water for 7 days, followed by 3 days without antibiotics, as in a previous study.23 They were fed a normal mouse chow with a regular salt content of 0.4%.

Groups of p47phox +/+ and −/− mice (n = 6–7) were anesthetized with isofluorane (1% to 2% in O2) 2 weeks before experiments. Radiotelemeters were inserted into a carotid artery.26 Basal recordings of MAP were made for 4 days, after which mice were anesthetized with isofluorane for insertion of osmotic minipumps (ALZET Corp, DURECT Corp, Cupertino, CA) to deliver Ang II (400 ng·kg⁻¹·min⁻¹) or vehicle subcutaneously for 2 weeks.7,28 Thereafter, the mice were euthanized and the kidney cortex was harvested. Cell membranes were separated to measure membrane-bound NADPH oxidase activity from lucigenin-enhanced chemiluminescence after the addition of 200 μmol·L⁻¹ of NADPH, as described.26 Other mice (n = 5–7 per group) were anesthetized with thiobarbitral (Inactin 50 mg·kg⁻¹·kg⁻¹) and ketamine (40 mg·kg⁻¹·kg⁻¹) and prepared for renal clearance studies26 12 to 14 days after Ang II or vehicle. The glomerular filtration rate (GFR) was the clearance of [⁹⁸]Hjulin, and the renal plasma flow (RPF) was the clearance [¹⁴C]paraaminophenurate corrected for renal extraction. Renal blood flow (RBF) was RPF factored by 1 hematocrit, and RVR was MAP factored by renal blood flow.

Afferent Arteriolar Responses
After 12 to 14 days of Ang II or vehicle infusions, mice were anesthetized, the kidneys removed, and an afferent arteriole dissected and perfused.6 Contractions to bath addition of Ang II (10⁻⁶ to 10⁻⁴ m) were recorded. The myogenic responses were studied in other arterioles during 20-mm Hg increases in renal perfusion pressure.29,30 The slope of active wall tension (difference between Ca²⁺-free and physiological solution) against perfusion pressure defined the myogenic response. Only 1 arteriole was used per animal. The inner luminal diameter and medial thickness were measured at 60-mm Hg perfusion pressure to compute the media:lumen ratio. The ROS generated in the afferent arterioles during incubation with 10⁻⁶ m Ang II or during increases in renal perfusion pressure from 40 to 80 mm Hg were assessed from the ratio of ethidium:di-hydroethidium fluorescence, as described and validated.6,30

Statistical Analysis
Values are presented as mean±SEM. Repeated-measures ANOVA was used to test concentration-dependent changes in MAP or arteriolar diameter. A 2×2 ANOVA with interaction was used to assess the effects of genotype, Ang II, and effects of genotype on the response to Ang II (interaction). Post hoc comparisons were performed using a Fisher test. Differences were considered to be statistically significant if P < 0.05.

Results
The mice were healthy and had similar body and kidney weight (Table S1, available in the online Data Supplement at http://hyper.ahajournals.org). Afferent arterioles from p47phox +/+ mice had readily detectable levels of p47phox mRNA, whereas it was absent from arterioles of p47phox −/− mice (Figure S1).

The basal levels of telemetric MAP were not different between strains, whereas the MAP measured during the daytime (Figure 1A) or nighttime (Figure 1B) increased after 2 to 3 days of Ang II infusion in p47phox +/+ mice but did not change with Ang II in p47phox −/− mice. The basal values of NADPH oxidase, MAP, renal blood flow, and RVR of vehicle-infused mice under anesthesia were similar in the 2 strains (Figure 2), but the GFR was lower in p47phox +/+ mice (P < 0.05). Two weeks of Ang II infusion in p47phox +/+ mice increased the NADPH oxidase (100±5–400±50 mm Hg; P < 0.0001), the MAP (77±2–91±2 mm Hg; P < 0.005), and the RVR (7.5±0.4–10±0.7 mm Hg/mL·min⁻¹·gram kidney weight⁻¹; P < 0.01). However, Ang II infusion in p47phox −/− mice did not change these variables. The basal afferent arteriolar diameter and the media:lumen ratio were similar in both strains and were unaffected by Ang II infusion (Table S2A).

Afferent arterioles isolated from vehicle-infused mice of both strains had graded contractions with Ang II (Figure 3).
mice, compared with p47phox

10

strain. Ang II (10

tration to cause a contraction (from 10

sensitivity, whether assessed from the lowest Ang II concen-

Ang II infusion in p47phox

maximum response (supplemental Table S2B). Two weeks of

(40–80 mm Hg) both increased afferent arteriolar ROS

This myogenic response was reduced by 48% in arterioles

changes in passive wall tension assessed in the absence of

a graded contraction (Figure 4A and 4B). There were no

Ang infusion

Figure 2. Mean±SEM values (n=6–9 per group) for mice studied under anesthesia showing renal cortical NADPH oxidase activity (A), arterial pressure (B), glomerular filtration rate (C), renal blood flow (D), and renal vascular resistance (E) in groups of p47phox

+/+

and −/− mice given a vehicle (open boxes) or Ang II (400·kg⁻¹·min⁻¹ SC) for 12 days. Compared with equivalent p47phox

+/+ mice: a, P=0.05.

After vehicle infusion, afferent arterioles from p47phox

−/− mice, compared with p47phox

+/+ mice, had a 40% reduced maximum response (supplemental Table S2B). Two weeks of Ang II infusion in p47phox

+/+ mice increased the Ang II sensitivity, whether assessed from the lowest Ang II concentration to cause a contraction (from 10⁻⁸ m during vehicle to 10⁻¹¹ m with Ang II infusion; Figure 3B) or from the ED₅₀ (Table S2B). In contrast, 2 weeks of Ang II infusion in p47phox

−/− mice actually reduced the maximum response to 10⁻⁶ m Ang II (Figure 3 and Table S2B) without changing the Ang II sensitivity.

An increase in perfusion pressure of afferent arterioles >40 mm Hg from vehicle-infused p47phox

+/+ mice led to a graded contraction (Figure 4A and 4B). There were no changes in passive wall tension assessed in the absence of calcium but an increase in active wall tension (Figure 4C). This myogenic response was reduced by 48% in arterioles from p47phox

−/− mice (Figure 4 and Table S2A). Ang II infusion did not change myogenic responses is either strain. Ang II (10⁻⁶ m) or increasing perfusion pressure (40–80 mm Hg) both increased afferent arteriolar ROS more in afferent arterioles from p47phox

+/+ than −/− mice (Figure 5).

Discussion

The main new findings were the p47phox

−/− mice had a normal basal renal cortical NADPH oxidase activity, MAP, and RVR under anesthesia and a normal conscious MAP during the daytime or nighttime but a lower GFR. However, unlike the p47phox

+/+ mice, the NADPH oxidase activity, MAP, and RVR of p47phox

−/− mice failed to increase during a slow pressor infusion of Ang II. Afferent arterioles from p47phox

−/− mice had a 38% reduced maximum responsiveness to Ang II and a 48% reduced myogenic response. Ang II and increased perfusion pressure increased afferent arteriolar ROS, but these responses were reduced in p47phox

−/− mice. Although Ang II infusion enhanced the maximum Ang II responsiveness of afferent arterioles from p47phox

+/+ mice by 26% and enhanced their sensitivity 1000-fold, Ang II infusion in p47phox

−/− mice actually reduced the maximum response of their arterioles to Ang II by 26% and did not change their sensitivity.
The normal basal NADPH oxidase activity in the kidney cortex from p47phox −/− mice confirms a previous study.27 Basal O2− generation from cultured VSMCs,11 and from aortas of p47phox −/− mice has been unchanged14 or increased.10,27 Apparently, p47phox is not required for basal O2− generation. The source of the p47phox-independent basal O2− generation in this study was not established but likely was from the NOX-4 component of NADPH oxidase, because it does not require p47phox for activity, and in VSMCs, NOX-4 is the major source of basal ROS but does not contribute to Ang II–stimulated ROS generation.31

Grote et al27 reported a higher basal MAP in young p47phox −/− mice that they attributed to an enhanced aortic angiotensin-converting enzyme activity, although angiotensin-converting enzyme gene expression in the kidney was unchanged. However, the absence of any rise in MAP with Ang II infusion in the p47phox −/− mice in our study suggests that excessive Ang II generation by angiotensin-converting enzyme likely did not account for altered responses of p47phox −/− mice in our study. Moreover, we did not confirm the difference in basal MAP between p47phox +/+ and −/− mice.

Unexpectedly, the p47phox −/− mice had lower GFRs in the basal state and during Ang II infusion. This contrasts with the effects of Tempol to increase the GFR of mice during a slow pressor infusion of Ang II.7 Because both Tempol7 and p47phox knockout prevented Ang II–induced oxidative stress, it suggests that the effects of p47phox −/− on the GFR may be dissociated from its antioxidant actions. We detected prominent expression of p47phox in the podocytes of rats,32 where it could regulate the GFR, although the mechanism is unclear. There were no differences in basal MAP between p47phox +/+ and −/− mice to explain the lower basal GFR in the p47phox −/− mice.

Previous studies in dogs and rats have reported that Ang II infusion did not change or diminish the myogenic components of renal autoregulation.34–37 This is the first direct study in isolated afferent arterioles. We detected no effect of 2 weeks of Ang II infusion on myogenic responses. This difference from the previous studies may relate to interactions between myogenic responses and tubuloglomerular feedback that were conducted in whole kidneys, because tubuloglomerular feedback responses are enhanced by Ang II infusion,38 and this can impair myogenic responses.39 The present study removed any confounding effects of tubuloglomerular feedback by studying isolated arterioles.

Our finding that Ang II infusion increased the Ang II responsiveness of afferent arterioles from p47phox +/+ mice confirms previous studies in rabbits,4 where the enhanced response was accompanied by a significant increase in the mRNA expression for p22phox but a significant reduction in mRNA expression for angiotensin type I receptors in the afferent arterioles. This suggests that enhanced responsiveness to Ang II in afferent arterioles of rodents infused with Ang II is attributed to activation of NADPH oxidase by p22phox and phosphorylated p47phox that enhances ROS generation. In the absence of this enhancing effect of ROS, the effects of Ang II to downregulate Ang type I receptors may become apparent as tachyphylaxis and an impaired response to Ang II.

Because oxidative stress determined BP in many rodent models,18 our findings of an unchanged basal level of NADPH oxidase activity in the kidneys may explain the unchanged basal MAP and RVR in p47phox −/− mice. Ang

Figure 4. Mean±SEM values (n=4–7) for afferent arteriolar diameter (A), change in diameter (B) and active wall tension (C) during increases in renal perfusion pressure. Data are shown for vessels from p47phox +/+ mice infused with vehicle (solid circles and continuous lines) or angiotensin II (open circles and broken lines) or p47phox −/− mice infused with vehicle (solid triangles and continuous lines) or angiotensin II (open triangles and broken lines). Significance of difference between groups: *P<0.05.

Figure 5. Mean±SEM values (n=5–7) for increases in ethidium: dihydroethidium ratio of afferent arterioles from p47phox +/+ mice (solid black) and p47phox −/− mice (open box). Data are shown for changes with 10−6 M angiotensin II or increased perfusion from 40 to 80 mmHg.
II infusion increased the excretion of lipid peroxidation products, ROS generation in kidneys, and in afferent arterioles of normal mice. Indeed, NADPH oxidase is a major source of O$_2^-$ in VSMCs, kidneys of rats infused with Ang II at a slow pressor rate, and the NADPH oxidase activity increased 4-fold with Ang II infusion in p47$^{phox}$ +/+ mice in this study. Because the NADPH oxidase activity was unchanged in the renal cortex of p47$^{phox}$ --/-- mice infused with Ang II, we concluded that p47$^{phox}$ was required for NADPH oxidase activation in the kidneys by Ang II. This extends previous reports of a reduced generation of O$_2^-$ in VSMCs or aortas from p47$^{phox}$ --/-- mice during stimulation with arachidonic acid. Ang II 10,11,14,25 platelet-derived growth factor, or phorbol myristate acetate. In another study, p47$^{phox}$ --/-- mice had a preserved, albeit delayed, increase in MAP in response to a direct pressor infusion of Ang II. This suggests that O$_2^-$ derived from p47$^{phox}$-dependent NADPH oxidase sets the sensitivity of the BP response to Ang II, whereas other actions of Ang II contribute to its maximal response. This is consistent with the substantial increase in sensitivity of afferent arterioles from p47$^{phox}$ +/+ mice to Ang II produced by an Ang II infusion, which was entirely lacking in those from p47$^{phox}$ --/-- mice.

Perspectives

Studies from gene-deleted mice have highlighted the importance of ROS in the regulation of BP. Superoxide dismutase 1 --/-- mice had an enhanced slow pressor response to Ang II, their afferent arterioles generated more ROS, and they had a 10 000-fold increased sensitivity to Ang II. The present studies demonstrated the essential role of p47$^{phox}$ in O$_2^-$ generation and in the hypertensive and renal vasoconstriction with a low-dose infusion of Ang II. Oxidative stress underlies many adverse cardiovascular and renal effects of Ang II. Renal vasoconstriction is implicated in the development of hypertension. Thus, p47$^{phox}$ is an attractive target to prevent hypertension and some of its complications.

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Disclosures

None.

References


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P47phox IS REQUIRED FOR AFFERENT ARTERIOLAR CONTRACTILE RESPONSES TO ANGIOTEINSENS II AND PERFUSION PRESSURE IN MICE

En Yin Lai¹, Glenn Solis¹, Zaiming Luo¹, Mattias Carlstrom¹, Kathryn Sandberg¹, Steven Holland³, Anton Wellstein², William J. Welch¹ and Christopher S. Wilcox¹

¹Hypertension, Kidney, and Vascular Research Center & Division of Nephrology and Hypertension, and ²Lombardi Cancer Center, Georgetown University Medical Center, Washington, D.C., U.S.A.; ³Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD., U.S.A.

Address for correspondence:
Christopher S. Wilcox, M.D. Ph.D.
Division of Nephrology and Hypertension
Georgetown University Medical Center
3800 Reservoir Road, NW
6 PHC Bldg, F6003
Washington, DC 20007
Email: wilcoxch@georgetown.edu
Phone: 1-202-444-9183
Fax: 1-877-625-1483
**Methods and Results**

**RNA isolation and real-time quantitative RT-PCR:** Total RNA was isolated from mouse afferent arterioles with RNeasy Microarray Tissue Mini Kit following the manufacturer’s instruction manual (Qiagen Valencia, CA). Reverse transcription of 0.5 μg of total RNA per sample was performed with iScriptTMcDNA Synthesis Kit (Bio-Rad, Hercules, CA). Primers and probes for rat p47(ID: mn00447921_m1) and 18S rRNA (ID: 4319413E) were purchased for gene expression assays (Applied Biosystems, Foster City, CA). The comparative \([\Delta\DeltaCT]\) method was used for relative quantification and statistical analysis. The results are shown in supplement figure S1. Data were available for afferent arterioles from four p47phox +/+ and four p47phox -/- mice. The mean value for p47phox mRNA/18S rRNA for p47phox +/+ mice was 1.01±0.06. The corresponding value for the p47phox -/- mice was 0.00±0.00. We conclude that the p47phox gene was fully knocked out in the afferent arterioles of p47phox -/- mice.
Table S1: Body and combined kidney weights

Mean ± SEM values. Data were obtained after 2 weeks of vehicle or angiotension II infusion. Comparing Ang II with vehicle: *, P<0.05.

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<th>p47_phox^{phox}-/-</th>
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Comparing equivalent p47_phox^{phox}+/+ and -/-: a, P<0.05
**Table S2**: Effects of Ang II infusion and p47phox genotype on afferent arteriolar diameter and media: lumen ratios and responsiveness and sensitivity to angiotensin II and myogenic responses.

Mean ± SEM values (n=4-7 per group). Comparing Ang II with vehicle infused: *P<0.05. Comparing equivalent p47phox +/+ with -/-;  

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<th>p47phox -/-</th>
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<td>Ang II</td>
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<td>(a) Arteriolar diameters</td>
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<td>(b) Angiotensin II contractions</td>
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<td>Maximum response (%)</td>
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<td>ED₅₀ (log 10⁻⁸ M)</td>
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<td>Threshold (mmHg)</td>
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<td>24.8±6.0</td>
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a, P<0.05.
Supplement figure S1: Mean ± SEM values (n=4 per group) for mRNA expression in afferent arterioles, relative to 185.