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P47^{phox} IS REQUIRED FOR AFFERENT ARTERIOLAR CONTRACTILE RESPONSES TO ANGIOTENSIN II AND PERFUSION PRESSURE IN MICE

En Yin Lai[1], **Glenn Solis**[1], **Zaiming Luo**[1], **Mattias Carlstrom**[1], **Kathryn Sandberg**[1], **Steven Holland**[3], **Anton Wellstein**[2], **William J. Welch**[1], and **Christopher S. Wilcox**[1]

[1] Hypertension, Kidney, and Vascular Research Center & Division of Nephrology and Hypertension, Georgetown University Medical Center, Washington, D.C., U.S.A.

[2] Lombardi Cancer Center, Georgetown University Medical Center, Washington, D.C., U.S.A.

[3] Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD., U.S.A.

Abstract

Myogenic and angiotensin contractions of afferent arterioles generate reactive oxygen species. Resistance vessels express NOX-2 and -4. Angiotensin II activates p47^{phox}/NOX-2 whereas it downregulates NOX-4. Therefore, we tested the hypothesis that p47^{phox} enhances afferent arteriolar angiotensin contractions. Angiotensin II infusion in p47^{phox} +/+, but not -/- mice, increased renal cortical nicotinamide adenine dinucleotide phosphate oxidase activity (7±1 to 12±1; P<0.01 vs 5±1 to 7±1; NS, $10^3 \cdot RLU \cdot min^{-1} \cdot \mu g\ protein^{-1}$), mean arterial pressure (77±2 to 91±2; P<0.005 vs 74±2 to 77±1; NS, mmHg) and renal vascular resistance (7.5±0.4 to 10.1±0.7; P<0.01 vs 7.9±0.4 to 8.3±0.4 NS, $mmHg/ml \cdot min^{-1} \cdot gkwt^{-1}$). Afferent arterioles from p47^{phox} -/- mice had a lesser myogenic response (3.1±0.4 vs 1.4±0.2 $dynes \cdot cm^{-1} \cdot mmHg^{-1}$; P<0.02) and a lesser (P<0.05) contraction to 10^{-6}M angiotensin II (diameter change +/+: 9.3±0.2 to 3.4±0.6 μm vs -/-: 9.9±0.6 to 7.5±0.4 μm). Angiotensin and increased perfusion pressure generated significantly (P<0.05) more reactive oxygen species in p47^{phox} +/+ than -/- arterioles. Angiotensin II infusion increased the maximum responsiveness of afferent arterioles from p47^{phox} +/+ mice to 10^{-6} M angiotensin II yet decreased the response in p47^{phox} -/- mice. The angiotensin infusion increased the sensitivity to angiotensin II only in p47^{phox} +/+ mice. We conclude that p47^{phox} is required to enhance renal nicotinamide adenine dinucleotide phosphate 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.

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

Conflict of interest
None.

Keywords

Nicotine adenine dinucleotide phosphate (NADPH) oxidase; hypertension; oxidative stress; reactive oxygen species (ROS)

INTRODUCTION

Blockade of angiotensin II (Ang II) reduced the blood pressure (BP) and renal vascular resistance (RVR) in many models of hypertension [1] and in humans with essential or renovascular 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 arteriole [4-6] and the kidney [7,8] which increase the vascular contractility and the renal vascular resistance (RVR) [4-6,9-18]. ROS are implicated in Ang II responses since tempol [18] 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].

Nicotinamide adenine dinucleotide phosphate (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 at least two neutrophil oxidases (NOX-2 and -4) are expressed in rodent microvessels with different regulation [8,9,12,20] and activation by cytosolic subunits [20]. NOX-2 is a prominent oxidase in small blood vessels and glomeruli where it interacts with $p22^{phox}$ in the membrane and $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac2 from the cytosol [12]. Ang II reduced the expression of NOX-4 [8] but increased the afferent arteriolar mRNA expression for $p22^{phox}$ [4] and increased the vascular membrane association [12,22-24] and c-Src-dependent phosphorylation and activation of $p47^{phox}$ [13], which assembled with $p22^{phox}$, NOX-2 and other cytosolic subunits to form a functional membrane oxidase [12]. Whereas knockout of $p47^{phox}$ -/- attenuated the increase in ROS [9-11] and rate of rise of BP [14,25] 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 $p47^{phox}$ expression in the kidney [8,26] and $p47^{phox}$ -/- mice had a maintained [25], or even increased [9,10,14,27], basal level of superoxide ($O_2^{\cdot -}$) generation in blood vessels, kidneys and vascular smooth muscle cells (VSMCs). Moreover, $p47^{phox}$ -/- mice had a normal basal BP [11,14,25,27]. Thus, the role of $p47^{phox}$ in basal and Ang II-stimulated regulation of BP and RVR is not completely understood. We tested the hypothesis that $p47^{phox}$ 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 $p47^{phox}$ +/+ vs -/- mice.

Experimental design

Male $p47^{phox}$ +/+ and -/- littermate mice aged 10-14 weeks were bred from ± founders and backcrossed at least 8 times to the C57BL/6 background [14]. Since 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 prior study [27]. They were fed a normal mouse chow with a regular salt content of 0.4%.

Groups of $p47^{phox}$+/+ and -/- mice (n=6-7) were anesthetized with isofluorane (1-2% in O_2) 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 (Direct Corp, Glenn) to deliver Ang II (400 $ng^{-1} \cdot kg^{-1} \cdot min^{-1}$) or vehicle (V) subcutaneously for 2 weeks [7,28]. Thereafter, the mice were sacrificed and the kidney cortex was harvested. Cell membranes were separated to measure membrane-bound NADPH oxidase activity from lucigenin-enhanced chemiluminescence after addition of 200 $\mu mol \cdot l^{-1}$ of NADPH, as described [26]. Other mice (n=5-7/group) were anesthetized with thiobarbital (Inactin 50 $mg^{-1} \cdot kg^{-1}$) and ketamine (40 $mg^{-1} \cdot kg^{-1}$) and prepared for renal clearance studies [26] 12-14 days after Ang II or vehicle. The glomerular filtration rate (GFR) was the clearance of [^{3}H]-inulin and the renal plasma flow was the clearance [^{14}C]-paraaminohippurate (PAH) corrected for renal extraction. Renal blood flow (RBF) was RPF factored by 1-hematocrit and RVR was MAP factored by RBF.

Afferent arteriolar responses

After 12-14 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^{-12} to 10^{-6} M) were recorded. The myogenic responses were studied in other arterioles during ≈ 20 mmHg increases in renal perfusion pressure [29,30]. The slope of active wall tension (difference between Ca^{2+}-free and physiologic solution) against perfusion pressure defined the myogenic response. Only one arteriole was used per animal. The inner luminal diameter and medial thickness were measured at 60 mmHg 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 mmHg was assessed from the ratio of ethidium to dihydroethidium 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 $P<0.05$.

Ethics

The experiments were approved by the Georgetown University Animal Care and Use Committee. They conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

RESULTS

The mice were healthy and had similar body and kidney weight (supplement table S1; please see http://hyper.ahajournals.org). Afferent arterioles from $p47^{phox}$ +/+ mice had readily detectable levels of $p47^{phox}$ mRNA whereas it was absent from arterioles of $p47^{phox}$ -/- mice (supplement figure S1; please see http://hyper.ahajournals.org)

The basal levels of telemetric MAP were not different between strains whereas the MAP measured during the daytime (Figure 1, panel A) or nighttime (Figure 1. panel B) increased after 2-3 days of Ang II infusion in $p47^{phox}$ +/+ mice, but did not change with Ang II in $p47^{phox}$ -/- mice.

The basal values of NADPH oxidase, MAP, RBF and RVR of vehicle-infused mice under anesthesia were similar in the two strains (Figure 2) but the GFR was lower in $p47^{phox}$ -/- mice ($P<0.05$). Two weeks of Ang II infusion in $p47^{phox}$ +/+ mice increased the NADPH oxidase (100±5 to 400±50; $P<0.0001$), the MAP (77±2 to 91±2 mmHg; $P<0.005$) and the

RVR (7.5±0.4 to 10.1±0.7 mmHg/ml·min^{-1}·gkwt^{-1}; P<0.01). However, Ang II infusion in p47^{phox} -/- 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 (supplement table S2a; please see http://hyper.ahajournals.org).

Afferent arterioles isolated from vehicle-infused mice of both strains had graded contractions with Ang II (Figure 3). After vehicle infusion, afferent arterioles from p47^{phox} -/- mice, compared to p47^{phox} +/+ mice, had a 40% reduced maximum response (supplement table 2b; please see http://hyper.ahajournals.org). Two weeks of Ang II infusion in p47^{phox} +/+ 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 ED_{50} (supplement table S2b; please see http://hyper.ahajournals.org). In contrast, two weeks of Ang II infusion in p47^{phox} -/- mice actually reduced the maximum response to 10^{-6} M Ang II (Figure 3 and supplement table 2b; please see http://hyper.ahajournals.org) without changing the Ang II sensitivity.

An increase in perfusion pressure of afferent arterioles above 40mmHg from vehicle-infused p47^{phox} +/+ mice led to a graded contraction (Figure 4A and B). 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 p47^{phox} -/- mice (supplement table S2a; please see http://hyper.ahajournals.org). Ang II infusion did not change myogenic responses is either strain.

Ang II (10^{-6} M) or increasing perfusion pressure (40 to 80 mmHg) both increased afferent arteriolar ROS more in afferent arterioles from p47^{phox} +/+ than -/- mice (Figure 5).

DISCUSSION

The main new findings were the p47^{phox} -/- 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 p47^{phox} +/+ mice, the NADPH oxidase activity, MAP and RVR of p47^{phox} -/- mice failed to increase during a slow pressor infusion of Ang II. Afferent arterioles from p47^{phox} -/- 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 p47^{phox} -/- mice. Whereas Ang II infusion enhanced the maximum Ang II responsiveness of afferent arterioles from p47^{phox} +/+ mice by 26%, and enhanced their sensitivity 1,000-fold, Ang II infusion in p47^{phox} -/- 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 p47^{phox} -/- mice confirms a prior study [27]. Basal $O_2^{\cdot -}$ generation from cultured VSMCs [9,11] and from aortas of p47^{phox} -/- mice has been unchanged [14] or increased [10,27]. Apparently, p47^{phox} is not required for basal $O_2^{\cdot -}$ generation. The source of the p47^{phox}-independent basal $O_2^{\cdot -}$ generation in this study was not established but likely was from the NOX-4 component of NADPH oxidase since it does not require p47^{phox} 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 al reported a higher basal MAP in young p47^{phox} -/- mice that they attributed to an enhanced aortic angiotensin converting enzyme (ACE) activity, although ACE gene expression in the kidney was unchanged [27]. However, the absence of any rise in MAP with Ang II infusion in the p47^{phox} -/- mice in our study suggests that excessive Ang II

generation by ACE likely did not account for altered responses of $p47^{phox}$ -/- mice in our study. Moreover, we did not confirm the difference in basal MAP between $p47^{phox}$ +/+ and -/- mice.

Unexpectedly, the $p47^{phox}$ -/- 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]. Since both tempol [7] and $p47^{phox}$ knockout prevented Ang II-induced oxidative stress, it suggests that the effects of $p47^{phox}$ -/- on the GFR may be dissociated from its antioxidant actions. We detected prominent expression of $p47^{phox}$ 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 $p47^{phox}$ +/+ and -/- to explain the lower basal GFR in the $p47^{phox}$ -/- mice.

Previous studies in dogs and rats have reported that Ang II infusion did not change [33], or diminish, the myogenic components of renal autoregualtion [34-37]. This is the first direct study in isolated afferent arterioles. We detected no effect of two weeks of Ang II infusion on myogenic responses. This difference from the prior studies may relate to interactions between myogenic responses and tubuloglomerular feedback (TGF) that were conducted in whole kidneys since TGF responses are enhanced by Ang II infusion [38] and this can impair myogenic responses [39]. The present study removed any confounding effects of TGF by studying isolated arterioles.

Our finding that Ang II infusion increased the Ang II responsiveness of afferent arterioles from $p47^{phox}$ +/+ mice confirms prior studies in rabbits [4] where the enhanced response was accompanied by a significant increase in the mRNA expression for $p22^{phox}$, but a significant reduction in mRNA expression for ATI-receptors, in the afferent arterioles. This suggests that enhanced responsiveness to Ang II in afferent arterioles of rodents infused with Ang II is due to activation of NADPH oxidase by $p22^{phox}$ and phosphorylated $p47^{phox}$ that enhances ROS generation. In the absence of this enhancing effect of ROS, the effects of Ang II to downregulate AT1-receptors may become apparent as tachyphylaxis and an impaired response to Ang II.

Since 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 $p47^{phox}$ -/- mice. Ang II infusion increased the excretion of lipid peroxidation products [7], ROS generation in kidneys [7] and afferent arterioles of normal mice [6]. Indeed, NADPH oxidase is a major source of $O_2^{\cdot -}$ in VSMCs [15], kidneys of rats infused with Ang II at a slow pressor rate [8] and the NADPH oxidase activity increased four-fold with Ang II infusion in $p47^{phox}$ +/+ mice in this study. Since 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 prior reports of a reduced generation of $O_2^{\cdot -}$ in VSMCs or aortas from $p47^{phox}$ -/- mice during stimulation with arachidonic acid [27], Ang II [10,11,14,25], platelet derived growth factor or phorbol myristate acetate [11]. In another study, $p47^{phox}$ -/- mice had a preserved, albeit delayed, increase in MAP in response to a direct pressor infusion of Ang II [14]. This suggests that $O_2^{\cdot -}$ 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. SOD-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 [6]. The present studies demonstrated the essential role of $p47^{phox}$ in $O_2^{\cdot -}$ generation and in the hypertension and renal vasoconstriction with a low dose infusion of Ang II. Oxidative stress underlies many adverse cardiovascular and renal effects of Ang II [17,18,40]. Renal vasoconstriction is implicated in the development of hypertension [41]. Thus, $p47^{phox}$ is an attractive target to prevent hypertension and some of its complications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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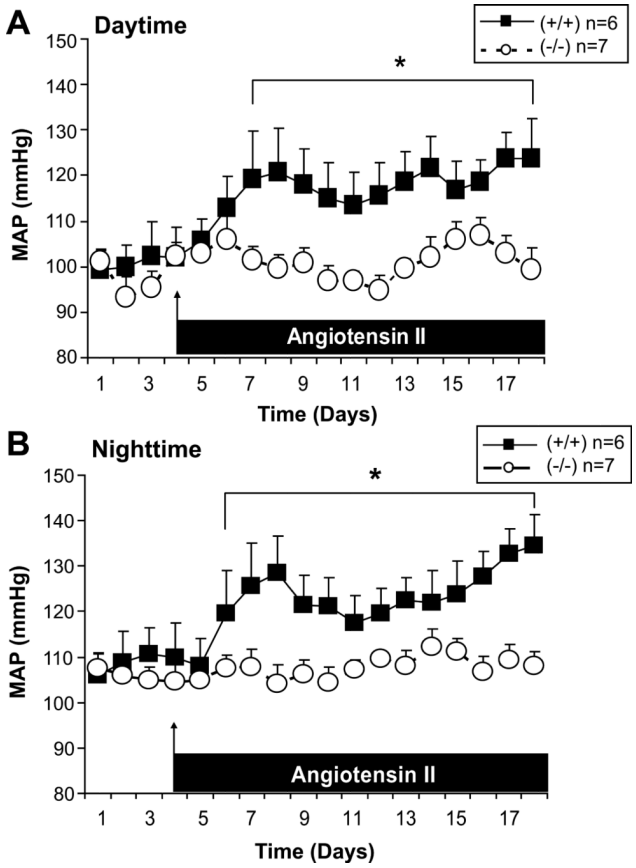

Figure 1.

Mean ± SEM values for telemetric mean arterial pressure of $p47^{phox}$ +/+ (solid squares and continuous lines; n = 6) and $p47^{phox}$ -/-(open circles and broken lines n = 7) mice before and during angiotensin II (400 ng · kg^{-1} · min^{-1} · sc) during the daytime (asleep) in panel A and the nighttime (awake) in panel B. Comparing groups: *, P<0.05.

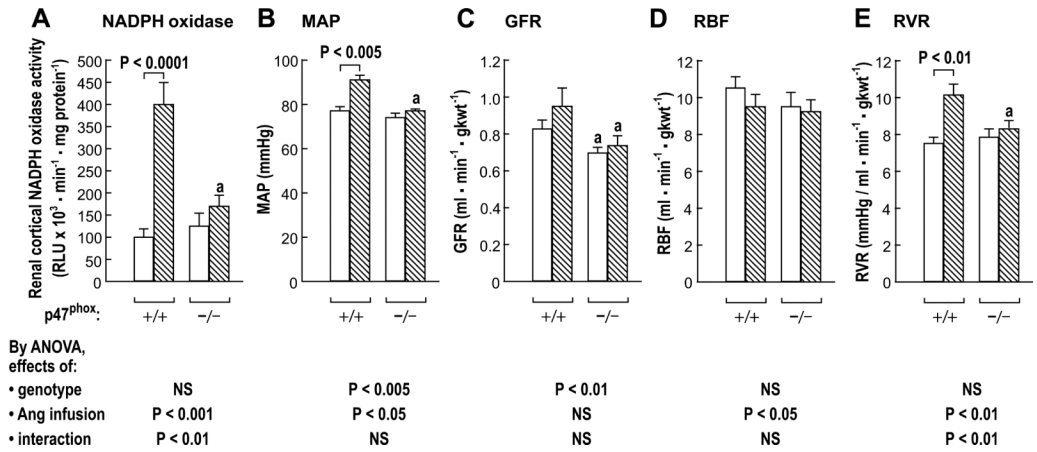

Figure 2.

Mean ± SEM values (n = 6-9 per group) for mice studied under anesthesia showing renal cortical NADPH oxidase activity (panel A), arterial pressure (panel B), glomerular filtration rate (panel C), renal blood flow (panel D), and renal vascular resistance (panel E) in groups of p47^{phox} +/+ and -/- mice given a vehicle (open boxes) or Ang II (400 · kg^{-1} · min^{-1} sc) for 12 days. Compared to equivalent p47^{phox} +/+ mice: a, P<0.05.

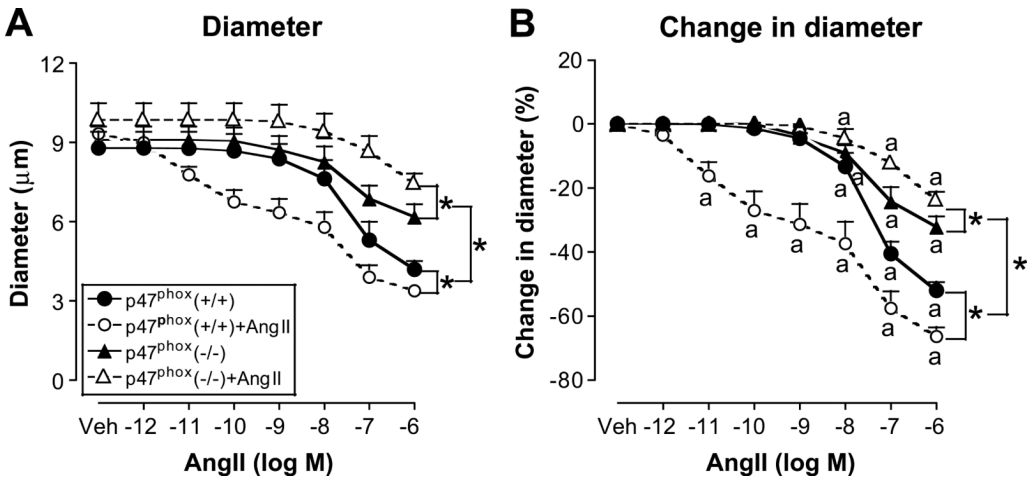

Figure 3.

Mean ± SEM values (n = 4 - 7) for afferent arteriolar diameter (Panel A) and changes in diameter from baseline (Panel B) during bath addition of angiotensin II to $p47^{phox}$ +/+ mice infused with vehicle (solid circles and continuous lines) or angiotensin II (open circles and broken lines) or $p47^{phox}$ -/- mice infused with vehicle (solid triangles and continuous lines) or angiotensin II (open triangles and broken lines). Significance of difference between groups: * $P<0.05$. Significance of difference from vehicle: a, $P<0.05$.

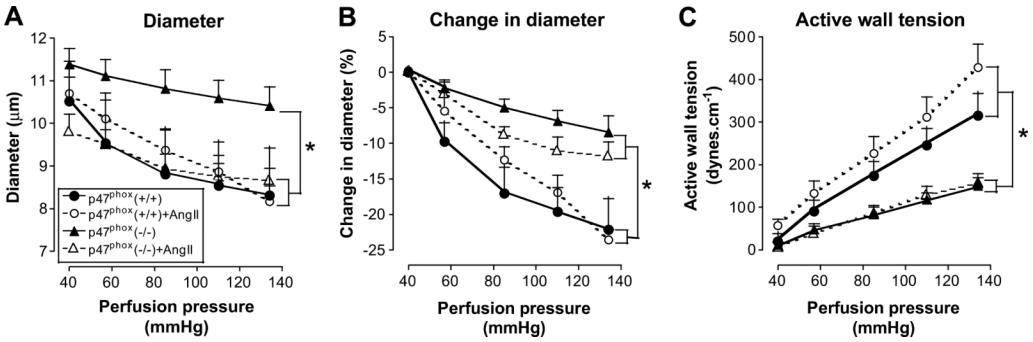

Figure 4.

Mean ± SEM values (n= 4 - 7) for afferent arteriolar diameter (panel A), change in diameter (panel B) and active wall tension (panel C) during increases in renal perfusion pressure. Data are shown for vessels from $p47^{phox}$ +/+ mice infused with vehicle (solid circles and continuous lines) or angiotensin II (open circles and broken lines) or $p47^{phox}$ -/- mice infused with vehicle (solid triangles and continuous lines) or angiotensin II (open triangles and broken lines). Significance of difference between groups: * $P<0.05$.

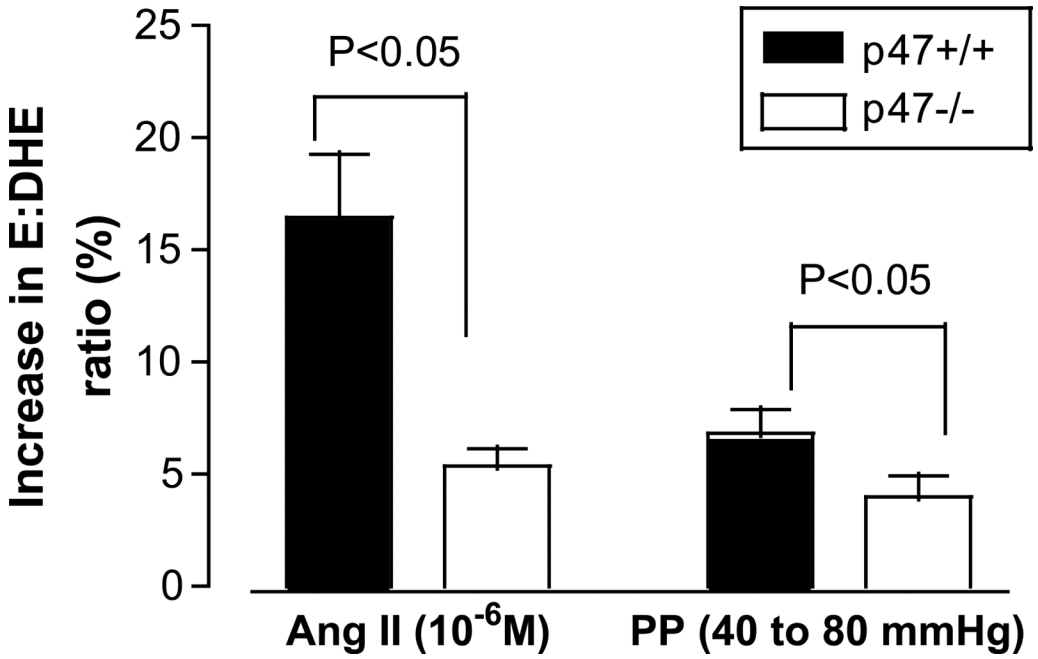

Figure 5.

Mean ± SEM values (n= 5 to 7) for increases in ethidium: dihydroethidium ratio of afferent arterioles from $p47^{phox}$ +/+ mice (solid black) and $p47^{phox}$ -/- mice (open box). Data are shown for changes with 10^{-6} M angiotensin II or increased perfusion from 40-80 mmHg.