

Role of p47^{phox} in Vascular Oxidative Stress and Hypertension Caused by Angiotensin II

Ulf Landmesser, Hua Cai, Sergey Dikalov, Louise McCann, Jinah Hwang, Hanjoong Jo, Steven M. Holland, David G. Harrison

Abstract—Hypertension caused by angiotensin II is dependent on vascular superoxide (O₂^{•-}) production. The nicotinamide adenine dinucleotide phosphate (NAD[P]H) oxidase is a major source of vascular O₂^{•-} and is activated by angiotensin II in vitro. However, its role in angiotensin II–induced hypertension in vivo is less clear. In the present studies, we used mice deficient in p47^{phox}, a cytosolic subunit of the NADPH oxidase, to study the role of this enzyme system in vivo. In vivo, angiotensin II infusion (0.7 mg/kg per day for 7 days) increased systolic blood pressure from 105±2 to 151±6 mm Hg and increased vascular O₂^{•-} formation 2- to 3-fold in wild-type (WT) mice. In contrast, in p47^{phox}^{-/-} mice the hypertensive response to angiotensin II infusion (122±4 mm Hg; *P*<0.05) was markedly blunted, and there was no increase of vascular O₂^{•-} production. In situ staining for O₂^{•-} using dihydroethidium revealed a marked increase of O₂^{•-} production in both endothelial and vascular smooth muscle cells of angiotensin II–treated WT mice, but not in those of p47^{phox}^{-/-} mice. To directly examine the role of the NAD(P)H oxidase in endothelial production of O₂^{•-}, endothelial cells from WT and p47^{phox}^{-/-} mice were cultured. Western blotting confirmed the absence of p47^{phox} in p47^{phox}^{-/-} mice. Angiotensin II increased O₂^{•-} production in endothelial cells from WT mice, but not in those from p47^{phox}^{-/-} mice, as determined by electron spin resonance spectroscopy. These results suggest a pivotal role of the NAD(P)H oxidase and its subunit p47^{phox} in the vascular oxidant stress and the blood pressure response to angiotensin II in vivo. (*Hypertension*. 2002;40:511-515.)

Key Words: oxidative stress ■ endothelium ■ angiotensin II ■ hypertension, experimental

Activation of the renin angiotensin system is critically involved in the pathogenesis of hypertension and atherosclerosis.¹ The recently completed Heart Outcomes Prevention Evaluation (HOPE) trial demonstrated a remarkable decrease in cardiovascular morbidity and mortality by ACE inhibition in individuals at increased risk for cardiovascular events.² A major mechanism whereby angiotensin II, the principal effector peptide of the renin-angiotensin system, may contribute to vascular pathology is stimulation of superoxide (O₂^{•-}) formation in vascular cells.^{3,4} We and others have shown that treatment with liposome-encapsulated superoxide dismutase (SOD) or the membrane-permeable SOD mimetic tempol markedly blunts the increase in blood pressure caused by angiotensin II administration, suggesting that stimulation of O₂^{•-} formation is critically involved in the blood pressure response to angiotensin II.^{5,6}

One source of O₂^{•-} that is stimulated by angiotensin II in endothelial and vascular smooth muscle cells is the nicotinamide adenine dinucleotide phosphate (NAD[P]H) oxidase.^{3,7,8} Several recent in vitro studies have suggested an important role of the p47^{phox} subunit of the NAD(P)H oxidase

for angiotensin II stimulated O₂^{•-} production in vascular smooth muscle cells.^{8,9} It is not known, however, whether activation of the NAD(P)H oxidase and its subunit p47^{phox} are critical for angiotensin II–stimulated O₂^{•-} production in endothelial cells. To address this issue, we studied mice lacking p47^{phox}, a cytosolic subunit of the NAD(P)H oxidase,¹⁰ and examined responses to angiotensin II administered chronically in vivo. We also cultured endothelial cells from these mice and examined the effect of angiotensin II on O₂^{•-} production in vitro.

Methods

Animals Studied

Male C57BL/6 mice were obtained from Jackson Laboratories (25 to 35 g; Bar Harbor, Me) and were used at the age of 6 to 8 months. Male mice lacking p47^{phox}¹⁰ were backcrossed at least ×7 to the C57BL/6 background and were used at the age of 6 to 8 months. For implantation of osmotic minipumps, the mice were anesthetized with intraperitoneal Avertin 2.5% (0.3 mL per 25 g of body weight, IP). The intrascapular region was shaved, and an osmotic minipump (Alzet Model 2002; Alza Corp) that contained angiotensin II was inserted via a 1-cm incision to permit subcutaneous infusion of

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From the Division of Cardiology, Emory University School of Medicine and Atlanta Veterans Administration Hospital (U.L., H.C., S.D., L.M., J.H., H.J., D.G.H.), Atlanta, Ga; and Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases, National Institutes of Health (S.M.H.), Bethesda, Md.

Correspondence to David G. Harrison, MD, Division of Cardiology, Emory University School of Medicine, 1639 Pierce Drive, 319 WMB, Atlanta, GA 30322. E-mail dharr02@emory.edu

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angiotensin II ([Val⁵]angiotensin II, infusion rate 0.7 mg/kg per day). Sham-operated animals underwent an identical surgical procedure, except that either no pump or an empty osmotic pump was implanted. On day 7 of angiotensin II infusion, the animals were killed by CO₂ inhalation, and their aortas were harvested for study. The Emory University Institutional Animal Care and Use Committee approved all animal experiments.

Blood Pressure Measurement

Systolic blood pressures were measured by a computerized tail-cuff system (Visitech Systems).¹¹ Before the osmotic pump was implanted, the mice were trained in the blood pressure device to accustom them to the procedure. On each day of blood pressure determination, 10 measurements were obtained and averaged for each mouse.

Measurements of Vascular Superoxide Production

Animals were euthanized by CO₂ inhalation. The aortas were rapidly removed and placed into chilled modified Krebs/HEPES buffer (composition in mmol/L: NaCl 99.01, KCl 4.69, CaCl₂ 2.50, MgSO₄ 1.20, KH₂PO₄ 1.03, NaHCO₃ 25.0, Na-HEPES 20.0, and glucose 5.6; pH 7.4), cleaned of excessive adventitial tissue, and cut into 4- to 5-mm ring segments with care taken not to injure the endothelium. Vascular O₂^{•-} production was determined using lucigenin-enhanced chemiluminescence as described before.¹² This method has recently been validated for O₂^{•-} measurements in vascular tissue when low concentrations of lucigenin (5 μmol/L) are used.^{13,14}

As a second approach to quantify vascular O₂^{•-} production, we employed dihydroethidium (HE) staining of intact vascular rings as described previously.¹⁵ Paired aortas from Angiotensin II-infused and sham animals were processed in parallel, and images were acquired with identical acquisition parameters. Reagents were purchased from Sigma-Aldrich.

Measurements of Superoxide Production in Cultured Endothelial Cells

Mouse aortic endothelial cells (MAECs) from wild-type and p47^{phox}^{-/-} mice were isolated using a matrigel culture as described previously and selected over vascular smooth muscle cells using heparin.¹⁶ Cells were maintained in DMEM (Gibco-BRL) containing 10% fetal calf serum (FCS, Hyclone Laboratories) supplemented with endothelial cell growth supplement (ECGS; 75 μg/mL; Sigma E-2759), heparin (10 U/mL), and antibiotics. Endothelial cells were used for studies at passage 3. On the day of confluence, the cell media was changed to DMEM with 5% serum overnight, and cells were studied the next day, one day post-confluency. Endothelial cell O₂^{•-} production was measured using electron-spin resonance spectroscopy (ESR) and the spin trap 1-hydroxy-3-carboxy-pyrrolidine (CP-H; Alexis Corporation). Cells were rinsed with ice-cold 50 mmol/L PBS buffer (pH 7.4) and removed from the plate by scraping. After centrifugation at 800g (7 minutes), the cells were resuspended in 400 μL PBS buffer and kept on ice. To inhibit iron-catalyzed oxidation of the spin trap, DTPA (0.2 mmol/L) was added to all samples. ESR measurements were performed in 50-μL glass capillaries (Corning). The ESR spectra were recorded using a Bruker EMX spectrometer (Bruker Corporation) and a super-high Q microwave cavity. O₂^{•-} formation was determined by following the oxidation of CP-H to paramagnetic 3-carboxy-proxyl (CP).^{17,18} The ESR instrumental settings were as follows: field sweep, 50 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 2 G; conversion time, 656 ms; time constant, 656 ms; 512 points resolution and receiver gain, 1 × 10⁵ (74 dB). Time scans were recorded using 1312 ms conversion time, 5248 ms time constant, and monitoring the ESR amplitude of low-field component of ESR spectrum of carboxy-proxyl nitroxide for 300 s.

Immunoblot Analysis of p47^{phox} and Angiotensin II Type 1-Receptor Expression in Endothelial Cells

Protein samples were prepared from mouse aortic endothelial cells and lysed directly in SDS sample buffer. Protein from endothelial

cell lysates (20 μg) was separated by SDS-PAGE, transferred to membranes, and probed with anti-p47^{phox} antibody (BD Transduction Laboratories) or angiotensin II type 1 (AT₁)-receptor antibody (Santa Cruz Biotechnology). For the p47^{phox} immunoblot analysis, mouse macrophage lysates (10 μg) were loaded as positive controls. Protein was visualized by chemiluminescence.

Data Analysis

All data are expressed as mean ± SEM. Comparisons between groups of animals or treatments were made by one-way ANOVA, followed by Student-Newman-Keuls test. Values of *P* < 0.05 were considered statistically significant.

Results

Effects of Angiotensin II Treatment on Vascular Superoxide Production in Wild-Type (C57/BL6) and p47^{phox}^{-/-} Mice

Angiotensin II infusion caused a 2- to 3-fold increase in vascular O₂^{•-} production in wild-type (C57/BL6) mice (Figure 1a). In contrast, in p47^{phox}^{-/-} mice, no increase in vascular O₂^{•-} formation was observed after treatment with angiotensin II (Figure 1a). To estimate O₂^{•-} production in mouse aortas in situ, we used HE staining. Conversion of HE by O₂^{•-} to ethidium results in nuclear fluorescence. Aortas from angiotensin II-treated wild-type mice consistently showed a markedly increased fluorescence, both in the endothelium and in vascular smooth muscle, indicating increased O₂^{•-} production (Figure 1b). In p47^{phox}^{-/-} mice, however, no increase in HE-detectable O₂^{•-} production was observed after angiotensin II infusion (Figure 1b).

Effect of Angiotensin II Treatment on Blood Pressure in Wild-Type and p47^{phox}^{-/-} Mice

In wild-type mice, angiotensin II infusion caused an increase in blood pressure from 105 ± 2 to 151 ± 6 mm Hg (Figure 2). Importantly, in p47^{phox}^{-/-} mice, the blood pressure response to angiotensin II was markedly blunted (Figure 2).

Effect of Angiotensin II on Superoxide Production in Cultured Endothelial Cells from Wild-Type and p47^{phox}^{-/-} Mice

To study the role of p47^{phox} in angiotensin II-induced O₂^{•-} formation in endothelial cells independent of changes in blood pressure we measured O₂^{•-} formation in cultured endothelial cells from wild-type and p47^{phox}^{-/-} mice. Whereas in wild-type endothelial cells, angiotensin II administration (10⁻⁶ mol/L) caused a substantial increase in CP-H oxidation, there was no change in CP-H oxidation in p47^{phox}-deficient endothelial cells in response to angiotensin II (Figures 3a and 3b). The increase of CP-H oxidation caused by angiotensin II in wild-type endothelial cells was completely inhibited by superoxide dismutase (50 U PEG-SOD), indicating that CP-H oxidation was caused by O₂^{•-} (Figure 3a).

Immunoblot Analysis of p47^{phox} and AT₁-Receptor Expression in Endothelial Cells

The p47^{phox} subunit of the NAD(P)H oxidase was expressed in aortic endothelial cells from wild-type mice but not in endothelial cells cultured from p47^{phox}-deficient mice (Figure 3c). The expression of the AT₁ receptor was similar in p47^{phox}-deficient and wild-type endothelial cells (Figure 3d).

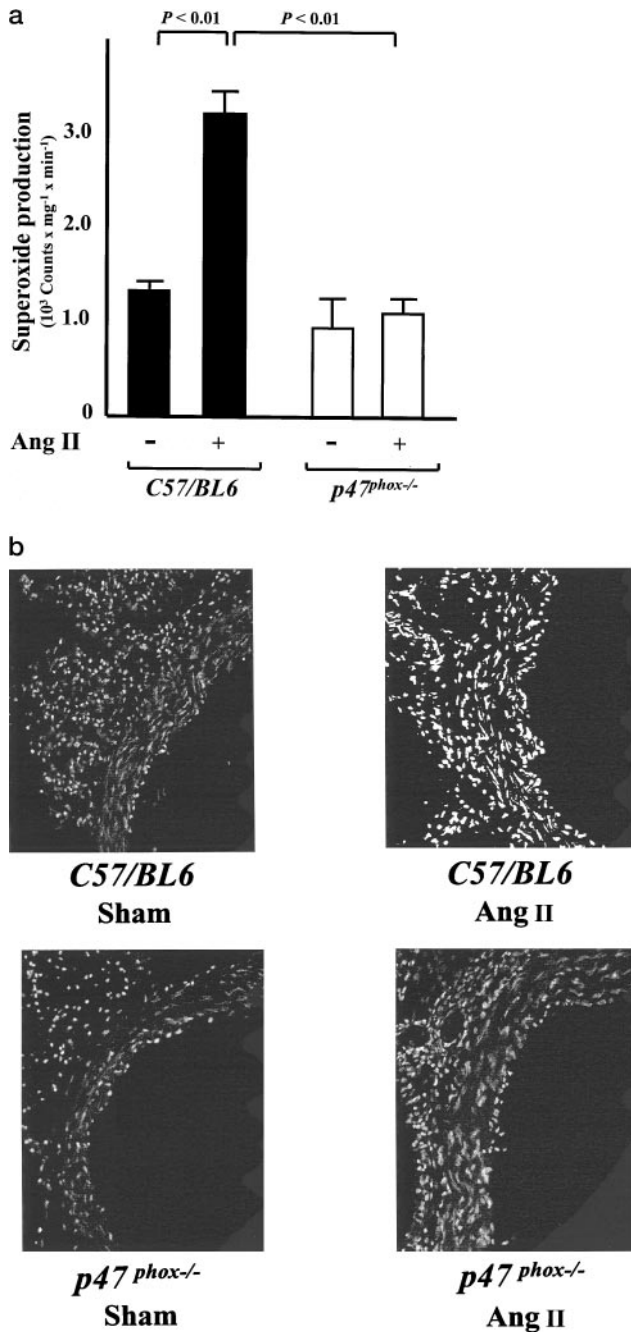


Figure 1. Effect of angiotensin II (Ang II) infusion on vascular superoxide production in mouse aortas from wild-type (C57/BL6) and p47^{phox}-/- mice. **a**, Superoxide production in sham and Ang II-treated mice as determined with lucigenin-enhanced chemiluminescence (5 μ mol/L; n=5 to 9). **b**, In situ detection of superoxide production with dihydroethidium (HE) in sham and Ang II-treated mice. Data are representative of 3 separate experiments.

Discussion

Our present results demonstrate a critical role of the NAD(P)H oxidase and its subunit p47^{phox} for the vascular oxidant stress response to angiotensin II. The increase in vascular O₂⁻ production in response to angiotensin II administration was diminished in p47^{phox}-/- mice and the amount of hypertension caused by angiotensin II was reduced

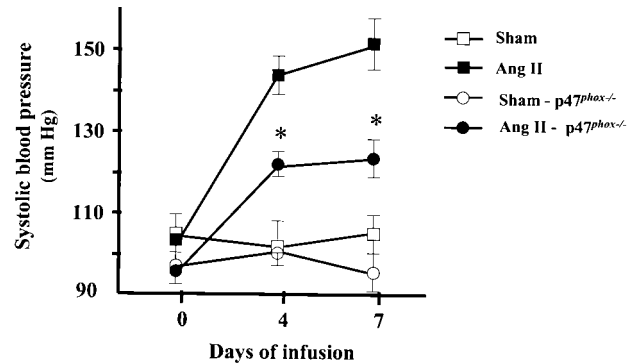


Figure 2. Effect of Ang II infusion on systolic blood pressure in wild-type (C57/BL6) and p47^{phox}-/- mice (n=5 to 9). *P<0.05 (ang II-infused wild-type vs ang II-infused p47^{phox}-/- mice).

in these animals compared with wild-type mice. Angiotensin II stimulated O₂⁻ production in cultured aortic endothelial cells from wild-type mice but not in p47^{phox}-deficient endothelial cells.

We have previously shown that angiotensin II administration in rats causes a substantial increase in vascular O₂⁻ formation and impairs endothelium-dependent vasodilation.⁴ Treatment with liposome-encapsulated superoxide dismutase (SOD) or the membrane-permeable SOD mimetic tempol blunts the increase in blood pressure caused by angiotensin II and preserves the bioavailability of nitric oxide, suggesting that the increase in vascular O₂⁻ formation is critical for the blood response to the octapeptide.^{5,6} An important source of O₂⁻ in endothelial and vascular smooth muscle cells is the NAD(P)H oxidase,^{3,19} which is stimulated by angiotensin II in both cell types.^{3,7-9} In addition, increased vascular NAD(P)H oxidase activity and increased expression of its subunit p22phox was observed in angiotensin II-treated rats in vivo,^{4,20} raising the question whether activation of this enzyme complex is critical for the oxidant stress and blood pressure response to angiotensin II. In the present studies, we used mice deficient in p47^{phox},¹⁰ a cytosolic subunit of the NAD(P)H oxidase, to address this question. Whereas angiotensin II administration caused a 2- to 3-fold increase of vascular O₂⁻ production in wild-type mice, no such increase was observed in p47^{phox}-deficient mice after angiotensin II treatment. Importantly, the blood pressure response to angiotensin II was markedly blunted in mice lacking p47^{phox} compared with wild-type mice, suggesting a pivotal role of NAD(P)H oxidase activation for the blood pressure increase caused by angiotensin II.

In addition to smooth muscle cells, a major source of vascular O₂⁻ stimulated by angiotensin II in vivo appears to be the endothelium. In wild-type, but not in p47^{phox}-deficient mice, there was a marked increase of endothelial O₂⁻ production evoked by angiotensin II as detected by HE fluorescent staining. Lavigne et al have recently demonstrated the requirement of p47^{phox} for angiotensin II-stimulated O₂⁻ production in vascular smooth muscle cells in vitro.⁸ In the present study, we therefore analyzed the role of p47^{phox} in angiotensin II-induced oxidant stress in endothelial cells. In cultured endothelial cells from wild-type mice, but not in p47^{phox}-deficient cells, there was a marked increase of O₂⁻

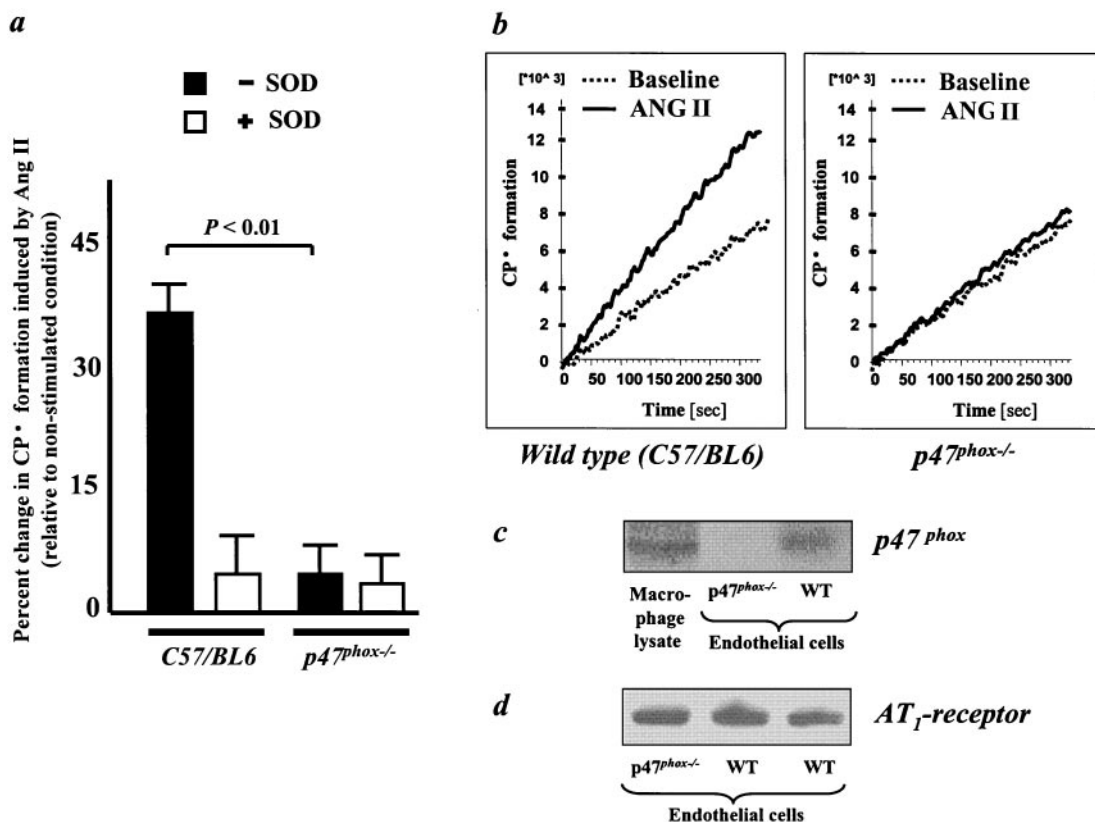


Figure 3. Determination of superoxide formation in cultured aortic endothelial cells from wild-type and p47^{phox}^{-/-} mice using ESR spectroscopy. **a**, Increase of CP-H oxidation by endothelial cells in response to Ang II; effect of superoxide dismutase (50 U PEG-SOD). **b**, Representative "time scan" of CP-H oxidation in wild-type and p47^{phox}^{-/-} deficient endothelial cells stimulated with Ang II. **c**, Western blot analysis of p47^{phox} expression in endothelial cells from wild-type (WT) and p47^{phox}^{-/-} mice. Protein extracted from mouse macrophage lysates was used as a positive control. **d**, Western blot analysis of AT₁-receptor expression in endothelial cells from WT and p47^{phox}^{-/-} mice. Data are representative of 3 separate experiments.

formation after exposure to angiotensin II as revealed by ESR spectroscopy, suggesting that activation of the NAD(P)H oxidase within the endothelium is dependent on p47^{phox}. One possible explanation for there being less O₂⁻ formation in response to angiotensin II in p47^{phox}^{-/-} deficient endothelial cells could be a reduced AT₁-receptor gene expression. Our Western blot analysis, however, showed similar expression of the AT₁ receptor in p47^{phox}^{-/-} deficient and wild-type endothelial cells.

Basal O₂⁻ production was similar in endothelial cells from wild-type and p47^{phox}^{-/-} mice, suggesting that p47^{phox} is not essential for basal O₂⁻ formation in endothelial cells. There are other sources of O₂⁻ (ie, xanthine oxidase, cytochrome p450, mitochondria), which could also account for basal O₂⁻ formation in p47^{phox}^{-/-} deficient cells. Our results are compatible with the recent findings of Li et al²¹ in cultured coronary microvascular endothelial cells from p47^{phox}^{-/-} mice, who found no reduction in basal O₂⁻ production, but a reduced response to TNF- α and phorbol ester in p47^{phox}^{-/-} deficient microvascular endothelial cells.²¹

In the present study, we observed expression of p47^{phox} in aortic endothelial cells from wild-type, but not from p47^{phox}^{-/-} deficient mice. The expression of the NAD(P)H oxidase subunit p47^{phox} has been demonstrated in other endothelial cell lines, ie, human umbilical vein endothelial cells and

murine microvascular endothelial cells.^{21,22} In neutrophils the importance of p47^{phox} for activation of the NAD(P)H oxidase is well documented because its phosphorylation appears to be the limiting step required for assembly of the active enzyme complex.²³ Furthermore, mutations of p47^{phox} are a cause of chronic granulomatous disease, an immune deficiency resulting from impaired phagocyte activity.²³ The results of the present studies suggest that p47^{phox} is equally vital to the activation of the NAD(P)H oxidase by angiotensin II within the endothelium.

Perspectives

In summary, the present study indicates that the NAD(P)H oxidase and its cytosolic subunit p47^{phox} play a pivotal role in mediating the hypertension caused by angiotensin II. Our results are in keeping with a recent study by Rey et al who demonstrated that treatment with a chimeric peptide designed to inhibit the association of p47^{phox} with the NAD(P)H oxidase membrane components diminished the blood pressure response to angiotensin II.²⁴ Our experiments also show that p47^{phox} and the NAD(P)H oxidase are essential for angiotensin II-stimulated O₂⁻ production in cultured aortic endothelial cells. Increased endothelial O₂⁻ production in response to angiotensin II may also be important for the proinflammatory and proatherogenic effects of the octapep-

tide. Stokes et al²⁵ have recently demonstrated that p47^{phox} deficiency diminished the hypercholesterolemia-induced leukocyte-endothelial cell adhesion, and we have previously shown that activation of the vascular NAD(P)H oxidase in hypercholesterolemia is dependent on angiotensin II.²⁶ Furthermore, when crossed with p47^{phox}^{-/-} mice, apolipoprotein E (Apo[e])-deficient mice have a rather marked reduction in atherosclerosis in the descending aorta compared with mice lacking only Apo(e).²⁷ Given these now very clear crucial roles in vascular disease, the NAD(P)H oxidase and p47^{phox} represent important targets for therapeutic intervention, and their specific inhibitors would very likely be useful in treatment of common disorders such as hypertension and atherosclerosis.

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