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Deletion of p47^{phox} Attenuates Angiotensin II–Induced Abdominal Aortic Aneurysm Formation in Apolipoprotein E–Deficient Mice

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Background—Angiotensin II (Ang II) contributes to vascular pathology in part by stimulating NADPH oxidase activity, leading to increased formation of superoxide (O₂⁻). We reported that O₂⁻ levels, NADPH oxidase activity, and expression of the p47^{phox} subunit of NADPH oxidase are increased in human abdominal aortic aneurysms (AAAs). Here, we tested the hypothesis that deletion of p47^{phox} will attenuate oxidative stress and AAA formation in Ang II–infused apoE^{-/-} mice.

Methods and Results—Male apoE^{-/-} and apoE^{-/-}p47^{phox}^{-/-} mice received saline or Ang II (1000 ng · kg⁻¹ · min⁻¹) infusion for 28 days, after which abdominal aortic weight and maximal diameter were determined. Aortic tissues and blood were examined for parameters of aneurysmal disease and oxidative stress. Ang II infusion induced AAAs in 90% of apoE^{-/-} versus 16% of apoE^{-/-}p47^{phox}^{-/-} mice (*P*<0.05). Abdominal aortic weight (14.1±3.2 versus 35.6±9.0 mg), maximal aortic diameter (1.5±0.2 versus 2.4±0.4 mm), aortic NADPH oxidase activity, and parameters of oxidative stress were reduced in apoE^{-/-}p47^{phox}^{-/-} mice compared with apoE^{-/-} mice (*P*<0.05). In addition, aortic macrophage infiltration and matrix metalloproteinase-2 activity were reduced in apoE^{-/-}p47^{phox}^{-/-} mice compared with apoE^{-/-} mice. Deletion of p47^{phox} attenuated the pressor response to Ang II; however, coinfusion of phenylephrine with Ang II, which restored the Ang II pressor response, did not alter the protective effects of p47^{phox} deletion on AAA formation.

Conclusions—Deletion of p47^{phox} attenuates Ang II–induced AAA formation in apoE^{-/-} mice, suggesting that NADPH oxidase plays a critical role in AAA formation in this model. (*Circulation*. 2006;114:404-413.)

Key Words: aneurysm ■ aorta ■ cardiovascular diseases ■ free radicals ■ inflammation

Abdominal aortic aneurysms (AAAs) cause considerable morbidity and mortality in the adult population¹ and are responsible for 1% to 3% of all deaths in men 65 to 85 years of age in developed countries. Although conventional treatments include surgery or percutaneous intervention in high-risk patients, effective medical therapies for AAAs have not been developed. In this regard, surprisingly little is known about the pathogenesis of AAAs. Recent studies suggest a prominent role for inflammation, matrix metalloproteinase (MMP) activation, and smooth muscle cell apoptosis in human AAA.^{2–6} Because reactive oxygen species (ROS) may regulate each of these processes, we and others have suggested that oxidative stress may contribute to the pathogenesis of AAAs.⁷

A major source of ROS in vascular tissue is the membrane-bound NADPH oxidase, which consists of transmembrane (eg, nox1, nox2, nox4, and p22^{phox}) and cytosolic (p47^{phox},

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p67^{phox}, and rac) subunits that assemble to form the functional oxidase.⁸ The expression and activity of NADPH oxidase in the vasculature are increased in various pathological states, including hypertension and atherosclerosis.⁹ With regard to AAAs, Miller et al⁷ have shown that NADPH oxidase activity and its p47^{phox} subunit are markedly upregulated in human aneurysmal aorta compared with adjacent nonaneurysmal tissue from the same individuals. Although these findings suggest a relationship between NADPH oxidase and AAA, it remains to be determined whether the enzyme contributes to the pathogenesis of the disease.

To investigate whether NADPH oxidase contributes to aneurysmal disease, we examined the effects of p47^{phox} gene deletion in the murine angiotensin II (Ang II) infusion model of AAA formation. In this model, infusion of Ang II in

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apolipoprotein E (apoE) – deficient male mice results in AAA formation in 90% to 100% of these animals.^{10,11} Moreover, the AAAs exhibit inflammatory cell infiltration, MMP activation, thrombus formation, and oxidative stress, all of which have been observed in human AAAs.^{7,11} We demonstrate that deletion of the p47^{phox} gene leads to a marked decrease in oxidative stress in response to Ang II infusion and has a profound impact on aneurysm development. These findings strongly support the hypothesis that oxidative stress, likely arising via NADPH oxidase activity, is a critical factor in the pathophysiology of AAA in this experimental model.

Methods

Experimental Animals

ApoE^{-/-}p47^{phox}^{-/-} mice were generated using apoE^{-/-}p47^{phox}^{+/-} breeding pairs (kindly provided by Dr Marshall Runge, University of North Carolina, Chapel Hill).¹² ApoE^{-/-} littermates were used as controls, and all mice were genotyped by PCR on tail clip samples. Three- to 6-month-old male apoE^{-/-} and apoE^{-/-}p47^{phox}^{-/-} mice were then subjected to a 4-week infusion of Ang II (1000 ng · kg⁻¹ · min⁻¹) via subcutaneous osmotic minipumps.¹³ Afterward, mice were euthanized; aortic diameter and weight were determined as described below; blood was drawn for lipid analysis and reduced glutathione (GSH) levels; and aortic tissue was harvested, weighed, and processed for histology and/or biochemical studies. Additional experiments were performed on mice coinjected with Ang II (1000 ng · kg⁻¹ · min⁻¹) with or without phenylephrine (PE; 12 500 ng · kg⁻¹ · min⁻¹) for 2 weeks. AAA was defined as ≥50% enlargement of maximal abdominal aorta diameter. The experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Iowa.

Implantation of Osmotic Minipumps

Alzet model 2004 osmotic minipumps (Durect Corporation, Cupertino, Calif) were loaded by incubation at 37°C for 24 hours with normal saline (NS; as a placebo control), Ang II (at concentrations to ensure delivery at 1000 ng · kg⁻¹ · min⁻¹), or Ang II plus PE (at concentrations to ensure delivery at 1000 and 12 500 ng · kg⁻¹ · min⁻¹, respectively) before implantation and were then inserted subcutaneously in the interscapular area.

Systolic Blood Pressure Measurement

Systolic blood pressure (SBP) was determined every other day, beginning 1 week before the implantation of the minipumps and continuing for the duration of the study, using a computerized tail-cuff system (BP-2000, Visitech Systems, Inc, Apex, NC). To avoid procedure-induced anxiety, mice were initially accustomed to the instrument for 5 consecutive days before the actual recorded measurements. Moreover, the first 10 of 30 blood pressure values recorded at each session were disregarded, and the remaining 20 values were averaged and used for analysis.

Aortic Tissue Collection and Measurement

After animals were euthanized, the abdominal and thoracic cavities were entered, blood was drawn from the right ventricle, and the aorta was irrigated with PBS through the left ventricle. The abdominal aorta was exposed with a dissection microscope (Olympus SZ-CTV, Center Valley, Pa), and the periadventitial tissue was carefully dissected away from the aorta wall. Maximal aortic diameter was determined with a digital caliper (Mitutoyo Absolute Digimatic, Kawasaki, Japan). The aortic root and heart were subsequently dissected out as described previously.¹³ The abdominal aorta (from the last intercostal artery to the ileal bifurcation) and the thoracic aorta were sectioned and weighed, and portions of these tissues were preserved at –80°C (for immunoassays), fixed in 4% paraformaldehyde (for immunohistochemistry), or homogenized/sonicated for biochemical assays.

Membrane Fraction Isolation

Membrane samples were prepared from whole-aorta homogenates as previously described.¹⁴ Briefly, homogenates were sonicated and centrifuged at 1000g for 4 minutes at 4°C. The pellet was mixed with 50 μ L lysis buffer (50 mmol/L Tris plus protease inhibitors) and then centrifuged at 30 000g for 20 minutes at 4°C. The supernatant was collected and centrifuged again at 100 000g for 1 hour at 4°C. The ensuing pellet (resuspended in PBS) represents the membrane fraction.

Intraperitoneal Leukocyte Harvesting in Mice

Mice were injected intraperitoneally with 2.5 mL of a 3% thioglycollate solution. After 5 hours, animals were euthanized (150 mg/kg pentobarbital), and the intact anterior peritoneal surface was exposed via a midline incision. Then, 3 to 5 mL harvest solution (0.02% EDTA in PBS) was gently injected intraperitoneally, the abdomen was gently massaged, and the peritoneal fluid was reaspirated. The harvesting step was repeated, and the resulting peritoneal fluids were combined and centrifuged at 200g for 10 minutes. The cell pellet was resuspended in PBS, counted, and kept on ice. A Wright stain from this cell suspension was used to determine the relative purity of the leukocyte fraction.

Assays for NADPH Oxidase Activity

For aortic tissue membrane fractions, lucigenin (5 μ mol/L) and NADPH (100 μ mol/L) were added to the samples, and subsequent light emission was recorded with a luminometer (Berthold FB12, Berthold Technologies, Bad Wildbad, Germany) every 2 minutes for 8 minutes. When required, diphenylene iodonium (10 μ mol/L) was added to the sample 10 minutes before the recording. For experiments with isolated peritoneal leukocytes, cell suspensions were stimulated by addition of phorbol myristate acetate (PMA; 100 nmol/L) 3 minutes before the assay. All measurements were performed in triplicate, and results were normalized per 1 mg protein (for membrane fractions) or per cell (for leukocyte suspensions).

Staining for Macrophages and Nitrotyrosine

Sections of abdominal aorta (5 μ m thick) on Fisher Superfrost Plus slides were deparaffinized and rehydrated. High-temperature antigen retrieval was done in a laboratory microwave (Ted Pella, Redding, Calif) with Vector Laboratory (Burlingame, Calif) antigen unmasking solution (No. H-3300). Nonspecific binding sites were blocked with 1.5% normal goat serum in PBS for 1 hour and then incubated at 4°C overnight with a primary antibody, either rabbit anti-mouse macrophage antibody (Accurate Chemical & Scientific Corporation, Westbury, NY, No. AIAD 31240, dilution 1:50) or rabbit anti-mouse nitrotyrosine antibody (Upstate No. 06–284, dilution 1:80, Upstate Cell Signaling, Charlottesville, Va). After washing with PBS, the sections were incubated with a fluorescently labeled secondary antibody (Molecular Probes GAR-Alexa 488) for 1 hour in the dark. The slides were again rinsed in PBS, stained briefly with the nuclear stain ToPro-3, and mounted in Vectashield (Molecular Probes, Carlsbad, Calif). Negative controls were performed simultaneously by omitting the primary antibody for each specimen. Visualization was performed with a Bio-Rad 1024 confocal laser scanning microscope (Bio-Rad, Hercules, Calif) equipped with a krypton-argon laser at wavelengths of 488 nm (antibody signal) and 647 nm (nuclear signal). Each image was collected by an investigator using identical microscope settings who was blinded to sample identity.

MMP-2 and MMP-9 Activity Assay

MMP-2 and MMP-9 activities were determined by zymography as previously described.¹⁵ Briefly, prepoured 10% polyacrylamide gels containing 1 mg/mL gelatin A were purchased from Bio-Rad. Equivalent amounts of samples were loaded based on protein content (Bradford assay, Bio-Rad). After electrophoresis, the gels were washed twice in a buffer containing 2.5% Triton X-100 and 50 mmol/L Tris-HCl (pH 8.0) for 30 minutes. The gels were then incubated overnight with a developing buffer containing 50 mmol/L Tris, 10 mmol/L CaCl₂, and 50 mmol/L NaCl at 37°C; then, they

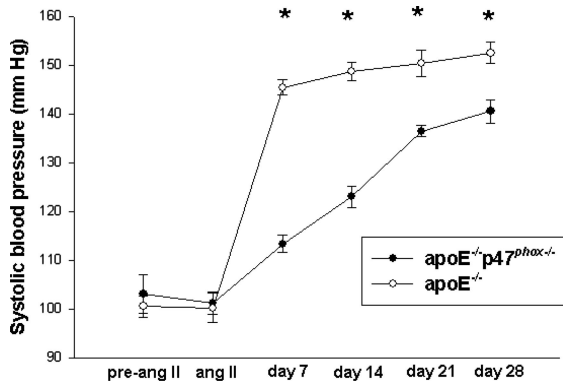


Figure 1. Ang II-induced hypertensive effect was blunted in mice lacking p47^{phox}. SBP was measured with a tail-cuff system (Visitech 2000). There was a significant and sustained increase in SBP in apoE^{-/-} mice (n=10) that was markedly inhibited in apoE^{-/-}p47^{phox}^{-/-} mice (n=6). The magnitude of the difference in the pressor response to Ang II between the 2 experimental groups diminished over time but remained significant throughout the duration of the study. *P<0.05 vs apoE^{-/-}p47^{phox}^{-/-} mice.

were stained with Coomassie Blue and analyzed with an Alpha-Innotech densitometer and Quantity One (Bio-Rad) software.

Quantification of Atherosclerosis in the Aortic Sinus

Aortic roots were sectioned, stained with hematoxylin, and quantified for atherosclerosis through the use of image analysis software as reported previously.¹⁶ The lesion area is defined by the internal elastic lamina and luminal boundary, and data are presented as the total lesion area in the number of sections quantified.

Quantification of Oxidative Stress

Whole-blood samples were assayed for GSH levels by the Radiation and Free Radical Research Core Facility at the University of Iowa with a spectrophotometric assay.¹⁷ Protein carbonyl formation was visualized with the OxyBlot protein oxidation detection kit (Chemicon International, Temecula, Calif), followed by quantitative densitometry (Quantity One 1-D analysis software, Bio-Rad).

Statistical Analysis

Results are expressed as mean±SEM unless otherwise noted. Differences between the 2 groups were analyzed by the Student *t* test, and differences between multiple groups were analyzed by 1-way ANOVA, followed by the Bonferroni *t* test. Whenever nonparametric analysis was indicated, ANOVA on ranks was used. Blood pressure data were analyzed with linear mixed-model analysis for repeated measures. Fisher's exact test was used to analyze categorical data. Values of P<0.05 were considered significant.

The authors had full access to the data and take responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results

Effects of Deletion of the p47^{phox} Gene on SBP, Lipid Profile, and Atherosclerosis in Ang II-Infused ApoE^{-/-} Mice

SBP was measured in conscious mice with a noninvasive tail-cuff system before (baseline) and during Ang II infusion. There was no difference in baseline SBP between apoE^{-/-} mice and apoE^{-/-}p47^{phox}^{-/-} mice. In apoE^{-/-} mice, Ang II infusion caused an increase in the SBP from 101±5 to 145±4 mm Hg (Figure 1), which was observed within 3 days after Ang II pump implantation and persisted throughout the duration of infusion. In apoE^{-/-}p47^{phox}^{-/-} mice, the Ang II-induced pressor response was blunted at all time points after the onset of infusion (Figure 1). Interestingly, the magnitude of the inhibitory effect of p47^{phox} gene deletion on the pressor response progressively diminished over the course of the 28-day infusion (P<0.05) but was never entirely abolished.

All animals developed severe hyperlipidemia, as expected in apoE^{-/-} mice. However, there were no significant differences between apoE^{-/-} mice and apoE^{-/-}p47^{phox}^{-/-} mice with regard to total cholesterol or triglycerides (see the Table). We also examined the degree of atherosclerosis at the level of the aortic root. As expected, all animals developed extensive atherosclerotic lesion formation. Although there was a trend toward decreased aortic root atherosclerosis in apoE^{-/-}p47^{phox}^{-/-} mice compared with apoE^{-/-} mice, it did not reach statistical significance.

Deletion of the p47^{phox} Gene Reduced the Incidence and Severity of Aneurysms in Ang II-Infused ApoE^{-/-} Mice

After 4 weeks of infusion of NS or Ang II, animals were euthanized, and aortas were isolated and examined for the presence of AAAs and thoracic aortic aneurysms. Representative pictures of aortas from these animals are shown in Figure 2A, and quantification of aneurysm formation is shown in Figure 2B. In animals infused with Ang II, suprarenal AAAs were present in 90% of apoE^{-/-} mice but in only 17% of the apoE^{-/-}p47^{phox}^{-/-} mice (P<0.05; Figure 2B). Moreover, 40% of the apoE^{-/-} mice infused with Ang II developed thoracic aortic aneurysm in addition to AAA, a finding that was observed in only 17% of the apoE^{-/-}p47^{phox}^{-/-} mice. Only 1 animal, in the apoE^{-/-} group, died of a ruptured AAA during the course of the study. As expected, no animals infused with saline (control) developed aneurysms (data not shown).¹¹

The effects of deletion of the p47^{phox} gene on maximal AAA diameter and weight were determined. Compared with

Lipid Profile, Aortic Root Atherosclerosis, and Blood GSH

	Lipid Profile, mg/dL		Aortic Root Atherosclerosis, μm^2	Blood GSH, nmol/mg protein
	Total Cholesterol	Triglycerides		
ApoE ^{-/-}	633±90 (n=5)	146±30 (n=5)	0.115±0.034 (n=6)	15.60 ±1.32 (n=6)
ApoE ^{-/-} p47 ^{phox} ^{-/-}	511±72 (n=4)	136±37 (n=4)	0.087±0.022 (n=4)	18.75±0.41* (n=3)

* P<0.05 vs apoE^{-/-}.

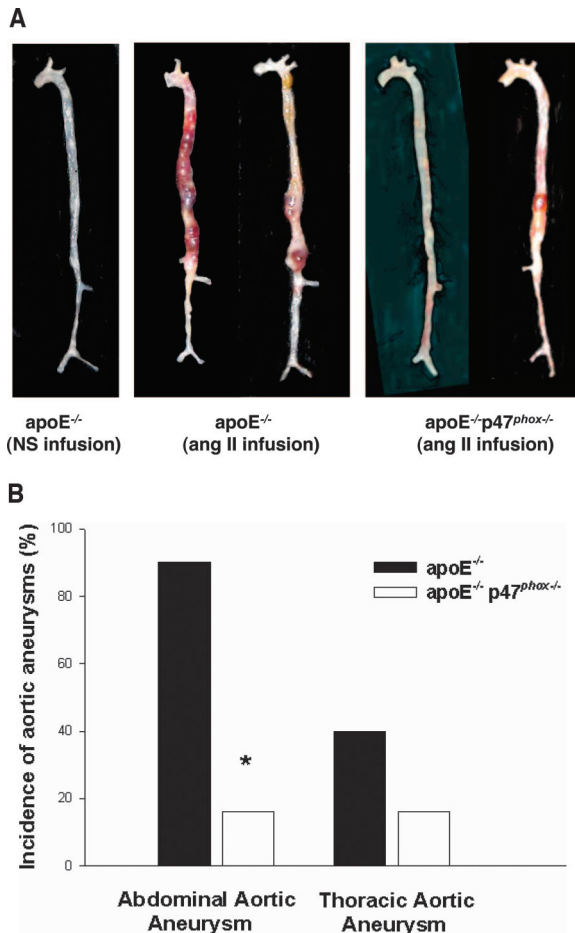


Figure 2. Gross morphology and incidence of AAAs. A, Representative photographs showing macroscopic features of aneurysms induced by Ang II. No animals infused with NS developed aneurysms (left specimen). Infusion of Ang II in apoE^{-/-} animals led to the development of AAA and less frequently thoracic aortic aneurysms (middle specimens). The incidence of both types of aneurysms was markedly decreased by inactivation of the p47^{phox} gene (right specimens). B, Incidence of aneurysms expressed as percent of animals in apoE^{-/-} (n=10) vs apoE^{-/-}p47^{phox}^{-/-} (n=6) mice. *P<0.05 vs apoE^{-/-}p47^{phox}^{+/+}.

the saline control, infusion of Ang II for 28 days resulted in a marked increase in both the maximal abdominal aorta diameter and the abdominal aorta weight in apoE^{-/-} mice, which was greatly attenuated in apoE^{-/-}p47^{phox}^{-/-} mice (P<0.05, respectively; Figure 3A and 3B). Moreover, in the apoE^{-/-} group, 40% of the animals displayed features of advanced AAA pathology with macroscopically visible thrombus (grade III to IV, as described by Daugherty et al¹⁸), as opposed to only 17% of the animals in the apoE^{-/-}p47^{phox}^{-/-} group (data not shown).

Deletion of the p47^{phox} Gene Attenuated NADPH Oxidase Activity

It is possible that deletion of genes that encode subunits of NADPH oxidase may be associated with compensatory up-regulation of expression of other subunits, thereby preserving oxidase function. Therefore, it was important to demonstrate the actual loss of this enzymatic activity in both aortic tissue and leukocytes from the double-knockout mice.

To confirm that the p47^{phox}-deficient mice lack a fully functional NADPH oxidase, we exposed mice to Ang II for 3 days, harvested whole aortas, and measured O₂⁻ formation in membrane preparations from aortic homogenates. Infusion of Ang II into apoE^{-/-} mice resulted in markedly increased aortic NADPH oxidase activity, which was strongly attenuated in apoE^{-/-}p47^{phox}^{-/-} mice (P<0.05; Figure 4A). Diphenyliodonium, an inhibitor of NADPH oxidase, blocked the O₂⁻ production in membrane preparations from both groups (Figure 4A), consistent with preformed, membrane-bound NADPH oxidase being the predominant source of O₂⁻ formation.

Leukocyte NADPH oxidase activity contributes significantly to ROS production and oxidative stress in the vasculature. Moreover, leukocytes play an important role in vascular inflammation, and infiltration of leukocytes into the aorta is an early event in AAA formation in this experimental model.¹⁹ To confirm that leukocytes from the p47^{phox}^{-/-} mice also demonstrate an impairment in NADPH oxidase activity, we harvested intraperitoneal leukocytes from apoE^{-/-} mice and apoE^{-/-}p47^{phox}^{-/-} mice and determined their ability to generate O₂⁻ after PMA stimulation. Treatment of leukocytes derived from apoE^{-/-} mice with PMA resulted in a rapid burst of O₂⁻ that was maximal at the earliest point examined (1.5 minutes) and remained above baseline for 4 minutes. In contrast, PMA failed to elicit a respiratory burst in leukocytes derived from apoE^{-/-}p47^{phox}^{-/-} mice (Figure 4B). These results confirm that deletion of p47^{phox} impairs NADPH oxidase activity in inflammatory cells.

Deletion of the p47^{phox} Gene Attenuated Oxidative Stress in Ang II-Infused ApoE^{-/-} Mice

If oxidative stress plays an important role in AAA formation in this model, then the improvements in AAA severity observed in the p47^{phox}-deficient mice should be associated with a decrease in oxidative stress. To determine whether deletion of p47^{phox}, in addition to abrogating NADPH oxidase activity, diminished oxidative stress, we examined the formation of nitrotyrosine in abdominal aortic tissues. Peroxynitrite, the product of the reaction of O₂⁻ and nitric oxide, reacts with and nitrates tyrosine groups on proteins. Thus, the relative level of immunoreactive nitrotyrosine in a tissue is a widely recognized marker of general oxidative stress. Ang II infusion caused a marked increase in nitrotyrosine immunostaining in the abdominal aorta of apoE^{-/-} mice, which was strongly attenuated in apoE^{-/-}p47^{phox}^{-/-} mice (Figure 5, middle). To confirm a decrease in overall oxidative stress, we determined levels of reduced GSH in whole blood. After 28 days of Ang II infusion, apoE^{-/-}p47^{phox}^{-/-} animals had significantly higher GSH levels than those seen in apoE^{-/-} animals expressing p47^{phox} (see the Table), consistent with a decrease in oxidative stress in the animals lacking p47^{phox}.

Deletion of the p47^{phox} Gene Attenuated Aortic Macrophage Infiltration in Ang II-Infused ApoE^{-/-} Mice

One mechanism whereby oxidative stress contributes to vascular disease is by enhancing vascular leukocyte infiltration, which is a key event in AAA formation in this

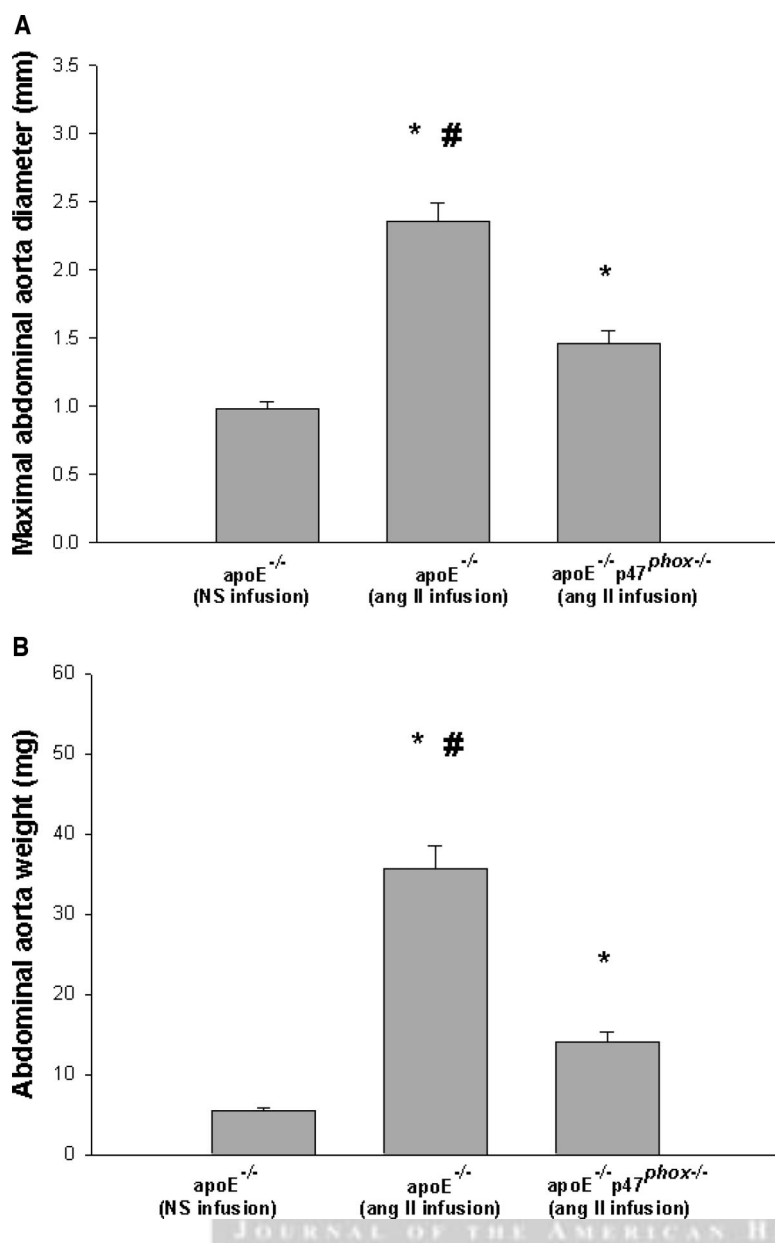


Figure 3. Ang II-induced AAA size was markedly reduced in mice lacking p47^{phox}. Abdominal aortic diameter (A) and weight (B) in saline-infused apoE^{-/-} mice (n=7), Ang II-infused apoE^{-/-} mice (n=10), and Ang II-infused apoE^{-/-} p47^{phox}^{-/-} mice (n=6). *P<0.05 vs saline infusion; #P<0.05 vs Ang II-infused apoE^{-/-} p47^{phox}^{-/-}.

experimental model.¹⁹ We therefore investigated whether deletion of p47^{phox} diminishes leukocyte infiltration into the abdominal aorta. Prominent leukocyte infiltration, particularly in the adventitia (Figure 5, left, arrows), was detected in abdominal aortic tissues from apoE^{-/-} mice infused with Ang II. Aortic leukocyte infiltration was markedly reduced in aortic tissues from Ang II-infused apoE^{-/-} p47^{phox}^{-/-} mice. We also examined the extent of macrophage infiltration in abdominal aortic tissue from our experimental animals using immunohistochemistry. As we have previously reported,¹³ extensive macrophage infiltration was detected in the abdominal aortas from Ang II-infused apoE^{-/-} mice (Figure 5, right). This macrophage infiltration was markedly attenuated in apoE^{-/-} p47^{phox}^{-/-} mice in response to Ang II infusion.

Deletion of the p47^{phox} Gene Attenuated MMP-2 Activity in Ang II-Infused ApoE^{-/-} Mice

MMPs, especially MMP-2 and MMP-9, are thought to play a critical role in the pathogenesis of AAA formation.^{2,20–26}

Because MMP activity may be partly redox regulated,²⁷ we examined MMP gelatinolytic activity in abdominal aortic tissue homogenates. As previously described,¹³ Ang II infusion in apoE^{-/-} mice significantly increased both MMP-2 and MMP-9 proteolytic activity compared with NS infusion (data not shown). Aortic MMP-2 activity was significantly reduced in Ang II-infused apoE^{-/-} p47^{phox}^{-/-} mice compared with apoE^{-/-} mice (Figure 6A and 6B). In contrast, we did not detect differences in aortic MMP-9 activity between the 2 groups of animals (data not shown).

Reduction in SBP Was Not Responsible for the Protective Effects of p47^{phox} Gene Deletion on Ang II-Induced AAA Formation

Because apoE^{-/-} p47^{phox}^{-/-} mice had a diminished pressor response to Ang II infusion and because hypertension is a documented risk factor for AAA formation, we determined whether a reduction in SBP was responsible for the

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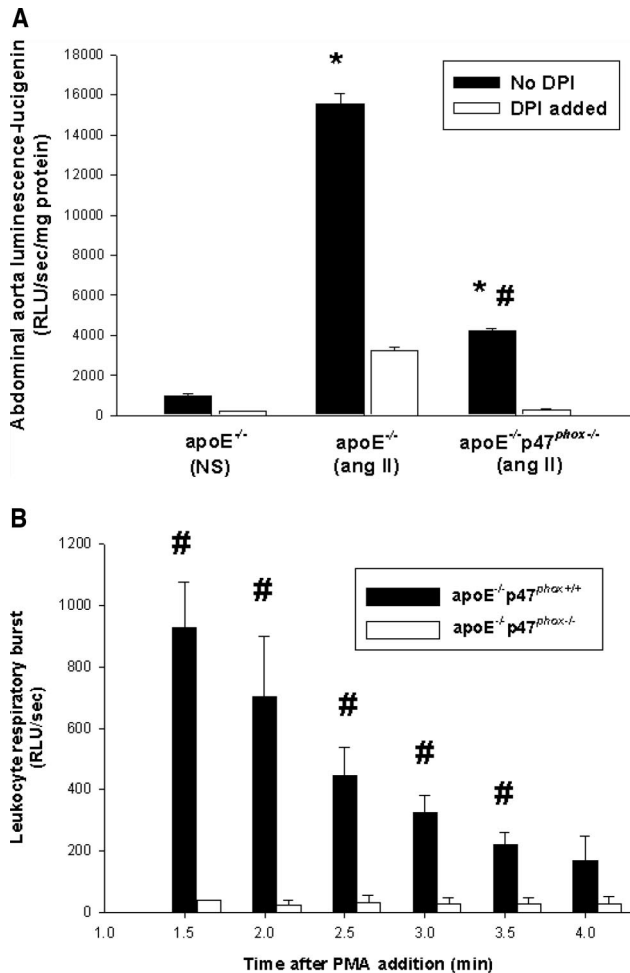


Figure 4. Aortic superoxide production and leukocyte respiratory burst are diminished in mice lacking p47^{phox}. Aortic membrane homogenates and peritoneal leukocyte suspensions were prepared as described in Methods. In apoE^{-/-} p47^{phox}^{-/-} animals, both aortic superoxide production (A) and leukocyte respiratory burst (B) were markedly suppressed compared with apoE^{-/-} animals. **P*<0.05 vs NS; #*P*<0.05 vs apoE^{-/-}; *n*=4 per group.

protective effects of p47^{phox} deletion in this experimental model. We conducted additional experiments in which apoE^{-/-} p47^{phox}^{-/-} mice were coinflused with PE and Ang II to elevate their SBP to a level similar to that observed in apoE^{-/-} mice infused with Ang II alone. PE causes hypertension by activating α -adrenergic receptors, thereby producing vasoconstriction that is independent of NADPH oxidase expression or activity. This experimental protocol was conducted for 2 rather than 4 weeks because SBP progressively rises in apoE^{-/-} p47^{phox}^{-/-} mice during prolonged Ang II infusion (Figure 1). An appropriate dose of PE (12 500 ng · kg⁻¹ · min⁻¹) was determined after extensive preliminary experiments. As shown in Figure 7A, apoE^{-/-} p47^{phox}^{-/-} mice coinflused with Ang II and PE demonstrated an increase in SBP that was virtually identical to that observed in apoE^{-/-} mice infused with Ang II only. Despite exhibiting similar levels of SBP, however, the apoE^{-/-} p47^{phox}^{-/-} mice were strongly protected against AAA formation (Figure 7B). To quantify oxidative stress

in this study, we measured protein carbonyl formation. As expected, infusion of Ang II in apoE^{-/-} mice increased aortic protein carbonyl formation over control levels, indicating increased oxidative stress (Figure 7C). In the aortas of apoE^{-/-} p47^{phox}^{-/-} mice coinflused with Ang II and PE, protein carbonyl formation was markedly diminished, confirming reduced oxidative stress. These findings indicate that a reduction in SBP was not responsible for the protective effects of p47^{phox} deletion on AAA formation and further suggest the importance of oxidative stress in the pathophysiology of AAA in this experimental model.

Discussion

Here, we demonstrate that deletion of the p47^{phox} gene has a marked protective effect against the formation of AAAs in apoE^{-/-} mice infused with Ang II. To the best of our knowledge, this is the first study that directly implicates NADPH oxidase in the pathogenesis of AAA.

The p47^{phox} subunit is an integral component of the membrane-bound NADPH oxidase, which is considered a primary source of ROS in vascular tissues.⁹ We have previously shown that in human aortic aneurysmal tissues, NADPH oxidase expression and activity and levels of the p47^{phox} subunit are markedly upregulated compared with adjacent nonaneurysmal aortic tissue obtained from the same patients.⁷ Furthermore, several experimental studies have implicated ROS in the development and progression of AAA.^{13,28,29} Although these findings suggest an association between NADPH oxidase and aneurysmal disease, they do not conclusively demonstrate that the enzyme plays a causal role in AAA formation.³⁰ To address this important distinction, we examined the effects of p47^{phox} deletion on AAA development in a well-established murine model of AAA formation: apoE-null (hyperlipidemic) male mice infused with Ang II for 4 weeks.¹¹ These mice develop AAAs that have some features in common with human disease, including prominent leukocyte infiltration, rupture of elastin bands, and formation of thrombus. As in humans, the incidence of AAA formation in this model is greater in males, and inflammation plays a critical role in the disease process because aneurysms were not observed in mice lacking the AT1a receptor on bone marrow-derived cells.¹⁰

In the present study, we show that inactivation of the p47^{phox} gene in apoE^{-/-} mice leads to a marked protective effect against AAA in this Ang II infusion model. Moreover, we found that inactivation of p47^{phox} attenuated ROS generation (in both aortic homogenates and leukocytes) and tissue markers of aortic oxidative stress. Furthermore, and potentially related to the decreased levels of oxidative stress,³¹ the p47^{phox}-null mice also showed reduced aortic macrophage infiltration and attenuation of MMP-2 activity. These findings are consistent with a paradigm of AAA development in which inflammation, at least partly modulated by oxidative stress, leads to increased proteolytic activity and aneurysm development. It is also consistent with our previous findings¹³ that oral treatment with vitamin E, although protective against AAA, led to a decrease in oxidative stress in the abdominal aorta where these aneurysms develop. Taken together, these findings suggest that it is the decrease in

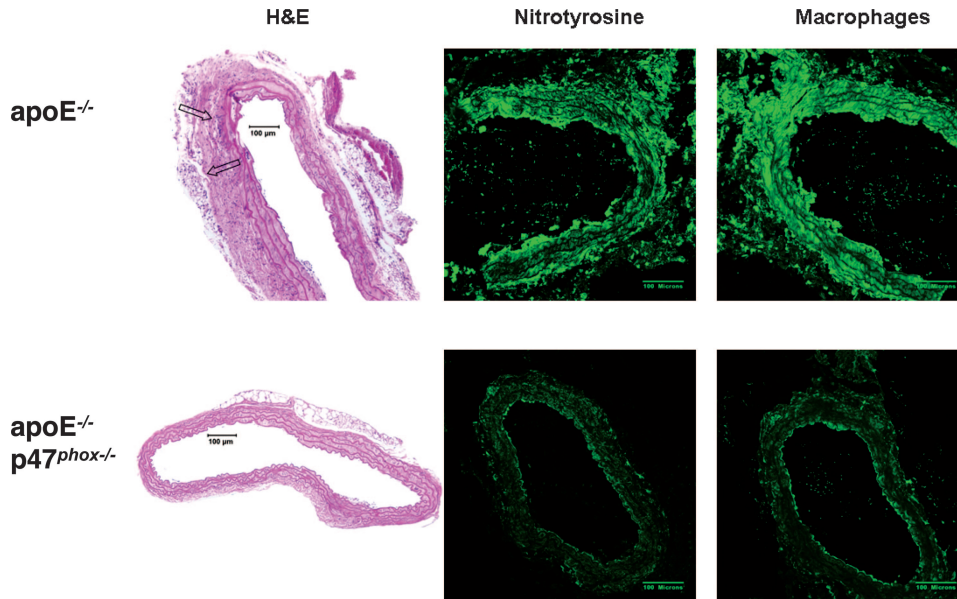


Figure 5. Ang II-induced nitrotyrosine formation and macrophage infiltration were prominently diminished in mice lacking p47^{phox}. Abdominal aortic tissues were harvested, and transversal sections were prepared and immunostained as described. Representative pictures from Ang II-treated, apoE^{-/-} (top row), and apoE^{-/-}p47^{phox}^{-/-} (bottom row) animals are shown, demonstrating that in the absence of functional p47^{phox} gene, inflammatory infiltrate is reduced (left, hematoxylin and eosin stain), nitrotyrosine formation is diminished (middle), and macrophage infiltration is attenuated (right). Arrows point to prominent, mostly adventitial, inflammatory infiltrates noted in the apoE^{-/-} group.

oxidative stress, rather than the specific means through which it is achieved, that is protective against AAA formation in this experimental model.

Infusion of Ang II produced a rapid and sustained increase in SBP in apoE^{-/-} mice, consistent with previous

reports. We observed that the Ang II pressor response was attenuated in apoE^{-/-}p47^{phox}^{-/-} mice, particularly early during the course of the infusion, which also has been reported previously.³² Normalization of the pressor response by coinfusion of PE with Ang II in apoE^{-/-}p47^{phox}^{-/-} mice did not alter the protective effects of p47^{phox} gene deletion on AAA formation, suggesting that the decreased incidence and severity of AAA observed in the apoE^{-/-}p47^{phox}^{-/-} mice were not due to reduced SBP. Furthermore, the question of whether increased SBP plays a significant role in murine AAA formation is debatable. When C57BJ/6 mice (the background strain for the apoE^{-/-} mice) are infused with Ang II, they develop AAAs at much lower frequency than apoE^{-/-} mice despite demonstrating a hypertensive response similar to that seen in Ang II-infused apoE^{-/-} mice (which develop AAAs at a rate of 90% to 100%¹¹). Moreover, the development of spontaneous AAAs in apoE^{-/-}eNOS^{-/-} mice was not prevented by normalization of blood pressure with hydralazine.³³ In contrast, vitamin E treatment had no effect on SBP yet protected against AAA formation in the Ang II infusion model.¹³ Finally, Ayabe et al³⁴ reported that unlike Ang II, norepinephrine did not induce aneurysm formation in apoE^{-/-} mice, even though both agents caused similar elevations in blood pressure. It is thus unlikely that hypertension is either necessary or sufficient to initiate aneurysm development in this experimental model.

We were unable to detect a significant difference in the extent of aortic root atherosclerosis in Ang II-infused, apoE^{-/-} animals with or without the p47^{phox} gene. Other investigators have shown that animals lacking the p47^{phox} gene product demonstrate significantly lower levels of atherosclerosis formation.¹² The limited number of animals studied and the variability in atherosclerosis related to the ages of our animals (3 to 6 months old, although closely

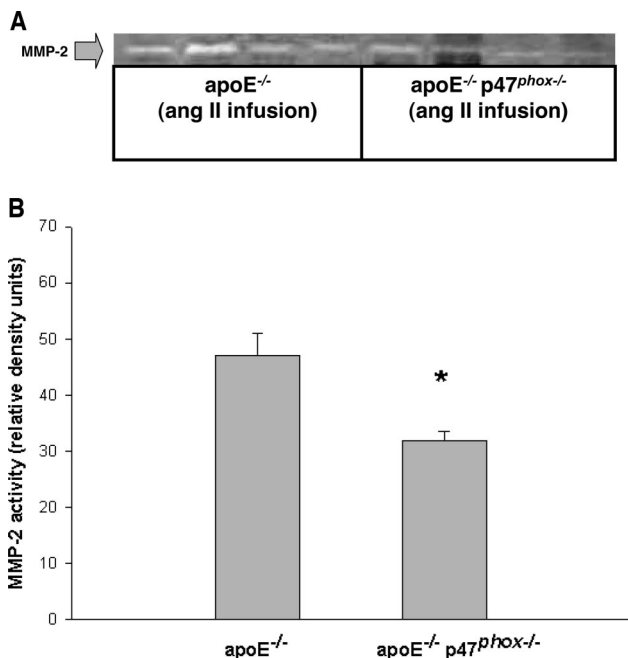


Figure 6. Ang II-induced MMP-2 proteolytic activity was inhibited in mice lacking p47^{phox}. Shown is a representative zymogram of aortic tissue homogenates demonstrating that in apoE^{-/-}p47^{phox}^{-/-} (right 4 lanes), MMP-2 activity (normalized per 1 mg protein) is significantly decreased compared with apoE^{-/-} animals (left 4 lanes) (A, B). B, Densitometric quantification of MMP-2 gelatinolytic activity in the 2 groups of animals (n=4 per group). *P<0.05 vs apoE^{-/-}.

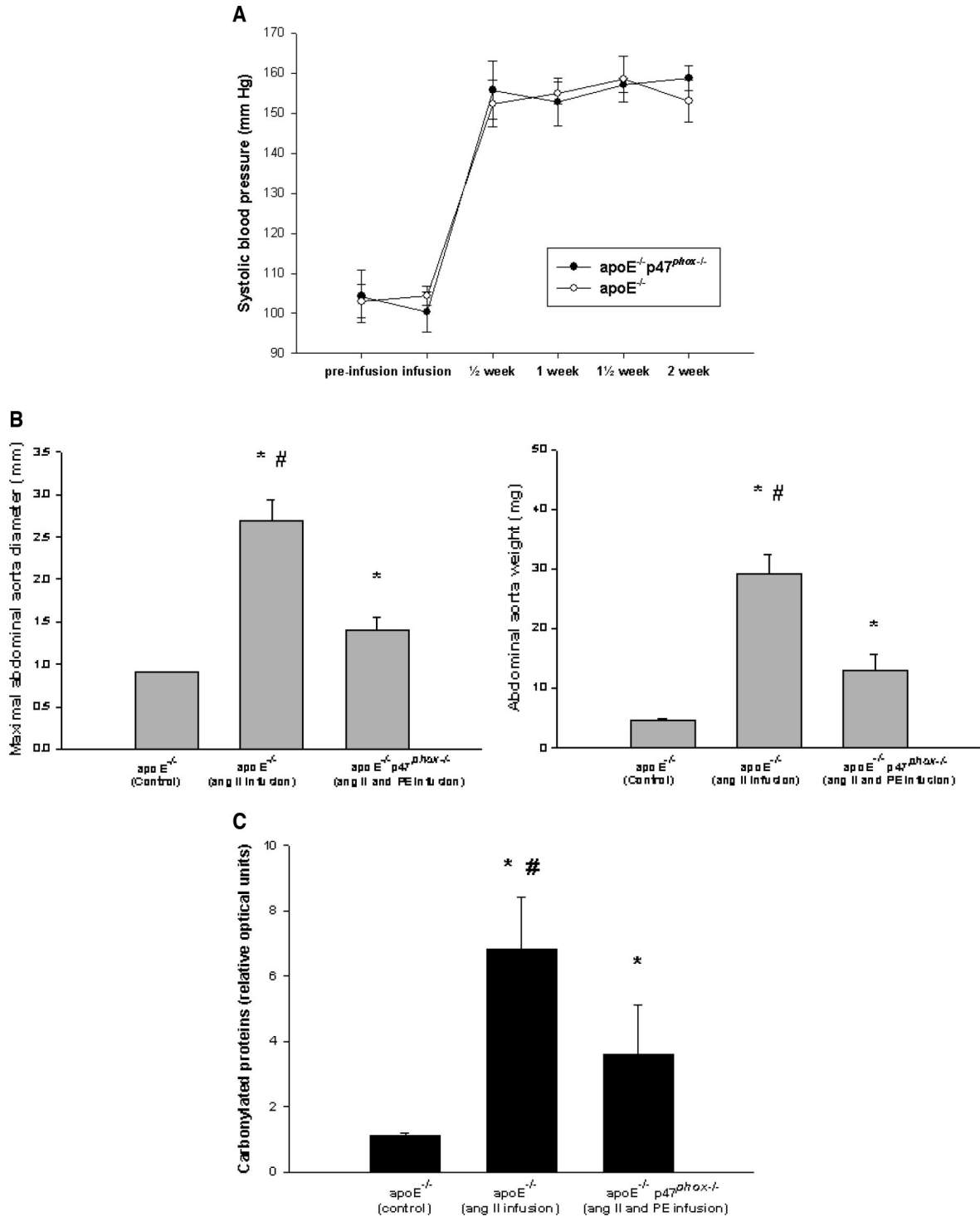


Figure 7. Attenuation of Ang II–induced AAA formation in mice lacking p47^{phox} was not due to reduced SBP. ApoE^{-/-} mice were infused with Ang II (1000 ng · kg⁻¹ · min⁻¹), whereas apoE^{-/-}p47^{phox}^{-/-} mice were infused with Ang II plus phenylephrine (1000 and 12 500 ng · kg⁻¹ · min⁻¹, respectively) for 2 weeks (n=4 per group). SBP (A) was measured by the tail-cuff method, as described in Figure 1. After 2 weeks, mice were euthanized, abdominal aortic weight and diameter were measured (B; n=4 per group), and aortic oxidative stress was quantified by protein carbonyl formation (C) and compared with control apoE^{-/-} mice that were not infused with Ang II (n=3 per group). #P<0.05 vs apoE^{-/-}; *P<0.05 vs control.

matched between experimental groups) might have contributed to the inability to detect a clear difference in atherosclerosis. In addition, it is important to point out that because of the experimental design, quantification of

atherosclerosis could not be performed in the abdominal aorta, the site of AAA development. However, a temporal study showed that atherosclerotic lesions were not detected in the abdominal aorta during the course of AAA devel-

opment in this experimental model (eg, during 28 days of Ang II infusion).¹⁹ Because deletion of p47^{phox} diminished AAA formation at the 14-day time point (Figure 7), our findings suggest that diminution of atherosclerosis was not primarily responsible for the attenuation of AAA formation observed in mice lacking p47^{phox}.

In summary, we demonstrate here that altering NADPH oxidase activity by deleting the p47^{phox} gene has a marked, protective effect against aortic aneurysmal disease. These findings suggest an important role for NADPH oxidase in the pathogenesis of AAA in this experimental model.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Abdominal aortic aneurysms (AAAs) are a major cause of morbidity and mortality in humans. Histological studies of AAAs suggest a prominent role for inflammation, matrix metalloproteinase activation, and smooth muscle cell apoptosis, processes that may be regulated by oxidative stress. Moreover, human AAAs exhibit locally increased levels of oxidative stress, NADPH oxidase activity, and expression of p47^{phox}, a cytosolic subunit of NADPH oxidase. In this study, we investigated the effects of deletion of p47^{phox} on AAA development in an established animal model of AAA formation (angiotensin II infusion in apolipoprotein E–null male mice). Deletion of p47^{phox}, which resulted in reduction in parameters of aortic oxidative stress, markedly attenuated AAA formation in this experimental model. Although the pressor response to angiotensin II was blunted in apoE/p47^{phox} double-knockout mice, attenuation of AAA formation was found to be independent of blood pressure reduction in these animals. Histology showed that deletion of p47^{phox} resulted in reduced aortic macrophage infiltration and matrix metalloproteinase-2 activation, consistent with a paradigm in which aortic oxidative stress triggers inflammation, increased aortic proteolytic activity, and aneurysm development. We conclude that oxidative stress, via NADPH oxidase activity, plays a pivotal role in AAA formation in this experimental model. Modulation of NADPH oxidase activity may represent a potential new therapeutic approach to treating AAAs.



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