Molecular Cardiology

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

Manesh Thomas, MD*; Dan Gavrila, MD*; Michael L. McCormick, PhD; Francis J. Miller, Jr, MD; Alan Daugherty, PhD, DSc; Lisa A. Cassis, PhD; Kevin C. Dellsperger, MD, PhD; Neal L. Weintraub, MD

\textbf{Background}—Angiotensin II (Ang II) contributes to vascular pathology in part by stimulating NADPH oxidase activity, leading to increased formation of superoxide (O$_2^-$). We reported that O$_2^-$ levels, NADPH oxidase activity, and expression of the \textit{p47}phox subunit of NADPH oxidase are increased in human abdominal aortic aneurysms (AAAs). Here, we tested the hypothesis that deletion of \textit{p47}phox will attenuate oxidative stress and AAA formation in Ang II–infused apoE–/– mice.

\textbf{Methods and Results}—Male apoE–/– and apoE–/–\textit{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 apo–/–\textit{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–/–\textit{p47}phox–/– mice compared with apoE–/– mice ($P<0.05$). In addition, aortic macrophage infiltration and matrix metalloproteinase-2 activity were reduced in apoE–/–\textit{p47}phox–/– mice compared with apoE–/– mice.

\textbf{Conclusions}—Deletion of \textit{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. (\textit{Circulation}. 2006;114:404-413.)

\textbf{Key Words:} aneurysm ■ aorta ■ cardiovascular diseases ■ free radicals ■ inflammation

Abdominal aortic aneurysms (AAAs) cause considerable morbidity and mortality in the adult population\textsuperscript{1} 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.\textsuperscript{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.\textsuperscript{7}

A major source of ROS in vascular tissue is the membrane-bound NADPH oxidase, which consists of transmembrane (eg, nox1, nox2, nox4, and p22phox) and cytosolic (p47phox, p67phox, and rac) subunits that assemble to form the functional oxidase.\textsuperscript{8} The expression and activity of NADPH oxidase in the vasculature are increased in various pathological states, including hypertension and atherosclerosis.\textsuperscript{9} With regard to AAAs, Miller et al\textsuperscript{10} have shown that NADPH oxidase activity and its \textit{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 \textit{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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From the Department of Internal Medicine (M.T., M.L.M., D.G., F.J.M., N.L.W.) and Free Radical and Radiation Biology Program, Department of Radiation Oncology (M.L.M., F.J.M., N.L.W.), University of Iowa, Iowa City; Veteran’s Administration Medical Center, Iowa City, Iowa (N.L.W.); Department of Internal Medicine (A.D.) and Graduate Center for Nutritional Sciences (L.A.C.), University of Kentucky, Lexington; and Department of Internal Medicine, University of Missouri, Columbia (K.C.D.).

*Drs Thomas and Gavrila contributed equally to this manuscript.

Correspondence to Dr Neal L. Weintraub, Department of Internal Medicine, University of Iowa, College of Medicine, 200 Hawkins Dr, E-315GH, Iowa City, IA 52242. E-mail neal-weintraub@uiowa.edu

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apoliipoprotein E (apoE)–deficient male mice results in AAA formation in 90% to 100% of these animals. \(^{10,11}\) Moreover, the AAs exhibit inflammatory cell infiltration, MMP activation, thrombus formation, and oxidative stress, all of which have been observed in human AAs. \(^{7,11}\) 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 confused 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, Visitec 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 periaortic 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 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 150 μL 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 μmol/L) and NADPH (100 μmol/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 μmol/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 mmol/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 μm 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 Laboratories (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. A1AD 31240, dilution 1:50) or rabbit anti-mouse nitrotyrosine antibody (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 CaCl\(_2\), and 50 mmol/L NaCl at 37°C; then, they were...
ANOVA, followed by the Bonferroni and differences between multiple groups were analyzed by 1-way tometry (Quantity One 1-D analysis software, Bio-Rad).

Whole-blood samples were assayed for GSH levels by the Radiation and Free Radical Research Core Facility at the University of Iowa.

The effects of deletion of the p47phox gene on maximal AAA diameter and weight were determined. Compared with
advanced AAA pathology with macroscopically visible thrombus. Inflammation of Ang II into apoE<sup>-/-</sup> mice resulted in markedly increased aortic NADPH oxidase activity, which was strongly attenuated in apoE<sup>-/-</sup>p47<sup>phox/-/-</sup> mice (P<0.05; Figure 4A). Diphenylidodium, an inhibitor of NADPH oxidase, blocked the O<sub>2</sub><sup>-</sup> production in membrane preparations from both groups (Figure 4A), consistent with preformed, membrane-bound NADPH oxidase being the predominant source of O<sub>2</sub><sup>-</sup> 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. To confirm that leukocytes from the p47<sup>phox/-/-</sup> mice also demonstrate an impairment in NADPH oxidase activity, we harvested intraperitoneal leukocytes from apoE<sup>-/-</sup> mice and apoE<sup>-/-</sup>p47<sup>phox/-/-</sup> mice and determined their ability to generate O<sub>2</sub><sup>-</sup> after PMA stimulation. Treatment of leukocytes derived from apoE<sup>-/-</sup> mice with PMA resulted in a rapid burst of O<sub>2</sub><sup>-</sup> 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<sup>-/-</sup>p47<sup>phox/-/-</sup> mice (Figure 4B). These results confirm that deletion of p47<sup>phox</sup> impairs NADPH oxidase activity in inflammatory cells.

**Deletion of the p47<sup>phox</sup> Gene Attenuated Oxidative Stress in Ang II–Infused ApoE<sup>-/-</sup> Mice**

If oxidative stress plays an important role in AAA formation in this model, then the improvements in AAA severity observed in the p47<sup>phox/-/-</sup> mice should be associated with a decrease in oxidative stress. To determine whether deletion of p47<sup>phox</sup>, 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 O<sub>2</sub><sup>-</sup> 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<sup>-/-</sup> mice, which was strongly attenuated in apoE<sup>-/-</sup>p47<sup>phox/-/-</sup> 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<sup>-/-</sup>p47<sup>phox/-/-</sup> animals had significantly higher GSH levels than those seen in apoE<sup>-/-</sup> animals expressing p47<sup>phox</sup> (see the Table), consistent with a decrease in oxidative stress in the animals lacking p47<sup>phox</sup>.

**Deletion of the p47<sup>phox</sup> Gene Attenuated Aortic Macrophage Infiltration in Ang II–Infused ApoE<sup>-/-</sup> 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...
We therefore investigated whether deletion of p47\textsuperscript{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\textsuperscript{-/-} mice infused with Ang II. Aortic leukocyte infiltration was markedly reduced in aortic tissues from Ang II–infused apoE\textsuperscript{-/-} p47\textsuperscript{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,\textsuperscript{13} extensive macrophage infiltration was detected in the abdominal aortas from Ang II–infused apoE\textsuperscript{-/-} mice (Figure 5, right). This macrophage infiltration was markedly attenuated in apoE\textsuperscript{-/-} p47\textsuperscript{phox}/-/- mice in response to Ang II infusion.

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

MMPs, especially MMP-2 and MMP-9, are thought to play a critical role in the pathogenesis of AAA formation.\textsuperscript{2,20–26} Because MMP activity may be partly redox regulated,\textsuperscript{27} we examined MMP gelatinolytic activity in abdominal aortic tissue homogenates. As previously described,\textsuperscript{13} Ang II infusion in apoE\textsuperscript{-/-} 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\textsuperscript{-/-} p47\textsuperscript{phox}/-/- mice compared with apoE\textsuperscript{-/-} 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\textsuperscript{phox} Gene Deletion on Ang II–Induced AAA Formation**

Because apoE\textsuperscript{-/-} p47\textsuperscript{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
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−/− p47phox−/− mice coinfused 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 p47phox 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 p47phox 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 p47phox subunit is an integral component of the membrane-bound NADPH oxidase, which is considered a primary source of ROS in vascular tissues. We have previously shown that in human aortic aneurysmal tissues, NADPH oxidase expression and activity and levels of the p47phox subunit are markedly upregulated compared with adjacent nonaneurysmal aortic tissue obtained from the same patients. Furthermore, several experimental studies have implicated ROS in the development and progression of AAA. 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. To address this important distinction, we examined the effects of p47phox 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. 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.

In the present study, we show that inactivation of the p47phox gene in apoE−/− mice leads to a marked protective effect against AAA in this Ang II infusion model. Moreover, we found that inactivation of p47phox 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, the p47phox-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 findings 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
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<sup>−/−</sup> mice, consistent with previous reports. We observed that the Ang II pressor response was attenuated in apoE<sup>−/−</sup>p47<sub>phox</sub><sup>−/−</sup> mice, particularly early during the course of the infusion, which also has been reported previously. Normalization of the pressor response by coinfusion of PE with Ang II in apoE<sup>−/−</sup>p47<sub>phox</sub><sup>−/−</sup> mice did not alter the protective effects of p47<sub>phox</sub> gene deletion on AAA formation, suggesting that the decreased incidence and severity of AAA observed in the apoE<sup>−/−</sup>p47<sub>phox</sub><sup>−/−</sup> 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<sup>−/−</sup> mice) are infused with Ang II, they develop AAAs at much lower frequency than apoE<sup>−/−</sup> mice despite demonstrating a hypertensive response similar to that seen in Ang II–infused apoE<sup>−/−</sup> mice (which develop AAAs at a rate of 90% to 100%). Moreover, the development of spontaneous AAAs in apoE<sup>−/−</sup>eNOS<sup>−/−</sup> mice was not prevented by normalization of blood pressure with hydralazine. In contrast, vitamin E treatment had no effect on SBP yet protected against AAA formation in the Ang II infusion model. Finally, Ayabe et al. reported that unlike Ang II, norepinephrine did not induce aneurysm formation in apoE<sup>−/−</sup> 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<sup>−/−</sup> mice with or without the p47<sub>phox</sub> gene. Other investigators have shown that animals lacking the p47<sub>phox</sub> gene product demonstrate significantly lower levels of atherosclerosis formation. 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
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 development.
opment in this experimental model (eg, during 28 days of Ang II infusion). Because deletion of p47\textsuperscript{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\textsuperscript{phox}.

In summary, we demonstrate here that altering NADPH oxidase activity by deleting the p47\textsuperscript{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.

**References**


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 p47phox, a cytosolic subunit of NADPH oxidase. In this study, we investigated the effects of deletion of p47phox on AAA development in an established animal model of AAA formation (angiotensin II infusion in apolipoprotein E–null male mice). Deletion of p47phox, 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/p47phox double-knockout mice, attenuation of AAA formation was found to be independent of blood pressure reduction in these animals. Histology showed that deletion of p47phox 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.