Atherosclerosis Is Attenuated by Limiting Superoxide Generation in Both Macrophages and Vessel Wall Cells

Aleksandr E. Vendrov, Zeenat S. Hakim, Nageswara R. Madamanchi, Mauricio Rojas, Chaitanya Madamanchi, Marschall S. Runge

Objective—We previously showed that NAD(P)H oxidase deficiency significantly reduces atherosclerosis in apoE−/− mice. The present study was designed to determine the relative contribution of monocyte/macrophage versus vascular wall cell NAD(P)H oxidase to atherogenesis in this model.

Methods and Results—Cell-specific NAD(P)H oxidase inhibition was achieved via allogenic, sex-mismatched bone marrow transplantation. Aortic atherosclerosis and superoxide production in apoE−/− mice (Control) with functional NAD(P)H oxidase in both monocytes/macrophages and vascular wall cells was compared with that in apoE−/− mice with nonfunctional monocyte/macrophage NAD(P)H oxidase (BMO) or nonfunctional vessel wall NAD(P)H oxidase (VWO). A significant decrease in superoxide production and atherosclerotic lesions was observed in BMO and VWO mice compared with control mice. Interestingly, BMO mice had significantly lower plasma oxidized LDL levels compared with control and VWO mice, whereas aortic sections of VWO mice showed decreased expression of cellular adhesion molecules compared with control and BMO mice. NAD(P)H oxidase deficiency also attenuated neointimal hyperplasia and mitogenic protein activation in apoE−/− mice after arterial injury.

Conclusions—We conclude that (1) both monocyte/macrophages and vessel wall cells play critical roles in atherogenesis; (2) decrease in atherosclerosis results from attenuated superoxide generation in monocyte/macrophages or vessel wall cells; and (3) superoxide generation may impact atherosclerosis, in part, by activating smooth muscle cell mitogenic signaling pathways. (Arterioscler Thromb Vasc Biol. 2007;27:2714-2721.)

Key Words: Adhesion molecules • signal transduction • oxidized lipids • reactive oxygen species • thrombin

Reactive oxygen species (ROS) derived from NAD(P)H oxidase have been strongly associated with experimental hypertension,1 cardiac hypertrophy,2 thrombosis,3 restenosis, and atherosclerosis.4 In humans, higher expression of NAD(P)H oxidase subunit proteins is associated with increased superoxide (O2−) production and severity of atherosclerosis.5 NAD(P)H oxidase is also an important source of increased O2− production in human diabetes mellitus, a risk factor for atherosclerosis.6 All vascular wall cells—endothelial cells, smooth muscle cells (SMCs), and fibroblasts—as well as monocytes/macrophages contain NAD(P)H oxidases which are activated under pathophysiological conditions. The resultant ROS induce redox-sensitive signaling pathways that contribute to atherogenesis.7

The phagocytic NAD(P)H oxidase contains the membrane-bound subunits gp91phox (Nox2) and p22phox, the catalytic site of the oxidase and the cytosolic components p47phox, p67phox, and G-protein Rac1 or Rac2.8 Vascular NAD(P)H oxidases are similar in structure to phagocytic NAD(P)H oxidase, but have a distinct molecular composition. Endothelial cells and adventitial fibroblasts possess all the components of the phagocytic oxidase, whereas SMCs predominantly express homologues of gp91phox, Nox1 and Nox4. Mouse SMCs also express a p67phox homologue, Nox1.9 The activation of vascular NAD(P)H oxidases is constitutive as well as inducible in a manner similar to the neutrophil enzyme by the translocation of the cytosolic components.10 Superoxide production by vascular oxidases is much less than that in macrophages.7

Increased ROS production from both monocytes/macrophages and in vascular wall cells has been implicated in atherosclerosis. Macrophage infiltration and ROS production were markedly increased in atherosclerotic plaques of patients with unstable angina pectoris whereas plaques from patients with stable angina had fewer macrophages and lower ROS production.11 However, ROS production in unstable plaques was derived from macrophages as well as SMCs. Similarly, the severity of atherosclerotic lesions correlated well with the activation of NAD(P)H oxidases in both macrophages and SMCs of human coronary arteries.5 In contrast, little or no macrophage infiltration was observed in experimental restenosis12 and atherosclerosis,13 which im-

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signaling pathways are attenuated in p47phox as described previously14 (see supplemental materials, available online at http://atvb.ahajournals.org). Bone marrow transplanted mice were maintained on rodent chow for 4 weeks and then fed a Western diet (42% fat, Harlan Teklad, TD 88137) for 12 weeks.

In the present study, we investigated the relative contribution of monocyte/macrophages and the vessel wall cell-derived ROS to atherosclerosis using bone marrow transplantation. Our data indicate that NAD(P)H oxidase–derived ROS generated from both monocytes/macrophages as well as vascular wall cells are important in the development of atherosclerotic lesions. Deficiency of NAD(P)H oxidase in monocytes/macrophages decreases plasma oxidized LDL levels, whereas deficiency of this enzyme in vascular wall cells attenuates the expression of cellular adhesion molecules in the aortas of apoE−/− mice. Thrombin-induced mitogenic signaling pathways are attenuated in p47phox−/− SMC compared with wild-type SMCs. Consistent with this, attenuation of neointimal hyperplasia after arterial injury and decrease in the activation of mitogenic signaling proteins in the neointima were observed in apoE−/−/p47phox−/− mice compared with wild-type. Together, these results suggest that ROS derived from monocytes/macrophages as well as vascular wall cells are involved in atherosclerosis, whereas NAD(P)H oxidase−dependent SMC mitogenic signaling pathways play an important role in restenosis.

Materials and Methods

Mice and Diet
ApoE−/−, p47phox−/−, and apoE−/−/p47phox−/− mice were obtained as described previously14 (see supplemental materials, available online at http://atvb.ahajournals.org). Bone marrow transplanted mice were maintained on rodent chow for 4 weeks and then fed a Western diet (42% fat, Harlan Teklad, TD 88137) for 12 weeks.

Bone Marrow Transplantation
Bone marrow transplantation (BMT) was performed as described in the supplemental materials.

Quantification of Atherosclerotic Lesions
Atherosclerosis in the aortas was measured by quantitation of oil red O–positive lesions (see supplemental materials).

Femoral Artery Injury and Morphometry
Transluminal femoral artery injury was performed as described4 (see supplemental materials).

Measurement of ROS Production in the Aorta
ROS levels in the aortas were measured by staining with dihydroethidium14 (see supplemental materials).

Fluorescence In Situ Hybridization
Frozen arterial sections were processed for in situ hybridization with Y chromosome paints (Applied Spectral Imaging) as described earlier.16 Images were captured with a charge-coupled device (CCD) camera.

Immunohistochemistry
Immunostaining of aortic and femoral artery cross sections was described online in the supplemental materials.

RNA Extraction and Real-Time RT-PCR
Detailed information regarding RNA extraction and real-time RT-PCR is available in the supplemental materials.

Analysis of Plasma Lipids
Plasma total cholesterol and triglyceride (TG) levels were measured by a quantitative enzymatic colorimetric assay (Stanbio Laboratory). Plasma Oxidized LDL levels were measured using the Oxidized LDL Competitive ELISA kit (Merodia).

Cell Culture and Western Analysis
Isolation of aortic SMCs and Western analysis were described online in the supplemental materials.

Statistical Analysis
Data are presented as mean±SEM. All data were analyzed using 1-way ANOVA followed by Newman-Keuls multiple comparison test. Differences were considered significant at P<0.05.
O2

mine which among the cell types with the capacity to produce
nor tissue-specific. Therefore, it was not possible to deter-

studies, the deficiency of NAD(P)H oxidase was neither cell-

mice (functional NAD(P)H oxidase in marrow-derived

monocytes/macrophages) had 66% less total mean athero-

use, using apoE

4.14 In these

and apoE

mice.4,14 In these

were similar in BMO and VWO aortas compared with control

in the control, BMO, and VWO mice aortas by incubating

fresh frozen sections of aortas with DHE. Aortas from control

mice had consistently increased DHE fluorescence compared

with aortas from BMO and VWO mice (Figure 1C and 1D).

Quantitative analysis of DHE fluorescence demonstrated a

increased ROS production and attenuated atherosclerotic lesion

formation in this murine model of atherosclerosis.

creased ROS production and attenuated atherosclerotic lesion

formation. To confirm the functional role of NAD(P)H oxidase in

monocytes/macrophages and vascular wall cells is nec-

essary for macrophage infiltration into atheroscle-

rotic lesions. A, Representative sections from fresh

frozen aortas (aortic arch) were stained for Mac-3. B, Adjacent sections from female mice were

stained with Y chromosome paint or Mac-3. Donor-derived Y chromosome positive cells (white

dots) are present in both macrophage enriched

noeintima (top left panel) and adventitia (bottom

left panel).

Results

Functional NAD(P)H Oxidase in Bone
Marrow–Derived Cells and the Vessel Wall Cells
Contributes to Aortic Atherosclerosis and
ROS Production

We have previously reported that reduced NAD(P)H oxid-
dase–derived O2

generation results in reduced atherosclero-
sis, using apoE

and apoE

mice.4,14 In these

studies, the deficiency of NAD(P)H oxidase was neither cell-
nor tissue-specific. Therefore, it was not possible to deter-
mine which among the cell types with the capacity to produce

O2

through activation of NAD(P)H oxidase were responsible for the observed decrease in atherosclerosis. To determine the

relative contribution of monocyte/macrophage NAD(P)H oxid-

dase to that of SMC and endothelial cells in atherosclerosis, we performed allologic sex-mismatched BMT from apoE

/p47phox

mice (control) showed that 7.5% of the aortic surface area was

covered with atherosclerotic lesions (Figure 1B). ApoE

mice that received bone marrow from apoE

/p47phox

mice, hence lacking functional NAD(P)H oxidase in bone

marrow–derived cells (monocytes/macrophages, BMO), had

53% less total mean lesion area (P<0.05) than the total mean

lesion area in apoE

mice. Similarly, apoE

/p47phox

mice (lacking functional NAD(P)H oxidase in aortic wall cells including endothelial cells, SMCs, and

fibroblasts, VWO) that received bone marrow from apoE

mice (functional NAD(P)H oxidase in marrow-derived

monocytes/macrophages) had 66% less total mean athero-
sclerotic lesion area than the total mean lesion area in apoE

mice (P<0.01; Figure 1B). These data indicate that

NAD(P)H oxidase activity in both monocytes/macrophages and aortic wall cells contributes to atherosclerotic lesion

formation.

To confirm the functional role of NAD(P)H oxidase in

monocytes/macrophages and vascular wall cells in athero-
sclerotic disease process, we measured O2

production in situ in the control, BMO, and VWO mice aortas by incubating

fresh frozen sections of aortas with DHE. Aortas from control

mice had consistently increased DHE fluorescence compared

with aortas from BMO and VWO mice (Figure 1C and 1D). Quantitative analysis of DHE fluorescence demonstrated a

63% decrease in O2

production in BMO and VWO aortas compared with control aortas (P<0.05). Consistent with decreased O2

production resulting from p47phox deficiency, no compensatory increases in the expression of other com-

ponents of the NAD(P)H oxidase were observed. Specifi-
cally, the expression of the catalytic subunits of Nox1 and

Nox4 isoforms and p22phox subunit of NAD(P)H oxidase

were similar in BMO and VWO aortas compared with control

aortas (supplemental Figure I). Together, these results indi-
cate that absence of a functional NAD(P)H oxidase in

monocytes/macrophages or vascular wall cells leads to de-

creased ROS production and attenuated atherosclerotic lesion

formation in this murine model of atherosclerosis.

Absence of a Functional NAD(P)H Oxidase in
Monocytes/Macrophages or Vascular Wall Cells
Results in Decreased Macrophage Infiltration Into
Atherosclerotic Lesions

To determine the effect of functional NAD(P)H oxidase on

histological composition of atherosclerotic lesions, fresh

frozen cross sections from the aortic arch of control, BMO,

and VWO mice were immunohistochemically stained for

presence of macrophages with anti–Mac-3 antibody (Figure
2A). Cross sections from control mice showed larger lesions enriched with macrophages (brown staining) and SMCs (staining not shown), whereas aortic sections from BMO and VWO mice had smaller lesions with fewer macrophages. These results indicate that a functional NAD(P)H oxidase is necessary in both the vessel wall and in the circulating cells for macrophage infiltration and SMC proliferation.

To confirm that monocytes/macrophages were derived from the donor bone marrow in the sex-mismatched allogenic transplantation used in the current study, cross sections from arterial arch in the control mice were stained with Y chromosome paint. As shown in Figure 2B, considerable number of Y chromosome–positive cells were present in macrophage enriched area of the neointima (top panel) and adventitia (bottom panel). These results indicate that absence of a functional NAD(P)H oxidase in either circulating inflammatory cells or vessel wall cells retards atherosclerotic lesion growth by curtailing the recruitment of macrophages to the endothelial surface during lesion initiation/progression. Further, these results confirm that macrophages infiltrate into the adventitia of the atherosclerotic lesions.17

NAD(P)H Oxidase in Bone Marrow–Derived Cells Plays a Critical Role in LDL Oxidation

One mechanism by which NAD(P)H oxidase promotes atherogenesis is through its effect on the composition of lipid levels.18,19 To assess whether the differences observed in the atherosclerotic burden among the 3 experimental mouse groups resulted from functionally important differences in plasma lipids, plasma cholesterol, triglyceride, and oxidized LDL (oxLDL) levels were determined in control, BMO, and VWO mice (Figure 3). Absence of NAD(P)H oxidase in either bone marrow–derived cells or vascular wall cells did not significantly affect plasma total cholesterol or triglyceride levels (Figure 3A and 3B). However, BMO mice had significantly lower (P<0.05) oxLDL levels compared with control and VWO mice (Figure 3C). These findings, together with the report that macrophage-mediated LDL oxidation is dependent on NAD(P)H oxidase,19,20 suggest that monocyte/macrophage oxidation of LDL contributes to the pathogenesis of atherosclerosis.

Expression of Cellular Adhesion Molecules at Luminal Surface of the Aorta Is Regulated by NAD(P)H Oxidase in the Vascular Wall Cells

Adherence of circulating blood monocytes to the vessel wall is one of the earliest events in atherogenesis, and an NAD(P)H oxidase inhibitor decreased atherosclerosis by attenuating cytokine-induced expression of cellular adhesion molecules (CAMs) and the adherence of monocytes to the endothelium.21 To determine whether the decrease in atherosclerosis observed in BMO and VWO mice resulted from inhibition of CAM expression, mRNA levels of vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, E-selectin, and P-selectin from aortas were analyzed by real-time RT-PCR (supplemental Figure II). VWO mice had significantly decreased expression of VCAM-1, ICAM-1 and P-selectin in their aortas compared with control and BMO mice. E-selectin expression levels were not significantly different among the 3 groups, though lower values were observed in the aortas of VWO mice.

We then performed immunohistochemical analysis of aortic cross sections to confirm the changes observed in mRNA expression of the CAMs (Figure 4). Immunoreactive VCAM-1 expression was abundant in the endothelium and medial SMCs of control and BMO mouse aortas, whereas it was sparse and confined to endothelium in the aortas of VWO mice. Similarly, ICAM-1 expression was low and restricted to endothelium in VWO mice compared with that in control and BMO mice wherein it was detected in both the endothelium and adventitia. Immunoreactive E-selectin was highly expressed in the endothelium and neointima of control and BMO mice, whereas it was absent in the aortic cross sections

Figure 3. Superoxide from bone marrow–derived circulatory cells plays a major role in LDL oxidation. Plasma total cholesterol (A), triglyceride (B), and oxLDL (C) levels (mean±SEM, *P<0.05) were measured in mice that were on Western diet for 12 weeks after allogenic sex-mismatched BMT.
of VWO mice. P-selectin was expressed in the endothelium of control and BMO mouse arteries and absent in the aorta of VWO mice. Together, these data indicate that NAD(P)H oxidases in monocytes/macrophages and vascular wall cells affect atherogenesis by at least 2 distinct mechanisms: (1) monocyte/macrophages induce LDL oxidation in a NAD(P)H oxidase–dependent manner; and (2) vascular wall cells express cell adhesion molecules in a NAD(P)H oxidase–dependent manner.

NAD(P)H Oxidase Deficiency Attenuates Neointimal Hyperplasia and Mitogenic Protein Activation in ApoE−/− Mice After Arterial Injury
NAD(P)H oxidase is necessary for neointimal hyperplasia after arterial injury. We previously demonstrated a significant decrease in neointimal lesions in p47phox−/− mice compared with wild-type mice in response to arterial injury.4 Here we investigated whether deficiency of apoE overrides the protection against neointimal hyperplasia afforded by a nonfunctional NAD(P)H oxidase. As expected, a statistically significant increase in neointimal hyperplasia (P<0.05) was observed in the femoral arteries of apoE−/− mice compared with that seen in sham operated mice, 4 weeks after injury (supplemental Figure III). Neointimal hyperplasia also increased in the femoral arteries of apoE−/−/p47phox−/− mice in response to injury, but the increase was less than that in apoE−/− mice and not statistically significant from that seen in sham operated apoE−/−/p47phox−/− mice.

Immunohistochemical staining of femoral artery cross sections for smooth muscle α-actin demonstrated that neointimal lesions are predominantly made up of SMCs4. To determine the molecular basis of SMC proliferation, we compared the activation of redox-sensitive mitogenic protein expression22,23—janus kinase 2 (JAK2), extracellular signal regulated kinase (ERK) 1/2, p38 mitogen-activated protein kinase (p38 MAPK), and signal transducer and activator of transcription 3 (STAT3)—in wire-injured femoral arteries of apoE−/− and apoE−/−/p47phox−/− mice (Figure 4). JAK2 and ERK1/2 phosphorylation increased substantially in the neointima of femoral arteries of apoE−/− mice than that in apoE−/−/p47phox−/− mice 4 weeks after injury. Similarly, significant increase in the expression of phosphorylated p38 MAPK and STAT3 was observed in the medial and neointimal SMCs of apoE−/− mice than that seen in apoE−/−/p47phox−/− mice. Together, these results indicate that NAD(P)H oxidase–stimulated activation of mitogenic signaling pathways is necessary for SMC proliferation after endothelial denudation injury in mice, even on a hypercholesterolemic background. Thus, SMC proliferation is likely an additional contributor to atherogenesis in apoE−/− mice impacted by NAD(P)H oxidase activity, together with LDL oxidation and adhesion molecule expression.

Confirmation of Redox-Sensitive Activation of Mitogenic Proteins in SMCs In Vitro
We next examined whether the NAD(P)H oxidase–dependent activation of SMC mitogenic proteins observed in vivo occurs in arterial SMCs in vitro. We treated SMCs isolated...
SMCs whereas no change in the phosphorylation of this protein was observed in p47phox−/− cells treated with thrombin (Figure 6C). Together, these results indicate NAD(P)H oxidase–derived ROS production modulates the activation of proteins involved in SMC proliferation and, in this manner, likely contributes to neointimal hyperplasia under pathophysiological conditions.

Discussion

Increased O$_2^-$ production plays an important role in the initiation and progression of atherosclerosis. Investigations in cultured cells, animal models, and human studies have led to the identification of NAD(P)H oxidases as a major source of O$_2^-$. Increased O$_2^-$ generation promotes atherosclerosis by several potential mechanisms. Superoxide anion promotes atherogenesis through the formation of oxidized lipids, particularly oxLDL. Monocytes/macrophages, the major inflammatory cell component of atherosclerotic lesions, induce LDL oxidation, a process previously shown to be dependent on NAD(P)H oxidase–derived O$_2^-$. In addition to LDL oxidation, NAD(P)H oxidase–derived O$_2^-$ has been implicated in endothelial dysfunction, increased expression of CAMs, and enhanced proliferation and migration of SMCs—factors that promote atherosclerosis. Superoxide also reacts with nitric oxide to produce peroxynitrite, a highly oxidizing atherogenic molecule which activates matrix metalloproteinases and facilitates vascular remodeling. Decreased peroxynitrite production and delayed vascular remodeling in response to increased shear stress has been observed in p47phox−/− mice compared with the wild-type mice.

We report here that NAD(P)H oxidase activity in monocytes/macrophages and vascular wall cells contributes equally to atherosclerotic lesion formation. Absence of functional NAD(P)H oxidase in bone marrow–derived monocytes/macrophages or vascular wall cells significantly decreased total aortic atherosclerotic lesion area in apoE−/− mice fed a Western diet. The protective effect against atherosclerosis in these 2 groups of mice correlated with decreased aortic O$_2^-$ production. These results extend our previous studies indicating that NAD(P)H oxidase plays an important role in atherogenesis. However, it is important to note that neither aortic atherosclerosis or O$_2^-$ production were abrogated, either in the present investigation or in our previous studies by the deficiency of p47phox in apoE−/− mice. The remnant ROS production in these mice might have come from other sources including Nox4-NAD(P)H oxidase, xanthine oxidase, cytochrome p450, and mitochondria. It is plausible that the remnant ROS production in the background of hypercholesterolemia could have resulted in the residual atherosclerosis observed in the apoE−/−/p47phox−/− mice.

Resting monocytes circulating in blood respond to an activating stimulus such as oxLDL, changes in vascular flow or infection, and adhere to activated endothelial cells in the vessel wall. The activated monocytes then typically extravasate into the subendothelial spaces where they mature into macrophages and contribute to lesion development by oxidizing LDL. Monocyte recruitment is critical for atheroscle-
rosis as lesion development is significantly decreased when monocytes are prevented from entering the vessel wall.30,31

The presence of fewer macrophages in atherosclerotic lesions in BMO and VWO mice is either a reflection of the smaller lesion size or a result of disruption of NAD(P)H oxidase activity in monocytes/macrophages or vascular wall cells, respectively. Absence of a functional NAD(P)H oxidase in the endothelial cells and SMCs in VWO mice may have contributed to decreased lesion size by attenuating expression of CAMs and the subsequent monocyte/macrophage infiltration.11 Nonfunctional NAD(P)H oxidase in bone marrow cells may have reduced the recruitment of monocytes into atherosclerotic lesions by significantly decreasing ox-LDL levels.32 It is also possible that absence of functional NAD(P)H oxidase decreases monocyte adhesive mechanisms at the endothelial interface. In this context, it is worth noting that an inhibitor of NAD(P)H oxidase attenuated the adherence of leukocytes on recombinant VCAM and P-selectin and reduced atherosclerotic lesion size in apoE-/- mice.21

Our current data on the presence of fewer macrophages in atherosclerotic lesions of BMO and VWO mice compared with that in control mice is seemingly contradictory to our previous observation that there was no difference in the homing of wild-type and p47phox-deficient macrophages to atherosclerotic lesions in apoE-/- mice.14 However, these results may have been influenced by the 2 different methods used in these studies. For example, alterations in the expression of CAMs that can occur during monocyte/macrophage isolation, labeling, and injection in homing studies may modify the interaction between circulating monocytes and the vascular wall.33

Neointimal hyperplasia in response to arterial injury was significantly less in apoE-/- mice that lack p47phox than in apoE-/- mice and was characterized by decreased levels of activated SMC mitogenic proteins, JAK2, ERK1/2, p38 MAPK, and STAT3 in neointimal SMCs (Figure 5).22,23 In vitro analysis confirmed the redox-sensitive regulation of these proteins in SMC (Figure 6). These data provide a molecular explanation for the observed decrease in proliferation of p47phox-/- SMCs compared with wild-type SMCs in response to agonist treatment.14 Our current results are in consonance with our previous findings that absence of p47phox protects against injury-induced arterial neointimal hyperplasia.4 In addition, these results indicate that absence of functional NAD(P)H oxidase protects against restenosis even on a hypercholesterolemic background.

In summary, the studies reported here confirm that NAD(P)H oxidase modulates the atherogenic phenotype in apoE-/- mice, and importantly, provide evidence for equal contribution of monocyte/macrophage and vessel wall NAD(P)H oxidase to aortic ROS production and atherosclerosis in apoE-/- mice. Furthermore, these data indicate that monocyte/macrophage NAD(P)H oxidase significantly affects LDL oxidation in vivo. Inhibition of NAD(P)H oxidase attenuates restenosis in response to arterial injury in apoE-/- mice, and the reduction in neointimal hyperplasia is possibly attained by the downregulation of SMC intracellular mitogenic signaling pathways activated by growth factors and cytokines.

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Disclosures

None.

References


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ONLINE DATA SUPPLEMENT

Animal Procedures
All mice were on C57BL/6 genetic background. ApoE and p47phox genotypes were determined by polymerase chain reaction (PCR) analysis of DNA extracted from tail biopsies. Animal care and procedures were performed according to the regulations of the Institutional Animal Care and Usage Committee of University of North Carolina at Chapel Hill. Animals were maintained in a 22°C room with a 12-hour light/dark cycle and given free access to food and water.

Bone marrow transplantation (BMT)
Bone marrow was harvested from 8-10-week-old male mice after euthanizing with CO2. Marrow cell were flushed from femurs and tibias with 2% BSA in PBS and erythrocytes were lysed using ACK lysis buffer (Biosource). Bone marrow cells were filtered, resuspended in PBS, and stored on ice until use. The cell number and viability was estimated using trypan blue. Age-matched female recipient mice were lethally irradiated (9 Gy) on the day of transplantation. Two million unfractionated bone marrow cells were injected into the retro-orbital plexus of each recipient mouse. The transplanted mice were maintained on Sufatrim in drinking water. Four weeks after BMT, successful engraftment was confirmed by determining the presence of Sry gene in the leukocytes of recipient mice using real-time PCR.

Quantification of atherosclerotic lesions
Atherosclerosis in the aortas was measured by quantitation of oil red O-positive lesions. At 12 weeks after feeding Western diet, the mice arterial trees were perfused with PBS and aortas were dissected from the heart to the iliac bifurcation. The aortas were cleared
of connective tissue and adventitial fat, and were fixed overnight in 4% paraformaldehyde (PFA). The aortas were rinsed in PBS, opened longitudinally, pinned flat on black wax in a dissecting pan, stained with oil red O, and counter stained with toulidine blue. The stained aortas were immersed in PBS and photographed using the Leica M420 Macroscope. The total aortic area and the lesion area were quantitated using NIH ImageJ software and the percent lesion area was calculated.

**Femoral artery injury and morphometry**

Ten-week-old mice were anesthetized with inhaled isoflurane. The femoral vessels were exposed by longitudinal groin incision and the superficial branch of femoral artery was dissected free from the adjacent nerve and vein. The distal portion of the vessel was encircled with 9-0 nylon suture; a vascular clamp was placed at the level of inguinal ligament. Hi-Torque Cross-It 200XT angioplasty guidewire (diameter 0.36 mm) (Guidant Corporation) was introduced into the arterial lumen through an arteriotomy made just proximal to the suture. After release of the clamp, the guidewire was advanced to the level of the aortic bifurcation and passed five times to denude the endothelium. The guidewire was then removed and the arteriotomy site ligated by tying the previously placed suture. The skin incision was closed with 6-0 silk sutures. The same injury procedure was carried out on contralateral side. Sham-operated arteries were dissected, temporarily clamped, and ligated without passing the guidewire. Animals were sacrificed and specimens collected at 28 days after injury.

The arterial tree was cleared by transcardial perfusion with 20 ml of PBS, followed by 20 ml of freshly prepared 4% PFA in PBS. Femoral arteries were post-fixed for 24 hours in
4% PFA at 4°C. Tissue samples containing the center-most 5 mm section distal to inguinal ligament were embedded in paraffin. Eight adjacent 5 µm sections were cut every 250 µm for the entire length of the artery and stained with combined Masson’s trichrome elastic stain. Morphometric analysis was performed using NIH ImageJ. Intimal and medial areas were determined in a blinded manner and intima/media (I/M) ratio was calculated.

**Measurement of ROS production in the aortas**

To measure *in situ* ROS production in the aortas, frozen cross sections of the aortas were stained with dihydroethidium (DHE) as described earlier.\(^1\) DHE reacts specifically with O\(_2^-\) and is converted to ethidium which intercalates DNA and the resultant fluorescence is an indirect measure of O\(_2^-\) levels.\(^2\) Fresh frozen sections (10 µM) from various regions of the aorta embedded in OCT medium (Sakura Finetek) were stained with 10 µM DHE in the dark for 5 min, rinsed, mounted and confocal imaging was performed using the Zeiss 510 laser scanning confocal microscope at excitation and emission settings of 485 and 585 nm, respectively. Results shown are typical of DHE staining from various regions of the aortas. Confocal images of DHE stained sections were quantified using NIH ImageJ software.

**RNA extraction and real-time RT-PCR**

Total RNA was extracted from whole aortas using MELT Total Nucleic Acid Isolation System (Ambion) following the manufacturer’s protocol. Reverse transcription was performed with 1 µg of total RNA using TaqMan Reverse Transcription Reagents Kit (Applied Biosystems). All TaqMan Gene Expression Assays were purchased from Applied Biosystems (VCAM-1: Mm00449197_m1, ICAM-1: Mm00516023_m1, E-
selectin: Mm00441278_m1, P-selectin: Mm00441295_m1, Nox1: Mm00549170_m1, Nox2: Mm00432775_m1, Nox4: Mm00479246_m1, p22phox (Cyba): Mm00514478_m1 and Eucaryotic 18s RNA: Hs00999999_s1). Real-time PCR was carried out using the ABI PRISM 7900 HT Sequence Detection System and TaqMan PCR Master Mix according to the manufacturer’s recommendations. Sequence Detection System software (v 2.1) (Applied Biosystems) was used for raw data processing and analysis. The data for each gene were analyzed by REST2005 (Relative Expression Software Tool)⁴. Expression level of each gene was determined by normalization to 18s ribosomal RNA levels.

**Immunohistochemistry**

Representative sections of aortas and femoral artery were stained using rat anti-mouse Mac-3, VCAM-1, E-selectin and P-selectin monoclonal antibodies and hamster anti-mouse ICAM-1 monoclonal antibody (BD Pharmingen), rabbit anti-phospho JAK2 monoclonal antibody (Epitomics), rabbit anti-phospho ERK1/2 polyclonal antibody, rabbit anti-phospho p38 MAPK polyclonal antibody, and rabbit anti-phospho STAT3 polyclonal antibodies (Cell Signaling Technology). Arterial cross sections were also stained with rabbit anti-Nox1, Nox2, Nox4 and p22phox polyclonal antibodies (Santa Cruz Biotechnology). Immunostaining was carried out using Vectastain Elite ABC Kit, Vector M.O.M. Immunodetection Kit and Vector DAB Substrate Kit (Vector Laboratories, Inc.) according to manufacturer recommendations. The sections were counterstained with Vector Methyl Green and permanently mounted with VectaMount Mounting Medium (Vector Laboratories, Inc.).

**Cell culture and Western analysis**
Vascular smooth muscle cells (SMC) were isolated from the aortas of 4-month-old male wild-type and p47phox−/− mice and cultured as described. The cells were growth-arrested for 72 hours prior to treatment with thrombin (1U/ml) for 1, 5, 10 and 30 minutes. Preparation of cell lysates and immunoblotting were performed as described. Antibodies used were phosphorylated ERK1/2, ERK1/2, phosphorylated p38 MAPK, p38MAPK, phospho JAK2 (Cell Signaling Technology), and JAK2 (Upstate Biotechnology).

References


SUPPLEMENTAL FIGURE LEGENDS

**Figure I.** Absence of p47phox does not affect the expression levels of Nox isoforms or p22phox in aortic wall. A, Real-time RT-PCR analysis of gene expression in aortas of mice fed Western diet for 12 weeks following allogenic sex-mismatched BMT (mean ± SEM, n=7). B, Representative cross sections of thoracic aortas were stained for immunoreactive Nox1, Nox4 and p22phox.

**Figure II.** Absence of functional NAD(P)H oxidase in arterial wall cells leads to decreased expression of VCAM-1, ICAM-1, E-selectin and P-selectin. Real-time RT-PCR analysis of gene expression in aortas of mice fed a Western diet for 12 weeks following allogenic sex-mismatched BMT (mean ± SEM, n=7, * p<0.01, **p<0.05).

**Figure III.** Absence of functional NAD(P)H oxidase attenuates neointima formation in response to vascular injury. A, Representative cross sections of femoral arteries from apoE−/− and apoE−/−/p47phox−/−, 28 days after arterial injury, were stained with Masson’s trichrome elastic stain. B, Morphometric analysis of femoral artery sections (mean ± SEM, n=9, * p<0.05 vs respective control).
Supplemental Figure I
Supplemental Figure II
Supplemental Figure III

A

B

Intima/media ratio

Sham  Injury  Sham  Injury

ApoE\(^{-/-}\)  ApoE\(^{-/-}/p47\text{phox}\)^{-/-}

0.00  0.25  0.50  0.75

*