NADPH Oxidase Deficiency Exacerbates Angiotensin II–Induced Abdominal Aortic Aneurysms in Mice

Yasuyoshi Kigawa, Takuro Miyazaki, Xiao-Feng Lei, Tomoya Nakamachi, Tatsunori Oguchi, Joo-ri Kim-Kaneyama, Matsuo Taniyama, Shohko Tsunawaki, Seiji Shioda, Akira Miyazaki

Objective—Although nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2) is reportedly essential for phagocyte host defenses, it has been found to aggravate atherosclerosis in apolipoprotein E (Apoe)-null mice through excess production of superoxide. We therefore assessed the role of NOX2 in an experimental model of abdominal aortic aneurysm (AAA) and assessed the mechanism of NOX2 action in AAA.

Approach and Results—AAA was induced in low-density lipoprotein receptor-null (Ldlr<sup>−/−</sup>) mice by infusing angiotensin II. Nox2 expression was elevated in the abdominal aortae of these mice during infusion of angiotensin II, with enhanced Nox2 expression mainly because of the recruitment of NOX2-enriched macrophages into AAA lesions. Unexpectedly, systemic Nox2 deficiency promoted AAA development but reduced the level of reactive oxygen species in AAA lesions. Nox2 deficiency stimulated macrophage conversion toward the M1 subset, enhancing expression of interleukin (IL)-1β and matrix metalloproteinase-9/12 mRNA. Administration of neutralizing antibody against IL-1β abolished AAA development in Nox2-deficient mice. Bone marrow transplantation experiments revealed that AAA aggravation by Nox2 deficiency is because of bone marrow–derived cells. Isolated bone marrow–derived macrophages from Nox2-null mice could not generate reactive oxygen species. In contrast, IL-1β expression in peritoneal and bone marrow–derived macrophages, but not in peritoneal neutrophils, was substantially enhanced by Nox2 deficiency. Pharmacological inhibition of Janus kinase/signal transducers and activators of transcription signaling inhibited excess IL-1β expression in Nox2-deficient macrophages, whereas matrix metalloproteinase-9 secretion was constitutively stimulated via nuclear factor-κB signals.

Conclusions—Nox2 deficiency enhances macrophage secretion of IL-1β and matrix metalloproteinase-9, disrupting tissue-remodeling functions in AAA lesions. These actions are unfavorable if NOX2 is to serve as a molecular target for AAA. (Arterioscler Thromb Vasc Biol. 2014;34:2413-2420.)

Key Words: aortic aneurysm, abdominal ▪ cytokines ▪ macrophages ▪ NADPH oxidase ▪ oxidative stress

Abdominal aortic aneurysm (AAA) is a major cause of cardiovascular deaths and the tenth leading cause of death in men >65 years of age in Western countries. The prevalence of AAA in the elderly has been estimated at 8.8%,<sup>2</sup> with a mortality rate of 80% after AAA rupture.<sup>3</sup> Although AAA rupture is life threatening, molecular targets for this disease have not yet been identified. AAA is often treated surgically, with artificial vessels or stent grafts.<sup>4</sup> To reduce surgical exposure and its accompanying side effects, various candidate molecules targeting AAA, including nicotinamide adenine dinucleotide phosphate oxidase (NOX) isozymes, are currently undergoing experimental trials.

NOX isozymes, which are composed of catalytic subunits (eg, NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2) and subcomponents (eg, p22<sup>vho</sup>, p47<sup>phox</sup>, p67<sup>phox</sup>, Rac1, and Rac2), stimulate the production of reactive oxygen species, including superoxide and hydrogen peroxide.<sup>5</sup> After the cloning of the gene that encodes NOX2 (Cybb),<sup>6,7</sup> the role of NOX systems could be investigated in phagocytic and host defense functions, including the respiratory burst.<sup>8</sup> Nonphagocyte NOX homologs were identified subsequently and characterized functionally in other cell types, including cells that make up vascular components.<sup>9</sup> NOX2 expression was observed recently in vascular endothelial and macrophage-derived foam cells in atheromas of apolipoprotein E (Apoe)-null mice, with a deficiency in Nox2 suppressing atherosclerotic development in mice.<sup>10</sup> In addition, suppression of Nox1 as well as the common cytosolic subcomponent p47<sup>phox</sup> inhibited AAA pathogenesis in mouse models.<sup>11,12</sup> Taken together, these findings indicated that excess NOX activation augments atherosclerosis and AAA.

A recent meta-analysis showed that antioxidants, such as vitamin E and β-carotene, failed to prevent the development of atherosclerosis and related cardiovascular events,<sup>13</sup> suggesting that antioxidants may be clinically ineffective in patients with atherosclerotic diseases. More specific clinical approaches,
targeting certain molecules, may be more promising because targeting redox-related *Nox* genes suppressed atherosclerotic disease in mice. However, the heterogeneity of biological redox systems complicates the understanding of the molecular basis of these diseases. For example, *Nox4*, which stimulates hydrogen peroxide production in vascular endothelial cells, has been reported to play an angiogenic and anti-inflammatory role under ischemic and inflammatory conditions, indicating that hydrogen peroxide may exhibit vasoprotective effects in certain situations. Moreover, genetic defects in phagocytic NOX systems have been found to induce chronic granulomatous disease, with most patients with chronic granulomatous disease experiencing systemic sterile hyperinflammation. Indeed, myeloid-specific deficiency of p47^(phox)^ was shown sufficient for aggravating zymosan-induced lung inflammation, suggesting that NOX systems in phagocytes may possess anti-inflammatory-like properties.

The roles of NOX systems in specific diseases, including the pathophysiological significance of NOX isozymes, except for NOX1 and p47^(phox)^, in AAA, remain unclear. We therefore sought to characterize the roles of NOX2 in AAA using a gene-targeting approach.

### Materials and Methods

An expanded materials and methods section is available in the online-only Supplement.

### Results

**Loss of Nox2 Exacerbates Angiotensin II–Induced AAA in Mice**

AAA was induced in high-cholesterol diet–fed *Nox2^+/y/Ldlr–/–* mice by infusing angiotensin II (AngII). Real-time reverse transcriptase polymerase chain reaction showed high levels of *Nox2* mRNA expression in the ascending and abdominal aorta after 28 days of AngII infusion (Figure 1A). In contrast, the levels of *Nox1* and *Nox4* mRNA were not altered significantly. Immunohistochemical analysis showed the presence of NOX2-positive cells in the adventitia of AAA lesions in *Nox2^+/y/Ldlr–/–* but not in *Nox2^–/–Ldlr–/–* mice (Figure 1B; Figure I in the online-only Data Supplement), with these cells corresponding to monocyte/macrophage antigen-2 (MOMA-2)–positive macrophages (Figure 1C). In contrast, NOX2 expression was negligible in vascular endothelial cells and smooth muscle cells.

Next, *Nox2^+/y/Ldlr–/–* and *Nox2^–/–Ldlr–/–* mice were examined for AAA. The maximal diameter and wet weight of AAAs were significantly greater in *Nox2^+/y/Ldlr–/–* than in *Nox2^–/–Ldlr–/–* mice (Figure 2A; Figure II in the online-only Data Supplement), although there were no statistically significant differences in ascending aortic lesions (Figure III in the online-only Data Supplement) and Kaplan–Meier survival curves (Figure 2B). Interestingly, the sizes of AngII-enhanced atherosclerotic lesions in the ascending/descending aortic arch and thoracic aorta of *Nox2^+/y/Ldlr–/–* and *Nox2^–/–Ldlr–/–* mice were comparable (Figure 2C), as was AngII-induced elevation of systolic blood pressure (Figure IV in the online-only Data Supplement). Real-time reverse transcriptase polymerase chain reaction showed that the expression of interleukin (IL)-1β mRNA (*Il1b*) in AAA lesions was greater in *Nox2^+/y/Ldlr–/–* than in *Nox2^–/–Ldlr–/–* mice.

### Figure 1. Nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2) is abundantly expressed in macrophages in adventitial regions of abdominal aortic aneurysm (AAA). AAAs were generated by infusing high-cholesterol diet–fed Nox2 wild-type mice (*Nox2^+/y/Ldlr–/–*) with angiotensin II (AngII). Saline infusion served as a control. A. NOX2 isozyme is selectively upregulated in the abdominal aorta during generation of AAA. Levels of NOX isozyme mRNAs in the ascending, descending, and abdominal aorta were quantified by real-time reverse transcriptase polymerase chain reaction and normalized relative to *Hprt1* mRNA expression in the same samples (n=5–14). ^*^P<0.05 vs time-matched saline control. B. Adventitial localization of NOX2 in AAA lesions. As a negative control anti-NOX2 antibody was incubated with AAA lesions in *Nox2*-deficient mice. C. NOX2 is localized to adventitial macrophages in AAA lesions in *Nox2* wild-type mice. Monocyte/macrophage antigen-2 (MOMA-2) served as a macrophage marker. A indicates adventitia, and L, vascular lumen.
(Figure 2D), and immunohistochemistry showed enrichment of IL-1β in MOMA-2–positive macrophages present in the adventitial regions of Nox2+/y/Ldlr−/− mice (Figure 2E; Figure V in the online-only Data Supplement). Immunohistochemical assays also showed that recruitment of MOMA-2–positive macrophages in the adventitial regions was equivalent in Nox2+/y/Ldlr−/− and Nox2−/y/Ldlr−/− mice (Figure 2F), and that the abundance of M1 macrophages (inducible nitric oxide synthase (iNOS)-positive/MOMA-2+ cells) in the adventitial regions was further potentiated by Nox2 deficiency (Figure 2G).

Real-time reverse transcriptase polymerase chain reaction showed that basic fibroblast growth factor (Fgf2) and type IV collagen (Col4) mRNA expression declined as a result of Nox2 deficiency, whereas the expression of transforming growth factor-β (Tgfb1), fibronectin (Fn1), and type I collagen (Col1a1) mRNAs remained unchanged (Figure 3A). Immunohistochemical staining revealed collagen IV localization in the adventitial regions of AAA in Nox2+/y/Ldlr−/− mice (Figure 3B; Figure VI in the online-only Data Supplement), but not in mice with a Nox2 deficiency. Dihydroethidium staining showed that oxidative stress in AAA lesions was much greater in Nox2+/y/Ldlr−/− than in Nox2−/y/Ldlr−/− mice (Figure 3C; Figure VII in the online-only Data Supplement). This was matched with higher superoxide levels in Nox2+/y/Ldlr−/− AAA lesions comparing with those in Nox2−/y/Ldlr−/− lesions (Figure 3D). Consistently, immunohistochemistry showed that Nox2 deficiency markedly diminished nitrotyrosine staining, indicating reductions in oxidative damage in AAA lesions by Nox2 deficiency (Figure 3E; Figure VIII in the online-only Data Supplement). Interestingly, in situ zymography indicated that gelatinase activity in adventitial regions was elevated by Nox2 deficiency, whereas that in medial lesions was not substantially influenced (Figure 3F; Figure IX in the online-only Data Supplement). Similarly, the levels of expression of matrix metalloproteinase 2 (Mmp2) and tissue inhibitor of MMP (Timp1, Timp2, and Timp3 mRNA were equivalent in AAA lesions of Nox2+/y/Ldlr−/− and Nox2−/y/Ldlr−/− mice. In contrast, Nox2 deficiency enhanced the expression of Mmp9 and Mmp12 mRNA (Figure 3G).

Contribution of IL-1β to exacerbation of AAA in Nox2-deficient mice was assessed. The AngII-infused Nox2-deficient
The contribution of bone marrow NOX2 to AAA pathogenesis was assessed by performing bone marrow transplantation experiments. Polymerase chain reaction–based genotyping validated bone marrow chimerism in recipient mice (Nox2+/y/Ldlr−/−) transplanted with Nox2+/y/Ldlr−/− bone marrow cells (chimeric mice), but not with Nox2−/y/Ldlr−/− bone marrow cells (nonchimeric mice; Figure 5A). Because both Nox2 wild-type and knockout alleles were detected in AAA lesions in the chimeric mice, the Nox-2-deficient bone marrow cells were likely recruited into AAA lesions originally composed of Nox2-expressing resident cells. After infusion of AngII, Nox2-chimeric mice showed a higher rate of rupture-associated mortality than nonchimeric mice (Figure 5B). Isolated abdominal aortas from survivors (8/19) showed significant expansion of AAA lesions compared with nonchimeric survivors (8/10; Figure 5C). The levels of expression of IL-1β (Il1b) mRNAs were higher in AAA lesions of chimeric than nonchimeric mice (Figure 5D), although their systolic blood pressure was equivalent (Figure XI in the online-only Data Supplement).

**Nox2 Deficiency Enhances IL-1β Secretion From Interferon-γ-Primed M1 Macrophages**

Murine bone marrow cells differentiate into classically (M1) and alternatively (M2) activated bone marrow–derived macrophages (BMMs) in response to stimulation with interferon (IFN)-γ and IL-4, respectively. Nox2 mRNA was induced by IFN-γ, but not by IL-4, in Nox2−/y/Ldlr−/− BMMs, but was abolished in Nox2+/y/Ldlr−/− BMMs (Figure 6A). Nox1 and Nox4 expression in BMMs was originally negligible and compensatory changes were not induced by Nox2 deficiency. Dichlorofluorescein assays indicated that the intracellular oxidative stress in Nox2−/y/Ldlr−/− BMMs, induced by IL-4 or IFN-γ, was inhibited by Nox2 deficiency or the addition of the free radical scavenger N-acetylcysteine (Figure 6B). Similarly, the induction of H₂O₂ by IL-4 or IFN-γ was abolished by Nox2 deficiency (Figure 6C).

Figure 3. Extracellular matrix deposition in angiotensin II (AngII)–induced abdominal aortic aneurysm (AAA) lesions is disrupted by nicotinamide adenine dinucleotide phosphate oxidase 2 (Nox2) deficiency. A, Nox2 deficiency reduces basic fibroblast growth factor and collagen IV mRNA expression levels in AAA lesions, as determined by real-time reverse transcriptase polymerase chain reaction. Results were normalized relative to Hprt1 mRNA expression and reported as fold changes (n=5–9). *P<0.05. B, Deposition of collagen IV in adventitial regions in AAA is inhibited by the loss of Nox2. C, Dihydroethidium-based fluorometry for detecting oxidative stress in AAA lesions. Serial sections supplemented with the free radical scavenger N-acetylcysteine (NAC) served as negative controls. D, Lucigenin-based chemiluminescence assay for measuring superoxide in AAA lesions. After chemiluminescence measurement in AAA lesions, cell-permeant radical scavenger Tyron was added to the reactants to confirm their baseline chemiluminescence. The baseline levels were subtracted from lucigenin chemiluminescence levels in AAA lesions, which were further normalized by their tissue weights (n=8). *P<0.05. E, Nox2 deficiency reduces nitrotyrosine expression in AAA lesions. F, Nox2 deficiency does not markedly affect gelatinase activity in medial regions of AAA. Serial sections supplemented with EDTA served as negative controls. G, Nox2 deficiency upregulates matrix metalloproteinase-9 (Mmp9) and Mmp12 mRNA expression (n=5–9). *P<0.05. A indicates adventitia; and L, vascular lumen.
IFN-γ-primed Nox2+/−/Ldlr−/− BMMs showed induction of the M1 markers inducible nitric oxide synthase (Nox2), IL-1β, IL-6, and TNF-α expression, but not TNF-α expression, being robustly stimulated by Nox2 deficiency (Figure 6D; Figure XIIA in the online-only Data Supplement), resulting in IL-1β secretion (Figure 6E). Overexpression of IL-1β by Nox2 deficiency is similarly detectable in TNF-α-stimulated or IFN-γ-stimulated peritoneal macrophages, but not in neutrophils (Figure 6F). IFN-γ treatment stimulated signal transducers and activators of transcription 1 (Stat1; Figure 6G) and inflammasome-related genes (Cas1, Nlrp1b, and Nlpr3; Figure XIIIB in the online-only Data Supplement) in BMMs without altering IFN-γ receptor mRNA expression levels (Ifngr1 and Ifngr2; Figure XIIIC in the online-only Data Supplement). This induction of Stat1, but not of inflammasome-related genes, was enhanced by Nox2 deficiency. Treatment of Nox2+/−/Ldlr− or Nox2+/−/Ldlr− BMMs with AG490, an inhibitor of Janus kinase/STAT signals, abolished IL-1β upregulation by IFN-γ (Figure 6H). IL-4-primed Nox2+/−/Ldlr− BMMs induced the M2 marker arginase-1 (Arg1), the expression of which was accelerated by Nox2 deficiency, although its rate of increase was not >2-fold (Figure 6I). Nox2 deficiency constitutively upregulated Mmp9 mRNA expression (Figure 6J), whereas the expression levels of Mmp12 and transforming growth factor-β (Tgfb1) mRNA in Nox2+/−/Ldlr− and Nox2+/−/Ldlr− BMMs were similar, even in response to cytokines (Figure XIID and XIIE in the online-only Data Supplement). The results of gelatin zymography were consistent with the Mmp9 mRNA expression (Figure 6J). Induction of MMP9 by Nox2 deficiency was suppressed by BAY11-7082, an inhibitor of nuclear factor-κB (NF-κB) signals, in the presence or absence of IFN-γ (Figure 6K). Expression levels of 1xB (Nfkbia, Njkibh, and Nfkbie) in BMMs were declined by the Nox2 deficiency even in the absence of cytokine stimulus (Figure 6L).

Discussion

The present study reveals that Nox2 deficiency results in harmful outcomes in an animal model of AAA. Because an absence of Nox2 has been found to upregulate proinflammatory responses (eg, production of IL-1β, IL-6, and TNF-α), thereby aggravating arthritis,13,14 systemic inflammatory response syndrome,19 as well as chronic granulomatous disease,20 Nox2 may possess anti-inflammatory-like effects even in inflammatory vascular diseases. Consistent with previous findings,20 we found that NOX2 contributes to reactive oxygen species generation in macrophages, although we also found that a Nox2 deficiency in macrophages potentiated the secretion of IL-1β and MMP9. IL-1β and MMPs was similarly upregulated in aggravated AAA lesions of Nox2-null mice, suggesting that a deficiency in Nox2 markedly disturbs proinflammatory cascades in macrophages present in AAA lesions.

IL-1β is normally synthesized by intracellular transcriptional systems as an immature, biologically inactive pro–IL-1β. This molecule is proteolytically converted to mature IL-1β by caspase-1–based inflammasomes.21 A deficiency in Nox2 or p47phox was found to significantly upregulate lipopolysaccharide (LPS)-induced IL-1β expression in lung, accompanied by the activation of activator protein-1 and NF-κB transcriptional systems.22 Moreover, a loss-of-function mutation in p47phox caused inflammatory skin lesions in mice concomitant with the upregulation of IL-1β, with both suppressed by the genetic reintroduction of wild-type p47phox.23 Monocytes from chronic granulomatous disease patients lacking p47phox have been found to produce higher amounts of IL-1β in response to LPS than monocytes from healthy subjects, an increase likely because of the upregulation of caspase-1–based inflammasomes.24 Thus, a lack of NOX systems enhances IL-1β production in monocytes/macrophages. Current results further showed that aggravation of AAA in Nox2-deficient mice, which is similarly inducible...
by myeloid-specific deficiency of Nox2, was resolved by systemic neutralization of IL-1β. It is noteworthy that NOX2 is expressed mainly in macrophages and neutrophils among the myeloid-derived cells, and that myeloid-specific suppression of Nox2 significantly upregulated IL-1β expression in AAA lesions; thus, it is likely that IL-1β derived from macrophages and neutrophils is responsible for accelerating AAA in Nox2-deficient mice. Our results consistently showed that IFN-γ–stimulated or TNF-α–stimulated peritoneal macrophages as well as IFN-γ–stimulated BMMs exhibited excess induction of IL-1β under the Nox2-null conditions, and the latter cells displayed acceleration of STAT1 mRNAs, but not of mRNAs encoded by inflammasome-related genes, such as Casp1 and Nlrp1a (Figure X in the online-only Data Supplement).

Figure 6. Nicotinamide adenine dinucleotide phosphate oxidase 2 (Nox2) deficiency converts M1 macrophages to an overactive state. Macrophage colony-stimulating factor (50 ng/mL)–differentiated bone marrow–derived macrophages (BMMs) were stimulated with interferon (IFN)-γ (10 ng/mL) and interleukin (IL)-4 (10 ng/mL) for 16 hours, to skew them toward M1 and M2 subsets, respectively. Peritoneal macrophages and neutrophils were stimulated with IFN-γ (50 ng/mL) and tumor necrosis factor (TNF)-α (10 ng/mL) for 16 hours.

A. Loss of Nox2 in BMMs is not compensated for by increased expression of other Nox isozymes. Levels of Nox mRNAs in BMMs were determined by real-time reverse transcriptase polymerase chain reaction, normalized relative to Gapdh expression and reported as fold changes (n=4). *P<0.05. B. Loss of Nox2 depletes oxidative stress in M1 and M2 macrophages. Macrophages were incubated with IFN-γ or IL-4, plus 10 mmol/L N-acetylcysteine (NAC) and the cells were assessed fluorometrically (n=3). *P<0.05. C. Loss of Nox2 depletes H2O2 generation in M1 and M2 macrophages. Amplex Red assays were performed in IFN-γ–polarized and IL-4–polarized BMMs (n=4–6). *P<0.05. D. Nox2 deficiency enhances differentiation into M1 macrophages and IL-1β mRNA expression (n=4). *P<0.05. E. Secretion of IL-1β by IFN-γ/lipopolysaccharide costimulated BMMs is elevated in the absence of Nox2. The concentration of IL-1β in the culture medium was determined by ELISA (n=3). *P<0.05. F. Peritoneal macrophages but not neutrophils exhibit overexpression of IL-1β mRNA by Nox2 deficiency. (n=3–4). *P<0.05. G. Loss of Nox2 induces signal transducers and activators of transcription 1 (Stat1) mRNA expression in IFN-γ–differentiated BMMs (n=4). *P<0.05. H. IFN-γ induction of IL-1β mRNA in Nox2+/y/Ldlr–/– and Nox2–/y/Ldlr–/– BMMs is mediated mainly through Janus kinase/STAT signals. Cells were incubated with the Janus kinase/STAT inhibitor AG490 at the indicated concentrations together with IFN-γ. Results are represented as fold changes compared with IFN-γ alone (n=4). *P<0.05 vs untreated. I. Nox2 deficiency constitutively increased matrix metalloproteinase-9 (Mmp9) mRNA expression (n=4). *P<0.05. J. Enhancement of Mmp9 activity in IFN-γ–primed BMMs by Nox2 deficiency. Gelatinase activity was determined by gelatin zymography. K. Upregulation of Mmp9 in BMMs by Nox2 deficiency is mediated mainly through nuclear factor-κB signals. Cells were incubated with 10 μmol/L of the nuclear factor-κB inhibitor Bay11-7082 (n=3). *P<0.05. L. Downregulation of inhibitor of κB in macrophage colony-stimulating factor–differentiated BMMs by Nox2 deficiency. Gapdh expression served as an internal control. (n=5–6). *P<0.05.
to macrophages, TNF-α–induced or IFN-γ–induced IL-1β expression was equivalent between the Nox2-wild-type and Nox2-deficient neutrophils. This is matched with previous report suggesting that Nox2 deficiency did not enhance but rather declined inflammasome-derived IL-1β in neutrophils.26 Taken together, excessive IL-1β derived from overactivated macrophages may be primary cue for exacerbation of AAA in Nox2-deficient mice. These deleterious roles of IL-1β in AAA are consistent with earlier results showing that the suppression of IL-1β or its receptor expression ameliorated AAA in mouse models.27

Macrophages in inflamed lesions originate from bone marrow cells.28 After emigration from bone marrow, monocytes patrol systemic blood vessels and are recruited into inflamed lesions by gradients of inflammatory cytokines and chemokines (eg, chemokine [C-C motif] ligand2). Mouse monocytes normally differentiate into M1- and M2-activated macrophages in response to T helper 1 (eg, IFN-γ and Toll-like receptor ligands) and T helper 2 (eg, IL-4 and IL-13) cytokines, respectively. Transplantation of aortic allografts into IFN-γ receptor null mice led to the formation of aneurysms,29 which may be because of excess secretion of MMP12 by IL-4–primed macrophages. Indeed, targeting of IL-4 signals reduced aneurysm formation. Conversely, the prevalence of M1 macrophages was found to be elevated in ruptured, but not intact, human cerebral aneurysms,30 suggesting that an imbalance between M1 and M2 macrophages can trigger extracellular matrix degradation and subsequent instability of AAA. We found that Nox2 deficiency promoted IFN-γ–primed conversion of BMMs to M1 macrophages, as well as potentiating the predominance of M1 macrophages in AAA lesions, suggesting that macrophages may be polarized preferentially into the M1 subset even in AAA lesions. Because most macrophages in AAA lesions express M1 markers, it was not surprising that a Nox2 deficiency contributes to the exacerbation of AAA, by skewing toward M1 macrophages.

Although MMPs play a key role in maintaining physiological extracelluar matrix turnover, excess MMP secretion during the inflammatory process has been found to augment AAA. In particular, macrophage-derived and vascular cell–derived MMP9 and MMP12 are considered major exacerbators of AAA,31,32 because they break down vascular structures, including the elastic lamellae and basement membrane. We found that the expression levels of MMP9 and MMP12, but not of MMP2, in AAA lesions, were elevated by Nox2 deficiency. Moreover, MMP9 in Nox2-deficient BMMs seemed to be constitutively upregulated via NF-κB signals. Our in situ zymography data suggested that Nox2 deficiency potentiated MMP activity in adventitial, but not medial, regions in AAs. Because Nox2 deficiency decreased the deposition of type IV collagen in AAA lesions, deficiency-induced excess MMP expression likely prevents type IV deposition, particularly in adventitial regions. Reducing the content of specific extracellular matrix may reduce vascular stiffness, thereby accelerating aortic dissection.

The results presented here highlight the potential concerns of targeting Nox2 to treat AAA. Although earlier study displayed that inhibition of Nox2 using small interfering RNA suppressed proinflammatory MMPs on macrophages,33 our present data show that Nox2 deficiency by gene targeting leads macrophages to hyperinflammatory phenotype. It is difficult to determine the mechanisms by which such opposite phenotypes arise; nevertheless, unexpected occurrence of hyperinflammation by Nox2 intervention is worthy to be attended. It is important to carefully monitor such hyperinflammatory phenotype when Nox2 intervention is conducted in future clinical study. This probably suggests the importance of choosing an appropriate NOX subtype(s) as a molecular target for AAA. Because Nox1 deficiency has been reported to ameliorate AAA, targeting this molecule may be successful in treating AAA. However, additional studies are needed to assess the impact of other vascular NOX subtypes on AAA.

Sources of Funding

This study was supported in part by a Grant-in-Aid for Scientific Research (26461368 to A. Miyazaki) and a Showa University Research Grant for Young Researchers, a Grant-in-Aid for Young Scientists (24790784) from the Japan Society for the Promotion of Science, a research grant from Takeda Science Foundation, a research grant from Banyu Life Science Foundation International, and a Dr Hiroshi Irisawa and Dr Aya Irisawa Memorial Research Grant from Japan Heart Foundation (all to T. Miyazaki).

Disclosures

None.

References

Nicotinamide adenine dinucleotide phosphate oxidase (NOX) systems are considered candidate molecular targets in the treatment of chronic inflammatory diseases, including abdominal aortic aneurysms because NOX-derived oxidative stress has been found to contribute to most inflammatory disorders. However, recent advances in gene-targeting techniques have shown that some NOX isozymes (eg, NOX4) exert vasoprotective roles in certain situations. Using a murine model of abdominal aortic aneurysm, we showed that deficiency augmented inflammatory disorders. However, recent advances in gene-targeting techniques have shown that some NOX isozymes (eg, NOX4) exert vasoprotective roles in certain situations. Using a murine model of abdominal aortic aneurysm, we showed that deficiency augmented inflammatory disorders. However, recent advances in gene-targeting techniques have shown that some NOX isozymes (eg, NOX4) exert vasoprotective roles in certain situations. Using a murine model of abdominal aortic aneurysm, we showed that deficiency augmented inflammatory disorders.

Significance


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Arterioscler Thromb Vasc Biol. 2014;34:2413-2420; originally published online September 4, 2014;
doi: 10.1161/ATVBAHA.114.303086

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/11/2413

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Supplemental Figures and Tables

NOX2 deficiency exacerbates angiotensin II-induced abdominal aortic aneurysms in mice

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Running title: NOX2 deficiency up-regulates IL-1β in macrophages

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Supplemental figure I. NOX2 is localized in adventitia in AAA lesions in Nox2*+/y/Ldlr−/− mice, but not in Nox2*−/−/Ldlr−/− mice. AAA was induced by AngII infusion. Arrows represent NOX2 expression. A: adventitia, L: vascular lumen.
Supplemental figure II. AngII-induced formation of AAA is accelerated by Nox2 deficiency.
Supplemental figure III. *Nox2* deficiency does not significantly affect the diameter in ascending aorta. Maximal diameter of AAA lesions between NOX2-wild-type and deficient mice was compared. Results were compared by Student’s t-test. There are no statistically significant differences between *Nox2*-wild-type and -deficient mice.
Supplemental figure IV. Systolic blood pressure and body weight in AAA mice. (A) Systolic blood pressure was monitored by using tail-cuff method (MK-2000; Muromachi Kikai Co, Ltd.; Tokyo, Japan). Values are represented as mean±SE (n=7). Multiple comparisons were conducted by two-way repeated-measures ANOVA. There are no statistically significant differences between Nox2-wild-type and -deficient mice. (B) Body weight in Nox2 wild type and deficient mice. Values are represented as mean±SE (n=4-7). Results were compared by two-way repeated-measures ANOVA. There are no statistically significant differences between those mice.
Supplemental figure V. Accumulation of IL-1β in AAA lesions in Nox2^{+/y}/Ldlr^{-/-} mice. Arrows represent IL-1β expression. A: adventitia, L: vascular lumen.
Supplemental figure VI. Impaired deposition of collagen IV in AAA lesions in Nox2<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice. Arrows represent collagen IV expression. A: adventitia, L: vascular lumen.
Supplemental figure VII. ROS generation in AAA lesions is abolished by Nox2 deficiency. The serial sections that were incubated together with ROS scavenger N-acetyl cysteine (NAC) served as a negative control. A: adventitia, L: vascular lumen.
Supplemental figure VIII. Adventitial nitrotyrosine expression in AAA lesions is diminished by Nox2 deficiency. A: adventitia, L: vascular lumen.
Supplemental figure IX. Adventitial gelatinase activity is accelerated by Nox2 deficiency. The serial sections that were incubated together with GQgelatin and EDTA served as a negative control. A: adventitia, L: vascular lumen.
Supplemental figure X. Systolic blood pressure and body weight in control IgG and anti-IL-1β groups. (A) Systolic blood pressure was monitored by using tail-cuff method (MK-2000; Muromachi Kikai Co, Ltd.; Tokyo, Japan). Values are represented as mean±SE (n=5-7). Multiple comparisons were conducted by two-way repeated-measures ANOVA. There are no statistically significant differences between control IgG and anti-IL-1β groups. (B) Body weight in control IgG and anti-IL-1β groups. Values are represented as mean±SE (n=5-7). Results were compared by two-way repeated-measures ANOVA. There are no statistically significant differences between those mice.
Supplemental figure XI. Systolic blood pressure and body weight in BMT mice. (A) Systolic blood pressure was monitored by using tail-cuff method (MK-2000; Muromachi Kikai Co, Ltd.; Tokyo, Japan). Values are represented as mean±SE (n=3). Multiple comparisons were conducted by two-way repeated-measures ANOVA. There are no statistically significant differences between chimeric and non-chimeric mice. (B) Body weight in BMT mice. Values are represented as mean±SE (n=3–5). Results were compared by two-way repeated-measures ANOVA. There are no statistically significant differences between those mice.
Supplemental figure XII. Changes in mRNA expression in BMMs. Bone marrow-derived cells were cultured for 3 days with M-CSF (50 ng/mL). Subsequently, the differentiated BMMs were stimulated with IFN-γ (10 ng/mL) or IL-4 (10 ng/mL). Complementary DNA from BMMs served as a template for real-time RT-PCR reactions. Primer sequences are represented in Supplementary Table S3. (A) mRNA expression of TNF-α. (B) mRNA expression of inflammasome-related genes. (C) mRNA expression of IFN-γ receptor subunits. (D) mRNA expression of MMP12. (E) mRNA expression of TGF-β. Values are represented as mean±SE (n=4). Multiple comparisons were
conducted by two-way non-repeated-measures ANOVA followed by Bonferroni’s post-hoc test. There are no statistically significant differences between Nox2-wild-type and -deficient BMMs.
Supplemental figure XIII. Schematic depiction of AAA aggravation by Nox2 deficiency. Nox2 deficiency activates adventitial macrophages, leading to excess IL-1β production and constitutive MMP9 secretion, via STAT1 and NF-κB signals, respectively. Over-active macrophages disturb inflammatory responses and ECM remodeling in adventitial regions, leading to aggravation of AAA.
Supplemental table I. Genotyping primers used in this study

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<th>Gene</th>
<th>Primer type</th>
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<td>Cybb wild</td>
<td>GCATTGCTTGCTCTTCTTGAAAGAGC</td>
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Supplemental table II. Antibodies used in immunohistochemical study.

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<td>MOMA-2</td>
<td>MOMA-2</td>
<td>AbD Serotec (Raleigh, NC)</td>
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<td>Collagen IV</td>
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<td>Bioworld Technology (Louis Park, MN)</td>
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<td>Cayman Chemical (Ann Arbor, MI)</td>
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<td>IL-1β</td>
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<td>Bioss corp. (Beijing, China)</td>
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## Supplemental table III. PCR Primers used in this study.

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<td>NOX2 (<em>Cybb</em>)</td>
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<tr>
<td>NOX4 (<em>Nox4</em>)</td>
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<td>CD68 (<em>Cd68</em>)</td>
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Supplemental Materials and Methods

NOX2 deficiency exacerbates angiotensin II-induced abdominal aortic aneurysms in mice

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Running title: NOX2 deficiency up-regulates IL-1β in macrophages

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Supplemental Material and Methods

Reagents

M-CSF (Leukoprol®) was purchased from Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan). Angiotensin II (014-18211) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Lipopolysaccharide (L6529; LPS), N-acetyl cysteine (A9165; NAC) and 2',7'-dichlorofluorescein diacetate (35845; DCF) were purchased from Sigma (St. Louis, MO). Dihydroethidium (D23107) and DQ gelatin (D12054) was purchased from Invitrogen (Carlsbad, CA). IL-4 (214-14) and IFN-g (315-05) were obtained from Peprotech Inc. (Rocky Hill, NJ). AG490 was purchased from Cayman Chemical (10010311; Ann Arbor, MI). Lucigenin was obtained from Santa Cruz Biothecnology Inc. (sc-202698: Santa Cruz, CA). All other reagents were high-purity grades obtained from commercial sources.

Animals

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Showa University. Mice were housed in climate-controlled (21 °C) specific pathogen-free facilities with a 12-h light-dark cycle, with free access to standard laboratory food (Picolab mouse diet 20; Laboratory Diet, Brentwood) and water. The Nox2−/− mice (C57/BL6J) were originally created by Dinauer et al.1 LDL receptor-deficient (Ldlr−/−) mice (C57/BL6J) were obtained from The Jackson Laboratory (2207; Bar Harbor, ME). Nox2−/−/Ldlr−/− mice were generated by intercrossing Nox2−/− mice with Ldlr−/− mice. Nox2+/−/Ldlr−/− and Nox2−/−/Ldlr−/− mice were maintained by homozygous breeding, since they have common genetic background. Genotypes in the mice were determined by standard PCR-based genotyping with specific primers (Table I in the online-only Data Supplement).
AngII-induced AAA model

Angiotensin II (AngII)–induced aneurysmal formation in atherogenic mice (Ldlr⁻/⁻ and Apoe⁻/⁻ mice) has been well documented previously.²⁻³ Male mice (10-12 week old) were fed high cholesterol diet (HCD; CRF-1 supplemented with 1.4% cholesterol and 0.4% cholic acid; Oriental Yeast; Tokyo, Japan) prior to AngII infusion. Two weeks following initiation of HCD, mice were subcutaneously implanted with osmotic mini pumps (model 2004; ALZA; Palo Alto, CA), which allow to infuse AngII into the mice at 1,000 ng/kg/min. Four weeks later, mice were sacrificed and aneurysmal diameter and weight were measured. Systolic blood pressure was monitored by using tail-cuff method (MK-2000; Muromachi Kikai Co, Ltd.; Tokyo, Japan).

Histochemistry

For immunohistochemical analysis, the isolated aortic specimens were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), and they were subsequently frozen, sectioned at 5 µm thickness and mounted on glass slides as previously described.⁴ Proteins of interest in the specimens were first labeled with primary antibody at 4°C overnight and then labeled with fluorescent dye-tagged secondary antibody. Primary antibodies used herein are shown in Supplementary table S2. For in situ zymography, unfixed aortic cryosections (5 µm thickness) were incubated with zymography buffer supplemented with fluorogenic gelatin substrate (DQ gelatin, D12054; Invitrogen) at 25 mg/ml for 30 min at room temperature. The serial sections that were incubated together with EDTA at 5 mM served as a negative control. For measuring oxidative stress in AAA lesions, unfixed aortic cryosections (5 µm thickness) were incubated with dihydroethidium (D23107; Invitrogen) at 5 µmol/L
for 30 min at 37°C. The serial sections that were incubated together with ROS scavenger N-acetyl cysteine (NAC) at 10 mmol/L served as a negative control. Fluorescence intensity was measured as an index of proteolytic activity and oxidative stress. Images were acquired utilizing conventional fluorescent microscopy (IX70, Olympus, Japan) or confocal microscopy (A1si, NIKON; Tokyo Japan) equipped with appropriate filter sets. Images were analyzed by using ImageJ software (National Institute of Health, Rockville, MD).

Lucigenin-based chemiluminescence assay

Lucigenin-based chemiluminescence assay was performed according to the previous literature.\textsuperscript{5} AAA lesions were dissected from animals in 5-mm long, and were weighed. Tissues were incubated for 30 min at 37°C in Hank’s balanced salt solution (HBSS) supplemented with 10 mmol/L diethyldithiocarbamate (197-01922; Wako Pure Chemical Industries), in order to eliminate endogenous superoxide dismutase activity. Tissues were then transferred to 96-well plate containing HBSS supplemented with 25 µmol/L lucigenin, and were minced and further incubated for 10 min at 37°C in the dark. Subsequently, chemiluminescence of the tissues were measured for 5 min in a 1-min interval utilizing microplate reader (Mithras LB 940, Berthold Thechnologies; Oak Ridge, TN). Following chemiluminescence measurement, cell-permeant radical scavenger Tyron (347-02741; Wako Pure Chemical Industries) at 10 mmol/L was added to the reactants in order to confirm their baseline chemiluminesce. Baseline levels were subtracted from lucigenin chemiluminescence levels in AAA lesions, which were further normalized by their tissue weight.

Quantification of atherosclerotic lesions
Atherosclerotic lesions in ascending/descending aortic arch and thoracic aortae were quantified as previously described.\textsuperscript{4} Aortic tree were carefully dissected, and were fixed with 4\% PFA in PBS. PFA-fixed thoracic aortae were stained with Oil red-O (Sigma) at 3 mg/mL in 60\% isopropanol for 30 min. Specimens were then washed with 60\% isopropanol and photographed for calculation of positive areas using ImageJ software.

**Administration of neutralizing antibody against IL-1β**

Systemic neutralization of IL-1β in Nox2-deficient mice was conducted according to the procedure for in vivo use of neutralizing antibody noted in the previous report.\textsuperscript{6} AngII-infused $\text{Nox2}^{+/y}/\text{Ldlr}^{-/-}$ mice received intraperitoneal administration of non-immune IgG (2 mg/kg; AB-108-C; R&D Systems; Minneapolis, MN) or neutralizing antibody against IL-1β (2 mg/kg; AB-401-NA; R&D Systems) twice a week. Four weeks later, mice were sacrificed and phenotypic analyses were conducted. Detailed experimental procedure is available in Figure 4A.

**Bone marrow transplantation**

In order to eliminate resident bone marrow cells, recipient mice ($\text{Nox2}^{+/y}/\text{Ldlr}^{-/-}$) were irradiated with X-ray at 8Gy for 20 min utilizing soft X-ray system (OM-150HTS, OHMiC, Tokyo, Japan). Next day, the bone marrow cells ($1 \times 10^7$ cells/animal), which were isolated from donor mice ($\text{Nox2}^{+/y}/\text{Ldlr}^{-/-}$ or $\text{Nox2}^{-/-}/\text{Ldlr}^{-/-}$), were intravenously injected into the recipient mice. Four weeks later, osmotic mini pumps were implanted into the mice, as described above, to be subjected to the AAA experiments. Successful replacement of bone marrow cells was validated by PCR-based genotyping in bone marrows and AAA lesions.
Real-time RT-PCR

Total RNA was isolated from aortic specimens or isolated BMMs with Trizol regent (Invitrogen) as previously described.\textsuperscript{4,7} Complementary DNAs converted from the isolated RNA involving ReverTra Ace reverse transcriptase (TOYOBO, Osaka, Japan) served as templates. Real-time RT-PCR was conducted with the GoTaq polymerase (Promega, Tokyo, Japan) utilizing ABI PRIZM 7900 Sequence Detection System (ABI Japan, Tokyo, Japan). Primer sequences used here are shown in Table III in the online-only Data Supplement.

Isolation of macrophages and neutrophils

For preparing bone marrow-derived macrophages (BMMs), murine bone marrow cells were collected by shearing femoral bone marrows with culture medium; subsequently, the cells were incubated in Dulbecco’s Modified Eagle Medium (DMEM; Sigma) supplemented with 10% serum, Penicillin-Streptomycin-Amphotericin B (Wako Pure Chemicals; Osaka, Japan) and M-CSF (50 ng/mL) for differentiation to macrophages. Following 3 days of culture, unbound cells were removed by washing with medium and differentiated BMMs were then stimulated with IFN-$\gamma$ (10 ng/mL) or IL-4 (10 ng/mL) for 16 hrs in the absence of M-CSF. Peritoneal macrophages and neutrophils were isolated from peritoneal exudate using conventional procedure.\textsuperscript{8,9} For preparing macrophages, $\text{Nox2}^{+/y}/\text{Ldlr}^{-/-}$ and $\text{Nox2}^{-/y}/\text{Ldlr}^{-/-}$ mice were intraperitoneally injected with 1 mL of 3% thioglycolate broth (Wako Pure Chemical) to induce peritoneal inflammatory cells. Four days later, peritoneal exudate cells (PECs) were collected with dMEM supplemented with 10% FBS, and seeded on 12-well culture plate. Following incubation for three hours, non-adherent cells were removed by washing with culture medium, and adherent cells served as
peritoneal macrophages. For preparing neutrophils, mice were intraperitoneally injected with 1 mL of 3% thioglycolate broth. Three hours later, PECs were collected with dMEM supplemented with 10% FBS, and seeded on 12-well culture plate. Following incubation for three hours, non-adherent cells were harvested as peritoneal neutrophils. Dominance of neutrophils was confirmed by detecting their polymorphic nuclei using DAPI staining.

**Measurement of intracellular ROS**

Oxidative stress in BMMs was measured as previously described. Briefly, cytokine-stimulated BMMs were subjected to 2',7'-dichlorofluorescein diacetate (35845; Sigma), a redox-sensitive fluorescent dye, at 5 µmol/L for 30 min at 37°C. Following washing 3 times with PBS, fluorescence intensity was measured utilizing microplate reader (Mithras LB 940, Berthold Thechnologies) as an index of intracellular oxidative stress. Alternatively, cells were incubated with 50 µmol/L Amplex Red reagent (A22188; Invitrogen; Carlsbad, CA), an H2O2-sensitive fluorescent dye and 0.1 U/mL horseradish peroxidase for 30 min at 37°C, and fluorescence intensity was measured as an index of H2O2.

**Measurement of IL-1β by ELISA**

Isolated BMMs were stimulated with IFN-γ at 10 ng/mL and lipopolysaccharide (LPS) at 1 µg/mL for 16 hrs. Non-concentrated conditioned medium, which was derived from the culture systems noted above, was subjected to the experiments. IL-1β in the conditioned medium was quantified by using conventional ELISA (900-M47; Peprotech; Rocky Hill, NJ) according to the manufacturer's instructions.

**Gelatin zymography**
Conditioned medium from BMM culture was pre-cleared by centrifugation to remove cell debris. Following centrifugation, the medium was concentrated by ultra filtration (3kDa, Millipore; Billerica, MA) and subsequently electrophoresed on SDS-PAGE gels containing 1 mg/mL gelatin (Sigma). Gels were washed in 2.5% Triton X-100 and incubated overnight in zymography buffer (50 mmol/L Tris pH 7.4, 10 mmol/L CaCl₂) at 37 °C, and were then stained with Coomassie brilliant blue. Subsequently, gel images were acquired utilizing conventional image scanner (Canon, Tokyo, Japan).

**Statistics**

Results are expressed as means±SE and statistical analyses were performed using Prism 5 (GraphPad Software; San Diego, CA). Equal valiance of statistical data was confirmed by F-test for two groups or Bartlett test for multiple groups. Student’s t-test was used for comparing two groups with equal variances; alternatively, Mann-Whitney U-test was applied for the data with unequal variances. Multiple comparisons were conducted with one-way or two-way non-repeated-measures ANOVA followed by a post hoc Bonferroni’s test, appropriately. Kaplan-Meier analysis with Wilcoxon’s test was employed to determine differences between groups in the mortality due to rupture. P values of <0.05 were considered statistically significant.
Supplemental References


7. Miyazaki T, Kimura Y, Ohata H, Hashimoto T, Shibata K, Hasumi K, Honda K. Distinct effects of tissue-type plasminogen activator and SMTP-7 on
