Impaired Superoxide Production Due to a Deficiency in Phagocyte NADPH Oxidase Fails to Inhibit Atherosclerosis in Mice

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Abstract—Superoxide, the reduced form of molecular oxygen, has been implicated in the genesis of vascular disease. One potential mechanism involves oxidation of low density lipoprotein into an atherogenic particle. A second involves reaction with nitric oxide to generate peroxynitrite, a highly oxidizing intermediate. A third involves regulation of signal transduction in artery wall cells. One well-characterized pathway for superoxide production resides in macrophages, the cellular hallmark of the early atherosclerotic lesion. Macrophages contain a membrane-bound NADPH oxidase that reduces oxygen to superoxide. In the current studies, we used mice that are deficient in the gp91-phox subunit of the NADPH oxidase—a model of chronic granulomatous disease (CGD)—to explore the role of superoxide in atherosclerotic vascular disease. Wild-type and CGD mice on the C57BL/6 background received a high-fat diet for 20 weeks to induce hypercholesterolemia. At the end of this period, the 2 strains of mice had comparable plasma lipid levels, and their atherosclerotic lesions were similar in size. We also crossed CGD mice with apolipoprotein E−/− mice to generate spontaneously hypercholesterolemic animals that lacked functional NADPH oxidase. After 24 weeks, the CGD-apoE−/− animals had lower plasma cholesterol and triglyceride levels than did the apoE−/− animals, but there was no difference in the extent of atherosclerotic plaque. Our findings suggest that superoxide generated by the NADPH oxidase of phagocytes does not promote atherosclerosis in mice with either diet-induced or genetic forms of hypercholesterolemia. (Arterioscler Thromb Vasc Biol. 2000;20:1529-1535.)

Key Words: peroxynitrite ■ oxidized LDL ■ lipid peroxidation

Superoxide generation by neutrophils, monocytes, and macrophages is of central importance in host defense and may provide oxidants involved in the pathogenesis of atherosclerotic vascular disease. The major biochemical agent for superoxide production by these cells is the NADPH oxidase, a membrane-associated electron transport chain. The integral membrane component of the oxidase, flavocytochrome b558, is composed of a large subunit, gp91-phox (glycoprotein91-phagocyte oxidase), and a small subunit, p22-phox. Multiple cytoplasmic subunits (p47-phox, p67-phox, p40-phox, Rac1, and Rac2) associate with the membrane species to provide the complete oxidase enzymatic activity. NADPH oxidase directly reduces molecular oxygen (O2) to superoxide (O2−), with NADPH but not NADH as a cofactor (reviewed in Reference 7) according to the following chemical equation:

\[
\text{NADPH} + 2\text{O}_2 \rightarrow \text{NADP}^+ + \text{H}^+ + 2\text{O}_2^- 
\]

Dismutation of superoxide generates hydrogen peroxide, and both molecules are used to generate other reactive oxygen intermediates such as hypochlorous acid, hydroxyl radical, and peroxynitrite ion. The key role of the NADPH oxidase system in host defenses against microbial pathogens is illustrated by the clinical features of chronic granulomatous disease (CGD). In this genetic disorder, defects in components of the oxidase impair superoxide production, leading to recurrent bacterial and fungal infections. The CGD phenotype has been reproduced in mice made deficient in gp91-phox or p47-phox.

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Several lines of evidence implicate superoxide in the pathogenesis of atherosclerosis. One potential route involves the modification of LDL to oxidized LDL, which, unlike native LDL, exerts a wide variety of atherogenic effects in vitro and in vivo. Significantly, superoxide dismutase inhibits LDL oxidation by cultured smooth muscle cells, endothelial cells, and activated phagocytes, all of which generate extracellular superoxide. Moreover, the lipids of LDL exposed to enzymatically generated superoxide are oxidized by reactions that require free or low-molecular-weight chelates of redox-active transition metal ions. This

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requirement suggests that reactive intermediates derived from superoxide mediate LDL oxidation. Collectively, these results suggest that superoxide generated by cells of the artery wall could convert LDL into an atherogenic form in vivo.

Excess superoxide is produced in arteries derived from cholesterol-fed rabbits, and endothelium-dependent vascular relaxation can be restored in such rabbits in part by treatment with superoxide dismutase or probucol. Furthermore, recent studies suggest that reactive oxygen intermediates also promote smooth muscle cell proliferation. For example, cultured vascular cells generate superoxide and hydrogen peroxide, and these intermediates have been proposed to regulate cell proliferation, hormone-induced hypertrophy, and apoptosis. The underlying mechanisms remain poorly understood, but they may involve elevation of intracellular calcium levels, activation of signaling pathways, and alteration of the redox status of critical intracellular or membrane-associated proteins.

Superoxide also reacts with nitric oxide (NO). Released by endothelial cells lining the artery wall, NO plays a critical role in smooth muscle cell relaxation and regulation of vasomotor tone. Its reaction with superoxide occurs at a nearly diffusion-controlled rate, and the product is peroxynitrite (ONOO−), a potent oxidizing intermediate:

\[ \text{O}_2^- + \text{NO} \rightarrow \text{ONOO}^- \]

This reaction may contribute to atherosclerotic vascular disease by several different mechanisms. One relates to the ability of superoxide to directly scavenge NO, which may account for the defect in vascular relaxation observed in hypercholesterolemic animals and humans. Another potential mechanism involves LDL oxidation by peroxynitrite. Evidence that this mechanism may operate in vivo comes from the detection of elevated levels of 3-nitrotyrosine in LDL isolated from human vascular lesions. Because 3-nitrotyrosine is the stable end product of the reaction between peroxynitrite and the aromatic ring of free tyrosine, its detection in LDL raises the possibility that NO, by virtue of its ability to form reactive nitrogen intermediates, promotes atherogenesis. This scenario counters the well-established antiatherogenic effects of low levels of NO.

Overall, the contribution of reactive oxygen species produced via NADPH oxidase has potential for wide relevance to the etiology of atherosclerosis. In this report, we used mice lacking gp91-phox to test the hypothesis that a deficiency in NADPH oxidase and therefore, in superoxide production protects against atherosclerosis in hypercholesterolemic mice. Surprisingly, our results suggest that phagocyte NADPH oxidase is not needed for the initiation or progression of atherosclerosis in this animal model.

Methods

Animals

Mice deficient in gp91-phox (CGD), the X-linked form of CGD, were backcrossed 6 times to C57BL/6J (Jackson Laboratories, Bar Harbor, Me) and maintained in a colony at the University of Washington, Seattle. Apolipoprotein E−/− mice, with a C57BL/6J background (Jackson Laboratories), were bred to CGD mice to create mice doubly deficient in both proteins (CGD-apoE−/−). Male mice of both apoE−/− and CGD-apoE−/− genotypes were readily obtained from related litters. Female apoE−/− mice obtained directly from the Jackson Laboratories were used as controls for female CGD-apoE−/− animals generated at the University of Washington because of difficulties in generating appropriate littermate controls.

All mice were maintained in a 22°C room with a 12-hour light/dark cycle and given free access to food and water. Food was withheld 4 hours before the collection of blood samples from the retro-orbital sinus into tubes containing EDTA. Plasma was stored at −70°C until analysis. Mice were killed immediately after blood collection by cervical dislocation, and each entire animal was perfused with 10 mL of antioxidant buffer (100 μmol/L DTPA and 100 μmol/L BHT in PBS, pH 7.4) via the left ventricle. Tissues were snap-frozen in LN2 and stored in antioxidant buffer at −70°C. Animal procedures were approved by the Animal Care and Use Committee of the University of Washington.

Diets

Mice were either maintained on pelleted rodent chow (4% fat, 24% protein, and 4.5% crude fiber by weight; Wayne Rodent BLOX 8694, Harlan Teklad) or fed an atherogenic diet (15% fat by weight, 1.25% cholesterol, and 0.5% sodium cholate; TD 90221, Harlan Teklad) as described. CGD and wild-type littermates were fed the pelleted rodent diet until they were 8 to 10 weeks of age. Mice then were fed the atherogenic diet for 20 weeks, at which time they were bled and killed. ApoE−/− and CGD-apoE−/− mice were fed pelleted rodent chow, bled, and killed at 24 weeks of age.

Plasma Lipids

Plasma cholesterol levels were determined by using a colorimetric kit (Diagnostic Chemicals Ltd) and cholesterol standards (Preciset No. 12552, Boehringer Mannheim). Plasma triglyceride levels were determined colorimetrically after removal of free glycerol (diagnostic kit No. 450032, Boehringer Mannheim). Plasma lipoproteins were separated by fast-performance liquid chromatography gel filtration on a Superose-6 column (Pharmacia LKB Biotechnology). A 100-μL aliquot of plasma from each of 3 mice per diet group was analyzed at a flow rate of 0.2 mL/min with PBS at 4°C. Aliquots (100 μL) from each 0.5-mL fraction were used for total cholesterol and triglyceride determinations.

Quantification of Atherosclerosis

Lesion sizes in the aortic sinus were measured as described. In brief, the upper sections of the heart were embedded in OCT medium and frozen. Every other section (10 μm thick) throughout the aortic sinus (400 μm) was taken for analysis. Sections were evaluated for lesions after being stained with oil red O and were quantified by using computer-assisted imaging and the Optimas Image Analysis software package (Bioscan Inc). Data are presented as lesion area normalized to the total number of sections examined for each mouse.

Superoxide Production

Intraperitoneal injection of thioglycollate (1 mL of 4% solution) was used to recruit peritoneal phagocytes. These cells were harvested by lavage (Hanks’ balanced salt solution [HBSS], Gibco BRL) 24 hours later. Cells were pelleted by centrifugation (300g at 5°C), washed once with HBSS, and resuspended in 1 mL of HBSS. Aliquots containing 5 × 10^6 cells were resuspended in 250 μL of HBSS containing 1 mg/mL ferricytochrome c (Sigma) and maintained at 37°C. Cells were stimulated by the addition of 400 mmol/L phorbol myristate acetate. Superoxide production was measured as the superoxide dismutase (10 μg/mL)–inhibitable reduction of cytochrome c, monitored as the change in absorbance at 550 nm with the use of a SpectraMAX microplate reader (Molecular Devices). Absorbance was read in 3-minute intervals up to 30 minutes and then in two 15-minute intervals up to 60 minutes.

mos1 mRNA Expression

Expression of aortic mos1 was evaluated by reverse transcription–polymerase chain reaction (RT-PCR) with the Titan One Tube RT-PCR system (Roche). In brief, total RNA was prepared from aortas (from proximal to abdominal aorta, inclusive) isolated from CGD-apoE−/− and apoE−/− mice by using TriPure reagent (Roche) and resuspended in 20 μL of deionized water. Amplification
Figure 1. Superoxide generation by peritoneal phagocytes. Thioglycollate-elicited peritoneal phagocytes were isolated from wild-type (n=7) and CGD (n=6) mice 2 to 4 months old. Absorbance at 550 nm reflects production of superoxide due to activity of ferricytochrome c. Absorbance was measured in the absence (filled symbols) or presence (open symbols) of superoxide dismutase (SOD). Superoxide production by cells isolated from CGD mice (square symbols) was essentially undetectable. Cells isolated from wild-type mice (circles) produced superoxide in the absence of SOD, and superoxide was nearly absent in the presence of SOD.

Statistics
Data are reported as mean±SEM. Multiple comparisons were performed with a 2-way ANOVA (SYSTAT, Inc). Post hoc analyses of multiple comparisons were corrected with Tukey’s test for additivity. Pearson’s correlation coefficient was used to assess correlations. Paired differences were determined by Student’s t test. P<0.05 was considered significant.

Results
Phagocytes From CGD Mice Fail to Generate Superoxide
Previous studies had demonstrated that peripheral blood neutrophils prepared from CGD mice failed to produce superoxide. To confirm that macrophages also fail to generate superoxide, we isolated peritoneal cells from animals treated with intraperitoneal thioglycollate for 48 hours. Under these conditions, macrophages represent >70% of the peritoneal inflammatory cell population. Isolated cells were activated with phorbol ester, and superoxide production was monitored as the superoxide dismutase–inhibitable reduction of ferricytochrome c. Cells isolated from wild-type mice (n=7) demonstrated a burst of superoxide production (Figure 1). In contrast, superoxide production by cells isolated from CGD mice (n=6) was essentially undetectable. These observations confirm that macrophages isolated from CGD mice fail to produce superoxide. They also strongly suggest that wild-type and CGD macrophages should differ markedly in their potential to produce superoxide in vivo.

Atherosclerosis in Wild-Type and CGD Mice
CGD mice appeared healthy and gained weight at the same rate as wild-type C57BL/6 mice. After 20 weeks of feeding the atherogenic diet, plasma cholesterol levels were nearly identical between wild-type and CGD genotypes within male (n=33 to 52) and female (n=16 or 17) groups (Figure 2A). Plasma triglyceride levels were nearly identical between genotypes for female mice (n=7 to 10) but were modestly reduced (∼30%, P<0.05) for male CGD (n=41) compared with male wild-type (n=27) mice (Figure 2B). There were no significant differences in the extent of atherosclerosis between wild-type and CGD mice for either male (n=33 to 39) or female (n=16 or 17) groups (Figure 2C).

Of note were sex differences for lesion sizes and plasma lipid levels. Lesions sizes for female mice were approximately 3-fold larger compared with those of males (P<0.02, Figure 2C). Although plasma total cholesterol levels were comparable between sexes, triglyceride levels were >2-fold higher for male mice compared with female mice (P<0.001, Figure 2B). It is well established that sex dimorphism in atherosclerosis occurs in the C57BL/6 strain.

There were no significant correlations between plasma lipid levels and size of lesions within each sex and genotype group by either linear or nonparametric analyses. Also, lesion sizes for male CGD (n=39) and wild-type (n=33) mice were nearly identical when considered as median values (3520 and 3940 μm², respectively). Median lesion sizes for female CGD
Atherosclerosis in ApoE−/− and CGD-ApoE−/− Mice

CGD-apoE−/− mice appeared healthy and had comparable body weights to those of apoE−/− mice (data not shown). However, nearly all of the CGD-apoE−/− mice showed evidence of modestly enlarged spleens and small lung granulomas at autopsy.

Plasma lipid levels were nearly identical between female apoE−/− and CGD-apoE−/− mice (Figures 2A and 2B). In contrast, plasma cholesterol and triglyceride levels for male CGD-apoE−/− mice (n=11) were 35% and 52% lower, respectively, than those of the male apoE−/− mice (n=11, P<0.005; Figures 3A and 3B). Analysis of plasma lipoproteins by high-resolution, size-exclusion fast-performance liquid chromatography confirmed this reduction in lipid levels (Figure 4, n=3 per profile). When considered as a percentage of total triglyceride, however, the relative distribution of triglyceride among VLDL, LDL, and HDL-size lipoproteins was equivalent between genotypes (Figure 4B). In contrast, the relative distribution of cholesterol among lipoproteins differed between genotypes (Figure 4A). HDL cholesterol was reduced by ~2-fold and LDL cholesterol (predominantly small LDL particles) was increased by ~60% for male CGD-apoE−/− mice.

Quantification of aortic sinus lesion sizes revealed no significant difference in the extent of atherosclerosis between apoE−/− and CGD-apoE−/− mice (Figure 3C). The mean lesion sizes for male apoE−/− and CGD-apoE−/− mice were 261 000±39 000 µm² (n=11) and 206 000±29 000 µm² (n=11), respectively. The mean lesion sizes for female apoE−/− and CGD-apoE−/− mice were 372 000±44 000 µm² (n=10) and 404 000±66 000 µm² (n=5), respectively. Because of the difference in lipid levels between apoE−/− and CGD-apoE−/− male mice, lesion areas were normalized to plasma total cholesterol levels for each of these animals. With this approach, male CGD-apoE−/− mice had a small (13%), nonsignificant increase in the extent of atherosclerosis compared with male apoE−/− mice. There were no significant correlations between lipid levels and size of lesions within each genotype and sex group as assessed by linear or nonparametric analyses. As with C57BL/6 mice fed the atherogenic diet, loss of gp91-phox failed to affect aortic lesion development in apox−/− mice.

Expression of mox1 mRNA

We tested the hypothesis that mox1 expression may be altered by the loss of gp91-phox. We found no overall differences in mRNA levels of mox1 in arteries between CGD-apoE−/− mice and apoE−/− mice (Figure 5). These results confirm that mox1 is expressed in the artery wall and raise the possibility that superoxide generated by this enzyme plays a role in atherosclerotic vascular disease.

Discussion

In vitro studies have implicated a number of potential mechanisms whereby superoxide might contribute to athero-
erosclerosis, we expected that mice with defective NADPH oxidase would have less atherosclerotic plaque than wild-type mice. Instead, we found that the lack of functional NADPH oxidase failed to produce the expected antiatherogenic effect. Most studies of superoxide have been carried out in vitro, which may have limited physiological relevance. For example, biochemical and pharmacological studies suggest that superoxide may not play a leading role in initiating or advancing atherosclerosis. Indeed, most studies of superoxide have been carried out in vitro, which may have limited physiological relevance. For example, biochemical and pharmacological studies suggest that reactive nitrogen species derived from NO play a pathogenic role in acute arthritis. However, experimental arthritis is more pronounced in mice that lack the inducible form of NO synthase than in control animals, suggesting that NO actually exerts anti-inflammatory effects in vivo.

A second explanation is that alternative pathways exist for superoxide production. For example, cultured nonphagocytic vascular wall cells and aortic tissue produce superoxide, monitored as lucigenin-dependent chemiluminescence. The biochemical mechanism may involve xanthine oxidase, mitochondrial oxidative phosphorylation, arachidonic acid metabolism, cytochrome P450, Mox1, or an NAD(P)H oxidase. The latter enzyme system has been only partially characterized. It bears similarities to the NADPH oxidase of phagocytes, although it does not require gp91-phox for activity. Its preference for NADH as a source of reducing equivalents serves as its distinguishing feature.

Mox1 has been recently characterized from human and rat tissues and is a homolog of gp91-phox. Cells transfected with mox1 generate superoxide, with consequences that include alteration of cell growth and diminished aconitase activity. The tissue distribution of mox1 differs from that of gp91-phox and includes rat aortic vascular smooth muscle cells. Thus, we tested whether mox1 mRNA could be found in mouse aortic tissue and whether or not expression was influenced by the loss of gp91-phox. We have shown for the first time that mox1 mRNA is present in mouse aortas. We also found no overall differences in the levels of mox1 mRNA between apoE−/− and CGD-apoE−/− mice, suggesting that the mox1 and NADPH oxidase superoxide-generating systems may be independently regulated. The presence of mox1 in aortic tissue raises the possibility that superoxide generated by this enzyme plays a role in atherogenesis. Collectively, data show that superoxide produced by NADPH oxidase found in the plentiful collection of phagocytes in mouse plaques fails to influence atherosclerosis in mice, but that multiple other sources of superoxide exist in arterial tissue, which may contribute to atherogenesis.

Cultured vascular wall cells can also generate superoxide by a pathway that involves autoxidizing thiols. For example, incubating smooth muscle cells in medium free of L-cystine (the disulfide form of L-cysteine) inhibited both superoxide production and LDL oxidation. Adding L-cystine back to the medium reversed both effects. This observation led to the proposal that smooth muscle cells take up L-cysteine and reduce it to a thiol, which moves out of the cell. Autoxidation of the thiol then generates superoxide, which mediates LDL oxidation when metal ions are present. The ability of thiols to oxidize LDL in the absence of cells supports this proposal. Moreover, cultured macrophages and endothelial cells use an L-cysteine–dependent pathway to generate extracellular thiol and to oxidize LDL. This pathway may be physiologically relevant because premature atherosclerosis and endothelial denudation are common in people with homocystinuria, a genetic disorder that greatly elevates levels of plasma homocysteine.

Tribble et al recently examined the physiological role of intracellular superoxide production in atherosclerosis by using transgenic mice that overexpress CuZn superoxide dismutase. These mice were backcrossed onto the C57BL/6 background and fed an atherogenic diet for 18 weeks. As in our experiments, there were no differences in lesion area between the transgenic and wild-type mice. In fact, superoxide dismutase activity was correlated positively with lesion size in the transgenic mice. The authors concluded that overexpression of superoxide dismutase failed to produce the expected antatherogenic effect. Because CuZn superoxide dismutase is a cytosolic enzyme, these observations suggest that intracellular sources of superoxide are unlikely to play a critical role in atherosclerosis, at least in this animal model.

Although our wild-type and CGD mice had lesions of similar size, we found 1 difference pertaining to the lack of functional NADPH oxidase. Both total cholesterol and...
triglyceride levels were lower in the CGD-apoE−/− mice than in the apoE−/− mice. Both groups of animals had been bred on the C57BL/6 background, making it unlikely that other genetic differences can account for the discrepancy in lipid levels. One complication is that most of our CGD-apoE−/− mice appeared to have lung granulomas. The mice were housed in modified, specific-pathogen-free conditions in microisolator cages, and long-term treatment with antibiotics did not seem warranted on the basis of observations in C57BL/6 mice lacking gp91-phox (vide infra and Reference 12). Despite the modest lung and spleen pathologies, the CGD-apoE−/− mice appeared healthy and had weight gains comparable to those in apoE−/− mice. Therefore, the differences in lipid levels were unlikely to be due to acute illness, which would be expected to elevate rather than lower triglyceride levels.46 It is noteworthy that reactive species have been implicated in signal transduction,23,47 raising the possibility that the NADPH oxidase modulates lipoprotein metabolism. Despite the reduced lipid levels seen in CGD-apoE−/− mice, lesion sizes were not reduced. Indeed, there was a nonsignificant 13% increase in atherosclerosis when lesion area was normalized to plasma cholesterol levels in the CGD-apoE−/− mice, making it unlikely that NADPH oxidase plays an important role in the etiology of atherosclerosis. In summary, our results suggest that superoxide production by the NADPH oxidase of phagocytes is not of central importance in the initiation or progression of atherosclerosis in hypercholesterolemic C57BL/6 mice. This observation mirrors findings from mice that overexpressed CuZn superoxide dismutase, suggesting that intracellular production of superoxide also is not pertinent to lesion formation. There-


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