Vascular Effects Following Homozygous Disruption of p47phox
An Essential Component of NADPH Oxidase

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Background—Evidence suggests that the vessel wall contains an oxidase similar, if not identical, to phagocytic NADPH oxidase. We tested the contribution of this specific oxidase to the progression of atherosclerosis and the regulation of blood pressure.

Methods and Results—An examination of aortic rings from wild-type mice and mice with homozygous targeted disruptions in p47phox revealed that p47phox knockout mice had a reduction in vascular superoxide production. However, analyses of apoE −/− p47phox +/+ and apoE −/− p47phox −/− strains of mice demonstrated no significant differences in atherosclerotic lesion sizes. Similarly, analyses of wild-type and p47phox knockout mice revealed no differences in either basal blood pressure or the rise in blood pressure seen after the pharmacological inhibition of nitric oxide synthase.

Conclusions—NADPH oxidase contributes to basal vascular superoxide production. However, the absence of a functional oxidase does not significantly affect the progression of atherosclerosis in the standard mouse apoE −/− model, nor does it significantly influence basal blood pressure. (Circulation. 2000;101:1234-1236.)

Key Words: apolipoproteins ■ atherosclerosis ■ blood pressure

The phagocytic NADPH oxidase generates superoxide through the assembly of a multi-subunit protein complex.1 Included in this complex are the 2 membrane-bound proteins gp91phox and p22phox, the recruited cytosolic oxidase proteins p47phox and p67phox, and the small GTPase rac2. Although initially the NADPH oxidase was thought to exist solely in phagocytes, recent evidence suggests that this oxidase may also function in nonphagocytic cells. Biochemically, NADH/NADPH activity has been detected in the intact species in smooth muscle cells,8 whereas the expression of constitutively active rac proteins in nonphagocytic cells augments the levels of reactive oxygen species.9 Similarly, all NADPH oxidase components have been detected by immunohistochemical staining of the vessel wall,7,10,11 and immunodepletion of p67phox from a vessel wall lysate reduces NADPH oxidase activity.7

Evidence also suggests a role for augmented NADPH oxidase activity in vascular disease. Consistent with this, the expression of certain components of the NADPH oxidase seems to be stimulated by angiotensin II and inflammatory cytokines.12,13 In addition, recent studies have also demonstrated that levels of p22phox are increased in human atherosclerotic plaque11 and in animal models of hypertension.14 In an effort to further address the precise role of NADPH oxidase, we analyzed the vascular effects of mice containing a targeted disruption of p47phox, an essential component of the phagocytic NADPH oxidase.15

Methods

Mouse Genotyping and Physiological Analysis

C57BL/6J apoE −/− mice were obtained from Jackson Laboratories (Bar Harbor, Maine). For studies in atherogenesis, p47phox −/− mice were first backcrossed 10 generations with C57BL/6J mice. Three backcrosses of C57BL/6J mice containing either a targeted deletion in apoE or p47phox allowed for the isolation of certain F3 mice (apoE −/− p47phox −/− or apoE −/− p47phox +/+ ) that were subsequently analyzed for atherosclerotic lesion size. Genotyping of animals was done with the apoE gene-specific primers 5′-GCCTAGCCGA-GGGAGAGCGG-3′ and 5′-TGGACTTGGGAGCTGTCAGC-3′ or the p47phox-specific primers 5′-ACATCAGAGCCCTCATCCTCC-3′ and 5′-CAACGCAGCAGCAGCTGGCAAG-3′. Mice were fed a standard chow diet of 18% protein and 4% fat and maintained in a pathogen-free environment, but they were not given prophylactic antibiotics.
Atherosclerotic lesion size was determined at 16 weeks of age in male mice. After formalin fixation, the heart and ascending aorta were embedded and analyzed as previously described. Briefly, for each of the 17 mice in each group, 10-μm sections through the aortic sinus were obtained. Mean lesion size was determined by a blinded observer using a computer analysis of Oil red-O stained areas obtained from averaging 5 sections per animal.

Cholesterol levels were obtained from blood obtained from the retro-orbital plexus and analyzed by a commercial enzymatic test according to the manufacturer’s recommendations (Boehringer Mannheim Diagnostics). Blood pressure was determined in conscious animals by inserting a pressure-transducing cannula (Micro-Med TXD-310) into the left carotid artery. Animals used for blood pressure analysis were either p47phox−/− male mice (C57/BL6x129) or their wild-type male littermates. To inhibit nitric oxide synthase activity, 10 mg/kg of NG-nitro-L-arginine methyl ester (L-NAME) was injected into the peritoneal cavity; blood pressure was then assessed over the next 60 minutes.17

Levels of vascular superoxide were determined using lucigenin (25 μmol/L) chemiluminescence. To inhibit cellular superoxide dismutase (SOD) activity, rings were pretreated with 10 mmol/L diethyldithiocarbamate, as previously described.7 Statistical comparisons between groups were made with a 2-tailed Student’s t test; P<0.05 was considered significant.

Results

Levels of superoxide were assayed from aortic rings derived from p47phox knockout mice or their wild-type littermates. Basal superoxide levels were low, and no significant differences were observed between the wild-type and p47phox−/− deficient mice (Figure 1). Because measured levels of superoxide represent the balance between production and degradation, we thought it possible that differences in superoxide production might be more readily apparent in the absence of cellular SOD activity. Thus, we pretreated rings with diethyldithiocarbamate to selectively inhibit SOD activity, as previously described.7 Under these conditions (Figure 1), significant differences were observed in superoxide levels, with p47phox−/− mice having an ≈50% decrease in superoxide levels.

We next sought to understand whether animals containing a targeted disruption of p47phox had altered in vivo vascular pathophysiology. Two lines of mice were analyzed: one contained a targeted disruption of apoE, and the other line contained both an apoE and p47phox disruption. Both the apoE−/− and p47phox−/− disrupted mice strains had been previously backcrossed for 10 generations into a C57BL/6J background to assure that these strains were otherwise genetically identical. The apoE−/− p47phox+/+ and apoE−/− p47phox−/− mice had equivalent serum cholesterol levels (data not shown). In addition, morphometric assessment of aortic lesion size revealed no differences between the 2 groups (Figure 2A).

Given the known role of superoxide in regulating the bioactivity of nitric oxide and, potentially, blood pressure, we next determined whether we could detect differences in blood pressure between p47phox−/− deficient mice and their wild-type counterparts. As shown in Figure 2B, basal blood pressure was indistinguishable between the 2 strains of mice. Similarly, treatment with L-NAME produced a similar increase in blood pressure in both wild-type and p47phox knockout mice.

Discussion

Our results demonstrate that the disruption of p47phox lowers vascular superoxide production. These results are, therefore,
similar to those recently described in vascular preparations obtained from gp91^phox^-deficient mice. However, our data also suggest that the absence of a functional phagocyte NADPH oxidase does not significantly affect the progression of atherosclerosis in the apoE^-/- mouse model, nor does it alter basal blood pressure.

This study may, therefore, seem to be in potential conflict with previous studies indicating that superoxide levels rise in early atherosclerosis and in models of hypertension. As such, under basal physiological conditions, levels of superoxide are not reduced by 50% in p47phox knockout animals. It is important to remember that almost all studies that have demonstrated an increase in superoxide production attributable to vascular NADPH oxidase activity have relied on biochemical assays or pharmacological inhibitors. These approaches cannot define the molecular components of the oxidase under study. Indeed, the existence of >1 NADPH oxidases is strongly supported by the recent isolation of mox1, a nonphagocytic homologue of gp91^phox^ that seems to generate superoxide without requiring p47^phox^.

In addition, although our results (Figure 1) suggest that an oxidase requiring p47^phox^ contributes to vascular superoxide production, this contribution was only evident after inhibiting SOD activity. As such, under basal physiological conditions, levels of superoxide were unchanged and, therefore, it is perhaps not as surprising that no effect on blood pressure or atherogenesis was evident. Similarly, the observation that superoxide levels were only reduced by 50% in p47^phox^- knockouted animals strongly suggests the existence of additional vascular oxidases. Potential sources include enzymes such as xanthine oxidases, lipoxygenases, and novel NADPH oxidases that do not require p47^phox^ for activity. The use of knockout animals represents a particularly promising approach to elucidate the relative contribution of these multiple superoxide-generating enzymes in vascular pathophysiology.

References

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