Augmented Superoxide Production By Nox2-Containing NADPH Oxidase Causes Cerebral Artery Dysfunction During Hypercholesterolemia

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**Background and Purpose**—We tested the hypothesis that elevated superoxide production by Nox2-NADPH oxidase occurs in cerebral arteries during hypercholesterolemia and causes decreased nitric oxide function.

**Methods**—Wild-type (WT), apolipoprotein E-deficient (ApoE−/−) and Nox2−/−/ApoE−/− mice were fed a high-fat diet for 7 to 14 weeks. Basal superoxide production by cerebral arteries was measured using L-012 (100 μmol/L)-enhanced chemiluminescence. Nitric oxide function was assessed in isolated middle cerebral arteries through the constrictor response to N⁵⁰-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L). Western blotting was used to measure protein expression of Nox2, p47phox, endothelial nitric oxide synthase, and superoxide dismutases (1–3).

**Results**—Morphology of cerebral arteries was similar in WT and ApoE−/− mice. In ApoE−/−, but not Nox2−/−/ApoE−/− mice, superoxide production by cerebral arteries was approximately 50% greater than in WT mice (P<0.05). Moreover, the magnitude of l-NAME-induced contractions of isolated middle cerebral arteries from ApoE−/− mice was <50% of that in WT mice (P<0.05), whereas in Nox2−/−/ApoE−/− mice, the contractile response was comparable to WT responses. In the presence of the superoxide scavenger, tempol (1 mmol/L), l-NAME-induced contractions of middle cerebral arteries were similar between WT and ApoE−/− mice. Expression of p47phox was approximately 2-fold higher in ApoE−/− versus WT mice, whereas Nox2, endothelial nitric oxide synthase, and superoxide dismutase isoforms were unchanged.

**Conclusions**—Elevated superoxide production and reduced basal nitric oxide-mediated relaxation occur in cerebral arteries of hypercholesterolemic mice even in the absence of lesions. These changes appear to be exclusively due to increased activity of Nox2-NADPH oxidase, possibly through increased expression of its regulatory subunit p47phox. (Stroke. 2010;41:784-789.)

**Key Words:** cerebral arteries ▪ hypercholesterolemia ▪ NADPH-oxidase ▪ reactive oxygen species ▪ vasomotor function

NADPH oxidases are an important source of superoxide in the cerebral circulation under both physiological and pathophysiological conditions.1–5 These enzymes comprise 2 membrane-bound subunits, Nox (catalytic subunit) and p22phox, up to 3 cytoplasmic subunits (p47phox/NoxO1 and p67phox/NoxA1), and a small G-protein (Rac1/Rac2).6 NADPH oxidases generate superoxide by transferring electrons from the substrate, NADPH, to molecular oxygen through the flavin-containing Nox catalytic subunit.6 In the cerebral vasculature, 3 isoforms of NADPH oxidase have been identified, namely Nox1-, Nox2-, and Nox4-containing NADPH oxidases.6 Recently, we reported that Nox2-containing NADPH oxidase contributes to activated (eg, angiotensin II- and NADPH-stimulated) superoxide production in the cerebral circulation of both rats and mice under physiological conditions.7,8 Moreover, excessive production of superoxide by Nox2-containing NADPH oxidase has been linked with cerebral artery dysfunction in experimental models of hypertension,9 Alzheimer disease,4 and aging.10

It is well established that hypercholesterolemia is a risk factor for coronary and peripheral vascular disease. In contrast, the relationship between cholesterol levels and stroke incidence is unclear.11,12 Several large cohort studies have reported little or no correlation between cholesterol levels and stroke incidence,13,14 whereas others have reported a positive correlation.15–17 However, some studies have reported that high cholesterol levels attenuate nitric oxide (NO·)-dependent relaxant responses in the cerebral circulation of experimental animals.18–20 Moreover, increased superoxide production by cerebral vascular NADPH oxidases may play a key role in...
compromised NO function during hypercholesterolemia. In the present study, we sought to test whether excessive production of superoxide by Nox2-containing NADPH oxidase occurs in the cerebral circulation of hypercholesterolemic apolipoprotein E-deficient (ApoE<sup>−/−</sup>) mice and if it causes decreased NO function.

**Methods**

All procedures were approved by the Institutional Animal Ethics Committee. Nox2-deficient (Nox2<sup>−/−</sup>) mice were originally generated in the laboratory of Professor Mary Dinauer and bred at Ozgene (Bentley DC, WA, Australia). Wild-type (WT) and ApoE<sup>−/−</sup> mice were obtained from the Animal Resources Centre (Canning Vale, WA, Australia). All mice studied were male and fully backcrossed to the C57BL/6J background. Nox2<sup>−/−</sup> mice were bred with ApoE<sup>−/−</sup> mice to generate a Nox2<sup>−/−</sup>/ApoE<sup>−/−</sup> double knockout colony and a genetically related ApoE<sup>−/−</sup> single knockout colony as previously described. Genotypes were determined by polymerase chain reaction amplification of tail DNA. From 5 weeks of age, mice were maintained on a high-fat diet (21% fat, 0.15% cholesterol; Specialty Feeds) for 7 to 14 weeks.

**Measurement of Total Plasma Cholesterol Levels**

Mice were anesthetized with isoflurane (Baxter Healthcare). Blood was collected into a heparinized tube and centrifuged at 4000 g (4°C) for 10 minutes. Plasma total cholesterol levels were then determined using a Roche MODULAR 917 (Roche Diagnostics, Indianapolis, Ind) enzymatic colorimetric assay.

**Histological Studies**

Middle cerebral arteries (MCAs) and aortae from WT and ApoE<sup>−/−</sup> mice were examined for the presence of atherosclerotic lesions. The basilar lobe with the MCA attached and the cerebellum with the basilar artery attached were mounted in an OCT Tissue-Tek mold and snap-frozen in liquid nitrogen. MCA and basilar arteries were then sectioned (10 μm) and incubated for 60 minutes with oil red O (0.5% in 60% isopropyl alcohol). Excess stain was removed with 60% isopropyl alcohol and sections were then counterstained with 25% hematoxylin. For sections (approximately 200 μm apart from each other along the length of the artery) per animal were viewed and digitized using an Olympus AX70 microscope equipped with a 40× oil immersion lens and a color DP70 Peltier cooled digital camera (Olympus, Tokyo, Japan). Aortic atherosclerotic lesion burden was assessed as previously described. Briefly, aortae were dissected in their entirety, cut open longitudinally along the ventral surface, and incubated with oil red O. Excess stain was removed with 60% isopropyl alcohol and en face images of each aortic segment were photographed. Atherosclerotic plaque area was expressed as a percentage of the total luminal surface area of the aorta.

**Measurement of Superoxide Production by Cerebral Arteries**

Experiments were carried out using pooled basilar arteries and MCAs. Basal superoxide production by cerebral arteries from WT, ApoE<sup>−/−</sup>, and Nox2<sup>−/−</sup>/ApoE<sup>−/−</sup> mice was measured by 100 μmol/L L-012-enhanced chemiluminescence as previously described. We have previously reported that superoxide is the major reactive oxygen species detected by L-012. Background counts were subtracted and superoxide production normalized to dry tissue weight.

**Assessment of NO Function in Cerebral Arteries**

MCAs from WT, ApoE<sup>−/−</sup>, and Nox2<sup>−/−</sup>/ApoE<sup>−/−</sup> mice were mounted between 2 micromanipulae in a pressure myograph (Living Systems Instrumentation Inc.). Arteries were superfused with warm (37°C), carbogen-bubbled (95% O₂, 5% CO₂) Krebs-bicarbonate solution (composition in mmol/L: NaCl 118, KCl 4.5, MgSO<sub>4</sub> 0.45, KH<sub>2</sub>PO<sub>4</sub> 1.03, NaHCO<sub>3</sub> 25, glucose 11.1, CaCl<sub>2</sub> 2.5). Intraluminal pressure was gradually increased to 60 mm Hg and maintained at this level with a pressure servo unit without further intraluminal perfusion. MCAs were allowed to equilibrate for 15 minutes before baseline diameters were measured. NO function was assessed through the constrictor response (measured from baseline diameter) to N<sup>ω</sup>-nitro-l-arginine methyl ester (L-NAME; 100 μmol/L; 30 minutes incubation). Contractile responses were recorded once they reached a steady level (approximately 30 minutes). In MCA from a separate group of WT and ApoE<sup>−/−</sup> mice, contractile responses to L-NAME were assessed in the presence of the superoxide scavenger, tempol (1 mmol/L; 10 minutes incubation). After equilibration and on completion of the experimental protocol, arteries were exposed to a high potassium physiological salt solution containing 122.7 mmol/L K<sup>+</sup> (KPSS; equimolar replacement of NaCl with KCl). These functional studies were performed on MCAs from each genotype on the same day.

**Western Blotting**

Experiments were carried out using pooled basilar arteries and MCAs. Protein expression of Nox2, p47phox, endothelial NO synthase (eNOS), superoxide dismutase isoforms (SOD1, SOD2 and SOD3), p47phox, endothelial NO synthase (eNOS), and superoxide dismutase isoforms (SOD1, SOD2 and SOD3) were measured by Western blotting.
SOD3), and β-actin was measured in cerebral arteries using Western Blotting as previously described. Immune-reactive bands were detected by enhanced chemiluminescence, quantified using a ChemiDoc XRS molecular imager (BioRad), and normalized to intensity of corresponding bands for β-actin.

Drugs
L-012 was purchased from Wako Pure Chemicals and all other drugs were from Sigma. L-012 was prepared in 100 mmol/L dimethyl sulfoxide and diluted in Krebs-HEPES solution such that the final concentration of dimethyl sulfoxide was ≤0.1%. l-NAME and tempol were dissolved in Krebs-bicarbonate solution.

Data Analysis
All results are presented as mean±SEM. Statistical comparisons were performed using 1-way analysis of variance with a Bonferroni multiple comparison post hoc test.

Results
Plasma Cholesterol and Cerebral Artery Morphology
Total plasma cholesterol levels were substantially higher in ApoE−/− (116±5 μmol/L, P<0.05) and Nox2−/−/ApoE−/− (122±4 μmol/L, P<0.05) mice than in WT mice (1.4±0.1 mmol/L). There were no detectable lesions or fatty deposits in either MCAs (Figure 1A–B) or basilar arteries (data not shown) from WT, ApoE−/−, or Nox2−/−/ApoE−/− mice (data not shown for Nox2−/−/ApoE−/− mice). In contrast, atherosclerotic lesions were prominent in the aorta of ApoE−/− mice (approximately 9% of total aortic surface, n=13; Figure 1C–D) but were undetectable in WT aorta (Figure 1B).

Superoxide Production by Cerebral Arteries
Basal superoxide production by cerebral arteries from ApoE−/− mice was approximately 50% greater than levels generated by arteries from WT mice (Figure 2, P<0.05). However, in Nox2−/−/ApoE−/−, cerebral artery superoxide production was not elevated (Figure 2, P<0.05) compared with WT.

Cerebral Artery NO− Function
During the equilibration period, vessels did not consistently develop significant tone.

Final baseline diameters of MCAs were similar between genotypes (WT, 116±5 μmol/L; ApoE−/−, 112±5 μmol/L; Nox2−/−/ApoE−/−, 122±4 μmol/L). The magnitude of l-NAME-induced contraction (measured from baseline diameter) of MCAs from ApoE−/− mice was <50% of that in WT (Figure 3A, P<0.05). By contrast, contractile responses to l-NAME in Nox2−/−/ApoE−/− mice were comparable to that in WT mice (Figure 3A), indicative of greater NO function in the absence of Nox2 despite the mice being hypercholesterolemic. By contrast, contractile responses to KPSS (also measured from baseline diameter) were similar among all genotypes (Figure 3B). In the presence of the superoxide scavenger, tempol (1 mmol/L), contractile responses to l-NAME (and KPSS) in MCAs from ApoE−/− mice were comparable to that in WT (Figure 4).

Cerebral Artery Protein Expression
Cerebral artery expression of Nox2 was similar in WT and ApoE−/− mice (Figure 5A). By contrast, in ApoE−/− mice, cerebral artery p47phox expression was approximately 2-fold higher versus WT mice (Figure 5B; P<0.05) but was similar to levels found in Nox2−/−/ApoE−/− mice (Figure 5B). Expression of eNOS and SOD isoforms (1, 2, and 3) were similar in WT, ApoE−/−, and Nox2−/−/ApoE−/− mice (Figure 6A–D).

Discussion
The present study provides novel insight into the effects of hypercholesterolemia on the cerebral circulation. The major findings are that, despite the absence of atherosclerotic lesions, cerebral arteries from hypercholesterolemic mice have (1) elevated Nox2-dependent superoxide production; (2) reduced NO function; and (3) increased expression of the NADPH oxidase organizer subunit, p47phox. Either genetic deletion of Nox2 or acute scavenging of superoxide with tempol abrogated the decreased NO function in cerebral arteries during hypercholesterolemia. Collectively, these findings represent the first evidence that augmented produc-
tion of Nox2-derived superoxide is a major mediator of cerebral artery dysfunction during hypercholesterolemia.

Under physiological conditions, reactive oxygen species such as superoxide and its downstream metabolites serve as important cell signaling molecules for the regulation of normal vascular function. However, a wealth of evidence indicates that excessive vascular superoxide production is associated with several risk factors for cerebrovascular disease. Several studies have reported that Nox2-containing NADPH oxidase contributes to activated (e.g., angiotensin II- and NADPH-stimulated) but not basal superoxide production in the cerebral circulation under physiological conditions. Moreover, it has been suggested that increased superoxide production by more than one NADPH oxidases may contribute to endothelial dysfunction in cerebral arterioles of hypercholesterolemic mice. However, it remains unclear whether superoxide production is elevated in the cerebral circulation of hypercholesterolemic mice. Using the chemiluminescence probe, L-012, we found in the present study that despite the absence of atherosclerotic lesions, basal superoxide production by cerebral arteries from hypercholesterolemic mice is elevated to more than one NADPH oxidases may contribute to basal superoxide production in the cerebral circulation under physiological conditions. Moreover, it has been suggested that increased superoxide production by cerebral arteries from hypercholesterolemic mice is approximately 50% greater than in normocholesterolemic WT mice. In genetically related hypercholesterolemic Nox2−/−/ApoE−/− mice, cerebral artery superoxide production was similar to levels found in arteries from WT mice. We have recently found that neither Nox2-NADPH oxidase nor Nox1-NADPH oxidase contributes to basal superoxide production under physiological conditions. The contribution of other NADPH oxidase isoforms and/or enzymatic sources to basal superoxide production remains to be determined. However, importantly, our data provide strong evidence that Nox2-containing NADPH oxidase is the key source of the augmented superoxide production by cerebral arteries during hypercholesterolemia.

A recent study found that acetylcholine-induced NO-dependent relaxant responses are impaired in cerebral arterioles of ApoE−/− mice. Similarly, Didion et al reported that constriction of the basilar artery to the soluble guanylate cyclase inhibitor 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) is reduced in hypercholesterolemic monkeys, which may reflect reduced basal production of NO. Moreover, some studies have reported impaired endothelium-dependent relaxant responses in the cerebral circulation of hypercholesterolemic rabbits. We and others have previously reported that Nox2 is predominantly localized to endothelial cells of mouse cerebral arteries. Increased superoxide production by Nox2 in cells that express NO synthase isoforms is likely to be of significance because superoxide reacts avidly with NO when both species are generated in the same biological environment.
compartment. Therefore, we next tested whether augmented Nox2-derived superoxide leads to decreased cerebral artery NO function. We assessed the basal NO function in pressurized isolated MCAs using L-NAME, whereby the magnitude of the constrictor response was indicative of the degree to which NO normally attenuates vascular tone. We found that contractions to L-NAME were substantially smaller in MCAs from ApoE−/−/− mice than responses in WT mice, suggesting that cerebral artery NO function is diminished during hypercholesterolemia. We next examined contractile responses to L-NAME in hypercholesterolemic Nox2−/−/−/ApoE−/−/− mice. Consistent with our superoxide data, L-NAME-induced contractions of MCAs from Nox2−/−/−/ApoE−/−/− mice were comparable to those observed in WT mice. Thus, these findings provide the first direct evidence that Nox2-containing NADPH oxidase plays a central role in impaired NO function of cerebral arteries during hypercholesterolemia.

A previous study reported that impaired acetylcholine-induced NO-dependent dilatation of cerebral arterioles in ApoE−/− mice can be restored to normal by the SOD mimetic, tempol.19 In this study, we extended this finding by assessing the effect of tempol on basal NO levels during hypercholesterolemia. We found that the magnitude of L-NAME-induced contractions was equivalent in cerebral arteries from WT and ApoE−/− mice when applied in the presence of tempol. This acute improvement by tempol of NO function in ApoE−/− mice suggests that superoxide might normally inactivate NO during hypercholesterolemia. It is conceivable that superoxide and/or a downstream reactive oxygen species might impair NO signaling by reducing the expression and/or activity of sGC.27,28 However, several studies have reported that responses to NO donors are normal in cerebral vessels from hypercholesterolemic animals, including ApoE−/−/− mice.19,23,25,26 Thus, smooth muscle signaling in response to NO is likely to be preserved in cerebral arteries during hypercholesterolemia and is unlikely to account for the smaller constriction to L-NAME in our ApoE−/−/− mice. Also, the relatively lower level of basal NO in ApoE−/−/− mice was unlikely to be attributable to reduced rates of NO generation and/or superoxide metabolism by SODs, because expression levels of endothelial NO synthase and all 3 SOD isoforms were similar between WT and ApoE−/−/− mice. To the best of our knowledge, no study has ever examined whether the ApoE gene is expressed and functionally important in cerebral vessels. Therefore, we cannot exclude the possibility that deletion of the ApoE gene, irrespective of the plasma cholesterol level, contributes to the changes we observed in this study.

To explore a possible molecular basis for our findings, we examined the effect of hypercholesterolemia on cerebral

Figure 6. Representative Western blots showing protein expression of (A) eNOS, (B) SOD1, (C) SOD2, and (D) SOD3 in cerebral artery (pooled basilar and middle cerebral) homogenates from WT, ApoE−/−, and Nox2−/−/−/ApoE−/− mice (top). Also shown is a summary of immunoreactive band intensity (bottom). Values are expressed as relative intensity normalized to β-actin intensity and are given as mean±SEM (n=4 to 6).
artery expression of the Nox2. Using Western blotting, we observed a Nox2-immunoreactive band at approximately 58 kDa in cerebral artery homogenates from WT and ApoE−/− mice, which was absent in homogenates from either Nox2−/− or Nox2−/−/ApoE−/− mice. Analysis of the intensity of this band, however, revealed no significant difference between WT and ApoE−/− mice. Thus, hypercholesterolemia does not influence expression levels of the Nox2 catalytic subunit in cerebral arteries. Numerous factors may regulate the activity of Nox2, including its association with the cytosolic organizer subunit p47phox. In ApoE−/− mice, cerebral artery expression levels of p47phox were approximately 2-fold higher than in WT mice but were similar to levels found in Nox2−/−/ApoE−/− mice. Greater quantities of the p47phox subunit in cerebral arteries during hypercholesterolemia might result in higher activity of Nox2 and thus may contribute to the augmentation in vascular superoxide levels.

In summary, the findings of this study reveal that even in the absence of atherosclerotic lesions, Nox2-dependent superoxide production is augmented in cerebral arteries of hypercholesterolemic mice. Moreover, this increase in Nox2-derived superoxide appears to impair NO function, possibly through the acute inactivation of NO, leading to endothelial dysfunction of cerebral arteries. The increased activity of Nox2 is associated with augmented expression of the cytosolic subunit, p47phox, during hypercholesterolemia. Thus, these findings demonstrate for the first time that Nox2-derived superoxide plays a central role in mediating cerebral endothelial dysfunction during hypercholesterolemia.

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Disclosures
None.

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