Cerebral Vascular Dysfunction During Hypercholesterolemia

Jiro Kitayama, MD; Frank M. Faraci, PhD; Steven R. Lentz, MD, PhD; Donald D. Heistad, MD

**Background and Purpose**—Studies of peripheral arteries in hypercholesterolemic animals suggest that increased generation of superoxide contributes to endothelial dysfunction, especially in the presence of atherosclerotic lesions. We hypothesized that vasomotor function is impaired in cerebral arterioles during hypercholesterolemia through a mechanism that involves oxidative stress.

**Methods**—Apolipoprotein E–deficient (apoE−/−) mice were fed a normal or a high-fat diet for >6 months. ApoE−/− mice fed a normal diet were used as normocholesterolemic controls. Responses of cerebral arterioles were examined in open cranial windows in vivo in anesthetized mice.

**Results**—In apoE−/− mice, intimal area was increased only in the proximal aorta on the normal diet and also markedly increased in the distal aorta on the high-fat diet. There were no increases in intimal area in the aortas of control mice or in the cerebral arterioles in any group. The dilator response of cerebral arterioles to ACh (10 μmol/L) in control mice (26±4% increase in diameter) was reduced in apoE−/− mice on either the normal (13±2%) or the high-fat (13±3%) diet (P<0.05 vs control). NADPH (10 μmol/L), a substrate for NADPH oxidase, produced dilator responses in control mice (8±4%) that were significantly increased in apoE−/− mice on the high-fat diet (16±2%, P<0.05 vs control). Tempol, a superoxide scavenger, and apocynin, an inhibitor of NADPH oxidase, significantly increased vasodilator responses to ACh and decreased vasodilation to NADPH in apoE−/− mice on the high-fat diet. Nitroprusside produced a similar dilatation in the cerebral arterioles of all groups.

**Conclusions**—Hypercholesterolemia is associated with oxidative stress and endothelial dysfunction in cerebral arterioles, despite the absence of atherosclerotic lesions. *(Stroke. 2007;38:2136-2141.)*

**Key Words:** hypercholesterolemia ▪ cerebral arterioles ▪ endothelium ▪ oxidative stress ▪ vasomotor function
function in cerebral arterioles in vivo in the absence of local atherosclerotic lesions through mechanisms that involve oxidative stress. We used apolipoprotein E-deficient (apoE−/−) mice for this study because they are a genetic model of hypercholesterolemia and atherosclerosis.20

Materials and Methods

Animal Preparation
Female apoE mice (n=18), which have a C57BL/6 genetic background, were used in this study. We used female mice because they develop more atherosclerosis than do males.21 The mice were originally obtained from Jackson Laboratories (Bar Harbor, Maine) and were bred and genotyped by polymerase chain reaction in our laboratory. At 3 weeks of age, apoE−/− mice were randomized to receive either a high-fat diet containing 20% fat and 0.2% cholesterol (TD.03159, Harlan Teklad) or a normal diet containing 5.7% fat (7912, Harlan Teklad). Age- and sex-matched heterozygous littermate (apoE+/−, n=11) mice, fed a normal diet, were used as normalcholesterolemic controls. Animals were studied at 7 to 13 months of age (mean ages of control mice, apoE−/− mice on the normal diet, and apoE−/− mice on the high-fat diet were 8±0, 9±2, and 8±0 months, respectively). All procedures followed institutional guidelines approved by the animal care and use committee at the University of Iowa.

Cranial Window
Mice were anesthetized with pentobarbital sodium (75 to 90 mg/kg IP). A catheter in the femoral artery was used to measure arterial pressure and to obtain blood. Mice were ventilated mechanically with supplemental O2. Depth of anesthesia was evaluated by applying pressure to a paw or tail and by observing changes in heart rate and blood pressure. Additional anesthetic (~20 mg/kg per hour) was administered when such changes occurred. Arterial blood gases were maintained within normal limits throughout each experiment (pH=7.31±0.01, PCO2=37±2 mm Hg, and PO2=116±7 mm Hg). Body temperature was maintained at 37°C with a heating pad. An open cranial window was made over the left parietal cortex, and a segment of a pial arteriole was exposed. After part of the dura was opened, the cranial window was suffused with artificial cerebrospinal fluid (aCSF) at 37°C. The ionic composition (in mmol/L) of aCSF was as follows: 132 NaCl, 2.95 KCl, 1.71 CaCl2, 0.65 MgCl2, 24.6 NaHCO3, and 3.69 t-glucose. The aCSF was bubbled continuously with appropriate gases to maintain normal levels of pH and PCO2. In CSF sampled from the cranial window, the pH was 7.36±0.01, PCO2 was 39±1 mm Hg, and PO2 was 125±2 mm Hg. The window was suffused with aCSF for at least 30 minutes before the experiment.

The diameter of the cerebral arteriole was recorded and measured with a microscope, videocassette recorder, and an image analyzer. The diameter of 1 arteriole per animal was measured under control conditions and during topical application of acetylcholine (ACh, 1 and 10 μmol/L) and sodium nitroprusside (SNP, 0.01 and 0.1 μmol/L). In addition, the effects of topical application of NADPH (1 and 10 μmol/L), a substrate for NADPH oxidase, were examined. The vasodilators were suffused over the cranioistomy for 5 minutes, and the internal diameter of the pial arteriole was measured.

In some mice, vasodilator responses were examined after superfusion of the cranial window with Tempol (1 mmol/L, a superoxide scavenger) for 30 minutes or apocynin (100 μmol/L, an inhibitor of NADPH oxidase) for 30 minutes. We examined the effects of ACh, SNP, and NADPH in 6 control and 5 apoE−/− mice on a high-fat diet during Tempol. Apocynin was also used in 6 control and 5 apoE−/− mice on a high-fat diet. Responses to vasoactive stimuli were not examined in the same mice before and after Tempol or apocynin. Topical application of these agents did not produce any changes in systemic arterial pressure or a prolonged change in arteriolar diameter after suffusion of the drug was stopped.

Measurement of Plasma Cholesterol
Blood was collected into a heparinized capillary tube from the clipped tail several days before the terminal cranial window study. Plasma total cholesterol level was determined with Softmax Pro analysis software (Molecular Devices), a cholesterol reference kit (Verichem), and cholesterol reagents (Infinity), which were adapted for a microplate assay. In brief, 2.5 μL of plasma samples and controls were loaded in a 96-well plate and mixed with 250 μL cholesterol reagents. After a 5-minute incubation at 37°C, the absorbance at 490 nm was read on a Spectra Max 190 (Molecular Devices) plate reader. When the values were >750 mg/dL, the samples were diluted in saline and measured again.

Histological Studies
After the cranial window study, the aorta and cerebral arterioles were examined in several mice for the presence of atherosclerotic lesions. In brief, after fixation of the aorta with 7% formalin and embedding in paraffin, the tissue was stained with van Gieson’s stain, van Gieson’s stains elastic fibers, and it was used to determine the size of the intimal area, which is enclosed by the internal elastic lamina.20 In cross sections of the ascending arch and descending and abdominal aortas, images were captured in a computer, and areas of intima and media were measured with National Institutes of Health Image J software. In the brain, the pial arteriole with pial arterioles attached was fixed in formalin, embedded in paraffin, stained with both hematoxylin and eosin, and stained with van Gieson’s. Three or four sections were examined per sample.

Statistical Analysis
All values are expressed as mean±SEM. Dilatation of pial arterioles to ACh, SNP, and NADPH is expressed as the percent change from baseline diameter. Single comparisons were made with an unpaired t test, and multiple comparisons were made with ANOVA followed by the Student-Newman-Keuls test to detect individual differences as appropriate. A probability value <0.05 was considered statistically significant.

Results
The body weights of control mice, apoE−/− mice on the normal diet, and apoE−/− mice on the high-fat diet were 23±0.4, 23±0.7, and 25±0.7 g, respectively. Plasma total cholesterol levels of control mice, apoE−/− mice on the normal diet, and apoE−/− mice on the high-fat diet were 62±4, 481±46, and 899±57 mg/dL, respectively. ApoE−/− mice have normal cholesterol levels compared with normal C57BL/6 mice (97±12 mg/dL), in which plasma cholesterol was measured in preliminary studies. The blood glucose level of each group was 119±10, 120±13, and 132±9 mg/dL (not significant), respectively. During anesthesia, mean arterial blood pressures of control mice, apoE−/− mice on the normal diet, and apoE−/− mice on the high-fat diet were 68±3, 65±3, and 67±3 mm Hg, respectively. These values were similar to those in a previous report.22

There were no atherosclerotic lesions in the aortas of control mice. In apoE−/− mice on the control diet, intimal area was increased only in the ascending aorta. In apoE−/− mice on the high-fat diet, intimal area was markedly increased in the ascending aorta, aortic arch, descending aorta, and abdominal aorta (Table). There were no detectable morphological
Aortic Intimal Areas of ApoE<sup>−/−</sup> Mice

<table>
<thead>
<tr>
<th></th>
<th>Ascending Aorta</th>
<th>Arch</th>
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<tr>
<td>Control (n=6)</td>
<td>0.00±0.00</td>
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<td>ApoE&lt;sup&gt;−/−&lt;/sup&gt;, normal diet (n=5)</td>
<td>0.21±0.08&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>ApoE&lt;sup&gt;−/−&lt;/sup&gt;, high-fat diet, (n=10)</td>
<td>0.39±0.13&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.19±0.04&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.13±0.05&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.18±0.06&lt;sup&gt;*&lt;/sup&gt;</td>
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Values are mean±SEM in mm<sup>2</sup>. *P<0.05 vs control.

Changes (increase in intimal area) in the cerebral arterioles in apoE<sup>−/−</sup> mice on either the control or high-fat diet (Figure 1).

Effects of Hypercholesterolemia on Cerebral Vasodilatation

The baseline diameter of cerebral arterioles was similar in the various groups of mice and averaged 26±1 µm. In both groups of apoE<sup>−/−</sup> mice (both diets), ACh produced significantly less dilatation of cerebral arterioles than in control mice (Figure 2A). In contrast, 1 µmol/L NADPH tended to produce greater dilator responses in apoE<sup>−/−</sup> mice (on both diets) than in control mice, and the high concentration of NADPH produced significantly more dilatation of cerebral arterioles in apoE<sup>−/−</sup> mice on the high-fat diet than in control mice (P<0.05; Figure 2B). SNP produced similar dilator responses of cerebral arterioles in control mice, apoE<sup>−/−</sup> mice on the control diet, and apoE<sup>−/−</sup> mice on the high-fat diet (Figure 2C).

Effects of Tempol and Apocynin on Cerebral Vasodilatation in Hypercholesterolemia

In control mice, Tempol and apocynin did not affect vasodilator responses to ACh, NADPH, and SNP (Figure 3). In apoE<sup>−/−</sup> mice on the high-fat diet, ACh-induced dilator responses of cerebral arterioles were significantly improved in the presence of Tempol (Figure 4A). In contrast, NADPH-induced dilator responses were significantly reduced by Tempol (Figure 4B). SNP-induced dilator responses were not affected by Tempol (Figure 4C). Apocynin showed similar effects on dilator responses to these agents in apoE<sup>−/−</sup> mice on the high-fat diet (Figure 4), but there were no significant effects in control mice (Figure 3).

Discussion

One major new finding in the present study is that responses to ACh in cerebral arterioles were reduced in apoE<sup>−/−</sup> mice on either a normal or a high-fat diet, compared with responses in control mice in vivo. Although apoE<sup>−/−</sup> mice on the high-fat diet demonstrated higher plasma cholesterol concentrations and more extensive atherosclerotic lesions in the aorta than did either control or apoE<sup>−/−</sup> mice on a normal diet, responses to ACh were impaired similarly in apoE<sup>−/−</sup> mice on both the normal and high-fat diet. Histological examination of tissues from hypercholesterolemic mice did not show morphological changes in the cerebral arterioles. These findings suggest that increased plasma cholesterol values, even without morphological changes in cerebral arterioles, are associated with endothelial dysfunction in cerebral arterioles.

A second new finding is that impaired endothelium-dependent dilator responses of cerebral arterioles in hypercholesterolemic mice were improved by treatment with Tempol, a superoxide scavenger, and apocynin, an inhibitor of NADPH oxidase. In addition, dilator responses to NADPH were enhanced in apoE<sup>−/−</sup> mice on the high-fat diet, and responses to NADPH were reduced by pretreatment with Tempol and apocynin. Thus, these results suggest that hypercholesterolemia may modulate cerebral arteriolar dysfunction, at least in part via NADPH oxidase–derived superoxide.

Vascular Effects of Hypercholesterolemia

ACh produces endothelium-dependent, NO-mediated dilator responses in cerebral blood vessels. Our finding that cerebral arteriolar responses to ACh are impaired during hypercholesterolemia is consistent with previous studies of the aorta<sup>15,20,24</sup> and carotid artery<sup>14</sup> from genetically altered...
hyperlipidemic mice. In extracranial vessels, impaired responses may be dependent on the presence of local atherosclerotic lesions. For example, vascular function is greatly impaired in the proximal aorta of mice with advanced atherosclerotic lesions, whereas in distal segments of the aorta with minimal evidence of atherosclerosis, relaxation to ACh is normal.20 On the other hand, endothelial function also may be impaired in vessels without morphological changes. For example, endothelial function is impaired in the common carotid arteries of apoE-deficient mice, although neither intimal thickening nor atherosclerotic lesions were found, in contrast to pronounced atherosclerotic lesions in the aorta.14

Several mechanisms contribute to endothelial dysfunction during hypercholesterolemia, including functional impairment of NOS due to a deficiency of substrate or cofactors,25 reduced protein expression and/or activity of eNOS,26,27 increased levels of asymmetric dimethylarginine (an endogenous inhibitor of NOS),28 and increased generation of superoxide with reduced bioavailability of NO.15,29 Among these mechanisms, there is considerable evidence that implicates production of reactive oxygen species (ROS) in the vessel wall as a key event predisposing to vascular changes. Previous studies of extracranial vessels of hypercholesteremic animal indicate that increased superoxide generation via activation of xanthine oxidase, NADPH oxidase, and eNOS contributes to generation of superoxide in the vascular wall.29–32

Superoxide in Cerebral Blood Vessels

There are several lines of evidence that suggest that vascular NADPH oxidase is functionally important in both physiology and pathophysiology, including during the development of atherosclerosis33 and in the regulation of vascular tone in cerebral vessels.16,17,34 Recent studies35,36 indicate that NADPH oxidase is present in cerebral vessels, and exogenous NADPH increases regional cerebral blood flow. These effects of NADPH on cerebral blood flow and on the generation of ROS in cerebral vessels are not observed in mice lacking gp91phox, a subunit of NADPH oxidase, or in the presence of a peptide inhibitor (gp91ds-tat) of gp91phox.35,36 Thus, activation of NADPH oxidase and increases in the ROS level appear to be important in the regulation of regional cerebral blood flow.

In the present study, NADPH-induced dilator responses of cerebral arterioles were greater in apoE−/− mice than in control mice. Cerebral arterioles dilate in response to ACh, a substrate for NADPH oxidase.18,19,34 The dilator response is thought to be mediated by superoxide and its derivative, H2O2, via activation of potassium channels in vascular smooth muscle cells.19,34 Another study, however, reported that increases in cerebral blood flow during NADPH are not attenuated by catalase.36 ROS may also inhibit channel function.37 Mechanisms that account for enhanced dilator responses of cerebral arterioles to NADPH in apoE−/− mice are unclear. A recent article indicated that exogenous NADPH increases regional cerebral blood flow not only via

A

B

C

Figure 2. A, Effects of hypercholesterolemia on ACh-induced dilator responses of cerebral arterioles in control mice (open bar, n=8), apoE−/− mice on the control diet (gray bar, n=7), and apoE−/− mice on the high-fat diet (black bar, n=8). Values are mean±SEM. *P<0.05 vs control. B, Effects of hypercholesterolemia on NADPH-induced dilator responses of cerebral arterioles in control mice (n=8), apoE−/− mice on the control diet (n=7), and apoE−/− mice on the high-fat diet (n=8). Values are mean±SEM. *P<0.05 vs control. C, Effects of hypercholesterolemia on dilator responses of cerebral arterioles to nitroprusside in control mice (n=8), apoE−/− mice on the control diet (n=7), and apoE−/− mice on the high-fat diet (n=8). Values are mean±SEM.

Figure 3. Effects of vehicle (n=11), Tempol (1 mmol/L, 30 minutes, n=6) and apocynin (100 μmol/L, 30 minutes, n=6) on dilator responses of cerebral arterioles in control mice to ACh (A), NADPH (B), and nitroprusside (C). Values for responses to vehicle are from the same animals as in Figure 1. Values are mean±SEM.
activation of NADPH oxidase but also by production of NO derived from vascular endothelial cells. During hypercholesterolemia, because the vasodilator response to ACh is impaired, it seems unlikely that the augmented vasodilator responses to NADPH are mediated by augmented production of NO. It is also possible that NADPH augments production of superoxide by uncoupled eNOS.

The dilator response to NADPH may be an index of NADPH oxidase activity of the vessels. Thus, greater responses to NADPH in hypercholesterolemic than in normocholesterolemic mice suggest that the activity of NADPH oxidase is greater in hypercholesterolemic mice. The effect of Tempol on the responses to ACh reflects superoxide generated by NADPH oxidase and other superoxide-generating enzymes. Thus, the relatively small effect of Tempol on the responses to ACh in normal mice suggests that not only is the activity of NADPH oxidase low in control vessels but also that levels of superoxide from other enzymes are low.

Thus, reduced dilator responses to ACh as well as enhanced dilator responses to NADPH in hypercholesterolemic mice may be mediated by increased generation of superoxide via activation of NADPH oxidase. This conclusion is supported by the finding that pretreatment with Tempol or apocynin enhanced dilator responses to ACh and inhibited NADPH-induced dilatation. Previous studies suggested that levels of superoxide dismutase are normal during hypercholesterolemia, and thus reductions in superoxide dismutase are not responsible for increased levels of superoxide. Thus, we speculate that increased generation of superoxide, at least in part via activation of NADPH oxidase, at least in part via activation of NADPH oxidase, at least in part via activation of NADPH oxidase, may play an important role in endothelial dysfunction of cerebral vessels in apoE-deficient mice. On the other hand, we cannot exclude the possibility that deletion of the apoE gene might alter dilator responses of cerebral arterioles and that the sensitivity to superoxide-mediated dilatation might be increased.

Responses to SNP, which are endothelium independent, were not significantly different in the groups that we studied but tended to be smaller in apoE−/− mice on the high-fat diet than in normal mice. Most studies have suggested that responses to nitroprusside are normal in vessels from atherosclerotic animals unless these vessels are severely atherosclerotic. There may be some inactivation of nitroprusside-induced NO by superoxide in apoE−/− mice. Cyclic GMP levels and nitroprusside-induced dilator responses may be lower in aortas from apoE-deficient mice than in control C57BL/6J mice. In cerebral arteries from atherosclerotic monkeys, exogenous NO-induced dilator responses were found to be intact, but the basal activity of soluble guanylate cyclase appeared to be diminished. Thus, we cannot exclude the possibility that the activity of soluble guanylate cyclase/cyclic GMP is reduced in cerebral arterioles during hypercholesterolemia.

Summary

In conclusion, cerebral arterioles develop endothelial dysfunction during hypercholesterolemia. In hypercholesterolemic mice, impaired responses to ACh, with improvement after Tempol or apocynin, and increased responses to topical application of NADPH, with reduced responses after a superoxide scavenger, suggest that superoxide and possibly increased activity of NADPH oxidase may contribute to impaired endothelial function during hypercholesterolemia.

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Disclosures

None.

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