Cerebral Vascular Dysfunction Mediated by Superoxide in Hyperhomocysteinemic Mice

Sanjana Dayal, PhD; Erland Arning, PhD; Teodoró Bottiglieri, PhD; Rainer H. Böger, MD; Curt D. Sigmund, PhD; Frank M. Faraci, PhD; Steven R. Lentz, MD, PhD

Background and Purpose—Hyperhomocysteinemia is an emerging risk factor for stroke, but little is known about effects of hyperhomocysteinemia on cerebral vascular function. We tested the hypothesis that chronic hyperhomocysteinemia in mice causes endothelial dysfunction in cerebral arterioles through a mechanism that involves superoxide.

Methods—Mice heterozygous for a targeted disruption of the cystathionine $\beta$-synthase gene ($\text{Cbs}^{+/+}$) and their wild type littermates ($\text{Cbs}^{+/+}$) were fed either a control diet or a high-methionine diet for 10 to 12 months.

Results—Plasma total homocysteine was elevated with the high-methionine diet compared with the control diet for both $\text{Cbs}^{+/+}$ (7.9±1.0 versus 5.0±0.5 $\mu$mol/L; $P<0.05$) and $\text{Cbs}^{+/−}$ (20.5±3.1 versus 8.2±0.9 $\mu$mol/L; $P<0.001$) mice. Dilatation of cerebral arterioles ($\sim$30 $\mu$m baseline diameter) was measured in vivo in response to the endothelium-dependent dilator acetylcholine or the endothelium-independent dilator nitroprusside. Vasodilatation to acetylcholine was impaired with the high-methionine diet compared with the control diet for both $\text{Cbs}^{+/+}$ and $\text{Cbs}^{+/−}$ mice ($P<0.01$). Dilatation of arterioles to acetylcholine was restored toward normal by the superoxide scavenger tiron ($P<0.05$). Vasodilatation to nitroprusside was not influenced by $\text{Cbs}$ genotype or diet. Dihydroethidium (DHE) staining for vascular superoxide was elevated in $\text{Cbs}^{+/−}$ mice fed the high-methionine diet and was inhibited by apocynin or $\text{N}^\omega$-nitro-L-arginine methyl ester, implicating NAD(P)H oxidase and nitric oxide synthase as potential sources of superoxide.

Conclusions—Superoxide is a key mediator of endothelial dysfunction in the cerebral circulation during diet-induced hyperhomocysteinemia. (Stroke. 2004;35:1957-1962.)

Key Words: cerebral n endothelium n homocysteine n stroke n superoxides

Hyperhomocysteinemia, or elevation of plasma total homocysteine (tHcy), is associated with increased risk of cardiovascular disease and stroke. Numerous epidemiological studies have suggested that hyperhomocysteinemia is an independent risk factor and that the relative risk for stroke is higher than that for coronary heart disease.1 A recent meta-analysis of individual participant data from 12 prospective studies found that a 25% elevation in plasma tHcy was associated with $\sim$10% higher risk of cardiovascular disease and $\sim$20% greater risk of stroke after adjustment for other known risk factors.2 Hyperhomocysteinemia has also been implicated as a risk factor for cerebral venous thrombosis3 and Alzheimer disease.4

Studies in animals and humans have demonstrated that hyperhomocysteinemia is associated with impaired endothelium-dependent dilatation of the aorta and other peripheral vessels.5 Hyperhomocysteinemic mice with heterozygosity for a targeted disruption of the cystathionine $\beta$-synthase ($\text{Cbs}$) gene have enhanced sensitivity to endothelial dysfunction in the aorta and mesenteric arterioles.6,7 Endothelial dysfunction in peripheral arteries during hyperhomocysteinemia is associated with decreased bioavailability of the endothelium-derived vasodilator, nitric oxide (NO).8 Proposed mechanisms include oxidative inactivation of NO and decreased production of NO due to inhibition of endothelial nitric oxide synthase (NOS) by asymmetric dimethylarginine (ADMA).8

Much less is known about the effects of hyperhomocysteinemia in cerebral blood vessels. Using mice heterozygous for disruption of the $\text{Cbs}$ gene, it was demonstrated recently that mild hyperhomocysteinemia promotes cerebral vascular hypertrophy and altered cerebral vascular mechanics.9 Zhang et al found that superfusion of the rat parietal cortex with a combination of $\text{Cu}^{2+}$ and a very high concentration (1 mmol/L) of homocysteine decreased cerebral blood flow and attenuated responses to endothelium-dependent vasodi-
In humans, acute methionine loading has been reported to impair cerebral vascular reactivity in older but not younger subjects. It is not known, however, if chronic hyperhomocysteinemia produces cerebral vascular dysfunction in vivo, or whether effects of hyperhomocysteinemia on cerebral blood vessels are mediated by oxidative stress.

In this study, we used the Cbs-deficient mouse model to test the hypothesis that chronic hyperhomocysteinemia produces endothelial dysfunction in cerebral arterioles in vivo. Our results indicate that cerebral vascular dysfunction occurs at levels of plasma tHcy that are associated with clinical hyperhomocysteinemia in humans, and that this effect is mediated in part by superoxide.

**Materials and Methods**

**Mice and Experimental Protocol**

Mice heterozygous for disruption of the Cbs gene were crossed to C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Me) for at least 9 generations and comparisons were performed between heterozygous (Cbs+/−) and wild-type (Cbs+/+) littersmates. Genotyping for the targeted Cbs allele was performed by polymerase chain reaction. At the time of weaning, mice were fed either a control diet (LM-485, Harlan Teklad), which contains 6.7 mg/Kg folic acid and 4.0 g/Kg L-methionine, or a high methionine diet (LM-485 diet supplemented with 0.5% methionine in drinking water). After 10 to 12 months on experimental diet, mice were used either for studies of DHE fluorescence data. Correlation coefficients were calculated using the Pearson product moment correlation. A value of P<0.05 was used to define statistical significance. Values are reported as mean±SE.

**Results**

**Effect of Cbs Genotype and Diet on Plasma Levels of tHcy and ADMA**

<table>
<thead>
<tr>
<th></th>
<th>Control Diet</th>
<th>High Methionine Diet</th>
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<tbody>
<tr>
<td></td>
<td>Cbs+/+</td>
<td>Cbs+/−</td>
</tr>
<tr>
<td></td>
<td>(n=15)</td>
<td>(n=17)</td>
</tr>
<tr>
<td>tHcy (µmol/L)</td>
<td>5.0±0.5</td>
<td>8.2±0.9†</td>
</tr>
<tr>
<td>Methionine (µmol/L)</td>
<td>27.5±3.5</td>
<td>22.4±3.2</td>
</tr>
<tr>
<td>ADMA (µmol/L)</td>
<td>0.37±0.03</td>
<td>0.47±0.03†</td>
</tr>
</tbody>
</table>

Values are mean±SE.

*P<0.05 vs mice of same genotype fed the control diet.

†P<0.05 vs Cbs+/+ mice fed the same diet.

**Plasma tHcy, Methionine, and ADMA**

Blood was collected in anesthetized mice by cardiac puncture into EDTA (final concentration 5 mmol/L) for measurement of plasma tHcy and ADMA. Plasma tHcy, defined as the total concentration of homocysteine after quantitative reductive cleavage of all disulfide bonds, was measured by high-performance liquid chromatography (HPLC) and SBDF (aminium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate) fluorescence detection. Plasma methionine was measured by HPLC with coulometric electrochemical detection as described. Plasma ADMA was determined by HPLC using precol umn derivatization with 0-phthalaldehyde.

**Dilatation of cerebral arterioles**

Dilatation of cerebral arterioles was measured as described. Briefly, mice were anesthetized with sodium pentobarbital and ventilated mechanically. A cranial window was made over the left parietal cortex, and a segment of a randomly selected pial arteriole (~30 µm in diameter) was exposed. The diameter of the cerebral arteriole was measured using a video microscope coupled to an image-shearing device under control conditions and during superfusion with acetylcholine (1 and 10 µmol/L) and nitroprusside (0.1 and 1 µmol/L). In some experiments the window was superfused with 10 mmol/L tiron (4,5-dihydroxy-1,3, benzene-disulfonic acid) for 30 minutes before superfusion with acetylcholine.

**Detection of Superoxide**

Dihydroethidium (DHE), an oxidative fluorescent dye, was used to detect superoxide in sections of common carotid artery or cerebral arterioles by laser scanning confocal microscopy as described. Some sections were preincubated for 30 minutes with either PBS, 250 U/mL polyethylene glycol-superoxide dismutase (PEG-SOD) (Sigma-Aldrich), 300 µmol/L apocynin (4-hydroxy-3-methoxy acetophenone) (Fluka), 100 µmol/L Nω-nitro-L-arginine methyl ester (L-NAME) (Sigma-Aldrich), or 20 µmol/L uric acid (Calbiochem) before incubation with DHE. Fluorescent images were analyzed with Scion Image software (more details available at http://www.scioncorp.com). Data are reported as the percentage of surface area of carotid sections within the upper 20% of fluorescence intensity.

**Statistical Analysis**

Two-way analysis of variance (ANOVA) followed by Tukey post hoc test was used to analyze the effects of Cbs genotype and diet on plasma tHcy, ADMA, and responses to vasodilators in cerebral arterioles. The Mann–Whitney rank sum test was used for comparisons of DHE fluorescence data. Correlation coefficients were calculated using the Pearson product moment correlation. A value of P<0.05 was used to define statistical significance. Values are reported as mean±SE.
Dilatation of cerebral arterioles to 1.0 μmol/L acetylcholine (Figure 1A and 1B). In mice fed the control diet and Cbs+/+ mice (n=9 and n=12, respectively) or the high methionine (High Met) diet (n=7 and n=10, respectively), before or after superfusion with 10 mmol/L tiron, *P<0.05 vs mice of the same genotype fed control diet. †P<0.05 vs mice of the same genotype and diet without tiron.

Vascular Superoxide

Superoxide production was measured in sections of carotid artery using DHE. In the presence of superoxide, DHE is oxidized to a fluorescent adduct that intercalates into DNA, producing a nuclear staining pattern.17 As observed previously,17,18 DHE fluorescence was observed throughout the vascular wall. DHE fluorescence was detected at similar levels in Cbs+/+ and Cbs+/− mice fed the control diet and Cbs+/+ mice fed high methionine diet (Figure 3), but fluorescence was elevated 2.7±0.5-fold in Cbs+/− mice fed the high methionine diet (P<0.01 versus Cbs+/+ mice fed the control diet). The fluorescence signal in Cbs+/− mice fed the high methionine diet was inhibited by 250 U/mL polyethylene glycol-superoxide dismutase (PEG-SOD) (Figure 4B), which demonstrates that superoxide was a major reactive oxygen species detected by DHE in these vessels.

To examine potential sources of vascular superoxide, sections of carotid artery from Cbs+/− mice fed the high methionine diet were preincubated with an inhibitor of NAD(P)H oxidase, apocynin (300 μmol/L), or an inhibitor of NOS, L-NAME (100 μmol/L), before DHE staining. Each of these inhibitors decreased DHE fluorescence by ∼40% (Figure 4C and 4D), which implicates both NAD(P)H oxidase and NOS as sources of vascular superoxide in hyperhomocysteinemic mice. DHE fluo-
Rerescence also was inhibited by preincubation with uric acid (20 μmol/L), an inhibitor of peroxynitrite-mediated oxidation 19 (Figure 4E).

Superoxide also was detected by DHE staining in cerebral arterioles (Figure 5). Although we were unable to obtain quantitative data because of the small size of the vessels, DHE fluorescence clearly was higher in Cbs+/+ or Cbs+/- mice fed the high methionine diet (Figure 5C and 5D) compared with the control diet (Figure 5A and 5B). DHE fluorescence in cerebral arterioles was markedly inhibited by PEG-SOD (Figure 5E).

Discussion
The data presented here demonstrate both cerebral vascular dysfunction and increased vascular superoxide in a murine model of hyperhomocysteinemia. Our results also demonstrate that endothelium-dependent responses in cerebral arterioles are restored toward normal by the superoxide scavenger, tiron. These findings suggest that superoxide is a key mediator of cerebral endothelial dysfunction during diet-induced hyperhomocysteinemia in vivo.

Despite strong epidemiological evidence linking hyperhomocysteinemia and stroke, 1,2 very few studies have examined functional effects of hyperhomocysteinemia in cerebral blood vessels. Using a rat model, Zhang et al 10 demonstrated that superfusion of the parietal cortex with exogenous homocysteine and Cu²⁺ decreased cerebral blood flow and attenuated responses to endothelium-dependent vasodilators. 10 Interestingly, the vascular effects of homocysteine/Cu²⁺ were pre-
vented by co-administration of superoxide dismutase, which suggested a potential role for superoxide. A major limitation of this study was that both exogenous Cu²⁺ and a very high concentration of exogenous homocysteine (1 mmol/L) were needed to observe these effects. Our current findings confirm and extend these observations in a murine model that produces levels of plasma tHcy similar to those in human subjects at increased risk for stroke because of moderate hyperhomocysteinemia. Our results also are in concordance with a recent study in which acute methionine loading was found to impair cerebral vascular reactivity in elderly human subjects.

There is growing evidence that hyperhomocysteinemia promotes the oxidative inactivation of endothelium-derived NO in peripheral arteries. Our findings with the superoxide scavenger, tiron, suggest that oxidative inactivation of NO also occurs in the cerebral microcirculation. DHE fluorescence in Cbs⁺/⁻ mice fed the high methionine diet was inhibited by apocynin (Figure 4), which suggests that one source of vascular superoxide in hyperhomocysteinemic mice is an NAD(P)H oxidase. Vascular NAD(P)H oxidases are expressed in endothelium and vascular smooth muscle and have been implicated as sources of superoxide in atherosclerosis, hypertension, and diabetes. Our results are consistent with the recent findings of Ungvari et al, who detected increased NADPH-dependent production of superoxide and increased expression of the NAD(P)H oxidase subunit nox1 in coronary arteries isolated from hyperhomocysteinemic rats.

We also found that DHE fluorescence was inhibited by L-NAME, which suggests that NOS may be another source of vascular superoxide in hyperhomocysteinemia. Biochemical “uncoupling” of NOS, which can be caused by oxidation of its cofactor tetrahydrobiopterin, results in the preferential production of superoxide rather than nitric oxide. Uncoupling of NOS during hyperhomocysteinemia may be mediated by peroxynitrite, a powerful oxidant that is formed through the reaction of superoxide with NO. We found that DHE fluorescence in Cbs⁺/⁻ mice fed a high methionine diet was decreased by uric acid (Figure 4), an inhibitor of peroxynitrite-mediated oxidation. We also considered the possibility that vascular superoxide is related to ADMA, an endogenous NOS inhibitor that may promote uncoupling of NOS. ADMA is derived from protein methylation reactions that produce homocysteine as a byproduct, and plasma ADMA is elevated during hyperhomocysteinemia in monkeys and humans. Only a modest effect of Cbs genotype on plasma ADMA was seen in the current study (Table 1), however, which suggests that plasma ADMA is unlikely to be a major mediator of endothelial dysfunction during hyperhomocysteinemia in mice.

We observed diet-induced impairment of endothelium-dependent dilatation of cerebral arterioles not only in Cbs⁺/⁻ mice with moderate hyperhomocysteinemia (plasma tHcy ≈ 20 μmol/L), but also in Cbs⁺/+ mice with very mild hyperhomocysteinemia (plasma tHcy ≈ 8 μmol/L) (Figure 4). This observation contrasts with previous findings using aortic rings from Cbs⁺/+ and Cbs⁺/+ mice in which only Cbs⁺/+ mice with plasma tHcy > 20 μmol/L exhibited impaired responses to acetylcholine. These results suggest that cerebral arterioles may be more sensitive than the aorta to the vascular effects of mild hyperhomocysteinemia. In support of this interpretation, hypertrophy of cerebral arterioles has been observed in Cbs⁺/+ and Cbs⁺/+ mice with mild hyperhomocysteinemia (plasma tHcy > 8 μmol/L). It is noteworthy, however, that responses of cerebral arterioles to acetylcholine were preserved in Cbs⁺/+ mice fed the control diet (Figure 1), despite the fact that these mice also had mild elevation of plasma tHcy (≈ 8 μmol/L). This finding raises the possibility that a methionine metabolite other than homocysteine may contribute to cerebral vascular dysfunction during dietary hyperhomocysteinemia. Another possibility is that mean daily levels of tHcy may have been higher in Cbs⁺/+ mice fed the high methionine diet than in Cbs⁺/+ mice fed the control diet because of augmented diurnal variation.

Several factors can be considered that may account for the greater influence of diet-induced hyperhomocysteinemia on cerebral arterioles as opposed to the aorta. First, the small size of cerebral arterioles (≈ 30 μm diameter) may enhance their susceptibility to oxidative damage. In this regard, it is interesting that small mesenteric arterioles in mice also appear to be highly sensitive to modest elevations of plasma tHcy. Second, the importance of endothelium-derived NO, as opposed to other mediators of endothelium-dependent dilatation, such as products of cyclooxygenase activity or an endothelium-derived hyperpolarizing factor (EDHF), may vary in different blood vessels. We consider it unlikely that the greater sensitivity to hyperhomocysteinemia of cerebral arterioles compared with aorta is related to endothelium-derived mediators other than NO because responses to acetylcholine in both aorta and cerebral arterioles in mice are almost completely dependent on NO. It also seems unlikely that increased production of EDHF compensates for the loss of endothelium-derived NO because hyperhomocysteinemia appears to inhibit vascular responses mediated by EDHF in renal vessels. However, we cannot exclude the possibility that responses to acetylcholine in hyperhomocysteinemic mice
are mediated in part by EDHF. Third, differences in experimental methods (dilatation of cerebral arterioles in anesthetized mice in vivo versus relaxation of aortic rings in vitro) may partly explain the greater apparent sensitivity of cerebral vessels to mild hyperhomocysteinemia. Although the contributing factors will require further study, our current findings clearly demonstrate that cerebral arterioles are sensitive to endothelial dysfunction caused by diet-induced hyperhomocysteinemia.

In summary, we have demonstrated that murine cerebral arterioles are susceptible to endothelial dysfunction during diet-induced hyperhomocysteinemia. Our data suggest that the mechanism of dysfunction is related to increased vascular superoxide, which may be produced by vascular NAD(P)H oxidase and NOS or both. The propensity of cerebral vessels to develop oxidative stress may account for the high prevalence of stroke, vascular dementia, and other cerebrovascular diseases in human subjects with hyperhomocysteinemia.

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References
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