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 (\(\text{Cbs}^+/\sim\)) 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 \(\mu\text{mol/L}; P<0.05\)) and \(\text{Cbs}^+/\sim\) (20.5±3.1 versus 8.2±0.9 \(\mu\text{mol/L}; P<0.001\)) mice. Dilatation of cerebral arterioles (≈30 \(\mu\text{m baseline diameter}\)) 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 \(\text{Cbs}^+/\sim\) 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 \(\text{Cbs}^+/\sim\) mice fed the high-methionine diet and was inhibited by apocynin or \(\text{N}-\text{nitro-L-arginine methyl ester}\), implicating \(\text{NAD(P)H oxidase}\) and \(\text{nitr}i\text{c oxide synthase}\) as potential sources of superoxide.

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

Key Words: cerebral ■ endothelium ■ homocysteine ■ stroke ■ superoxides
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 crossedbred 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+/+) littermates. 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 0.5% methionine in drinking water). After 10 to 12 months on experimental diet, mice were used either for studies of vasomotor responses in cerebral arterioles or for measurement of plasma tHcy, ADMA, and responses to vasodilators in cerebral arterioles.

Responses in 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 superfusion was superimposed 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-methoxyacetophenone) (Fluka), 100 μmol/L NO-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.scion-corp.com). Data are reported as the percentage of surface area of carotid sections within the upper 20% of fluorescence intensity.

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 (ammonium 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 precolumn derivatization with O-phthalaldehyde.

Statistical Analysis
Two-way analysis of variance (ANOVA) followed by Tukey posthoc 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.

Results

Effect of Cbs Genotype and Diet on Plasma tHcy
Plasma tHcy was higher in Cbs+/− mice than in Cbs+/+ mice (P<0.001), with a significant genotype effect observed for both the control diet and the high methionine diet (Table 1). The overall effect of diet was highly significant (P<0.001), with higher levels of plasma tHcy for the high methionine diet compared with the control diet. Plasma methionine was elevated only in Cbs+/− mice fed the high-methionine diet (P<0.05). Plasma ADMA was higher in Cbs+/− mice than in Cbs+/+ mice for the control diet (P<0.05), but not for the high methionine diet (Table 1). Diet or Cbs genotype did not affect body weight (data not shown).

Responses in Cerebral Arterioles
Mean arterial pressure of anesthetized mice was similar in Cbs+/+ and Cbs+/− mice (73±6 versus 70±4 mm Hg for the control diet and 73±5 versus 70±3 mm Hg for the high methionine diet, respectively). Acetylcholine produced
concentration-dependent dilatation of cerebral arterioles of both Cbs+/+ and Cbs+/− mice (Figure 1A and 1B). In mice fed the control diet, responses to acetylcholine tended to be diminished in Cbs+/− mice compared with Cbs+/+ mice (13±1.2 versus 16±2.5% for 1.0 μmol/L acetylcholine and 22±1.8 versus 27±3.5% for 10 μmol/L acetylcholine), but these differences did not reach statistical significance (P>0.10). In mice fed the high methionine diet, responses to acetylcholine were significantly impaired in both Cbs+/+ and Cbs+/− mice compared with mice of the same genotypes fed the control diet (P<0.05). This effect of the high methionine diet was lost after cerebral arterioles were pretreated with the superoxide scavenger tiron (10 mmol/L) (Figure 1A and 1B). Responses to 1.0 μmol/L acetylcholine improved significantly after pretreatment with tiron in both Cbs+/+ and Cbs+/− mice fed the high methionine diet (P<0.05). Responses to 10 μmol/L acetylcholine also improved after pretreatment with tiron in mice fed the high methionine diet; this effect was significant for Cbs+/+ mice (P<0.05), but not for Cbs+/− mice (P>0.10). Concentration-dependent responses to nitroprusside were not affected by Cbs genotype or diet (P>0.10) (Figure 2).

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. As observed previously, 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-
Rescience also was inhibited by preincubation with uric acid (20 μmol/L), an inhibitor of peroxynitrite-mediated oxidation (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, very few studies have examined functional effects of hyperhomocysteinemia in cerebral blood vessels. Using a rat model, Zhang et al demonstrated that superfusion of the parietal cortex with exogenous homocysteine and Cu2+ decreased cerebral blood flow and attenuated responses to endothelium-dependent vasodilators. Interestingly, the vascular effects of homocysteine/Cu2+ were pre-
vented by co-administration of superoxide dismutase, which suggested a potential role for superoxide.\textsuperscript{10} A major limitation of this study was that both exogenous Cu\textsuperscript{2+} 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.\textsuperscript{1} 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.\textsuperscript{11}

There is growing evidence that hyperhomocysteinemia promotes the oxidative inactivation of endothelium-derived NO in peripheral arteries.\textsuperscript{8} Our findings with the superoxide scavenger, tiron, suggest that oxidative inactivation of NO also occurs in the cerebral microcirculation. DHE fluorescence in \( \text{Cbs}^{+/–} \) 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.\textsuperscript{20} Our results are consistent with the recent findings of Ungvari et al,\textsuperscript{21} 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 tetrahydrobipterin, 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.\textsuperscript{22} We found that DHE fluorescence in \( \text{Cbs}^{+/–} \) mice fed a high methionine diet was decreased by uric acid (Figure 4), an inhibitor of peroxynitrite-mediated oxidation.\textsuperscript{19} We also considered the possibility that vascular superoxide is related to ADMA, an endogenous NOS inhibitor that may promote uncoupling of NOS.\textsuperscript{23} ADMA is derived from protein methylation reactions that produce homocysteine as a byproduct, and plasma ADMA is elevated during hyperhomocysteinemia in monkeys and humans.\textsuperscript{23} Only a modest effect of \( \text{Cbs} \) 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 \( \text{Cbs}^{+/–} \) mice with moderate hyperhomocysteinemia (plasma tHcy \( \approx 20 \mu \text{mol/L} \), but also in \( \text{Cbs}^{+/+} \) mice with very mild hyperhomocysteinemia (plasma tHcy \( \approx 8 \mu \text{mol/L} \)). This observation contrasts with previous findings using aortic rings from \( \text{Cbs}^{+/+} \) and \( \text{Cbs}^{+/–} \) mice in which only \( \text{Cbs}^{+/–} \) mice with plasma tHcy \( > 20 \mu \text{mol/L} \) exhibited impaired responses to acetylcholine.\textsuperscript{7} 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 \( \text{Cbs}^{+/+} \) and \( \text{Cbs}^{+/–} \) mice with mild hyperhomocysteinemia (plasma tHcy \( > 8 \mu \text{mol/L} \)).\textsuperscript{9} It is noteworthy, however, that responses of cerebral arterioles to acetylcholine were preserved in \( \text{Cbs}^{+/–} \) mice fed the control diet (Figure 1), despite the fact that these mice also had mild elevation of plasma tHcy (\( \approx 8 \mu \text{mol/L} \)). 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 \( \text{Cbs}^{+/+} \) mice fed the high methionine diet than in \( \text{Cbs}^{+/–} \) mice fed the control diet because of augmented diurnal variation.\textsuperscript{14}

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 (\( \approx 30 \mu \text{m} \) 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.\textsuperscript{6} 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.\textsuperscript{13} 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.\textsuperscript{24} However, we cannot exclude the possibility that responses to acetylcholine in hyperhomocysteinemic mice

![Figure 5. Detection of superoxide by DHE fluorescence in cerebral arterioles. A through D, Representative laser-scanning fluorescent confocal micrographs of sections of cerebral arterioles from \( \text{Cbs}^{+/+} \) mice fed the control diet (A), \( \text{Cbs}^{+/+} \) mice fed the control diet (B), \( \text{Cbs}^{+/+} \) mice fed the high methionine diet (C), and \( \text{Cbs}^{+/+} \) mice fed the high methionine diet (D). E, \( \text{Cbs}^{+/–} \) mice fed the high methionine, section pretreated with 250 U/mL PEG-SOD. Bar, 10 \( \mu \text{m} \).](image-url)
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


Cerebral Vascular Dysfunction Mediated by Superoxide in Hyperhomocysteinemic Mice
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