Folate dependence of hyperhomocysteinemia and vascular dysfunction in cystathionine β-synthase-deficient mice

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Lentz, Steven R., Rochelle A. Erger, Sanjana Dayal, Nobuyo Maeda, M. René Malinow, Donald D. Heistad, and Frank M. Faraci. Folate dependence of hyperhomocysteinemia and vascular dysfunction in cystathionine β-synthase-deficient mice. Am J Physiol Heart Circ Physiol 279: H970–H975, 2000.—Hyperhomocysteinemia is a risk factor for stroke, myocardial infarction, and venous thrombosis. Moderate hyperhomocysteinemia is associated with impaired endothelial function, but the mechanisms responsible for endothelial dysfunction in hyperhomocysteinemia are poorly understood. We have used genetic and dietary approaches to produce hyperhomocysteinemia in mice. Heterozygous cystathionine β-synthase-deficient mice (CBS+/−), which have a selective defect in homocysteine transsulfuration, and wild-type (CBS+/+) littermates were fed either a control diet or a diet that is relatively deficient in folate acid for 6 wk. Plasma total homocysteine was 5.3 ± 0.7 μM in CBS+/+ mice and 6.4 ± 0.6 μM in CBS+/- mice (P = 0.3) given the control diet. Plasma total homocysteine was 11.6 ± 4.5 μM in CBS+/+ mice and 25.1 ± 3.2 μM in CBS+/- mice (P = 0.004) given a low-folate diet. In mice fed the control diet, relaxation of aortic rings in response to the endothelium-dependent vasodilator acetylcholine did not differ significantly between CBS+/+ mice and CBS+/- mice. In contrast, in mice fed a low-folate diet, maximal relaxation to acetylcholine was markedly impaired in CBS+/- mice (58 ± 5%) compared with CBS+/+ mice (84 ± 4%) (P = 0.01). No differences in comparison with the endothelium-independent vasodilator sodium nitroprusside were observed among the four groups of mice. These data indicate that CBS-deficient mice are predisposed to hyperhomocysteinemia during dietary folate deficiency, and moderate hyperhomocysteinemia is associated with marked impairment of endothelial function in mice. acetylcholine; atherosclerosis; endothelium; homocysteine; thrombomodulin

Hyperhomocysteinemia is associated with increased risk for stroke, myocardial infarction, and venous thrombosis (3, 7, 27). Previous studies in humans and nonhuman primates have demonstrated that experimental moderate hyperhomocysteinemia (plasma total homocysteine concentration of 10–30 μM) produces endothelial dysfunction, including impaired endothelium-dependent vasodilatation (1, 4, 5, 16, 25, 38) and decreased thrombomodulin anticoagulant activity (22, 25). However, the precise mechanisms by which hyperhomocysteinemia predisposes blood vessels to endothelial dysfunction are poorly understood (20).

Several potential mechanisms have been proposed to explain endothelial dysfunction during hyperhomocysteinemia, including direct endothelial injury due to increased oxidative stress (26, 30), homocysteine-induced reductive stress leading to altered gene expression (33), altered cellular methylation (19), and direct effects on coagulation or fibrinolysis (13, 21). Experimental evidence for some of these proposed mechanisms has been obtained from studies of endothelial cells exposed to exogenous homocysteine in vitro, but these mechanisms have not been examined definitively using a physiologically relevant model of hyperhomocysteinemia in vivo.

One approach to examine mechanisms of endothelial dysfunction in vivo is to study effects of hyperhomocysteinemia in genetically altered strains of mice. The first well-characterized genetic model of hyperhomocysteinemia in mice was developed by Watanabe et al. (39), who created a targeted deletion of the cystathionine β-synthase (CBS) gene by homologous recombination. Because CBS is the rate-limiting enzyme in homocysteine transsulfuration (10), CBS-deficient mice are predisposed to hyperhomocysteinemia. When homozygous CBS-deficient mice (CBS−/−) are fed a standard laboratory diet, they develop markedly elevated levels of plasma total homocysteine (~200 μM), and they also exhibit growth retardation, hepatic dysfunction, and shortened survival. Because of the severity of the phenotype, however, CBS−/− mice may have limited utility for investigation of specific effects of hyperhomocysteinemia on endothelial function. In contrast, heterozygous CBS-deficient mice (CBS+/−) may be a more useful experimental model of hyperhomocysteinedependent vasodilatation (1, 4, 5, 16, 25, 38) and decreased thrombomodulin anticoagulant activity (22, 25). However, the precise mechanisms by which hyperhomocysteinemia predisposes blood vessels to endothelial dysfunction are poorly understood (20).

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teinemia because they have normal growth and viability despite mildly elevated concentrations of plasma total homocysteine (6–15 μM) (39). Concentrations of plasma total homocysteine in CBS +/- mice are quite similar to those in humans with heterozygous CBS deficiency (31), which suggests that partial genetic impairment of homocysteine transsulfuration produces similar effects on homocysteine metabolism in humans and mice.

CBS +/- mice do not develop spontaneous atherosclerotic or other vascular lesions when fed a normal diet (39), but a recent preliminary report suggests that they may have subtle abnormalities of endothelial vasomotor function (6). It is uncertain, however, whether this genetic model will be useful to dissect mechanisms of endothelial dysfunction in hyperhomocysteinemia. Because homocysteine metabolism is influenced by both genetic and dietary factors (27), we suspected that the vascular phenotype of CBS-deficient mice may vary depending on dietary conditions. Therefore, we tested the hypothesis that CBS +/- mice are predisposed to hyperhomocysteinemia and endothelial dysfunction by dietary folate deficiency, which impairs homocysteine remethylation. Our results demonstrate that dietary folate deficiency elevates plasma total homocysteine concentration and produces marked endothelial dysfunction in CBS +/- mice.

MATERIALS AND METHODS

Mice and experimental protocol. To minimize the potential influence of differences in genetic background, CBS-deficient mice (39) were crossbred to C57BL/6J mice (The Jackson Laboratory) for at least eight generations, and comparisons were performed between heterozygous CBS-deficient (CBS +/-) mice and wild-type (CBS +/+ ) littermates. Genotyping for the targeted CBS allele was performed by polymerase chain reaction (39). At the time of weaning, mice were fed either a control diet that contains 0.75 mg folic acid/100 g (LM-485, Harlan Teklad) or a diet that is relatively deficient in folic acid (0.15 mg/100 g) for 6 wk. The low-folate diet contained succinylsulfathiazole (0.1 mg/100 g) to decrease folate availability from intestinal bacteria. At 9–10 wk of age, mice were euthanized with pentobarbital sodium (75 mg ip), plasma was collected into EDTA (final concentration 5–10 mM) for measurement of total homocysteine and folate, and the thoracic aorta was removed for ex vivo studies. The experimental protocol was approved by the University of Iowa and Veterans Affairs Animal Care and Use Committees.

Vascular responses. After removal of loose connective tissue, the proximal aorta was cut into multiple 3- to 4-mm rings. Rings were suspended in an organ chamber containing oxygenated Krebs buffer maintained at 37°C and connected to a force transducer to measure isometric tension (contraction and relaxation) (2, 25). Contraction dose-response curves were generated by cumulative additions of the thromboxane A2 analog U-46619 (0.03–3 μM). Other rings were contracted submaximally using U-46619, and relaxation dose-response curves were generated by cumulative additions of the endothelium-dependent vasodilator acetylcholine (10−8 to 10−5 M) or the endothelium-independent vasodilator sodium nitroprusside (10−8 to 10−5 M). We have used these methods previously in mouse vessels and demonstrated that responses to acetylcholine are mediated by nitric oxide (2, 9, 18).

Aortic thrombomodulin activity. Thrombomodulin activity (thrombomodulin-dependent activation of protein C) was measured using a modification of a two-stage assay described previously (25). In the first stage, 0.15 μM human protein C (generously provided by Dr. Hans Peter Schwarz, Immuno) and 2.6 nM human thrombin (Enzyme Research Laboratories) were incubated for 30 min at 37°C with rings of proximal thoracic aorta 1.0 mm in length. In the second stage, the amidolytic activity of activated protein C was measured spectrophotometrically using the chromogenic substrate S-2366 (Kabi Pharmacia Hepar). Replicate assays were performed with four to five rings from each aorta. Reference curves were generated using rabbit lung thrombomodulin (American Diagnostica). One unit of activity was defined as the amount of activated protein C generated in the presence of 1.0 nM rabbit thrombomodulin. This assay detects thrombomodulin activity on the luminal endothelium, because denudation of the vessels decreased protein C activation by >90%.

Plasma assays. Plasma total homocysteine concentration was measured by high-performance liquid chromatography and electrochemical detection as described previously (28, 29). Total homocysteine was defined as the amount of homocysteine obtained after treatment of the sample with a reducing agent that converts free and bound sulfoxides into their respective thiols (32). Hyperhomocysteinemia was defined as the elevation of plasma total homocysteine. Plasma levels of folate were measured by an automated chemiluminescence immunoassay (Chiron Diagnostics ACS:180).

Statistical analysis. Comparisons were performed using the unpaired two-tailed Student’s t-test. A value of P < 0.05 was used to define statistical significance. Values are reported as means ± SE.

RESULTS

Experimental hyperhomocysteinemia. Experimental hyperhomocysteinemia was produced by combining dietary folate deficiency with genetic deficiency of CBS. Deficiency of folate was used to limit homocysteine remethylation, and heterozygous CBS deficiency was employed to examine effects of impaired homocysteine transsulfuration (Fig. 1). Beginning at the time of

![Fig. 1. Production of hyperhomocysteinemia in mice. Dietary deficiency of folate produces hyperhomocysteinemia by limiting homocysteine remethylation, whereas genetic deficiency of cystathionine β-synthase (CBS) produces hyperhomocysteinemia by interfering with homocysteine transsulfuration. SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine.](http://alphea.aphysiology.org/)
weaning (about 3 wk of age), CBS +/- and CBS +/+ littermates were fed either control diet or low-folate diet for 6–7 wk. In mice fed control diet, plasma total homocysteine concentration did not differ significantly between CBS +/- and CBS +/+ mice (Table 1). In mice fed low-folate diet, plasma total homocysteine concentration was markedly elevated in CBS +/- mice compared with CBS +/+ mice (P = 0.004) (Table 1). Plasma concentrations of folate were compared with CBS +/- or CBS +/- mice fed low-folate diet compared with CBS +/- or CBS +/+ mice fed control diet (P = 0.001) (Table 1).

Vasomotor responses. The endothelium-dependent vasodilator acetylcholine and the endothelium-independent vasodilator nitroprusside each produced dose-dependent relaxation of aortic rings from all mice. In mice fed control diet, no differences in relaxation to acetylcholine were observed between CBS +/- and CBS +/+ mice (Fig. 2A). Maximal relaxation to the highest dose of acetylcholine was 81 ± 4% in CBS +/+ mice and 75 ± 5% in CBS +/- mice (P = 0.4). Similarly, no differences in relaxation to nitroprusside (Fig. 3B) or contraction to the thromboxane A2 analog U-46619 (Fig. 2C) were observed between CBS +/- and CBS +/+ mice fed control diet.

In mice fed low-folate diet, relaxation to acetylcholine was markedly impaired in CBS +/- mice compared with CBS +/+ mice (Fig. 3A). Maximal relaxation to acetylcholine was 58 ± 9% in CBS +/- mice compared with a relaxation of 84 ± 4% in CBS +/+ mice (P = 0.01). Relaxation to nitroprusside did not differ significantly between CBS +/- and CBS +/- mice fed low-folate diet (Fig. 3B), although maximal relaxation to nitroprusside tended to be lower in CBS +/- mice than in CBS +/+ mice (P = 0.14). No differences in contraction to U-46619 were observed between CBS +/- and CBS +/+ mice fed low-folate diet (Fig. 3C).

Aortic thrombomodulin activity. Previous studies in monkeys suggested that diet-induced hyperhomocysteinemia was associated with impaired aortic thrombomodulin-dependent activation of anticoagulant protein C in the aorta and carotid artery (25). Therefore, we measured thrombomodulin activity ex vivo in aortas obtained from mice fed control or low-folate diets. No differences in aortic thrombomodulin activity were observed between CBS +/- and CBS +/+ mice fed either control or low-folate diets (Fig. 4).

Table 1. Effect of diet and CBS genotype on plasma concentrations of total homocysteine and folate

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<tr>
<td>Total homocysteine, μM</td>
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<td>Folate, ng/ml</td>
<td>63.9 ± 13.1</td>
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Values are means ± SE; n, number of mice. CBS, cystathionine β-synthase; CBS +/- mice, wild-type mice; CBS +/- mice, heterozygous CBS-deficient littermates. *P < 0.05 vs. control diet; †P < 0.05 vs. CBS +/-.

DISCUSSION

In this study, we have combined genetic and dietary approaches to produce moderate hyperhomocysteinemia in mice. We found that dietary folate deficiency produced a greater elevation in plasma total homocysteine concentration in CBS +/- mice than in CBS +/+ mice. Endothelial vasomotor dysfunction was observed only in mice with a combined defect in homocysteine remethylation (produced by dietary folate deficiency) and homocysteine transsulfuration (produced by heterozygous deficiency of CBS).

The mean plasma total homocysteine concentration in CBS +/- mice fed the low-folate diet was 25 μM,
which is similar to plasma total homocysteine levels obtained after oral methionine loading in humans (12). Several recent studies have demonstrated that methionine loading produces acute impairment of endothelium-dependent relaxation of conduit or resistance vessels in human subjects (1, 4, 5, 16, 38). In each of these studies, acute impairment of endothelial function was detected within 2–8 h after ingestion of methionine, which increased plasma total homocysteine concentrations to 20–30 μM. Our observation that responses to the endothelium-dependent vasodilator acetylcholine were impaired in CBS+/− mice that were fed the low-folate diet indicates that experimental hyperhomocysteinemia also produces adverse effects on endothelial function in mice. These findings are consistent with our previous studies in which endothelial vasomotor dysfunction was detected in cynomolgus monkeys with diet-induced hyperhomocysteinemia (25). Because impaired vasomotor responses were observed only in the group of mice with the highest levels of plasma total homocysteine, it cannot be determined from these data whether the CBS +/− genotype sensitizes to vascular function independently of its effects on plasma total homocysteine concentration.

In contrast to the marked endothelial vasomotor dysfunction observed in CBS +/− mice fed low-folate diet, no impairment of endothelial function was detected in CBS +/+ mice fed low-folate diet, even though these mice had mildly elevated concentrations of plasma total homocysteine (∼12 μM). This finding in mice differs somewhat from previous findings in cynomolgus monkeys, in which endothelial dysfunction was detected in the presence of very mild elevation of plasma concentrations of total homocysteine (∼11 μM) (25). Thus it appears that higher concentrations of plasma total homocysteine may be required to produce endothelial vasomotor dysfunction in mice than in monkeys. This apparent difference in concentration dependence may be partly due to the fact that fasting homocysteine levels were measured in monkeys, whereas nonfasting levels were measured in mice. It also is possible that the differential sensitivity to hyperhomocysteinemia between monkeys and mice is due to species-specific differences in antioxidant mechanisms (35) or other mechanisms that regulate endothelium-dependent relaxation. Despite these differences in concentration dependence, it is clear that endothelial vasomotor dysfunction is a consistent and reproducible consequence of hyperhomocysteinemia in experimental animals and humans. Previous studies using pharmacological approaches and gene-targeting have demonstrated that relaxation of the mouse aorta in response to acetylcholine is mediated by nitric oxide produced by endothelial nitric oxide synthase (15, 17, 18). These ex vivo findings are consistent with earlier observations that homocysteine decreases bioavailabil-

Fig. 3. Vasomotor responses of aortas from CBS +/+ mice (filled symbols; n = 9) or CBS +/+ mice (open symbols; n = 8) fed low-folate diet. A: relaxation to acetylcholine. B: relaxation to nitroprusside. C: contraction to the thromboxane A2 analog U-46619. *P < 0.05 vs. CBS +/+ mice. Four rings were studied from each animal.

Fig. 4. Aortic thrombomodulin activity. Thrombomodulin-dependent activation of protein C in proximal aorta was measured in CBS +/+ mice (open bars; n = 9) and CBS +/+ mice (hatched bars; n = 8–14) fed either control diet or low-folate diet. Four to five rings were studied from each animal.
ity of nitric oxide in cultured endothelial cells (36, 37). In addition to preventing pathological vasoconstriction, endothelium-derived nitric oxide also inhibits platelet aggregation and leukocyte adhesion. Thus decreased bioavailability of nitric oxide is a plausible mechanism for increased risk of thrombosis and atherosclerosis in hyperhomocysteinemia.

If oxidative inactivation of endogenous endothelium-derived nitric oxide is a major mechanism for endothelial dysfunction in experimental hyperhomocysteinemia (26), one might also expect to see abnormal vascular responses to exogenous nitrovasodilators, such as nitroprusside. We observed that CBS +/− and CBS +/+ mice tended to differ in responses to nitroprusside when they were fed the low-folate diet (Fig. 4B). Although this difference did not reach statistical significance, the observation is consistent with previous findings that vasodilator responses to nitroprusside are modestly impaired in monkeys with diet-induced hyperhomocysteinemia or hypercholesterolemia (8, 22, 25). These observations suggest that hyperhomocysteinemia may lead to oxidative inactivation of nitric oxide derived from both exogenous and endogenous sources.

In addition to producing impairment of endothelial vasomotor function, hyperhomocysteinemia may adversely affect anticoagulant properties of endothelium. Thrombomodulin is an endothelial surface protein that functions as a critical cofactor for activation of anticoagulant protein C (24). The thrombomodulin/protein C system and endothelium-dependent regulation of vasomotor tone are two distinct but complementary properties of endothelium that may protect vessels from thrombotic complications of vascular disease. Thrombomodulin-dependent activation of protein C can be inhibited by exogenous homocysteine in cultured human endothelial cells (14, 23, 34), and monkeys with diet-induced hyperhomocysteinemia have decreased thrombomodulin activity in the aorta and carotid artery (25). We did not, however, detect any differences in aortic thrombomodulin activity between CBS +/+ and CBS +/− mice fed either control or low-folate diets. These results imply that human, macaque, and murine thrombomodulin may differ in sensitivity to hyperhomocysteinemia. In this regard, it is noteworthy that human thrombomodulin contains a critical methionine residue that is sensitive to oxidation, which results in loss of thrombomodulin activity (11). Although murine thrombomodulin also contains a methionine residue in this position, it is not known whether its activity is altered by oxidative stress.

In summary, a folate-deficient diet produces hyperhomocysteinemia and impaired endothelial function in heterozygous CBS-deficient mice. Because of the increasing use of transgenic and gene-targeting techniques in mice, the availability of this murine model should facilitate future studies of mechanisms of vascular dysfunction in hyperhomocysteinemia.

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REFERENCES


