Endothelial Dysfunction and Elevation of S-Adenosylhomocysteine in Cystathionine β-Synthase–Deficient Mice

Sanjana Dayal, Teodoro Bottiglieri, Erland Arning, Nobuyo Maeda, M. René Malinow, Curt D. Sigmund, Donald D. Heistad, Frank M. Faraci, Steven R. Lentz

Abstract—Hyperhomocysteinemia is associated with increased risk for cardiovascular events, but it is not certain whether it is a mediator of vascular dysfunction or a marker for another risk factor. Homocysteine levels are regulated by folate bioavailability and also by the methyl donor S-adenosylmethionine (SAM) and its metabolite S-adenosylhomocysteine (SAH). We tested the hypotheses that endothelial dysfunction occurs in hyperhomocysteinemic mice in the absence of folate deficiency and that levels of SAM and SAH are altered in mice with dysfunction. Heterozygous cystathionine β-synthase–deficient (CBS+/−) and wild-type (CBS+/+) mice were fed a folate-replete, methionine-enriched diet. Plasma levels of total homocysteine were elevated in CBS+/− mice compared with CBS+/+ mice after 7 weeks (27.1±5.2 versus 8.8±1.1 μmol/L; P<0.001) and 15 weeks (23.9±3.0 versus 13.0±2.3 μmol/L; P<0.01). After 15 weeks, but not 7 weeks, relaxation of aortic rings to acetylcholine was selectively impaired by 35% (P<0.05) and thrombomodulin anticoagulant activity was decreased by 20% (P<0.05) in CBS+/− mice. Plasma levels of folate did not differ between groups. Levels of SAH were elevated ~2-fold in liver and brain of CBS+/− mice, and correlations were observed between plasma total homocysteine and SAH in liver (r=0.54; P<0.001) and brain (r=0.67; P<0.001). These results indicate that endothelial dysfunction occurs in hyperhomocysteinemic mice even in the absence of folate deficiency. Endothelial dysfunction in CBS+/− mice was associated with increased tissue levels of SAH, which suggests that altered SAM-dependent methylation may contribute to vascular dysfunction in hyperhomocysteinemia. (Circ Res. 2001;88:1203-1209.)

Key Words: acetylcholine ■ endothelium ■ homocysteine ■ methylation ■ thrombomodulin

Elevation of plasma concentration of total homocysteine (tHcy) is considered to be a clinical risk factor for cardiovascular disease.1–3 Mild or moderate hyperhomocysteinemia (plasma tHcy concentration of 15 to 50 μmol/L) is found in up to 40% of patients with myocardial infarction, stroke, or venous thrombosis.1–3 Although strong associations between hyperhomocysteinemia and cardiovascular events have been observed in many retrospective and some prospective studies, a few prospective studies have failed to confirm this association.5 One potential explanation for the inconsistent results of the prospective studies is that hyperhomocysteinemia itself may not be the causative agent in vascular dysfunction, but instead may be a marker for another risk factor.4–6

One factor that is often associated with hyperhomocysteinemia is deficiency of folate, because folate is required for efficient remethylation of homocysteine to methionine (Figure 1). In addition to causing hyperhomocysteinemia, folate deficiency may impair endothelial function through effects on endothelial nitric oxide synthase (eNOS), a major mediator of endothelium-dependent relaxation.7 In a previous study,8 we examined the influence of folate on endothelial function in mice with heterozygous deficiency of the cystathionine β-synthase (CBS) gene. We found that both the plasma concentration of tHcy and the severity of endothelial dysfunction in aorta were strongly influenced by the folate content of the diet.8 Whether endothelial dysfunction in CBS-deficient mice was caused by hyperhomocysteinemia, folate deficiency, or another factor related to homocysteine metabolism was not determined.

Another proposed mechanism for endothelial dysfunction in hyperhomocysteinemia is that hyperhomocysteinemia may be a marker for altered methylation of cellular substrates that utilize S-adenosylmethionine (SAM) as a methyl donor.9 SAM serves as a donor for methyl transfer reactions that produce a variety of methylated products, including proteins,
nucleic acids, phospholipids, and other substrates (Figure 1). The major byproduct of these methyltransferase reactions is S-adenosylhomocysteine (SAH), which undergoes hydrolysis to form homocysteine. SAH is a potent inhibitor of methyltransferases, which suggests that elevation of SAH in hyperhomocysteinemia could lead to inhibition of methyltransfer reactions in endothelial cells. In support of this hypothesis, incubation of cultured endothelial cells with exogenous homocysteine was found to increase intracellular levels of SAH and inhibit methylation of p21^ras , resulting in decreased cell proliferation. The influence of hyperhomocysteinemia on tissue levels of SAH has not been well characterized, however, and it is not known whether elevated levels of SAH are associated with endothelial dysfunction.

In the present study, we have utilized a folate-replete, methionine-enriched diet with CBS-deficient mice to ask two related questions. First, does endothelial dysfunction occur in hyperhomocysteinemic mice in the absence of folate deficiency? Second, are tissue levels of SAM and SAH altered in hyperhomocysteinemic mice with endothelial dysfunction?

Our results indicate that endothelial dysfunction does occur during chronic hyperhomocysteinemia in mice with normal folate status. Our findings also demonstrate that hyperhomocysteinemic mice with endothelial dysfunction have elevated levels of SAH and a decreased SAM/SAH ratio. These findings are compatible with the hypothesis that altered SAM-dependent methylation may contribute to endothelial dysfunction in hyperhomocysteinemia.

Materials and Methods

Mice and Experimental Protocol

To minimize the potential influence of differences in genetic background, CBS-deficient mice were crossed to C57BL/6J mice (The Jackson Laboratory, Bar Harbor, Maine) for at least eight generations, and comparisons were performed between heterozygous CBS-deficient (CBS1/2) mice and wild-type (CBS1/1) littermates. Homozygous CBS-deficient mice were not studied because they exhibit growth retardation, hepatic dysfunction, and shortened survival. Genotyping for the targeted CBS allele was performed by polymerase chain reaction. At the time of weaning, mice were fed LM-485 chow (Harlan Teklad), which contains 7.5 mg/kg folic acid and 4.0 g/kg l-methionine, and were provided with drinking water that was either unsupplemented (control) or supplemented with 0.5% l-methionine (high methionine). The terms “control diet” and “high-methionine diet” refer to the combination of solid chow and drinking water consumed by each group. After either 7 or 15 weeks of experimental diet, mice were euthanized with sodium pentobarbital (75 mg IP). The breeding schedule was designed to allow for the study of two mice (littermates) of the appropriate age on each study day. For the 7-week time point, the ages of the mice ranged from 9 to 10 weeks, with mean values of 6.7 to 6.8 weeks on experimental diet. For the 15-week time point, the ages of the mice ranged from 17 to 20 weeks, with mean values of 14.8 to 15.1 weeks on experimental diet. Blood was collected in EDTA (final concentration 5 mmol/L) for measurement of plasma levels of tHcy, methionine, and folate, and the thoracic aorta, lung, liver, and brain were removed for ex vivo studies. The experimental protocol was approved by the University of Iowa and Veterans Affairs Animal Care and Use Committees.

Vasomotor Responses

After removal of loose connective tissue, the proximal aorta was cut into multiple 3- to 4-mm rings that were suspended in an organ chamber containing oxygenated Krebs buffer maintained at 37°C. Rings were contracted submaximally using the thromboxane analogue U46619, and relaxation dose-response curves were generated by cumulative addition of the endothelium-dependent vasodilator acetylcholine or the endothelium-independent vasodilators sodium nitroprusside or papaverine, as described previously. We have used these methods previously and demonstrated that responses to acetylcholine in mouse aorta are mediated by eNOS.

Thrombomodulin Activity

Thrombomodulin activity (thrombomodulin-dependent activation of protein C) was measured using a two-stage assay described previously. Activity was measured in rings of proximal thoracic aorta 1.0 mm in length or in lysates of lung tissue prepared by homogenization in 0.02 mol/L Tris-HCl and 0.1 mol/L NaCl (pH 8). 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 nmol/L rabbit thrombomodulin. The thrombomodulin activity in lung homogenates was expressed as units/mg of total protein.

Other Assays

Plasma tHcy, defined as the total concentration of homocysteine after quantitative reductive cleavage of all disulfide bonds, was measured by high-performance liquid chromatography and electrochemical detection as described previously. Plasma levels of methionine were measured by high-performance liquid chromatography coupled to coulometric electrochemical detection. The method was modified from that described by Martin et al by increasing the electrochemical potential to +1100 mV to detect methionine. Plasma levels of folate were measured by an automated chemiluminescence immunoassay (Chiron Diagnostics).

Levels of SAM and SAH in liver and brain tissue were measured by high-performance liquid chromatography coupled to a UV detector as described previously. Tissues were collected in ice-cold perchloric acid (0.4 mol/L) immediately after the mice were euthanized, and samples were deproteinized by homogenization and centrifugation within 1 hour. The clear supernatant fraction was stored at −80°C until the time of analysis.

Statistical Analysis

Comparisons between genotypes (CBS1/2 versus CBS1/1 mice) or between diets (control diet versus high-methionine diet) were performed within each age group using the unpaired, two-tailed Student’s t test. Responses to vasodilators in aorta were analyzed using two-way repeated-measures ANOVA with Bonferroni multiple-comparison analysis at specific concentrations of vasodilator. Correlation coefficients were calculated using the Pearson method. A
**Results**

In a previous study, moderate hyperhomocysteinemia (plasma tHcy ≈25 μmol/L) was produced by feeding heterozygous CBS-deficient mice a diet that was deficient in folate. To produce hyperhomocysteinemia without folate deficiency, mice in the present study were fed a folate-replete diet that contained supplemental methionine in drinking water. Beginning at the time of weaning (~3 weeks of age), CBS+/− mice and CBS+/– littermates were fed either the control diet or the high-methionine diet for up to 15 weeks. All mice appeared to be healthy, and body weight did not differ between CBS+/− and CBS+/– mice or between mice fed the control and high-methionine diets (not shown).

**Plasma Levels of tHcy, Methionine, and Folate**

After 7 weeks, plasma tHcy was mildly elevated in CBS+/− mice fed the control diet (P<0.05 vs CBS+/+ mice) and markedly elevated in CBS+/− mice fed the high-methionine diet (P<0.001 versus CBS+/+ mice) (Table 1). Similar differences in levels of plasma tHcy between CBS+/− and CBS+/+ mice were observed after 15 weeks (Table 1). Plasma methionine concentration tended to be higher in mice fed the high-methionine diet than in mice fed the control diet and higher in CBS+/− mice than in CBS+/+ mice, but these differences did not reach statistical significance (Table 1). Plasma folate did not differ between groups.

**Vasomotor Responses in Aorta**

Acetylcholine, nitroprusside, and papaverine each produced dose-dependent relaxation of aortic rings. After 7 weeks, despite marked differences in plasma tHcy, no differences in relaxation to acetylcholine or nitroprusside were observed between CBS+/− and CBS+/+ mice fed either diet (not shown). After 15 weeks, relaxation to acetylcholine did not differ significantly between CBS+/− mice and CBS+/+ mice and CBS+/− mice fed the control diet (Figure 2A) but was impaired in CBS+/− mice compared with CBS+/+ mice fed the high-methionine diet (Figure 2B). Maximal relaxation to the highest dose of acetylcholine was 90±3% in CBS+/+ mice and 73±8% in CBS+/− mice (P<0.05). No differences in relaxation to nitroprusside (Figures 2C and 2D) or papaverine (not shown) were observed between CBS+/+ and CBS+/− mice fed either diet. Vessels from all groups of mice contracted similarly to the thromboxane analogue U46619 (not shown).

**Thrombomodulin Activity**

Thrombomodulin anticoagulant activity was measured in aortic arch and lung. After 7 weeks, no differences in thrombomodulin activity were observed between CBS+/+ and

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**Table 1. Effect of Diet and CBS Genotype on Plasma Concentrations of tHcy, Methionine, and Folate**

<table>
<thead>
<tr>
<th></th>
<th>Control Diet</th>
<th>High-Methionine Diet</th>
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<tr>
<td>Number of mice</td>
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<tr>
<td>7 weeks</td>
<td>10</td>
<td>12</td>
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<td>15 weeks</td>
<td>8</td>
<td>10</td>
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<tr>
<td>tHcy, μmol/L</td>
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<tr>
<td>7 weeks</td>
<td>2.5±0.3</td>
<td>6.2±1.1*</td>
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<tr>
<td>15 weeks</td>
<td>4.1±0.4</td>
<td>6.3±0.9</td>
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<tr>
<td>Methionine, μmol/L</td>
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<tr>
<td>15 weeks</td>
<td>22.8±1.6</td>
<td>30.6±2.5</td>
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<tr>
<td>Folate, ng/mL</td>
<td></td>
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<tr>
<td>15 weeks</td>
<td>46.7±7.2</td>
<td>48.5±5.5</td>
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Values are mean±SE.

*P<0.05 vs CBS+/+.

†P<0.05 vs control diet.

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**Figure 2.** Relaxation of aorta to acetylcholine or nitroprusside in mice fed a control diet (A and C) or a high-methionine diet (High Met; B and D) for 15 weeks. ●, CBS+/+ mice (n=8 to 14); ○, CBS+/− mice (n=10 to 11). *P<0.05 vs CBS+/+ mice.

**Figure 3.** Thrombomodulin activity in aorta (A) or lung (B) of mice fed a control diet or a high-methionine diet (High Met) for 15 weeks. White bars, CBS+/+ mice (n=8 to 14); black bars, CBS+/− mice (n=10 to 11). *P<0.05 vs CBS+/+ mice.
CBS+/-; mice fed either diet (not shown). After 15 weeks, thrombomodulin activity in aortic arch was ~20% lower in CBS+/-; mice compared with CBS+/+; mice fed the control diet (Figure 3A). Similar decreases (20% to 25%) in thrombomodulin activity in aortic arch also were observed in CBS+/-; or CBS+/+; mice fed the high-methionine diet compared with CBS+/+; mice fed the control diet (Figure 3A). No differences in thrombomodulin activity in lung were observed between CBS+/+; and CBS+/-; mice fed either diet (Figure 3B).

**Tissue Levels of SAM and SAH**

After 7 weeks, levels of SAM in liver did not differ significantly between groups, but levels of SAH in liver were elevated ~2-fold in CBS+/-; mice fed the high-methionine diet compared with CBS+/+; mice fed the control diet (P<0.001) (Table 2). CBS+/-; mice fed the high-methionine diet also had a lower SAM/SAH ratio in liver compared with CBS+/+; mice fed the control diet (P<0.005). After 15 weeks, levels of SAH in liver were elevated and SAM/SAH ratio was decreased in all three groups with elevated plasma tHcy (CBS+/+; mice fed the high-methionine diet, CBS+/-; mice fed the control diet, and CBS+/-; mice fed the high-methionine diet) (Table 2).

**Figure 4.** Correlations between plasma tHcy and SAH in liver (A) or SAH in brain (B) of mice fed a control diet or a high-methionine diet for 7 or 15 weeks. ○, CBS+/+; mice fed the control diet; ●, CBS+/-; mice fed the control diet; △, CBS+/-; mice fed the high-methionine diet; ●, CBS+/-; mice fed the high-methionine diet.

Levels of SAM and SAH also were measured in brain. Compared with levels in liver, levels of SAM in brain were 2- to 4-fold lower, levels of SAH were 10- to 15-fold lower, and SAM/SAH ratio was 3- to 5-fold higher (Table 3). After 7 weeks, levels of SAH in brain were elevated in CBS+/-; mice fed the high-methionine diet (P<0.05). Like SAM/SAH ratio in liver, SAM/SAH ratio in brain was decreased in all three groups with elevated plasma tHcy (CBS+/+; mice fed the high-methionine diet, CBS+/-; mice fed the control diet, and CBS+/-; mice fed the high-methionine diet).

**Discussion**

By providing supplemental methionine to CBS+/-; mice, we have produced moderate hyperhomocysteinemia (plasma tHcy ~25 μmol/L) in the absence of folate deficiency. Hyperhomocysteinemic mice had normal vascular responses after 7 weeks but developed impaired endothelium-dependent relaxation and decreased thrombomodulin anticoagulant activity in aorta after 15 weeks. This finding demonstrates that endothelial dysfunction can occur during hyperhomocysteinemia in mice, even in the absence of folate deficiency. Another major finding of this study is that endothelial dysfunction in CBS+/-; mice was associated not only with elevation of plasma tHcy but also with elevation of SAH and decreased SAM/SAH ratio in liver and brain. This finding suggests a possible...
role for altered SAM-dependent methylation reactions in the vascular pathology of hyperhomocysteinemia.

Endothelial dysfunction has been observed in animal models of experimental hyperhomocysteinemia in several species, including nonhuman primates,16 and rats.22–24 In a previous study, we found that endothelium-dependent relaxation in aorta was preserved in CBS+/– mice with normal folate levels and very mild hyperhomocysteinemia (plasma tHcy = 6 μmol/L) but impaired in folate-deficient CBS+/– mice with higher levels of plasma tHcy (≈25 nmol/L).8 Abnormal endothelial function in heterozygous CBS-deficient mice also was reported independently by Eberhardt et al.25 who detected evidence for endothelial dysfunction in aorta and mesenteric microvessels in CBS+/– mice with mild hyperhomocysteinemia (plasma tHcy 9 to 10 μmol/L). One potential explanation for the greater impairment of responses in the studies of Eberhardt et al than in our studies is that the diets may have contained different amounts of folate, methionine, or other constituents that influence tHcy levels. Plasma levels of folate were not reported by Eberhardt et al.

The precise mechanisms by which hyperhomocysteinemia produces endothelial dysfunction are incompletely defined. Impairment of endothelium-dependent relaxation in hyperhomocysteinemia appears to be mediated in part by decreased bioavailability of endothelium-derived nitric oxide, which may be caused either by decreased production or increased degradation of nitric oxide.26 Homocysteine-induced oxidative inactivation of nitric oxide has been observed in studies of cultured endothelial cells,27 and indirect evidence for increased oxidative inactivation of nitric oxide during hyperhomocysteinemia has been obtained in some studies in animals.23 In support of this mechanism, CBS-deficient mice have been reported to exhibit increased generation of superoxide anion in aorta.25 In our study, vasodilator responses to nitroprusside were normal in CBS+/– mice fed the high-methionine diet, which suggests that the sensitivity of soluble guanylyl cyclase in vascular smooth muscle to nitric oxide was relatively normal. Normal responses to nitroprusside also were observed in previous studies of CBS-deficient mice.5,25

Expression of eNOS does not appear to be decreased during hyperhomocysteinemia,25 but production of nitric oxide by eNOS may be limited by endogenous inhibitors such as asymmetric dimethylarginine (ADMA).28,29 ADMA is derived from the hydrolysis of proteins that are methylated on arginine residues by SAM-dependent protein arginine methyl transferases.30 It has been proposed, therefore, that hyperhomocysteinemia due to methionine loading may increase production of ADMA through increased SAM-dependent arginine methylation.29 Alternatively, hyperhomocysteinemia may produce elevation of ADMA by inhibiting its metabolism by the enzyme dimethylarginine dimethylaminohydrolase.30,31 Our observations in hyperhomocysteinemic mice are consistent with the latter mechanism, because we observed elevation of SAH without elevation of SAM, and chronically elevated levels of SAH would be expected to inhibit most methyltransferases.32 The effect of hyperhomocysteinemia on the specific protein arginine methyltransferase that produces ADMA is not known, however, so this question will require further study.

In comparison with our previous findings in CBS+/– mice fed a low-folate diet,8 CBS+/– mice fed high-methionine had almost identical levels of plasma tHcy but differed in the duration of hyperhomocysteinemia required to produce endothelial dysfunction. When fed a low-folate diet, CBS+/– mice developed marked impairment to endothelium-dependent vasodilators in aorta after 6 to 7 weeks.8 In the present study, when CBS+/– mice were fed a high-methionine diet, aortic responses to endothelium-dependent vasodilators were preserved after 7 weeks but became impaired after 15 weeks. These findings suggest that the vascular phenotype of hyperhomocysteinemic mice is dependent on both the duration of hyperhomocysteinemia and the underlying defect in homocysteine metabolism.

The more rapid onset of endothelial dysfunction in CBS+/– mice with folate deficiency compared with CBS+/– mice given supplemental methionine suggests that the homocysteine remethylation pathway may be particularly important for maintaining normal vasomotor responses during hyperhomocysteinemia. Endothelial cells appear to lack both CBS activity and betaine-homocysteine methyltransferase and therefore must rely on the folate-dependent enzyme methionine synthase for homocysteine remethylation.6 For this reason, endothelial cells may be particularly sensitive to folate deficiency, and intracellular levels of homocysteine may increase disproportionately in endothelial cells compared with liver when mice are fed a folate-deficient diet. This effect may be exacerbated in CBS-deficient mice, because increased plasma tHcy (derived from liver and other tissues) may lead to increased uptake of Hcy by endothelial cells. Alternatively, effects of folate deficiency unrelated to hyperhomocysteinemia, such as direct impairment of endothelial nitric oxide production7 may have contributed to endothelial dysfunction in CBS+/– mice fed the low-folate diet.

Another potential mechanism by which vascular function may be impaired in hyperhomocysteinemia is through altered methylation of cellular substrates that utilize SAM as a methyl donor.9 An inverse correlation between hepatic SAM concentration and plasma tHcy has been observed in folate-deficient rats13 and in mice deficient in methylenetetrahydrofolate reductase.52 Incubation of cultured endothelial cells with exogenous homocysteine increases intracellular levels of SAH and inhibits carboxymethylation of p21WAF1, resulting in decreased cellular proliferation.11 In uremic patients, hyperhomocysteinemia is associated with increased levels of SAH and decreased protein methylation in erythrocytes.14 A recent study in healthy young women demonstrated that elevation of plasma tHcy was associated with elevation of SAH in plasma and lymphocytes and with hypomethylation of lymphocyte DNA.35

Our findings in CBS-deficient mice are concordant with these clinical observations, as we found that hyperhomocysteinemic mice with endothelial dysfunction had increased tissue levels of SAH in liver and brain. For technical reasons, we were unable to measure levels of SAH in aorta in mice. Nevertheless, our findings are compatible with the hypothesis that altered SAM-dependent methylation may contribute to vascular dysfunction in hyperhomocysteinemia. For example,
hypothesis that elevation of tHcy, through its effects on SAH, is a marker of altered methylation reactions in endothelial cells.

CBS−/− mice fed the high-methionine diet for 15 weeks not only had impaired endothelium-dependent vasomotor function but also had decreased thrombomodulin anticoagulant activity. Thrombomodulin activity was decreased in aorta but not in lung. Thrombomodulin activity can be inhibited by exogenous homocysteine in cultured endothelial cells and we have observed decreased thrombomodulin activity in carotid artery and aorta of monkeys with moderate hyperhomocysteinemia. Clinical evidence for impairment of thrombomodulin-dependent activation of protein C in hyperhomocysteinemia has been unconvincing, however. Our observations in monkeys and mice suggest that thrombomodulin may be more susceptible to hyperhomocysteinemia in large conduit vessels such as aorta than in small vessels such as those found in the pulmonary microvasculature. One potential mechanism for the differential effects of hyperhomocysteinemia on thrombomodulin activity in large and small vessels is that oxidative stress may be more prominent in large vessels. Thrombomodulin activity is possibly inhibited through oxidative mechanisms in vivo, because thrombomodulin contains a critical oxidation-sensitive methionine residue near its thrombin binding domain. Another possible mechanism is that hyperhomocysteinemia may alter the expression or activity of the endothelial cell protein C receptor, which is expressed mainly on large vessels.

In summary, moderate hyperhomocysteinemia was produced in CBS-deficient mice with normal folate status. In comparison with previous findings in CBS−/− mice with folate deficiency, a longer duration of hyperhomocysteinemia was needed to produce endothelial dysfunction in aorta in CBS−/− mice without folate deficiency. The development of endothelial dysfunction in mice was associated with increased levels of SAH and decreased SAM/SAH ratio in liver and brain. These findings provide support for the hypothesis that, in addition to hyperhomocysteinemia itself, related factors such as folate deficiency and altered SAM-dependent methylation reactions may contribute to vascular dysfunction. Additional studies will be needed to identify specific methylation products that may be altered in hyperhomocysteinemia.

Acknowledgments

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References


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