Vascular complications of cystathionine β-synthase deficiency: future directions for homocysteine-to-hydrogen sulfide research

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Submitted 18 June 2010; accepted in final form 20 October 2010

Beard RS Jr, Bearden SE. Vascular complications of cystathionine β-synthase deficiency: future directions for homocysteine-to-hydrogen sulfide research. Am J Physiol Heart Circ Physiol 300: H13–H26, 2011. First published October 22, 2010; doi:10.1152/ajpheart.00598.2010.—Homocysteine (Hcy), a cardiovascular and neurovascular disease risk factor, is converted to hydrogen sulfide (H2S) through the transsulfuration pathway. H2S has attracted considerable attention in recent years for many positive effects on vascular health and homeostasis. Cystathionine β-synthase (CBS) is the first, and rate-limiting, enzyme in the transsulfuration pathway. Mutations in the CBS gene decrease enzymatic activity, which increases the plasma Hcy concentration, a condition called hyperhomocysteinemia (HHcy). Animal models of CBS deficiency have provided valuable insights into the pathological effects of transsulfuration impairment and of both mild and severe HHcy. However, studies have also highlighted the complexity of HHcy and the need to explore the specific details of Hcy metabolism in addition to Hcy levels per se. There has been a relative paucity of work addressing the dysfunctional H2S production in CBS deficiency that may contribute to, or even create, HHcy-associated pathologies. Experiments using CBS knockout mice, both homozygous (−/−) and heterozygous (+/−), have provided 15 years of new knowledge and are the focus of this review. These murine models present the opportunity to study a specific mechanism for HHcy that matches one of the etiologies in many human patients. Therefore, the goal of this review was to integrate and highlight the critical information gained thus far from models of CBS deficiency and draw attention to critical gaps in knowledge, with particular emphasis on the modulation of H2S metabolism. We include findings from human and animal studies to identify important opportunities for future investigation that should be aimed at generating new basic and clinical understanding of the role of CBS and transsulfuration in cardiovascular and neurovascular disease.

transsulfuration; arteriosclerosis; thrombosis; disease risk factors

HOMOCYSTEINE (Hcy) is an intermediate amino acid derived from methionine catabolism. Hcy has two fates in mammals: remethylation to methionine or transsulfuration to cysteine. The elevation of Hcy levels in plasma, hyperhomocysteinemia (HHcy), is an independent and graded risk factor for both cardiovascular (CVD) and neurovascular (NVD) diseases (4, 58, 61a, 66) and neurovascular (NVD) diseases (4, 58, 61a, 66, 92, 102, 131, 138, 153, 187). HHcy is caused by genetic deficiencies in the enzymes responsible for remethylation or transsulfuration of Hcy, nutritional deficiencies in vitamins serving as cofactors for these enzymes, and transient HHcy by dietary overload of methionine-rich foods (e.g., meats, dairy products, sesame seeds). Clinically, HHcy is determined by the plasma Hcy concentration ([Hcy]) in three categories: “mild” (15–30 μmol/l), “moderate” (30–100 μmol/l), and “severe” (>100 μmol/l). Vitamin dependence of the respective pathways has led to the hypothesis that increasing the dietary intake of these cofactors will lead to a reduction in Hcy levels and a subsequent improvement in disease outcomes.

This hypothesis has lost traction after the failure of several large-scale randomized control trials (RCTs) that demonstrated that vitamin therapy did lower Hcy levels but failed to rescue CVD or NVD outcomes (6, 15, 39, 71, 100, 154).

However, there is debate over the design, interpretation, and conclusions of these RCTs with the suggestion that they are insufficient to disregard the “Hcy/CVD hypothesis” based on 1) substantial evidence from laboratory investigations demonstrating Hcy’s ability to activate pathways involved in atherogenesis and thrombosis, 2) animal studies indicating that HHcy in the presence of dyslipidemia enhances atherogenesis, and 3) the validity of these trials due to inappropriate windows of exposure and followup, focus on subjects with established disease (i.e., secondary prevention studies), and measurements of composite CVD end points instead of specific events, such as stroke (34). A key question, however, is whether lowering [Hcy] adequately addresses the underlying etiology of the disease process being studied. Several metabolic pathways intersect at the production-catabolism of Hcy. Information from the available RCTs suggests that one of these pathways, transsulfuration, may be underexplored in the context of HHcy. Only two of these RCTs supplemented the three B vitamins...
individuals in study groups rather than “cocktail” supplementation (15, 39). This is important because each vitamin places an emphasis on different pathways in addition to lowering [Hcy]; specifically, folic acid and cobalamin (vitamin B12) facilitate remethylation, whereas pyridoxine (vitamin B6) supports transsulfuration. When folic acid and vitamin B12 are supplemented, [Hcy] is reduced, but CVD persists.

In contrast, vitamin B6 alone did not lower [Hcy] in recent clinical trials [the Norwegian Vitamin Trial and Western Norway B Vitamin Intervention Trial (15, 39)]. Given that lowering Hcy through remethylation may not improve disease outcomes, and vitamin B6 alone failed to decrease [Hcy], the brunt of the deleterious effects of HHcy may manifest through impaired transsulfuration capacity or flux (Fig. 1). This review calls special attention to this branch of Hcy metabolism in future studies.

This idea is further supported by the observation that the methionine-loading test (MLT) is a better marker for vascular disease than the fasting Hcy level. The MLT is a method used to raise [Hcy] acutely by saturating cystathionine synthesis (or cellular cystine uptake) is the rate-limiting step for glutathione production, a ubiquitous antioxidant. Thus, transsulfuration is the endogenous pathway for coupling toxic Hcy removal with protective H2S and cysteine production. CGL has the capacity to metabolize homocysteine in vitro (dashed line from homocysteine to CGL).

Transsulfuration

Methionine → Homocysteine → Folate → CBS → CGL → Cysteine → Hydrogen sulfide (H2S)

Hyperhomocysteinemia
- Endothelial dysfunction
- Promotes inflammation
- Promotes free radical formation
- Impairs angiogenesis
- Promotes arteriosclerosis
- Increases risk of stroke

H2S
- Protects endothelium
- Suppresses inflammation
- Scavenges free radicals
- Promotes angiogenesis
- Supresses atherosclerosis?
- Reduces risk of stroke?

Fig. 1. Established vascular phenotypes associated with transsulfuration. Cystathionine β-synthase (CBS) is the first enzyme of the transsulfuration pathway and has the unique property of the irreversible catalysis of homocysteine. Hydrogen sulfide (H2S) is produced during transsulfuration by both CBS and cystathionine γ-lyase (CGL). CBS activity determines the balance between the vascular damage of hyperhomocysteinemia and the protective potential of endogenous H2S production. CGL has the capacity to metabolize homocysteine in vitro (dashed line from homocysteine to CGL).

CBS DEFICIENCY

CBS (EC 4.4.1.22) is the first, and rate-limiting, enzyme in the transsulfuration pathway. CBS has the capacity to convert Hcy to H2S and the cysteine precursor cystathionine. Cysteine synthesis (or cellular cystine uptake) is the rate-limiting step for glutathione production, a ubiquitous antioxidant. Thus, transsulfuration is the endogenous pathway for coupling toxic Hcy removal with protective H2S and cysteine production. CBS is the only enzyme available for terminal removal of Hcy since the other metabolic pathways are cyclic and allow its reconstitution. Deficiencies in CBS activity caused by CBS genetic mutations are the most frequent cause of familial HHcy (90) and the underlying cause of the homozgyous CBS genetic disorder homocystinuria, which is characterized by severe HHcy.

The gene for CBS is evolutionarily well conserved and located on the 21q22.3 region of human chromosome 21 (64, 117). The homologous mouse gene is found on chromosome 17 (117). The mouse gene is traditionally distinguished by using lowercase nomenclature (cbs), whereas the human CBS
gene is denoted as Cbs. Cbs comprises 23 exons, with 6 exons involved in alternative splicing (89). The targeted exons in CBS-deficient mice are homologous to exons 3, 4, and 8 of the human gene (173). The CBS gene codes for 551 and 561 amino acid residues in the human and mouse, respectively. These genes produce ~67-kDa proteins that aggregate as tetramers (50, 91). CBS can be cleaved by trypsin or TNF-α into a more catalytically active ~50-kDa core that associates as a dimer (80, 91, 188). The translation of CBS incorporates pyridoxine and heme prosthetic groups in mammals, but the heme group is absent in yeast and protozoa (75). The function of the heme ligand is still not entirely clear but appears to be important in redox sensitivity of CBS activity (10). The canonical CBS-mediated reaction is a pyridoxine-dependent condensation of Hcy with serine to yield the cysteine precursor cystathionine.

There is now a critical need for understanding how specific CBS mutations modify the capacity for H2S production in addition to lowering [Hcy]. Armed with this information and complementary arsenals for modulating the pathway, clinicians may be able to bring the fight against associated pathologies from “bench to bedside.” Given the many CBS mutations (see below), this knowledge will be necessary for the design of patient-centered treatments.

**Human CBS Genetic Deficiency**

Human genetic studies have identified at least 153 point mutations, insertions, deletions, and one small indel present in the CBS locus. Not all of the point mutations have been directly tested for activity; those that have (~20) significantly reduce CBS activity (90). The pyridoxine-responsive I278T and pyridoxine-insensitive G307S mutations are located on exon 8 and are the most common CBS mutations (90). While the I278T mutation is pan-ethnic, accounting for as much as 50% of mutations in The Netherlands (83), the G307S mutation seems to be highest in those with Celtic heritage. In Ireland, 71% of patients with homocystinuria have the G307S mutation (48). The most common insertion is a 68-bp insertion in exon 8 (844ins68) and occurs in ~5% of Caucasians (84, 148, 158); although this insertion does not alter CBS activity, there is no information regarding its H2S-generating capacity. To the best of our knowledge, there are no documented gain-of-function mutations.

Homozygous CBS deficiency is relatively rare (estimates very widely but average roughly 1 in 300,000), with the major clinical manifestations of premature arteriosclerosis, thromboembolism, mental retardation, ectopia lentis, hepatosteatosis, and skeletal abnormalities (116). The extent to which heterozygous, polymorphic, or unidentified forms of homozygous CBS mutations contribute to the epidemiology of CVD is a topic of ongoing investigation. The prevalence of heterozygous CBS deficiency is as high as 1:6,400 in Norway [screening for 6 mutations (133)] and 1:15,500 in the Czech Republic [screening for 10 mutations (72)]; the prevalence is probably much higher if all mutations/insertions/deletions are considered.

There is a great deal of allelic variance with geographic location, amino acid consequence, and varying degrees of phenotypic outcomes. The OMIM database of the National Center for Biotechnology Information curates a small percentage of this variance and categorizes the different mutations by loss of function and associated phenotypes (see MIM identifier no. 236200). Despite the growing volume of information concerning CBS mutations in humans, there remains a significant gap in translational knowledge. The mechanistic etiology of disease states associated with CBS deficiency is likely to depend on the H2S-producing activity of the resulting mutated proteins. A greater focus needs to be placed on not simply the Hcy-metabolizing activity of the various CBS mutations but especially on developing a better understanding of how these mutations alter H2S production.

In 1968, Kilmer McCully first recognized the relationship between CVD and HHcy while working with children who had homocystinuria due to homozygous CBS deficiency (110). These patients have severely elevated plasma [Hcy] and develop atherothrombotic disease at a young age. There are two major allelic variance types in CBS deficiency: vitamin B6 responsive and vitamin B6 nonresponsive (116). Future clinical trials should be careful to appreciate these response subtypes as they may help in elucidating the transsulfuration-specific pathologies. Neonatal screening for common CBS mutations has given clinicians the opportunity to screen and identify patients that would benefit from diet modifications. Without treatment, homozygous vitamin B6-responsive patients have significantly less severe pathologies (Table 1), which is attributed to a small amount of residual CBS activity in this group (116).

Determining the prevalence of CBS deficiency in the general population and associations with CVD and NVD is an unresolved challenge and will be more difficult as the list of CBS mutations grows. Table 1 shows the generalized phenotypes associated with the two broad deficiency categories: heterozygous and homozygous. For specific correlations between individual gene mutations and respective disease states, the reader should consult the extensive database curated by Dr. Jan Kraus at http://cbs.1f1.cuni.cz/. Besides genetic screening, other approaches have been used to identify heterozygous CBS patients, e.g., the MLT and CBS activity assays in cultured fibroblasts (115). With the increasing availability of rapid DNA sequencing techniques, future trials should incorporate ways of correlating CBS genetic mutations with HHcy to determine the prevalence of human heterozygous CBS deficiency. However, quantifying CBS capacity (i.e., H2S or transsulfuration) may be more important given the additional potential for posttranslational, epigenetic, or micro-RNA modifications (discussed below) that would not be reflected in simple genetic variance screening. This approach may shed light on the variability in patient outcomes after interventions. For example, classifying patients by functional etiology rather than [Hcy] or DNA mutations per se may help in interpreting intervention outcomes and provide additional information for developing therapeutic strategies.

**Murine CBS Deficiency: Heterozygous and Homozygous Knockouts**

CBS-deficient mice have been substantially characterized. cbs−/− mice are born at the 1:2 ratio expected from +/−...
Asomatosis, skeletal abnormalities, and ocular disease (173). Human interactions compared with human homozygous patients, e.g., hepatoste-
vs. 6.1 mice have 30 times the normal level of plasma Hcy [203.6 higher in cbs+/− mice and 40-fold higher in cbs−/− mice (173). The slightly elevated plasma [Hcy] in cbs+/− mice provides a good starting point for assessing the effect of subclinical elevations and borderline HHcy prevalent in the general population (73, 74). Perhaps of greater interest is the substantial vascular dysfunction observed in cbs+/− mice considering the subclinical elevation in [Hcy]. It remains unknown how cbs+/− affects endogenous H2S production and signaling. Data from these murine models clearly indicate that subclinical elevations in [Hcy] may be of substantial clinical relevance. Translational studies that quantify [Hcy], CBS activity, and H2S bioavailability and associate these with disease states and progression are needed if we are to convert the evidence from these murine models to therapeutic practice.

**Endothelial Dysfunction in CBS Deficiency**

Endothelial dysfunction (defined here as a decrease in a vessel’s response to vasoconstricting/dilating substances produced by, or acting through, the endothelium) is the most consistently reported finding in HHcy. Most animal models of HHcy exhibit endothelial dysfunction, and studies using CBS-deficient murine models have produced similar results. Large vessels (the aorta and carotid artery) and smaller resistance vessels (mesenteric, celiac, and cerebral arteries) have all been used to experimentally demonstrate endothelial dysfunction in cbs+/− and cbs−/− mice (40, 74, 174). These experiments consistently demonstrate that when an endothelium-dependent vasodilator (bradykinin, ACh, and β-methacholine) is applied to an isolated vessel from a cbs+/− mouse, there is a decreased dilatory response compared with controls. Paradoxically, vessels of cbs+/− mice can constrict in response to endothelium-dependent vasodilators (174). This vasocostriction in response to vasodilators has also been observed in HHcy rats (high-methionine diet) (9). The vasomotor dysfunction is primarily endothelial specific, as opposed to smooth muscle dysfunction, because the vessels respond normally to sodium nitroprusside, an exogenous nitric oxide (NO) donor (174).

The phenotype of endothelial dysfunction observed in CBS-deficient mice and other animal models of HHcy is largely attributed to redox imbalance and decreased bioavailability of NO (for a review, see Ref. 97). There are three hypotheses for decreased NO bioavailability in cbs+/− mice: 1) quenching (the higher load of free radicals in CBS-deficient mice, such as superoxide, reacts with endothelium-derived NO, rendering it unavailable to dilate smooth muscle), 2) decreased production [e.g., endothelial NO synthase (eNOS) activity is impaired via its regulatory phosphorylation sites (143)], or 3) quenching and decreased production of NO occur simultaneously. Recent evidence suggests that H2S functions in concert with NO for biological activity by joining together to form a unique nitrosothiol (177) or by displacing NO from S-nitrosothiols to liberate NO for downstream signaling (122). Thus, a fourth

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**Table 1. Phenotypes of humans with CBS genetic deficiencies**

<table>
<thead>
<tr>
<th>Cardiovascular system</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygote</td>
<td>Early thrombotic events (&lt;50 yr old) (51% peripheral veins, 32% cerebrovascular accidents, 11% peripheral arteries, 4% myocardial infarctions, and 2% other)</td>
</tr>
<tr>
<td>Heterozygote</td>
<td>Abdominal aortic aneurysm</td>
</tr>
<tr>
<td></td>
<td>Coronary artery disease</td>
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<tr>
<th>Nervous system</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Homozygote</td>
<td>Mental retardation</td>
</tr>
<tr>
<td></td>
<td>Psychiatric disorders</td>
</tr>
<tr>
<td></td>
<td>Seizures</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Skeletal System</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygote</td>
<td>Marfanoid habitus</td>
</tr>
<tr>
<td></td>
<td>Osteoporosis</td>
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</table>

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<thead>
<tr>
<th>Ocular system</th>
<th>References</th>
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<tbody>
<tr>
<td>Homozygote</td>
<td>Ectopia lentis</td>
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<tr>
<th>Skin</th>
<th>References</th>
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<tbody>
<tr>
<td>Homozygote</td>
<td>Hypopigmentation</td>
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<table>
<thead>
<tr>
<th>Other</th>
<th>References</th>
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<tbody>
<tr>
<td>Homozygote</td>
<td>Pancreatitis</td>
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<td></td>
<td>Spontaneous pneumothorax</td>
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The reader is referred to the extensive cystathionine β-synthase (CBS) mutation database curated by Dr. Jan Kraus for data linking specific gene mutations to respective disease states: http://cbs.lf1.cuni.cz/ and http://cbs.lf1.cuni.cz/search_res.php

by +/− mouse mating but have a high mortality rate after the third and fourth postnatal weeks (168). Homozygous knockout mice have 30 times the normal level of plasma Hcy [203.6 ± 65.3 vs. 6.1 ± 0.8 μM (173)] and exhibit similar disease manifestations compared with human homozygous patients, e.g., hepatosteatosis, skeletal abnormalities, and ocular disease (173). Human patients with CBS deficiency do not exhibit phenotypes as severe as cbs−/− mice, likely because most human homozygous mutations maintain at least some level of CBS activity. To the best of our knowledge, all of the research in cbs+/− and cbs−/− mice has been with the same line of mice on the C57Bl/6 background originally generated and reported by Watanabe et al. (173) with one exception, where Akahoshi et al. (5) backcrossed into BALB/cA, C3H/HeJ, and DBA/2J strains. They found that C5H/HeJ cbs−/− mice lived longer and escaped severe lipidosis and hepatic steatosis compared with the more commonly used C57Bl/6 background, suggesting that in vivo phenotypes resulting from CBS deficiency depend on the genetic environment and related compensations. Future studies using this and other background strains are needed. It has also been demonstrated that better maternal care increases the lifespan of cbs−/− mice. By decreasing litter size and increasing weaning to 6 wk, there is an increased likelihood of some cbs−/− mice surviving to over 10 wk of age (74).
hypothesis emerges, where impaired H₂S production in CBS deficiency reduces some aspect of H₂S-dependent NO signaling. Moreover, it has been recently reported that much of the activity of H₂S may be through protein sulphydrylation (118), much like NO acts through S-nitrosylation. The role of CBS deficiency in reducing the cellular sulphydryl-modifying potential through reduced generation of H₂S remains unexplored. We propose that groundbreaking discoveries will come from focused and definitive studies into the interplay between NOS and transsulfuration and between S-nitrosylation and S-sulphydrylation. In support of this concept, we discovered that CBS (and cystathionine γ-lyase) are secreted into the bloodstream by the liver and endothelium, where transsulfuration actively generates H₂S from Hcy (13). This extracellular H₂S and transsulfuration capacity protected the endothelium from HHcy and from redox stress challenges (serum starvation and hypoxia-reoxygenation). NO binds to the heme of CBS, thereby inhibiting its activity (146). As Hcy levels rise and NO bioavailability declines, this inhibition would also tend to decline, allowing CBS-mediated production of H₂S. However, when the HHcy is due to CBS deficiency, this compensatory benefit may not manifest. Discussed later in this review is the complementary hypothesis that Hcy may act through protein N-sulfhydration (67). These interrelations have yet to be fully explored.

Aortas from cbs⁺⁻ mice have ~43% lower cGMP levels after stimulation with ACh compared with their WT littermates (40). Elevated cGMP levels in response to ACh stimulation are an indirect measure of the activation of eNOS. In resistance microvessels, we have previously reported significantly lower basal expression of eNOS and the pS1179eNOS-to-eNOS (activation site) ratio in mice with diet-induced HHcy with no difference in the pT495eNOS-to-eNOS (deactivation site) ratio (101). S1179 eNOS phosphorylation was also significantly less in these vessels when they were stimulated with ACh, both ex vivo and in situ. Jiang et al. (74) proposed that endothelial dysfunction in aortic and cremasteric vessels of cbs⁺⁻ mice is not attributed to redox imbalance; rather, eNOS activity is decreased because of PKC-mediated phosphorylation of T495 on eNOS. The discrepancies in eNOS phosphorylation between cbs⁺⁻ and diet-induced HHcy further emphasize the need to carefully consider the murine model used and its relation to the human etiology under investigation. In general, these findings are consistent with the “decreased production” hypothesis. Nevertheless, there is substantial support for the “quenching” hypothesis, and it is important to appreciate that these mechanisms need not be mutually exclusive.

Superoxide production is higher in aortas of cbs⁺⁻ mice compared with WT littermates (175). Superoxide reacts with NO to form peroxynitrite, which may nitrosylate protein tyrosine residues. 3-Nitrotyrosine labeling (a marker of nitrosylated proteins) is also increased in cbs⁺⁻ vessels (94). Glutathione peroxidase (GPx)-1 activity is impaired in cbs⁺⁻ mice, and increasing glutathione pools and GPx-1 activity can partially restore endothelial function in these mice (175). Experimental increases in the activity of SOD or the free radical scavenger 4,5-dihydroxybenzene 1,3-disulfonate (tiron) reverse the endothelial dysfunction observed in these mice (175).

Overall, there seem to be multiple adaptations occurring in the endothelium of CBS-deficient and HHcy mice, with multiple mechanisms contributing to the dysfunction. However, there is a relative lack of information regarding the direct effect of Hcy versus impaired transsulfuration capacity on endothelial function, and the molecular mechanisms, aside from general redox imbalance, are virtually unknown. As pointed out below, studies contrasting the effects of Hcy with those of H₂S may shed some light on this subtle but important issue. We propose that shifting the balance toward an elevation in [Hcy] and, perhaps more importantly, away from H₂S robs cells of necessary protein sulphydrylation.

Vascular Remodeling in CBS Deficiency

HHcy is strongly correlated with neointimal hyperplasia and stroke (41, 98). Moderate HHcy is associated with an increased incidence of carotid artery stenosis (140). Hcy promotes vascular smooth muscle proliferation and hyperplasia in vivo (minipigs with HHcy) and in vitro (rat aortic smooth muscle cells) (137, 157). Hypertrophy of cerebral vessels is a risk factor for stroke (63). Several morphological changes have been observed in vessel walls of CBS-deficient mice. Cerebral arteriolar walls from cbs⁺⁻ mice are ~25% thicker than those from WT littermates (12). These vessel walls had lower ratios of nondistensible (collagen and basement membrane) to distensible (elastin and smooth muscle) components. Hence, the hypertrophy in cbs⁺⁻ vessel walls was associated with an increase in compliance, consistent with a weaker myogenic response. These findings were also accompanied by an increase in vessel distensibility during maximal dilatation with EDTA (12). Increased wall thickness, elevated blood pressure, increased extracellular matrix fiber deposition, and fragmented elastic fibers have been documented in aortas of cbs⁺⁻ mice on a methionine-enriched diet compared with WT littermates (123). These findings were partially attributed to matrix remodeling by increased expression and activities of matrix metalloproteinases (matrix metalloproteinase-2 and -9). Interestingly, diet supplementation of these mice with 3-deazaadenosine, a Hcy scavenger, mitigated these effects (123). As noted, vessel walls have increased neointima formation and hyperplasia in several animal models of HHcy. Rats treated with H₂S demonstrated attenuated neointima formation and hyperplasia in balloon-injured carotid arteries (112). Given the importance of the metabolic activity of CBS highlighted in this review and the Hcy-H₂S (substrate-product) balance of transsulfuration, the therapeutic potential of H₂S would be greatly enhanced by a better understanding of how the enzymes of the pathway may be regulated pharmacologically in vivo. Although we have learned much from experimental approaches using a known exogenous treatment with H₂S-liberating compounds such as NaHS, there is now a relatively greater need to understand the benefits of modulating the capacity for endogenous H₂S metabolism.

Impaired Angiogenesis

Hcy impairs the proliferation of endothelial cells in cell culture (109, 144) and angiogenesis in animal models (66, 96), which likely contributes to its overall impact in several vasculopathies. cbs⁺⁻ mice have impaired angiogenesis, as demonstrated by the decreased compensation of blood flow and total capillary density in a hindlimb ischemia model (16). Also, cbs⁺⁻ mice on a high-methionine diet have decreased reendothelialization after balloon injury to the carotid artery (152).
In a rat model of HHcy, angiogenesis was also impaired in response to hindlimb ischemia (38). Conversely, treatment of rats with the H$_2$S donor NaHS promoted angiogenesis after hindlimb ischemia (169). The molecular mechanisms by which Hcy reduces endothelial cell proliferation are poorly understood.

Jamaluddin et al. (70) have linked hypomethylation of the cyclin-A gene to the impaired endothelial proliferation in HHcy. Guillen et al. (51) found several genes (Fsp27, Cd36, Sdc1, Syt1, and Hsd3b5) upregulated in the livers of 9-day-old $cbs^{-/-}$ pups. Among these, Cd36 sensitizes endothelial cells to the antimigration and antitube formation effects of thrombospondin-1 (29). Whereas Hcy impairs the proliferation of endothelial cells, it paradoxically promotes the proliferation of smooth muscle cells (157). These effects are consistent with the pattern of proliferation and growth in atherosclerotic plaques. It is intriguing that H$_2$S has the opposite effect: promoting the proliferation of endothelial cells (19) while impairing the proliferation of smooth muscle cells (37, 182). This set of observations underscores the main theme of this review. Specifically, beyond the effects of Hcy alone, does an imbalance in the Hcy-to-H$_2$S ratio in CBS deficiency produce the observed vascular phenotypes? For example, is a doubling of Hcy and halving of H$_2$S more toxic than a fourfold increase in Hcy? The answers are likely to be more complex than a simple ratio, but an appreciation of this interplay in future mechanistic studies will likely bring greater insights into the underlying pathologies. It no longer seems appropriate to simply study one aspect of this pathway in isolation.

**Dyslipidosis, Thrombosis, and Arteriosclerosis in CBS Deficiency**

Compared with $cbs^{-/-}$ mice, $cbs^{+/-}$ mice represent a good model for observing the more common epidemiological cause of HHcy due to impaired transsulfuration, and they exhibit a host of vascular pathologies common to atherothrombotic diseases. However, $cbs^{+/-}$ mice do not spontaneously develop atherosclerosis or embolisms when fed a standard chow diet. WT ($cbs^{+/-}$) mice fed a high-methionine diet to induce HHcy only develop atherosclerosis when fed a Western-type (standard chow + 0.15% cholesterol and 21% fat) or atherogenic (standard chow + 1.25% cholesterol, 15% fat, and 0.5% cholic acid) diet (184). Regardless, both CBS deficiency and CBS deficiency plus methionine exacerbate atherosclerosis in mice fed a Western-type or atherogenic diet (12, 152). This observation has prompted the hypothesis that Hcy itself does not cause atherosclerosis; rather, it accelerates atherosclerosis by inducing factors such as endothelial dysfunction, altered liver lipid metabolism, and proinflammatory/thrombotic signals. This has also led to the cross-breeding of $cbs^{+/-}$ mice with atherogenic mouse models such as apolipoprotein (Apo; ApoE and ApoA1) knockout mice. ApoE is important in catabolism of triglyceride-rich lipoprotein constituents and is inversely correlated with CVD, Alzheimer’s disease, immunodysregulation, and cognition (106).

While $cbs^{+/-}$ mice show no differences from $cbs^{+/-}$ in hepatic triglycerides, plasma cholesterol, or plasma triglycerides, they do exhibit an ~44% greater accumulation of hepatic cholesterol (176). Hcy increases intracellular cholesterol in human aortic smooth muscle cells but not human umbilical vein endothelial cells (HUVECs) in vitro (176). In fact, there is also a decrease in LDL uptake by HUVECs treated with Hcy. It is unknown whether the lack of uptake of LDL by the endothelium contributes to the preferential accumulation of lipids in the intima of developing atherosclerotic plaques or promotes oxidation to oxLDL, a significant contributor to plaque formation. It has been recently demonstrated that ox-LDL promotes endothelial stiffening by modulating the cholesterol structure of the cell membrane, which could be counteracted with an excess of cholesterol in vitro (145). It remains to be determined whether dysregulation of lipid metabolism, particularly in the vascular wall, in CBS deficiency plays a role in vascular stiffening and plaque formation. ApoE$^{-/-}$ mice spontaneously develop severe dyslipidosis and atherosclerotic lesions (88). ApoE$^{-/-}$ mice cross-bred with $cbs^{+/-}$ and $^{/-}$ mice have accelerated development of atherosclerotic lesions compared with ApoE$^{-/-}$ mice. In $cbs^{-/-}$ ApoE$^{-/-}$ mice, aortic lesions are twice the size of those in ApoE$^{-/-}$ mice at 6 mo of age; $cbs^{+/-}$ ApoE$^{-/-}$ mice have an ~30% increase in lesion size compared with ApoE$^{-/-}$ mice at 1 yr of age (167). The accelerated formation of atherosclerotic lesions in these mice is associated with an increased uptake of acetylated LDL by affected macrophages, increased plasma total cholesterol, increased accumulation of cholesterol esters and triglycerides in lesions, and decreased HDL cholesterol (167). ApoA1 is the major protein constituent of HDL and is responsible for HDL’s cardiovascular-protective properties of promoting cholesterol efflux from tissues to the liver for excretion. Double heterozygous $cbs^{+/-}$ APOA1$^{+/-}$ mice develop hypertension compared with WT mice (aorta systolic pressure: 124 ± 7.7 vs. 109 ± 11.2 mmHg, $P < 0.05$) and cardiac muscle hypertrophy (20, 21). Simvastatin, a statin-class drug commonly used to treat hypercholesterolemia and CVD, restored systolic pressure to control values and significantly lowered plasma [Hcy] (5.8 ± 0.8 vs. 4.9 ± 0.7 μM, $P < 0.05$) (22); these effects were independent of plasma lipids but related to increased NO production.

As noted, humans with homozygous CBS deficiency suffer from premature vascular thrombotic disease. However, since $cbs^{-/-}$ mice have a high rate of neonatal lethality due to liver failure, they are limited as a model for observing the progression of vascular disease into adulthood. Nevertheless, the pathologies present in young $cbs^{-/-}$ mice may prove important in understanding the pathologies present in humans with both mild and severe HHcy. Table 2 shows the major vascular phenotypes from CBS-deficient mouse models. Namekata et al. (119) assessed common markers of dyslipidosis in $cbs^{-/-}$ mice and found significantly elevated levels of triglycerides and nonesterified fatty acids in both the liver and serum. Liver thiolase is critical for the $β$-oxidation of fatty acids in the liver, and $cbs^{-/-}$ mice have impaired thiolase activity. ApoB100 is the primary protein constituent of LDL and VLDL cholesterol, and its elevation in serum is a better indicator of heart disease than total cholesterol and LDL (59). In $cbs^{-/-}$ mice, there is an approximately fourfold increase in serum ApoB100 and a significant increase in VLDL but a decrease in LDL. The increase in VLDL and decrease in LDL suggests an impaired capacity to convert VLDL to LDL. In addition to decreased HDL-cholesterol, $cbs^{-/-}$ mice also have impaired lecithin-cholesterol acyltransferase (LCAT) function. LCAT expression in the liver and activity in the serum is decreased in $cbs^{-/-}$ mice (119).

To elucidate the potential mechanisms for the dyslipidosis observed in $cbs^{-/-}$ mice, Hamelet et al. (53) found several...
Table 2. Vascular phenotypes from murine models of CBS deficiency

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<td>Cbs&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>vessels</td>
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<th>Cerebrovascular dysfunction</th>
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<tr>
<td>Cerebral arteriolar remodeling</td>
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<td>Altered blood-brain barrier in permeability*</td>
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*<sup>cbs</sup> is the murine CBS gene and <sup>Cbs</sup> is the human CBS gene; thus, <sup>cbs</sup><sup>−/−</sup> <sup>Cbs</sup><sup>−/−</sup> mice are <sup>cbs</sup><sup>−/−</sup> mice with heterozygous <sup>Cbs</sup> gene expression. Apo, apolipoprotein; Hcy, homocysteine. *With concomitant diet-induced hyperhomocysteinemia.

upregulated genes in the livers of <sup>cbs</sup><sup>−/−</sup> mice that are markers of endoplasmic reticulum stress and altered cholesterol/fatty acid metabolism. Additionally, there was an overexpression of genes involved in reverse cholesterol transport. In <sup>cbs</sup><sup>−/−</sup> mice, there is also decreased expression of DYRK1A, which is an important antiapoptotic factor in the liver (55). Maclean et al. (103) recently generated a line of mice with one allele replaced by the human gene (specifically, this is a <sup>cbs</sup><sup>−/−</sup> <sup>Cbs</sup><sup>−/−</sup> model) on the C57Bl/6j background. In this model, CBS activity was low and Hcy levels remained severely elevated (83-fold higher than WT mice), which was attributed to use of the human, rather than the murine, promoter. However, the replacement did rescue the hepatic steatosis, and the neonatal lethality of complete knockout mice demonstrating a low, threshold requirement for CBS activity in liver lipid metabolism independent of [Hcy].

In <sup>cbs</sup><sup>−/−</sup> mice, which also do not suffer from dyslipidosis or hepatic disease but have only slightly elevated [Hcy], there is a 50% increase in catalase activity that may compensate for potential redox imbalance. There are no changes in GPx-1, glutathione reductase, or glutathione-S-transferase activities in the livers of <sup>cbs</sup><sup>−/−</sup> mice (56). However, these findings contrast another study (174) showing that <sup>cbs</sup><sup>−/−</sup> mice do have decreased liver GPx-1 activity, which was restored by supplementing their drinking water with the intracellular cysteine donor L-2-oxothiolizidine-4-carboxylic acid (174). Zhang et al. (183) created humanized <sup>cbs</sup><sup>−/−</sup> mice by replacing the CBS gene with one of the known human mutant forms, S466L. They reported that these mice have severe elevations in plasma [Hcy] (~214 μM) without hyperlipidemia; this mutation did create a proinflammatory state with enhanced monocyte/macrophage activation and exacerbated atherosclerotic lesion formation when crossed with ApoE<sup>−/−</sup> mice. Thus, CBS deficiency may contribute to atherosclerosis independent of the lipid profile per se. This is supported by observations in CBS-deficient humans who develop arterial fibrosis and intimal thickening that is atherosclerotic but need not be artherosclerotic. Clearly, more work is needed to dissect the mechanisms by which impaired CBS activity contributes to a predisposition for atherosclerosis and vascular inflammation. Respective mutations may be linked to different aspects of the complex atherosclerotic environment.

Neurological and Neurovascular Disorders in CBS Deficiency

HHcy is a risk factor for several neurological diseases with markedly different histological presentations, i.e., Lewy body formations in Parkinson’s disease and amyloid β-plaques in Alzheimer’s disease. Amyloidosis is accelerated in a mouse model of Alzheimer’s disease (Tg2576 mice) when they are fed a HHcy diet (low folate, vitamin B6, and vitamin B12) (185). Hcy is a major risk factor for dementia (4, 8, 26, 49, 102, 111, 141, 165). Even mildly elevated Hcy (>14 μmol/l) creates a twofold increase in the risk of dementia with a significant correlation between [Hcy] and cognitive impairment (47, 141). It remains unknown whether CBS-deficient mice have normal cognitive ability and normal brain histology. Plasma [Hcy] steadily increases with age (42, 127, 136, 164). Hcy also increases blood-brain barrier (BBB) permeability (77, 93, 99), which may be an important factor in the etiology of small vessel disease and dementia (17, 27, 36, 76, 85, 105, 147, 155, 161, 172, 179). Moreover, Hcy is a risk factor for cerebral small vessel disease via endothelial dysfunction (57) and is an independent risk factor for silent brain infarcts and white
matter lesions, as found in the Rotterdam Scan study (165), Northern Manhattan study (181), and Framingham Offspring study (142). Indeed, the magnitude of white matter lesion changes in cerebral small vessel disease is directly related to the level of HHcy (180). BBB disruption may be part of the etiology of lacunar stroke (170–172). HHcy is linked to lacunar infarction and stroke (65, 81). Thus, Hcy is a significant factor in a spectrum of cerebrovascular pathologies that are related through a chronic increase in BBB permeability, subcortical damage, stroke subtypes, and progressive dementia. However, there is little understanding of the mechanisms by which Hcy triggers endothelial damage and BBB permeability. When fed a HHcy diet (low folate, vitamin B12, vitamin B6, and choline), cbs+/− mice have increased BBB permeability compared with WT littermates (77). Treatment with muscimol (γ-aminobutyric acid receptor agonist) or sodium sulfide (H2S donor) can rescue some of the BBB permeability observed in these mice (93, 159). The latter finding supports the concept that modulating flux through transsulfuration may be a viable, and therapeutically well tolerated, method for shifting the Hcy-H2S balance to one that supports an intact and healthy BBB. Neither of these treatments identifies the mechanism(s) by which the BBB is affected. The role of HHcy and transsulfuration capacity in cerebrovascular damage and in the etiology of Hcy-associated neurological disease remains to be fully explored.

The need to understand HHcy in the context of CBS impairment is further highlighted by the importance of transsulfuration capacity in neuronal long-term potentiation (LTP). Autopsied brains from Alzheimer’s disease patients who showed impairment of LTP and memory loss have a significant decrease in [H2S], which was thought to be a consequence of decreased CBS activity (44). H2S derived from CBS expressed in neurons of the hippocampus is an important neuromediator in LTP (82). Eto et al. (45) reported that brains from cbs+/− mice have lower [H2S] with concomitant LTP alterations (increased excitatory postsynaptic potential slopes) in the hippocampus compared with WT controls. Glutamate receptors are a critical component in synaptic plasticity of the hippocampus. Both Hcy and H2S modulate neuronal glutamate receptors (24, 186), in particular, ionotropic and metabotropic glutamate receptors.

From a clinical perspective, perhaps the largest gap in knowledge regarding the etiology of Hcy-associated pathologies is the lack of definitive molecular mechanism by which Hcy triggers damage and dysfunction. For example, almost nothing is known about whether extracellular or plasma-derived Hcy has receptor-dependent functions in non-neural cells. Rather, much of the interest in Hcy has suggested or assumed activity after intracellular transport through cysteine transporters with various affinities for Hcy (18, 73). It is imperative that future efforts be put toward dissecting the pathways necessary for the effects of Hcy, with a particular focus on pharmacological targets that may provide for therapeutic interventions, e.g., identifying potential receptors for Hcy on vascular cells and dissecting their downstream pathways.

Nonvascular Complications in CBS Deficiency

Although this review is focused on the vascular complications, other organ systems are affected by CBS deficiency. Therefore, we include findings in nonvascular systems (shown in Table 3) with the hopes this knowledge may serve as a platform from which to identify common molecular mechanisms associated with these pathologies.

Reproductive Complications in CBS Deficiency

In addition to the high neonatal mortality in cbs−/− mice, there is a high rate of infertility (52). Human epidemiological and meta-analyses have reported that HHcy is a risk factor for pregnancy complications such as preeclampsia, spontaneous abortion, and abruptio placenta (132, 160). Guzman et al. (52) showed that infertility of female cbs−/− mice is a consequence of uterine failure and not failure of other reproductive organs. The independent or codependent effects of HHcy versus transsulfuration impairment remain to be resolved. Given that angiogenesis is impaired in cbs+/− mice, as demonstrated in a hindlimb ischemia model (16), it will be clinically important to dissect the microvascular mechanisms linking CBS deficiency to placental and fetal development.

Proinflammatory States in CBS Deficiency

Since HHcy is associated with vascular and neurological diseases that are associated with proinflammatory and pro-

### Table 3. Nonvascular phenotypes from murine models of CBS deficiency

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<th>Skeletal system</th>
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<th>Reproductive system</th>
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thrombotic states, there is a great deal of interest in identifying markers of these conditions in the context of HHcy. Diet-induced (acute methionine load) HHcy in rats induces thromboxane biosynthesis, platelet aggregation, and tissue factor expression and activity. However, cbs+/− mice do not develop spontaneous thrombosis (32). Thus, CBS deficiency or subclinical elevation in [Hcy] may sensitize the cardiovascular system to enhance disease progression. This was demonstrated in the context of HHcy for atherosclerosis on the ApoE−/− background (60). P-selectin is an important factor produced by the endothelium that aids in leukocyte extravasation under inflammatory conditions. cbs+/- mice on a HHcy diet have increased P-selectin on the endothelial surface and a concomitant increase in serum soluble P-selectin (77). A more thorough investigation of serum cytokines and proinflammatory molecules in CBS-deficient mice would help to better understand the baseline proinflammatory environment created by this condition.

**Skin and Skeletal Phenotypes of CBS Deficiency**

In addition to osteoporosis, Marfan syndrome-like traits are present in some humans with CBS homozgyous deficiency (139). Robert et al. (135) found that cbs+/- and cbs−/− mice have shorter hindlimbs compared with WT littermates. Furthermore, cbs−/− mice develop kyphoscoliosis and shorter long bones. These findings appear to be partly attributed to impairment in endochondral growth. Another key phenotype of CBS deficiencies is abnormal collagen homeostasis (78). Whether this contributes to the connective tissue abnormalities and high prevalence of HHcy in rheumatoid arthritis is unexplored. Moreover, some patients with homocystinuria have other skin abnormalities, such as hypopigmentation (129). Similarly, cbs−/− mice have hyperkeratosis, wrinkled skin, and an abnormally thin dermal layer (134). Fibrosis and collagen disruption have also been reported in other studies using various tissues from CBS-deficient mice, and this phenotype is not tissue specific. It is important to recognize the parallel between connective tissue abnormalities in these studies and those found in the cardiovascular system (e.g., Ref. 12). Future studies should consider if and how extracellular matrix abnormalities contribute to the CBS-deficient phenotypes and whether these are systemic affects that underlay the multidimensional and multigorgan dysfunctions observed.

**Hcy Metabolites**

Although most of the research to date has focused on Hcy per se, there are alternative metabolites of Hcy. Both genetic and nutritional disorders in Hcy metabolism lead to elevated levels of Hcy-thiolactone in the plasma. Hcy-thiolactone has the ability to react with protein lysine residues, forming an isopeptide bond leading to a product termed N-homocysteinylated proteins. N-Homocysteinylated is a neo-self-antigen with the ability to trigger autoantibodies. Serum levels of anti-N-homocysteinylated antibodies have been positively correlated with levels of total plasma Hcy (162). In CBS-deficient human patients, plasma N-homocysteinylated proteins are approximately twofold greater than control subjects (68). In stroke patients, anti-N-homocysteinylated autoantibodies were ~50% higher than in healthy controls (162). There is an approximately eightfold increase in plasma N-homocysteinylated proteins in cbs−/− mice compared with WT littermates (69). The ability of impaired transsulfuration and HHcy to trigger an autoimmune response could explain why the condition is correlated with so many pathologies and why decreasing [Hcy] has not altered disease progression in most RCTs. Indeed, there is a paucity of information concerning the potential autoimmune responses triggered in HHcy.

**Micro-RNA and Epigenetics**

CBS mRNA contains dozens of sites predicted for microRNA regulation (Microcosm Targets http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) in rodents and humans. To date, numerous studies have correlated various DNA mutations with [Hcy] and disease risk or progression. An important and likely fruitful area for future investigation is the posttranscriptional regulation of CBS and transsulfuration activity. Another plausible explanation for why lowering Hcy in RCTs has been ineffective in reoccurring vascular events is the hypothesis that altered metabolism of Hcy alters DNA methylation, leading to certain genes being turned on or off. Since DNA methylation persists in DNA transcription and cell division, removing (or lowering) Hcy after vascular/neurological disease development may be too late. The H19 differentially methylated domain (DMD) is used as a marker of global DNA methylation. Recently, Lentz et al. (35) examined cbs+/- mice fed a HHcy diet (increased methionine and decreased folic acid, vitamin B6, and vitamin B12) and found that liver samples contained hypomethylated H19 DMD, whereas brain and aortic samples contained hypermethylated H19 DMD. The CBS-deficient mouse is a unique model for exploring the effect of Hcy on DNA methylation because, unlike dietary induction of HHcy, CBS deficiency elevates Hcy in a way that does not manipulate the substrates, cofactors, and enzymes that are involved in the methionine/DNA methylation cycle. This presents an opportunity to specifically test the role of impaired Hcy-H2S metabolism in the epigenetics of vascular dysfunction. It is also of interest that endothelial cells isolated from patients with heterozygote CBS homocystinuria are more sensitive to Hcy in vitro, as shown by decreased viability and increased platelet adherence (33). These data support the idea that genetic background modulates endothelial responses to Hcy; the extent to which this heightened sensitivity depends on genomic regulation at the level of methylation deserves serious attention. For example, hypomethylation of cyclin-A in HHcy reduces endothelial cell proliferation (70). Whether this hypomethylation and impaired proliferation persists even when [Hcy] is lowered remains to be determined. Future studies are needed to determine if CBS deficiency produces changes in DNA methylation in animals on standard chow. Additionally, there is an urgent need for systematic, discovery-driven interrogation of the genome for sites modified by methylation in animals on standard chow. Additionally, there is an urgent need for systematic, discovery-driven interrogation of the genome for sites modified by methylation in model systems of Hcy-CBS-H2S dysfunction and a translational approach to understanding how these may be modified with exogenous H2S or modulation of CBS activity.

**Summary**

CBS deficiency from genetic mutations in humans represents a global health problem with multiple phenotypic outcomes. Nearly all organ systems are affected by deficiencies in this enzyme. Decades of mixed results from clinical trials...
aimed at ameliorating HHcy-mediated disease have left clinicians with a statistically powerful disease marker but no promising treatment guidelines. This review raised the idea that the underlying etiology for the HHcy may be as important as HHcy itself in understanding disease risk and designing appropriate therapies. We emphasize the need for greater efforts toward understanding the role of transsulfuration in reducing Hcy and increasing H2S concentrations. The development of CBS knockout mice has given researchers a critical tool for investigating the underlying pathologies associated with impaired transsulfuration as well as the resulting HHcy. The majority of research conducted using this mouse model has attempted to uncover the relations among elevated [Hcy] and various pathologies. Advancement of the field will require focused approaches to dissecting the mechanistic etiology of vascular damage with a specific goal of identifying the molecular mechanisms involved in driving the respective pathologies.

Therapeutic advances can only come from a thorough understanding of the molecular mechanisms of vascular regulation by Hcy, by CBS deficiency, by H2S, and by their combinations. Studies of micro-RNA regulation of transsulfuration activity, DNA methylation, and epigenetic modifications are sought. Because lowering of [Hcy] has met with little success in altering disease progression, identifying the first steps in Hcy-mediated damage, specifically, whether Hcy acts through cell surface receptors or only after entering the cell cytosol, deserves serious attention. Furthermore, the assumption that these pathologies are solely related to HHcy should be approached with caution. It is important for investigators to recognize that lack of transsulfuration products, particularly H2S, are likely to play a substantial role in the phenotypes observed in CBS deficiency. There are ongoing clinical trials investigating the therapeutic potential of H2S (for a review, see Ref. 128), and translational scientists should strive to understand the efficacy of exogenous H2S while discovering ways of modulating endogenous production. Our recent discovery of active transsulfuration in the extracellular space (blood plasma) calls particular attention to understanding systemic and cellular compartmentalization of transsulfuration activity. Whenever possible, future studies should attempt measurements of transsulfuration activity in addition to Hcy levels to better understand the role of this pathway in measured outcomes. Understanding the basic mechanisms behind the association of CBS deficiency and vascular damage will greatly increase our ability for future treatment options of this condition. In clinical trials, when groups have been supplemented with vitamin B6 alone, [Hcy] was not lowered, suggesting that there is little information related to lowering Hcy through transsulfuration and its specific relation to disease outcomes. This review calls on those in the HHcy field to revisit a broader vision of HHcy as a family of disorders, with underlying etiologies holding the key to therapeutic advances in the respective disease states.

GRANTS

R. S. Beard was supported by National Center for Research Resources Grant 5P20-RR-016454 (IDeA Network of Biomedical Research Excellence Program). S. E. Bearden was supported by ISU PRC1019.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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