Extracellular transsulfuration generates hydrogen sulfide from homocysteine and protects endothelium from redox stress

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Bearden SE, Beard RS Jr, Pfau JC. Extracellular transsulfuration generates hydrogen sulfide from homocysteine and protects endothelium from redox stress. Am J Physiol Heart Circ Physiol 299: H1568–H1576, 2010. First published September 3, 2010; doi:10.1152/ajpheart.00555.2010.—Homocysteine, a cardiovascular and neurocognitive disease risk factor, is converted to hydrogen sulfide, a cardiovascular and neuronal protectant, through the transsulfuration pathway. Given the damaging effects of free homocysteine in the blood and the importance of blood homocysteine concentration as a prognosticator of disease, we tested the hypotheses that the blood itself regulates homocysteine-hydrogen sulfide metabolism through transsulfuration and that transsulfuration capacity and hydrogen sulfide availability protect the endothelium from redox stress. Here we show that the transsulfuration enzymes, cystathionine β-synthase and cystathionine γ-lyase, are secreted by microvascular endothelial cells and hepatocytes, circulate as members of the plasma proteome, and actively produce hydrogen sulfide from homocysteine in human blood. We further demonstrate that extracellular transsulfuration regulates cell function when the endothelium is challenged with homocysteine and that hydrogen sulfide protects the endothelium from serum starvation and from hypoxia-reoxygenation injury. These novel findings uncover a unique set of opportunities to explore innovative clinical diagnostics and therapeutic strategies in the approach to homocysteine-related conditions such as atherosclerosis, thrombosis, and dementia.

HOMOCYSTEINE (Hcy) is a sulfur-containing amino acid formed in an intermediate step during the metabolism of methionine to cysteine (Cys). Hyperhomocysteinemia (HHcy) is an elevated blood concentration of Hcy and is categorized in the clinical setting as mild (16–30 μmol/l), moderate (31–100 μmol/l), or severe (>100 μmol/l). In recent years, several large clinical trials have established elevated levels of Hcy in the blood as a significant, independent risk factor for venous thromboembolism, hypercoagulability, cardiovascular disease, and stroke (1, 40). The proinflammatory effects of Hcy include endothelial cell expression of leukocyte adhesion molecules, secretion of proinflammatory cytokines and chemokines, release of matrix-degrading enzymes, and activation of procoagulants (22). Elevated Hcy leads to vascular inflammation (reviewed in Refs. 33 and 41) and accelerates the progression of atherosclerosis (10). Hcy increases blood-brain barrier permeability (12, 17) and is a risk factor for neurodegenerative disease (25, 34, 35), especially Alzheimer disease (AD) (6, 19, 31). Even mildly elevated Hcy (>14 μmol/l) creates a twofold increase in risk of AD with a significant correlation between blood Hcy concentration and cognitive impairment (8, 31).

Current therapies for elevated Hcy are limited to vitamin supplements, which serve as cofactors in the pathways of Hcy metabolism. These therapies lower Hcy levels but generally do not alter disease outcomes (summarized in Ref. 11). Without a better understanding of the mechanisms that regulate Hcy endogenously, therapeutic options will remain limited. The pathways for Hcy metabolism have been well characterized. Hcy may be remethylated to methionine, a reversible process, or it may be condensed with serine in the transsulfuration pathway, an irreversible process. The transsulfuration pathway is composed of two enzymes, cystathionine β-synthase (CBS), which converts Hcy to cystathionine, and cystathionine γ-lyase (CGL), which converts cystathionine to Cys. There are over 150 known mutations in the human CBS enzyme, and homozygous CBS deficiency is the most common cause of severe HHcy and homocystinuria (14). CBS and CGL also produce hydrogen sulfide (H2S) from thiol substrates (Hcy and Cys). The importance of H2S in cardiovascular homeostasis has made this pathway a popular target for study. For example, H2S has attracted attention for its role in metabolic regulation (3), free radical biology (28), cardioprotection (7, 23), vascular relaxation (13), vascular oxygen sensing (26, 27), and vascular inflammation (33). A major limitation in the ability to control Hcy-H2S metabolism in the clinical setting is our poor understanding of the compartmentalization and regulation of these enzymes in vivo.

Approximately 1–4% of the total Hcy is free in the blood (i.e., in reduced form), while the rest is bound to protein sulfur residues or exists as simple disulfides with other Hcy or Cys molecules (36). Regulation of reduced Hcy is critical, since this form is the species responsible for endothelial dysfunction in vivo (4) and for the prothrombotic effects in blood (20, 30, 37). Endothelial cells isolated from normal and CBS-deficient humans have similar rates of Hcy export (38), which suggests that the endothelium is not a source of the HHcy in these patients. Rather, the endothelium is subject to the effects of elevated Hcy delivered in the blood stream and generated by other tissues (e.g., production in liver or deficient clearance by kidneys). A tonic level of transsulfuration activity in the blood could serve to support homeostasis of the reduced form of Hcy and of plasma aminothiols (21, 36). Indeed, the low percentage of free Hcy in the blood is consistent with a highly regulated mechanism to minimize endothelial exposure to this toxic molecule.

Despite the wealth of attention to blood levels of Hcy in cardiovascular and neurocognitive disease and the growing recognition of the importance of H2S in physiology, it remains unknown whether Hcy levels and H2S production are regulated within the blood. Specifically, is there an endogenous mecha-
nism for regulating Hcy-H$_2$S metabolism in the blood, per se, and does this impact the overall health of the endothelium? Thus, we tested the following hypotheses: 1) that transsulfuration enzymes are present in blood, 2) that blood transsulfuration enzymes can produce H$_2$S, 3) that H$_2$S can protect the vascular endothelium from redox stress, and 4) that the concentration of transsulfuration enzymes in the blood is important for maintaining endothelial function when Hcy levels increase.

**MATERIALS AND METHODS**

All experimental procedures were approved by the respective human (Institutional Review Board) and animal (Institutional Animal Care and Use Committee) research committees of Idaho State University (ISU). Human plasma/serum was obtained from ISU health clinics from samples already being collected for routine tests, including demographic and medical data but stripped of personal identifiers, as exempted from written informed consent by the ISU Human Subjects Committee.

All reagents used were purchased from Fisher Scientific or Sigma-Aldrich. For all experiments in this report, the Hcy used was dl-homocysteine purchased from Sigma-Aldrich.

**Subjects/Mice**

Human plasma and serum were obtained from healthy subjects (Caucasian, nonsmokers with no clinical diagnosis of disease) at 18–71 yr of age and used for activity assays (described below) or stored at −80°C for biochemical analyses. Mice (all C57Bl/6 background) were maintained in an in-house breeding colony on a 12:12-h light-dark cycle with food and water ad libitum. CBS-deficient mice (CBS$^{-/-}$) were originally obtained as a gift from Dr. Steven Lentz, University of Iowa in 2008. Mice were bred CBS$^{-/-}$ to CBS$^{+/+}$ so that offspring provided both CBS$^{+/+}$ mice and wild-type littermate controls (CBS$^{-/-}$) and have been backcrossed at least 10 generations. The etiology of HHcy is complex and includes both genetic and dietary factors. To compare how the etiology of HHcy may differentially alter expression patterns for transsulfuration enzymes in the blood, we compared expression patterns between the CBS$^{-/-}$ model and a dietary model of high methionine, low folate as previously reported (18).

**Western Blotting**

Sample proteins were denatured in 5× Laemmli sample buffer with (reducing) or without (nonreducing) 10% β-mercaptoethanol and separated by SDS-PAGE, blotted to polyvinylidene difluoride membranes, and probed using polyclonal rabbit anti-CGL (Sigma Genosys custom antibody) or anti-CBS (H-300; Santa Cruz Biotechnology) antibodies at 1:2,000 overnight at 4°C. Immunopositive bands were quantified (Versadoc; Bio-Rad) following detection with either 2 antibody detection (Pierce) after horseradish peroxidase-conjugated secondary antibody detection (Vector Laboratories) or 2) colorimetric detection (BCIP/NBT; Invitrogen) after AP-conjugated secondary antibody detection (Vector Laboratories). In some cases, samples were cleared of IgG (ProteinA sepharose beads; Santa Cruz Biotechnology) and albumin (Affi-Gel; Bio-Rad). This was done to remove possible interference from these abundant proteins running near the same molecular weight as the proteins of interest but was found to be unnecessary and omitted in later experiments. For Western blotting of proteins in media, samples were concentrated 25-fold by membrane filtration (30 kDa mol mass cutoff; Amicon) or precipitated with 25% (wt/vol) trichloroacetic acid, then separated and probed for CBS and CGL as above.

**Transsulfuration Activity Assays**

Plasma and serum from healthy human subjects were collected into Vacutainer tubes (Becton-Dickinson) and stored at 4°C overnight.

**Assay 1.** For H$_2$S production, assay buffer was 200 μmol/l pyridoxal-5'-phosphate (PLP) cofactor and 25 μM Na$_2$S in MOPS buffer. Samples (90% assay volume) were incubated in buffer with 2 mmol/l each Hcy and Cys (Hcy + Cys) or Cys only (Cys). Control assays were buffer + Cys (Ctrl 1), buffer + Hcy + Cys (Ctrl 2), buffer + sample (Ctrl 3), or methionine as substrate (Ctrl 4). CBS condenses Hcy and Cys to form H$_2$S and can also produce H$_2$S from these substrates independently; although CGL was long thought to require Cys to form H$_2$S, it has recently been reported that CGL can also use Hcy as a substrate (5). Activity assays were run at 37°C; off-gassed H$_2$S was trapped in center-well filter discs containing zinc acetate followed by colorimetric detection as previously detailed (32).

**Assay 2.** To confirm specificity of activity of CBS and CGL enzymes, an additional set of experiments were run with the same assay conditions but using inhibitors of CBS (hydroxylamine, 2 mmol/l) or CGL (propargylglycine, 2 mM) activity.

**Assay 3.** The activity of CBS was specifically determined in a third set of assays using serine (2 mmol/l) and sodium sulfide (Na$_2$S, 2 mmol/l) and 200 μmol/l PLP as substrates dissolved in MOPS (10% final volume) and quantifying Cys production (a reaction catalyzed only by CBS) by the ninhydrin assay as described (9). Because the enzymatically produced Cys might then be acted on during the assay by the same enzymes, we also inhibited CGL activity in parallel assays (propargylglycine, 2 mmol/l, dissolved in the substrate buffer) and predicted a greater Cys yield from the assay.

**Assay 4.** A fourth set of assays was completed to test the capacity of this pathway to break down substrate (in addition to the three assays measuring product). Cys was added as substrate (2.2 mmol/l in MOPS with 200 μmol/l PLP cofactor, 10% final volume) to samples, and the concentration of remaining Cys was quantified at time points up to 2 h later using the ninhydrin assay. Controls for this set of experiments were Cys in buffer (2 mmol/l in MOPS with 200 μmol/l PLP) and vehicle alone (i.e., MOPS buffer without Cys).

**Cell Secretion of Transsulfuration Enzymes**

Primary liver cell culture. C57BL/6 mice were killed by cervical dislocation and perfused transcardially with 2% BSA in cold PBS containing heparin and sodium nitroprusside. Livers were removed, minced with scissors, rinsed with cold PBS four times, and rocked on ice with 0.05% collagenase type 4 in PBS for 20 min. The resulting slurry was poured through a 40-μm cell strainer (BD Biosciences). Cells were then washed three times in cold PBS with centrifugation (300 g, 5 min) between washes. The final pellet was resuspended in serum-free medium 199, and cells were plated into 25-cm$^2$ flasks in 4 ml for a final 10$^5$ cells per flask and incubated at 37°C in a humidified incubator with 5% CO$_2$-balance air. Cell viability was quantified by trypan blue exclusion. Conditioned media was collected at the indicated times by withdrawing 600 μl, centrifugation at 17,000 g for 15 min at 4°C, and storage of 500 μl supernatant at −20°C for analysis.

**Endothelial cells.** The mouse microvascular endothelial cell line, bEnd.3 (24), was grown and maintained in DMEM plus 10% FBS at 37°C in 5% CO$_2$-balance air. Upon reaching confluence, media was changed to 5% FBS and then serum-free DMEM at 24-h intervals. Cells were washed four times with serum-free media followed by incubation in serum-free media for 1, 20, or 40 h. Media was removed and centrifuged at 5,000 g for 15 min to remove any contaminating cells or debris (although none was noted on microscopy of the media); the upper 75% of the supernatant was removed and centrifuged again; and the upper 75% of this supernatant was removed and used for detection of secreted CBS and CGL as described in Western Blotting.

**Endothelial Cell Culture and Stress Responses**

Human umbilical vein endothelial cells (HUVECs; from ATCC, Manassas, VA) were grown to confluence in 24-well or 96-well plates in endothelial growth medium (endothelial basal medium-2 with growth factor bullet kit;Lonza). Cells were maintained in a humid-
fied incubator at 37°C throughout experiments (Forma Series 2). At confluence, media was changed to endothelial basal medium-2 plus 10% FCS. Four days later, cells were given one of two treatments: 1) serum starved (endothelial growth medium-2 without serum) for 24 h or 2) subjected to 5% CO₂-0.5% O₂-balance N₂ for 18 h followed by 5% CO₂-balance air for 6 h. H₂S was added to the media in a bolus of 10% final media volume after dissolving Na₂S to the desired molar concentration. Treatment was at time 0 for the serum starvation condition and at the onset of reoxygenation for the hypoxia/reoxygenation condition.

**Cell viability.** Cell viability was quantified by MTT assay over the final 2 h of each experiment using untreated cells as controls.

**DNA damage.** DNA damage was quantified by ELISA for 8-hydroxydeoxyguanosine (8-OHdG) using a kit from Oxis International (Foster City, CA). 8-OHdG is produced during the repair of DNA following damage.

**Whole Cell ELISA for 3-Nitrotyrosine**

Cells were fixed in fresh 4% paraformaldehyde (in PBS, pH 7.4) at 4°C for 1 h and then permeabilized and blocked with 1% Triton-1% BSA (in PBS, pH 7.4). Immunolabeling was as follows: rabbit anti-3-nitrotyrosine (3-NT, 1:1,000, A21285; Invitrogen) overnight at 4°C, 5 × 10 min washes with PBS, secondary anti-rabbit (1:1,000, AP-1000; Vector Laboratories) alkaline phosphatase conjugate at room temperature, repeat washes, and colorimetric detection (Blue Phos; KPL, Gaithersburg, MD).

**Cell Monolayer Permeability**

HUVECs were seeded at confluence in ThinCert inserts (Greiner BioOne) coated with 0.1% gelatin. Later (2 days), cells were treated as described for respective experiments along with 1% sodium fluorescein in the upper chamber. Media (50 µl) was removed from the lower chamber each hour and diluted 10-fold, and fluorescence was quantified with a plate reader (excitation 485/20, emission 528/20, Synergy HT; BioTek). Background fluorescence of media alone was subtracted from each reading before graphing and statistical comparisons.

**Statistics**

All experiments were conducted at least in quadruplicate; higher n are indicated with respective experiments. Linear regression was used to evaluate trend data, and groupwise data were compared by ANOVA with Tukey post hoc comparisons. Alpha was set at 0.05 for statistical significance.

**RESULTS**

**Transsulfuration Enzymes are Present in Plasma and Serum**

By denaturing SDS-PAGE, CBS was detected at two sizes, as shown in Fig. 1, at full length (~63 kDa) and possibly the highly active, evolutionarily conserved catalytic core (~45 kDa) (15), although a second band near this size has previously been considered nonspecific using a different antibody (29). When tertiary and quaternary structure were maintained using nonreducing conditions, both CBS and CGL were found predominantly in oligomeric form, consistent with reports in other tissues (Fig. 1D). There were no differences in the concentrations of CBS or CGL between plasma and serum, demonstrating the potential for future studies or diagnostics from either fraction.

**Blood Transsulfuration Enzyme Activity**

Figure 2A illustrates the discovery that Hcy and Cys are substrates for the production of H₂S in the blood. The production of H₂S was catalyzed by CBS and CGL in blood since H₂S was not produced by substrates in buffer alone (Ctrl 1 and 2). Moreover, H₂S was not released in control conditions since blood samples alone (in reaction buffer) did not off-gas detectable amounts of H₂S (Ctrl 3). Methionine is the precursor to Hcy production, but the enzymes for this conversion are not thought to occur in the blood (methionine adenosyltransferase and adenosylhomocysteinase). Ctrl 4 used methionine as substrate and showed no appreciable H₂S production, which is consistent with the presence of transsulfuration alone, i.e., the ability to metabolize Hcy but not the capacity to produce Hcy de novo from methionine. Figure 2B demonstrates that inhibiting CBS reduced H₂S production by ~27% (P < 0.05) while
inhibiting both CBS and CGL reduced $H_2S$ production by $\sim 53\%$ ($P < 0.05$). To specifically test the activity of CBS, we used an assay to test the unique ability of CBS to produce Cys from serine and $Na_2S$. Figure 2C shows significant production of Cys from these substrates; moreover, inhibiting CGL from its ability to subsequently catabolize Cys significantly increased the amount of Cys detected at the end of the 2-h assay period. The inference from this experiment is that Cys was actively consumed as it was being made. To test this specifically, we provided Cys as the only substrate and quantified its metabolism. Figure 2D shows that a significant amount of added Cys was rapidly removed (3-min time point; Fig. 2D), which we attribute primarily to production of Cys disulfides (e.g., $2 \text{Cys} \rightarrow \text{Cys, Cys } + \text{albumin, etc.}$), which are not detected by this assay. Over the course of the next 2 h, Cys was metabolized so that the values were not different from serum alone by the end of the 2-h assay. Control assays show no change in Cys concentration over the 2-h period, demonstrating that Cys is not spontaneously degraded or otherwise converted to an undetectable form.

$H_2S$ can Protect the Vascular Endothelium from Redox Stress

Exogenous $H_2S$ following serum starvation and hypoxia/reoxygenation conferred significant protection as shown by improved cell viability (MTT) and reduced DNA damage (8-OHdG) (Fig. 3).

The Concentration of CBS

The concentration of CBS in blood of male and female subjects (Fig. 4, A and B) was more variable than that of CGL; specifically, the standard deviation of the Western blot band densities was $18.9\%$ for CBS and only $0.3\%$ for CGL while the ratio of the variance of CBS to variance of CGL was $4,541$.

Serum was immunoprecipitated to remove vascular cell adhesion molecule [VCAM; pseudo-immunoprecipitated (IP)] condition as a nonspecific control or for CBS and CGL enzymes from the serum, followed by 10-fold dilution in media and overnight (15 h) incubation with Hcy (200 $\mu$M) to allow enzymatic metabolism of Hcy. The next morning, this solution was diluted another fivefold (50-fold final dilution of serum) and incubated on confluent HUVECs for 24 h. Hence, conditions were untreated cells (control), cells treated with Hcy that had been incubated overnight with serum with CBS and CGL removed (IP; little capacity to metabolize the Hcy), and cells treated with Hcy that had been incubated overnight with serum containing CBS and CGL (pseudo-IP; intact capacity to metabolize Hcy). In the first set of experiments, cells were lysed and Western blot was performed for iNOS and claudin-5 expression, which are markers of cell stress and intercellular adhesion, respectively. Control cells showed little or no iNOS expression and high claudin-5 expression; pseudo-IP resulted in a small but detectable iNOS expression with reduced clau-
demonstrated significant nitrosylation when transsulfuration enzymes were removed (IP) but not under control or pseudo-IP conditions (Fig. 5B). In the third set of experiments, HUVEC monolayer permeability was tested (Fig. 5D). Removal of transsulfuration enzymes by IP resulted in significantly greater monolayer permeability when challenged with Hcy compared with a much smaller change in permeability (compared with a control monolayer) when the enzymes were able to metabolize the Hcy (pseudo-IP). Thus, extracellular transsulfuration capacity was a critical and significant mechanism responsible for protecting endothelium from Hcy treatment as evidenced by protection from the induction of iNOS expression and protein nitrosylation as well as protection from the loss of claudin-5 expression and monolayer permeability.

**Endothelial Cells and Hepatocytes Secrete Transsulfuration Enzymes**

Mouse microvascular endothelial cells secreted both CBS and CGL into culture media over the 40-h experiment period (Fig. 6). Primary mouse hepatocytes secreted both CBS and CGL into culture media over the 40-h experiment period (Fig. 6). For both endothelial and liver cells, this increase was due to cell secretion rather than cell death since cell viability remained 97% throughout these experiments (MTT assay).

**Role of Etiology of HHcy in Blood Transsulfuration Enzymes**

Two etiologies of HHcy were compared for their effects on expression of blood transsulfuration enzymes: high-methionine and low-folate diet or genetic deficiency in CBS (CBS+/−/−). Expression of CBS increased under dietary HHcy but decreased in CBS+/−/− while expression of CGL changed in the opposite direction, with decrease in dietary HHcy and increase in CBS+/− (Fig. 7).

**DISCUSSION**

The first major discovery of these studies is that the transsulfuration enzymes, CBS and CGL, are active in human
blood, where they produce H$_2$S from Hcy and Cys (Figs. 1 and 2). Second, we demonstrate that a poorly understood product of transsulfuration, H$_2$S, protects endothelium from redox stress (Fig. 3). Third, extracellular transsulfuration protects endothelium from deleterious effects of extracellular Hcy treatment, suggesting that blood transsulfuration may be involved in protecting the endothelium from increases in circulating Hcy concentration (Fig. 5). Fourth, the endothelium itself, along with the liver, secrete the transsulfuration enzymes into their environment.

CBS was found at two sizes in human plasma (Fig. 1), the smaller of which is consistent with an evolutionarily conserved, highly catalytic core (15). However, from mouse liver cells, CBS was secreted only in the full-length form. It is possible that human liver secretes both forms in vivo, either

Fig. 5. Effect of CBS concentration in media bathing endothelial cells during Hcy treatment. CBS and CGL enzymes were removed from human serum by immunoprecipitation (IP); serum was then incubated with 200 μM Hcy for 15 h at 37°C and used to spike HUVEC media (10% final volume). As controls, pseudo-IP was performed using an antibody to vascular cell adhesion molecule (VCAM) or cells were left untreated (Control = CTRL). A: iNOS expression is induced by Hcy treatment, and this is exacerbated when transulfuration capacity is diminished. *P < 0.05 for all pairwise comparisons. B: protein nitrosylation (3-nitrotyrosine; 3-NT) is increased by Hcy treatment but rescued when extracellular transsulfuration activity is intact. *P < 0.05 compared with control and VCAM. C: claudin-5 expression is reduced by Hcy treatment and is further reduced when transsulfuration capacity is diminished. *P < 0.05 for all pairwise comparisons. D: consistent with the barrier properties of claudin-5, monolayer permeability is significantly greater when cells are treated with Hcy-spiked media void of transsulfuration enzymes compared with the VCAM-IP (pseudo-IP) control condition where transsulfuration enzymes were active and able to reduce the Hcy burden. P < 0.05 compared with control (*) and compared with control and VCAM (#). A-D: n = 4/group.

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Fig. 6. Secretion of CBS and CGL enzymes by liver and endothelial cells. A: representative Western blots of conditioned media proteins from mouse hepatocytes or endothelial cells incubated for 1, 20, or 40 h. B: optical density of bands shown in A. P < 0.05 compared with 1 h (*) and for all pairwise comparisons (#).
constitutively or in response to changing stimuli. Alternatively, the protein may be cleaved to its shorter form following release from liver cells. Tumor necrosis factor-α has this capacity (44), which may play a role in coupling inflammatory reactions with H2S production. Endothelial cells secreted both enzymes as well; in this case, CBS was secreted at both sizes (Fig. 6). As the barrier between blood and tissue, constantly bathed by the blood, endothelium may reduce endogenous exposure to free Hcy by maintaining a level of transsulfuration capacity in the blood. Protection of endothelium by extracellular transsulfuration during a Hcy challenge is supported by the data presented in Fig. 5. Neither CBS nor CGL contain an NH2-terminus sequence that targets them for secretion (a “classical” secretory pattern). However, many proteins are secreted without such a sequence, i.e., in a nonclassical fashion. SecretomeP is a program that predicts nonclassically secreted proteins (2), where a score greater than 0.5 indicates a likelihood of secretion. For example, interleukin-1 is a well-known nonclassically secreted protein and scores 0.61. CBS and CGL score 0.65 and 0.64, respectively. The Human Plasma Protein Project database documents the presence of amino acid sequences that are consistent with CBS and CGL in human plasma. Our data now demonstrate that active CBS and CGL are part of the nonclassical secretome. Although these findings demonstrate release of CBS and CGL, they cannot discern whether there was active synthesis and secretion during the study period or whether the enzymes secreted were from an intracellular pool already present at the start of the experiments. Specific studies using labeled methionine would be needed to make this discrimination.

Clinical interventions for HHcy have focused on dietary supplementation with the cofactors of remethylation, folic acid and B12, or transsulfuration, B6. While there remains much debate over the proper study design, time frame, outcome measures, and data interpretation, these trials have shown minimal success in modulating risk or progression of disease. Because Hcy is a toxic, proinflammatory, and prothrombotic molecule, alternative approaches and a deeper understanding of therapeutic options are needed. In this regard, it is important to note that two of the large-scale clinical trials concerned with lowering Hcy levels with vitamin therapy have included groups that did not experience a lowering of plasma Hcy levels. Because CBS incorporates B6 into its structure as it is made, it may not be surprising that additional B6 would fail to enhance activity of this pathway. Nevertheless, this demonstrates that these trials failed to enhance flux through transsulfuration. Lowering of Hcy levels with folate and B12 demonstrates that the folate and remethylation cycles were activated. We propose that increasing transsulfuration activity has been untested in the context of remediating the damaging effects of HHcy. The present data are consistent with the idea that this pathway is important for protecting the vascular endothelium from both oxidative stress (serum starvation and hypoxia-reoxygenation; Fig. 3) and the direct effects of Hcy (Fig. 5). Whether this is a direct effect of H2S, per se, or modulation of protein sulfhydration balance remains to be determined. To specifically test whether the endothelium is more vulnerable to Hcy...
when extracellular transsulfuration capacity is diminished, CBS and CGL were immunoprecipitated from serum before incubation with Hcy. When transsulfuration capacity was intact (pseudo-IP using VCAM antibody), serum incubated overnight with Hcy was only mildly stressful to endothelium as demonstrated by a slight increase in iNOS expression, no change in protein nitrosylation, a smaller decrease in claudin-5 expression, and a smaller change in monolayer permeability. This finding is consistent with Fig. 2 in demonstrating that serum transsulfuration reduces Hcy load and extends this idea to show beneficial outcomes of extracellular transsulfuration on endothelial health and function. However, when transsulfuration capacity was diminished by immunoprecipitation (CBS + CGL-IP), iNOS expression increased concomitant with protein nitrosylation while claudin-5 expression declined concomitant with increased monolayer permeability. These data suggest that a robust transsulfuration capacity in the blood can protect the endothelium from a variety of stress environments and cellular responses, and these findings document a critical role of extracellular transsulfuration at the blood-endothelial interface.

Given that HHcy may result from both genetic and dietary causes, we explored how two common models of HHcy affect expression of transsulfurating enzymes in blood. Figure 7 illustrates the findings that both CBS and CGL expression are significantly altered by HHcy regardless of the etiology. However, the effects are opposite for both enzymes: dietary HHcy increased CBS expression while decreasing CGL expression; genetic deficiency of the CBS enzyme decreased expression of CBS while increasing CGL expression. These findings underscore the need for more focused approaches to understanding the complexity of HHcy-related diseases based on etiology and not simply the concentration of Hcy per se. Of particular importance is a better understanding of the role of dysfunction in H2S metabolism in HHcy.

Some of the major findings of this study are summarized in Fig. 8. Collectively, our findings demonstrate that concentrations of free Hcy can be regulated directly in the blood and that this balance is intimately tied with the production of H2S and endothelial protection via the transsulfuration pathway. Specifically, we have shown 1) that transsulfurating enzymes are present in blood, 2) that blood transsulfurating enzymes can produce H2S, 3) that H2S can protect the vascular endothelium from redox stress, and 4) that functional extracellular transsulfuration at the blood-endothelial interface is important for maintaining endothelial function when Hcy levels increase. We further show that the etiology of HHcy can have very different effects on expression of transsulfuration enzymes in this compartment. Measurement and modulation of this pathway may provide innovative opportunities for improving human health. For example, clinical interventions aimed at modulating Hcy metabolism and H2S production through transsulfuration activity may provide a convenient and well-tolerated approach. In addition, the ease of blood collection presents the possibility of these enzymes as diagnostic markers. Much work remains to be done though we anticipate that a better understanding of the regulation of CBS and CGL activity in the blood may generate new weapons in the arsenal to combat Hcy-related diseases such as atherosclerosis, thrombosis, and dementia.

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**DISCLOSURES**

None

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