ANTIOXIDANTS & REDOX SIGNALING
Volume 28, Number 4, 2018

DOI: 10.1089/ars.2017.7009

Discoveries

ORIGINAL RESEARCH COMMUNICATION

Biogenesis of Hydrogen Sulfide and Thioethers by Cystathionine Beta-Synthase

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Abstract

Aims: The transsulfuration pathway enzymes cystathionine beta-synthase (CBS) and cystathionine gamma-lyase are thought to be the major source of hydrogen sulfide (H_2S). In this study, we assessed the role of CBS in H_2S biogenesis.
Results: We show that despite discouraging enzyme kinetics of alternative H_2S-producing reactions utilizing cysteine compared with the canonical condensation of serine and homocysteine, our simulations of substrate competitions at biologically relevant conditions suggest that cysteine is able to partially compete with serine on CBS, thus leading to generation of appreciable amounts of H_2S. The leading H_2S-producing reaction is condensation of cysteine with homocysteine, while cysteine desulfuration plays a dominant role when cysteine is more abundant than serine and homocysteine is limited. We found that the serine-to-cysteine ratio is the main determinant of CBS H_2S productivity. Abundance of cysteine over serine, for example, in plasma, allowed for up to 43% of CBS activity being responsible for H_2S production, while excess of serine typical for intracellular levels effectively limited such activity to less than 1.5%. CBS also produced lanthionine from serine and cysteine and a third of lanthionine coming from condensation of two cysteines contributed to the H_2S pool.
Innovation: Our study characterizes the H_2S-producing potential of CBS under biologically relevant conditions and highlights the serine-to-cysteine ratio as the main determinant of H_2S production by CBS *in vivo*.
Conclusion: Our data clarify the function of CBS in H_2S biogenesis and the role of thioethers as surrogate H_2S markers. *Antioxid. Redox Signal.* 28, 311–323.

Keywords: enzyme kinetics, alternative reactions, homocystinuria, animal model, sulfur metabolism

Introduction

CYSTATHIONINE BETA-SYNTHASE (CBS) (EC 4.2.1.22) plays a crucial role in cysteine biosynthesis from the essential amino acid methionine *via* cystathionine (Cth) in the transsulfuration pathway (35). Cystathionine is generated by the CBS-catalyzed condensation of serine (Ser) and homocysteine (Hcy) and is then cleaved by cystathionine gamma-lyase (CGL) to yield cysteine (Cys). CBS is a member of the large β-family of pyridoxal-5′-phosphate (PLP)-dependent enzymes and its catalytic core has a high degree of sequence and structural conservation with cysteine synthases and O-acetylserine(thiol)lyases, the CBS orthologs in prokaryotes, plants, and nematodes (17, 39, 52, 57). CBS catalyzes primarily β-replacement reactions (Fig. 1) (4, 38). Despite catalyzing mostly one reaction type, CBS can utilize a wide range of substrates. The substrate specificity of CBS was originally investigated by Braunstein *et al.* (5). They demonstrated that in addition to cystathionine formation from serine and homocysteine (canonical activity), partially purified rat liver CBS catalyzed the production of thioethers from serine and substituted analogs of serine, cysteine, and S-alkyl derivatives of cysteine upon their incubation with a variety of

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Innovation

We have developed sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) and high performance liquid chromatography (HPLC) methods to simultaneously measure formation of thioethers and hydrogen sulfide (H_2S) from mixtures of cystathionine beta-synthase (CBS) substrates. In the presence of physiologically relevant concentrations of serine, cysteine, and homocysteine, the serine-to-cysteine ratio represents the major factor determining H_2S-producing capacity of CBS. At low serine concentration, up to ~30–40% of cystathionine and lanthionine originates from homocysteine+cysteine and cysteine+cysteine condensation, respectively, thus contributing significantly to H_2S production. In contrast, higher serine concentrations typical for tissues favored thioether synthesis without production of H_2S, thus advocating caution in using thioethers as surrogate markers of H_2S production.

sulfhydryl compounds, such as β-mercaptoethanol and cysteamine (Fig. 1). They and others showed that CBS is also capable of H_2S production *in vitro* by both cysteine desulfuration (β-elimination reaction) and its condensation with a number of other sulfhydryl compounds (β-replacement reactions) (7, 42, 58, 59).

H_2S used to be considered a toxic gas until the discovery that it regulates a number of physiological processes (16). Moreover, H_2S has been implicated in various diseases ranging from hypertension, atherosclerosis through lipid metabolism, diabetes, and inflammation to neuromodulation (53, 60). Despite its importance, there is still no consensus on the physiological levels of H_2S in blood and/or tissues or on the most reliable technique for measuring H_2S in biological samples. In contrast to the initial studies reporting up to 300 μM H_2S in circulation (37), the current conservative estimate of the physiological H_2S levels in circulation is in the higher nanomolar to lower micromolar range (60). Although it has been demonstrated that the main source of H_2S in mammals is cysteine (23, 50, 55), the identity of the enzyme(s) responsible for H_2S biogenesis from this amino acid has not been fully ascertained. Unlike the remaining gaseous signaling molecules CO and NO, whose synthesis is known to be highly regulated, a number of housekeeping enzymes, apart from CBS, could utilize cysteine as a substrate to yield H_2S: CGL, cysteine aminotransferase coupled to mercaptopyruvate sulfurtransferase (9, 50), and cysteine lyase (5, 6). Nonenzymatic pathways leading to H_2S production have been considered as well (9). Several laboratories investigated the capacity of different tissues to produce H_2S upon incubation with cysteine (40, 50). Although results were obtained with nonphysiologically high concentrations of substrate, CBS and CGL seem to be the major catalysts of

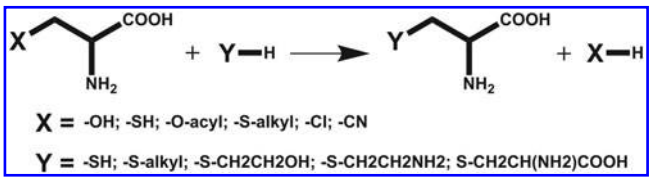

FIG. 1. Generic reaction catalyzed by CBS. CBS, cystathionine beta-synthase.

H_2S formation from cysteine in rat liver and kidney tissue extracts. More recently, Kruger's and Banerjee's groups have obtained results giving further evidence that CBS is involved in the production of H_2S (11, 26). As accurate determination of H_2S is notorious for complexity and inaccuracy due to its volatility, reactivity, and susceptibility to oxidation (18), thioethers lanthionine (Lth) and homolanthionine (Hlth) have emerged as surrogate markers of H_2S synthesis by CBS and CGL, respectively (12, 16, 29, 47).

The goal of this study was to characterize the canonical and alternative CBS reactions kinetically and to evaluate the role of CBS in the formation of H_2S. We studied reactions utilizing substrates that may be of physiological relevance. In addition to using saturating concentrations of substrates, we evaluated the H_2S-producing activity of CBS using physiological levels as well. Moreover, we determined and compared the CBS efficacy to generate H_2S using cysteine at varying concentrations of the competing canonical CBS substrate, serine. Furthermore, we correlated our *in vitro* and *in situ* results with *in vivo* assessment of thioethers as surrogate markers of H_2S formation in wild-type monkeys and homocystinuric CBS knockout (KO) mice.

Results

CBS enzyme kinetics

We determined the kinetic parameters of several different reactions catalyzed by CBS. The first reaction was the canonical one condensing Hcy and Ser to yield Cth and water. The second one uses Cys in place of Ser to give Cth and H_2S. The third reaction catalyzes the formation of H_2S by either desulfuration of Cys or beta-replacement of Cys with another molecule of Cys. We have determined the sum of these two reactions since we measured H_2S, which is one of the two products of each of these reactions. Finally, in the fourth reaction, we examined the possibility of CBS to utilize H_2S instead of its formation by condensing Ser with H_2S to yield Cys and water. Kinetic parameters were determined in the absence and the presence of S-adenosylmethionine (AdoMet), an allosteric activator of CBS. Table 1 summarizes the CBS enzyme kinetics. First, the presence of AdoMet does not significantly affect the affinity of the enzyme for its substrates. Instead, it increases V_{max} of the reaction, thus confirming the previous observations that AdoMet is a V-type allosteric activator of CBS (49). Interestingly, the H_2S production from Cys alone seems to be significantly less sensitive to AdoMet stimulation than any other reaction catalyzed by CBS. Second, it is clear that CBS has higher affinity for Ser than for Cys. While the k_m for Ser was around 1.5 mM in both the canonical and H_2S-consuming alternative reactions, the affinity of the enzyme for Cys was ~6- and ~10-fold lower for H_2S production from Cys alone and the condensation of Cys with Hcy, respectively. Third, the highest reaction velocity (V_{max}) was obtained for condensation of Cys with Hcy, which was roughly twofold higher compared with the canonical reaction. However, taking into account the affinity of CBS for the respective substrates, it is quite clear that the enzyme's catalytic efficiency (*i.e.*, k_{cat}/K_m) in the canonical reaction is the highest of all the studied reactions: ~2 to 5-fold compared with the condensation of Cys and Hcy, ~15 to 100-times compared with the H_2S production from Cys alone, and ~10 to 200-fold when related to Cys production from Ser and H_2S. Thus, these results suggest

Table 1. Kinetic Parameters Determined for Canonical and Alternative Reactions Catalyzed by Human Cystathionine Beta-Synthase

L-serine + L-homocysteine → L-cystathionine + H_2O[a]				
	L-Ser	*L-Ser + AdoMet*	*L-Hcy*	*L-Hcy + AdoMet*
V_{max} (μmol.mg^{-1}.h^{-1})	218 ± 40	823 ± 58	277 ± 40	756 ± 75
K_m (m*M*)	1.41 ± 0.35	2.13 ± 0.98	1.04 ± 0.24	1.00 ± 0.06
k_{cat} (s^{-1})	3.67 ± 0.67	14.01 ± 0.80	4.66 ± 0.67	12.70 ± 1.25
k_{cat}/K_m (mM^{-1}.s^{-1})	2.63 ± 0.15	7.61 ± 2.84	4.61 ± 0.94	12.74 ± 1.22
L-cysteine + L-homocysteine → L-cystathionine + H_2S				
	L-Cys	*L-Cys + AdoMet*	*L-Hcy*	*L-Hcy + AdoMet*
V_{max} (μmol.mg^{-1}.h^{-1})	415.9 ± 11.8	1839.2 ± 78.4	479.2 ± 12.9	1571.4 ± 27.8
K_m (m*M*)	13.41 ± 0.03	15.92 ± 1.47	4.31 ± 0.05	4.48 ± 0.06
k_{cat} (s^{-1})	7.09 ± 0.20	31.34 ± 1.34	8.17 ± 0.22	26.78 ± 0.47
k_{cat}/K_m (mM^{-1}.s^{-1})	0.53 ± 0.02	1.98 ± 0.10	1.93 ± 0.09	6.02 ± 0.14
L-cysteine + H_2O → L-serine + H_2S (and/or: L-cysteine + L-cysteine → L-lanthionine + H_2S)				
		L-Cys		*L-Cys + AdoMet*
V_{max} (μmol.mg^{-1}.h^{-1})		62.5 ± 0.3		82.8 ± 0.6
K_m (m*M*)		8.11 ± 0.48		8.41 ± 0.82
k_{cat} (s^{-1})		1.06 ± 0.01		1.41 ± 0.01
k_{cat}/K_m (mM^{-1}.s^{-1})		0.13 ± 0.01		0.18 ± 0.01
L-serine + H_2S → L-cysteine + H_2O				
	L-Ser	*L-Ser + AdoMet*	*H_2S*	*H_2S + AdoMet*
V_{max} (μmol.mg^{-1}.h^{-1})	19.9 ± 10.6	41.0 ± 7.9	20.6 ± 9.7	46.1 ± 10.7
K_m (m*M*)	1.27 ± 0.23	1.32 ± 0.33	5.02 ± 0.25	4.70 ± 0.07
k_{cat} (s^{-1})	0.39 ± 0.18	0.62 ± 0.13	0.35 ± 0.16	0.78 ± 0.18
k_{cat}/K_m (mM^{-1}.s^{-1})	0.28 ± 0.09	0.47 ± 0.08	0.07 ± 0.03	0.17 ± 0.04

[a]Data for the canonical reaction were published previously (17). All values are the average of three independent determinations ± standard deviations.

H_2S, hydrogen sulfide.

that CBS is indeed capable of alternative reactivity *in vitro*, particularly the H_2S production from Cys and Hcy. At the same time, CBS appears to be better poised to utilize substrates in the canonical, that is, non-H_2S-yielding, reaction.

H_2S-forming activity of WT and KO mouse livers

Next, we examined the ability of crude mouse liver extracts to catalyze H_2S formation from either Cys+Hcy or Cys in the absence and presence of AdoMet and different inhibitors of PLP-dependent enzymes (Fig. 2). We utilized livers from wild-type (WT) mice as well as from CBS-knockout (KO) mice completely lacking CBS activity (62). Comparison of WT and KO activities demonstrates that CBS is responsible for most of the H_2S-forming activity in the liver. However, CBS H_2S-producing activity depends on the presence of both substrates Cys and Hcy (Fig. 2A) as the use of Cys as a sole substrate (Fig. 2B) resulted in very low and essentially identical H_2S formation by WT and KO liver extracts, thus pointing to the role of CGL in H_2S biogenesis from Cys. When the two extracts were assayed in the presence of 1 m*M* propargylglycine (PAG), a potent and selective inhibitor of CGL (3), the activity of the WT extract was reduced by about 20% (0.715 ± 0.027 and 0.570 ± 0.022 U/mg of protein of the WT extract in the absence and presence of 1 m*M* PAG, respectively), while the activity of the KO liver extract was nearly obliterated. The sum of the WT+PAG and KO activities adds up to the WT activity suggesting that the WT+PAG value represents CBS contribution, while the KO value corresponds to the CGL contribution to H_2S formation. This result is further supported by a clearly detectable response to AdoMet in WT extract, but not in the KO extract. Interestingly, no response to AdoMet was detected when the liver extracts were assayed in the presence of Cys alone corroborating a similar phenomenon observed in CBS kinetics and further implicating the CGL role in H_2S formation (Table 1). When the extracts were assayed in the presence of 1 m*M* aminooxyacetic acid (AOAA), a potent inhibitor of PLP-dependent enzymes, the H_2S-forming activity of the extracts was approaching zero. The interpretation of these results is that CBS accounts for the bulk of the H_2S-forming activity of a mouse liver extract when examined *in vitro* and that the presence of a second thiol (Hcy under physiological conditions) is crucial for H_2S biogenesis by CBS from cysteine.

Competition of serine and cysteine on CBS

Since Ser and Cys do not occur in cells in isolation, we examined condensation of Ser+Hcy and Cys+Hcy in the

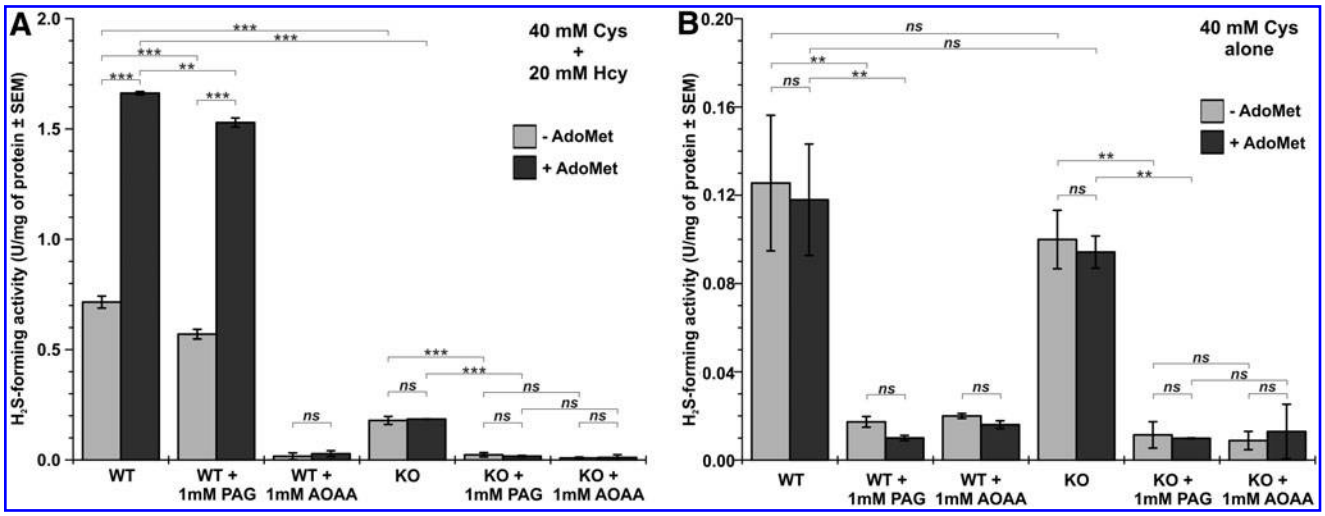

FIG. 2. H_2S-forming activity of WT and CBS KO mouse liver extract. Production of H_2S was determined in the presence of 40 m*M* Cys and 20 m*M* Hcy **(A)** or 40 m*M* Cys as a sole substrate **(B)** The absence or presence of AdoMet, a CBS allosteric activator, is represented by *light* or *dark gray color*, respectively. Liver homogenates were assayed in the absence or presence of 1 m*M* PAG, a potent and selective cystathionine gamma-lyase inhibitor, or 1 m*M* AOAA, a generic inhibitor of pyridoxal-5′-phosphate-dependent enzymes. Error bars indicate SEMs from four independent measurements. Please note the difference in scales of y-axes between **(A)** and **(B)**. Significance is designated by *asterisks* as $^{**}p < 0.01$, and $^{***}p < 0.001$ with ns being non-significant. AOAA, aminooxyacetic acid; H_2S, hydrogen sulfide; KO, knockout; PAG, propargylglycine; SEM, standard error of the mean; WT, wild-type.

presence of a competing substrate, that is, Cys and Ser, respectively. First, we examined the impact of increasing Ser concentrations on H_2S formation from Cys+Hcy in the presence and absence of AdoMet. The experiment was carried out at fixed concentrations of Cys and Hcy of 40 and 20 m*M*, respectively, to reach and maintain the most favorable (V_{max}) conditions for H_2S formation, while avoiding possible substrate inhibition (49). As can be seen in Figure 3A, gradually increasing Ser concentration leads to an exponential-like decrease in H_2S production due to substrate (Ser)-induced inhibition. Thus, at ~13.6 and 3.2 m*M* Ser in the absence and the presence of AdoMet, respectively, H_2S production was down to 50% of its initial value, and at equimolar concentration of competing substrates (40 m*M*), the H_2S formation was nearly eliminated. In the complementary experiment, we looked at the impact of increasing Cys concentrations on the canonical reaction. Again, we used the most favorable substrate concentrations to reach the highest activity as dictated by reaction kinetic parameters: 10 m*M* each of Ser and Hcy. Figure 3B shows that gradually increasing Cys concentration resulted in a very small linear decrease in formation of [^{14}C]-labeled Cth from Ser. Both linear and nonlinear fittings showed a systemic

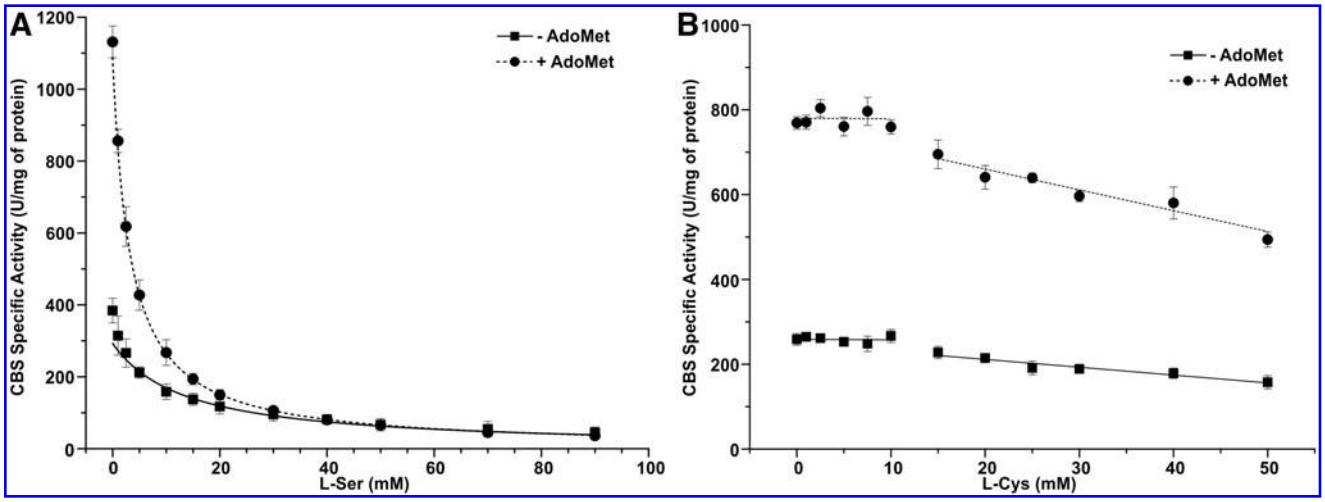

FIG. 3. Substrate competition in CBS-catalyzed reactions under optimal V_{max} conditions. (A) Exponential-like decay in an alternative H_2S-forming CBS condensation of Cys+Hcy in the presence of increasing Ser concentration. **(B)** No impact of added Cys up to 10 m*M* equimolar concentration on the canonical condensation of Ser+Hcy, followed by a slow linear decrease of Cth formation up to 50 m*M* Cys concentration. Competitions were performed in the absence (*squares*, *solid line*) and the presence (*circles*, *dashed line*) of CBS allosteric activator AdoMet. Points represent an average value from three independent determinations and error bars indicate SEMs.

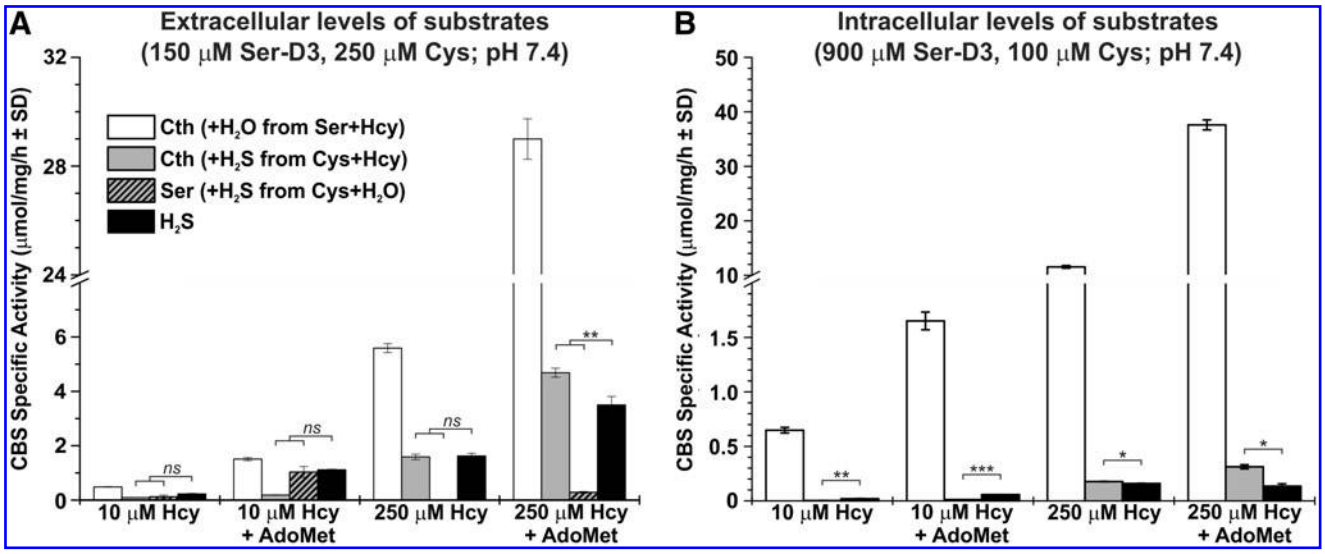

FIG. 4. Substrate competition on CBS under physiologically relevant conditions. CBS specific activities determined at the physiological pH 7.4 when simulating **(A)** the extracellular levels of substrates (150 μM Ser-D3, 250 μM Cys) and **(B)** the intracellular levels of substrates (900 μM Ser-D3, 100 μM Cys). The assays were performed in the absence or presence of the 500 μM allosteric activator AdoMet as CBS allosteric activator and contained either 10 or 250 μM Hcy to mimic physiological and homocystinuric conditions. Data on Lth production from Ser+Cys and Cys+Cys are not shown due to very low specific activities (for details, see Supplementary Table S1). Error bars indicate SEMs from three separate measurements. *Solid* and *hatched gray columns* indicated H_2S production inferred from synthesis of cystathionine or serine, while *solid black columns* show H_2S levels determined by the monobromobimane method. Significance is designated by *asterisks* as $*p<0.05$, $**p<0.01$, and $***p<0.001$ with ns being non-significant.

deviation of the model from the data. Proper fitting was only achieved after splitting the data set into two groups: up to the 10 mM equimolar concentration of the competing substrates and above, that is, 15–50 mM Cys. Figure 3B shows that up to 10 mM cysteine, there was no detectable inhibition of cystathionine formation from Ser. Only at 15 mM Cys, we started to see a gradual linear inhibition, which even at 50 mM Cys did not exceed 40% of the initial CBS activity.

Since the experiments shown in Figure 3 were carried out at supraphysiological substrate concentrations, we verified our observation under physiologically relevant conditions. We decided to simulate both the extracellular and intracellular substrate concentrations as they vary greatly (41, 54) under normal conditions and in genetic defects with Hcy accumulation (homocystinurias). Thus, we determined simultaneous production of thioethers, Ser, and H_2S by CBS at the physiological pH 7.4 in the absence and presence of AdoMet using 150 μM Ser and 250 μM Cys for extracellular levels or 900 μM Ser and 100 μM Cys for intracellular concentrations of substrates with 10 and 250 μM Hcy mimicking normal and homocystinuric conditions (Fig. 4, Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/ars). In this set of experiments, we used deuterated Ser to differentiate whether the thioethers Cth and Lth originated from either Ser or Cys. This approach, in turn, allowed us to correlate anticipated and experimentally determined H_2S production. The results show that the production of H_2S by CBS substantially depends on the concentration ratio between Ser and Cys. Figure 4A illustrates that extracellular levels of Ser and Cys allow for significant H_2S production: 14.6–43.0% of total CBS activity (see Supplementary Table S1 for details). Interestingly, this activity was more pronounced under normal rather than homocystinuric conditions. In addition, H_2S production from Cys desulfuration to Ser was only detected during the simulation of normal extracellular conditions. On the other hand, Figure 4B shows that H_2S-generating CBS activity under intracellular concentrations of substrates was substantially diminished to 0.4–1.5% of total CBS activity. The production of Lth from either Ser+Cys or Cys+Cys represents the less favored reaction of the enzyme with only $\sim$3% and $\sim$0.2% of total CBS activity under normal and homocystinuric conditions, respectively. About a third of the Lth pool was attributed to condensation of two cysteines and thus H_2S production. Since quantification of Lth coming from Cys+Cys reaction was complicated by high blanks, we carried out additional experiments using deuterated Cys (data not shown), which confirmed the preference of CBS for Ser. Taken together, these data suggest that CBS capability to generate H_2S substantially depends on the concentration ratio of Ser and Cys and that the condensation of Cys+Hcy is the leading alternative H_2S-generating reaction.

Thioethers as surrogate markers of H_2S biogenesis in vivo

We also investigated production of thioethers Cth, Lth, and Hlth as surrogate markers of H_2S biogenesis *in vivo* using wild-type monkeys (Fig. 5) and homocystinuric CBS KO mice (Fig. 6). Figure 5 shows plasma levels of thioethers in monkeys injected with PEG human truncated CBS (htCBS) at three different dose levels (1, 3, and 10 mg/kg). Baseline levels before treatment were compared with the levels at 32 h post sixth injection when steady-state levels of PEG htCBS in circulation have been reached. Plasma Cth levels increased 4.4-, 6.0-, and 9.5-fold from baseline levels (0.98 ± 0.07 μM)

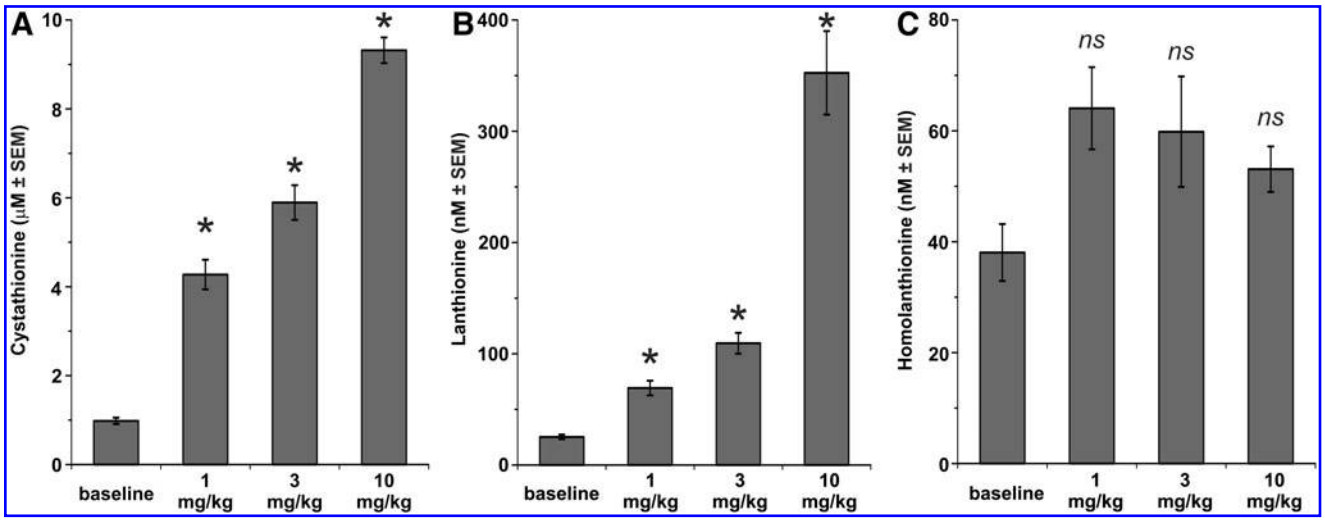

FIG. 5. Plasma levels of thioethers after administration of PEG htCBS to wild-type monkeys. Plasma cystathionine **(A)**, lanthionine **(B)**, and homolanthionine **(C)** levels before (*baseline*) and 32 h after the sixth subcutaneous injection of PEG htCBS administered every 72 h at three different doses (1, 3, and 10 mg/kg) when steady-state levels of PEG htCBS in plasma were reached. Error bars indicate SEMs from 4 animals in each dose group (12 animals for *baseline*). htCBS, human truncated CBS. Significance is designated by *asterisks* as $*p < 0.05$ with ns being non-significant.

in the 1, 3, and 10 mg/kg dosing groups, respectively (Fig. 5A). Plasma Lth baseline levels were ~39-fold lower than that of Cth (25.3 ± 2.1 n*M*) and, similarly to Cth levels, increased 2.7, 4.3, and 13.9 times in 1, 3, and 10 mg/kg dosing groups, respectively (Fig. 5B). Hlth concentration in monkey plasma was 38.1 ± 5.1 n*M* before treatment and six consecutive injections of 1, 3, and 10 mg/kg of body weight of PEG htCBS resulted in a modest increase of 1.9-, 1.6-, and 1.4-fold over the baseline levels (Fig. 5C). While changes in Cth and Lth plasma levels were significantly different from baseline and between each group ($p < 0.02$), a slight increase in Hlth plasma levels was found to be nonsignificant. Biogenesis of Cth and Lth by CBS was higher with increasing doses, but not entirely proportional. Taken together, we conclude that CBS is responsible for *in vivo* production of Cth and Lth, but not Hlth.

Figure 6 shows plasma levels and concentrations in the liver, kidney, and brain tissue homogenates of thioethers in either untreated homocystinuric CBS KO mice or the ones receiving PEG htCBS (3× a week, 7.5 mg/kg of body weight *via* subcutaneous injections) compared with their healthy WT littermates. The lack of CBS activity in untreated CBS KO mice resulted in a virtual absence of Cth in plasma and tissues with the exception of residual levels in the brain (Fig. 6A). Treatment with PEG htCBS led to about 47-fold increase of Cth in plasma and kidney and its normalization in the liver compared with healthy WT mice. Cth appeared to accumulate in the brain of WT mice at levels of about 70 nmol/g. While the treatment increased the brain Cth sevenfold compared with untreated CBS KO mice, these levels were still 8× less than in WT mice. A similar pattern as for Cth was observed for Lth in plasma and tissues (Fig. 6B). The data show substantial accumulation of Lth in the brain of WT mice, about 5 and 7 times more than in liver and kidney, respectively, and ~29 times more compared with 55.5 n*M* plasma Lth. Administration of PEG htCBS resulted in fivefold increase in plasma Lth and normalization of its concentration in the kidney, but levels of this thioether remained unchanged in the liver and brain compared with untreated CBS KO mice. As *in vivo* data from wild-type monkeys suggested that CBS is not a generator of Hlth, Figure 6C further supports this notion showing virtual absence of Hlth in plasma of WT mice and essentially no increase in its plasma concentration with PEG htCBS treatment of CBS KO mice despite the high Hcy substrate levels. Furthermore, lack of CBS activity in CBS KO mice resulted in 10-, 11-, and 14-fold increase of Hlth concentration in the liver, kidney, and brain, respectively, compared with the levels observed in WT mice. Administration of PEG htCBS led to complete normalization of liver Hlth levels and ~2-fold decrease in kidney and brain compared with untreated CBS KO mice. To put the data on thioethers in the context of normal and homocystinuric conditions, Figure 6D shows plasma and tissue levels of Hcy as a limiting cosubstrate for production of Cth and Hlth. There was a substantial accumulation of Hcy in plasma and tissues (26- and ~6-fold, respectively) of untreated CBS KO mice compared with WT controls. Treatment with PEG htCBS resulted in a significant decrease or complete normalization of Hcy to WT levels. Taken together, PEG htCBS in circulation of homocystinuric mice directly resulted in substantial production of Cth and Lth and indirectly limited the CGL-catalyzed Hlth biogenesis by decreasing the availability of Hcy.

Discussion

PLP enzymes play an important role in cellular metabolism due to their potential to utilize different substrates (2, 44). Catalytic promiscuity is particularly frequent among PLP-dependent enzymes due to their common mechanistic features. The first step in all PLP-dependent reactions is the formation of the external aldimine and divergence in reaction specificity occurs from this point. External aldimine is formed when the amino group of the substrate displaces the active site lysine residue from the internal aldimine. When considering the control and specificity of CBS *in vivo*, the determining factors regulating the choice and use of substrate are not clear at this point.

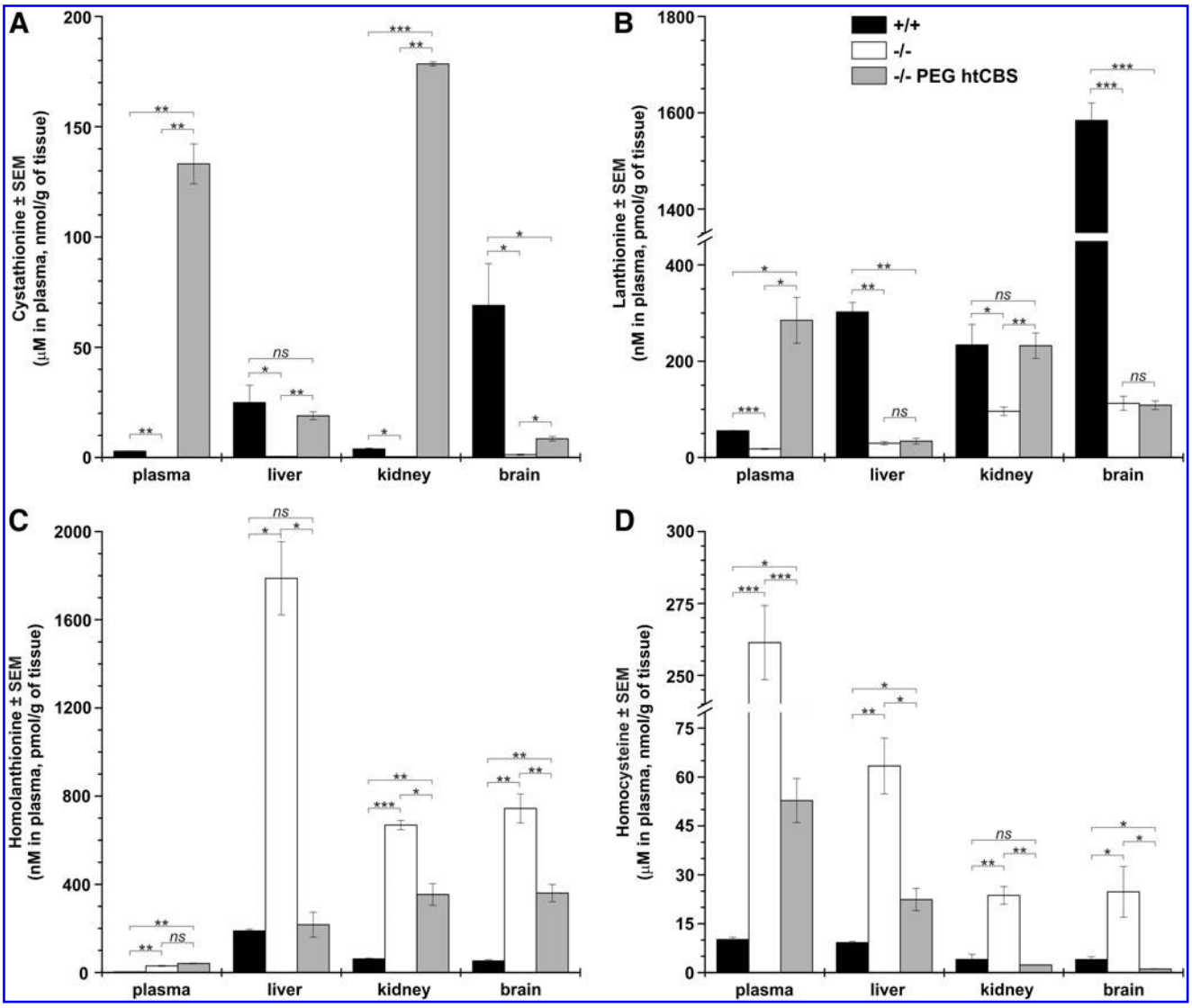

FIG. 6. Thioethers as surrogate markers of H_2S biogenesis. Cystathionine **(A)**, lanthionine **(B)**, homolanthionine **(C)**, and total plasma and nonprotein-bound tissue homocysteine **(D)** concentrations in plasma and liver, kidney, and brain tissue homogenates, respectively, in 18–19-day-old CBS KO mice receiving no treatment (–/–; *white bars*) or injected 3 × a week with 7.5 mg/kg of body weight of PEG htCBS *via* subcutaneous injections from 2 days of age (–/– PEG htCBS; *gray bars*) compared with untreated, age-matched, healthy WT littermates (+/+; *black bars*). Error bars indicate SEMs from three mice. Significance is designated by *asterisks* as $*p<0.05$, $**p<0.01$, and $***p<0.001$ with ns being non-significant.

There has recently been increased interest in the study of H_2S biogenesis and the potential roles of this molecule in human physiology. It has been found that this molecule has important regulatory and signaling functions in humans (1, 14, 20, 43, 53, 61, 63). Endogenous H_2S production has been suggested to occur among other sources as a result of the β-replacement reaction catalyzed by CBS, in which Cys is condensed with Hcy to form H_2S and Cth (6, 11, 28, 49). The results we obtained by comparing the effects of Cys *versus* Ser in isolation each with Hcy are similar to those reported previously. Specifically, in the study performed by Chen *et al.* (11), the k_{cat}/K_m values they observed for the classical Ser+Hcy and alternative Cys+Hcy reactions were 5.9 and 0.72 $mM^{-1}.s^{-1}$, respectively, while we obtained values of 2.63 and 0.53 $mM^{-1}.s^{-1}$, respectively. Banerjee's laboratory reported a very similar value for the classical reaction of 1.92 $mM^{-1}.s^{-1}$, while a somewhat higher value of 2.88 $mM^{-1}.s^{-1}$ was obtained for the alternative reaction (49). Higher affinity of CBS for Ser rather than Cys and unfavorable enzyme kinetics for alternative reactions suggest that CBS use of Cys in H_2S biogenesis is limited and outcompeted by Ser.

The availability of the CBS KO mouse model allowed us to evaluate the relative contribution of CBS to the overall H_2S production. Figure 2 showed that CBS KO mouse liver extract had ~25% of H_2S-forming activity when compared with WT liver meaning that in a normal mouse liver extract, the majority of H_2S-forming activity is due to CBS when 20 m*M* Hcy and 40 m*M* Cys were used. This ~25% of the remaining H_2S-forming activity in the CBS KO liver extract presumably corresponds to CGL because the WT liver activity in the presence of PAG, an inhibitor of CGL, is also lower by ~20% compared with WT liver extract assayed in the absence of the inhibitor. Essentially, no H_2S was produced by the KO liver extract in the presence of CGL

inhibitor PAG, thus further supporting this conclusion. The use of 40 m*M* Cys alone resulted in a very low H_2S-producing activity (cca 50% of KO liver extract in the presence of Cys and Hcy). Together with the observation that WT and KO liver has essentially identical H_2S-forming capacity using Cys as a sole substrate, the results suggested that it must correspond to CGL contribution. These results are in contrast to the previously reported study addressing the significance of transsulfuration enzymes in H_2S biogenesis (26), where the authors reported that the capacity for liver H_2S production using similar substrate concentrations was approximately equal for CBS and CGL. From the broader perspective considering that Cys is much more likely to be available as a substrate than Hcy in a healthy individual, the data suggest that CBS would not contribute substantially to the total H_2S pool. Indeed, if accounted for physiologically relevant concentrations of substrate and adjusting for the differences in CBS *versus* CGL levels in the liver, it was estimated that only 3% of H_2S production could be attributed to CBS (26).

All the past enzyme kinetic studies have studied the H_2S-forming activity when Cys and Hcy or Cys alone were the substrates. Cys, however, does not occur in any mammalian cells in the absence of Ser. To further complicate the CBS-dependent H_2S biogenesis, one needs to consider the omnipresence of Ser in addition to the physiologically relevant concentrations of both competing substrates, Ser and Cys. Thus, we carried out a sequence of H_2S-generating experiments in the presence of Ser, Cys, and Hcy together. We very quickly realized that Ser at less than 1/10 of Cys concentration (3.2 m*M vs.* 40 m*M*) inhibits H_2S formation to $\sim$50% and virtually eliminates it at an equimolar level both in the presence and absence of AdoMet (Fig. 3A). On the other hand, inhibition of the canonical reaction by Cys did not occur until the Cys level reached the 15 m*M* concentration in the reaction (*i.e.*, above 10 m*M* equimolar) (Fig. 3B). The concentration of Cys in the extracellular compartment, such as plasma, is equal or up to fourfold higher compared with Ser (total Cys: 200–361 μM, Ser: 97–267 μM) (41); however, the situation inside the cell is substantially different. While the intracellular Cys concentrations are narrowly maintained between 26 and 119 nmol/g of liver on a normal diet (54), 20–100 nmol/g of liver on a high-protein diet (51), or 5–101 μM inside the leukocytes and fibroblasts (13), the intracellular Ser concentrations are significantly higher, but vary greatly at 957 ± 166 nmol/g of liver (54) or $\sim$1.7 m*M* inside the HCT116 cells cultured in standard DMEM with equal Ser and Cys concentrations of 400 μM in the medium (10). When we performed the substrate competition study using physiologically relevant extracellular and intracellular substrate concentrations (Fig. 4, Supplementary Table S1), we observed a substantial effect of Ser and Cys concentrations on the enzyme's choice of alternative reactions. While 1.7 times higher Cys than Ser concentration, simulating extracellular substrate levels, yielded up to 43% of total production in H_2S, a ninefold excess of Ser over Cys, mimicking intracellular substrate levels, resulted in less than 1.5% of CBS activity being involved in H_2S generation. Surprisingly, when 10 μM Hcy was used to model normal physiological conditions, the leading H_2S-forming reaction catalyzed by CBS was Cys desulfuration compared with all other studied conditions where Cys+Hcy condensation was the preferred one. In addition, formation of Lth by CBS was found to be very limited compared with Cth, thus contributing minimally to the H_2S pool. Previously, one study estimated that about 5% of the Cth formed in a mouse liver was derived from Cys (11). However, the authors used 580 μM Hcy as physiological intracellular concentration for competition studies, which would indicate a severe CBS deficiency rather than normal conditions (and thus a lack of CBS enzyme to generate any Ser- or Cys-derived Cth). Taken together, the enzyme kinetics and substrate competition studies suggest that high intracellular Ser:Cys concentration ratio (>8) efficiently, but not completely, suppresses H_2S formation from the most kinetically relevant Cys+Hcy condensation on CBS. Furthermore, our results also suggest that the Ser:Cys concentration ratio may be the main factor determining CBS-catalyzed H_2S biogenesis. Therefore, it is reasonable to expect that differences in Ser and Cys levels in individual tissues may result in variable amount of H_2S produced by CBS in these tissues.

Previous studies suggested that the thioethers, particularly Lth and Hlth, could be used as surrogate markers for assessment of H_2S production (16, 29, 47). Previous studies concluded that CBS is exclusively responsible for the *in vitro* production of Lth (49), while CGL efficiently cleaves it (49) and is responsible for the biosynthesis of Hlth using two molecules of Hcy, particularly in CBS-deficient homocystinuria (12, 16, 46). Our results are in agreement with CBS being responsible for the production of Lth. However, our data advocate for cautious interpretation of thioethers as surrogate markers for H_2S production because we determined that about two-thirds of the generated Lth originated from Ser+Cys rather than Cys+Cys condensation and thus this fraction cannot contribute to the H_2S pool. Our *in vivo* data further support the role of CBS as Lth generator; however, compared with Cth production, the Lth biogenesis by CBS is lower by 1–2 orders of magnitude. This observation correlates with the results in cultured cells and human plasma, where both Lth and Hlth were found to be lower by 2–3 orders of magnitude compared with Cth levels (16, 29). Interestingly, Cth and Lth substantially accumulate in the brains of WT mice. While there is no clear consensus on CGL activity in brain tissue (1, 22, 56), our metabolite data suggest that CGL is either not present in appreciable amounts or its activity is significantly diminished in the brain leading to the accumulation of Cth and Lth compared with the liver, kidney, or plasma. Thus, we can summarize that despite Ser being a much preferred substrate for CBS over Cys and the discouraging enzyme kinetics (Table 1) and simulation studies (Figs. 3 and 4), CBS generates appreciable amounts of H_2S *in vivo* (Figs. 5 and 6).

Hlth was found to be virtually unchanged in plasma of wild-type monkeys upon administration of PEG htCBS and highly elevated in CBS KO mouse liver, kidney, and brain tissues compared with WT, thus further lending support to the notion that CGL, but not CBS, is responsible for Hlth biogenesis. The presence of Hlth was reported in plasma and urine of CBS-deficient patients (29, 45) and it was proposed that CGL might be responsible for its production in homocystinuric patients (12). Thus, our data are in support of this hypothesis. Repeated injections of PEG htCBS in CBS KO mice resulted in decreased Hlth levels in tissues. Since Hlth is not a substrate for either CBS or CGL and CGL is the sole source of Hlth (12), the decreased Hlth levels in tissues of

PEG htCBS-treated KO mice are a consequence of lower availability of Hcy. It was shown that exogenous PEG htCBS in circulation serves as a sink for Hcy produced in CBS-deficient tissues (8). Therefore, depletion of Hcy availability in the cell by PEG htCBS treatment resulted in decreased or normalized Hlth and H_2S levels produced by endogenous CGL, thus possibly representing additional benefit of treatment homocystinuria with PEG htCBS.

In conclusion, enzyme kinetics and substrate competition studies under optimal as well as physiologically relevant conditions showed that Ser is the preferred substrate for CBS over Cys and that the most kinetically feasible condensation of Cys+Hcy leading to H_2S production is outcompeted by the presence of Ser in a concentration-dependent manner. While a high Ser:Cys ratio, typical for a tissue, substantially limits production of H_2S from CBS-catalyzed Cys+Hcy condensation to 1.5% of total CBS activity, a small excess of Cys over Ser, as seen in plasma, channels up to 43% of total CBS activity leading to H_2S generation. Interestingly, production of Lth was not substantially affected by the Ser:Cys ratio with roughly a third of the Lth pool coming from Cys+Cys condensation and thus contributing to the H_2S pool. However, considering Lth levels 1–2 orders of magnitude lower than that of Cth, its contribution to the total H_2S pool is limited. The recent years have witnessed fast-paced progress in the understanding of H_2S biology, its biogenesis, homeostasis, signaling, and molecular targets, while there are still significant gaps in our knowledge of H_2S physiology and controversies associated with biological effects of H_2S (24, 25, 60). Considering the prominent role of CBS and H_2S in brain signaling and development (43) and cancer (21), we hope that our study clarifies the role of CBS in H_2S production and emphasizes the function of thioethers as surrogate markers of H_2S biogenesis.

Materials and Methods

Chemicals

Unless stated otherwise, all materials were purchased from Sigma or Fisher Scientific. L-[U-^{14}C]-serine was obtained from Perkin Elmer Life Sciences.

CBS enzyme preparation

The expression and purification of wild-type, full-length, and truncated human CBS were performed essentially as described previously (33, 34, 36). Preparation of PEGylated htCBS (PEG htCBS) is described elsewhere (8). Supplementary Figure S1 illustrates the stability of PEG htCBS in plasma at 37°C for a period of 7 days and its catalytic activity and potential to produce H_2S.

Animals, study design, and study approval

All animal procedures and animal protocols were approved by the IACUC of the University of Colorado Denver, which is an Association for Assessment and Accreditation of Laboratory Animal Care-accredited (No. 00235), Public Health Service-assured (No. A 3269-01), and US Department of Agriculture-licensed (No. 84-R-0059) institutions. Briefly, heterozygous CBS KO mice (62) were obtained from The Jackson Laboratory and further propagated in-house. Mice were maintained on the 2918 standard extruded diet (Envigo). Genotype was routinely determined by using the quantitative real-time polymerase chain reaction as described elsewhere (8). Mice were treated from the second day after birth until days 18–19 with subcutaneous injections of either PEG htCBS or phosphate-buffered saline (PBS). The dose of 7.5 mg/kg was administered subcutaneously three times a week. A 24 h after the last injection, mice were euthanized by CO_2 and cervical dislocation right after taking a blood sample from the submandibular vein using lancet into a tube containing lithium heparin (Terumo). Mice abdominal and thoracic cavities were open and cardiac perfusion using PBS was performed to clear the tissues from blood. Subsequently, the liver, kidney, and brain were harvested, cut into a smaller pieces, and flash-frozen in liquid nitrogen. No toxic effects were observed during PEG htCBS administration. On the contrary, PEG htCBS injections from day 2 of age rescued CBS KO mice from a premature death (8).

A study using wild-type Cynomolgus monkeys (*Macaca fascicularis*) was performed at Charles River Laboratories Edingburgh Ltd. (Tranent, United Kingdom), which is a good laboratory practice-complaint facility approved and validated for a variety of preclinical and clinical laboratory services. Briefly, at the initiation of dosing, six male and six female monkeys were 27–30 months old and weighed between 2.7 and 4.1 kg. PMI Nutrition International Certified Primate Diet No. 5S48 (25% protein) was provided as a daily ration (200 g) throughout the study and the animals had access to water *ad libitum* from the public supply. PEG htCBS in three different dose levels (1, 3, and 10 mg/kg) was given by subcutaneous injection on the back on six occasions (every 72 h). Blood was collected from the femoral vein into lithium heparin before treatment initiation (baseline) and before and 32 h after each injection. No toxic side effects of the treatment with PEG htCBS were observed as indicated by no significant changes in body weights, hematology and coagulation markers, clinical chemistry, organ weights, and gross and histopathology findings (data not shown).

Preparation of liver extracts

Adult WT and KO mice were euthanized using carbon dioxide, followed by cervical dislocation. Blood from tissues was removed by transcardial perfusion using ice-cold phosphate-buffered saline. Liver was dissected, cut into smaller pieces, which were immediately flash-frozen in liquid nitrogen. Four volumes of ice-cold lysis buffer (30 m*M* potassium phosphate pH 6.5, 1 m*M* beta-mercaptoethanol containing protease inhibitor cocktail for use with tissue extracts from Sigma, cat# P8340) were added to one volume of tissue (volume per weight) and liver was homogenized using a glass handheld homogenizer. The suspension was clarified by centrifugation at 15,000 *g*, 4°C, for 15 min. Clear supernatant was transferred into a fresh tube and protein concentration was determined by using Bradford reagent (Thermo Scientific) according to the manufacturer's protocol.

CBS activity assays

The CBS activity in the classical reaction was determined by a previously described radioisotope assay using L-[^{14}C]-serine as the labeled substrate (30). Briefly, a purified enzyme (420 ng) was assayed in a 100-μl reaction for 30 min at 37°C. The reaction mixture contained 100 m*M* Tris-HCl pH 8.6, 10 m*M* Ser, 0.2 m*M* PLP, 0.3 μCi L-[^{14}C]-Ser, and 0.5 mg/ml

bovine serum albumin (BSA). The reaction was performed in the absence or presence of AdoMet at the final concentration of 0.5 m*M*. The reaction mixture with enzyme was incubated at 37°C for 5 min and the reaction was initiated by addition of 200 m*M* Hcy solution to a final concentration of 10 m*M*. The reaction was terminated by an immediate cooling of the mixture in ice water and the product was separated from the substrates by paper chromatography. Spots corresponding to Cth were cut out, submerged in Opti-Fluor scintillation fluid (Perkin-Elmer), and the radioactivity was determined in a scintillation counter (Beckman). For enzyme kinetics, 500–2500 ng of the enzyme was used. The assay was carried out for 3, 6, and 9 min to ensure linearity, where one substrate was kept constant at saturating concentrations (10 m*M*), while the other substrate was varied. The assay was terminated by mixing a 20-μl aliquot of the reaction mixture with 5 μl of performic acid (30% H_2O_2:100% formic acid = 1:9).

The activities in the H_2S-generating alternative reactions were determined by using a colorimetric detection of H_2S described earlier (27) with the following modifications. The reaction mixture (200 μl) contained 200 m*M* Tris-HCl pH 8.6, 40 m*M* Cys, 20 m*M* Hcy (omitted in the cysteine β-elimination/β-replacement reaction), 0.5 m*M* PLP, and 0.5 mg/ml BSA. The reaction was performed in the absence or presence of AdoMet at the final concentration of 0.5 m*M*. The mixture was incubated at 37°C for 2 min and the reaction was initiated by addition of CBS enzyme (2.5 μg) and carried out at 37°C for 6 min. The reaction was terminated by a 40-fold dilution of a 25-μl assay aliquot in water and mixing it with 100 μl N,N-dimethyl-p-phenylenediamine reagent and 100 μl ferric chloride solution. The samples were stored in the dark at room temperature for 20 min for color development. The concentration of H_2S was determined from the absorbance at 650 nm using a standard curve prepared from sodium sulfide solutions of known concentration. For enzyme kinetics, 4 μg of the enzyme was used. The assay was carried out for 6 min to ensure linearity, where one substrate was kept constant at saturating concentrations (40 m*M* Cys or 20 m*M* Hcy), while the other substrate concentration varied.

The Cys-producing (H_2S-consuming) activity was determined by using a colorimetric detection of generated Cys as described earlier (19) with the following modifications. The reaction mixture (200 μl) contained 200 m*M* Tris-HCl pH 8.6, 15 m*M* sodium sulfide, 10 m*M* Ser, 0.5 m*M* PLP, and 0.5 mg/ml BSA. The reaction was performed in the absence or presence of AdoMet at the final concentration of 0.5 m*M*. The mixture was incubated at 37°C for 2 min and the reaction was initiated by addition of the enzyme (10 μg) and carried out at 37°C for 6 min. Incubation was terminated by removing a 50-μl assay aliquot and mixing it with equal amounts of glacial acetic acid and acidic ninhydrin reagent. After 10 min of boiling and immediate cooling, the color was stabilized by addition of 850 μl of denatured ethanol. The concentration of Cys was determined from the absorbance at 560 nm using a standard curve prepared from Cys solutions of known concentration.

For all the canonical and alternative reactions, one unit of activity is defined as the amount of CBS that catalyzes the formation of 1 μmol of product in 1 h at 37°C under standard assay conditions.

The H_2S-forming activity of WT and CBS KO liver extracts was determined by the above described assay. Briefly, 200 μg of liver extract was assayed for 30 min in the absence or presence of 0.25 m*M* AdoMet, 1 m*M* PAG, and/or 1 mM AOAA. Substrate concentrations used were to favor H_2S production by both CBS and CGL: 40 m*M* Cys and 20 m*M* Hcy or 40 m*M* Cys alone in the complete absence of Ser.

CBS substrate competition assays

Substrate competitions under saturating V_{max} optimal conditions were examined by using either the H_2S-producing CBS activity assay, when increased concentration of Ser (0–90 m*M*) was competing with a set concentration of 40 m*M* Cys, or the radiometric CBS activity assay, when increased concentration of Cys (0–50 m*M*) was competing with a set concentration of 10 m*M* Ser. Data analysis and fitting were performed using the Origin Pro software.

In addition, we tested competition between Cys and Ser in a combinatorial design under several physiologically relevant conditions. Concentration of substrates was adjusted to simulate the extracellular conditions (150 μ*M* Ser and 250 μ*M* Cys) or the intracellular milieu (900 μ*M* Ser and 100 μ*M* Cys). To mimic the physiological state and homocystinuric conditions, we used 10 or 250 μ*M* Hcy, respectively. The reactions were carried out in the absence or presence of AdoMet (final concentration 0.5 m*M*). Physiological pH 7.4 was achieved by using PBS. The activity of purified human CBS WT enzyme (600 ng) was assayed in 60-μl reactions in 2-ml screw cap vials at 37°C for 30 min. The reaction mixture contained PBS pH 7.4, 0.1 m*M* DTPA, 0.2 m*M* PLP, 1 mg/ml BSA, L-[D3]-Ser (150 or 900 μ*M*), L-Cys (250 or 100 μ*M*), and L-Hcy (10 or 250 μ*M*). The L-Hcy stock solution was freshly prepared from L-homocysteine thiolactone as described previously with substitution of dithiothreitol for tris-(2-carboxyethyl)-phosphine (30). The reaction mixtures were maintained in ice water bath until the addition of enzyme, the vials were flushed with nitrogen immediately after the start of a reaction, closed, and subsequent manipulations were performed through the septum using Hamilton syringes. Blank samples for each series of experiments were prepared and processed identically and the enzyme solution was substituted by the same volume of water. Reactions were terminated by addition of 30 μl of 0.5 *M* HCl and samples were incubated at room temperature for 20 min to allow the release of H_2S into the headspace of the vial. The liquid layer of the assay mixture was removed to quantify the reaction products Cth, [D2]-Cth, Lth, [D2]-Lth, and Ser using LC-MS/MS methods, as described previously (29, 31, 47). H_2S was determined from the gaseous phase as described below by modification of a previously published method (48).

Metabolite analyses

Various metabolites, including thioethers Cth, Lth, and Hlth, were determined in mouse plasma and perchloric acid extracts of tissues using HPLC and LC-MS/MS methods as described previously (29, 32, 47) with examples of assay chromatograms shown in Supplementary Figures S2 and S3. Concentrations of thioethers in monkey plasma were determined by gas chromatography–mass spectrometry (Thermo DSQII) as N-propyl ester heptafluorobutyramide derivatives in negative chemical ionization mode with selected-ion monitoring (15). Lth and Hlth were measured relative to a norleucine internal standard, whereas Cth was quantified relative to [D4]-Cth internal standard (Cambridge Isotope Laboratories). Lth and Hlth were quantified relative to the Cth response curve.

H_2S determination

Calibration samples for H_2S determination were processed identically to the CBS assay mixtures in closed system using the 2-ml screw cap septum vials flushed with nitrogen and Hamilton syringes. Stock 30 m*M* Na_2S solution was prepared freshly for each series by dissolving anhydrous Na_2S (Alfa Aesar) in alkaline TRIS buffer (100 m*M* TRIS-HCl pH 9.5, 0.1 m*M* DTPA). The stock solution was diluted with the alkaline TRIS buffer in a stepwise manner to yield final concentrations of 0–60 μM sulfide in a final volume of 60 μl. Sodium sulfide solutions were incubated at 37°C for 30 min and subsequently 30 μl of 0.5 *M* HCl was added. Release of gaseous H_2S into the headspace of the vial proceeded at room temperature for 20 min. The liquid was removed from the vial and gaseous H_2S in the headspace was subsequently recaptured into alkaline solution and converted into sulfide dibimane by addition of 150 μl of 3 m*M* monobromobimane (mBrB) prepared by dilution of a 100 m*M* stock solution in acetonitrile with an alkaline buffer (100 m*M* TRIS-HCl pH 9.5, 0.1 m*M* DTPA). The recapturing and derivatization reaction proceeded at room temperature for 30 min and this process was terminated by adding 23 μl of 70% sulfosalicylic acid. The concentration of sulfide dibimane was determined by HPLC with fluorescence detection. Used column was ProntoSIL 120-3-C18 AQ 3.0 μm, 200 × 4.0 mm. Mobile phase A was 0.1% formic acid and mobile phase B was 0.1% formic acid in 40% acetonitrile. The flow was 0.45 ml/min and the gradient was as follows (time point and % of mobile phases A/B): 0 min 95/5%, 1 min 95/5%, 8 min 0/100%, 19 min 0/100%, 20 min 95/5%, and 29 min end of the analysis. Fluorescence detector was set at 390 nm (excitation wavelength) and 475 nm (emission wavelength), and retention time of sulfide dibimane was around 15.5 min (see the example of chromatograms Supplementary Fig. S4). A blank sample prepared for each calibration curve was treated identically and sodium sulfide solution was replaced by the alkaline TRIS buffer. All assays were performed in triplicates, data shown are means and standard deviations.

Statistical analysis

All data are presented as mean ± standard error of the mean and compared using an unpaired two-tailed Student's *t*-test. When comparing multiple groups, the data were first analyzed by multivariate ANOVA, followed by Tukey's *post hoc* analysis to determine significance. Significance is designated by asterisks as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ with *ns* being nonsignificant. For *in vivo* metabolite comparison, one-way ANOVA with Bonferroni–Holm post hoc test was used to determine significance. Data from substrate competition experiment under optimal V_{max} conditions were plotted using OriginLab's OriginPro software using the substrate inhibition model for fitting.

Acknowledgments

The authors thank Richard Carrillo for preparation of PEG htCBS and Insun Park for help with mice. This work was supported, in part, by the American Heart Association Scientist Development Grant (16SDG30040000 to T.M.) and research funding from Orphan Technologies, Ltd., (to J.P.K.). J.K., J.S., M.K., and V.K. were supported by Grant No. 16-30384A from the Czech Health Research Council and received institutional support from projects RVO-VFN 64165 and PROGRES Q26.

Author Contributions

J.P.K. conceived the idea. T.M. and J.P.K. designed experiments and directed work. J.K., J.S., M.K., and V.K. performed the metabolite analyses in mouse plasma and tissues and selected *in vitro* reactions, as well as H_2S determinations. M.A.R. and J.F.G. determined thioethers in monkey plasma and selected *in vitro* reactions. T.M. performed all the remaining experiments, analyzed data, prepared figures, and cowrote the manuscript with J.P.K. All authors contributed to data presentation and manuscript revisions.

Author Disclosure Statement

The private company Orphan Technologies, Ltd., partially funded the research presented here (to J.P.K.). T.M. provides consultation services to Orphan Technologies, Ltd. T.M. and J.P.K. are inventors on patent describing purification and use of PEG htCBS as an enzyme replacement therapy for homocystinuria (U.S. patent numbers 9,034,318 and U.S. 9,243,239). All other authors have no competing financial interests to disclose.

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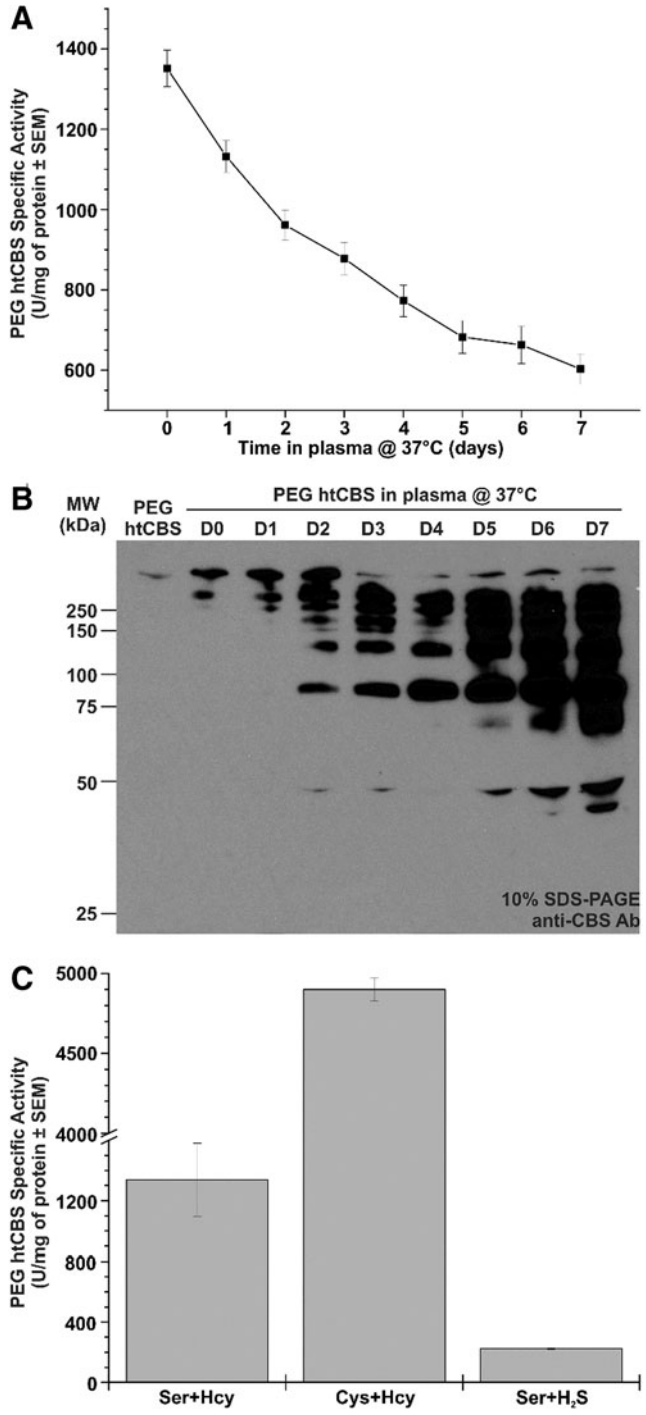
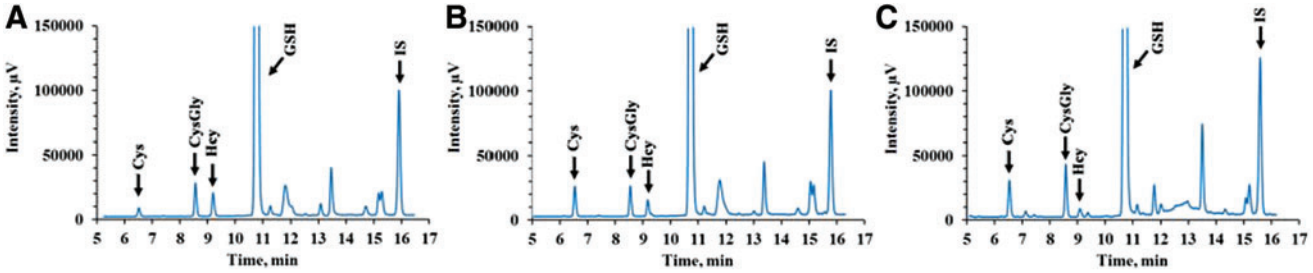
Date of first submission to ARS Central, January 25, 2017; date of final revised submission, August 22, 2017; date of acceptance, August 31, 2017.

Abbreviations Used

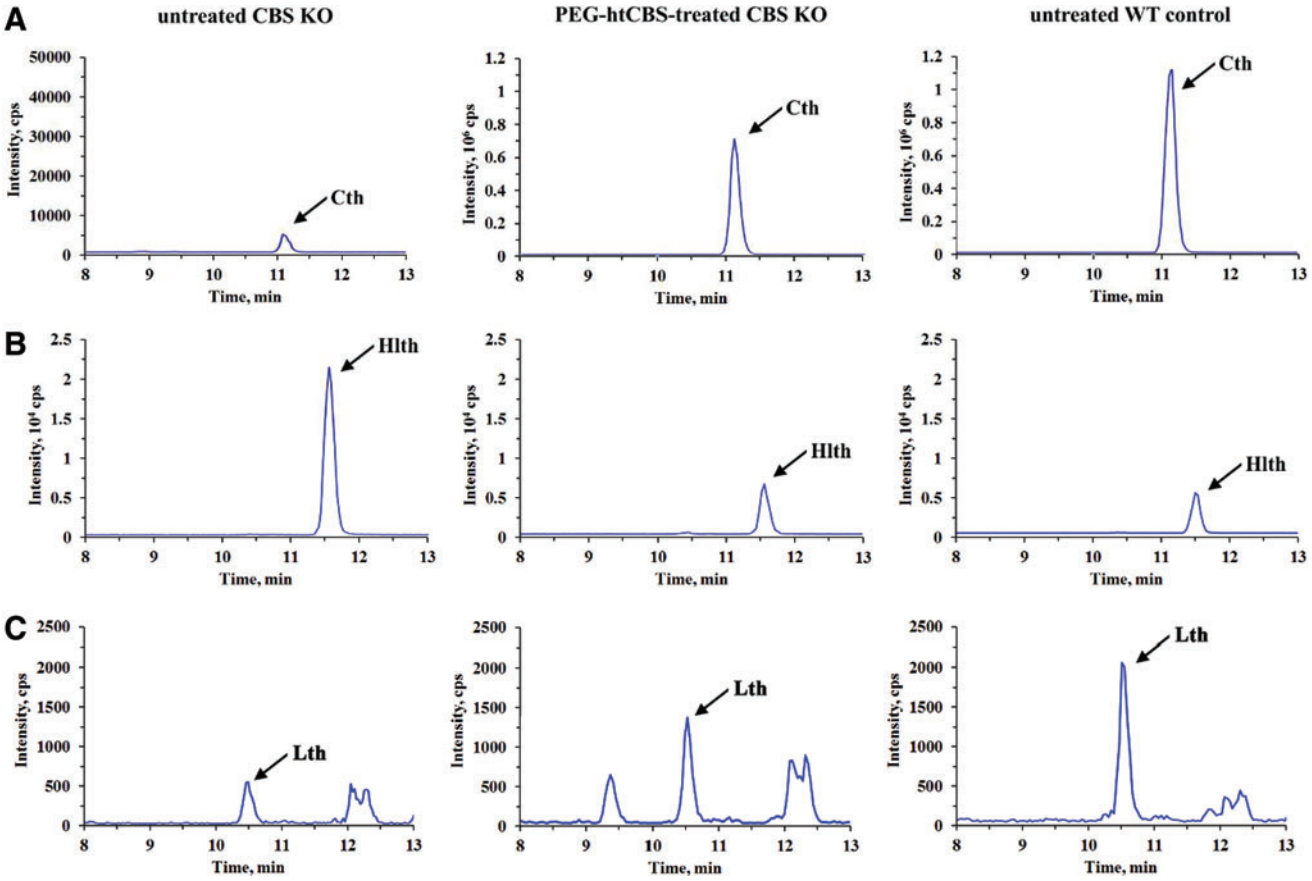
AdoMet = S-adenosylmethionine
AOAA = aminooxyacetic acid
BSA = bovine serum albumin
CBS = cystathionine beta-synthase
CGL = cystathionine gamma-lyase
Cth = cystathionine
Cys = L-cysteine
H_2S = hydrogen sulfide
Hcy = L-homocysteine
Hlth = homolanthionine
HPLC = high performance liquid chromatography
htCBS = human truncated CBS
KO = knockout
LC-MS/MS = liquid chromatography-tandem mass spectrometry
Lth = lanthionine
PAG = propargylglycine
PBS = phosphate-buffered saline
PLP = pyridoxal-5′-phosphate
SEMs = standard error of the mean
Ser = L-serine
WT = wild-type



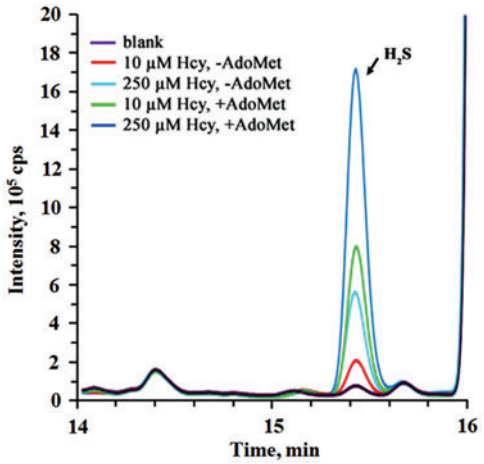
SUPPLEMENTARY FIG. S1. PEG htCBS stability in plasma and its catalytic activity. **(A, B)** Impact of isothermal incubation of PEG htCBS at 37°C in human plasma at a final concentration of 0.5 mg/ml for a period of 7 days on enzymes's specific activity and conjugate stability, respectively. **(C)** Specific activities of PEG htCBS in the canonical condensation of Ser+Hcy and two alternative reactions: an H_2S-yielding Cys+Hcy and H_2S-utilizing Ser+H_2S. Error bars indicate SEMs from at least three independent measurements. CBS, cystathionine beta-synthase; H_2S, hydrogen sulfide; htCBS, human truncated CBS; SEM, standard error of the mean.



SUPPLEMENTARY FIG. S2. HPLC analysis of aminothiols in liver extracts. Examples of chromatograms from aminothiol analyses of liver extracts from untreated CBS KO **(A)**, –/– PEG htCBS-treated CBS KO **(B)**, and untreated WT mice **(C)**. Samples were analyzed after reduction with tris (2-carboxyethyl) phosphine and derivatization with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate on HPLC system (Shimadzu LC-10A, Japan) with fluorescence detection (excitation 385 nm, emission 415 nm). Peaks: cysteine (Cys), cysteinylglycine (CysGly), homocysteine (Hcy), glutathione (GSH), and N-(2-mercaptopropionyl)-glycine (internal standard [IS]). HPLC, high performance liquid chromatography; KO, knockout; WT, wild-type.



SUPPLEMENTARY FIG. S3. LC-MS/MS analysis of thioethers in liver extracts. Signals of cystathionine (Cth, **A**), homolanthionine (Hlth, **B**), and lanthionine (Lth, **C**) in liver extracts from untreated CBS KO (*right*), –/– PEG htCBS-treated CBS KO (*middle*), and untreated WT mice (*left*). The samples were derivatized before LC-MS/MS analysis using the EZ:faast kit (Phenomenex). The detection of analytes was performed using the positive electrospray ionization technique and selected multiple reaction monitoring on the API 4000 triple quadrupole mass spectrometer (Applied Biosystems). The precursor → product mass transitions for the derivatization products of Cth, Hlth, and Lth were 479.3 → 230.3, 493.3 → 230.3, and 465.3 → 216.3, respectively. KO, knockout; LC-MS/MS, liquid chromatography tandem mass spectrometry; WT, wild-type.



SUPPLEMENTARY FIG. S4. Examples of chromatograms from HPLC analysis of H_2S in CBS competitive assays. Conditions of CBS assays simulated extracellular substrate concentrations (150 μM Ser, 250 μM Cys) at pH 7.4 (phosphate-buffered saline buffer). Concentrations of Hcy mimic normal (10 μM Hcy) and homocystinuric conditions (250 μM Hcy). Assays were performed in the absence and presence of 500 μM AdoMet. The *arrow* indicates the peak of sulfide dibimane, the reaction product of H_2S with monobromobimane.

Supplementary Data

Supplementary Table S1. Cystathionine Beta-Synthase Catalytic Activity Using Simulated Physiological Substrate Concentrations and pH 7.4 Under Normal and Homocystinuric Conditions

		CBS specific activity (μmol of product/mg of protein/h ± SD)[a]									*CBS specific activity (%)*		
Substrate concentrations	*500 μM AdoMet*	*[D2]-Cth (from [D3]-Ser+Hcy)*	*Cth (from Cys+Hcy)*	*[D2]-Lth (from [D3]-Ser+Cys)*	*Lth (from Cys+Cys)*	*Ser (from Cys+H_2O)*	*Determined H_2S (HPLC)*	*Anticipated H_2S (Σ from Cys)*	*Σ from Ser*	*Σ*	*From Cys*	*From Ser*	*Lth fraction*
150 *μM* [D3]-Ser	−	0.486 ± 0.006	0.0951 ± 0.0015	0.0230 ± 0.0019	<LLQ	0.125 ± 0.058	0.230 ± 0.022	0.22	0.509	0.729	30.2	69.8	3.2
250 *μM* Cys	+	1.51 ± 0.06	0.187 ± 0.008	0.108 ± 0.006	<LLQ	1.03 ± 0.20	1.12 ± 0.02	1.22	1.616	2.834	43.0	57.0	3.8
10 *μM* Hcy													
150 *μM* [D3]-Ser	−	5.59 ± 0.17	1.59 ± 0.10	0.0195 ± 0.0028	<LLQ	<LLQ	1.62 ± 0.10	1.59	5.609	7.199	22.1	77.9	0.3
250 *μM* Cys	+	29.0 ± 0.7	4.69 ± 0.17	0.0428 ± 0.0089	<LLQ	0.287 ± 0.029	3.50 ± 0.31	4.97	29.041	34.014	14.6	85.4	0.1
250 *μM* Hcy													
900 *μM* [D3]-Ser	−	0.648 ± 0.026	0.0024 ± 0.0010	0.0174 ± 0.0015	<LLQ	<LLQ	0.0183 ± 0.0028	0.0024	0.665	0.668	0.4	99.6	2.6
100 *μM* Cys	+	1.65 ± 0.08	0.0111 ± 0.0001	0.0587 ± 0.0013	<LLQ	<LLQ	0.0562 ± 0.0013	0.011	1.710	1.721	0.6	99.4	3.4
10 *μM* Hcy													
900 *μM* [D3]-Ser	−	11.5 ± 0.2	0.177 ± 0.002	0.0132 ± 0.0012	<LLQ	<LLQ	0.159 ± 0.001	0.177	11.551	11.728	1.5	98.5	0.1
100 *μM* Cys	+	37.6 ± 0.9	0.312 ± 0.19	0.0607 ± 0.0019	<LLQ	<LLQ	0.134 ± 0.022	0.312	37.660	37.972	0.8	99.2	0.2
250 *μM* Hcy													

[a]All values are the averages of three independent determinations. <LLQ means that the respective products were detected, but were below lower limit of quantification for the respective assay. CBS, cystathionine beta-synthase; H_2S, hydrogen sulfide; HPLC, high performance liquid chromatography.