Heme-dependent Metabolite Switching Regulates H₂S Synthesis in Response to Endoplasmic Reticulum (ER) Stress

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Substrate ambiguity and relaxed reaction specificity underlie the diversity of reactions catalyzed by the transsulfuration pathway enzymes, cystathionine β-synthase (CBS) and γ-cystathionase (CSE). These enzymes either commit sulfur metabolism to cysteine synthesis from homocysteine or utilize cysteine and/or homocysteine for synthesis of H₂S, a signaling molecule. We demonstrate that a kinetically controlled heme-dependent metabolite switch in CBS regulates these competing reactions where by cystathionine, the product of CBS, inhibits H₂S synthesis by the second enzyme, CSE. Under endoplasmic reticulum stress conditions, induction of CSE and up-regulation of the CBS inhibitor, CO, a product of heme oxygenase-1, flip the operating preference of CSE from cystathionine to cysteine, transiently stimulating H₂S production. In contrast, genetic deficiency of CBS leads to chronic stimulation of H₂S production. This metabolite switch from cystathionine to cysteine and/or homocysteine renders H₂S synthesis by CSE responsive to the known modulators of CBS: S-adenosylmethionine, NO, and CO. Used acutely, it regulates H₂S synthesis; used chronically, it might contribute to disease pathology.

Hydrogen sulfide (H₂S) is a signaling molecule that regulates physiological processes ranging from neomodulation (1) to cardioprotection (2) and inflammation (3). Two enzymes in the transsulfuration pathway, cystathionine β-synthase (CBS) and γ-cystathionase (CSE), produce H₂S (4, 5). In humans, the canonical role of these enzymes is to commit sulfur metabolism to cysteine synthesis from homocysteine or utilize cysteine and/or homocysteine for synthesis of H₂S, a signaling molecule. We demonstrate that a kinetically controlled heme-dependent metabolite switch in CBS regulates these competing reactions where by cystathionine, the product of CBS, inhibits H₂S synthesis by the second enzyme, CSE. Under endoplasmic reticulum stress conditions, induction of CSE and up-regulation of the CBS inhibitor, CO, a product of heme oxygenase-1, flip the operating preference of CSE from cystathionine to cysteine, transiently stimulating H₂S production. In contrast, genetic deficiency of CBS leads to chronic stimulation of H₂S production. This metabolite switch from cystathionine to cysteine and/or homocysteine renders H₂S synthesis by CSE responsive to the known modulators of CBS: S-adenosylmethionine, NO, and CO. Used acutely, it regulates H₂S synthesis; used chronically, it might contribute to disease pathology.

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Results

To investigate ER stress-induced regulation of the transsulfuration pathway, we monitored the incorporation of radiolabel from [³⁵S]methionine into two downstream pathway products, GSH and sulfate in HEK293 cells during ER stress induced by thapsigargin (Fig. 1a). Flux through the canonical reactions, which leads to GSH production, initially increased in response to ER stress, but declined subsequently (Fig. 1b, supplemental Fig. S1a), begging

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the question as to why the flux to GSH decreased despite increased CSE expression (Fig. 1c). CSE levels remained elevated 50 h after thapsigargin treatment, whereas CBS levels were unchanged (Fig. 1c) as reported previously (12, 13). No change was seen in xCT, the cysteine/glutamate antiporter (Fig. 1c), which was reportedly induced in murine islets and MIN6 cells, albeit at a higher (1 μM) concentration of thapsigargin (12). Total GSH levels also declined in thapsigargin-treated cells for the duration of the experiment (supplemental Fig. S1b). In contrast, radiolabel incorporation into sulfate, an H₂S oxidation product, continued to increase in thapsigargin-exposed cells (Fig. 1d, supplemental Fig. S1c), and total sulfate also increased (supplemental Fig. S1d). Increased synthesis of sulfate is consistent with enhanced production and oxidation of H₂S. Similar results were obtained with HeLa cells for incorporation of radiolabel into GSH, although the GSH pool was more sensitive to ER stress in this cell line (supplemental Fig. S2, a and b).

We hypothesized that the change in the kinetics of radiolabeling reflected cellular switching from the canonical to H₂S-producing reactions. This metabolite switching mechanism could operate under ER stress conditions due to the induction of heme oxygenase-1 (16), a source of CO, which binds to the heme cofactor in CBS and inhibits its activity (17–20). We hypothesized that low cystathionine and increased homocysteine resulting from CBS inhibition promote H₂S synthesis by CSE (Fig. 1a). The catalytic efficiency of CSE is significantly greater for cysteine synthesis from cystathionine (kcat/Km = 82,000 M⁻¹ s⁻¹) than for H₂S synthesis from cysteine (159 M⁻¹ s⁻¹) and from homocysteine (492 M⁻¹ s⁻¹) (5). Hence, under conditions where CBS is active, the canonical transsulfuration reactions for converting homocysteine to cysteine are expected to predominate. Conversely, inhibition of CBS and diminished cystathionine levels are expected to increase the efficiency of H₂S generation from cysteine and homocysteine catalyzed by CSE.

As a test of our model, in HEK293 cells, we overexpressed the constitutively expressed heme oxygenase-2 (HO-2), which also releases CO. HO-2 overexpression decreases incorporation of radiolabel from [³⁵S]methionine into GSH in control and thapsigargin-treated cells (Fig. 2a). The decrease in GSH levels observed in untransfected cells exposed to thapsigargin was not seen in HO-2-overexpressing cells (Fig. 2b), suggesting a protective antioxidant effect as discussed below. Radiolabel incorporation into sulfate was decreased in HO-2-overexpressing cells and was unaffected by thapsigargin treatment (Fig. 2c), although total sulfate levels increased in treated cells (Fig. 2d). The decreased incorporation of radiolabel despite the
 increased production of sulfate in HO-2-overexpressing cells (± thapsigargin) can be explained by the increased expression of the cystine transporter xCT under these conditions (supplemental Fig. S3). The consequent increased import of unlabeled cystine from the extracellular medium by HO-2-overexpressing cells leads to radiolabel dilution in the cysteine pool. We speculate that the higher xCT levels reflect an adaptation to decreased cysteine synthesis via the transsulfuration pathway in HO-2-overexpressing cells. Up-regulation of xCT and consequent import of the GSH substrate, cysteine, would also explain why the GSH pool size is unaffected while radiolabel incorporation into GSH from methionine is inhibited in HO-2-overexpressing cells. The increased synthesis of sulfate versus GSH from methionine in HO-2-overexpressing versus control cells is consistent with CBS inhibition and increased H₂S synthesis under these conditions (Fig. 1a).

Consistent with our model, we found that CSE-dependent H₂S synthesis by murine liver lysate is inhibited at increasing concentrations of cystathionine (between 50 and 1,000 μM) in the presence of 1 mM cysteine (Fig. 3a). CSE is the dominant source of H₂S under these conditions (4). H₂S synthesis diminished up to 250 μM cystathionine; however, the inhibition was reversed at higher concentrations. The CSE-catalyzed cleavage of cystathionine to cysteine likely contributes to the U-shaped dependence because the concentration of cysteine, an H₂S-producing substrate, rises with increasing concentration of cystathionine. This result is consistent with the model that the supply of cystathionine by CBS steers CSE activity away from H₂S synthesis within a certain concentration window. Despite the similar catalytic efficiencies (k_{cat}/K_{m} = 2.650 M^{-1} s^{-1} for serine versus 2.882 M^{-1} s^{-1} for cysteine), serine is expected to inhibit H₂S production by CBS due to its lower K_{d} than cysteine (4). Indeed, the addition of serine, the canonical substrate for CBS, inhibited H₂S production by CBS in liver lysate in the presence of high concentrations of cysteine and homocysteine, which supported H₂S synthesis by CSE (supplemental Fig. S4). These results, combined with the higher cellular concentration of serine versus cysteine, indicate that CBS is poised to catalyze the canonical transsulfuration reaction in vivo.

Next, we tested metabolite switching in an animal model of CBS deficiency (Fig. 3b, inset) in which plasma homocysteine levels are ~40-fold higher than in wild-type controls (21). Our kinetic studies have predicted a graded increase in CSE-derived H₂S with increasing homocysteine concentrations, and linked the resulting H₂S synthesized to homolanthionine (5), a metab-
olite found in urine of homocystinuric individuals but not in normal individuals (22). We measured H₂S production in liver from CBS knock-out mice at physiologically relevant concentrations of cysteine and homocysteine. The rate of H₂S synthesis was ~2-fold higher in liver lysates from Cbs⁻/⁻ mice as compared with Cbs⁺/⁺ controls (Fig. 3b). Under these conditions, the concentration of cystathionine in tissue homogenates is negligible due to dilution, whereas inhibitory concentrations of cystathionine are produced from the exogenously supplied substrates of CBS, which is present only in wild-type tissue.

Discussion

This is the first report of heme-dependent metabolite switching that can transiently regulate H₂S production via the transulfuration pathway (Fig. 3c). Under basal conditions, when CBS is active, cystathionine is synthesized and kinetic control favors its conversion by CSE to cysteine. Diminished cystathionine and increased homocysteine via inhibition of CBS, e.g. by CO produced in response to ER stress, favor increased H₂S synthesis by CSE. Similarly, we predict that conditions that increase NO levels, decrease S-adenosylmethionine, an allosteric activator and stabilizer of CBS (23), or disable CBS via mutations, as in CBS-dependent homocystinuria, will enhance H₂S production by CSE. CSE is a major source of H₂S in liver at physiologically relevant substrate concentrations (24) and in the cardiovascular system (25). Our model suggests that the activity of CBS dampens H₂S production by CSE. H₂S plays an important role in the cardiovascular system, and we speculate that the low CBS in endothelial cells is a mechanism for promoting H₂S synthesis by CSE.

Although the metabolite switch from cystathionine to cysteine and/or homocysteine could be protective in an acute response, its chronic operation, as in homocystinuria in a background of high homocysteine, is likely to have pathological consequences. Interestingly, cystathionine administration to homocystinuric mice attenuated liver injury and steatosis without protecting against the pathological effects of ER stress due to high homocysteine (26), which can now be explained by the metabolite switching model. We speculate that other conditions in which regulation of H₂S synthesis is perturbed include type 1 diabetes, Down syndrome, and caloric restriction. Patients with insulin-dependent diabetes without nephropathy have decreased plasma homocysteine (27), whereas inhibitory concentrations of cystathionine are produced from the exogenously supplied substrates of CBS, which is present only in wild-type tissue.

The metabolite switching model helps explain why large randomized controlled trials for lowering homocysteine had limited success in reducing cardiovascular disease outcomes in patients (32–34) and suggests instead that a strategy targeting H₂S might be effective. In light of the metabolite switching mechanism for regulating H₂S, the current approaches for treating the orphan disease, homocystinuria, and chronic diseases such as type I diabetes, where H₂S dysregulation and ER stress are implicated (12, 35), should be reevaluated.

Experimental Procedures

Animal Tissues—Livers from Cbs⁻/⁻ (Tg-I278T) and wild-type mice were a generous gift from Dr. Warren Kruger (Fox Chase Cancer Center, Philadelphia, PA). Briefly, the mice express a human Cbs transgene carrying the pathogenic I278T mutation under the control of a zinc-inducible promoter (36) to overcome the neonatal lethality associated with the Cbs⁻/⁻ genotype (21). The livers were harvested from female mice maintained on zinc-free water for 4 months, frozen, and shipped to our laboratory.

Cell Culture and Metabolic Labeling—HEK293 cells were grown in 10-cm dishes in minimum essential medium (Lonza) supplemented with 10% FBS and 2 mM L-glutamine until they reached 60–80% confluency. ER stress was induced by adding thapsigargin to the culture medium to a final concentration of 0.5 μM, and cells were grown until the indicated times before sample collection. For metabolic labeling studies, 5–10 μCi of [35S]methionine (PerkinElmer) was added per 5 ml of medium 4–5 h prior to sample collection.

GSH Determination—For analysis of GSH and GSSG, an aliquot of cell suspension was mixed with an equal volume of metaphosphoric acid solution (135 mM metaphosphoric acid, 5 mM EDTA, and 150 mM NaCl). After a freeze-thaw cycle, precipitated proteins were removed by centrifugation (10,000 × g for 5 min). To the resulting protein-free supernatant, iodoacetate was added to a final concentration of 10 mM, to block free thiol groups. The pH was adjusted to 8–9 with potassium carbonate, and the reaction mixture was incubated for 1 h at room temperature in the dark. An equal volume of 2,3-dinitrofluorobenzene (1.5% v/v in absolute ethanol) was added to derivatize amino groups and incubated at room temperature for 4 h in the dark before HPLC analysis. The second aliquot of the cell suspension was used to measure the protein concentration using the Bradford reagent (Bio-Rad) and for Western blotting analysis.

HPLC Analysis—Derivatized samples were analyzed by HPLC using a Bondapak–NH₂ 300 × 3.9-mm column (Waters) with a methanol/acetate gradient as described previously (37). Radiolabel incorporation into GSH and GSSG was determined by scintillation counting of the corresponding HPLC fractions. The results were normalized to protein concentration to deter-
mine GSH and GSSG concentrations as described previously (38).

**Sulfate Analysis**—Sulfate concentration in the culture medium was measured using a turbidity assay as described previously (39). To measure radioactivity associated with sulfate, BaCl2 and HCl were added to a final concentration of 50 mM and 0.5 N, respectively, to 3 ml of culture medium to precipitate sulfate. After 20 min of incubation at room temperature, the barium sulfate precipitate was collected by centrifugation and washed with the same precipitating solution in water. The pellet was dissolved in 1 M NaOH, and the radioactivity was measured in a scintillation counter.

**H2S Production Assay**—Reactions for H2S production were prepared in polypropylene syringes as described previously (20) with minor modifications. Reactions containing tissue homogenate and the substrates (cysteine and homocysteine or cysteine alone as indicated in the figure legends in a total liquid volume of 1 ml) were mixed in 20-ml syringe barrels. Syringes were sealed with plungers and immediately made anaerobic by flushing the headspace with nitrogen using a three-way stopcock, and then left under nitrogen in a final volume (liquid + gas) of 20 ml. Syringes were incubated at 37 °C with gentle shaking (80 rpm) for the times indicated in the figure legends. Control reactions containing only tissue homogenate or only substrates were prepared in parallel. Aliquots (200 μl) from the gas were collected through a septum attached to the stopcock, and then injected into an HP 6890 gas chromatograph. A standard curve was prepared using pure H2S gas from Cryogenic Gases with a stock concentration of 40 ppm. The amount of H2S in the injected volume was calculated from the peak areas using the calibration coefficient obtained from the standard curve.

**Western Blotting Analysis**—Anti-HO-2 antibody (LSBio Inc.) was used at a 1:1000 dilution. Anti-xCT antibody (Santa Cruz Biotechnology) was used at a 1:1000 dilution, and anti-CBS and anti-CSE antibodies were raised in chicken against human proteins and affinity-purified in our laboratory using the respective recombinant human proteins. Frozen tissue was homogenized in 100 mM HEPES, pH 7.4, in an ice bath using a glass homogenizer.

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