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ORIGINAL ARTICLE

Primary hepatocytes from mice lacking cysteine dioxygenase show increased cysteine concentrations and higher rates of metabolism of cysteine to hydrogen sulfide and thiosulfate

Halina Jurkowska · Heather B. Roman · Lawrence L. Hirschberger · Kiyoshi Sasakura · Tetsuo Nagano · Kenjiro Hanaoka · Jakub Krijt · Martha H. Stipanuk

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Abstract The oxidation of cysteine in mammalian cells occurs by two routes: a highly regulated direct oxidation pathway in which the first step is catalyzed by cysteine dioxygenase (CDO) and by desulfhydration-oxidation pathways in which the sulfur is released in a reduced oxidation state. To assess the effect of a lack of CDO on production of hydrogen sulfide (H_2S) and thiosulfate (an intermediate in the oxidation of H_2S to sulfate) and to explore the roles of both cystathionine γ-lyase (CTH) and cystathionine β-synthase (CBS) in cysteine desulfhydration by liver, we investigated the metabolism of cysteine in hepatocytes isolated from *Cdo1*-null and wild-type mice. Hepatocytes from *Cdo1*-null mice produced more H_2S and thiosulfate than did hepatocytes from wild-type mice. The greater flux of cysteine through the cysteine desulfhydration reactions catalyzed by CTH and CBS in hepatocytes from *Cdo1*-null mice appeared to be the consequence of their higher cysteine levels, which were due to the lack of CDO and hence lack of catabolism of cysteine by the cysteinesulfinate-dependent pathways. Both CBS and CTH appeared to contribute substantially to cysteine desulfhydration, with estimates of 56 % by CBS and 44 % by CTH in hepatocytes from wild-type mice, and 63 % by CBS and 37 % by CTH in hepatocytes from *Cdo1*-null mice.

Keywords Cysteine · Cysteine dioxygenase · Cystathionine γ-lyase · Cystathionine β-synthase · Hydrogen sulfide · Thiosulfate · Mice · Hepatocytes

H. Jurkowska · H. B. Roman · L. L. Hirschberger · M. H. Stipanuk (✉)
Division of Nutritional Sciences, Cornell University, 227 Savage Hall, Ithaca, NY 14853, USA
e-mail: mhs6@cornell.edu

H. Jurkowska
Chair of Medical Biochemistry, Jagiellonian University Medical College, Kraków, Poland

K. Sasakura · T. Nagano · K. Hanaoka
Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

J. Krijt
First Faculty of Medicine and General University Hospital, Institute of Inherited Metabolic Disorders, Charles University in Prague, Prague, Czech Republic

Introduction

Cysteine catabolism is necessary to deal with the sulfur of methionine as well as that of pre-formed cysteine. Methionine sulfur is transferred to serine via the transmethylation plus transsulfuration pathways, forming cysteine. As shown in Fig. 1, the oxidation of cysteine sulfur occurs by both direct oxidation and desulfhydration-oxidation pathways in mammals (Kabil et al. 2011; Stipanuk and Ueki 2011; Stipanuk et al. 2009). Mammalian cysteine dioxygenase (CDO, encoded by the *Cdo1* gene) catalyzes the conversion of cysteine to cysteinesulfinate and, thus, initiates a pathway of cysteine catabolism in which its sulfur is oxidized at the first step (Stipanuk 2004; Stipanuk and Ueki 2011; Stipanuk et al. 2009). CDO concentration and activity are robustly regulated in response to the sulfur amino acid content of the diet (Bella and Stipanuk 1995; Dominy et al. 2006, 2008; Stipanuk et al. 2004) and act to maintain cellular cysteine levels within a homeostatic range. One important consequence of CDO's role in maintenance of low cellular cysteine levels is the prevention of excess metabolism of cysteine to hydrogen sulfide (H_2S, which exists as an equilibrium mixture of HS^- and H_2S at physiological pH) by the alternative cysteine

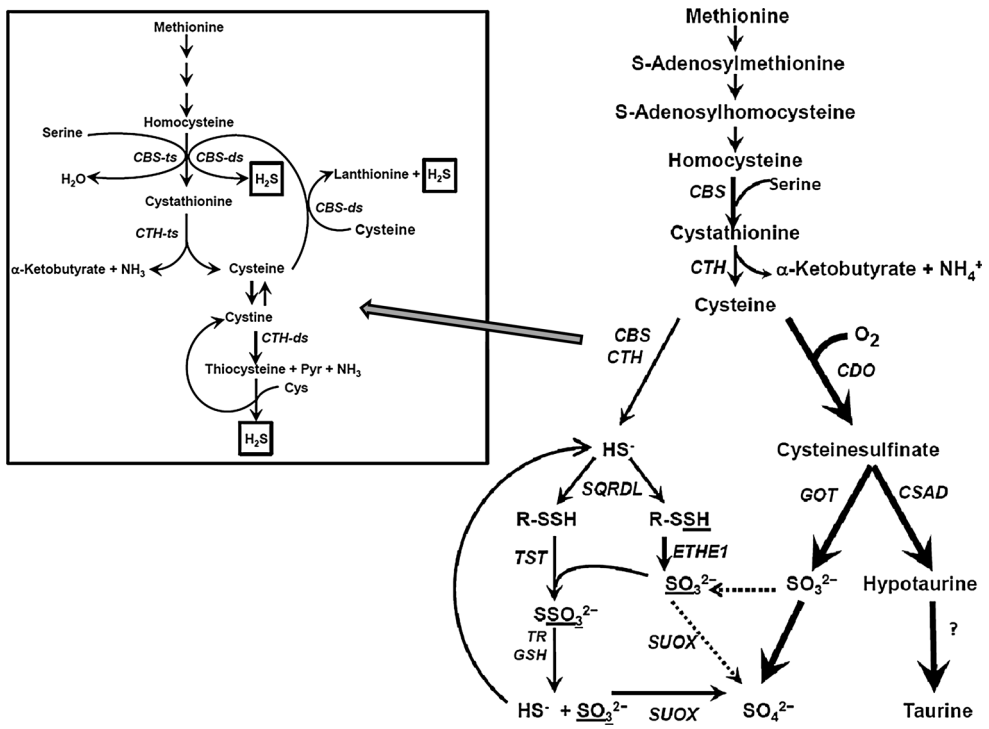

Fig. 1 Metabolism of methionine sulfur and cysteine by direct oxidation and desulfhydration-oxidation pathways. The direct oxidation pathway is usually the major route for disposal of excess cysteine, as indicated by the *heavy lines* and *arrows* in the pathway shown on the *right*. Little is known about the reaction by which hypotaurine is converted to taurine. The *dotted lines* indicate that sulfite produced in the direct oxidation pathway may be incorporated into thiosulfate, and the sulfite produced by mitochondrial sulfide oxidation may be directly oxidized to sulfate without incorporation into thiosulfate. Details of the major desulfhydration reactions catalyzed by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CTH) are shown in the *inset* on the *left*. CBS-ts, CBS-catalyzed transsulfuration; CBS-ds, CBS-catalyzed cysteine desulfhydration; CTH-ts, CTH-catalyzed transsulfuration; CTH-ds, CTH-catalyzed cysteine desulfhydration; CDO, cysteine dioxygenase; CSAD, cysteinesulfinic acid decarboxylase; ETHE1, mitochondrial persulfide dioxygenase; GOT, aspartate (cysteinesulfinate) aminotransferase; SQRDL, sulfide quinone reductase-like protein; SUOX, sulfite oxidase; TR, thiosulfate reductase; TST, thiosulfate sulfur transferase

desulfhydration pathways. Cysteine concentrations in tissues of *Cdo1*-null mice are increased to twofold to fourfold wild-type levels (Roman et al. 2013; Ueki et al. 2011).

Cysteine is also catabolized by desulfhydration pathways in which the sulfur can be released as H_2S. Cellular cysteine concentrations are a major determinant of flux of cysteine to H_2S due to the fact that the desulfhydration enzymes [i.e., cystathionine γ-lyase (CTH) and cystathionine β-synthase (CBS)] function physiologically at substrate concentrations far below their K_m values (Chiku et al. 2009; Singh et al. 2009; Chen et al. 2004; Uren et al. 1978; Yamanishi and Tuboi 1981; Kraus et al. 1978). As shown in Fig. 1, the major desulfhydration reaction catalyzed by CTH in the mouse is the net conversion of cysteine to H_2S, pyruvate and ammonia. Also shown in Fig. 1 is the major desulfhydration reaction catalyzed by CBS, the condensation of cysteine and homocysteine to form cystathionine with release of H_2S. CBS also catalyzes a minor cysteine desulfhydration reaction in which two molecules of cysteine are condensed to form lanthionine with release of H_2S; this reaction is useful because, unlike cystathionine, lanthionine is a product of cysteine desulfuration but not of the transsulfuration pathway.

The H_2S formed by cysteine desulfhydration is further oxidized in the mitochondria. As shown in Fig. 1, sulfide oxidation is initiated by action of mitochondrial sulfide quinone reductase-like protein (SQRDL, encoded by *Sqrdl*), which forms an enzyme-bound persulfide that is then either converted to sulfite by persulfide dioxygenase (encoded by the *Ethe1* gene) or transferred to sulfite by a sulfur transferase (probably thiosulfate sulfurtransferase, encoded by *Tst*) to form thiosulfate (Hildebrandt and Grieshaber 2008). Thus, two molecules of H_2S are converted to one molecule of thiosulfate. Thiosulfate is subsequently cleaved by a GSH-dependent thiosulfate reductase (TR) activity to yield sulfite + H_2S (Stipanuk 2004; Uhteg and Westley 1979), and the sulfite is oxidized to sulfate by sulfite oxidase (encoded by *Suox*), as is also the case for sulfite generated in the CDO-mediated pathway, while the sulfur atom released as H_2S must undergo another cycle of sulfide oxidation (Stipanuk 2004; Uhteg and Westley 1979). Although thiosulfate has been reported

to be an obligate intermediate in the oxidation of sulfide to sulfite (Huang et al. 1998; Koj et al. 1967; Szczepkowski et al. 1961), it seems likely that some of the sulfite produced by mitochondrial oxidation of H_2S is further oxidized to sulfate by sulfite oxidase without ever being incorporated into thiosulfate. Mitochondrial electron transport is required for sulfide oxidation, with the disulfide bond in SQRDL being regenerated by transfer of the electrons that originated in H_2S to FAD and hence to ubiquinone. Electron transfer from sulfide is coupled to O_2 consumption and ATP synthesis, making H_2S an inorganic fuel for the cell (Lagoutte et al. 2010).

We recently disrupted the *Cdo1* gene in C57BL/6 mice and reported that these mice exhibit increased levels of urinary thiosulfate and evidence of toxicity from excess production of H_2S, as well as increased tissue cystathionine and lanthionine levels that are most likely the consequence of excess cysteine desulfhydration by CBS-catalyzed reactions (Roman et al. 2013; Ueki et al. 2011). In this whole mouse model, H_2S production clearly exceeded the capacity of the *Cdo1*-null animal to oxidize sulfide to sulfate, the major excretory form of sulfur in mammals (Hildebrandt and Grieshaber 2008; Roman et al. 2013). Because taurine supplementation had little effect on the clinical phenotype of *Cdo1*-null mice, including impairments in postnatal growth and survival and skeletal and connective tissue abnormalities, we hypothesized that excess production of H_2S plays a dominant role in producing many of the phenotypic manifestations of a lack of CDO (Roman et al. 2013; Ueki et al. 2011).

To further evaluate the consequences of loss of CDO on cysteine metabolism, we investigated the metabolism of cysteine in hepatocytes isolated from *Cdo1*-null and wild-type mice. This approach allowed us to more directly assess the effect of a lack of CDO on H_2S and thiosulfate production and to explore the roles of both CTH and CBS in cysteine desulfhydration by liver cells.

Materials and methods

Primary hepatocytes and cell culture

Cdo1-null (*Cdo1*$^{-/-}$) and wild-type (*Cdo1*$^{+/+}$) mice for this study were generated by crossing C57BL/6 *Cdo1*$^{+/-}$ male and female mice as described previously (Roman et al. 2013; Ueki et al. 2011). All experimental procedures involving live animals were conducted with the approval of the Cornell University Institutional Animal Care and Use Committee (#2009-0138). Mice (42–100 days of age) were euthanized with an overdose of isoflurane, and hepatocytes were quickly isolated by a modification of the method described by Dominy et al. (2012). The liver was perfused with prewarmed calcium/magnesium-free Hank's balanced salt solution (CMF-HBSS) containing 25 mM HEPES, 500 mM EDTA, 100 units/ml penicillin, and 100 μg/ml streptomycin for 6 min at a flow rate of 3 ml/min followed by perfusion with pre-warmed CMF-HBSS containing 25 mM HEPES, 2 mM $CaCl_2$, 100 units/ml penicillin, 100 μg/ml streptomycin and 0.75 mg/ml collagenase (Sigma-Aldrich C8051) for an additional 6 min. After removal and mechanical dispersion of liver tissue, cells were suspended in ice-cold plating medium, passed through a 70 μm cell strainer (Corning 352350), and pelleted by centrifugation at $60\times g$ for 3 min at 4 °C. After resuspension of the cell pellet in Percoll (Sigma-Aldrich P4937) diluted 1:1 in plating medium, cells were pelleted by centrifugation at $800\times g$ for 4 min at 4 °C and subsequently washed three times in plating medium with centrifugation at $60\times g$ for 3 min at 4 °C to remove red blood cells. Viability of isolated hepatocytes was determined by trypan blue exclusion and was routinely greater than 90 %.

Isolated hepatocytes were plated on collagen-coated 6-well plates (4×10^4 cells per cm^2) in DMEM (Gibco #12800-017, containing 1 mM pyruvate, 25 mM glucose and 4 mM glutamine) supplemented with 10 % (vol/vol) fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 1 μM dexamethasone, 0.1 μM insulin and an additional 1 mM sodium pyruvate and then incubated at 37 °C in an atmosphere of 5 % CO_2. At 2 h after plating, the plating medium was replaced with fresh culture medium consisting of DMEM supplemented with 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.2 % (wt/vol) of bovine serum albumin fraction V (instead of FBS),100 nM dexamethasone and 1 nM insulin (Sigma-Aldrich #I9278). After 24 h, culture medium was replaced with treatment medium (total volume of 3.0 ml per well). In the first series of experiments, treatment medium was identical to the culture medium except that it also included 0.05 mM bathocuproine disulfonic acid (BCS, Sigma-Aldrich), and 0.3 mM L-cysteine (Cys, MP Biomedicals, Inc.) was added as indicated. In the second series of experiments, all treatment media contained 0.3 mM Cys and 0.05 mM BCS, and either 1 mM or 2 mM DL-propargylglycine (PPG, Sigma-Aldrich) was added as indicated.

Hepatocytes were cultured in the treatment medium for 24 or 48 h prior to harvest; because primary hepatocytes do not grow or proliferate in culture and because we wanted to measure the accumulation of exported metabolites, medium was not changed between 24 and 48 h. For measurement of metabolite levels in the medium, the medium was removed prior to harvesting the hepatocytes and immediately frozen and stored at −80 °C for later analysis. For measurement of metabolite levels in cells, hepatocytes were harvested by treating with 0.25 % trypsin (Gibco #25200), washing with

ice-cold PBS to remove trypsin, suspending the released hepatocytes in ice-cold PBS and centrifuging the cell suspension at 1,600×*g* for 10 min at 4 °C to obtain the pelleted cells, which were immediately frozen and stored at −80 °C for later analysis. For assay of enzyme activities, cells were washed twice with ice-cold PBS, harvested into 0.5 ml of PBS, centrifuged at 1,600×*g* for 10 min at 4 °C to obtain the pelleted cells, which were stored at −80 °C until assays were performed. For measurement of enzyme abundance by Western blotting, hepatocytes were washed twice with ice-cold PBS and harvested into lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 % Nonidet P-40) supplemented with 1× Complete Protease Inhibitor Cocktail (Roche). The lysate was frozen and stored at −80 °C until analysis.

Production of hydrogen sulfide from cysteine

Production of H_2S from cysteine was measured in cells cultured as described above except 24-well plates were used, with a cell plating density of 3.75×10^4 per cm^2, and 25 μM HSip-1 (stock, 6 mM HSip-1 in H_2O) was added to the treatment medium (total volume of 1.0 ml per well). HSip-1 was synthesized as described previously (Sasakura et al. 2011). After 24 and 48 h of culture, 100 μl of medium from each well was transferred to an opaque 96 well plate, and the fluorescence of HSip-1 was measured using a Tecan SPECTRAfluor Plus plate reader at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Fluorescence was corrected for that in wells that contained an identical reaction mixture but no cells. Confirmation of the proportionality of the assay was done by evaluating HSip-1 fluorescence for wells containing medium and all additions (but no cells) and to which various amounts of Na_2S monohydrate (≥98 % purity, Sigma-Aldrich #S2006) had been added at zero time; standard curves generated in this way were used to approximate the amount of H_2S that was trapped by the probe in the hepatocyte studies. Confirmation of the specificity of the HSip-1 assay for H_2S was done by evaluating the effectiveness of 5 mM PPG in inhibiting H_2S production in HEK293T cells transfected with pCMV6-Entry vector encoding the C-terminal Myc- and DDK-tagged ORF clone of murine *Cth* (Origene #MR206244) or with empty vector.

Determination of cell viability

The effect of culture conditions on cell viability was assessed by measuring the leakage of lactate dehydrogenase (LDH) from dead or dying cells using a Cytotoxicity Detection Kit (Roche) as described previously (Jurkowska et al. 2011). Only cell preparations and treatments that yielded LDH leakage less than 10 % were used.

Measurement of total aminothiols, cystathionine and lanthionine

Frozen hepatocytes were thawed on ice and resuspended in 0.1 M phosphate buffer, pH 7.5, containing 2 mM *tris*(2-carboxyethyl) phosphine (TCEP) to reduce disulfide bonds linking thiols to protein sulfhydryl groups or to each other. The mixture was then sonicated for three 5-s intervals at 4 °C, and the homogenates were used for HPLC determinations and for determination of the level of protein. Frozen samples of culture medium were thawed on ice, and 2 μl of 200 mM TCEP (in 125 mM borate buffer, pH 9.0) was added to 200 μl of medium to yield a final concentration of 2 mM TCEP for reducing disulfide bonds.

For the determination of total cysteine, reduced homogenate or medium was mixed with an equal volume of 5 % (wt/vol) trichloroacetic acid, and the mixture was centrifuged for 15 min at 15,000×*g* at 4 °C to obtain the acid supernatant. Tissue total cysteine was measured by HPLC as described previously (Ueki et al. 2012).

For the measurement of cystathionine and lanthionine, the prepared homogenate or medium was mixed with one volume of 5 % (wt/vol) sulfosalicylic acid, and the mixture was centrifuged at 15,000×*g* for 15 min at 4 °C to obtain the acid supernatant. Cystathionine was measured by HPLC as described previously (Ueki et al. 2012). Lanthionine was determined by LC–MS/MS as previously described (Roman et al. 2013).

Measurement of thiosulfate

Thiosulfate was determined in culture medium by the method of Shih et al. (1979). Prior to analysis, the samples of culture medium were deproteinated using Amicon Ultra 0.5-ml centrifugal filters with a 3 kDa cutoff (Millipore) with centrifugation for 20 min at 14,000×*g*.

Enzyme activity assays

On the day of analysis, frozen hepatocyte pellets were resuspended in 0.1 M phosphate buffer, pH 7.5, containing 1× Complete Protease Inhibitor Cocktail (Roche) and sonicated for three 5-s intervals at 4 °C. After centrifugation at 1,600×*g* for 10 min, the supernatant was used for the determination of the activity of sulfurtransferases (mercaptopyruvate sulfurtransferase or MPST, CTH and TST). The activity of MPST was assayed according to method of Valentine and Frankenfeld (1974) with some modifications described by Wróbel et al. (2004). CTH activity was determined according to Matsuo and Greenberg (1958) as modified by Czubak et al. (2002). TST (rhodanese) activity was assayed by the method of Sörbo (1957) as described by Wróbel et al. (2004).

Western blotting

Cell lysates were centrifuged at 20,000×*g* for 15 min at 4 °C. Aliquots containing 25 µg of total protein were separated by SDS-PAGE (12 %, w/v, polyacrylamide), and then, the protein bands were transferred onto a 0.45-µm Immobilon-FL PVDF membrane (Millipore Corp.). Membranes were blocked using blocking buffer for near infrared fluorescent westerns (LI-COR Biosciences) and blotted for immunoreactive proteins. Sources and dilutions of primary antibodies were as follows: anti-CBS (1: 800; Proteintech Group), anti-CTH (1: 800; Proteintech Group), anti-MPST (1: 800; Santa Cruz Biotechnology), anti-SQRDL (1: 1,000; Proteintech Group), anti-TST (1: 1,000; Proteintech Group, USA), ETHE1 (1:400; Thermo Scientific) and anti-actin (1: 1,000; Cell Signaling Technology). An infrared fluorescent dye-labeled secondary antibody (IRDye, LI-COR Biosciences) and the Odyssey direct infrared imaging system and software (LI-COR Biosciences) were used to visualize and quantify the relative abundance of each protein. Protein abundances were normalized by actin abundance and then expressed as fold of the wild-type control value.

Protein assay

The protein level of cell homogenates or cell lysates was determined by the BCA Protein Assay Kit (Thermo Scientific/Pierce) using bovine serum albumin (BSA) as a standard.

Statistical analysis

Results were analyzed as a full factorial least squares model using JMP version 10 (SAS, Cary, NC, USA). Post hoc individual pairwise comparisons of least squares means in the model were made using Tukey's comparisons; comparisons were considered significant at $p < 0.05$. Values for cysteine, cystathionine, thiosulfate and H_2S were log transformed prior to statistical analysis.

Results

In order to measure H_2S in hepatocytes and in the medium, we needed an assay that would trap H_2S as it was evolved in the cell culture system. We explored the use of HSip-1, a highly selective and sensitive fluorescent probe based on azamacrocyclic copper(II) ion complex chemistry (Sasakura et al. 2011). HSip-1 was added to the medium at the start of the treatment period, and fluorescence developed as the probe trapped the H_2S released by the cells. This approach makes use of the fact that H_2S is membrane permeable and that as H_2S leaves the cell and is trapped by the probe, remaining HS^- is protonated to maintain the HS^-/H_2S chemical equilibrium such that release of H_2S from the cell is promoted (Kolluru et al. 2013; Nagy et al. 2014; Cuevasanta et al. 2012). As shown in Fig. 2a, HSip-1 fluorescence was proportionate to the amount of sodium sulfide (Na_2S) added to the culture medium at zero time, and this HSip-1 fluorescence was stable between 24 and 48 h. Additionally, HEK293T cells that overexpressed CTH, one of the enzymes that catalyzed cysteine desulfhydration, produced 4- to 6-fold as much H_2S as control cells transfected with an empty vector, as shown in Fig. 2b. To further validate the effectiveness and specificity of HSip-1, we tested the effect of addition of PPG, an irreversible inhibitor of CTH (Abeles and Walsh 1973; Rao et al. 1990; Stipanuk and Beck 1982; Washtien and Abeles 1977). PPG reduced H_2S fluorescence by 95 % for HEK293T cells expressing recombinant CTH and by 65 % for control cells transfected with the empty vector (Fig. 2b). Residual H_2S production is most likely attributable to the cysteine desulfhydration activity of CBS.

The HSip-1 probe was then used to measure the accumulation of H_2S in the medium when wild-type and *Cdo1*-null hepatocytes were incubated in standard DMEM or in DMEM supplemented with 0.3 mM Cys. As shown in Fig. 3a, H_2S released into the medium by *Cdo1*-null hepatocytes was greater than that for wild-type hepatocytes, with *Cdo1*-null cells cultured in standard DMEM releasing 2.8-fold as much H_2S and *Cdo1*-null cells cultured in DMEM + 0.3 mM Cys releasing 1.7-fold as much H_2S as did the wild-type hepatocytes over 48 h of culture. H_2S level was also influenced by the concentration of total cysteine in the medium, with H_2S accumulation tending to be higher in cells cultured in medium to which 0.3 mM Cys had been added (0.7 mM total cysteine) compared to standard DMEM (0.2 mM cystine, or 0.4 mM total cysteine). [Because the solubility limit of cystine is ~0.1 mM, we added the additional cysteine as the free thiol, along with BCS, in an effort to increase the total soluble cysteine concentration in the medium.] Although exogenous Na_2S added to the medium is not the same as H_2S evolved slowly over time, we nevertheless used Na_2S standard curves to approximate the amount of H_2S trapped by HSip-1. Using this approach, we show that H_2S trapped in the medium over a 48-h period ranged from about 200 to 1,000 nmol/mg cellular protein. We also attempted to measure intracellular levels of H_2S using diacetylated HSip-1 (HSip-1 DA, Sasakura et al. 2011), but were unable to detect fluorescence above background for our cell extracts.

Thiosulfate accumulation in the medium followed a similar pattern as that for H_2S except that the fold difference between wild-type and *Cdo1*-null hepatocytes was less than for H_2S. As shown in Fig. 3b, the amount of thiosulfate that accumulated in the medium of *Cdo1*-null

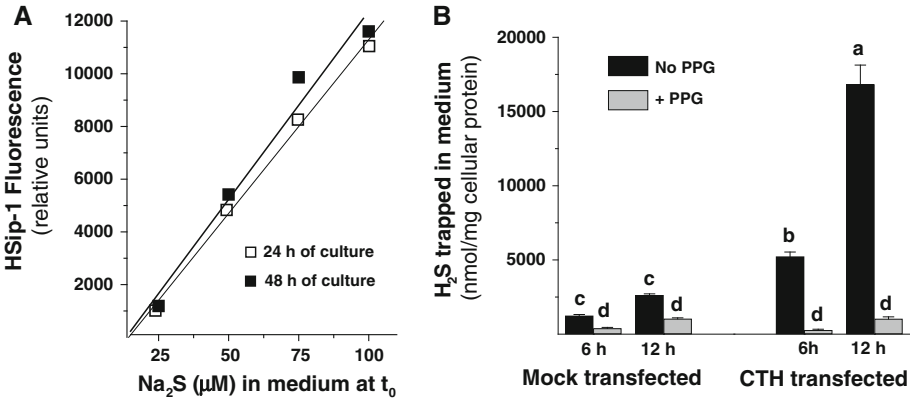

Fig. 2 HSip-1 method for assay of H_2S production over time. **a** An example of a standard curve for HSip-1 fluorescence as a function of amount of Na_2S added to the medium at time zero. DMEM to which 0.3 mM Cys, 0.05 mM BCS, 0.03 mM HSip-1, and varying levels of added Na_2S monohydrate, but no cells, had been added was maintained under standard culture conditions. Aliquots of medium were removed after 24 and 48 h of culture to measure HSip-1 fluorescence. Fluorescence developed over time and was stable between 24 and 48 h. Fluorescence readings were corrected for blank cultures that did not contain any Na_2S. **b** Demonstration of specificity of HSip-1 assay for quantitation of H_2S. HEK293T cells were transfected with CTH cDNA or empty vector and then incubated in standard DMEM with 0.05 mM BCS and 0.03 mM HSip-1, and with/without 5 mM DL-propargylglycine (PPG). Fluorescence was read after removing 100 μl of medium from each well at 6 h and again at 12 h. Fluorescence readings were corrected for blank cultures that did not contain cells. Values are mean ± SEM for 3 replicates. Values not denoted by the *same letter* are significantly different ($p \leq 0.05$) by Tukey's mean comparison procedure

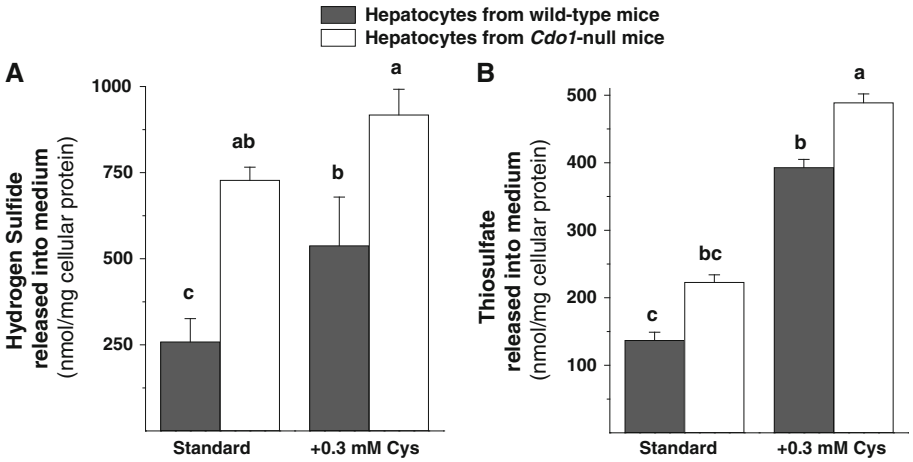

Fig. 3 **a** H_2S production in hepatocytes from wild-type and *Cdo1*-null mice as assessed by trapping and measurement of H_2S released into the medium using the HSip-1 probe. Hepatocytes were cultured in standard DMEM or in DMEM supplemented with 0.3 mM Cys for 48 h. Medium also contained 0.05 mM BCS and 25 μM HSip-1. Fluorescence was read after removing 100 μl of medium from each well at 48 h. Fluorescence readings were corrected for blank cultures that did not contain cells. **b** Thiosulfate production in hepatocytes from wild-type and *Cdo1*-null mice as assessed by accumulation of thiosulfate in the medium. Hepatocytes were cultured in standard DMEM or in DMEM supplemented with 0.3 mM Cys for 48 h. Medium also contained 0.05 mM BCS. Results are the mean ± SEM from 6 to 7 independent experiments. Values for a metabolite that are not denoted by the *same letter* are significantly different ($p \leq 0.05$) by Tukey's mean comparison procedure

hepatocytes over 48 h was 1.6-fold that for wild-type cells when hepatocytes were cultured in standard DMEM and 1.2-fold when hepatocytes were cultured in +Cys medium. Thiosulfate production also increased in response to addition of supplemental cysteine to the medium in both wild-type and *Cdo1*-null hepatocytes. Thiosulfate accumulation in the medium ranged from about 100 to 500 nmol/mg protein. As for intracellular H_2S levels, intracellular thiosulfate levels were too low to measure with our assay.

The cysteine desulfhydration enzymes do not appear to be highly regulated in response to the absence of CDO. Neither CTH, CBS nor MPST abundance differed significantly in hepatocytes isolated from wild-type versus *Cdo1*-null mice, as shown in Fig. 4a, c. Neither CTH nor MPST activity was different between *Cdo1*-null and wild-

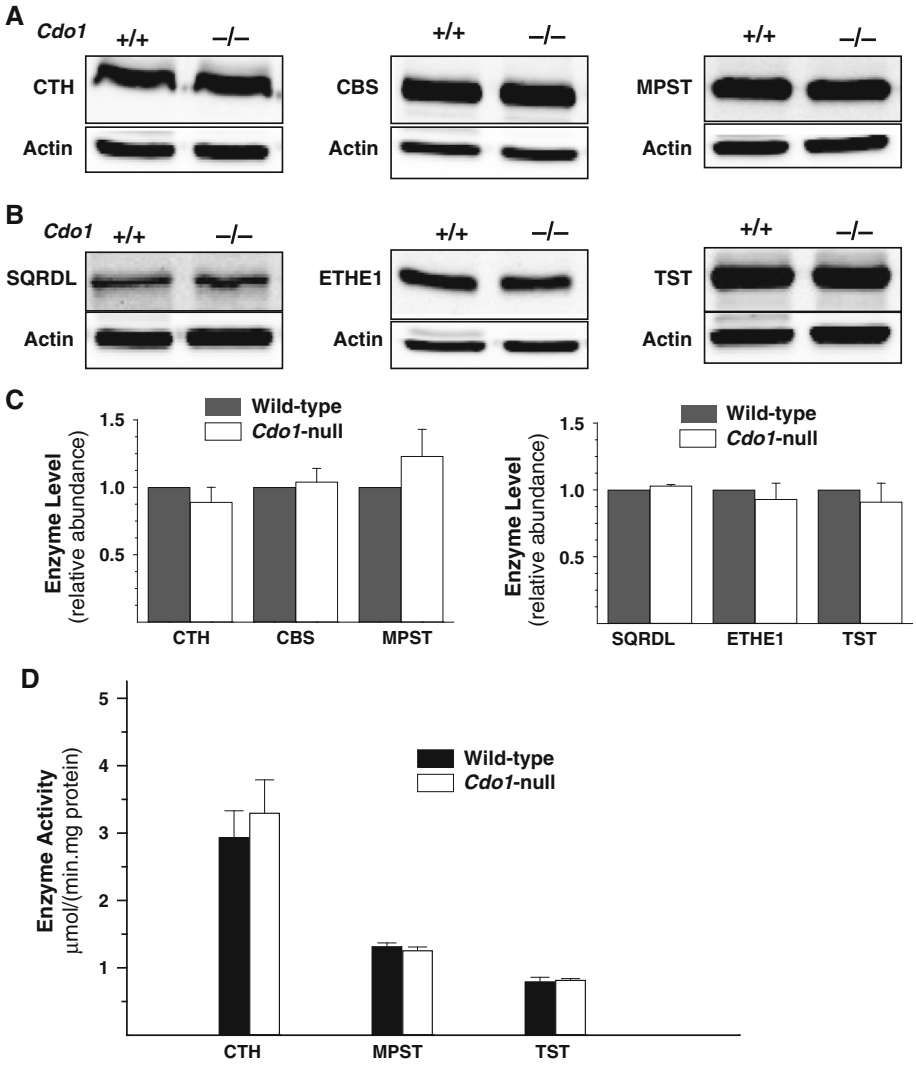

Fig. 4 Relative amounts of enzymes involved in cysteine desulfhydration and sulfide oxidation in cultured hepatocytes of wild-type and *Cdo1*-null mice. **a** Western blots of enzymes involved in cysteine desulfhydration: (cystathionine γ-lyase (CTH), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (MPST). **b** Western blots of mitochondrial enzymes involved in sulfide oxidation/sulfide quinone reductase (SQRDL), persulfide dioxygenase (ETHE1) and thiosulfate sulfurtransferase (TST, rhodanese). Representative blots are shown. **c** Densities of bands in Western blots were normalized using β-actin. Values in the bar graphs represent mean ± SEM for 3 separate experiments. **d** Activities of CTH, MPST and TST measured in hepatocytes isolated from wild-type and *Cdo1*-null mice after the cells had been cultured in standard DMEM for 24 h. Activities were measured using V_{max} assays

type hepatocytes (Fig. 4d). The activity of CBS was not assayed by a V_{max} assay due to the large differences the levels of allosteric regulators, such as *S*-adenosylmethionine, have on its activity. On the other hand, cellular total cysteine concentrations in *Cdo1*-null hepatocytes were 2.2- to 2.9-fold those in wild-type hepatocytes (Fig. 5), and the increased amount of H_2S released by hepatocytes into the medium by *Cdo1*-null hepatocytes is presumably due to the increase in substrate concentration for the desulfhydration enzymes, consistent with these enzymes having K_m*s* that are substantially above intracellular cysteine concentrations. It does not appear that the increased amount of H_2S produced by *Cdo1*-null hepatocytes is due to a lower capacity for H_2S oxidation, because the relative abundances of mitochondrial enzymes involved in sulfide oxidation (SQRDL, ETHE1 and TST) were similar in hepatocytes from *Cdo1*-null and wild-type mice, as shown in Fig. 4b, c. The activity of TST in cultured hepatocytes was also assayed in vitro and was observed to be similar for cells from wild-type and *Cdo1*-null mice (Fig. 4d).

To assess the relative roles of CTH and CBS in catalysis of cysteine desulfhydration in hepatocytes, we first assessed the effect of PPG on H_2S and thiosulfate accumulation in the medium (Fig. 6). PPG is widely used as a specific inhibitor of CTH both in vitro and in vivo (Washtien and Abeles 1977; Abeles and Walsh 1973; Rao et al. 1990; Stipanuk and Beck 1982). Although H_2S accumulation was slightly lower in cells treated with 2 mM PPG, the 13 % (wild-type) and 21 % (null) apparent decreases were not statistically significant, perhaps due to the relatively high variability of the H_2S assay method. However, addition of either 1 or 2 mM PPG significantly decreased thiosulfate accumulation in the medium for incubations of both wild-type and *Cdo1*-null cells, with thiosulfate accumulation being 27 and 30 % lower, respectively, for cells treated with 2 mM PPG. The H_2S and thiosulfate results together suggest that CTH is not the major enzyme that catalyzes

cysteine desulfhydration in murine liver. These estimates may be influenced, however, by the fact that PPG treatment increased cysteine levels to 2.0-fold (24 h) basal levels in hepatocytes from wild-type mice but not in hepatocytes from *Cdo1*-null mice in which they were already increased (Fig. 7). After 48 h of incubation, cellular cysteine levels in PPG-treated wild-type cells were not significantly greater than those in the non-treated wild-type cells.

Both intracellular and medium cystathionine levels were greater in *Cdo1*-null cells than in wild-type hepatocytes, with cellular cystathionine levels being 62 % higher and medium cystathionine levels being 55 % higher (Fig. 8a, b). The higher cystathionine levels are consistent with a role of CBS in cysteine desulfhydration, with rates of cysteine desulfhydration by CBS being greater in hepatocytes from *Cdo1*-null mice. Lanthionine levels in the medium at 24 h were 50 % higher in *Cdo1*-null hepatocytes compared to wild-type hepatocytes (Fig. 8c). Lanthionine levels in the cell extracts were too low to measure. Although the majority of the cystathionine generated in cells is rapidly hydrolyzed to cysteine, α-ketobutyrate and ammonia, and the amount of cystathionine that accumulated in the medium was low relative to H_2S and thiosulfate production, the higher level cystathionine that accumulated both intracellularly and in the medium for cultures of *Cdo1*-null hepatocytes compared to wild-type hepatocytes supports an important role of CBS in cysteine desulfhydration in murine liver. This conclusion involves the assumption that the increase in cystathionine in *Cdo1*-null cells is due to an increase in desulfhydration and not an increase in transsulfuration, but the similar accumulation of lanthionine, which can be produced by a CBS-catalyzed cysteine desulfhydration reaction but not by transsulfuration reactions, provides further support for our conclusion.

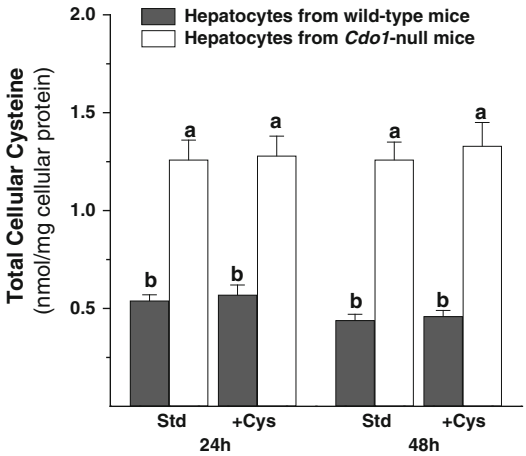

Fig. 5 Total cellular cysteine levels in hepatocytes from wild-type and *Cdo1*-null mice. Hepatocytes were cultured in standard DMEM or in DMEM supplemented with 0.3 mM Cys for 24 h or 48 h. Results are the mean ± SEM from 3 to 5 independent experiments. Values for a metabolite that are not denoted by the *same letter* are significantly different ($p \leq 0.05$) by Tukey's mean comparison procedure

Discussion

Previous work with the *Cdo1*-null mouse provided indirect evidence for enhanced flux of cysteine through desulfhydration reactions, leading to production of excess levels of H_2S. Enhanced flux of cysteine through desulfhydration pathways was associated with increased urinary excretion of thiosulfate, suggesting that H_2S and thiosulfate were being produced at rates that exceeded the capacity for the further oxidation of thiosulfate to sulfate. Thiosulfate is formed from H_2S in the mitochondria by the transfer of enzyme-bound persulfide sulfur from SQRDL to sulfite, which can be formed from another molecule of SQRDL-bound persulfide sulfur by ETHE1-catalyzed dioxygenation (Linden et al. 2012; Tiranti et al. 2009). Thiosulfate accumulates when the rate of thiosulfate formation exceeds

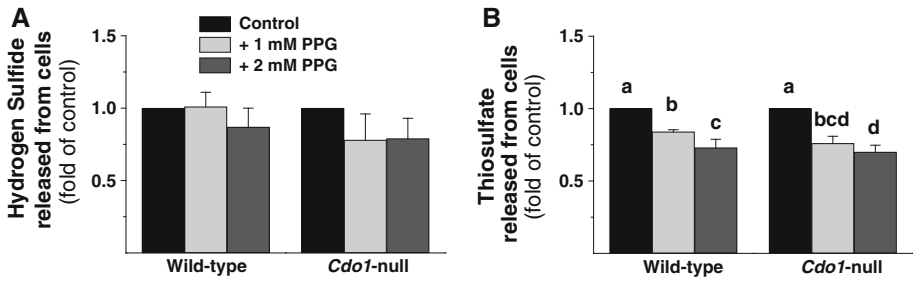

Fig. 6 **a** H_2S production in hepatocytes from wild-type and *Cdo1*-null mice as assessed by trapping and measurement of H_2S released into the medium with the HSip-1 probe. **b** Thiosulfate production in hepatocytes from wild-type and *Cdo1*-null mice as assessed by accumulation of thiosulfate in the medium. Hepatocytes were cultured in DMEM supplemented with 0.3 mM Cys and with 1 mM PPG, 2 mM PPG or no PPG for 24 h. Results are the mean ± SEM from 3 independent experiments. Values for thiosulfate that are not denoted by the *same letter* are significantly different ($p \leq 0.05$) by Tukey's mean comparison procedure; values for H_2S did not reach statistical significance for individual comparisons

the capacity of the cell for further oxidation of thiosulfate by GSH-dependent thiosulfate reductase and sulfite oxidase (Roman et al. 2013; Stipanuk 2004; Uhteg and Westley 1979). A second observation implying the excess production of H_2S in the *Cdo1*-null mouse was the inhibition of mitochondrial cytochrome *c* oxidase activity and the enhanced degradation of cytochrome *c* oxidase subunits due to destabilization of the complex. The inhibition of cytochrome *c* oxidase activity by H_2S and the consequent accelerated degradation of cytochrome *c* oxidase subunits by H_2S is well established (Di Meo et al. 2011; Roman et al. 2013). With the isolated hepatocyte model, we have now been able to directly show that hepatocytes from *Cdo1*-null mice produce more H_2S and thiosulfate than those from wild-type mice, as judged by measurement of the H_2S and thiosulfate released into the medium. The concurrent increase in the release of both H_2S and thiosulfate into the medium by hepatocytes from *Cdo1*-null mice supports the validity of the HSip-1 method for assessment of the production of H_2S as well as the validity of using thiosulfate accumulation as a proxy for H_2S production under conditions where SQRDL and ETHE1 actions are not blocked.

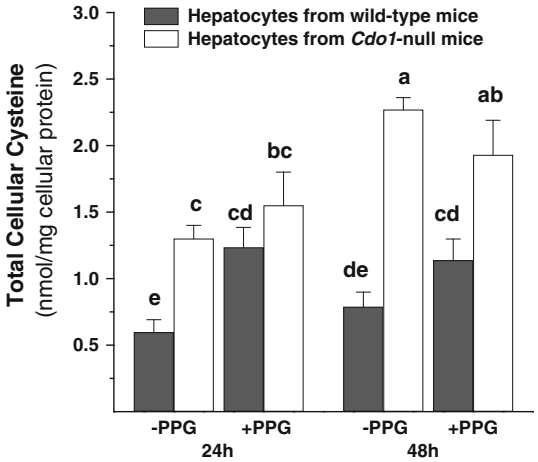

Fig. 7 Effect of PPG on total cellular cysteine levels in hepatocytes from wild-type and *Cdo1*-null mice. Hepatocytes were cultured in DMEM supplemented with 0.3 mM Cys with or without 2 mM PPG for 24 h or 48 h. Results are the mean ± SEM from 3 independent experiments. Values for a metabolite that are not denoted by the *same letter* are significantly different ($p \leq 0.05$) by Tukey's mean comparison procedure

The comparison of fluorescence yield when H_2S produced from Na_2S added to the medium was trapped versus when H_2S produced within hepatocytes was trapped suggests that H_2S production by hepatocytes is substantial. By our estimates, the amount of H_2S trapped in the medium over 48 h ranged from 200 to 1,000 nmol/mg cellular protein, which roughly equates to 23–115 μmol/g liver over 48 h or a minimum rate of production of 8–40 nmol min^{-1} g $hepatocytes^{-1}$. Thiosulfate accumulation in the medium ranged from about 100 to 500 nmol/mg protein, which roughly equates to 11.5–57.5 μmol/g liver over 48 h or a minimum rate of production of 4–20 nmol min^{-1} g $hepatocytes^{-1}$. Assuming both sulfur atoms of thiosulfate originate from H_2S, the amount of thiosulfate that accumulated in the medium equates to production of an additional 8–40 nmol min^{-1} g $hepatocytes^{-1}$. The actual rate of H_2S production undoubtedly exceeds the sum of that accounted for by H_2S and thiosulfate released into the medium (i.e., 16–80 nmol min^{-1} g $hepatocytes^{-1}$), because most of the H_2S and thiosulfate would presumably be completely oxidized to sulfate in the mitochondria of the cells and not released into the medium. Although there are many-fold disagreements among published values for liver hydrogen sulfide concentration (Ang

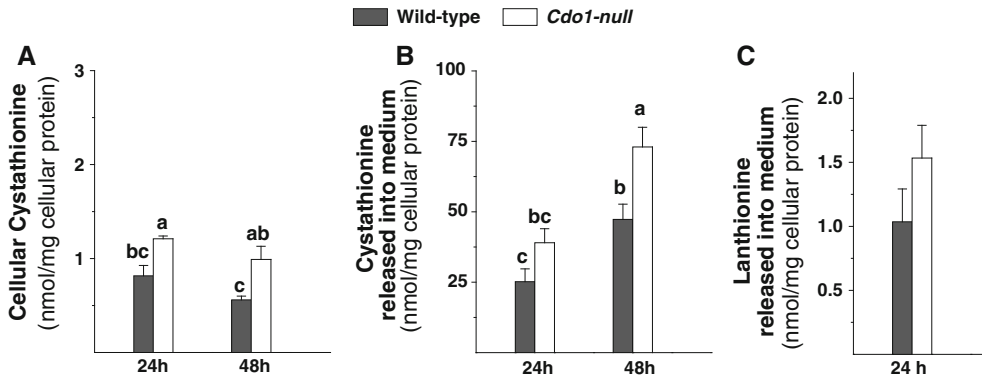

Fig. 8 Cellular (**a**) and medium (**b**) cystathionine levels and medium lanthionine levels (**c**) for cultures of hepatocytes from wild-type and *Cdo1*-null mice that were cultured in DMEM supplemented with 0.3 mM Cys. Cystathionine was measured at both 24 h and 48 h; lanthionine was measured only at 24 h. Results are the mean ± SEM from 3 to 5 independent experiments. Values for cystathionine that are not denoted by the *same letter* are significantly different ($p \leq 0.05$) by Tukey's mean comparison procedure; values for lanthionine did not reach statistical significance

et al. 2012; Dorman et al. 2002; Levitt et al. 2011), the in vivo production of H_2S in *Cdo1*-null mice clearly is sufficient to partially inhibit cytochrome *c* oxidase in liver and other tissues (Roman et al. 2013), and cytochrome *c* oxidase inhibition has been demonstrated to require a 1 μM (~1 nmol/g) concentration of H_2S (Nicholson et al. 1998). Thus, our whole animal data are consistent with low nanomolar tissue concentrations of H_2S in *Cdo1*-null mice. It would appear that H_2S levels represent a balance between the rate of H_2S production and H_2S removal and that the increased rate of H_2S production in *Cdo1*-null cells exceeds the capacity for removal such that steady-state levels of H_2S are increased in tissues of mice lacking CDO.

We proposed that the increased metabolism of cysteine through cysteine desulfhydration pathways in the *Cdo1*-null mouse is mainly a consequence of the increased cellular cysteine concentrations that result from the lack of CDO and hence the block in metabolism of cysteine via cysteinesulfinate-dependent pathways (Roman et al. 2013). These studies with isolated hepatocytes provide further support for this conclusion. The accumulation of H_2S and thiosulfate in medium both increased with addition of Cys to the standard culture medium. In addition, hepatocytes from *Cdo1*-null mice maintained onefold to twofold higher intracellular levels of cysteine than did hepatocytes from wild-type cells, and these higher levels of cysteine were associated with higher rates of H_2S and thiosulfate formation as assessed by measurement of these compounds in the medium.

Differences between *Cdo1*-null and wild-type hepatocytes cannot be attributed to differences in the abundances of desulfhydration enzymes or sulfide oxidizing enzymes. As observed for liver (Roman et al. 2013), there was no difference in the abundance of either CTH or CBS protein between hepatocytes from mice of the two genotypes. Although it is unlikely that MPST is a major contributor to cysteine desulfhydration in hepatocytes (Stipanuk 1986, 2004), its abundance was also measured and found not to differ in hepatocytes from wild-type and *Cdo1*-null mice. Additionally, there was no difference in measured CTH or MPST activity in cultured hepatocytes from *Cdo1*-null and wild-type mice. Similarly, the lack of any differences in the abundance of SQRDL, ETHE1 or TST suggests that the capacity for sulfide removal is similar for *Cdo1*-null and wild-type hepatocytes.

Both CTH and CBS are thought to play substantial roles in cysteine desulfhydration, but dramatic tissue differences in their relative roles have been demonstrated (Shibuya et al. 2009; Kabil et al. 2011; Yang et al. 2008; Roman et al. 2013). Based on thiosulfate measurements, PPG appeared to block total cysteine desulfhydration by 27 % in wild-type hepatocytes and 30 % in *Cdo1*-null hepatocytes, indicating that, under the range of substrate concentrations we used, CTH catalyzed less than half of the total cysteine desulfhydration in hepatocytes. However, the actual contribution of CTH in wild-type cells might be greater than observed because PPG treatment also resulted in a doubling of the cellular cysteine levels in the wild-type, but not the in the *Cdo1*-null cells. An increase in the cellular cysteine concentration would be expected to favor cysteine desulfhydration, in this case by CBS which is not inhibited by PPG, in wild-type cells. The K_{m1} of mammalian CBS for cysteine (6–7 mM for human CBS) is much above normal intracellular levels (Chen et al. 2004; Singh et al. 2009). Thus, PPG treatment of wild-type hepatocytes might increase CBS-catalyzed desulfhydration at the same time that CTH-catalyzed desulfhydration is blocked, resulting in an underestimation of the true contribution of CTH in wild-type hepatocytes. If we assume that the amount of lanthionine that accumulated in the medium during the incubation of hepatocytes with cysteine is proportionate to CBS activity, we can estimate that CBS-mediated desulfhydration increased by about 30 % for wild-type cells treated with PPG [i.e., 4.6 ± 0.4 (mean ± SEM) compared to 3.6 ± 0.5 nmol/mg cellular protein]. This would mean that the residual non-CTH cysteine desulfhydration activity (73 % for wild-type hepatocytes) would actually be just 56 % under non-PPG conditions. Thus, we estimate that CTH and CBS make roughly equal contributions to cysteine desulfhydration in murine wild-type hepatocytes.

On the surface, the conclusion that CBS makes a large contribution (i.e., 56 % in wild-type hepatocytes or 63 % in *Cdo1*-null hepatocytes) to hepatic desulfhydration is at odds with the report of Kabil et al. (2011) that CBS-catalyzed desulfhydration accounts for 3 % or less of the total H_2S production in mouse liver. The latter estimate was based on determinations of the molar ratios of CBS to CTH in mouse liver (Kabil et al. 2011) and simulations of the contributions of both enzymes to H_2S production (Chiku et al. 2009; Singh et al. 2009). The use of human enzymes as standards, the use of antibodies against human enzymes as probes in the quantification of mouse enzymes and the use of kinetic parameters for human enzymes, especially those for CTH substrate affinities, in the kinetic simulations applied to the mouse liver enzymes could underlie the dramatically lower estimate made by Kabil et al. (2011) for CBS's role in cysteine desulfhydration in mouse liver. Rat CTH reportedly uses cystine for generation of H_2S by conversion of cystine to thiocysteine + pyruvate + NH_3, with thiocysteine reacting with cysteine or another thiol (RSH) to form cystine or CySSR with elimination of H_2S (Cavallini et al. 1962), whereas human CTH uses cysteine as substrate, converting it directly to pyruvate + H_2S + NH_3 (Chiku et al. 2009). Our results for mouse hepatocytes clearly support a substantial role of CBS in hepatic cysteine desulfhydration and

agree with the clear role of CBS-catalyzed cysteine desulfhydration reported for liver and pancreas of wild-type and *Cdo1*-null mice, which were based on accumulation of both cystathionine and lanthionine in the expected ratio for CBS-catalyzed desulfhydration reactions in various tissues of *Cdo1*-null mice (Roman et al. 2013). In summary, we have demonstrated that mouse hepatocytes produce both H_2S and thiosulfate from cysteine and that the amounts of H_2S and thiosulfate formed by cysteine desulfhydration pathways are greater in hepatocytes from *Cdo1*-null mice than in those from wild-type mice. The rates of flux through the cysteine desulfhydration reactions catalyzed by CTH and CBS appear to be the consequence of the higher cysteine levels in cells lacking CDO and hence the capacity for cysteine catabolism through the cysteinesulfinate-dependent pathways. Furthermore, CBS, as well as CTH, makes a substantial contribution to cysteine desulfhydration in murine hepatocytes.

The demonstration that CTH and CBS use cysteine as substrate and catalyze cysteine desulfhydration reactions under physiological conditions and the observation that the rate of flux of cysteine through desulfhydration pathways is highly responsive to changes in cellular cysteine concentrations have clinical relevance because H_2S has been shown to be an essential regulatory molecule affecting numerous body systems as well as a potent cellular toxin (Cuevasanta et al. 2012; Olson 2012; Predmore et al. 2012; Whiteman et al. 2011; Whiteman and Winyard 2011; Yang et al. 2008). Thus, the control of cellular cysteine concentrations is likely to be important for the proper regulation of H_2S signaling pathways as well as for the prevention of H_2S toxicity. Loss-of-function mutations of *CDO1* as well as increased levels of H_2S in synovial fluid have been linked to severity and rapid progression of rheumatoid arthritis as well as several neurodegenerative conditions in humans (Bradley et al. 1994; Whiteman et al. 2011). Additionally, *CDO1* has been shown to be a tumor suppressor gene that is silenced by promoter methylation in various human cancers (Brait et al. 2012); the tumorigenic effects of *CDO1* silencing could stem from the consequent increases in cellular levels of H_2S. The connection between cysteine levels and rates of H_2S production underscores the critical importance of regulating cellular cysteine levels and suggests that increased cysteine levels are likely to be associated with excess H_2S-mediated signaling or toxicity. On the other hand, modulation of cysteine levels may be a clinically practical approach to modulating tissue H_2S levels.

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Conflict of interest No conflict of interests, financial or otherwise, are reported by the authors.

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