Reactive cysteine persulfides and S-polythiolation regulate oxidative stress and redox signaling

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Using methodology developed herein, it is found that reactive persulfides and polysulfides are formed endogenously from both small molecule species and proteins in high amounts in mammalian cells and tissues. These reactive sulfur species were biosynthesized by two major sulfurtransferases: cystathionine β-synthase and cystathionine γ-lyase. Quantitation of these species indicates that high concentrations of glutathione persulfide (perhydropersulfide >100 μM) and other cysteine persulfide and polysulfide derivatives in peptides/proteins were endogenously produced and maintained in the plasma, cells, and tissues of mammals (rodent and human). It is expected that persulfides are especially nucleophilic and reducing. This view was found to be the case, because they quickly react with H_2O_2 and a recently described biologically generated electrophile 8-nitroguanosine 3′,5′-cyclic monophosphate. These results indicate that persulfides are potentially important signaling/effector species, and because H_2S can be generated from persulfide degradation, much of the reported biological activity associated with H_2S may actually be that of persulfides. That is, H_2S may act primarily as a marker for the biologically active of persulfide species.

thiol redox | hydrogen sulfide | electrophilic signaling | polysulfidomics

Hydrogen sulfide (H_2S) has been suggested to be an endogenous small molecule signaling species (1) by unknown mechanisms. Our laboratory recently showed that the presence of hydrogen sulfide anion (HS^-) may be responsible for the regulation and metabolism of various important electrophilic species [e.g., 8-nitroguanosine 3′,5′-cyclic GMP (8-nitro-cGMP)] (2). However, these studies also indicated that reactive intermediates other than HS^- likely react with the electrophiles of interest. These previous studies alluded to the generation of a more reactive sulfur species capable of reacting with electrophiles, such as 8-nitro-cGMP. As reported herein, it was determined that reactive sulfur intermediates, such as hydropersulfides (RSSH) and polysulfides [$RS(S)_nH$ and $RS(S)_nSR$], are formed in appreciable amounts during sulfur amino acid metabolism and possess important chemical and biological properties. Some of these sulfide species have long been known as sulfane sulfur compounds, which were suggested to exist endogenously in mammalian systems (1, 3–5). Reports also indicated that a hydropersulfide moiety with the general molecular formula RSSH may be formed on specific protein cysteine (Cys) residues, most typically of sulfur-transferring enzymes (i.e., sulfurtransferases) during enzymatic reactions (1, 5). Although such persulfide chemical reactivity is thought to be involved in the catalytic activity of particular enzymes (e.g., rhodanese, Cys desulfurases, and sulfide:quinone oxidoreductase) (6, 7), the more general physiological function and occurrence of Cys persulfides (CysSSH) and related species in cells and tissues, especially mammals, were unclear. Moreover, the exact chemical nature and physiological relevance of these biological polysulfur derivatives, especially sulfane sulfur compounds, remain uncertain.

Herein, an MS-based metabolomic method for the analysis of low-molecular weight persulfides/polysulfides and a proteomic analysis combined with a Tag-Switch assay to detect S-polythiolated protein adducts have been developed. Using these techniques, the endogenous formation and presence of Cys hydropersulfide derivatives as well as their possible physiological functions were investigated. The roles of the enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) in Cys hydropersulfide generation were evaluated. Moreover, the antioxidant, cytoprotective, and redox signaling properties of these persulfide species in cells were assessed. Finally, the effect of various sulfides on cellular redox/electrophilic signaling associated with the endogenous electrophile 8-nitro-cGMP was examined.

Results

Polysulfidomics: Metabolomic Analysis for Unique Polysulfur Biology. No established or convenient methodology exists to study polysulfur

Significance

Reactive sulfur-containing compounds, such as L-cysteine hydropersulfide (CysSSH), reportedly form in mammals. However, the biological relevance of these reactive sulfur species remains unclear. We determined that CysSSH was synthesized from cystine by cystathionine β-synthase and cystathionine γ-lyase, which in turn may contribute to high levels of glutathione hydropersulfide (>100 μM) and other CysSSH derivatives of peptides/proteins formed in cells, tissues, and plasma from mice and humans. Compared with glutathione and hydrogen sulfide, CysSSH derivatives were superior nucleophiles and reductants and capable of regulating electrophilic cell signaling mediated by 8-nitroguanosine 3′,5′-cyclic monophosphate. Altogether, it is proposed that reactive Cys persulfides and S-polythiolation have critical regulatory functions in redox cell signaling.

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species [e.g., RSS_nH and $RS(S)_nR$; R = L-Cys and glutathione (GSH), $n \geq 1$]. Thus, to identify endogenous formation of polysulfur-related products or metabolites, a method based on the MS of biologically relevant synthetic polysulfur compounds was developed. The reaction of oxidized thiols with H_2S to generate persulfides and derived species was used to synthesize various polysulfides and/or hydropolysulfides ($RS(S)_nH$) (*SI Appendix*, Figs. S1 and S2 and Tables S1 and S2). Interestingly, it was also found that dihydropolysulfides can be formed by autoxidation of H_2S at high (millimolar) concentrations. These species are, however, unstable under aerobic conditions. Importantly, Cys hydropersulfide and polysulfide formation were found to be much more efficient with CSE-catalyzed enzymatic generation using cystine as a substrate than chemical synthesis by H_2S (Fig. 1*A* and *SI Appendix*, Fig. S1) (see below). Because reduced polysulfides, such as perhydropersulfide (GSSH) and trihydropersulfides (GSSSH), are metastable, they were alkylated by monobromobimane (Br-bimane) before analysis by MS (*SI Appendix*, Fig. S2 *B* and *C*). MS-based polysulfur metabolomics (i.e., polysulfidomics) can now be performed with a wide repertoire of stable isotope-labeled sulfide derivatives as internal standards (*SI Appendix*, Fig. S2*D* and Tables S1 and S2), which will allow identification and quantification of reactive sulfides and polysulfides in cells and tissues.

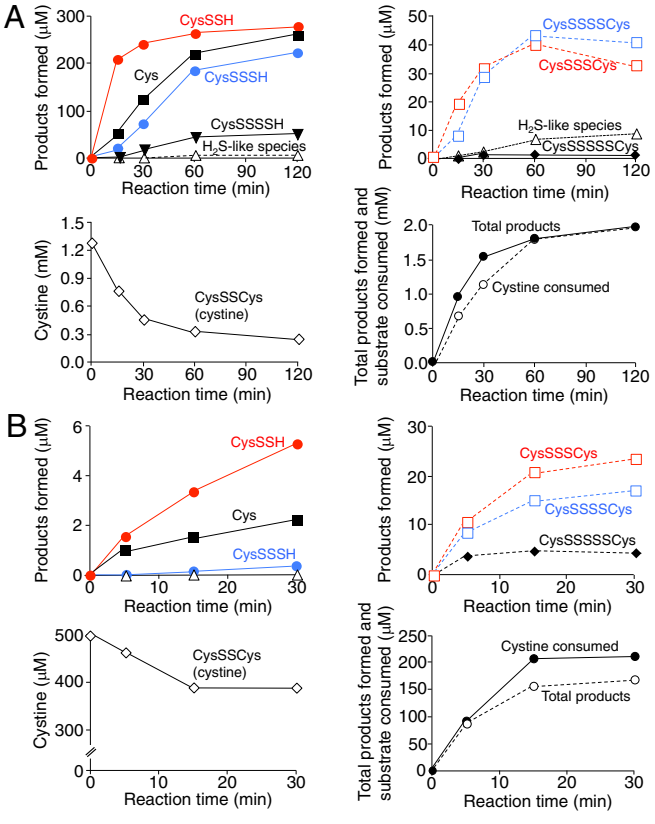

Fig. 1. Enzymatic generation of CysSSH and polysulfides catalyzed by CSE and CBS. Yields of Cys polysulfides by conversion of CysSSCys by (*A*) CSE and (*B*) CBS. (*A*) CysSSCys (1.25 mM) was incubated with 50 μg/mL CSE in 30 mM Hepes buffer (pH 7.5) containing 50 μM pyridoxial phosphate at 37 °C. (*B*) CysSSCys (0.5 mM) was incubated with 5 μg/mL CBS in 30 mM Hepes buffer (pH 7.5) containing 50 μM pyridoxial phosphate and 0.1 mM *S*-adenosyl methionine at 37 °C. H_2S-like species represent not only H_2S/HS^- but also other unidentified products that may directly or indirectly generate bis-S-bimane through reaction with Br-bimane and originate from Cys persulfide derivatives. Details are in *SI Appendix*.

Cys Persulfides as Primary Products of CBS and CSE. CBS was first characterized by its ability to catalyze the coupling of homo-Cys (HCys) and serine to make cystathionine (1). CSE can then use cystathionine, converting it to Cys and α-ketobutyrate (*SI Appendix*, Fig. S3*A*). If cystine (CysSSCys) is used as a substrate for CSE instead of cystathionine, CSE may generate Cys hydropersulfide (CysSSH) as an unstable intermediate (8). However, the physicochemical nature and biological relevance of CysSSH remain unclear.

A study of CBS and CSE activities with various substrates revealed that CysSSCys is the preferential CSE/CBS substrate for generation of Cys persulfide (*SI Appendix*, Fig. S3 *C* and *D*). With CysSSCys as a substrate for recombinant CSE, CysSSH was a major product, which was previously reported (Fig. 1*A* and *SI Appendix*, Fig. S3*C*) (8). Recombinant CBS also catalyzed generation of primarily CysSSH from CysSSCys (Fig. 1*B*). This result is, to our knowledge, the first report of direct CysSSH formation from CysSSCys through CBS catalysis. Product analysis by us and others (1) revealed that both CSE and CBS most likely undergo a CS lyase-like reaction to form CysSSH (*SI Appendix*, Fig. S3*B*). Although both enzymes may produce H_2S (9), presumably by decomposition of Cys polysulfides (possibly depending on the redox status of the reaction milieu) (1, 9), this study and earlier studies indicate that CysSSCys metabolism by CSE and CBS results in initial and significant CysSSH generation.

Generation of CysSSH [and Cys trisulfide (CysSSSH)] in A549 and other cells depended greatly on levels of CSE and/or CBS proteins, because overexpression of CSE or CBS led to dramatic increases in CysSSH and CysSSSH, and CBS knockdown caused reduction of CysSSH (Fig. 2 and *SI Appendix*, Figs. S4, S5, and S6*H* and Table S3). Sulfur transfer reactions from CysSSH (and derived species) to GSH to form GSSH also occurred in a manner depending on CSE and CBS expression in the cells (compare with Fig. 3*A*). Imaging profiles with a fluorescent probe SSP2 (10) correlated well with CysSSH and GSSH levels determined by MS-based polysulfidomics (Fig. 2 and *SI Appendix*, Fig. S6*H*). In fact, the specificity of this SSP probe for polysulfide-dependent fluorescent responses was confirmed by using a reagent known to prevent or react with Cys polysulfides (*SI Appendix*, Fig. S6 *A*–*G*). Metabolic profiles determined by polysulfidomics are, thus, consistent with the proposal that CSE and CBS are largely responsible for endogenous Cys persulfide biosynthesis. Thus, it is established that persulfide/polysulfide generation is highly prevalent in cells through CBS and CSE activity and that levels of these species (i.e., CysSSH, GSSH, etc.) are significant.

Maintenance of Cys S-Polythiolation Induced by Cys Persulfide and Recycling by Glutathione Reductase. Thiol redox exchange reactions with GSH [and oxidized glutathione (GSSG)] should be a primary fate of cellular CysSSH (and derived species). That is, reactions of intracellular CysSSH with large GSH excesses should result in significant generation of GSSH- and GSH-based polysulfides (Fig. 3*A* and *SI Appendix*, Fig. S5*B*). HPLC used to separate and detect bimane-conjugated persulfides determined that glutathione reductase (GSR) reduced glutathione polysulfides (e.g., GSSG, GSSSG, and GSSSSG) to generate GSH-based GSSH, GSSSH, and tetrahydropersulfide (GSSSSH) (Fig. 3*B*). Because GSH is the single most prevalent thiol in cells (1–10 mM) (11), it is reasonable that GSSH is the most abundant Cys-based persulfide among various polysulfide derivatives. The generation of GSSH can be explained by a putative mechanism involving polysulfur exchange (Fig. 3*A*). A high GSSH content may, therefore, be maintained by CBS and CSE generating CysSSH and GSR regenerating GSSH and $GS(S)_nH$ from oxidized glutathione polysulfides in cells. Thus, it is expected that polythiolated glutathione adducts would be the most prevalent in biological systems.

Endogenous Cys Persulfide Formation in Cells and in Vivo in Tissues. To test the idea that CysSSH is generated in vivo, the levels of hydropolysulfide species in animal tissues were determined. CysSSH, HCys persulfide (HCysSSH), GSSH, and GSSSH were

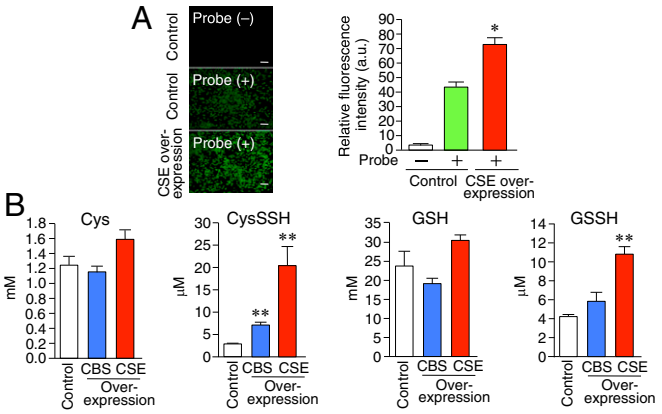

Fig. 2. Cellular formation of reactive sulfur species, mainly Cys and GSH polysulfides, identified by cellular fluorescence imaging and polysulfur metabolomics. (*A*) SSP2-induced fluorescence imaging of A549 cultured cells. (Scale bars: 50 μm.) (*B*) Intracellular levels of Cys and GSH polysulfides in A549 cells overexpressing CBS or CSE as assessed by LC-MS/MS. Data represent means ± SDs ($n = 3$). *$P < 0.05$ versus control; **$P < 0.01$ versus control.

present in mouse heart, liver, and brain (*SI Appendix*, Fig. S7*A* and Table S4). GSSH concentrations in tissues were >100 μM in the brain and about 50 μM in other major organs, including heart and liver. Because the same or higher amounts of HCysSSH compared with those amounts of HCys were observed, HCysSSH seems to be the major HCys derivative in such organs. We also investigated various thiols, persulfides, and polysulfides in human and mouse plasma and found appreciable levels of Cys, HCys, GSH, persulfides, and polysulfides derived from these thiols (i.e., CysSSH, CysSSCys, and CysSSSCys and corresponding GSH and HCys species, except for HCysSSHCys in mouse plasma) (*SI Appendix*, Fig. S7*B*). Preliminary polysulfidomics also revealed remarkable amounts of endogenous Cys persulfide derivatives in human tissues. Thus, endogenous production of numerous polysulfur-containing Cys derivatives occurs in the cells and tissues of mammals.

To elucidate the metabolic pathway involved in CysSSH biosynthesis in cells and in vivo, we applied polysulfidomics to clarify the contribution of amino acid transporters and dietary intake of CysSSCys and methionine to Cys polysulfide formation in whole organisms. Two major amino acid transporters [cystine/glutamine transporter (xCT) and sodium-coupled neutral amino acid transporter 2] (12, 13) were responsible for the intracellular supply of CysSSCys that was functionally linked to CysSSH biosynthesis (*SI Appendix*, Fig. S8). Cys persulfide biosynthesis depended on dietary CysSSCys and methionine as well, which was confirmed by in vivo metabolic profiling of levels of sulfide derivatives in mouse cardiac tissues (Fig. 4). The logical conclusion is that Cys persulfide metabolism is regulated by a dynamic supply and efficient metabolism of CysSSCys and methionine. Thus, there is little question that high-level generation of endogenous and small per- and polysulfides occurs. It is expected that these species are capable of sulfur exchange with protein thiols to give per- and polysulfur protein species. This idea was examined.

Protein S-Polythiolation and Its Proteomic Analysis. Thiol redox exchange reactions between low-molecular weight species and protein thiols to give protein Cys-S-polythiolation are clearly possible. Preliminary studies indicate facile sulfur atom transfer from CysSSH to, for example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*SI Appendix*, Fig. S9 *C* and *D*). To examine this reaction further in cells, a Tag-Switch assay was developed, in which nucleophilic protein thiol species (thiols and hydropersulfides) were tagged with the electrophile methylsulfonyl benzathiazole (MSBT) (14). Among all of the tagged thiol species, only those species with the reactive sulfur residue present in polysulfides could be selectively modified by a biotin-cyano (CN)–labeled probe. Treatment with an avidin-conjugated enzyme-linked chemiluminescence probe then allows detection (*SI Appendix*, Fig. S9*A*). This method coupled with liquid chromatography (LC)-MS/MS readily identified GSSH (*SI Appendix*, Fig. S9*B*). Several protein bands showed strong positive Tag-Switch-Tag responses in control A549 cells, with band intensities greatly increasing after overexpression of CBS and CSE (Fig. 5*A*). Specific CN-biotin labeling of Cys residues in proteins was further verified by means of LC-MS/MS as illustrated in Fig. 5*B*. Formation of Cys-CN-biotin was clearly identified in whole-cell lysates and even with a protein band gel electrophoresed from cellular proteins, which were simultaneously detected by avidin-based Western blotting (Fig. 5*C*) as well as a model protein GAPDH herein (*SI Appendix*, Fig. S9*D*). The proteomic analysis indeed identified several polythiolated proteins (Fig. 5*D* and *SI Appendix*, Table S5). Several proteins involved in sulfur metabolism and known to possess protein-bound Cys persulfides were detected along with other polythiolated proteins that are unrelated to sulfur metabolism, such as protein disulfide isomerase, heat shock proteins, aldo-keto reductase, GAPDH, enolase, and phosphoglycerate kinase. These proteins or enzymes may, thus, function as sensors and effectors regulated by protein polythiolation as a posttranslation modification.

Antioxidant Activities of Hydropersulfides: Reactions with H_2O_2. The results presented thus far indicate that polysulfur species (persulfides and polysulfides) are highly prevalent both as small molecule species and in proteins. Thus, it is now reasonable to begin to examine their chemical reactivity as a means of defining their chemical biology and potential use. Hydropersulfides were previously proposed to be more nucleophilic and superior reducing agents compared with their thiol counterparts (5, 15). Thus, the ability of persulfides, specifically GSSH, to react with electrophilic oxidants was investigated. GSSH and its related polysulfides were generated in reaction mixtures containing GSR and the appropriate polysulfide (i.e., GSSSG) (*SI Appendix*). Marked H_2O_2 scavenging developed only under reaction conditions that generated GSSH (GSSSG and GSR) (Fig. 6*A*). In

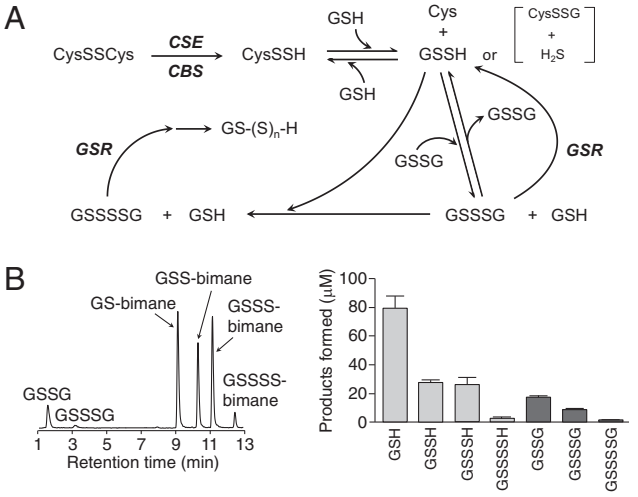

Fig. 3. Mechanism of intracellular maintenance of various Cys and GSH polysulfides. (*A*) Schematic representation of the Cys polysulfur cycle involving CBS, CSE, and GSR for intracellular maintenance of various Cys and GSH polysulfides. (*B*) Regeneration of GSSH and GSSSH from GSSSG catalyzed by GSR. GSSSG (0.1 mM) was reacted with GSR (1 U/mL) and NADPH (0.2 mM) in 20 mM Tris·HCl buffer (pH 7.4) at room temperature for 5 min followed by incubation with 5 mM Br-bimane at 37 °C for 15 min. Shown are the (*Left*) LC-MS elution profile and (*Right*) quantitative data for the reaction. Data represent means ± SDs ($n = 3$).

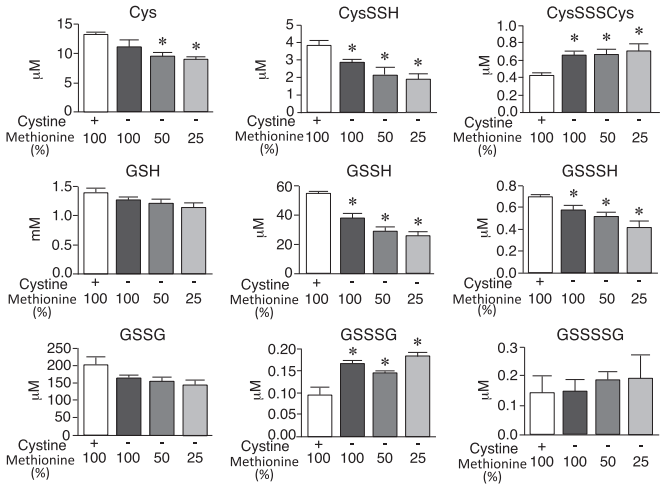

Fig. 4. Polysulfur metabolomics in vivo. Effects of dietary sulfur sources on levels of various Cys-related polysulfides in mouse heart as determined by LC-MS/MS. Mice were maintained with the diets containing different amounts of cystine and methionine for 4 wk. Data are means ± SDs ($n = 3$). *$P < 0.05$ versus control diet [+, cystine; methionine at 100% (wt/wt)].

the GSR reaction with GSSG as a substrate, which produced GSH concentrations similar to or higher than those concentrations of GSSH generated from GSSSG and GSR, no measurable H_2O_2 decomposition occurred. HPLC and MS of the GSSH reaction with H_2O_2 indicated that GSSH was readily oxidized by H_2O_2 to give primarily GSSG and GSSSG. CSE overexpression also led to protection against H_2O_2-mediated cell death, consistent with the idea that persulfides are efficient and potentially important peroxide scavengers (Fig. 6*B*). These findings, therefore, show that the extra sulfur in hydropolysulfides is an extremely reactive reductant and can be a potent antioxidant in cells and in vivo.

Interaction of Hydropersulfides with Electrophiles and Redox Signal Regulation. The above work indicates that persulfides are especially reducing, capable of rapid reaction with the electrophile H_2O_2. A reasonable mechanism for the reaction of GSSH with H_2O_2 involves GSSH acting initially as a nucleophile. That is, persulfides can act as nucleophilic reductants. Thus, to further examine the inherent nucleophilicity of persulfides, the reaction with another endogenously generated electrophile was examined. Previous work by us has shown endogenous generation of 8-nitro-cGMP, an electrophilic species made by nitration of guanine, and the ability of this species to modify nucleophilic Cys residues in proteins (16). A facile reaction between hydropersulfides and 8-nitro-cGMP should, therefore, take place. In fact, a reaction between hydropersulfides (e.g., GSSH and GSSSH) and 8-nitro-cGMP in a cell-free mixture showed efficient and direct generation of the expected adduct, indicating that hydropersulfides reacted directly with 8-nitro-cGMP to give the dialkyl polysulfide (*SI Appendix*, Fig. S10). Generation of 8-SH-cGMP from the dialkyl persulfide intermediate likely occurs through additional reaction with hydropersulfides (Fig. 7*A* and *SI Appendix*, Fig. S10); 8-SH-cGMP did not form to a significant extent with only H_2S present, consistent with the idea that hydropersulfides are responsible for generation of 8-SH-cGMP but not H_2S. This unique electrophile thiolation chemistry is supported by our in vivo finding of appreciable amounts of a thiolation adduct of 8-nitro-cGMP, 8-SH-cGMP, in major organs in mice (Fig. 7*B* and *SI Appendix*, Fig. S11); 8-SH-cGMP levels in tissues were generally much higher than levels of cGMP (except in kidney). Comparing production of 8-SH-cGMP with that of GSH persulfides revealed a similar relationship in different organs (Fig. 7*B* vs. *SI Appendix*, Table S4). These data, thus, suggest a possible physiological interaction of hydropersulfides with 8-nitro-cGMP in terms of in vivo metabolism (2, 17). Significant amounts of 8-SH-cGMP, formed presumably after initial adduct generation from hydropersulfides and 8-nitro-cGMP, were also found in C6 glioma cells (Fig. 7*C*). The biological relevance of a pathway for 8-SH-cGMP formation through hydropersulfide generation was supported by the suppression of intracellular generation of 8-SH-cGMP from 8-nitro-cGMP and CysSSH as well in C6 rat cells with CBS knockdown (Fig. 7*D*). Consistent with previous reports (5, 15), these data support the idea that persulfides possess significant nucleophilic character.

Discussion

The methods described herein identify the product of CBS- and CSE-mediated metabolism of CysSSCys as CysSSH. Little or no H_2S was detected, which indicates that CysSSH and derived species (and not necessarily H_2S) may be major endogenous sulfides formed during metabolism of sulfur-containing amino acids. These results are consistent with the idea that H_2S is generated primarily as a byproduct of persulfide degradation. This interpretation may indicate a primary signaling/regulatory function associated with persulfides (because of their prevalence and established biosynthesis through both CSE and CBS) and that H_2S may be a marker for persulfide formation (although this view does not preclude possible physiological function for H_2S). The release of H_2S from persulfides can occur through reduction of persulfides by other thiol-based reductants or through the actions of GSR, because both pathways are known to release H_2S from persulfides (18, 19). Clearly, H_2S is known to react with oxidized thiol species to generate persulfides (15). However, this study indicates that enzyme-mediated persulfide formation by CSE- and CBS-mediated CysSSCys metabolism is facile and a more likely source of biological persulfides. As mentioned previously, generation of CysSSH from CSE-mediated metabolism is known (8), and the K_m for cystine with CSE has been reported to be 0.03–0.07 mM (rat liver CSE), which is significantly lower than the K_m values for L-cystathionine (0.8–3.5 mM)

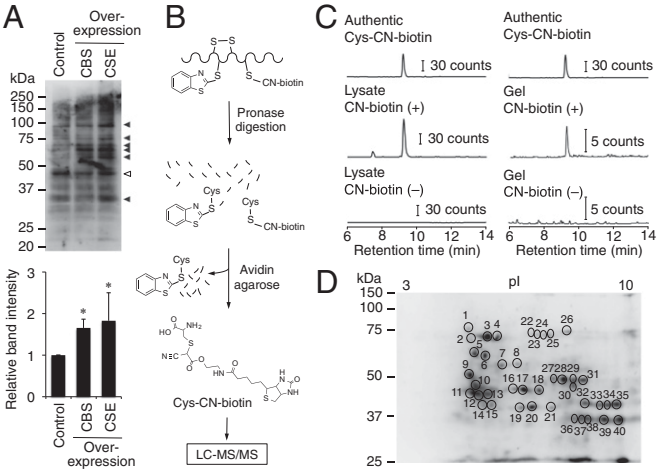

Fig. 5. Unique proteomic analysis based on the Tag-Switch-Tag strategy for protein-bound reactive sulfur labeling. (*A*) Protein-Cys polysulfurs identified as (*Upper*) biotin-labeled bands in Western blotting and (*Lower*) quantitative data by densitometric analysis. Data are means ± SDs ($n = 3$). *$P < 0.05$ versus control. (*B*) Schematic diagram for identification of protein-bound Cys-CN-biotin. (*C*) LC-MS/MS identification of Cys-CN-biotin (*m/z* 474.1). Lysates of A549 cells overexpressing CSE reacted with CN-biotin [CN-biotin (+)] or without CN-biotin [CN-biotin (–)] were analyzed as (*Left*) whole-cell lysates (Lysate) or (*Right*) after gel electrophoresis (Gel). Representative results analyzing the gel at the position indicated by the white arrowhead in *A* are shown. (*D*) Protein-Cys polysulfurs identified as spots on membranes transferred from 2D gels. Some labeled spots in the 2D image were subjected to polysulfur proteomics, which identified several polythiolated proteins (*SI Appendix*, Table S5).

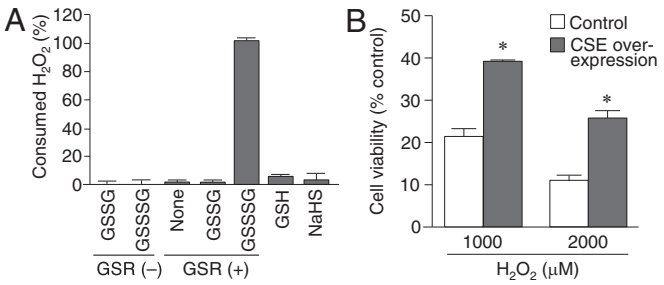

Fig. 6. The antioxidant effect of GSSH. Potent antioxidant ability of GSSH as determined by scavenging of H_2O_2. (*A*) Direct scavenging of H_2O_2 by GSSH in vitro. H_2O_2 (0.1 mM) was reacted with GSSG or GSSSG (0.2 mM each) in the absence or presence of GSR (1 U/mL) and NADPH (0.4 mM) at 37 °C for 30 min. (*B*) Effects of CSE overexpression on H_2O_2-mediated cell death. A549 cells with or without CSE overexpression were treated with H_2O_2 (1,000 or 2,000 μM) for 24 h. Cell viability was determined and expressed as a percentage of control without H_2O_2 treatment. Data represent means ± SDs ($n = 4$). *$P < 0.05$ versus control.

and L-homoserine (15–20 mM) (20). Thus, cystine seems to be a preferred substrate compared with other known substrates for CSE.

Although previous reports describe H_2S as a major signaling molecule in mammals (1, 9), results herein suggest that the Cys-based persulfides may be the actual signaling species. Moreover, because exogenous H_2S reacts with oxidized thiols in biological systems to give persulfides (15), the pharmacology reported for H_2S may also be, at least partially, because of Cys hydropersulfide generation. Toohey (1) postulated that sulfane sulfur [a sulfur atom bound to another sulfur or a sulfur atom with six valence electrons and no charge (sometimes referred to as S^0)] and not H_2S is the primary biological effector, and our results at least partially support this postulate, because hydropersulfides and polysulfides contain sulfane sulfur. Earlier reports presumed that a specific form of biological sulfur storage existed in cells and proposed that it was sulfane sulfur, which is a substance found endogenously (1) and may be a precursor to biological H_2S in the presence of thiols.

It is worth noting that the chemistry and biology of per- and polysulfides have received recent attention. For example, it was reported that protein persulfide generation can occur through the reaction of oxidized impurities of H_2S donor solutions (i.e., polysulfides) and that this reaction may be responsible for the biological actions of H_2S donors (21). Others have shown that CSE activity can lead to persulfide formation, although this finding was mechanistically ill-defined (22), and another recent report alludes to polysulfide formation from H_2S being responsible for signaling in the brain (23). It needs to be stressed, however, that the work described herein indicates that the Cys per- and polysulfides are synthesized directly (from CSE or CBS), highly prevalent, and likely signaling effector species.

The high GSSH levels detected in cells and tissues indicate that it is the dominant biological polysulfide. The generation of GSSH likely results from the reaction of GSH with CysSSH. The intracellular concentrations of GSSH of 10 μM to >100 μM likely account for the sulfane sulfur levels previously reported (1). Such GSSH levels were maintained by cystine (CysSSCys) uptake machinery propelled by appropriate amino acid transporters, such as xCT (13) and sodium-coupled neutral amino acid transporter 2 (12), and Cys-related metabolism maintained by dietary methionine and cystine, which was determined by polysulfidomics (Fig. 4 and *SI Appendix*, Fig. S8).

We found that numerous proteins contain persulfides or polysulfides, which indicates a ubiquitous and possible regulatory function associated with polythiolation of proteins. The generation of these polythiolated protein species can be envisioned to occur through reaction of protein thiols with the high levels of GSSH (*SI Appendix*, Fig. S9 *C* and *D*). The occurrence of hydropersulfides in mammalian systems was previously described (24). However, the persulfide assay used in that study (a modified biotin switch assay) has been questioned (25), and our present results also question the validity of this previous work. The assay of Mustafa et al. (24) relied on the nucleophilicity of a protein thiol for reaction with an electrophile (*S*-methyl methanethiosulfonate) and assumed that a protein hydropersulfide would not react. However, results presented here and elsewhere (15, 25) indicate that hydropersulfides are extremely nucleophilic and should react readily with electrophiles. Indeed, the assay developed herein relies on the established nucleophilicity of persulfides. Regardless, using the Tag-Switch assay, this study confirms protein S-polythiolation in cells (Fig. 5). Another important aspect of endogenous persulfides is the potential for interference of the above-mentioned biotin-switch assay for S-nitrosothiols. This interpretation is further discussed in *SI Appendix*.

Antioxidant activity associated with GSH is typically mediated by specific enzymes, such as GSH-dependent peroxidase and GST (26). Without catalytic assistance, GSH is a relatively inert sulfhydryl with low nucleophilicity, reacting poorly with electrophilic oxidants (e.g., H_2O_2). However, GSSH has strong nucleophilic/antioxidant activity (15). This idea is strongly supported by the results herein, which reveal a potent H_2O_2-scavenging activity of GSSH (Fig. 6). Thus, Cys persulfide derivatives, most typically GSSH, may provide a primary and potent antioxidant defense in cells.

Previous work from this laboratory has established electrophilic signaling associated with 8-nitro-cGMP (17). The overall signaling and fate of 8-nitro-cGMP depends heavily on the biological environment in which it is formed, and given the observed nucleophilicity of hydropersulfides, the presence of polythiolation pathways becomes critical for regulation of electrophilic signaling. The magnitude of electrophilic signaling in cells can be tempered or altered in the presence of highly nucleophilic Cys-related polysulfides, especially GSSH. Herein, trapping of 8-nitro-cGMP by hydropersulfides (GSSH) initially generates

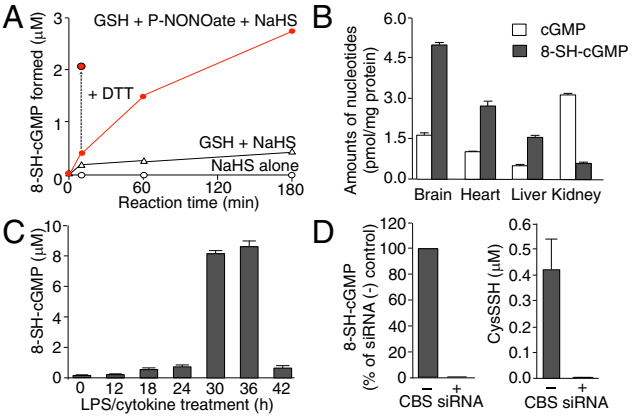

Fig. 7. The interaction of GSSH with 8-nitro-cGMP. (*A*) 8-SH-cGMP formation by chemical polythiolation, with formation of polysulfide cGMP as an intermediate molecular species (*SI Appendix*, Fig. S10), which led to effective 8-SH-cGMP formation from 8-nitro-cGMP. 8-Nitro-cGMP (1 mM) was reacted with NaHS (0.1 mM) in the absence or presence of GSH (0.1 mM), P-NONOate (0.1 mM), or both in 10 mM Tris·HCl buffer (pH 7.4) at 37 °C. Addition of 10 mM DTT enhanced 8-SH-cGMP formation. (*B*) In vivo endogenous formation of 8-SH-cGMP in organs in normal mice. Both 8-SH-cGMP and cGMP were quantified by using LC-MS/MS of the methanol extract of each organ. (*C*) Endogenous 8-SH-cGMP formation dependent on CBS expression in C6 cells in culture as identified by LC-MS/MS. C6 cells were treated with LPS, IFN-γ, TNF-α, and IL-1β to induce endogenous NO generation, leading to the formation of 8-nitro-cGMP. (*D*) Effects of CBS knockdown on production of (*Left*) 8-SH-cGMP and (*Right*) CysSSH in C6 cells treated with LPS plus cytokines as in *C* for 36 h assessed by LC-MS/MS. Error bars indicate means ± SDs ($n = 3$).

the disulfide adduct 8-GSS-cGMP. Additional decomposition of this species forms the thiol 8-SH-cGMP, which may function physiologically as a cyclic nucleotide similar to parental cGMP but with unique redox/nucleophilic properties (2). Interestingly, 8-SH-cGMP is the most prevalent cGMP-containing species (including cGMP itself) in the brain, heart, and liver of mice (Fig. 7*B*), indicating possible biological relevance to this species. The enhanced nucleophilicity of persulfides may also have implications to other electrophilic signaling pathways (e.g., those pathways involving nitroplipids or 4-hydroxynonenal) (27).

In summary, the results of this study indicate the following findings.

i) Using an LC-MS/MS assay developed herein, it was determined that the enzymes CSE and CBS are capable of directly generating a persulfide, CysSSH, when using cystine as a substrate.
ii) The generation of CysSSH in biological systems was observed and found to lead to subsequent formation of GSH-based per- and polysulfides (e.g., GSSH, GSSSH, GSSSG, etc.).
iii) Significant levels (50–100 μM) of per- and polysulfides are detected in mammalian cells, tissues, and plasma.
iv) Using a Tag-Switch assay, per- and polysulfides were found in numerous proteins (which likely formed through interaction with small molecule persulfides).
v) Persulfides are uniquely potent reductants and nucleophiles with greater reactivity than thiols and capable of scavenging oxidants and cellular electrophiles.

This study indicates the prevalence, relevance, and unique chemical reactivity of persulfides and related polysulfur species. It is likely that the physiological use of persulfides is based on their unique reactivity (as nucleophiles and reductants). In this regard, it is especially important to note that the generation of this potent nucleophilic reductant relies on the presence of cystine, an oxidized species. Direct formation of the highly reducing persulfide will, therefore, occur primarily in the presence of a thiol oxidant. This persulfide formation is a unique situation and may represent a rapid and important immediate response to the presence of such an oxidant. Clearly, the speculation above indicates that levels of cystine may regulate the generation of cellular persulfides. Intracellular cystine levels may be controlled by the presence of oxidants and cellular uptake as well as reaction with other reductants, such as GSH (which may be low under oxidizing conditions), and the relatively low K_m for cystine may allow CSE to compete with other cystine degrading pathways. Thus, examination of the factors controlling intracellular cystine concentrations will be extremely important in the future.

Materials and Methods

LC-MS/MS Analysis for Polysulfidomics. Samples containing the enzymatic reaction solution, cell lysates, and tissue homogenates were mixed with a methanol solution containing 5 mM Br-bimane and incubated at 37 °C for 15 min. After centrifugation, aliquots of the supernatants were diluted 10–100 times with distilled water containing known amounts of isotope-labeled internal standards. We used an Agilent 6430 Triple Quadrupole LC/MS (Agilent Technologies) to perform LC-electrospray ionization-MS/MS. The ionization was achieved by using electrospray in the positive mode, and polysulfide derivatives were identified and quantified by means of multiple reaction monitoring.

Tag-Switch Assay and Proteomics of Protein S-Polythiolation. Biotinylation of polysulfides was performed by Tag-Switch-Tag labeling of polysulfides using MSBT as the Tag reagent and CN-biotin as the Switch-Tag reagent. S-thiolated proteins were also biotinylated by this labeling method. Lysates of A549 cells were reacted with 10 mM MSBT in 20 mM Tris·HCl (pH 7.4) at 37 °C for 30 min. These solutions were then reacted with 20 mM CN-biotin at 37 °C for 30 min, after which time protein samples were subjected to Western blotting with avidin-HRP. Protein samples were subjected to isoelectric focusing and separated by SDS/PAGE followed by silver staining or Western blotting with avidin-HRP. Spots detected were subjected to in-gel digestion and LC-MS/MS-based proteomics for S-thiolated proteins.

Additional details are provided in *SI Appendix, SI Materials and Methods*.

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