The Reaction of Hydrogen Sulfide with Disulfides: Formation of a Stable Trisulfide and Implications to Biological Systems

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Running title: Dialkyl trisulfide formation and use as a precursor to persulfides

Abbreviations: Cystathionine gamma-lyase (CSE), cystathionine beta-synthase (CBS), cystine (Cys-SS-Cys), cysteine (Cys-SH), glutathione (GSH), glutathione disulfide (GSSG), glutathione trisulfide (GSSSG), cysteine trisulfide (Cys-SSS-Cys), 2-mercaptoethanol (2ME), 2-hydroxyethyl disulfide (2HED), 2-hydroxyethyl trisulfide (2HET), 2-ME hydropersulfide (2MESSH), 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS), iodoacetamide (IAM), *N*-acetyl penicillamine (NAP), *N*-ethylmaleimide (NEM), β-(4-hydroxyphenyl)ethyl iodoacetamide (HPE-IAM), *S*-methoxycarbonyl penicillamine disulfide (MCPD), MCPD hydropersulfide (MCP-SSH).

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Abstract

Background and Purpose: The signaling associated with hydrogen sulfide (H_2S) remains to be established and recent studies have alluded to the possibility that H_2S-derived species play important roles. Of particular interest are hydropersulfides (RSSH) and related polysulfides (RSS_nR, n>1). This work elucidates the fundamental chemical relationship between these sulfur species as well as examine their biological effects.

Experimental Approach: Using standard analytical techniques (^{1}H-NMR and mass spectrometry), the equilibrium reactions between H_2S, disulfides (RSSR), RSSH, dialkyltrisulfides (RSSSR) and thiols (RSH) were examined. Their ability to protect cells from electrophilic and/or oxidative stress was also examined using cell culture.

Key Results: H_2S, RSSR, RSSH, RSSSR and RSH are all in a dynamic equilibrium. In a biological system, these species can exist simultaneously and thus it is difficult to discern which species is(are) the biological effector(s). Treatment of cells with the dialkyl trisulfide cysteine trisulfide (Cys-SSS-Cys) results in high intracellular levels of hydropersulfides and protection from electrophilic stress.

Conclusion: In aqueous systems, the reaction between H_2S and RSSR results in the formation of equilbria whereby H_2S, RSH, RSSR, RSSH and RSSSR are present. In a biological system, any of these species can be responsible for the observed biological activity. These equilibrium species can also be generated via the reaction of RSH with RSSSR.

Implications: Due to these equilibria, Cys-SSS-Cys can be a method for generating any of the other species. Importantly, HEK293T cells treated with Cys-SSS-Cys results in increased levels of hydropersulfides, allowing examination of the biological effects of RSSH.

Introduction

Hydrogen sulfide (H_2S) is an endogenously generated small molecule bioregulator with a wide array of reported physiological functions [for recent reviews, see Kashfi and Olson, 2013; Polhemus and Lefer, 2014; Kimura, 2014]. For example, H_2S has been proposed to have a role in long-term potentiation, smooth muscle relaxation, inhibition of inflammation, activation of K_{ATP} channels and protection against ischemia-reperfusion injury, just to name a few. However, it has also been hypothesized that some of the effects attributed to H_2S may instead be due to the presence of hydropersulfides (RSSH) [for example, Ida et al., 2014; Ono et al., 2014] since RSSH can be in equilibrium with H_2S in the presence of an oxidized thiol (i.e. a disulfide, RSSR) via **Reaction 1** [Cavallini et al., 1970; Francoleon et al., 2011; Vasas et al., 2015] and, possibly more importantly, the biosynthesis of H_2S can occur via RSSH intermediacy (*vide infra*). (Note: the term RSSH will be used to generally describe both the neutral RSSH and the anionic RSS^- forms, unless specifically noted).

$$H_2S + RSSR \rightleftharpoons RSSH + RSH \qquad (1)$$

Although the forward and reverse reactions shown in **Reaction 1** can be slow (at least in a purely chemical system) [Cuevasanta et al., 2015], it appears that H_2S and RSSH are intimately linked and likely to be present simultaneously via **Reaction 1** in a biological system. This makes the assignment of the compound responsible for the biological activity difficult and/or indicates that multiple effectors can be responsible for the observed effects. Furthermore, the chemistry represented by **Reaction 1** is likely to be greatly oversimplified in that other reactions can potentially emanate from this initial equilibrium [for example, see Bogdandi et al., 2018, this issue]. For example, RSSH species are superior nucleophiles compared to the corresponding RSH species and can react further with other electrophiles in solution (i.e. RSSR) to give a dialkyltrisulfide (RSSSR, **Reaction 2**) [for example, Ono et al., 2014; Bogdandi et al., 2018 this issue].

$$RSSH + RSSR \rightleftharpoons RSSSR + RSH \qquad (2)$$

Clearly, other chemical reactions/equilibria are possible (*vide infra*), making the simple reaction of H_2S with RSSR potentially more complex than previously recognized.

It is important to note that the generation of RSSSR is not dependent on the presence of H_2S in a biological system. For example, RSSH can be formed via the conversion of cystine (Cys-SS-Cys) to the corresponding cysteine hydropersulfide (Cys-SSH) and pyruvate or serine via the actions of cystathionine gamma-lyase (CSE) or

cystathionine beta-synthase (CBS) (**Reaction 3**) [for example, Yamanishi and Tuboi, 1981; Stipanuk, 1986; Ida et al., 2014].

Cys-SS-Cys → Cys-SSH + pyruvate (CSE) or serine (CBS) (3)

In this system, subsequent reaction of the highly nucleophilic Cys-SSH product with the electrophilic enzyme substrate Cys-SS-Cys would generate the corresponding cysteine trisulfide (or thiocystine, Cys-SSS-Cys, **Reaction 2**) in the absence of H_2S. Of course, H_2S can eventually be formed in a biological system where thiols will undoubtedly be present via the simple reverse of **Reaction 1** and the eventual formation of an equilibrium mixture of all species is likely. More recently, it has been reported that Cys-SSH can also be generated from cysteine (Cys-SH) via the actions of cysteine t-RNA synthases, leading to translational incorporation of Cys-SSH into proteins along with release of free Cys-SSH [Akaike et al., 2017].

Numerous studies have utilized **Reaction 1** to generate RSSH *in situ* and there seems to be little doubt that RSSH can be generated in this way [for example, Cavallini et al., 1970; Francoleon et al., 2011; Saund et al., 2015]. However, the potential for **Reaction 1** to lead to further chemistry (such as **Reaction 2**) has not been adequately accounted for in these previous studies and it is certain that other sulfur species besides those shown in the equilibrium reaction may be present. It is also possible that biologically generated RSSH participates in numerous other equilibria as well and a complete description of the chemical biology of hydropersulfides may require consideration of these other processes. Thus, this study examines **Reaction 1** and evaluates the possibility of further chemistry occurring that may be relevant to biologically generated RSSH species. Importantly, a companion paper in this issue [Bogdandi et al., 2018] describes aspects of the equilibrium reactions mentioned above. The paper herein serves to examine the general scope of these equilibria with other RSH/RSSR as well as further investigate the generation and potential biological utility of an important species associated with this chemistry, specifically RSSSR.

Materials and Methods

Chemicals and Solutions: Cystine (Cys-SS-Cys), cysteine (Cys-SH), glutathione (GSH), glutathione disulfide (GSSG), 2-mercaptoethanol (2ME), 2-hydroxyethyl disulfide (2HED), 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS), iodoacetamide (IAM), *N*-acetyl penicillamine (NAP), sulfasalazine and *N*-ethylmaleimide (NEM) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium hydrosulfide (NaSH) was purchased from Strem Chemicals, Inc. (Newburyport, MA). *S*-Benzyl-L-cysteine was purchased from Wako Chemicals (Osaka, Japan). β-(4-Hydroxyphenyl)ethyl iodoacetamide (HPE-IAM) was

purchased from Molecular BioSciences (Boulder, CO). All other chemicals were purchased from commercial sources and were the highest purity available. Glutathione trisulfide (GSSSG) was a gift from Prof. Herbert Nagasawa and synthesized via previously published procedures [Savige et al., 1964].

Instrumentation: All NMR analyses were performed using an Agilent 400 MHz NMR spectrometer running on vnmrj software. Water suppression for all samples was achieved using a (^{1}H) PreSat method in the vnmrj software. All NMR solvents were 10% D_2O in 100 mM phosphate buffer (pH 7.4) containing 1 mM DSS as an internal standard.

Mass spectra of chemical studies were recorded using a Thermo Triple Quadrupole Electrospray Ionization Mass Spectrometer (ESI-MS) in positive ion mode coupled with XCalibur 2.1 software. All samples were directly injected via syringe pump at a flow rate of 5 µl/min using N_2 as a sheath gas and Ar as a collision gas. Ion optics were optimized using authentic samples of disulfides. The capillary temperature for all samples was 250°C and spray voltages were 3000 V for 2HED samples and 2500 V for GSSG samples. Mass spectral analysis of cell lysates was obtained using a triple quadrupole (Q) mass spectrometer LCMS-8050 (Shimadzu) coupled to the Nexera UHPLC system (Shimadzu).

Methods:

Synthesis of cysteine trisulfide (Cys-SSS-Cys, thiocystine): Cys-SSS-Cys was synthesized following the general method of Savige et al. [1964] as described briefly below. Cystine-*S*-monoxide was synthesized by dissolving Cys-SS-Cys in 1M H_2SO_4 (approx. 10 mL of H_2SO_4 solution/gram of cystine) in an ice bath. Two equivalents of peracetic acid were then added followed by stirring (on ice) for two hours. After stirring, the pH was adjusted to 4 using pyridine. Cystine-S-monoxide was then precipitated from solution via the addition of ethanol (approximately 3 times the volume of the initial H_2SO_4 solution). The precipitate was filtered and dried. The cystine-S-monoxide was then dissolved in 1M H_2SO_4 (approximately 10 mL of H_2SO_4 solution per gram of cystine-S-monoxide). Two equivalents of solid NaSH was then added to a separate flask and the cystine-S-monoxide solution added to the NaSH. After addition the flask was sealed with a septum and stirred at room temperature for 1 hour. The pH was then adjusted to 3.5 using pyridine. A 50/50 mixture of ethanol/THF was then added (approximately 2-3 times the volume of the initial H_2SO_4 solution) to precipitate Cys-SSS-Cys. The precipitate was filtered, washed with 50/50 ethanol/THF and dried. Analysis was carried out using ^{1}H-NMR and ESI/MS. Typical purity of the resulting product was >90% (by NMR) with cystine as the only detectable impurity. Cys-SSS-Cys: ^{1}H-NMR (400 MHz, D_2O) δ 4.015 (dd, J = 8.0, 4.0 Hz, 1H), δ 3.42 (dd, J =

15.2, 4.0 Hz, 1H), δ 3.23 (dd, *J* = 15.2, 8.0 Hz, 1H). Mass spectral analysis (ESI-MS) m/z [M + H]$^+$ calc. for $C_6H_{12}N_2O_4S_3$, 273.37; found 272.98.

Synthesis of S-methoxycarbonyl penicillamine disulfide (MCPD): The hydropersulfide donor MCPD used previously by our lab [Millikin et al., 2016; Bianco et al. 2016] was synthesized via the method of Artaud and Galardon [2014].

NMR Analysis of the NaSH/RSSR Equilibrium: Stock solutions of 2HED (200 mM) and NaSH (50 and 100 mM) were prepared in 100 mM phosphate buffer (pH 7.4). DSS (internal standard) stock solutions (10 mM) were prepared in D_2O. For all experiments, DSS was diluted in an NMR tube filled with buffer to a final concentration of 1 mM. For reactions between 2HED and NaSH, the stock solution of 2HED was added to the DSS containing NMR tube to a final concentration of 10 mM. NaSH was then added to the NMR tube to final concentrations of 5, 10 or 15 mM. All solutions were equilibrated at room temperature for 30 minutes, followed by ^{1}H-NMR analysis.

For cyanolysis reactions, stock solutions of 2HED and NaSH were diluted in an NMR tube containing buffer with 1 mM DSS to final concentrations of 20 and 10 mM, respectively. The solution stood at room temperature for 30 minutes. At this time, a stock solution of sodium cyanide (100 mM in buffer) was diluted into the NMR tube to a final concentration of 10 mM. The cyanolysis reaction was allowed to proceed at room temperature for 1 hour, followed by ^{1}H-NMR analysis.

ESI-MS Analysis of the NaSH/RSSR Equilibrium: A stock solution of NaSH (50 mM) was prepared in 50 mM ammonium bicarbonate buffer (pH 7.4). Disulfide solutions were further diluted in the same buffer to final concentrations of 1 mM before ESI-MS analysis. For the reaction of 2HED and NaSH, 2HED was diluted in buffer to a final concentration of 1 mM. NaSH was then added to a final concentration of 0.5 mM and the mixture allowed to stand at room temperature for 30 minutes prior to ESI-MS analysis.

NMR Analysis for the Competitive Trapping of Hydropersulfides: Stock solutions of 2HED, NaSH and DSS were prepared as stated above (NMR Analysis of the NaSH/RSSR Equilibrium). For reactions of 2HED, 10 mM NaSH was added to an NMR tube of 20 mM 2HED in 100 mM phosphate buffer (pH 7.4) with 1 mM DSS, and the mixture equilibrated for 30 minutes at room temperature. IAM or NEM (1, 2, 3, 4 or 5 mM final concentration) was then added and the reaction proceeded at room temperature for 1 hour, followed by ^{1}H-NMR analysis.

ESI-MS Analysis for the Competitive Trapping of Hydropersulfides: Stock solutions of 2HED and NaSH were prepared as above (ESI-MS Analysis of the NaSH/RSSR Equilibrium). For reactions, 0.5 mM NaSH was added to a solution of 1 mM 2HED in 50 mM ammonium bicarbonate buffer (pH 7.4) and the mixture equilibrated for 30 minutes at room temperature. IAM (0.5 mM) was then added and the reaction proceeded for 1 hour at room temperature, followed by ESI-MS analysis.

ESI-MS Analysis of the Trisulfide Equilibrium: Stock solutions of GSSSG (25 mM) and NAP (25 mM) were prepared in 50 mM ammonium bicarbonate buffer (pH 7.4). For reactions, 250 µM NAP was added to a solution of 250 µM GSSSG in the same buffer and the reaction proceeded at room temperature for 1 hour, followed by ESI-MS analysis.

ESI-MS Analysis for the reaction of RSSH and RSSR: A stock solution of GSSG (100 mM) was prepared in 50 mM ammonium bicarbonate buffer (pH 7.4). A stock solution of MCPD (20 mM) was prepared in deionized (DI) water. For the reaction, 200 µM MCPD was added to 1 mM GSSG and the reaction proceeded at room temperature for 30 minutes followed by ESI-MS analysis.

Cell culture and treatments for Cys-SSS-Cys uptake assay: Human embryonic kidney (HEK) 293T cells (CRL-11268, American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (DMEM, high-glucose; Sigma-Aldrich) that was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, under standard cell culture conditions (37 °C, humidified, 5% CO_2/95% air).

Analysis of intracellular hydropersulfides and other polysulfides with cultured HEK293T cells using LC-ESI-MS/MS analyses: Intracellular hydropersulfide levels in cultured HEK293T cells were quantified by LC-MS/MS with beta-(4-hydroxyphenyl)ethyl iodoacetamide (HPE-IAM) as a trapping agent for hydropersulfides and polysulfides as reported recently [Numakura et al., 2017; Akaike et al, 2017]. Briefly, the cultured cells were washed with PBS twice and homogenized in a cold methanol solution containing 1 mM HPE-IAM, after which the cell lysates were incubated at 37°C for 20 min. After centrifugation, aliquots of the supernatants of the lysates were diluted 20 times with 0.1% formic acid containing known amounts of isotope-labeled internal standards, which were then analyzed via LC-ESI-MS/MS for per/polysulfide determination. A triple quadrupole (Q) mass spectrometer LCMS-8050 (Shimadzu) coupled to the Nexera UHPLC system (Shimadzu) was used to perform LC-ESI-MS/MS. Per/polysulfide derivatives were separated by means of Nexera UHPLC with a YMC-Triart C18 column (50 × 2.0 mm inner diameter) under the following elution conditions:

mobile phases A (0.1% formic acid) with a linear gradient of mobile phases B (0.1% formic acid in methanol) from 5% to 90% for 15 min at a flow rate of 0.2 ml/min at 40 °C. MS spectra were obtained with each temperature of the ESI probe, desolvation line, and heat block at 300 ºC, 250 ºC, and 400 ºC, respectively; and the nebulizer, heating, and drying nitrogen gas flows were set to 3, 10, and 10 liters/min, respectively. Various per/polysulfide derivatives were identified and quantified by means of multiple reaction monitoring. Experiments were carried out 5 separate times (each time in triplicate). For each experiment, the values from the triplicates were averaged to give a single value. Experimental results are presented as the averages of the five individual experiments for an n=5. Experimental protocols did not require sample blinding or randomization since sample analyses were not subjective and not susceptible to experimental bias.

Examination of Cys-SSS-Cys pretreatment on cell viability: HEK293T cells were plated at 3 x 10^4 cells/well in a 96-well plate. The following day, media was removed and replaced with DMEM w/o Cys/Met (Gibco) alone or with 1 mM Cys-SSS-Cys. After 1.5 hrs, media was replaced and cells treated with H_2O_2 (Fisher Sci) or NEM (Sigma) for another 6 hrs in complete media. For WST8 assays (Cayman Chemical Co.), the reagent was added for the final 2 hrs and then the absorbance read at 450 nm. For Sytox Green assays (Life Technologies), the reagent was added after the 6 hrs and then the fluorescence (485/528 nm) measured. All treatments were performed in triplicate and samples read on a Synergy HT plate reader (BioTek Instruments). Experiments were carried out 5 separate times. For each experiment, the values from the triplicates were averaged to give a single value. Experimental results are presented as the averages of the five individual experiments for an n=5. Experimental protocols did not require sample blinding or randomization since sample analyses were not subjective and not susceptible to experimental bias.

Data and statistical analysis: Statistical analyses were performed with GraphPad Prism software ver. 6.0 (GraphPad Software, San Diego, CA). Results are presented as means from 5 experiments run separately. For statistical comparisons, a two-way ANOVA was utilized followed by Bonferroni post-tests, with significance set at $P < 0.05$. Post-hoc tests were run only if F achieved $P<0.05$ and there was no significant variance inhomogeneity. In general, experimental design and statistical analyses of the biological experiments follow the recommendations prescribed by Curtis et al. [2015].

Nomenclature of targets and ligands: Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY [Harding *et al.*, 2018],

and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 [Alexander *et al.*, 2017].

Results

Analysis of the NaSH/RSSR Equilibrium: Bogdandi and coworkers [2018, this issue] have described the dynamic equilibrium that establishes between numerous sulfur and polysulfur-containing species. For example, the reaction of GSSG with H_2S results in the formation of GSSH, GSH and GSSSG as equilibrium partners with the initial reactants (**Reactions 1** and **2**). To determine whether these reactions are specific to GSSG or are generally applicable to other disulfides, a series of studies analogous to those reported by Bogdandi et al. [2018, this issue] using a chemically simple disulfide have been carried out. Thus, the reaction of NaSH with 2-hydroxyethyl disulfide ($HOCH_2CH_2SSCH_2CH_2OH$, 2HED), the disulfide of the thiol 2-mercaptoethanol ($HOCH_2CH_2SH$, 2ME) was examined. Increasing additions of NaSH (5-15 mM) to 2HED (10 mM) caused subsequent concentration-dependent increases in the formation of two new species after equilibration (**Figure 1a**). Comparison of the newly formed species with standard samples (data not shown) indicates these species are 2ME (δ = 2.67-2.70 and 3.68-3.72 ppm) and 2-hydroxyethyl trisulfide ($HOCH_2CH_2SSSCH_2CH_2OH$, 2HET) (δ = 3.08-3.11 and 3.91-3.94 ppm). Of additional note is that the ratio of formed 2ME:2HET is 2:1, which would be expected for formation of 2HET via **Reactions 1** and **2**, as described below). Interestingly, addition of 10 mM NaCN to an equilibrated sample of 20 mM 2HED + 10 mM NaSH (**Figure 1b**, bottom spectrum) causes the disappearance of 2HET (**Figure 1b**, top spectrum), supporting the reaction of CN^- with RSSSR.

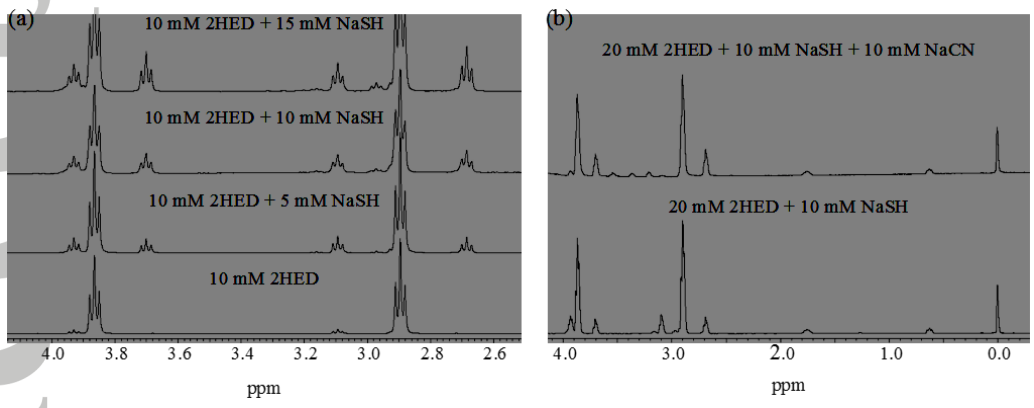


Figure 1: (**a**) Selected region for the stacked ^{1}H-NMR spectra of equilibrated samples (post 30 min) of 10 mM 2HED with increasing additions of NaSH (0, 5, 10 and 15 mM). The newly formed species correspond to 2HET (δ = 3.08-3.11 and 3.91-3.94 ppm) and 2ME (δ = 2.67-2.70 and 3.68-3.72 ppm) and increase in concentration with increasing additions of NaSH. (**b**) Stacked ^{1}H-NMR spectra of an equilibrated sample (post 30 min) of 20 mM 2HED + 10

mM NaSH (bottom spectrum) and addition of 10 mM NaCN (top spectrum) to this equilibrated sample. Addition of NaCN eliminates 2HET (δ = 3.08-3.11 and 3.91-3.94 ppm), making it absent in the top spectrum. All spectra were measured in 100 mM phosphate buffer (pH 7.4) with 10% D_2O and 1 mM DSS as an internal reference.

Data collected from NMR analysis (**Figure 1**) indicate the addition of NaSH to 2HED results in the formation of RSH and RSSSR species after equilibration (consistent with the work of Bogdandi and coworkers [2018, this issue] with GSSG), indicating the general nature of this chemistry. ESI-MS analysis of the 2HED/NaSH reaction also confirms the generation of the corresponding 2HET, which is consistent with the results of NMR analysis here and of Bogdandi and coworkers for GSSG/NaSH [2018, this issue] (data not shown).

Analysis for the Competitive Trapping of Hydropersulfides: The results above and those of Bogdandi et al., [2018, this issue] indicate that addition of NaSH to RSSR results in the formation of the corresponding RSH and RSSSR species as a general reaction. It has been widely accepted that the initial reaction of H_2S with RSSR results in an equilibrated mixture with the corresponding RSH and RSSH species (**Reaction 1**). However, lack of detection of RSSH in the equilibrated systems herein (described above) implies that under the conditions of these experiments the RSSH species are subject to further reaction. This is also consistent with the detection of RSSSR (*vide supra*). It is therefore reasonable to consider that RSSH is fleeting and participates in a second equilibrium that ultimately results in RSSSR formation (**Reaction 2**). If indeed a highly nucleophilic RSSH is fleeting due to subsequent reaction with electrophiles present in the reaction mixture (e.g. RSSR, **Reaction 2**), it is expected that RSSSR formation could be avoided or lessened by the presence of an alternate electrophile capable of trapping the intermediate RSSH (**Reaction 4**). In other words, trapping of RSSH by an exogenous electrophile (**Reaction 4**) would be competitive with trapping of RSSH by RSSR (**Reaction 2**) (as reported by Bogdandi for GSSG [2018, this issue]), and thus by increasing the concentration of this exogenous electrophile, a leftward shift in the equilibrium shown in **Reaction 2** would be expected and RSSSR levels would decrease.

$$RSSH + E^+ \rightarrow RSS\text{-}E + H^+ \quad (E^+ = \text{electrophile}) \qquad (4)$$

Indeed, increased addition of the electrophile iodoacetamide (IAM) (0.5-2.5 mM) to an equilibrated sample of 20 mM 2HED + 10 mM NaSH caused subsequent decreases in the concentration 2HET formed (δ = 3.08-3.11 and 3.91-3.94 ppm) (**Figure 2a**). Additionally, increasing additions of the alternate electrophile *N*-ethylmaleimide (NEM) (0-5 mM) also caused subsequent decreases in the amount of 2HET formed (**Figure 2b**, black

diamonds), albeit to a lesser extent compared to IAM (**Figure 2b**, black squares). Again, as was the case for GSSH [Bogdandi et al., this issue], it appears that the hydropersulfide of 2ME (2MESSH) preferentially reacts with 2HED in the presence of NEM, and this preference occurs to a lesser extent in the presence of IAM.

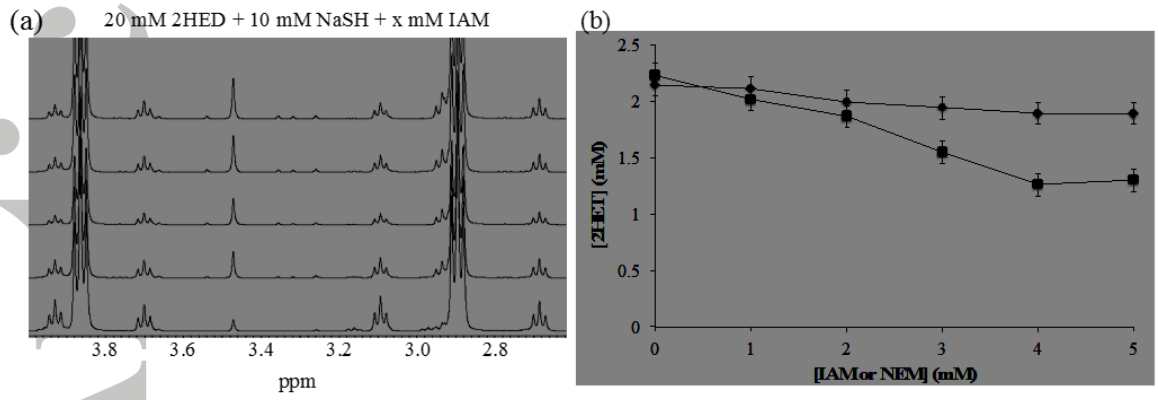


Figure 2: Selected region for stacked ^{1}H-NMR spectra of 20 mM 2HED + 10 mM NaSH with increasing additions of IAM (1-5 mM, bottom to top spectrum, respectively). Increasing additions of IAM causes subsequent decreases in the concentration of 2ME (δ = 2.67-2.70 and 3.68-3.72 ppm) and 2HET (δ = 3.08-3.11 and 3.91-3.94 ppm) because IAM competes with 2HED for the trapping of the intermediate 2ME-SSH. (b) Plot for [2HET] as a function of increasing concentrations of exogenous electrophiles IAM (black squares) or NEM (black diamonds) (0-5 mM). Increasing addition of both electrophiles causes a subsequent decrease in the concentration of 2HET formed. All ^{1}H-NMR experiments were performed in 100 mM phosphate buffer (pH 7.4) with 10% D_2O and 1 mM DSS as an internal reference.

ESI-MS Analysis for the Competitive Trapping of Hydropersulfides: The above data indicate that exogenous addition of an electrophile competes with RSSR for the trapping of RSSH. To verify this further, the products formed after addition of 0.5 mM IAM to an equilibrated sample of 1 mM 2HED + 0.5 mM NaSH were analyzed via ESI-MS. The spectrum shown in **Figure 3** indicates that exogenously added IAM to an equilibrated solution of 2HED + NaSH traps 2MESSH, resulting in the 2MESSH-acetamide trapped adduct ($[2MESS+AM+H]^+$, m/z = 168.05). It should also be noted that 2HET is no longer present in the ESI-MS spectrum of 2HED + NaSH treated with IAM, indicating that trapping of 2MESSH by IAM causes a leftward shift in **Reaction 2** with subsequent loss of 2HET, consistent with the data presented in **Figure 2**.

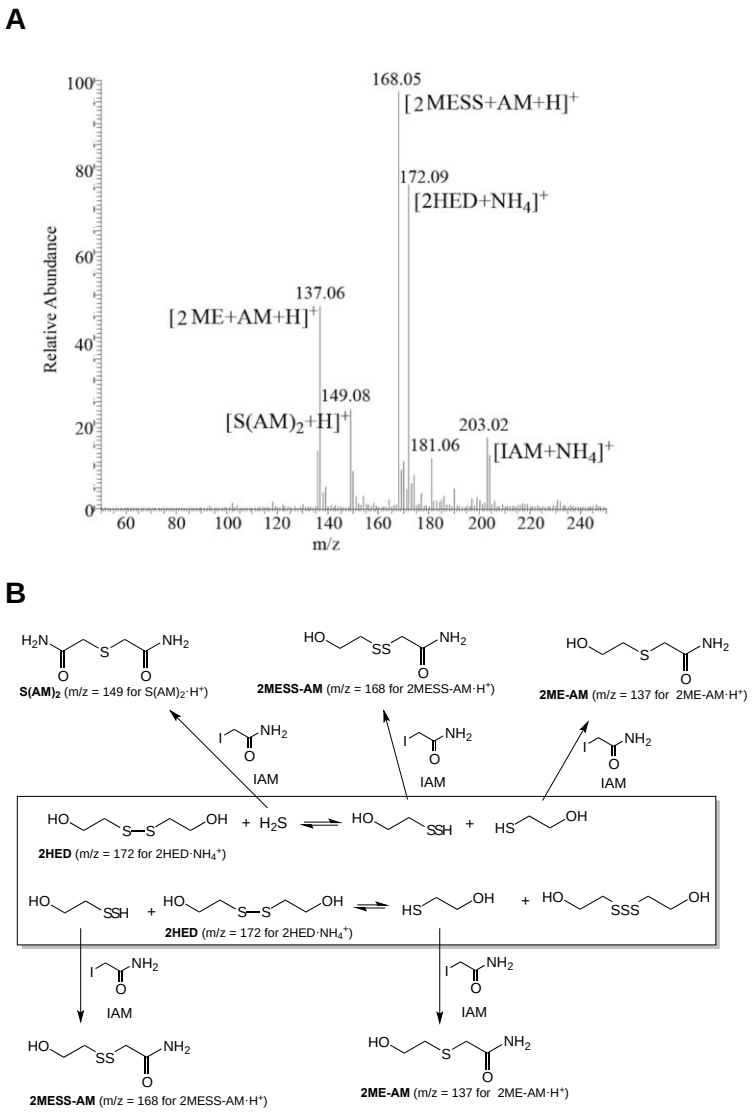


Figure 3: A) ESI-MS spectrum resulting from the addition of 0.5 mM IAM to an equilibrated solution (post 30 min) of 1 mM 2HED + 0.5 mM NaSH. Addition of IAM reacts with, and traps HS^- ($[S(AM)_2+H]^+$), 2ME ($[2ME+AM+H]^+$) and 2MESSH, ($[2MESS+AM+H]^+$). **B**) Scheme of equilibrium and trapped species.

ESI-MS Analysis of the Trisulfide Equilibrium: Summation of **Reaction 1** and **2** leads to the overall equilibrium of **Reaction 5**.

$$2RSSR + H_2S \rightleftharpoons RSSSR + 2RSH \qquad (5)$$

Consistent with the relevance of **Reaction 5**, **Figure 1a** shows that treatment of 2HED with NaSH forms both RSSSR and RSH in an approximate 1:2 ratio, respectively. Additionally, the equilibrium of **Reaction 5** implies that reaction of RSH with RSSSR should lead to formation of the corresponding RSSR and H_2S (the reverse of **Reaction 5**). To test this, 250

µM GSSSG was reacted with 250 µM of the thiol N-acetyl penicillamine (NAP) and analyzed by ESI-MS. **Figure 4a** shows that treatment of GSSSG with NAP (post 3 hours) results in the mixed NAP-SS-G disulfide ([NAP+GS+H]$^+$, m/z = 497.18) with no other alkyl polysulfur species formed, consistent with the reverse of **Reaction 5**.

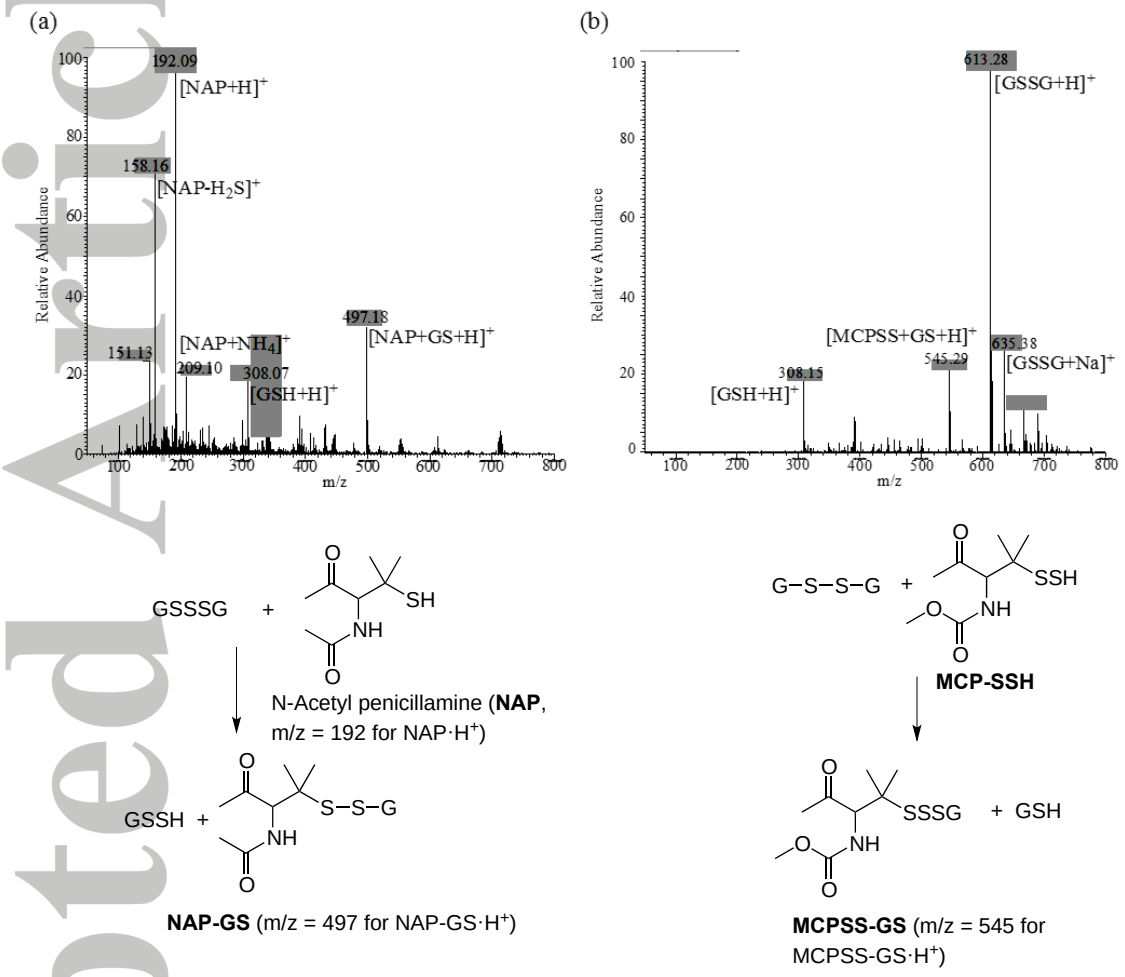


Figure 4: ESI-MS spectrum for the reaction of (a) 250 µM NAP + 250 µM GSSSG (post 3 hours). The mixed disulfide ([NAP+GS+H]$^+$, m/z = 497.18) is the only resulting species from this reaction. (b) Reaction of 200 µM MCP-SSH + 1 mM GSSG. The mixed trisulfide ([MCPSS+GS+H]$^+$, m/z = 545.29) and GSH ([GSH+H]$^+$, m/z = 308.15) are the only resulting species from the reaction. All spectra were recorded in 50 mM ammonium bicarbonate buffer (pH 7.4). Relevant chemistry and mass assignments are shown directly below the spectra.

ESI-MS Analysis for the reaction of RSSH and RSSR: To this point, RSSH intermediates resulting from reaction of NaSH with RSSR have not been directly detected. As an indication of their presence, RSSH intermediates have been trapped via addition of the electrophiles IAM and NEM (**Figures 2** and **3**). To further confirm reaction of RSSH with RSSR, ultimately leading to formation of the corresponding RSSSR (**Reaction 2**), a model

H_2S-independent RSSH donor MCPD was used, as previously described by us [Millikin et al., 2016]. The ESI-MS spectrum corresponding to the decomposition of MCPD (releasing the MCP-SSH hydropersulfide) in the presence of GSSG is shown in **Figure 4b**. As displayed in the spectrum, reaction of MCP-SSH with GSSG leads to the mixed MCP-SSS-G trisulfide (**Figure 4b**, $[MCPSS+GS+H]^+$, m/z = 545.29). No other MCP-SSH derived polysulfur species are detected, which is consistent with the primary fate of RSSH in the presence of RSSR being RSSSR formation (**Reaction 2**). Interestingly, the persulfide donor MCPD is also a disulfide and yet no adduct associated with the persulfide reacting with the precursor donor disulfide species is detected. Thus, GSSG appears to outcompete this reaction since the only trisulfide that was readily detectable was the mixed trisulfide with GSSG (MCPSS+GS). It can also be envisioned that the persulfide formed in this experiment could have also reacted with a protonated hydropersulfide resulting in the corresponding trisulfide, RSSSR and HS^- ($RSS^- + RSSH \rightarrow RSSSR + HS^-$). However, the pK_a of RSSH has been reported to be 6.2 – 4.3 [Everett and Wardman, 1995; Cuevasanta et al., 2015]. Thus, under the conditions of these experiments (pH 7.4), this reaction is unlikely to be competitive since the predominant species will be the nucleophilic anionic RSS^- species with lesser amounts of the electrophilic protonated RSSH species present.

Dialkyltrisulfides as biological sources of hydropersulfides: Thus far, the results presented are consistent with the idea that RSSSR is in a dynamic equilibrium with RSSR, H_2S, RSH and RSSH via **Reactions 1** and **2**. Moreover, RSSH levels are kept low in this system and higher order polysulfides are not readily generated (a rationale for this is given below). These results indicate that the equilibria set up via **Reactions 1** and **2** (and summarized by **Reaction 5**) can be approached starting from either RSSR/H_2S or RSSSR/RSH (**Reaction 5**). That is, RSSH can be generated starting with either equilibrium pair. In order to examine this in a biological system, cells were treated with cysteine trisulfide (Cys-SSS-Cys), with the hope that uptake would occur, thus establishing an intracellular RSSH equilibrium. Indeed, it was found that HEK293T cells treated with 0.2, 0.5 and 1 mM Cys-SSS-Cys for 1.5 hours results in a concentration-dependent and significant increase in intracellular levels of Cys-SSH and also GSSH (**Figure 5**). Importantly, treatment with Cys-SS-Cys alone did not result in increased RSSH levels, indicating that the observed increases in intracellular RSSH were not due to CSE/CBS-mediated conversion of Cys-SS-Cys to Cys-SSH.

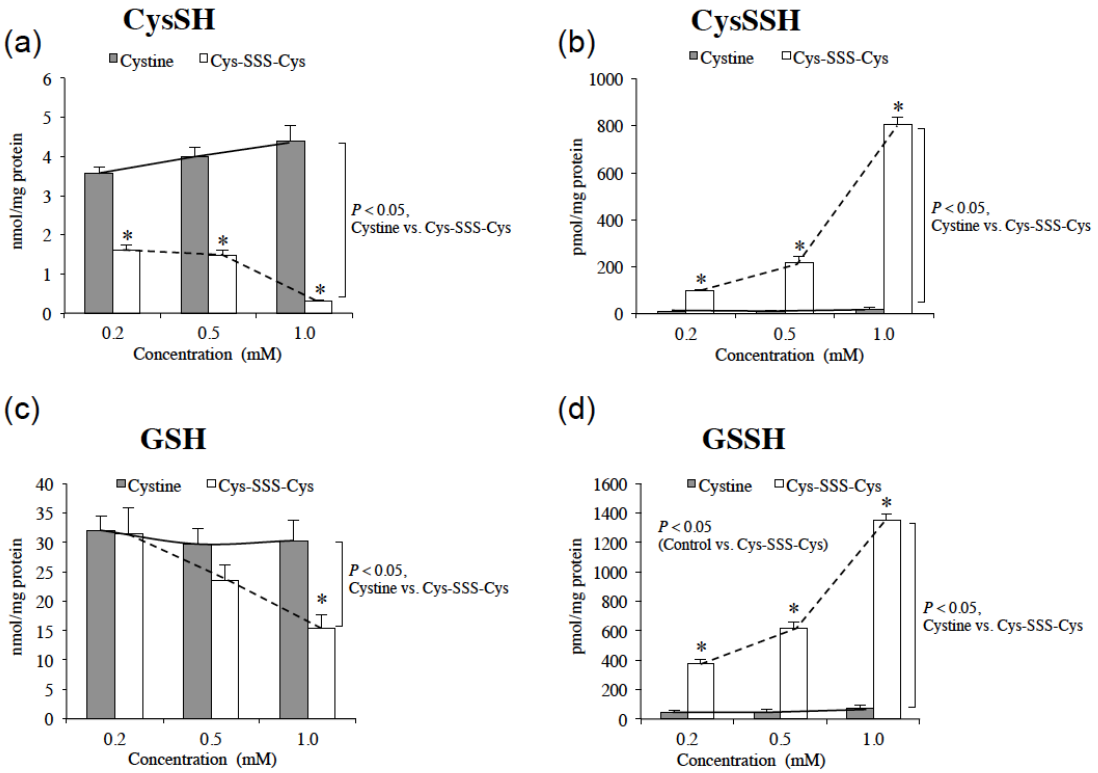


Figure 5: RSH and RSSH levels in HEK293T cells pretreated with either cystine (Cys-SS-Cys) or cysteine trisulfide (Cys-SSS-Cys). (**a**) Cysteine levels resulting from pretreatment with Cys-SS-Cys or Cys-SSS-Cys; (**b**) Cysteine hydropersulfide (Cys-SSH) levels in cells pretreated with Cys-SS-Cys or Cys-SSS-Cys; (**c**) Glutathione levels in cells pretreated with Cys-SS-Cys or Cys-SSS-Cys and (**d**) Glutathione hydropersulfide (GSSH) levels in cells pretreated with Cys-SS-Cys or Cys-SSS-Cys. Each experiment was carried out 5 separate times (each time in triplicate). For each experiment, the values from the triplicates were averaged to give a single value. Data represents average ± SD (n = 5), *P< 0.05. Statistical analysis was performed as described in the Methods section.

Dialkyltrisulfide-mediated cellular protection against oxidative and electrophilic stress:

The results shown in **Figure 5** indicate that HEK293T cells pre-treated with Cys-SSS-Cys results in significant increases in intracellular Cys-SSH and GSSH. It has been postulated that due to their unique chemistry [for example, Ono et al., 2014; Millikin et al., 2016] a possible function of intracellular RSSH species is to protect cells from oxidative and/or electrophilic stress. Thus, Cys-SSS-Cys represents a novel method for examining the ability of increased intracellular RSSH levels in HEK293T cells to protect against electrophilic and/or oxidative stress. To test this, HEK293T cells were pre-treated with 1 mM Cys-SSS-Cys for 1.5 hours followed by exposure to either 0-1 mM H_2O_2 (oxidative/electrophilic stress) or 0-1 mM NEM (electrophilic stress). After 6 hours of treatment with the stressor, cell viability was assessed using a WST8 assay [Ishiyama et al., 1997]. The data indicate that pretreatment with Cys-SSS-Cys results in significant protection against NEM-mediated

toxicity (**Figure 6b**) but only marginal effects on H_2O_2 toxicity were observed (**Figure 6a**). Since viability measured by WST8 is actually an indicator of cellular reductive capacity (NADH/NADPH levels), another method for cell viability was used that interrogates another cell function in order to help confirm these results. Thus, the membrane impermeable nucleic acid dye, Sytox Green was also used as a measure of cytotoxicity (a measure of cell membrane integrity [Jones and Singer, 2001]). Cells were again treated as before, and Sytox Green added after 6 hours (**Figure 6c**). As before, pretreatment with Cys-SSS-Cys protected cells against NEM-mediated toxicity confirming the previous results using WST8.

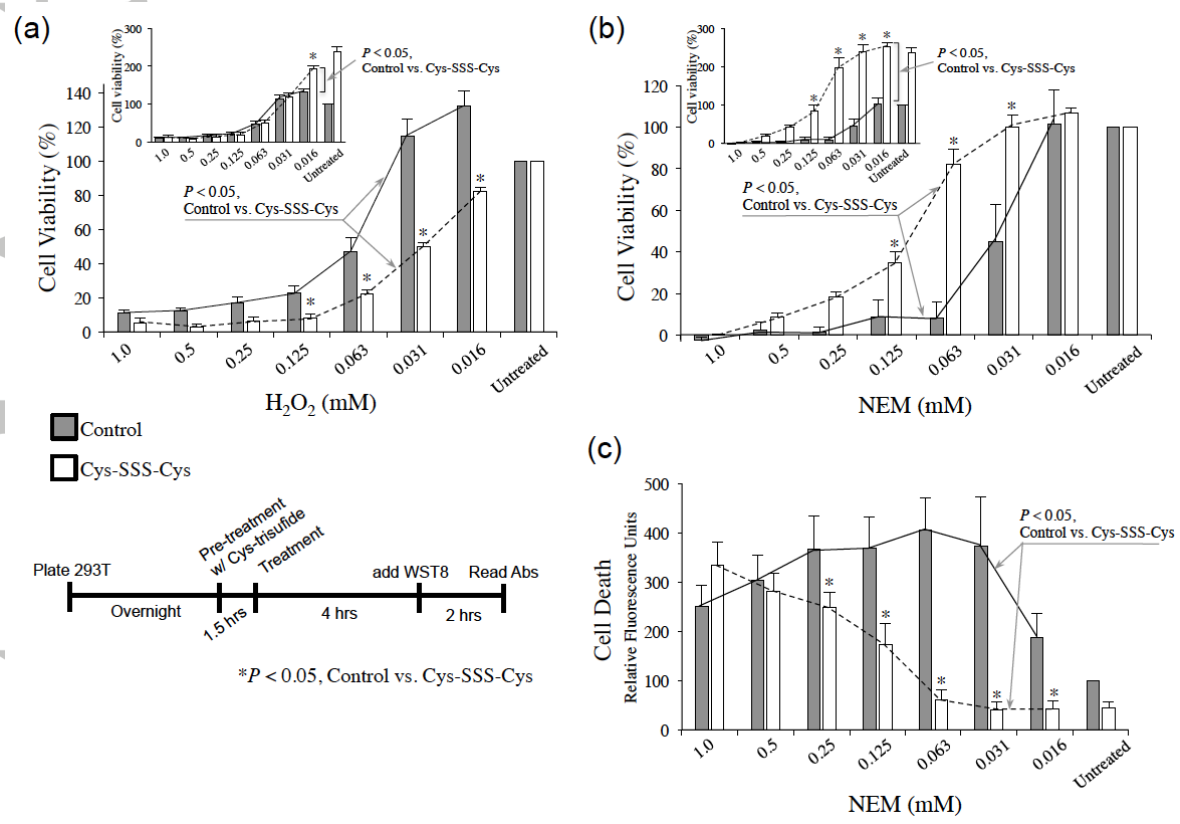


Figure 6: HEK293T cells were pre-incubated with 1mM cysteine trisulfide (Cys-SSS-Cys) for 1.5 hrs. Media was then replaced and cells treated with the indicated amount of H_2O_2 (a) or NEM (b,c) for 6 hrs. The cell viability indicator, WST-8, was used for (a) and (b), while the cell death indicator Sytox Green was used in (c). For (a) and (b), graphs show data normalized to 100% viability for either untreated control or Cys-SSS-Cys only background, whereas insets show data normalized to 100% viability only in untreated controls. Each experiment was carried out 5 separate times (each time in triplicate). For each experiment, the values from the triplicates were averaged to give a single value. Experimental results are presented as the averages ± SEM of the five individual experiments, *P < 0.05. Statistical analysis was performed as described in the Methods section.

Discussion

Recent studies have alluded to the possible importance of RSSH species in biological signaling and in cellular protection against oxidative and/or electrophilic stress [for example, Ono et al., 2014; Ida et al., 2014; Millikin et al., 2016; Saund et al., 2015]. It is becoming increasingly apparent that H_2S, RSSH and other polysulfides are all biologically prevalent and their chemistries intimately intertwined [for example, Ida et al., 2014; Doka et al., 2016]. As mentioned previously, numerous studies have utilized the reaction of H_2S with RSSR (**Reaction 1**) to generate RSSH for study in both chemical and biological systems. However, the work presented herein indicates this may be an oversimplified view of this reaction. It is clear that the RSSH product of **Reaction 1** is very nucleophilic (most likely as the RSS^- anion) and reacts further with the electrophilic RSSR to form RSSSR (**Reaction 2**). It is important to note that RSSH is more acidic than the corresponding RSH by approximately 1-2 pK_a units (and possibly more [Cuevasanta et al., 2015]), indicating that a higher percentage of the nucleophilic anionic RSS^- species will exist compared to the thiolate (RS^-). It is also important to note that the higher levels of RSS^- (compared to the protonated and electrophilic RSSH) will greatly decrease the rate of the reverse of **Reaction 1** since an electrophilic RSSH is required in this reaction [Cuevasanta et al., 2015].

RSSSR generated from **Reaction 2** is likely to be even more electrophilic than the corresponding RSSR (*vide infra*) indicating it may react further under these reaction conditions. In the case of a disulfide, RSSR, nucleophilic attack at either sulfur atom releases RSH/RS^- as the leaving group (**Reaction 6**). In the case of RSSSR, there are two types of sulfur atoms, a sulfane sulfur atom and non-sulfane sulfur atoms (the bolded atoms of R**S**S**S**R). Nucleophilic attack at the sulfane sulfur atom results in an RS^- leaving group (**Reaction 7**) and attack at a non-sulfane sulfur atoms results in expulsion of an RSS^- leaving group (**Reaction 8**).

$$RSSR + Nuc^- \rightarrow RS\text{-}Nuc + RS^- \quad (6)$$

$$RSSSR + Nuc^- \rightarrow RSS\text{-}Nuc + RS^- \quad (7)$$

$$RSSSR + Nuc^- \rightarrow RS\text{-}Nuc + RSS^- \quad (8)$$

Previous studies examining the nucleophilic cleavage of mixed disulfides (**Reaction 6** where the R groups are different), found that the better leaving group (the weaker RS^- base) governed the regiochemistry [Hiskey and Carroll, 1961]. That is, the sulfur atom attacked was the one that had the better leaving group attached. Extrapolating this idea to the nucleophilic attack on RSSSR, it would be expected that attack at the non-sulfane sulfur

sites would predominate since RSS^- is a significantly weaker base than RS^- and thus a better leaving group [for example, Nagy, 2013; Cuevasanta et al., 2015]. Thus, in the reactions examined herein, the attack of a highly nucleophilic RSS^- species on RSSSR is non-productive since it gives back the same reactants (**Reaction 9**).

$$R'SSSR' + RSSH \rightleftharpoons R'SSSR + R'SSH \quad (9)$$

(note: prime notation given to follow reactants)

Of course other nucleophiles are present in solution that can also react with the electrophilic RSSSR species, namely H_2S and RSH. The reaction of HS^- with RSSSR should also occur on the non-sulfane sulfur atoms, leading to the generation of RSSH (**Reaction 10**) and the reaction of RS^- with RSSSR will lead to the formation of the corresponding RSSR and RSSH (**Reaction 11**).

$$RSSSR + H_2S \rightleftharpoons 2\ RSSH \quad (10)$$

$$RSSSR + R'SH \rightleftharpoons RSSR' + RSSH \quad (11)$$

(note: prime notation given to follow reactants)

In the case of the reaction of H_2S with RSSR (**Reaction 1**) and subsequent reactions (**Reactions 2**, **9**, **10** and **11**) formation of the equilibrium partners RSSR, H_2S, RSH, RSSSR and RSSH are expected without the generation of higher order polysulfides (i.e. $RSSS_nR$ or RSS_nH ($n > 1$). Although chemical reactions can be envisioned that could lead to higher order polysulfides (**Reactions 12** and **13**), based on the results and arguments presented here, these reactions appear unfavorable since the sulfane sulfur of RSSSR would have to be attacked.

$$RSSSR + RSSH \rightleftharpoons RSSSSR + RSH \quad (12)$$

$$RSSSR + H_2S \rightleftharpoons RSSSH + RSH \quad (13)$$

Figure 7 schematically depicts the rationale explaining the lack of formation of higher order polysulfides (e.g. tetrasulfides, RSSSSR) from the reaction of RSS^- with RSSSR. In all cases the favored reaction involves nucleophilic attack at the non-sulfane sulfur atoms (**Figure 7**, pathway **b**).

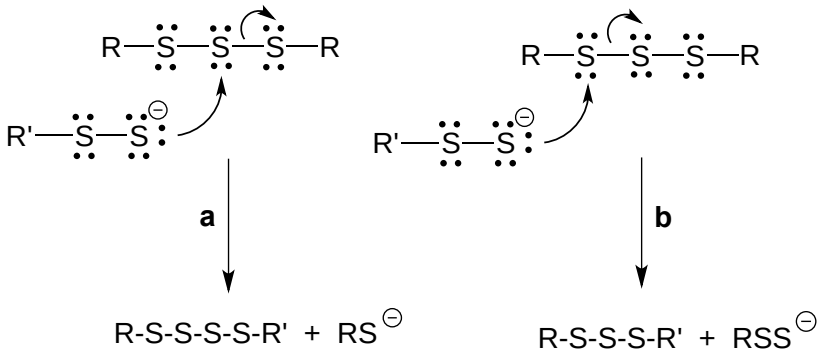
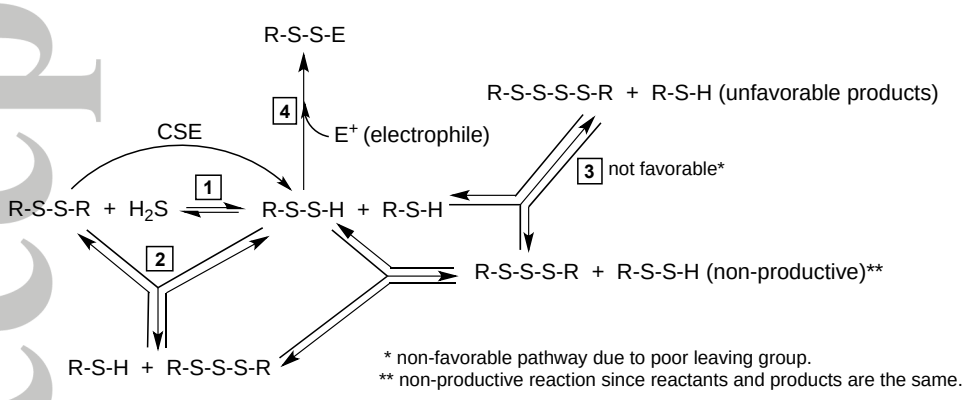


Figure 7: Reaction of a nucleophilic hydropersulfide with a trisulfide. (**a**) nucleophilic attack at the sulfane sulfur atom, (**b**) nucleophilic attack at a non-sulfane sulfur atom.

Another factor that may favor nucleophilic attack on the non-sulfane sulfur atoms of RSSSR may involve electron pair repulsion between the attacking nucleophile and the lone pair electrons on the sulfur atoms of RSSSR. Nucleophilic attack on the central sulfane sulfur atom of RSSSR by RSS^- (**Figure 7**, pathway **a**) would be inhibited by repulsion between the nucleophile and the lone pairs of electrons on both of the two adjacent sulfur atoms whereas nucleophilic attack on the non-sulfane sulfur atoms (**Figure 7**, pathway **b**) involves repulsion from only one adjacent sulfur atom. Regardless of which effect predominates, both favor nucleophilic attack on the non-sulfane sulfur sites of an RSSSR species in this purely chemical system. **Scheme 1** illustrates the likely integrated chemistry that describes the results herein.



Scheme 1: Integrated reaction pathways.

Support for the chemistry depicted in **Scheme 1** is as follows:

1) Equilibrium 1 is a previously established reaction, which has been used by several labs to generate RSSH species *in situ* [Rao and Gorin, 1959; Cavalini et al., 1970; Francoleon et al., 2011].

2) Equilibrium 2 is evidenced by results presented in this study and the study of Bogdandi et al., [2018, this issue]. Both NMR and mass spectral analysis indicate that the reaction of a disulfide (e.g. 2HED) with H_2S results in detectable levels of thiol (2ME) and the corresponding trisulfide (2HET). Interestingly, RSSH species are not detected using either method of analysis, indicating that they may be at extremely low levels under the conditions of these experiments.

3) Reaction 3 (tetrasulfide, RSSSSR formation, **Reaction 12**, **Figure 7a**) appears unfavorable (*vide supra*) and, expectedly, RSSSSR species are not observed.

4) Reaction 4 the trapping of RSSH by an exogenous electrophile, occurs readily and serves as evidence for the proposed equilibria (although RSSH species are not directly detected).

It is important to note that care must be taken when extrapolating the chemical results described herein to a biological system. The kinetics of all the reactions examined (e.g. the forward and reverse reactions of **Reactions 1** and **2**) are not firmly established and it is possible (if not likely) that competing reactions of the equilibrium species with other available biological molecules can occur. For example, RSSH species are highly redox active [Bianco et al., 2017; Francoleon et al., 2011] and can be intercepted by mild biological oxidants before it can participate in the equilibrium reactions described herein. Also, enzymatic conversion of, for example, RSSSR species via reduction processes to other species can occur, thus removing trisulfides from the equilibrium reactions. It is also worth noting that previous studies have examined the kinetics associated with the forward process of **Reaction 1** (the reaction of H_2S with a disulfide) and the rate constants are reported to be highly variable and dependent on the nature of the reactants [Vasas et al., 2015; Cuevasanta et al., 2015]. For example, second order rate constants ranging from approximately 10^3 $M^{-1}s^{-1}$ to 10^{-2} $M^{-1}s^{-1}$ for the reaction of H_2S with 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and 2HED, respectively, are reported. Since RSSH species are considered to be superior nucleophiles compared to thiols (and H_2S), it is expected that they can react faster with electrophilic species compared to the corresponding thiol or H_2S. Indeed, Cuevasanta and coworkers [2015] estimate that RSSH species can be as much as 20-fold more reactive than the corresponding thiol at physiological pH.

Treatment of HEK293T cells with Cys-SSS-Cys led to a significant increase in the intracellular hydropersulfides Cys-SSH and GSSH (**Figure 5**). It is established that the uptake of Cys-SS-Cys in mammalian cells occurs via the activity of the antiporter xc^-, which exports glutamate while it takes up Cys-SS-Cys from extracellular solution [Conrad and Sato, 2012; Lewerenz et al., 2013]. However, it is not known whether Cys-SSS-Cys is also transported via xc^-. Regardless, Cys-SSS-Cys may represent a possible pharmacological/research tool for raising intracellular hydropersulfides (although more cell types need to be examined to determine if this is a general phenomenon). Importantly, trisulfides (e.g. Cys-SSS-Cys) are stable and can be stored and used similarly to disulfides (e.g. Cys-SS-Cys). In our hands, Cys-SSS-Cys is stable to acidic conditions, can be stored at room temperature, is stable to decomposition for months and is more soluble (approximately 10x) than Cys-SS-Cys in aqueous solution. On the other hand, hydropersulfides are inherently unstable to disproportionation and cannot be easily stored [Rao and Gorin, 1959].

Consistent with the idea that hydropersulfides can protect against specific stressors, HEK293T cells pretreated with Cys-SSS-Cys (which leads to increased intracellular levels of hydropersulfides, **Figure 5**) were significantly protected from the toxicity of the electrophile NEM (**Figure 6b**). However, a marginal effect (if any, depending on how samples are normalized) was observed with H_2O_2 treatment (**Figure 6a**). In the case of NEM, the increased levels of the highly nucleophilic hydropersulfides may be capable of intercepting/scavenging an otherwise toxic electrophile before it is able to modify critical cellular sites. Alternatively, hydropersulfide formation on critical protein thiols could be protected from irreversible electrophilic modification since modification will occur on the sacrificial sulfane sulfur atom, which can be reductively removed [Ono et al., 2014; Millikin et al., 2016]. Importantly, persulfide-mediated protection against thiophilic metal toxins has also been reported [Abiko et al., 2015]. Although much more work remains to determine whether generation of per- and/or poly-sulfides represents a protective mechanism against oxidative and/or electrophilic stress, the utility of Cys-SSS-Cys as a means of pharmacologically altering intracellular hydropersulfide levels may be an important tool in this regard.

Differences in the observed protection by Cys-SSS-Cys between H_2O_2 and NEM is intriguing. It is well established that NEM toxicity is primarily the result of thiol protein alkylation [for example, Vimard et al., 2011]. Thus, considering the high nucleophilicity of RSSH it is not surprising that increased intracellular RSSH results in protection against NEM. Although H_2O_2-mediated physiology (i.e. signaling) can either directly or indirectly involve oxidative thiol modification [for example, Winterbourn, 2013; Jones, 2008] the toxicity of H_2O_2 is thought to primarily involve metal-mediated generation of potent and

indiscriminate oxidants (i.e. via the Fenton reaction) [Winterbourn, 1995]. Importantly, the oxidants generated in this way are thought to be too reactive to be trapped or scavenged since they are immediately reactive and do not diffuse away from their site of generation. Thus, RSSH may have little effect on Fenton-type oxidants.

It is important to note that intracellular increases in RSSH species may also lead to higher intracellular levels of RSSR, RSH and H_2S via the equilibrium processes described above. Thus, it is naïve and even ill-advised to conclude categorically that the observed protection is the sole result of increased intracellular RSSH levels. Since treatment of cells with Cys-SSS-Cys does not dramatically increase levels of RSH species (**Figure 5**) the observed protection is clearly not due to RSH. However, it remains possible that the protection results, at least partially, from increased intracellular H_2S levels. Indeed, based on the results herein and those of Bogdandi and coworkers [2018, this issue], it is currently difficult to distinguish between the actions of RSSH from H_2S in a biological system since they are equilibrium partners and should be present simultaneously. To be sure, there are chemical rationale for predicting protective functions associated with RSSH species [for example, Ono et al., 2014; Millikin et al., 2016], but this remains to be unequivocally established.

Finally, it is interesting to note that treatment of cells with Cys-SSS-Cys alone leads to an increase in apparent "viability" (as measured by the WST8 assay) (**Figure 6a** and **6b**) in control samples. That is, Cys-SSS-Cys treatment on its own appears to result in an increase in cellular reducing equivalents/reduction capacity (what WST8 ultimately measures [Ishiyama et al., 1997]), which is construed as an increase in viability. It is noteworthy that hydropersulfides are considered to be good reductants (superior to, for example, thiols and akin to ascorbate and/or tocopherols [for example, Bianco et al., 2016; Chauvin et al., 2017]). Thus, in spite of the fact that RSSH species are formally oxidized with respect of the corresponding RSH, they are significantly more reducing and may possibly be responsible for the greater reducing capacity observed. Interestingly, Akaike and coworkers [2017] reported that hydropersulfide generation in mitochondria is an important aspect of mitochondrial regulation and activity and thus it is intriguing to speculate that since NADH (one of the primary species assayed by WST8) is used by mitochondria as an electron source, the apparent increases in NADH levels in Cys-SSS-Cys pre-treated cells may be the result of other reductants, possibly RSSH species, serving as alternative mitochondrial electron sources (proposed previously [Akaike et al., 2017]) allowing NADH levels to rise.

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Conflict of Interest: The authors declare no conflicts of interest.

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