Redox Biology 12 (2017) 325–339

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

# Catalase as a sulfide-sulfur oxido-reductase: An ancient (and modern?) regulator of reactive sulfur species (RSS)

Kenneth R. Olson[a,*], Yan Gao[a], Eric R. DeLeon[a,b], Maaz Arif[a], Faihaan Arif[a], Nitin Arora[a], Karl D. Straub[c,d]

[a] Indiana University School of Medicine - South Bend, South Bend, IN 46617, USA
[b] Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA
[c] Central Arkansas Veteran's Healthcare System, Little Rock, AR 72205, USA
[d] Departments of Medicine and Biochemistry, University of Arkansas for Medical Sciences, Little Rock, AR 72202, USA

ARTICLE INFO

*Keywords:*
Hydrogen sulfide
Polysulfide
Garlic
Reactive oxygen species
Thioredoxin
Evolution
*Aspergillus niger*

ABSTRACT

Catalase is well-known as an antioxidant dismutating $H_2O_2$ to $O_2$ and $H_2O$. However, catalases evolved when metabolism was largely sulfur-based, long before $O_2$ and reactive oxygen species (ROS) became abundant, suggesting catalase metabolizes reactive sulfide species (RSS). Here we examine catalase metabolism of $H_2S_n$, the sulfur analog of $H_2O_2$, hydrogen sulfide ($H_2S$) and other sulfur-bearing molecules using $H_2S$-specific amperometric electrodes and fluorophores to measure polysulfides ($H_2S_n$; SSP4) and ROS (dichlorofluorescein, DCF). Catalase eliminated $H_2S_n$, but did not anaerobically generate $H_2S$, the expected product of dismutation. Instead, catalase concentration- and oxygen-dependently metabolized $H_2S$ and in so doing acted as a sulfide oxidase with a $P_{50}$ of 20 mmHg. $H_2O_2$ had little effect on catalase-mediated $H_2S$ metabolism but in the presence of the catalase inhibitor, sodium azide (Az), $H_2O_2$ rapidly and efficiently expedited $H_2S$ metabolism in both normoxia and hypoxia suggesting $H_2O_2$ is an effective electron acceptor in this reaction. Unexpectedly, catalase concentration-dependently generated $H_2S$ from dithiothreitol (DTT) in both normoxia and hypoxia, concomitantly oxidizing $H_2S$ in the presence of $O_2$. $H_2S$ production from DTT was inhibited by carbon monoxide and augmented by NADPH suggesting that catalase heme-iron is the catalytic site and that NADPH provides reducing equivalents. Catalase also generated $H_2S$ from garlic oil, diallyltrisulfide, thioredoxin and sulfur dioxide, but not from sulfite, metabisulfite, carbonyl sulfide, cysteine, cystine, glutathione or oxidized glutathione. Oxidase activity was also present in catalase from *Aspergillus niger*. These results show that catalase can act as either a sulfide oxidase or sulfur reductase and they suggest that these activities likely played a prominent role in sulfur metabolism during evolution and may continue do so in modern cells as well. This also appears to be the first observation of catalase reductase activity independent of peroxide dismutation.

## 1. Introduction

Reactive oxygen species (ROS) are produced from one-electron reductions of oxygen that sequentially form superoxide ($O_2^{\bullet-}$), hydrogen peroxide ($H_2O_2$), hydroxyl radical ($HO^{\bullet}$) and ultimately terminate in water;

$$O_2\ (-e^-) -> O_2^{\bullet-}\ (-e^-) -> H_2O_2\ (-e^-) -> HO^{\bullet}\ (-e^-) -> H_2O \quad (1)$$

Reactive sulfide species (RSS) are chemically, biochemically and physiologically similar to ROS [1] and can be produced from sequential one-electron oxidations of hydrogen sulfide ($H_2S$) to form a thiyl radical ($HS^{\bullet}$), hydrogen persulfide ($H_2S_2$) and persulfide radical ($HS_2^{\bullet-}$) before terminating in elemental sulfur ($S_2$); the latter usually cyclizing to $S_8$;.

$$H_2S\ (+e^-) -> HS^{\bullet}\ (+e^-) -> H_2S_2\ (+e^-) -> HS_2^{\bullet-}\ (+e^-) -> S_2 \quad (2)$$

While ROS have pathophysiological consequences when in excess, there is considerable evidence that $H_2O_2$, and perhaps $O_2^{\bullet-}$ are important homeostatic signaling entities under normal circumstances [2–18]. As chalcogens with six valence electrons, oxygen and sulfur would be expected to exhibit some commonalities in their biological actions and this has become quite apparent in regard to signaling via cysteine sulfur (Cys-S) in regulatory proteins. Peroxidation of Cys-S produces the sulfenyl, Cys-SOH and persulfidation (a.k.a. sulfhydration) produces a cysteine persulfide (Cys-S-SH; [19]). In the few regulatory systems where both peroxidation and persulfidation have

* Correspondence to: Indiana University School of Medicine -South Bend, Raclin Carmichael Hall, 1234 Notre Dame Avenue, South Bend, IN 46617, USA.
*E-mail address:* olson.1@nd.edu (K.R. Olson).

http://dx.doi.org/10.1016/j.redox.2017.02.021
Received 23 January 2017; Received in revised form 11 February 2017; Accepted 24 February 2017
Available online 01 March 2017

been examined in detail the effector responses appear to be identical [20–25] with the added caveat that, unlike $H_2O$, $H_2S$ can also reduce protein disulfide bonds and effect enzyme activity [26]. In addition, we have shown that many of the methods used to measure ROS are sensitive to RSS and often more so [27]. This further confounds issues of the relative biological importance of ROS versus RSS in terms of tissue production, metabolism and intracellular signaling.

Cells have purportedly developed a number of "antioxidant" mechanisms to regulate ROS and guard against their toxicity. Catalase is one of the earliest known and best characterized of the antioxidant enzymes catalyzing the dismutation of peroxide to water and oxygen;

$$2H_2O_2 -> 2H_2O + O_2 \quad (3)$$

However, because catalase appears to have appeared in evolution long before oxygen was present and at a time when RSS were more likely to be involved in cellular metabolism [1], (also see discussion), we wondered if catalase could also dismutate persulfide, i.e.;

$$2H_2S_2 -> 2H_2S + S_2 \quad (4)$$

While we observed that catalase did indeed remove persulfides from solution we also observed, unexpectedly, that catalase also removed $H_2S$ from solution. Because sulfur in $H_2S$ is in its most reduced state (−2), $H_2S$ dismutation is impossible and the most logical scenario is that $H_2S$ is oxidized. We also observed that under certain conditions catalase generated $H_2S$ from other sulfur-bearing molecules. Thus catalase appears to be a "primordial" sulfur oxidoreductase. In the present study we examine these aspects of catalase-mediated sulfur metabolism and attempt to place them into an evolutionary perspective where these functions most likely evolved and suggest how they may still play a homeostatic role in modern animals.

## 2. Materials and methods

### 2.1. Chemicals

SSP4 (3',6'-Di(O-thiosalicyl)fluorescein), $Na_2S_2$, $Na_2S_3$ and $Na_2S_4$ were purchased from Dojindo molecular Technologies Inc. (Rockville, MD). Thioredoxin was purchased from ThermoFisher Scientific (Grand Island, NY). Carbon monoxide (CO, 1 mM), carbonyl sulfide (COS, 20 mM) and sulfur dioxide ($SO_2$, 1.4 M) solutions were prepared by bubbling pure gas through a sintered glass aerator into buffer for 20–30 min. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Phosphate buffer (in mM): 137 NaCl, 2.7, KCl, 8 $Na_2HPO_4$, 2 $NaH_2PO_4$, pH 7.4.

Sorensen's buffer in (mM): 200 $Na_2HPO_4$, 200 $NaH_2PO_4$, ratio sdjusted to pH 6, 7 or 8.

### 2.2. Polysulfide measurement

The polysulfide-specific fluorophore, SSP4 was used to measure polysulfides. Samples and test compounds were aliquoted into black 96 well plates in a darkened room and fluorescence was measured on a SpectraMax M5e plate reader (Molecular Devices, Sunnyvale, CA). Typically, fluorescence was measured every 10 min over 90 min. In order to reduce the potential loss of $H_2S$ due to volatilization in these and other experiments the cover of the well plate was lined with parafilm in an attempt to seal off the wells.

### 2.3. Amperometric measurement of $O_2$, $H_2O_2$ and $H_2S$

Amperometric $O_2$ and $H_2O_2$ sensors, ISO-OXY-2 and ISO-HPO-2, respectively, were purchased from WPI (World Precision Instruments, Sarasota, FL). They are designed for tissue culture with 2 mm dia replaceable membrane sleeves and a reported detection limit of 0.1% (ISO-OXY-2) and < 100 nM (ISO-HPO-2). It should be noted that the ISO-HPO-2$H_2O_2$ sensor cannot be used when $H_2S$ is present as it is 24 times more sensitive to $H_2S$ than it is to $H_2O_2$ [27].

$H_2S$ amperometric sensors with a sensitivity of 14 nM $H_2S$ gas (~100 nM total sulfide) were constructed in-house as described previously [28]. The sensors were connected to WPI TBR 4100 Free Radical Analyzers and data was archived on a laptop PC with software provided by the manufacturer and exported into Microsoft Excel. The $H_2S$ sensor was calibrated periodically throughout each day with fresh standards made up in anoxic phosphate buffer (pH 7.4). This sensor does not respond to polysulfides or other oxidized forms of sulfur.

A reaction chamber with a side ports for the $H_2S$ and $O_2$ sensors and a 1-cm wide by 2 cm deep central well was purchased from WPI (NOCHM-4). A polycarbonate stopper with a hole in the stopper permitted venting the head space air when the stopper was lowered into the chamber and provided an access port for sample injection with a Hamilton microliter syringe. The chamber was placed on a magnetic stirrer and stirred with a Teflon micro stir bar. Compounds of interest were injected through the stopper and the reactions monitored for 10–30 min or longer if necessary.

### 2.4. Oxygen sensitivity of $H_2S$ oxidation by catalase

To determine if catalase-mediated inactivation of $H_2S$ was an oxidative process buffer containing catalase was deoxygenated by passing 100% $N_2$ into the chamber via a 21 ga needle inserted into the stopper until $O_2$ was removed as indicated by the $O_2$ electrode. This decreased the rate of $H_2S$ consumption confirming that this was an oxidative process. Preliminary experiments showed that $H_2S$ oxidation was not affected by a 6% $O_2$ balance $N_2$ mixture. In order to examine $O_2$ tensions below this the 6% $O_2$/bal $N_2$ gas was mixed with 100% $N_2$ using a Wösthoff Digamix gas mixing pump (H. Wösthoff Messtechnik GmbH, Bochum, Germany). Samples were gassed as above and $Po_2$ was continuously monitored. The partial pressure of $O_2$ at which catalase oxidation was halved ($P_{50}$) was determined from the graph of percent $H_2S$ consumption vs percent $O_2$ in chamber. Oxygen concentration in μM was determined from the prevailing barometric pressure ($P_B$) measured in the laboratory with a mercury barometer, water vapor pressure ($P_{H2O}$, 17.5 mmHg at 20 °C) and the oxygen solubility coefficient (α, at 300 mosm $L^{-1}$ and 20 °C =1.7196 μmol $L^{-1}$ $mmHg^{-1}$; [29]; $O_2$ (μM) =α•0.209•($P_B$-$P_{H2O}$)).

### 2.5. pH sensitivity of catalase-mediated $H_2S$ oxidation

In order to determine if the catalase preferentially reacts with dissolved $H_2S$ or the hydrosulfide anion ($HS^-$) the rate of $H_2S$ oxidation in Sorensen's buffer at pH 6, 7 and 8 was monitored with the amperometric $H_2S$ sensor. As the $pKa_1$ of the reaction, $H_2S$ <-> $HS^-$ is 6.98 at 20 °C [30], this allowed us to adjust the $H_2S$:$HS^-$ ratio from 90:10 to 10:90.

Vetrano et al. [31] have shown that catalase oxidizes dichlorofluorescein (DCF) and we observed DCF oxidation competes with $H_2S$ oxidation (Olson unpublished). In these experiments we first measured DCF oxidation by catalase at pH 6,7 and 8 in 96 well plates in the absence of $H_2S$ to determine the pH sensitivity of catalase. By repeating these experiments in the presence of $H_2S$ it was then possible to identify the sulfide species that competes with DCF in the catalase-mediated oxidation process.

### 2.6. Sodium azide

In preliminary experiments we monitored $H_2O_2$ concentration with the $H_2O_2$ sensor and found that catalase dismutation of 10 μM $H_2O_2$ could be completely inhibited by 50 mM sodium azide ($NaN_3$; not shown). This concentration of azide was used in all further experi-

ments. The effects of azide on $H_2S$ metabolism were measured by adding 10 μM $H_2S$ to catalase in the presence or absence of 50 mM azide. As this appeared to decrease the rate of catalase-mediated $H_2S$ oxidation we then examined the possibility that another electron acceptor, $H_2O_2$, might take the place of $O_2$. In these experiments 10 or 100 μM $H_2O_2$ was added 2–5 min after $H_2S$ in the presence of catalase with or without azide.

### 2.7. Aspergillus niger catalase

Catalase from the fungus *Aspergillus niger* was used to investigate limited aspects of sulfide:sulfur metabolism. This enzyme does not contain or readily utilize NADPH cofactors and only forms Compound I.

### 2.8. Data Analysis

Data was analyzed and graphed using QuatroPro (Corel Corporation, Ottawa Ont, Canada) and SigmaPlot 13.0 (Systat Software, Inc., San Jose, CA). Statistical significance was determined using one-way ANOVA and the Holm-Sidak test (SigmaPlot 13.0). Results are given as mean + or ± SE; significance was assumed when $p \leq 0.05$.

## 3. Results

### 3.1. Catalase as a sulfide oxidase

#### 3.1.1. Effects of catalase on mixed polysulfide

When dissolved, $K_2S_n$ forms a mixture of polysulfides, $H_2S_n$, where n=1–8, i.e., $H_2S$, $H_2S_2$, $H_2S_3$... $H_2S_8$. As shown in Fig. 1A, 20 μM $H_2S_n$ alone in solution concentration-dependently increased SSP4 fluorescence and this was almost completely prevented by adding 25 μM catalase. As we presumed this was due to catalase dismutation of the polysulfide to $H_2S$ (Eq. (4)) we then measured $H_2S$ directly and in real time with the amperometric sensor. As shown in Fig. 1B, dissolving $K_2S_n$ produced $H_2S$ which slowly out-gassed through the hole in the stopper. However, when catalase was added the $H_2S$ concentration began to decrease more rapidly and increasing catalase concentration from 10 to 25 μM decreased the $H_2S$ concentration from ~4 μM to essentially nil in approximately 7 min. Addition of a second 25 μM catalase (50 μM total) did not affect $H_2S$ concentration confirming that $H_2S$ was not produced from the remaining polysulfides. These results clearly show that under aerobic conditions catalase metabolizes polysulfides. They also suggest that polysulfide metabolism is either not a dismutative process (in that no $H_2S$ is produced), or if $H_2S$ is produced it is also metabolized by catalase or tightly bound to it.

It should be noted that the exact ratio of polysulfide species produced when $K_2S_n$ is dissolved is not known. We show in Fig. 1B that dissolving 20 μM of the salt produced ~4 μM $H_2S$. If there is equal distribution of the remaining 16 μM of $S_2$-$S_7$ sulfur species there would be 72 μM sulfur. If catalase dismutates these polysulfides there would be enough catalase at 25 μM (100 μM heme) to bind one sulfide per heme. Subsequent studies were aimed at resolving this issue.

#### 3.1.2. Catalase reactions with $H_2S$

Catalase from 0 to 50 μM concentration-dependently increased the rate of 10 μM $H_2S$ removal from buffer when measured amperometrically (Fig. 2A, B). Under aerobic conditions administering $H_2S$ in five consecutive 10 μM doses cumulatively increased $H_2S$ concentration (Fig. 2C), whereas in the presence of 25 μM catalase, ten consecutive 20 μM $H_2S$ injections were continuously removed and did not accumulate (Fig. 2D). The average rate of removal of these 10$H_2S$ injections in the presence of catalase (−1.81 ± 0.03 μmoles $H_2S$/min) remained relatively constant over the course of the experiment (Fig. 2D, E). This suggests that $H_2S$ does not remain bound to catalase thereby affecting its activity. This is supported by the fact that at the end of the experiment the $H_2S$ concentration (200 μM) was twice as much as the four heme groups in 25 μM catalase (100 μM). In another experiment, 13 consecutive $H_2S$ additions were applied with the same results (not shown).

#### 3.1.3. $O_2$ sensitivity of catalase-mediated $H_2S$ oxidation

Because $H_2S$ sulfur is in its most reduced form (−2), catalase metabolism of $H_2S$ is likely an oxidative process. This possibility was examined by measuring $H_2S$ metabolism at different oxygen tensions. The oxygen sensitivity of 25 μM catalase-mediated metabolism of $H_2S$ (measured amperometrically) is shown in Fig. 3A. There was a progressive decrease in the rate of consumption of 10 μM $H_2S$ as the percent $O_2$ fell below 4.8%. The rate of $H_2S$ consumption was halved at ~2.7% $O_2$ (Fig. 3B) which at average barometric pressure (745 mmHg) and 100% humidity ($P_{H2O}$ =17.5 mmHg) resulted in an apparent $P_{50}$ of ~20 mmHg.

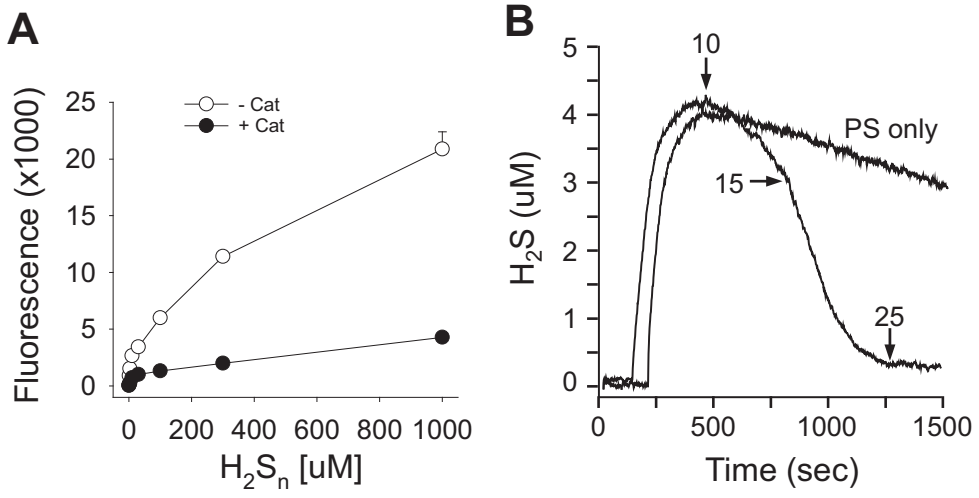


**Fig. 1.** (**A**) Concentration-dependent polysulfide ($H_2S_n$) mediated SSP4 fluorescence in the presence or absence of 25 μM catalase. Catalase essentially inhibits fluorescence over a wide range of polysulfide concentrations. Mean +SE, n =4, many error bars are within symbols. (**B**) Amperometric measurement of $H_2S$ concentration in 20 μM polysulfide in the absence of catalase (PS only) or after additions of 10, 15 and 25 μM catalase (total catalase, 10, 25 and 50 μM). In the absence of catalase $H_2S$ slowly out-gases from the chamber. Addition of 10 μM catalase increases the rate of $H_2S$ disappearance and this is further increased by a second addition of 15 μM catalase which completely removes all $H_2S$. Doubling the total catalase concentration to 50 μM by a third addition of catalase did not affect $H_2S$ concentration.

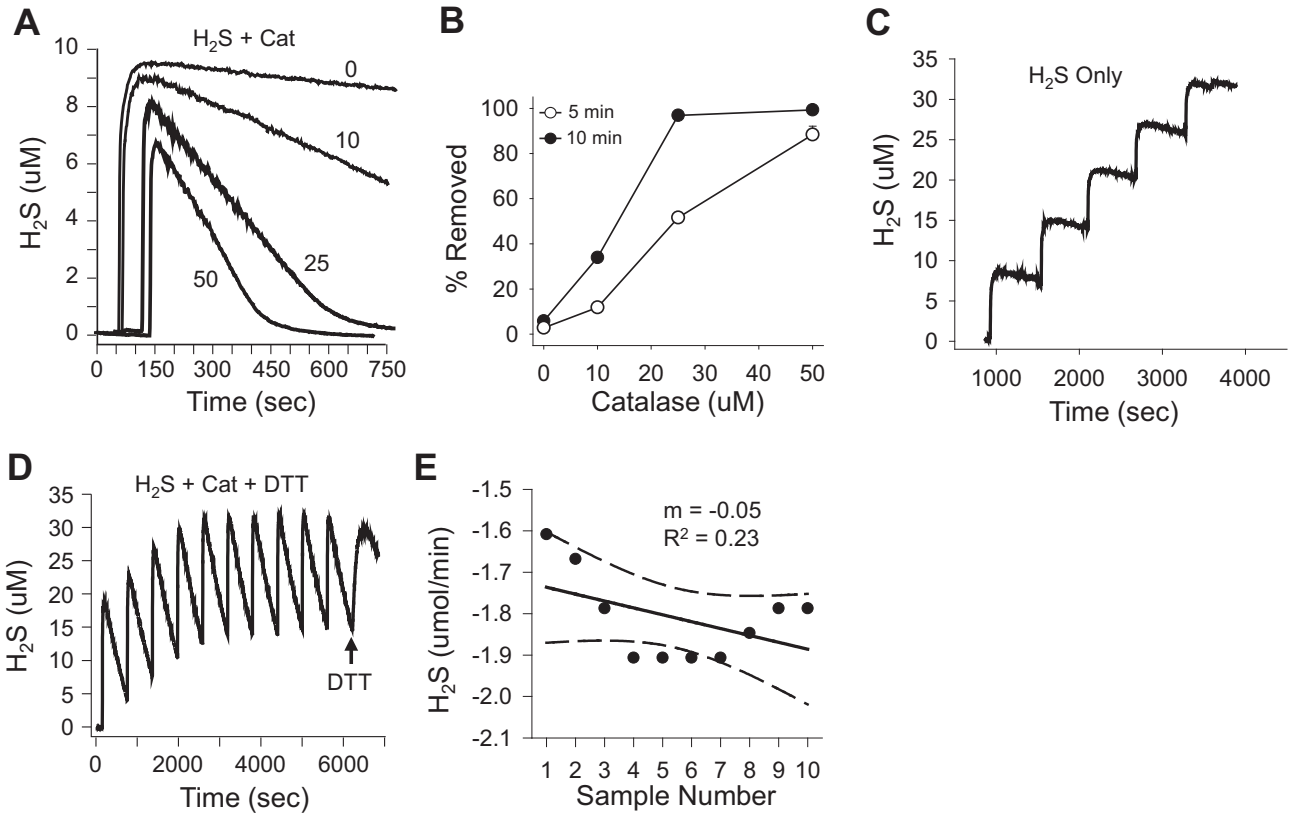


**Fig. 2.** Amperometric measurements of $H_2S$ metabolism by catalase. (**A, B**) Catalase from 0 to 50 μM concentration dependently increased the rate of disappearance of 10 μM $H_2S$; (**A**) representative traces, (**B**) average percent removed at 5 (open circles) and 10 (black circles) min. Mean +SE ($n$ =3), all values with catalase > 0 are significantly different from each other ($p < 0.001$). Repetitive injections of 10 μM $H_2S$ in the absence of catalase cumulatively increase $H_2S$ concentration (**C**), whereas in the presence of 25 μM catalase ten consecutive 20 μM $H_2S$ injections are efficiently removed (**D**). Addition of 1 mm DTT after $H_2S$ injections (**D**, arrow) further increases $H_2S$ concentration. (**E**) Plot of the decay slope of $H_2S$ removal in (from **D**) as a function of injection number showing random variation in slope.

*3.1.4. pH sensitivity of catalase-mediated $H_2S$ and DCF oxidation*

Because the amperometric sensor only measures dissolved $H_2S$ gas and this decreases as pH increases the sensor was calibrated at pH 6, 7 and 8 and $H_2S$ consumption was corrected accordingly (Fig. 4A, B). Metabolism of 10 μM $H_2S$ by 25 μM catalase at 5 and 10 min after $H_2S$ injection, measured amperometrically and corrected for pH, increased from pH 6 to pH 7 but did not change from pH 7–8 even though the $H_2S/HS^-$ ratio decreased another 10 fold (Fig. 4C).

The pH sensitivity of $H_2S$ metabolism by catalase cannot be determined solely from amperometric measurements because both catalase activity and the $H_2S$:$HS^-$ ratio can be affected by pH. As a surrogate for the effect of pH on catalase during $H_2S$ metabolism, we measured pH sensitivity of catalase-mediated oxidation of DCF. As shown in Fig. 4D, DCF fluorescence increased nearly 3 fold as pH was increased from 6 to 7 and another 3 fold from pH 7–8. Background DCF fluorescence also increased as a function of pH (291, 357, 533 at pH 6, 7 and 8, arbitrary fluorescence units) but this 1.8-fold increase was far less than the 9.1-fold increase in the presence of catalase. The failure of $H_2S$ metabolism to increase between pH 7 and 8, commensurate with increased catalase activity, suggests that dissolved $H_2S$ is

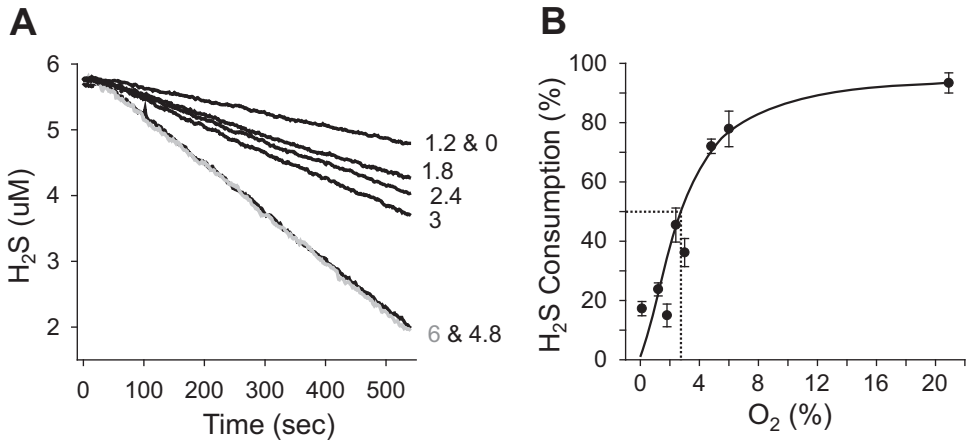


**Fig. 3.** Oxygen sensitivity of $H_2S$ metabolism by 25 μM catalase. **A**) Decrease in $H_2S$ concentration at various % $O_2$. (**B**) Rate of $H_2S$ consumption as a function of % $O_2$. The rate of $H_2S$ consumption is halved at ~2.7% $O_2$, which at average barometric pressure (745 mmHg) and 100% humidity ($P_{H2O}$ =17.5 mmHg) results in an apparent $P_{50}$ of ~20 mmHg. (**A**) Representative traces from single amperometric measurements. (**B**) Average $H_2S$ consumption; mean ± SE (n=3), line fit by eye.

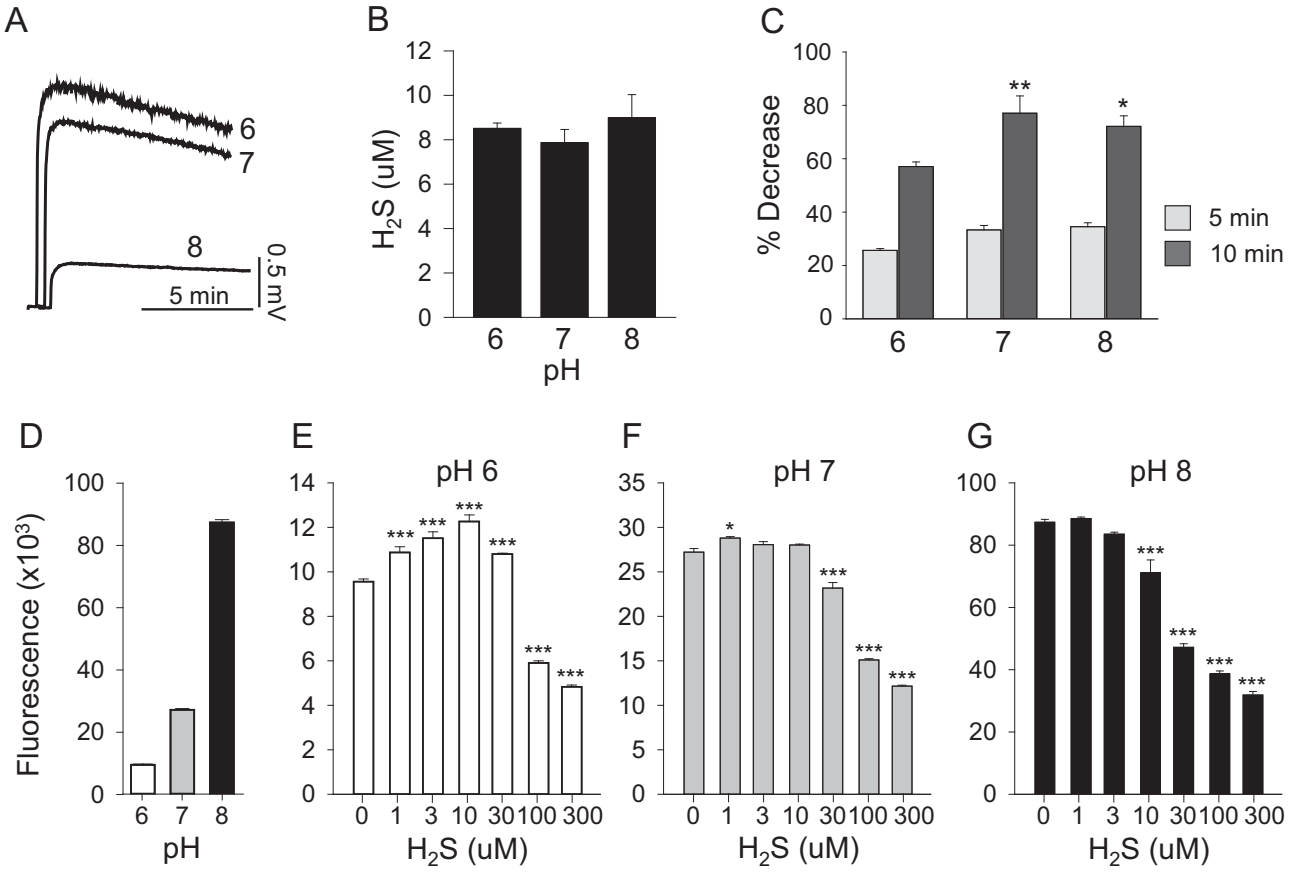


**Fig. 4.** Effect of pH on catalase-mediated H2S oxidation measured amperometrically (**A-C**) and on interactions with DCF oxidation (**D–G**). (**A**) Sensor response to 10 μM $H_2S$ standard (output in mV) decreases as pH increases due to pH-dependent decrease in dissolved $H_2S$ gas. (**B**) Sensor response to 10 μM $H_2S$ in presence of 25 μM catalase corrected for pH effect on dissolved $H_2S$. Peak $H_2S$ is similar after correction but less than 10 μM due to initial $H_2S$ consumption. (**C**) Percent $H_2S$ consumption by 25 μM catalase at 5 and 10 min after $H_2S$ injection. **D**. Catalase-mediated oxidation of 10 μM DCF increased over nine-fold as pH was increased from 6.0 to 8.0. **E-G**. Effect of $H_2S$ on catalase-mediated DCF oxidation at pH 6.0, 7.0 and 8.0. At pH 6.0, low $H_2S$ concentrations increased DCF oxidation and higher concentrations inhibited it. As pH increased, the stimulatory effect was lost and $H_2S$ became solely inhibitory. Mean ± SE (n=3, **B**, **C**; n=4, **D-G**); *, $p < 0.5$; **, $p < 0.01$; ***, $p < 0.001$.

preferred over $HS^-$ as a substrate for catalase.

Knowing the effect of pH on DCF oxidation (Fig. 4D) and the fact that $H_2S$ appears to be a competitive inhibitor of DCF oxidation (Olson, unpublished observation), we then examined the interaction between $H_2S$ and DCF at different pH in order to confirm if dissolved $H_2S$ was more reactive than $HS^-$. At pH 6.0, $H_2S$ from 1 to 30 μM concentration-dependently increased catalase-mediated DCF oxidation and decreased it at 100 and 300 μM (Fig. 4E). At pH 7.0, 1 μM $H_2S$ slightly, but significantly increased DCF oxidation, whereas oxidation was concentration-dependently decreased from 30 to 300 μM $H_2S$ (Fig. 4F). At pH 8.0, 10–300 μM $H_2S$ concentration-dependently inhibited DCF oxidation and there was no stimulatory effect of $H_2S$ (Fig. 4G). These results suggest that $H_2S$ is metabolized by catalase and that $HS^-$ might inhibit this process.

### *3.1.5. Effects of sodium azide on $H_2S$ metabolism*

As shown in Fig. 5A and C, addition of 10 μM $H_2O_2$ produced a slight transient decrease in 10 μM $H_2S$ concentration in the presence of 25 μM catalase in both normoxia and hypoxia. However, when catalase was inhibited with sodium azide, 10 μM $H_2O_2$ removed approximately 80% of the 10 μM $H_2S$ in less than 1 min (Fig. 5B, C). Hypoxia in combination with azide inhibition slightly, but significantly *($p < 0.05$),* enhanced the effect of $H_2O_2$ (Fig. 5C). By comparison, three consecutive 100 μM injections of $H_2O_2$ did not completely remove 10 μM $H_2S$ from solution when catalase was not inhibited with azide in hypoxia (Fig. 5D), although each $H_2O_2$ injection reduced the $H_2S$ concentration by approximately 25%. Without azide catalase dismutation of $H_2O_2$ was clearly evident in this experiment as $O_2$ production increased with each addition of $H_2O_2$ followed by a decrease in oxygen presumably due to catalase-induced consumption of oxygen by sulfide (Fig. 5E). $H_2O_2$ did not affect $H_2S$ concentration in the presence of azide, but without catalase, (Fig. 5F). In the absence of $H_2O_2$ azide also decreased the rate of catalase oxidation of $H_2S$ (not shown).

### *3.1.6. Catalase-mediated polysulfide production from $H_2S$ and catalase-mediated $H_2S$ metabolism*

Although catalase metabolized polysulfides from the $K_2S_n$ salt (Fig. 1), it is possible that catalase also generated polysulfide intermediates during $H_2S$ oxidation and that these were different from those in $H_2S_n$ or metabolized by catalase as they were produced. In order to examine these possibilities we compared the production and metabolism of putative polysulfides and potential polysulfide oxides from $H_2S$ and $H_2S_n$ with SSP4 fluorescence. As shown in Fig. 6A and B, 300 μM $H_2S$ in combination with either 300 μM $H_2O_2$ or $H_2O_2$ +50 mM azide produced the greatest increase in SSP4 fluorescence in the absence of catalase, the effects of azide appeared to be negligible. There was little polysulfide in $H_2S$. With low catalase there was a small increase in fluorescence from $H_2S$ that was approximately 20% of that produced from $H_2S$ plus $H_2O_2$, but other combinations of compounds did not increase fluorescence. In the presence of 40 μM catalase, fluorescence from $H_2S$ was increased to 70% of that produced from $H_2S$ plus $H_2O_2$ in the absence of catalase. Fluorescence from $H_2S$ with 40 μM catalase was not significantly increased by adding $H_2O_2$ but it was decreased by nearly 25% by azide. Catalase alone did not affect SSP4 fluorescence.

The mixed polysulfide ($H_2S_n$, where n=1–8; 300 μM) produce the

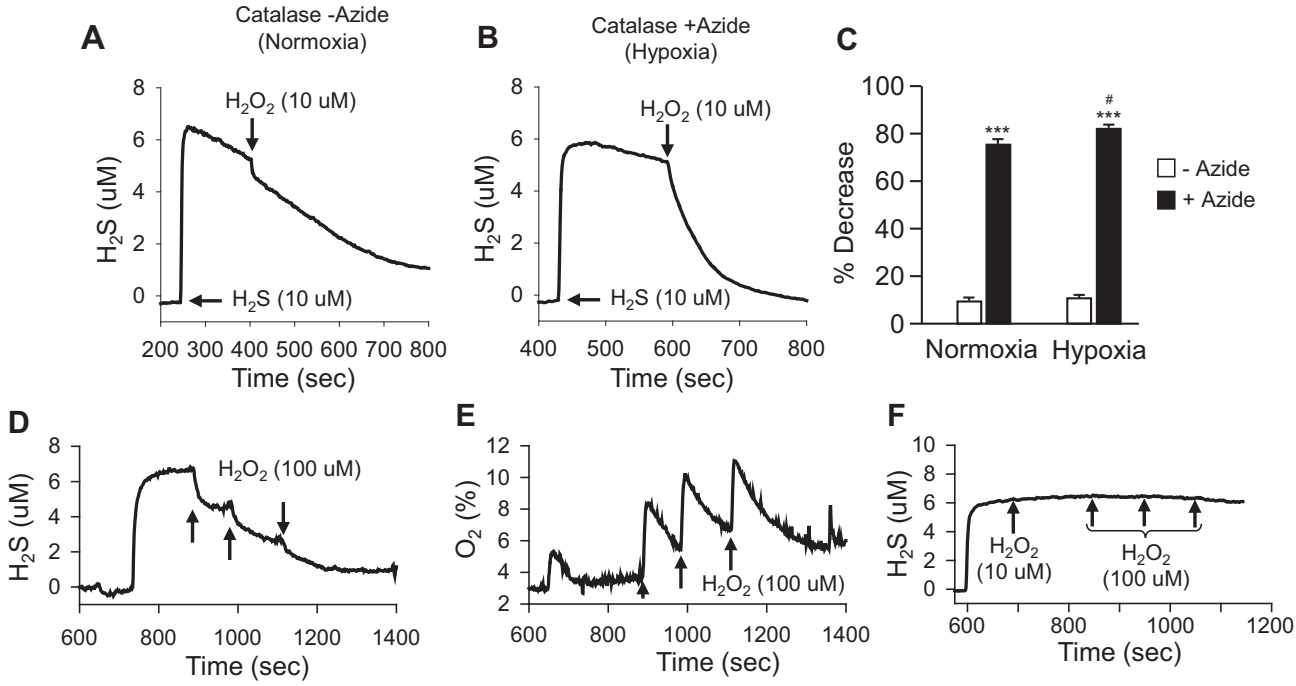


**Fig. 5.** Sodium azide (50 mM) inhibition of catalase promotes $H_2S$ oxidation by $H_2O_2$. (**A**) In normoxia with 25 μM catalase but without sodium azide, 10 μM $H_2S$ (measured amperometrically) rapidly decreased while addition of 10 μM $H_2O_2$ produced a slight, rapid decrease bur did not change slope. (**B**) In hypoxia, azide and catalase, 10 μM $H_2O_2$ nearly completely removed 10 μM $H_2S$ within 1 min. (**C**) Summary of azide effects. $H_2O_2$ (10 μM) had little effect on $H_2S$ concentration in the presence of catalase without azide (white bars) in either normoxia or hypoxia but it decreased $H_2S$ concentration by around 80% when catalase was inhibited by azide and this was slightly augmented in hypoxia. (**D**) In hypoxia, each of three 100 μM $H_2O_2$ injections reduced $H_2S$ by ~20% in the presence of catalase without azide but increased $O_2$ (**E**). (**F**) With azide but without catalase $H_2O_2$ did not affect $H_2S$ concentration. Mean +SE (n=3); *** effect of azide inhibition at same $O_2$ *($p < 0.001$)*, # effect of $O_2$ *($p < 0.05$)*.

greatest increase in SSP4 fluorescence and this was partially inhibited to the same extent by either 300 μM $H_2O_2$ or 300 μM $H_2O_2$ +50 mM azide, again suggesting effect was due to $H_2O_2$ not azide (Fig. 6C, D). Polysulfide fluorescence was reduced by more than 80% by 10 μM catalase and completely eliminated by 40 μM catalase. The inhibitory effect of catalase was unaffected by addition of $H_2O_2$, azide or $H_2O_2$ plus azide.

Collectively, the above results suggest that while catalase interacts with $H_2S$ to increase SSP4 fluorescence (Fig. 6A, B), this fluorescence is not due to generation of a "classical" polysulfide because catalase efficiently prevented fluorescence from the mixed polysulfide $H_2S_n$ (Fig. 6C, D). These results are consistent with those of Fig. 1 and confirm that catalase potently metabolizes polysulfides.

### 3.2. Catalase as a sulfur reductase

#### 3.2.1. Catalase releases $H_2S$ from dithiothreitol (DTT)

Although we observed that catalase efficiently removed polysulfides (Fig. 1), we also noticed that after exposing 25 μM catalase to ten consecutive 20 μM $H_2S$ injections (200 μM total) subsequent addition of 1 mM DTT produced an additional increase in $H_2S$ (Fig. 2D). This suggests that either DTT released $H_2S$ from polysulfides that were formed by catalase-mediated oxidation of $H_2S$, which seems unlikely given that catalase rapidly metabolizes polysulfides (Figs. 1, 6), or that catalase directly generates $H_2S$ from DTT. In order to evaluate these possibilities 1 mM DTT was added 10 min after $H_2S$ was metabolized by increasing concentrations of catalase. As shown in Fig. 7A, the amount of $H_2S$ released appeared to be correlated with the catalase concentration as both 20 and 50 μM catalase removed all of the $H_2S$ in this time period. To directly examine the role of catalase in $H_2S$ release from DTT, these experiments were repeated without pre-exposure to $H_2S$. This also produced a catalase concentration-dependent increase in $H_2S$ release (Fig. 7B) confirming that catalase reacts directly with DTT to generate $H_2S$.

Although 50 μM catalase metabolizes nearly 90% of a 10 uM $H_2S$ injection in 5 min (Fig. 1A), the $H_2S$ released from the interaction of 50 μM catalase with 1 mM DTT remained in the reaction chamber essentially unchanged even after nearly 90 min (Fig. 7C). This poses a conundrum, does DTT and $H_2S$ compete for the same site on the enzyme, or is there so much $H_2S$ released it overwhelms the capacity of catalase to remove this $H_2S$ in the observation interval? In order to examine these possibilities, lower DTT concentrations were employed. As shown in Fig. 7D, $H_2S$ released by 50 μM catalase metabolism of 100 μM DTT in normoxia initially increased then abruptly decreased. However, in the absence of oxygen $H_2S$ concentration increased to more than twice as much as that produced in normoxia and $H_2S$ remained elevated for the duration of the experiment. These effects were observed when either 50 or 100 μM of DTT was employed, although the magnitude and duration of the response was greater with the latter (Fig. 7E). These studies show that catalase directly releases $H_2S$ from DTT independent of the presence of oxygen and that in the presence of oxygen $H_2S$ production and metabolism appear to occur concurrently.

In order to verify that DTT could nevertheless release $H_2S$ from polysulfides, 1 mM DTT was added to dissolved polysulfide salts, $Na_2S_2$, $Na_2S_3$ $Na_2S_4$. As shown in Table 1, $H_2S$ was spontaneously released from these polysulfides; the amount of $H_2S$ released essentially doubled with each additional sulfur indicating that DTT quantitatively reduced these polysulfides back to $H_2S$.

#### 3.2.2. CO inhibition of $H_2S$ production from DTT

In order to determine if catalase-mediated $H_2S$ production from DTT involves the catalase heme iron the buffer was sparged with CO gas for 20 min (which also removed $O_2$) then the buffer was added to the chamber followed by 50 μM catalase then 100 μM DTT. $H_2S$ production was measured amperometrically and compared to catalase-mediated $H_2S$ production in $N_2$ sparged buffer. As shown in Fig. 7F, 1 mM CO reduced $H_2S$ production by 70% demonstrating that

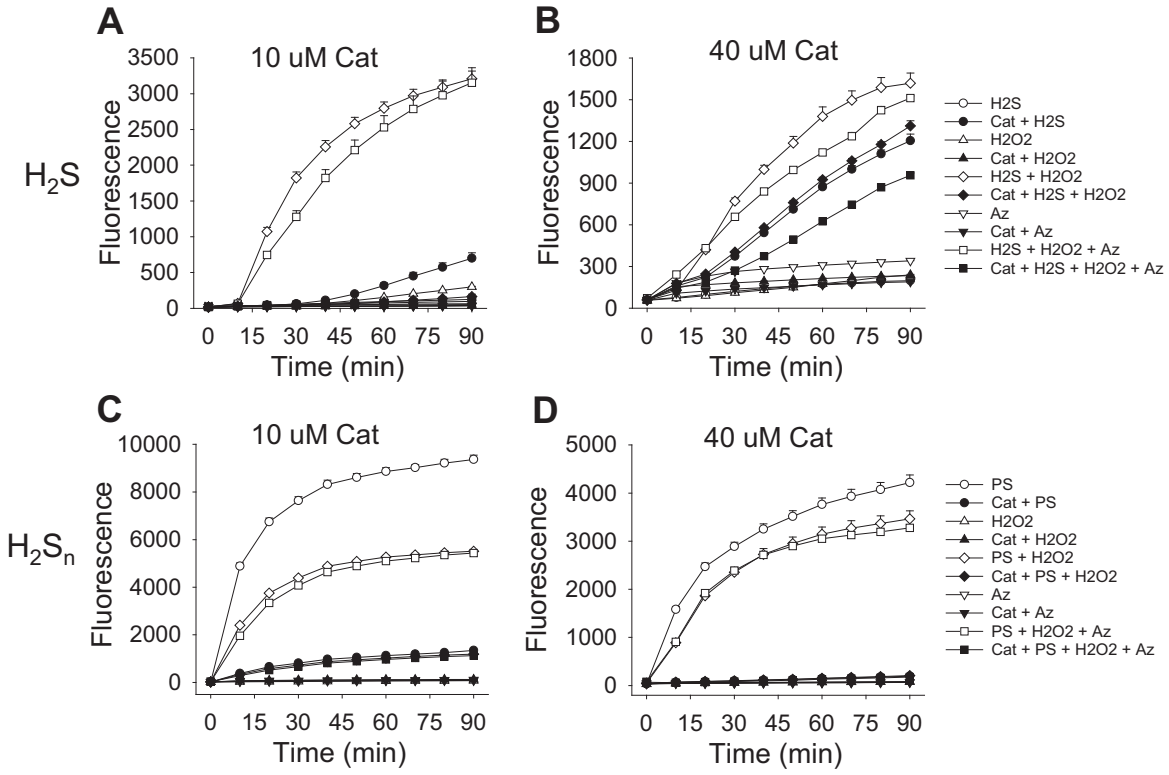


**Fig. 6.** (**A, B**) Effects of combinations of $H_2S$ (300 μM), $H_2O_2$ (300 μM) and sodium azide (Az, 50 mM) with or without 10 μM (**A**) or 40 μM (**B**) catalase (Cat) on putative polysulfide production measured by SSP4 fluorescence. $H_2S$ in combination with either $H_2O_2$ or $H_2O_2$ + azide produced the greatest increase in fluorescence. Low (10 μM) catalase produced a small amount of fluorescence from $H_2S$. Conversely, substantial fluorescence was produced by $H_2S$ and 40 μM catalase. $H_2O_2$ did not significantly increase fluorescence production from $H_2S$ and 40 μM catalase, whereas fluorescence was decreased by azide. Catalase alone did not affect SSP4 fluorescence. (**C, D**) Effects of combinations of the mixed polysulfide $H_2S_n$ (PS; 300 μM), $H_2O_2$ (300 μM) and sodium azide (50 mM) with or without 10 μM (**C**) or 40 μM (**D**) catalase on SSP4 fluorescence. In the absence of catalase, polysulfide alone produced the greatest increase in fluorescence and fluorescence was decreased by $H_2O_2$ alone and to the same extent by $H_2O_2$ plus azide. Polysulfide fluorescence was reduced by more than 80% by 10 μM catalase and completely eliminated by 40 μM catalase. The inhibitory effect of catalase was unaffected by addition of $H_2O_2$, azide or $H_2O_2$ plus azide. Traces of SSP4 or SSP4 plus catalase did not affect fluorescence and have been omitted for clarity. Mean +SE, n =4, many error bars are within symbols. The difference in total fluorescence in **A**, **B** or **C**, **D** was due to different lot numbers of SSP4.

the heme iron in catalase is also involved in the reductive component of catalase metabolism of sulfur-bearing molecules.

### *3.2.3. Catalase-mediated $H_2S$ production from other sulfur compounds*

The ability of catalase to release $H_2S$ from DTT prompted an inquiry into the effects of catalase on other sulfur-bearing compounds. In these experiments, buffer in the reaction chamber was sparged with 100% $N_2$ for 20 min, non gaseous sulfur compounds were then added and sparged another 10 min with $N_2$, the stopper was then lowered to eliminate headspace and catalase was added. $H_2S$ release was measured amperometrically for at least 10 min after the catalase was added. This procedure allowed us to determine if any $H_2S$ was released from the sulfur-bearing compound in the absence of catalase. With gaseous sulfur compounds, COS and $SO_2$, the buffer was sparged with $N_2$ as above, the stopper was then lowered to eliminate headspace and minimize volatilization of COS or $SO_2$, and the dissolved gas was added. After the response had stabilized (within a few min) catalase was added and $H_2S$ release measured as above. The effects of $SO_2$ on catalase oxidation of DCF were also examined.

As shown in Fig. 8, 50 μM catalase produced $H_2S$ from 100 μM garlic oil and 100 μM diallyl disulfide (DATS) in hypoxia, whereas in normoxia considerably less $H_2S$ was produced. Addition of 100 μM $H_2O_2$ to $H_2S$ released by DATS plus catalase produced a rapid decrease in $H_2S$ concentration in hypoxia but not in normoxia (Fig. 8B).

A considerable amount of $H_2S$ was released when 100 μM polysulfide ($K_2S_n$) was dissolved but only several μM more were released when 50 μM catalase was added (Fig. 8C). This suggests that catalase does not release $H_2S$ from polysulfides or of it does the $H_2S$ is removed by a non-oxidative process, which seems unlikely. $H_2S$ was also released from the spontaneous decomposition of 30 μM COS, however, when catalase was added the $H_2S$ concentration decreased in normoxia but did not change in hypoxia (Fig. 8D). These results suggest that catalase oxidized $H_2S$ that was formed from COS decomposition but catalase did not react directly with COS to produce $H_2S$. Catalase did not produce $H_2S$ when incubated with 100 μM cysteine, cystine, glutathione, oxidized glutathione, thiosulfate ($S_2O_3^{2-}$) or metabisulfite ($S_2O_5^{2-}$) in either normoxia or hypoxia (not shown).

Sulfur dioxide ($SO_2$) did not spontaneously produce appreciable amounts of $H_2S$, whereas there was a concentration-dependent increase in $H_2S$ release in the presence of catalase (Fig. 9A) with a threshold $SO_2$ less than 1 mM. The release of $H_2S$ from $SO_2$ was unaffected by the presence or absence of oxygen, although $H_2S$ concentration appeared to decrease more rapidly in hypoxia (Fig. 9B). Addition of DTT produced a further increase in $H_2S$. Sulfite ($SO_3^{2-}$), supposedly in equilibrium with dissolved $SO_2$, produced a minimal amount of $H_2S$ in the presence of catalase in hypoxia (inset, Fig. 9B). These results suggest that $SO_2$ gas, not the cognate anion, $SO_3^{-2}$, reacts with catalase.

$SO_2$ alone concentration-dependently oxidized 20 μM DCF, between 300 μM and 3 mM (Fig. 9C) but not at higher or lower concentrations (not shown). $SO_2$ concentration-dependently inhibited catalase oxidation of DCF with an $EC_{50}$ between 300 μM and 1 mM and DCF oxidation was essentially completely inhibited by $SO_2$ > 3 mM (Fig. 9C).

DCF is commonly used an an indicator of ROS and DCF oxidation is often assumed to be an indicator of $H_2O_2$ [32]. DCF is oxidized by

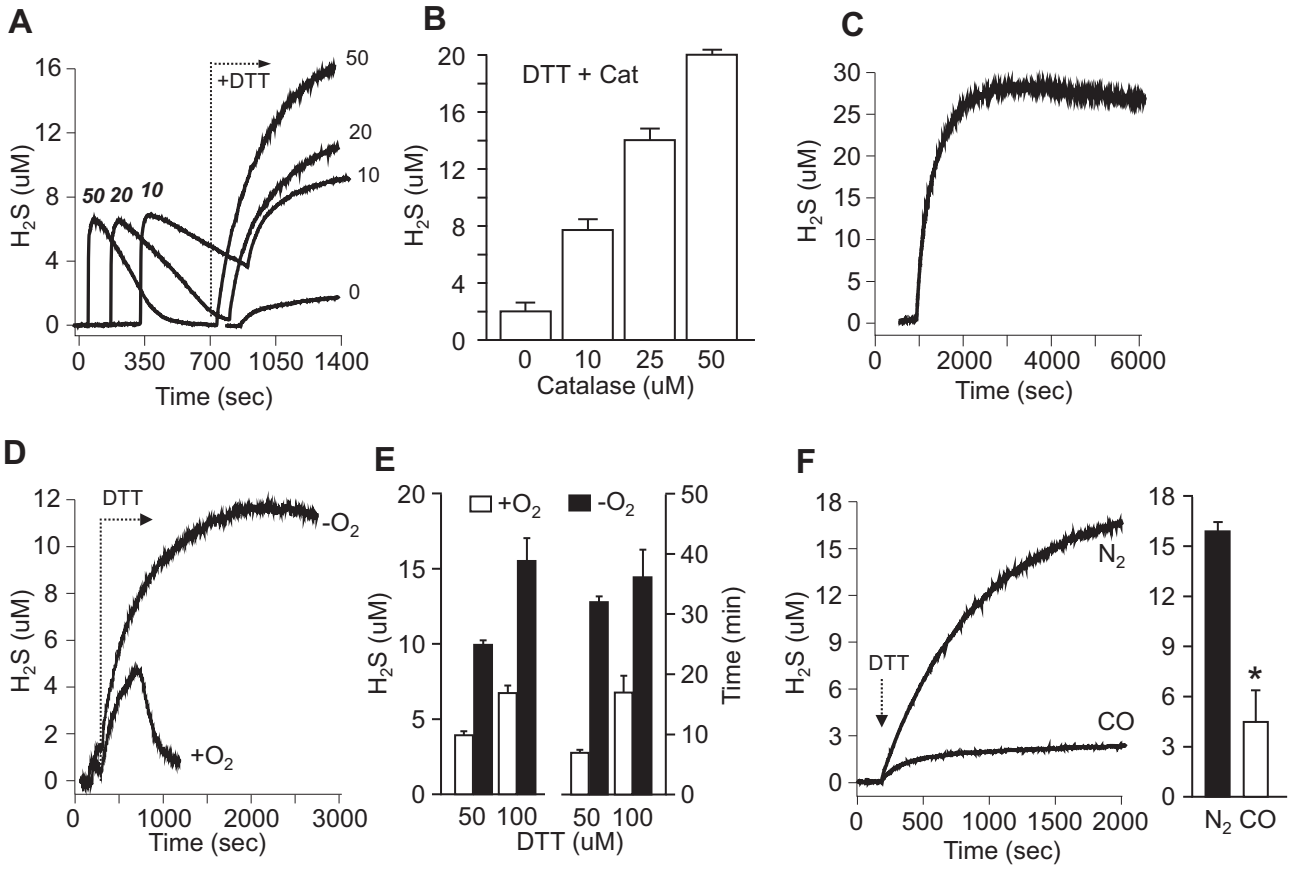


**Fig. 7.** Catalase directly releases $H_2S$ from 1 mM DTT and this is inhibited by 1 mM carbon monoxide (CO). (**A**) Representative amperometric traces of $H_2S$ release from DTT alone (0) or when 1 mM DTT was added 10 min after 10 μM $H_2S$ was metabolized by 10, 20 or 50 μM catalase. Numbers in bold/italic indicate catalase concentration after $H_2S$ addition; plain numbers are from same curve as above showing $H_2S$ generated 10 min after DTT addition in same experiment. (**B**) Effect of catalase concentration on $H_2S$ release from 1 mM DTT without prior addition of $H_2S$. All values are significantly ($p < 0.001$) different from each other (mean +SE, $n$ =3 replicates). (**C**) $H_2S$ released from 50 μM catalase metabolism of 1 mM DTT remains relatively constant for nearly 90 min. (**D**) Amperometric traces of $H_2S$ released from 50 μM catalase metabolism of 100 μM DTT in normoxia and hypoxia. In normoxia, $H_2S$ concentration increases then there is a relatively abrupt decrease in concentration. In hypoxia $H_2S$ concentration continues to increase until it is twice that in normoxia and it does not abruptly decrease. (**E**) $H_2S$ released from 50 μM catalase metabolism of 50 and 100 μM DTT in normoxia and hypoxia and duration of $H_2S$ response which is shorter in normoxia (mean +SE, $n$ =3 replicates; all hypoxia are significantly ($p < 0.001$) different from respective normoxia and all 50 μM DTT are significantly ($p < 0.001$)different from 100 μM DTT). (**F**) Production of $H_2S$ from 100 μM DTT by 50 μM catalase in hypoxia ($N_2$) and in hypoxia with 1 mM carbon monoxide (CO). Left, typical amperometric traces; right, average $H_2S$ production (mean +SE, n=3; *$p$=0.006).

**Table 1**
$H_2S$ (μM) released spontaneously (spont) upon dissolving 10 μM $H_2S_2$, $H_2S_3$ or $H_2S_4$ standards in normoxia and after subsequent addition of 1 mM DTT (+DTT).

| | $H_2S_2$ | $H_2S_3$ | $H_2S_4$ |
|---|---|---|---|
| $H_2S$ (spont) | 9.1 ± 0.36* | 5.9 ± 0.51 | 5.7 ± 0.62 |
| + DTT | 3.3 ± 1.12[a,b,] | 7.7 ± 0.84[a,c] | 16.8 ± 1.28[b,c] |
| Total $H_2S$ from polysulfide | 12.4 | 13.6 | 22.5 |

Measured with amperometric $H_2S$ sensor. Mean ± SE; n =10–12 (H2S), 5–6 (DTT). * significantly different from either $H_2S_3$ or $H_2S_4$ ($p < 0.001$). a; significantly different from same letter at ($p$=0.015); b, c, significantly different from same letter at ($p < 0.001$). +DTT corrected for $H_2S$ released by DTT alone (3.9 μM).

catalase alone and as shown in Fig. 9D, 20 μM DCF is concentration-dependently oxidized by $H_2O_2$ with an $EC_{50}$ of approximately 1.5 mM and maximal oxidation at 10 mM $H_2O_2$. This is slightly less than the amount of oxidation produced by 20 μM catalase alone. This suggests that even a small amount of active catalase could give the impression of significant ROS production.

These results show that in the absence of oxygen, catalase acts as a selective sulfur reductase capable of directly producing reduced sulfur in the form of $H_2S$ from a variety of oxidized sulfides. They also suggest that in the presence of oxygen or another electrophile, such as $H_2O_2$, catalase oxidizes $H_2S$ concurrently with its formation.

### 3.2.4. Contribution of NADPH and NADH to $H_2S$ production from DTT and thioredoxin

NADPH is a well-known co-factor for bovine catalase that has been proposed to prevent formation of inactive compound II and this effect can be enhanced by addition of non-bound NADPH [33]. In these studies we examined the possibility that exogenous NADPH can provide a pathway for the reaction of DTT and the endogenous thiol, thioredoxin with catalase. As shown in Fig. 10A, the addition of NADPH increased $H_2S$ release from DTT and catalase by ~50%. NADPH nearly doubled $H_2S$ release from DTT after three consecutive additions of catalase (Fig. 10B). $H_2S$ was not released from DTT and NADPH in the absence of catalase (not shown). Conversely, NADH did not augment $H_2S$ release from DTT in the presence of catalase compared to the alkaline vehicle that was necessary to dissolve NADPH (Fig. 10C). The alkaline-induced increase in catalase activity is consistent with our observations that alkalinity increases catalase oxidase activity (Fig. 4) indicating that both the oxidase and reductase activities of catalase are pH dependent.

In addition, as shown in Fig. 10D, approximately 1 μM of $H_2S$ is transiently produced when 10 μM Trx is added to 50 μM catalase in hypoxia. Reversing the order of addition of Trx and catalase produced a similar amount of $H_2S$ that also was not sustained (not shown). We next examined the contribution of NADPH to this reaction and in the presence of NADPH nearly 7 μM $H_2S$ were produced from the same

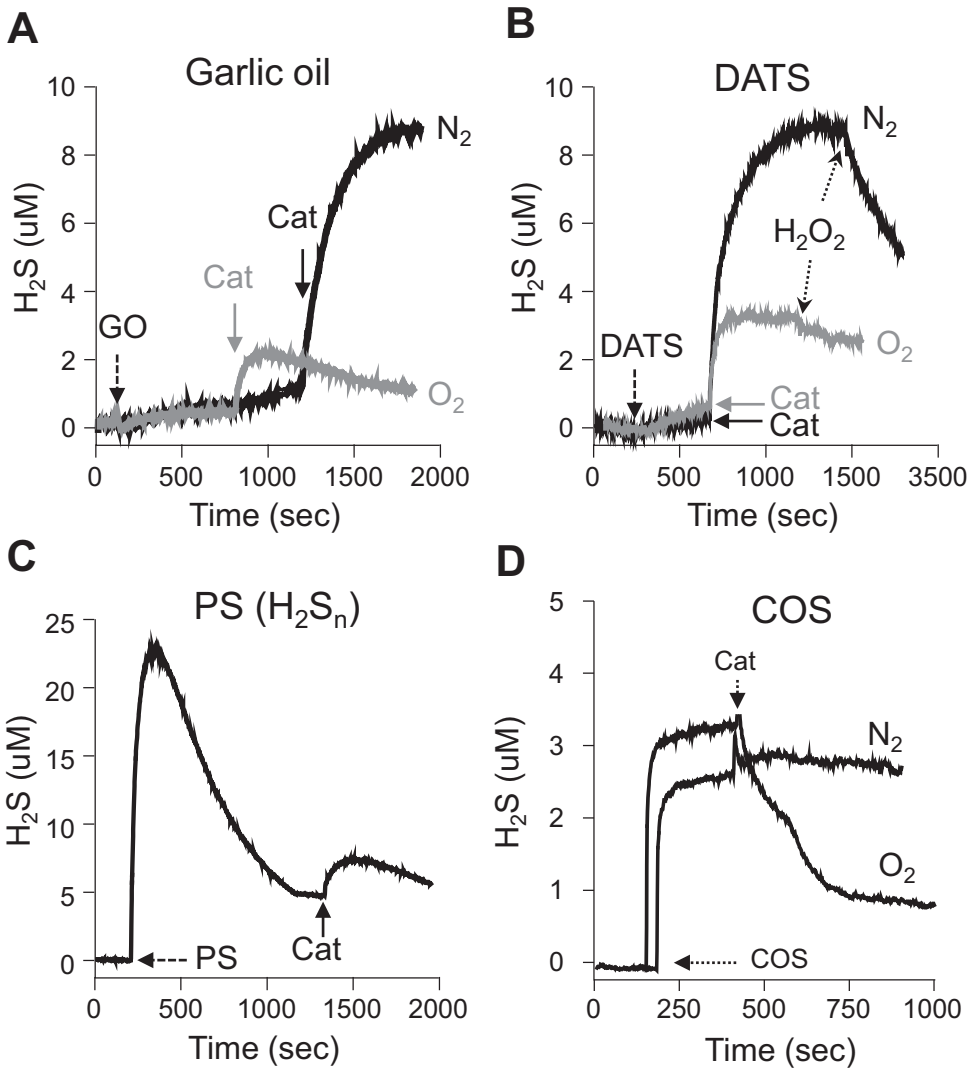


**Fig. 8.** Amperometric traces showing catalase-mediated $H_2S$ production from sulfur-bearing molecules. Under hypoxic ($N_2$) conditions catalase (Cat, 50 μM) produced $H_2S$ from (**A**) 100 μM garlic oil (GO) or (**B**) 100 μM diallyl disulfide (DATS). $H_2S$ production from garlic oil and DATS was considerably reduced in normoxia ($O_2$). Peroxide ($H_2O_2$; 100 μM) rapidly reduced $H_2S$ generated from DATS in hypoxia. (**C**) Considerable $H_2S$ is produced when 100 μM of the polysulfide (PS) salt $K_2S_n$ is dissolved (producing the mixed polysulfide $H_2S_n$); whereas relatively little $H_2S$ is recovered after subsequent addition of 50 μM catalase in hypoxia. (**D**) $H_2S$ was released from the spontaneous decomposition of carbonyl sulfide (COS) and metabolized by catalase in normoxia but not hypoxia. Catalase did not increase $H_2S$ production from COS in either normoxia or hypoxia.

reactants (Fig. 10C). Accounting for some $H_2S$ volatility or degradation, the amount of $H_2S$ released in the presence of NADPH is nearly equivalent to the amount of Trx used. It is clear that Trx is an effective endogenous substrate for catalase and that NADPH is required. Conversely, NADH did not augment $H_2S$ release from thioredoxin (not shown) which is similar to its lack of effect on DTT.

*3.2.5. Sulfur metabolism by catalase from Aspergillus niger*

Because NADPH is not a cofactor in catalase from the fungus *Aspergillus niger* and it does not form compound II [34,35] we conducted several studies to determine if this enzyme is capable of oxidizing $H_2S$ or DCF and/or if it can generate $H_2S$ from Trx. As shown in Fig. 11A and B, 20 μM of *A. Niger* catalase enhanced the rate of $H_2S$ disappearance compared to $H_2S$ alone indicative of an oxidative capability, although this was only about 20–30% of that produced by bovine catalase. *A. Niger* catalase also oxidized DCF and again its catalytic activity was only about 20% of that of bovine catalase (Fig. 11C). However, when 400 μM NADPH and 10 μM Trx were added to 20 μM *A. Niger* catalase there was no $H_2S$ production (not shown). These experiments show that catalase from *A. niger* possesses oxidative activity and they also suggest that the lack of the NADPH binding site prevents the transfer of reducing equivalents and subsequent formation of $H_2S$ from sulfur-donating compounds.

## 4. Discussion

Vetrano et al., [31] provided the first evidence for an oxidase function of catalase, other than peroxide, using the xenobiotic 10-acetyl-3,7-dihydrophenoxazine (ADP). They also showed that catalase could oxidize DCF and the endogenous compounds, indole and β-phenethylamine. Furthermore, they and others have suggested catalase may not be a major contributor to $H_2O_2$ metabolism in that the $H_2O_2$ turnover rate is extremely high, the affinity of catalase for $H_2O_2$ is very low (Km ≥10 mM) and at low and presumably "physiological" $H_2O_2$ concentrations peroxidatic activity would be expected to predominate [31,36–38]. This raises the question of the actual catalytic role of catalases under normal conditions, are they dismutative, peroxidatic or oxidative? Furthermore, are there other endogenous compounds that are preferred, or more physiologically relevant catalase substrates?

Our experiments show that catalase can act as a sulfide/sulfur oxidase and that this is not accomplished by a "classical" dismutation reaction. We also show that catalase can act as a reductase and generate $H_2S$ from a

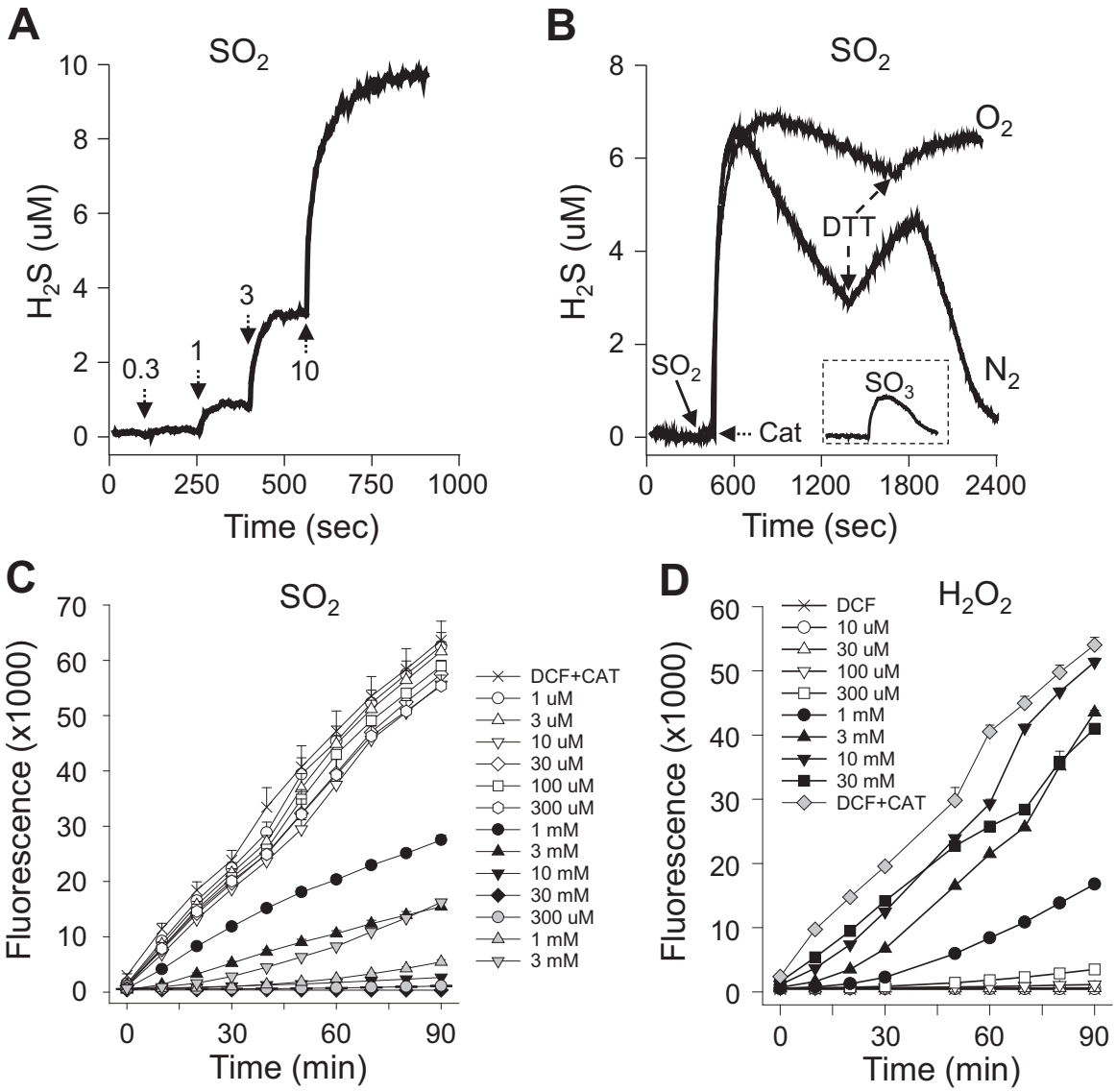


**Fig. 9.** (**A**) Amperometric traces showing $H_2S$ was $SO_2$ concentration (arrows)- and catalase (50 μM)-dependently released in hypoxia or (**B**) from 7 mM $SO_2$ in both normoxia and hypoxia; $H_2S$ release appeared to be somewhat prolonged in normoxia. Addition of 1 mM DTT produced a further increase in $H_2S$ in both conditions. Inset in (**B**) shows that only a small amount of $H_2S$ was released from 7 mM sodium sulfite ($SO_3$, shown in same concentration-time scale. (**C**) effects of $SO_2$ on DCF (20 μM) oxidation in the absence (gray symbols) or presence of 20 μM catalase (open or solid symbols at low and high $SO_2$ concentrations, respectively). $SO_2$ concentration-dependently oxidized DCF between 300 μM and 3 mM $SO_2$ in the absence of catalase, whereas $SO_2$ inhibited catalase oxidation of DCF at concentrations > ~300 μM and completely inhibited oxidation at 10 mM. (**D**) Comparison of DCF oxidation by increasing concentrations of $H_2O_2$ (open and solid symbols) or 20 μM catalase (gray diamonds). More DCF is oxidized by 20 μM catalase than by 30 mM $H_2O_2$.

variety of sulfur-bearing molecules. This to our knowledge is the first demonstration of catalase as a peroxide-independent reductase. Collectively, our results suggest that catalase can play a role in sulfur metabolism, sulfur signaling, and through oxidation of DCF, may inadvertently led to misinterpretation of redox stress.

### 4.1. Physiological functions of catalase-mediated sulfur metabolism

#### 4.1.1. Detoxification

The toxicity of $H_2S$ and other sulfur compounds are well known [39] and sulfur toxicity may have been an even greater problem early in evolution when these sulfur molecules were more prevalent [1]. In fact, the ability of catalase to metabolize $H_2S$ and polysulfides may have taken its origin in these sulfidic environments. The identity of potential electron acceptors in these early anoxic environments is unclear, although the ability of the modern bovine catalase to utilize $H_2O_2$ suggests some flexibility, perhaps ancient enzymes used other sulfur or nitrogen oxides that were present.

#### 4.1.2. Response to hypoxia

However, the catalase-mediated production of $H_2S$ from sulfur-bearing molecules under hypoxic conditions versus oxidation of $H_2S$ in normoxia is suggestive of a regulatory system that is responsive to oxygen availability. The two most logical candidates that would benefit from this catalase activity are tissue (especially vascular) oxygen sensing and protection from ischemia and reperfusion injury.

*4.1.2.1. Oxygen sensing.* There is growing evidence that $H_2S$ serves as an $O_2$ sensor in a variety of tissues including vascular and non-vascular smooth muscles, chemoreceptors, and airway epithelia. Here $H_2S$ transduces the hypoxic response by the oxygen-dependent balance between constitutive $H_2S$ production and $H_2S$ oxidation. Thus hypoxia increases $H_2S$ and this in turn activates a variety of downstream $H_2S$-mediated, homeostatic effector responses [40].

Prior work from our lab has provided substantial evidence that during hypoxia tissue $H_2S$ production increases and this constitutes an

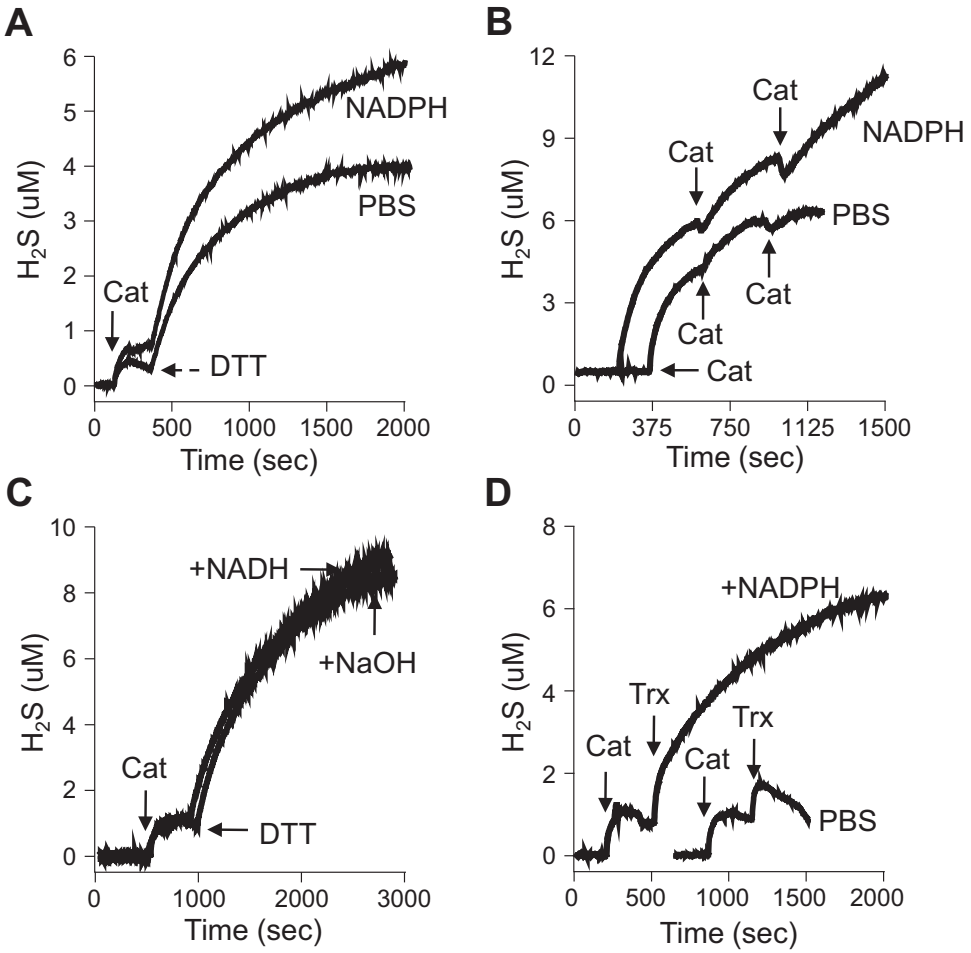


**Fig. 10.** Amperometric measurements illustrating the effect of NADPH and NADH on catalase-mediated $H_2S$ release from DTT and thioredoxin (Trx) in hypoxia. (**A**) Catalase (50 μM) releases more $H_2S$ from 50 μM DTT in the presence of 400 μM NADPH than in phosphate buffer (PBS). (**B**) Release of $H_2S$ following multiple additions of 50 μM catalase is sustained in the presence of 400 μM NADPH but not in PBS. (**C**) NADH (400 μM) does not augment $H_2S$ release from 50 μM catalase and 50 μM DTT compared to alkaline vehicle (NaOH). (**D**) $H_2S$ release from 50 μM catalase and 10 μM thioredoxin is greatly increased by 400 μM NADPH compared to PBS.

important mechanism in vascular oxygen sensing [40]. While the hypoxia-induced increase in $H_2S$ is believed to result from decreased oxidative metabolism in tissue, decreased uptake and metabolism of $H_2S$ by RBC could amplify the response as RBCs avidly remove $H_2S$ from the circulation under normoxic conditions [28]. Although, catalase, which is abundant in RBC is thought to be the main

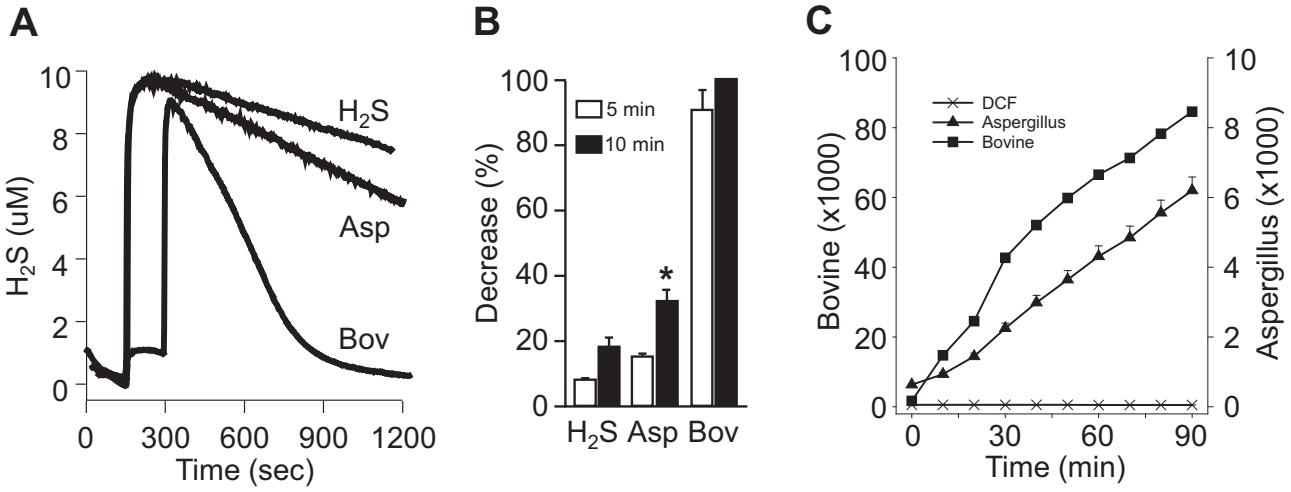


**Fig. 11.** Oxidase function of catalase from *Aspergillus niger*. (**A**) Amperometric measurements of a 10 μM $H_2S$ standard and 10 μM $H_2S$ with 20 μM *A. niger* catalase (Asp) and 20 μM bovine catalase (Bov) in hypoxia and (**B**) average decrease in $H_2S$ at 5 (open bars) and 10 (solid bars) min (mean +SD, *n=3*, $H_2S$ and Bov, *n=2* Asp; * significantly greater than respective control ($p=0.006$); all Bov are greater than $H_2S$ or Asp ($p < 0.001$). (**C**) Oxidation of 20 μM DCF *A*. Niger or bovine catalase. DCF is readily oxidized by *A*. Niger catalase compared to DCF alone, however, bovine catalase is 10-fold more efficacious (mean 3 replicates +SE).

mechanism of $H_2O_2$ metabolism [41], this process is not oxygen sensitive, whereas $H_2S$ metabolism is. In fact, the $P_{50}$ for the oxyhemoglobin dissociation, typically ~25 mmHg, is strikingly similar to the 20 mmHg $P_{50}$ for catalase oxidation of $H_2S$ (Fig. 3). Thus, hypoxia would be expected to nearly simultaneously increase unloading of $O_2$ and inhibit $H_2S$ oxidation. As RBCs are also capable of generating $H_2S$ [42], catalase would directly affect $H_2S$ levels in these cells in a $P_{O_2}$-sensitive manner. The increase in $H_2S$ would not only convey its cytoprotective effects to the tissues but promote vasodilation to help restore $O_2$ delivery. Furthermore, the tissue acidosis that usually accompanies ischemia would also decrease catalase oxidase activity (Fig. 4) and further decrease $H_2S$ oxidation.

*4.1.2.2. Protection from ischemia and reperfusion injury*. The location of catalase in peroxisomes in most cells except RBC may also provide anti-ischemic regulatory opportunities and the kidney may especially benefit from this. $H_2S$ and sulfides are now well recognized for their role in protecting kidneys from ischemic reperfusion injury and in ischemic conditioning [43,44]. Unlike most tissues, from 7 to 80 times more $H_2S$ is derived from D-cysteine than L-cysteine in the kidney and brain and in these tissues uptake of D-cysteine and conversion to bound sulfane sulfur is faster than that of L-cysteine. It has also been demonstrated that D-cysteine, but not L-cysteine, protects the kidney from ischemia-reperfusion injury [45].

Although it is not clear how hypoxia is coupled to $H_2S$ formation, peroxisomal D-cysteine appears to be the primary source of $H_2S$ production [45]. Here D-cysteine is metabolized to 3-mercaptopyruvate (3-MP) by D-amino acid oxidase. It has been proposed that 3-MP is then transferred to the mitochondrion via peroxisome-mitochondrial vesicular trafficking and the mitochondrial enzyme 3-mercaptopyruvate sulfur transferase (3-MST) accepts the sulfur thereby forming a sulfane sulfur (3-MST-S) on the enzyme. This sulfur can be released as $H_2S$ by intracellular antioxidants and diffuse back to the peroxisome, or it can be transferred to other intracellular thiols as a polysulfide. Assuming catalase is involved in peroxisomal $H_2S$ and polysulfide metabolism, the oxygen sensitivity of this enzyme would provide the oxygen "sensing" transducer that couples ischemic insult to the appropriate homeostatic responses. Catalase may also generate $H_2S$ directly during hypoxia from other sulfur-bearing sources (see below).

### *4.1.3. Physiological functions of catalase-mediated $H_2S$ production from sulfur-bearing compounds*

Catalase-mediated generation of $H_2S$ from a variety of exogenous and endogenous sulfur-bearing molecules suggests an important, and heretofore unrecognized function of this enzyme. This has both physiological implications and the potential for creating experimental artefacts.

*4.1.3.1. DTT*. DTT is a commonly used reductant in many experimental situations. Its ability to reduce poly- and persulfides is well known [46–49] and we clearly show its efficacy in generating $H_2S$ from polysulfides in buffer (Table 1). However, we also show that catalase generates significant quantities of $H_2S$ directly from DTT (Fig. 7) and that it is likely that this is also an enzymatic process involving the heme center as it is inhibited by CO (Fig. 7F). Inadvertent $H_2S$ production from DTT could have profound effects on experiments in which DTT is added to cells to serve as a thiol reductant. This could be further confounded in experiments where oxygen tension is varied. Furthermore, even the general interpretation of ROS production in cells could be an artifact of DCF oxidation by catalase.

*4.1.3.2. Garlic compounds*. Garlic oil (GO) and its main active ingredient, diallyl trisulfide (DATS) are well known for their garlic-related health benefits and this has been attributed to their ability to release $H_2S$. In buffer $H_2S$ can be released non-enzymatically from GO and DATS by relatively high concentrations of cysteine or glutathione (GSH; [50,51]). Benavides et al. [50] have also shown that addition of garlic or DATS to RBCs releases $H_2S$ and this is augmented by glucose. They propose that garlic reacts with exofacial thiols on the RBC membrane to cross the membrane and then react with GSH to produce $H_2S$ and oxidized GSH (GSSG). Glucose metabolism by the RBC provides NADPH to reduce GSSG to GSH and sustain the reaction. Our experiments suggest an alternative mechanism catalyzed by catalase. It is expected that this would also be sustained by NADPH which is consistent with our results showing NADPH increases catalase-mediated $H_2S$ release from DTT and thioredoxin (Fig. 10).

*4.1.3.3. $SO_2$*. $SO_2$ is released in large quantities from volcanoes [52,53] and early organisms could have exploited this in catalase-mediated redox reactions possibly setting the precedence for this enzyme (see below). $SO_2$ is also endogenously generated in vertebrate tissues including the vasculature and its production appears to be physiologically regulated [54–57]. Like $H_2S$, $SO_2$ is toxic in excess [58,59], but at lower concentrations it vasodilates arteries and protects tissues against ischemic conditions, reperfusion injury and oxidative stress [57,60–67]. $SO_2$ is reportedly rapidly hydrated in the lung and with a pKa of ~9 form sulfite ($SO_3^{-2}$) and bisulfite ($HSO_3^{-1}$) in a 3:1 Molar ratio [68]. However, $SO_2$ gas is a far stronger vasodilator than either sulfite or bisulfite [57] suggesting that the gaseous form is the biologically active moiety. Furthermore, only about 10% of the dissolved $SO_2$ is ionized in aqueous solution [69].

We show that $H_2S$ can be released from $SO_2$ by catalase (Fig. 9A,B) which suggests that perhaps the ultimate biologically active "form" is actually $H_2S$, or that $H_2S$ at least contributes to the biological activity of $SO_2$. This is further supported by our observation that $H_2S$ is not released from sulfite (Fig. 9B) which could explain why sulfite is not as efficacious a vasodilator as gaseous $SO_2$ [57].

While significant amounts of $H_2S$ are released from 7 mM $SO_2$ by catalase, 1 mM $SO_2$ halves the ability of catalase to oxidize DCF (Fig. 9C). We did not examine the concentration-dependency of catalase-mediated $H_2S$ production from $SO_2$ but it would be interesting if there was a regulatory feedback inhibition on $H_2S$ production. The production of $H_2S$ from $SO_2$ may involve a dismutation reaction of $SO_2$ such as the following over-all reaction;

$$2SO_2 + 2H_2O -> H_2S + O_2 + H_2SO_4 \quad (5)$$

*4.1.3.4. Thioredoxin*. Thioredoxin (Trx) is an evolutionarily conserved antioxidant present in essentially all living organisms [70,71]. The redox-active site of Trx contains two cysteines in a Cys-X-X-Cys sequence where X=amino acids [72] which is similar to DTT (S-C-C-C-C-S, where C = carbon). The ability of catalase to release $H_2S$ from Trx (Fig. 10D) suggests that Trx is an endogenous substrate of catalase and that the mechanism of $H_2S$ generation is similar to that of DTT. This is further supported by the increased release of $H_2S$ from both DTT and Trx in the presence of NADPH. The nearly stoichiometric production of $H_2S$ from Trx in the presence of NADPH suggests that Trx could be a significant source of $H_2S$ production. This could provide another link to hypoxic responses where NADPH would be expected to increase and thereby increase $H_2S$.

### *4.1.4. Mechanism of catalase-mediated $H_2S$ release from DTT and Trx*

It is not clear how catalase mediates the release of $H_2S$ from DTT.

$H_2S$ can be released from thiols by elimination reactions catalyzed by cystathionine β synthase or cystathionine γ lyase, e.g., RSH + R'SH −> R-S-R' + $H_2S$, or RSH + $H_2O$ −> ROH + $H_2S$. To our knowledge no such function for catalase has been described. If $H_2S$ is released by a redox reaction an additional oxidant would be required as the formal oxidation state of sulfur in DTT and $H_2S$ (and reduced thioredoxin) is −2. The oxidant does not appear to be oxygen as $H_2S$ release is enhanced in hypoxia (Fig. 7D). However, the ability of CO to reduce $H_2S$ production from DTT by nearly 80% (Fig. 7F) suggests that the reaction does occur at the heme iron, which supports a redox mechanism. Clearly additional studies are necessary to identify the process involved.

#### 4.1.5. Role of NADPH

Bovine catalase is an evolutionarily recent mono-functional clade 3 catalase with NADPH tightly bound to each of its four subunits [35,73]. The NADPH is believed to prevent formation of inactive compound II from compound I when $H_2O_2$ concentrations are low (reviewed in [74]). Because bound NADPH is too large to enter the catalytic pore (which restricts access to molecules larger than $H_2O_2$) it is believed that electrons are transferred from unbound NADPH to bound NADPH and then tunneled to the heme [75].

Our experiments showing that NADPH is required for $H_2S$ generation from molecules as large as DTT, DATS and Trx raises the question of how (or where) do the reactants access the catalytic site. Vetrano et al. [31] examined the crystal structure of bovine catalase and identified a potential large binding pocket adjacent to the β-barrel region abutting the heme environments that would accommodate and orient large and diverse electronegative substrates such as DCF through interaction with $Arg^{74}$, $Arg^{111}$, $Arg^{364}$ and $Phe^{131}$. This would appear to be the likely site of sulfur metabolism by bovine catalase as well as provide access for two-electron donation by unbound NADPH. $SO_2$ which is only weakly bound to water may find its way directly to the heme by the channel used by $H_2O_2$.

Catalase from *A. niger* is a large subunit mono-functional clade 2 enzyme that does not have the narrow catalytic pore nor does it bind NADPH or form compound II during low concentrations of $H_2O_2$ [73,74]. We did not find evidence that *A. niger* catalase could generate $H_2S$ from Trx even in the presence of NADPH. This does not completely rule out the possibility of *A. niger* catalase as a sulfur reductase as other substrates and electron donors may be involved. This could not be pursued in the present studies due to the limited availability of this catalase. However, it is evident that *A. niger* catalase, is similar to bovine catalase in its ability to oxidize $H_2S$ and DCF, albeit at a slower rate (Fig. 11). This is not surprising as the rate of $H_2O_2$ dismutation is also slower for *A. niger* catalase [76].

#### 4.1.6. Product(s) of $H_2S$ oxidation

Our studies suggest that the majority of the reaction products of $H_2S$ oxidation are most likely elemental sulfur or sulfur oxides ($SO_n$ or $S_nO_n$) because there was minimal recovery of $H_2S$ by DDT after catalase-mediated $H_2S$ oxidation when $H_2S$ was measured amperometrically and because catalase readily consumes polysulfides. DTT, which unfortunately also liberates a small amount of $H_2S$ when dissolved (Table 1; [47]) clearly liberated $H_2S$ from polysulfide standards ($H_2S_n$, n=2–4; Table 1) but was unable to do so after $H_2S$ reaction with catalase (Fig. 2).

In an attempt to 'trap' polysulfides as they are formed, we added combinations of $H_2S$, $H_2O_2$ and azide with or without catalase to SSP4, a fluorophore that irreversibly reacts with polysulfides (Fig. 6) and other sulfanes. The greatest SSP4 fluorescence was produced by the combination of $H_2S$ and $H_2O_2$ in the absence of catalase. Catalase plus $H_2S$ also increased SSP4 fluorescence and this was slightly increased by addition of $H_2O_2$ in the presence of 40 μM catalase and slightly decreased with $H_2O_2$ and 10 μM catalase. Overall, $H_2O_2$ appeared to have little consistent effect on catalase mediated formation of polysulfide from $H_2S$ suggesting that $H_2O_2$ was dismutated so rapidly that it did not have time to react with $H_2S$. Azide inhibited fluorescence produced by $H_2S$ in the presence of catalase but not fluorescence produced by $H_2S$ and $H_2O_2$ in the absence of catalase. This is consistent with azide inhibition of catalase-mediated $H_2S$ consumption measured amperometrically and suggests that this is operating at the site of $O_2$ consumption. However, when catalase was added to mixed polysulfides derived from $K_2S_n$, it completely inhibited SSP4 fluorescence irrespective of the presence of $H_2O_2$ or azide (Fig. 6C). This suggests that the 'polysulfides' produced by catalase and $H_2S$ were not of the form $H_2S_n$, even though they remained reactive with SSP4 in the absence of catalase. Thus, if polysulfides are formed they must either rapidly decompose under aerobic conditions, as suggested by Wedmann et al. [77], or they are further metabolized by catalase.

#### 4.1.7. Limitations to the above studies

While these studies point out the potential for catalase to greatly affect sulfur metabolism in cells a few limitations to the study need to be identified. First, the concentrations of $H_2S$ and polysulfides that we used likely exceed intracellular levels. This is especially the case for the plate reader experiments where the sensitivity of the fluorophores predetermined the lower limit of concentration. Furthermore the actual concentrations of these sulfur moieties in cells and their distribution has not been resolved. Second, the purity of polysulfides is an issue especially once in solution and exposed to oxygen. Therefore, the measurements of $H_2S$ produced from polysulfides (Table 1) are only estimates, although there are clear differences that correlate with the number of sulfur atoms. Finally, there is always some uncertainty regarding the specificity of the fluorophores.

#### 4.1.8. Catalase and evolution

Life began around 3.8 billion years ago (bya) and was chemolithotrophic; the energy that sustained and drove early evolution was obtained from inorganic reducing compounds in the environment. Although still somewhat controversial, a strong case can be made that $H_2S$ emanating from hydrothermal vents provided this energy as well as creating the chemical backbone for the earliest catalysts and cell walls [1]. Escape from the chemotrophic existence probably came within the next few hundred million years (~3.6 bya) in the form of anoxigenic photosynthesis [78,79]. It is also possible that $H_2S$ would have been the likely substrate in this reaction [1];

$$H_2S + CO_2 + h\nu \rightarrow (CH_2O)_n + H_2O + S_{(n)} \quad (6)$$

thereby generating a variety of reactive sulfide species that would need metabolic attention. It took over a billion years for organisms (namely cyanobacteria) to develop sufficiently efficient light gathering antennae to replace $H_2S$ with the far more abundant $H_2O$ and its oxidization product became $O_2$ [79]. Although this "great oxidation event" (GOE) may have transiently increased atmospheric $O_2$ up to 1%, the oceans remained anoxic and became more sulfidic [80]. Eukaryotes appeared around 1.5 bya and upon engulfing cyanobacteria, created the first "plants." The combined efforts of cyanobacteria and plants accelerated $O_2$ production but it took nearly another billion years, to 0.6 bya before the vast quantities of ferrous iron and sulfide, which previously "mopped up" $O_2$ were oxidized. Only then did $O_2$ begin to accumulate in the oceans and rise to present day levels in the atmosphere. It is generally thought that during this period of Earth's oxygenation organisms either retreated to anoxic environments, died or developed strategies to remove excess $O_2$. To deal with the latter, the "ox-tox" hypothesis, posits that this led to the appearance of the classical antioxidant mechanisms including SOD, catalase, glutathione reductase, peroxiredoxins and thioredoxins, molecules that were designed to rapidly dispose of ROS [81].

We propose an alternate scenario; the metabolic "machinery" that was necessary to deal with RSS had to have developed early in

evolution commensurate with $H_2S$-based anoxigenic photosynthesis. These pathways were then fine-tuned over a billion years of evolution before organisms began producing oxygen. Just as the transition from $H_2S$- to $H_2O$-based photosynthesis merely required better light-gathering capabilities, the transition from RSS to ROS based metabolism probably required only slight enzymatic modifications. It is not surprising, then, that many (if not all) of the "antioxidant" mechanisms, including superoxide dismutases, thioredoxins, peroxiredoxins and catalases appeared early in evolution [70,72,82–88], probably well before either oxygen or ROS were a serious threat.

Manganese catalase, most likely the ancestral catalase, appeared at least 3 billion years ago, nearly three-quarters of a billion years before the GOE. It has been proposed that the function of Mn catalase was to generate oxygen from peroxide dismutation and thereby provide oxygen to early aerobes [79,89]. We have questioned this hypothesis based on the perceived difficulty of finding a sufficient and reliable source of peroxide to sustain life compared to the ready availability of RSS [1]. Our identification of the ability of catalase to generate $H_2S$ from sulfur-bearing molecules under reducing (hypoxic) conditions and to paradoxically oxidize them under oxidizing conditions bespeaks of an enzyme that initially dealt with RSS. It also supports the hypothesis that some of these "primordial" functions have been retained in present-day organisms to metabolize and regulate RSS.

Collectively, our results suggest that our view of catalase as just an antioxidant enzyme may be too myopic. The next challenge will be in identifying additional endogenous sulfur substrates and products that may be under homeostatic control by this and other "redox-regulatory" enzymes.

## Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors (http://Www.Nature.Com/Nature).

## Author contributions

K.R.O. designed the study, M.A. F.A. N.A, E.R.D. and Y.G. performed the experiments. K.R.O., E.R.D and K.D.S. analyzed the data interpreted the results. K.R.O. wrote the manuscript and all authors discussed the results and commented on the manuscript.

## Acknowledgments

This research was supported by National Science Foundation Grant No. IOS 1446310.

## References

[1] K.R. Olson, K.D. Straub, The role of hydrogen sulfide in evolution and the evolution of hydrogen sulfide in metabolism and signaling, Physiology 31 (1) (2016) 60–72.
[2] N. Brandes, H. Tienson, A. Lindemann, V. Vitvitsky, D. Reichmann, R. Banerjee, U. Jakob, Time line of redox events in aging postmitotic cells, Elife 2 (2013) e00306.
[3] D.I. Brown, K.K. Griendling, Regulation of signal transduction by reactive oxygen species in the cardiovascular system, Circ. Res. 116 (3) (2015) 531–549.
[4] Y.R. Chen, J.L. Zweier, Cardiac mitochondria and reactive oxygen species generation, Circ. Res. 114 (3) (2014) 524–537.
[5] E.T. Chouchani, V.R. Pell, A.M. James, L.M. Work, K. Saeb-Parsy, C. Frezza, T. Krieg, M.P. Murphy, A unifying mechanism for mitochondrial superoxide production during ischemia-reperfusion injury, Cell Metab. 23 (2) (2016) 254–263.
[6] M.L. Circu, T.Y. Aw, Reactive oxygen species, cellular redox systems, and apoptosis, Free Radic. Biol. Med. 48 (6) (2010) 749–762.
[7] M.L. Circu, T.Y. Aw, Intestinal redox biology and oxidative stress, Semin. Cell Dev. Biol. 23 (7) (2012) 729–737.
[8] S.B. Gibson, Investigating the role of reactive oxygen species in regulating autophagy, Methods Enzym. 528 (2013) 217–235.
[9] R.L. Goncalves, C.L. Quinlan, I.V. Perevoshchikova, M. Hey-Mogensen, M.D. Brand, Sites of superoxide and hydrogen peroxide production by muscle mitochondria assessed ex vivo under conditions mimicking rest and exercise, J. Biol. Chem. 290 (1) (2015) 209–227.
[10] J.D. Lambeth, A.S. Neish, Nox enzymes and new thinking on reactive oxygen: a double-edged sword revisited, Annu. Rev. Pathol. 9 (2014) 119–145.
[11] X. Li, P. Fang, J. Mai, E.T. Choi, H. Wang, X.F. Yang, Targeting mitochondrial reactive oxygen species as novel therapy for inflammatory diseases and cancers, J. Hematol. Oncol. 6 (2013) 19.
[12] K.M. Holmstrom, T. Finkel, Cellular mechanisms and physiological consequences of redox-dependent signalling, Nat. Rev. Mol. Cell Biol. 15 (6) (2014) 411–421.
[13] A. Olschewski, E.K. Weir, Redox regulation of ion channels in the pulmonary circulation, Antioxid. Redox Signal. (2014).
[14] E. Radi, P. Formichi, C. Battisti, A. Federico, Apoptosis and oxidative stress in neurodegenerative diseases, J. Alzheimer's Dis. 42 (2014), 2014, pp. S125–S152.
[15] M. Schieber, N.S. Chandel, ROS function in redox signaling and oxidative stress, Curr. Biol. 24 (10) (2014) R453–R462.
[16] E. Schulz, P. Wenzel, T. Munzel, A. Daiber, Mitochondrial redox signaling: interaction of mitochondrial reactive oxygen species with other sources of oxidative stress, Antioxid. Redox Signal. 20 (2) (2014) 308–324.
[17] K. Sharma, Obesity and diabetic kidney disease: role of oxidant stress and redox balance, Antioxid. Redox Signal. 25 (4) (2016) 208–216.
[18] C.C. Winterbourn, Are free radicals involved in thiol-based redox signaling?, Free Radic. Biol. Med. (2014).
[19] C.M. Lo, K.S. Carroll, The redox biochemistry of protein sulfenylation and sulfinylation, J. Biol. Chem. 288 (37) (2013) 26480–26488.
[20] M.R. Filipovic, Persulfidation (S-sulfhydration) and H2S, Handb. Exp. Pharmacol. 230 (2015) 29–59.
[21] H. Kimura, Signaling molecules: hydrogen sulfide and polysulfide, Antioxid. Redox Signal. 22 (2015) 362–376.
[22] P. Nagy, Mechanistic chemical perspective of hydrogen sulfide signaling, Methods Enzymol. 554 (2015) 3–29.
[23] K. Ono, T. Akaike, T. Sawa, Y. Kumagai, D.A. Wink, D.J. Tantillo, A.J. Hobbs, P. Nagy, M. Xian, J. Lin, J.M. Fukuto, Redox chemistry and chemical biology of HS, hydropersulfides, and derived species: implications of their possible biological activity and utility, Free Radic. Biol. Med (2014).
[24] B.D. Paul, S.H. Snyder, Protein sulfhydration, Methods Enzym. 555 (2015) 79–90.
[25] J.I. Toohey, A.J. Cooper, Thiosulfoxide (sulfane) sulfur: new chemistry and new regulatory roles in biology, Molecules 19 (8) (2014) 12789–12813.
[26] Z. Palinkas, P.G. Furtmuller, A. Nagy, C. Jakopitsch, K.F. Pirker, M. Magierowski, K. Jasnos, J.L. Wallace, C. Obinger, P. Nagy, Interactions of hydrogen sulfide with myeloperoxidase, Br. J. Pharmacol. 172 (6) (2015) 1516–1532.
[27] E.R. DeLeon, Y. Gao, E. Huang, M. Arif, N. Arora, A. Divietro, S. Patel, K.R. Olson, A case of mistaken identity: are reactive oxygen species actually reactive sulfide species?, Am. J. Physiol Regul. Integr. Comp. Physiol. 310 (7) (2016) (R549-560).
[28] N.L. Whitfield, E.L. Kreimier, F.C. Verdial, N. Skovgaard, K.R. Olson, Reappraisal of H2S/sulfide concentration in vertebrate blood and its potential significance in ischemic preconditioning and vascular signaling, Am. J. Physiol Regul. Integr. Comp. Physiol. 294 (6) (2008) R1930–R1937.
[29] R.G. Boutilier, T.A. Heming, G.K. Iwama, Physiochemical Parameters for Use in Fish Respiratory Physiology, Academic Press, 1984 (City).
[30] K.R. Olson, A practical look at the chemistry and biology of hydrogen sulfide, Antioxid. Redox Signal. 17 (1) (2012) 32–44.
[31] A.M. Vetrano, D.E. Heck, T.M. Mariano, V. Mishin, D.L. Laskin, J.D. Laskin, Characterization of the oxidase activity in mammalian catalase, J. Biol. Chem. 280 (42) (2005) 35372–35381.
[32] M. Karlsson, T. Kurz, U.T. Brunk, S.E. Nilsson, C.I. Frennesson, What does the commonly used DCF test for oxidative stress really show?, Biochem. J. 428 (2) (2010) 183–190.
[33] G.F. Gaetani, A.M. Ferraris, P. Sanna, H.N. Kirkman, A novel NADPH:(bound) NADP+ reductase and NADH:(bound) NADP+ transhydrogenase function in bovine liver catalase, Biochem. J. 385 (Pt 3) (2005) 763–768.
[34] K. Kikuchi-Torii, S. Hayashi, H. Nakamoto, S. Nakamura, Properties of Aspergillus niger catalase, J. Biochem. 92 (5) (1982) 1449–1456.
[35] H.N. Kirkman, G.F. Gaetani, Catalase: a tetrameric enzyme with four tightly bound molecules of NADPH, Proc. Natl. Acad. Sci. USA 81 (14) (1984) 4343–4347.
[36] G. Cohen, P. Hochstein, Generation of hydrogen peroxide in erythrocytes by hemolytic agents, Biochemistry 3 (1964) 895–900.
[37] D.E. Heck, M. Shakarjian, H.D. Kim, J.D. Laskin, A.M. Vetrano, Mechanisms of oxidant generation by catalase, Ann. N.Y. Acad. Sci. 1203 (2010) 120–125.
[38] H.S. Jacob, S.H. Ingbar, J.H. Jandl, Oxidative hemolysis and erythrocyte metabolism in hereditary acatalasia, J. Clin. Investig. 44 (1965) 1187–1199.
[39] D.H. Truong, M.A. Eghbal, W. Hindmarsh, S.H. Roth, P.J. O'Brien, Molecular mechanisms of hydrogen sulfide toxicity, Drug Metab. Rev. 38 (4) (2006) 733–744.
[40] K.R. Olson, Hydrogen sulfide as an oxygen sensor, Antioxid. Redox Signal. (2014).
[41] S. Mueller, H.D. Riedel, W. Stremmel, Direct evidence for catalase as the predominant H2O2 -removing enzyme in human erythrocytes, Blood 90 (12) (1997) 4973–4978.
[42] D.G. Searcy, S.H. Lee, Sulfur reduction by human erythrocytes, J. Exp. Zool. 282 (3) (1998) 310–322.
[43] E.M. Bos, H.G. Leuvenink, P.M. Snijder, N.J. Kloosterhuis, J.L. Hillebrands, J.C. Leemans, S. Florquin, G.H. van, Hydrogen sulfide-induced hypometabolism prevents renal ischemia/reperfusion injury, J. Am. Soc. Nephrol. 20 (9) (2009) 1901–1905.
[44] E.M. Bos, P.M. Snijder, H. Jekel, M. Weij, J.C. Leemans, M.C. van Dijk, J.L. Hillebrands, T. Lisman, G.H. van, H.G. Leuvenink, Beneficial effects of gaseous hydrogen sulfide in hepatic ischemia/reperfusion injury, Transpl. Int. 25 (8) (2012) 897–908.
[45] N. Shibuya, S. Koike, M. Tanaka, M. Ishigami-Yuasa, Y. Kimura, Y. Ogasawara, K. Fukui, N. Nagahara, H. Kimura, A novel pathway for the production of hydrogen sulfide from D-cysteine in mammalian cells, Nat. Commun. 4 (2013) 1366.

[46] R. Miyamoto, K.I. Otsuguro, S. Yamaguchi, S. Ito, Contribution of cysteine aminotransferase and mercaptopyruvate sulfurtransferase to hydrogen sulfide production in peripheral neurons, J. Neurochem (2014).
[47] K.R. Olson, E.R. DeLeon, Y. Gao, K. Hurley, V. Sadauskas, C. Batz, G.F. Stoy, Thiosulfate: a readily accessible source of hydrogen sulfide in oxygen sensing, Am. J. Physiol Regul. Integr. Comp. Physiol. 305 (2013) R592–R603.
[48] N. Shibuya, M. Tanaka, M. Yoshida, Y. Ogasawara, T. Togawa, K. Ishii, H. Kimura, 3-Mercaptopyruvate sulfurtransferase produces hydrogen sulfide and bound sulfane sulfur in the brain, Antioxid. Redox Signal. 11 (2009) 703–714.
[49] S. Tanabe, Development of assay methods for endogenous inorganic sulfur compounds and sulfurtransferases and evaluation of the physiological functions of bound sulfur, Yakugaku Zasshi 128 (6) (2008) 881–900.
[50] G.A. Benavides, G.L. Squadrito, R.W. Mills, H.D. Patel, T.S. Isbell, R.P. Patel, V.M. Darley-Usmar, J.E. Doeller, D.W. Kraus, Hydrogen sulfide mediates the vasoactivity of garlic, Proc. Natl. Acad. Sci. USA 104 (46) (2007) 17977–17982.
[51] E.R. DeLeon, Y. Gao, E. Huang, K.R. Olson, Garlic oil polysulfides: H2s- and O2-independent prooxidants in buffer and antioxidants in cells, Am. J. Physiol Regul. Integr. Comp. Physiol. 310 (11) (2016) R1212–1225.
[52] A. Bekker, M.E. Barley, M.L. Fiorentini, O.J. Rouxel, D. Rumble, S.W. Beresford, Atmospheric sulfur in Archean komatiite-hosted nickel deposits, Science 326 (5956) (2009 2009) 1086–1089.
[53] H. Keppler, Experimental evidence for the source of excess sulfur in explosive volcanic eruptions, Science 284 (5420) (1999) 1652–1654.
[54] M. Balazy, I.A. bu-Yousef, D.N. Harpp, J. Park, Identification of carbonyl sulfide and sulfur dioxide in porcine coronary artery by gas chromatography/mass spectrometry, possible relevance to EDHF, Biochem. Biophys. Res. Commun. 311 (3) (2003) 728–734.
[55] S.X. Du, H.F. Jin, D.F. Bu, X. Zhao, B. Geng, C.S. Tang, J.B. Du, Endogenously generated sulfur dioxide and its vasorelaxant effect in rats, Acta Pharmacol. Sin. 29 (8) (2008) 923–930.
[56] L. Luo, S. Chen, H. Jin, C. Tang, J. Du, Endogenous generation of sulfur dioxide in rat tissues, Biochem. Biophys. Res. Commun. 415 (1) (2011) 61–67.
[57] Z. Meng, J. Li, Q. Zhang, W. Bai, Z. Yang, Y. Zhao, F. Wang, Vasodilator effect of gaseous sulfur dioxide and regulation of its level by Ach in rat vascular tissues, Inhal. Toxicol. 21 (14) (2009) 1223–1228.
[58] A. Hansell, C. Oppenheimer, Health hazards from volcanic gases: a systematic literature review, Arch. Environ. Health 59 (12) (2004) 628–639.
[59] X.B. Wang, J.B. Du, H. Cui, Sulfur dioxide, a double-faced molecule in mammals, Life Sci. 98 (2) (2014) 63–67.
[60] S. Chen, J. Du, Y. Liang, T. Ochs, D. Liu, L. Zhu, X. Tang, C. Tang, H. Jin, Sulfur dioxide inhibits excessively activated endoplasmic reticulum stress in rats with myocardial injury, Heart Vessels (2011).
[61] S. Chen, J. Du, Y. Liang, R. Zhang, C. Tang, H. Jin, Sulfur dioxide restores calcium homeostasis disturbance in rat with isoproterenol-induced myocardial injury, Histol. Histopathol. 27 (9) (2012) 1219–1226.
[62] S. Chen, S. Zheng, Z. Liu, C. Tang, B. Zhao, J. Du, H. Jin, Endogeous sulfur dioxide protects against oleic acid-induced acute lung injury in association with inhibition of oxidative stress in rats, Lab. Investig. 95 (2) (2015) 142–156.
[63] S.X. Du, H.F. Jin, D.F. Bu, X. Zhao, B. Geng, C.S. Tang, J.B. Du, Endogenously generated sulfur dioxide and its vasorelaxant effect in rats, Acta Pharmacol. Sin. 29 (8) (2008) 923–930.
[64] P. Huang, Y. Sun, J. Yang, S. Chen, A.D. Liu, L. Holmberg, X. Huang, C. Tang, J. Du, H. Jin, The ERK1/2 signaling pathway is involved in sulfur dioxide preconditioning-induced protection against cardiac dysfunction in isolated perfused rat heart subjected to myocardial ischemia/reperfusion, Int. J. Mol. Sci. 14 (11) (2013) 22190–22201.
[65] H. Jin, Y. Wang, X. Wang, Y. Sun, C. Tang, J. Du, Sulfur dioxide preconditioning increases antioxidative capacity in rat with myocardial ischemia reperfusion (I/R) injury, Nitric Oxide 32 (2013) 56–61.
[66] Y. Liang, D. Liu, T. Ochs, C. Tang, S. Chen, S. Zhang, B. Geng, H. Jin, J. Du, Endogenous sulfur dioxide protects against isoproterenol-induced myocardial injury and increases myocardial antioxidant capacity in rats, Lab. Investig. 91 (1) (2011) 12–23.
[67] X.B. Wang, X.M. Huang, T. Ochs, X.Y. Li, H.F. Jin, C.S. Tang, J.B. Du, Effect of sulfur dioxide preconditioning on rat myocardial ischemia/reperfusion injury by inducing endoplasmic reticulum stress, Basic Res. Cardiol. 106 (5) (2011) 865–878.
[68] Z. Meng, Y. Li, J. Li, Vasodilatation of sulfur dioxide derivatives and signal transduction, Arch. Biochem. Biophys. 467 (2) (2007) 291–296.
[69] M. Falk, P.A. Giguere, On the nature of sulphurous acid, Can. J. Chem. 36 (7) (1958) 1121–1125.
[70] S.V. Novoselov, V.N. Gladyshev, Non-animal origin of animal thioredoxin reductases: implications for selenocysteine evolution and evolution of protein function through carboxy-terminal extensions, Protein Sci. 12 (2) (2003) 372–378.
[71] C.H. Williams, L.D. Arscott, S. Muller, B.W. Lennon, M.L. Ludwig, P.F. Wang, D.M. Veine, K. Becker, R.H. Schirmer, Thioredoxin reductase two modes of catalysis have evolved, Eur. J. Biochem. 267 (20) (2000) 6110–6117.
[72] J. Lu, A. Holmgren, The thioredoxin antioxidant system, Free Radic. Biol. Med. 66 (2014) 75–87.
[73] M. Zamocky, B. Gasselhuber, P.G. Furtmuller, C. Obinger, Molecular evolution of hydrogen peroxide degrading enzymes, Arch. Biochem. Biophys. 525 (2) (2012) 131–144.
[74] H.N. Kirkman, G.F. Gaetani, Mammalian catalase: a venerable enzyme with new mysteries, Trends Biochem. Sci. 32 (1) (2007) 44–50.
[75] L.P. Olson, T.C. Bruice, Electron tunneling and ab initio calculations related to the one-electron oxidation of NAD(P)H bound to catalase, Biochemistry 34 (22) (1995) 7335–7347.
[76] J. Switala, P.C. Loewen, Diversity of properties among catalases, Arch. Biochem. Biophys. 401 (2) (2002) 145–154.
[77] R. Wedmann, S. Bertlein, I. Macinkovic, S. Boltz, J.L. Miljkovic, L.E. Munoz, M. Herrmann, M.R. Filipovic, Working with "HS": facts and apparent artifacts, Nitric Oxide (2014).
[78] M.F. Hohmann-Marriott, R.E. Blankenship, Evolution of photosynthesis, Annu. Rev. Plant Biol. 62 (2011) 515–548.
[79] J. Raymond, R.E. Blankenship, The origin of the oxygen-evolving complex, Coord. Chem. Rev. 252 (2008) 377–383.
[80] N.J. Planavsky, C.T. Reinhard, X. Wang, D. Thomson, P. McGoldrick, R.H. Rainbird, T. Johnson, W.W. Fischer, T.W. Lyons, Earth history. low mid-Proterozoic atmospheric oxygen levels and the delayed rise of animals, Science 346 (6209) (2014) 635–638.
[81] C.G. Kurland, S.G. Andersson, Origin and evolution of the mitochondrial proteome, Microbiol. Mol. Biol. Rev. 64 (4) (2000) 786–820.
[82] D.V. Dibrova, D.A. Cherepanov, M.Y. Galperin, V.P. Skulachev, A.Y. Mulkidjanian, Evolution of cytochrome bc complexes: from membrane-anchored dehydrogenases of ancient bacteria to triggers of apoptosis in vertebrates, Biochim. Biophys. Acta 1827 (11–12) (2013) 1407–1427.
[83] S.C. Grace, Phylogenetic distribution of superoxide dismutase supports an endosymbiotic origin for chloroplasts and mitochondria, Life Sci. 47 (21) (1990) 1875–1886.
[84] E.M. Hanschmann, J.R. Godoy, C. Berndt, C. Hudemann, C.H. Lillig, Thioredoxins, glutaredoxins, and peroxiredoxins–molecular mechanisms and health significance: from cofactors to antioxidants to redox signaling, Antioxid. Redox Signal. 19 (13) (2013) 1539–1605.
[85] M.A. Inupakutika, S. Sengupta, A.R. Devireddy, R.K. Azad, R. Mittler, The evolution of reactive oxygen species metabolism, J. Exp. Bot. 67 (21) (2016) 5933–5943.
[86] B. Knoops, E. Loumaye, V. Van Der Eecken, Evolution of the peroxiredoxins, Subcell. Biochem 44 (2007) 27–40.
[87] A.F. Miller, Superoxide dismutases: ancient enzymes and new insights, FEBS Lett. 586 (5) (2012) 585–595.
[88] X.H. Zhang, H. Weissbach, Origin and evolution of the protein-repairing enzymes methionine sulphoxide reductases, Biol. Rev. Camb. Philos. Soc. 83 (3) (2008) 249–257.
[89] K.M. Kim, T. Qin, Y.Y. Jiang, L.L. Chen, M. Xiong, D. Caetano-Anolles, H.Y. Zhang, G. Caetano-Anolles, Protein domain structure uncovers the origin of aerobic metabolism and the rise of planetary oxygen, Structure 20 (1) (2012) 67–76.