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Investigations on the role of hemoglobin in sulfide metabolism by intact human red blood cells

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ABSTRACT

In addition to their role as oxygen transporters, red blood cells (RBCs) contribute to cardiovascular homeostasis by regulating nitric oxide (NO) metabolism via interaction of hemoglobin (Hb) with nitrite and NO itself. RBCs were proposed to also participate in sulfide metabolism. Although Hb is known to react with sulfide, sulfide metabolism by intact RBCs has not been characterized so far. Therefore we explored the role of Hb in sulfide metabolism in intact human RBCs. We find that upon exposure of washed RBCs to sulfide, no changes in oxy/deoxyhemoglobin (oxy/deoxyHb) are observed by UV-vis and EPR spectroscopy. However, sulfide reacts with methemoglobin (metHb), forming a methemoglobin-sulfide (metHb-SH) complex. Moreover, while metHb-SH is stable in cell-free systems even in the presence of biologically relevant thiols, it gradually decomposes to produce oxyHb, inorganic polysulfides and thiosulfate in intact cells, as detected by EPR and mass spectrometry. Taken together, our results demonstrate that under physiological conditions RBCs are able to metabolize sulfide via intermediate formation of a metHb-SH complex, which subsequently decomposes to oxyHb. We speculate that decomposition of metHb-SH is preceded by an inner-sphere electron transfer, forming reduced Hb (which binds oxygen to form oxyHb) and thiyl radical (a process we here define as "reductive sulfhydration"), which upon release, gives rise to the oxidized products, thiosulfate and polysulfides. Thus, not only is metHb an efficient scavenger and regulator of sulfide in blood, intracellular sulfide itself may play a role in keeping Hb in the reduced oxygen-binding form and, therefore, be involved in RBC physiology and function.

1. Introduction

The main physiological functions of RBCs are to transport oxygen (O_2) and nutrients to the tissues and remove metabolic waste products including carbon dioxide (CO_2) from the tissues. Besides these roles, RBCs exert other non-canonical functions such as those for nitric oxide (NO) metabolism and glutathione transport [1–3]. Hemoglobin (Hb) is the most abundant protein in RBCs, constituting > 99% of the total cellular protein pool, and Hb was demonstrated to play a central role not only in canonical O_2/CO_2 exchange, but also in non-canonical NO metabolism [4]. Hb is responsible for NO scavenging (a reaction involving oxyHb and leading to formation of nitrate and metHb), NO transport via formation of nitrosylhemoglobin (involving the reduced deoxy form) and S-nitrosohemoglobin (although the latter is a matter of ongoing debate, reviewed in [3]), and NO production from nitrite under hypoxic conditions [4,5]. Thus, RBCs are considered to play a fundamental role in regulating systemic NO bioavailability and thereby controlling vascular tone and conferring cardioprotection [1–3].

There is accumulating evidence that, similar to NO, hydrogen sulfide (H_2S) also plays a central role in the cardiovascular system and beyond, exerting actions not unlike those of NO. Moreover, it is now evident that H_2S chemically and functionally interacts with NO at multiple levels, which has given rise to the notion of a physiological and pharmacological "H_2S/NO cross-talk" [6–8]. In the course of these (rather complex) reactions, numerous other reactive sulfur species including sulfur radicals, nitrosopersulfide and polysulfides are formed [9,10]; the latter of which were shown to have potent biological activity in their own right [8,9,11,12]. It has been argued that sulfur,

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nitrogen and oxygen metabolites are interlinked at a fundamental cell regulatory level, forming a so-called 'reactive species interactome', which allows the cells to sense and adapt to changes in environmental conditions and thereby maintain optimal biological fitness [13]. Thus, not only the enzymatic formation of H_2S, but also its effective removal and/or metabolism is likely to be of fundamental importance. In this context, RBCs have been shown to metabolize H_2S, preventing H_2S accumulation and associated toxicity in the blood [14–17]. Furthermore, several investigators proposed that Hb plays a central role in the binding and oxidative metabolism of H_2S in these cells [18–21].

The early pioneering studies of the last century, and even before that (see [22]; this issue), focused largely on the chemical interactions of H_2S with heme proteins, including Hb, and the toxicological implications of human/animal exposure to H_2S [17,19,20]. Results of those studies indicated that under various conditions, H_2S forms distinct reversible and irreversible (i.e. sulfhemoglobin) complexes with Hb. By 1933, Keilin had already identified metHb (the oxidized ferric iron(III) form of Hb), rather than the reduced ferrous Hb (iron(II) species), as the predominant form of Hb that interacts with H_2S in a reversible manner [23]. While there seems to be little doubt that the reaction of metHb with H_2S in vitro results in H_2S oxidation, the formation of oxidized products such as polysulfides (RS_nSR, $n \geq 1$) and/or thiosulfate ($HS_2O_3^-$) appears to have been characterized only recently [18]. Importantly, although much effort has been spent studying the interaction of H_2S and Hb [19–21,23], all previously performed studies seem to have only utilized isolated human Hb, and/or human RBC lysates [18]. It is well known that NO scavenging by Hb in intact RBCs is approximately 1000-fold slower than that of free Hb [24], and these differences may have a fundamental impact on our understanding of the physiological role of RBCs; whether the same also holds true for H_2S is currently unknown.

To the best of our knowledge, the fate of H_2S in RBCs, in particular the interaction of H_2S with Hb in intact human RBCs, has not yet been investigated. Therefore, the aim of the present study was to analyze the role of Hb in H_2S metabolism in intact human RBCs. Here we report for the first time that under aerobic conditions H_2S reduces metHb to oxyHb via formation of a metHb-SH intermediate complex; in the course of this reaction $HS_2O_3^-$ and polysulfides are produced and released into the extracellular space. Thus, our findings point to a potential role for naturally occurring metHb in the metabolism of H_2S in human RBCs.

2. Materials and methods

2.1. Reagents and solutions

Disodium sulfide (Na_2S), human hemoglobin (Hb), diethylamine NONOate diethylammonium salt (DEA/NO), iodoacetamide (IAM), cysteine (CySH), glutathione (GSH) and G-25 Sephadex resin, salts and all other materials, unless specified otherwise, were all purchased from Sigma Aldrich/Merck (Munich, Germany). Blood collection tubes were from BD Bioscience (Heidelberg, Germany). Stock solutions of Na_2S (10 mM) were prepared in $HBSS^+$ (1.26 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.41 $MgSO_4$, 0.33 mM KCl, 0.44 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 138 mM NaCl, 0.34 mM Na_2HPO_4 and 5.56 mM D-glucose pH 7.4), placed on ice and used within 5 min after mixing. DEA/NO (50 mM) stock solutions were prepared in 0.01 M NaOH, kept on ice and used within 1 h. Hb (5 mg) was dissolved in 1 mL $HBSS^+$ buffer; the solution was mixed well by vortexing, followed by centrifugation for 5 min at 10,000 × *g* and 4 °C to remove non-dissolved Hb precipitates. MetHb concentration was determined by measuring the absorbance at λ_{max} = 408 nm (ε = 179,000 cm^{-1} M^{-1}) [25]. Hb was then diluted with $HBSS^+$ to a final concentration of ~10 μM Fe(III).

2.2. Collection of human blood

Venous blood was collected form young (20–40 years old), healthy volunteers following written informed consent to participate. Procedures were conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of the Heinrich Heine University (ClinicalTrials.gov Identifier: NCT02272530).

2.3. Isolation of human RBCs from whole blood

Fresh blood was collected and anti-coagulated with EDTA in BD vacutainers. Blood was centrifuged for 10 min at 800 × *g* and 4 °C. After removal of the plasma and buffy coat, cells were washed by resuspension in $HBSS^+$ (pH 7.4), followed by centrifugation for 10 min at 300 × *g* and 4 °C. The supernatant was aspirated, discarded, and this washing process was repeated twice more. Washed cells were resuspended in $HBSS^+$ at a concentration 0.4 or 40% hematocrit (Ht). The concentration of oxyHb in RBC suspensions was estimated by measuring the absorbance at λ_{max} = 415 nm (ε = 125,000 cm^{-1} M^{-1}) [25].

2.4. Treatment of human RBC suspensions with Na_2S and QTOF LC-MS analysis

Washed native RBCs and/or metRBCs (i.e. cells in which Hb had been fully oxidized to metHb by pretreatment with DEA/NO; see 2.6) were treated at a Ht of 40% with Na_2S (0 or 100 μM) in $HBSS^+$ at 37 °C. After 0, 5 and 30 min of treatment, aliquots from each cell sample were removed and centrifuged for 1 min at 4000 × *g* and 4 °C. The supernatant was collected and diluted 1:4 (v/v) with a solution of 12.5 mM IAM in methanol. Cells were lysed by 1:4 (v/v) dilution in a solution of 12.5 mM IAM in methanol, followed by sonication for 2 min. Supernatant and cell lysate samples were kept at room temperature for 30 min to allow for the reaction of IAM with sulfide-derived species. All samples were then centrifuged at 10,000 × *g* for 10 min at 4 °C. The resulting supernatants of all samples were collected and analyzed via QTOF LC-MS. QTOF LC-MS analysis was performed on an Agilent 1290 Infinity UPLC System with a binary pump and auto-sampler coupled to an Agilent 6550 iFunnel Q-TOF System. Samples were separated on a Zorbax Eclipse Plus C18 RRHD 2.1 × 50 mm 1.8 μm column. Mobile phase A was 0.1% formic acid in Millipore water and mobile phase B was acetonitrile. Separation was accomplished using the following conditions: 99% solution A from 0 to 2 min, followed by a gradient decreasing from 99 to 1% solution A over minutes 7–12. The flow rate was 0.6 mL/min and the temperature was held constant at 20 °C. Analytes were ionized via electrospray ionization (ESI) operating in positive ion mode; other conditions were as follows: gas temperature 220 °C, drying gas flow 12 L/min, nebulizer pressure 35 psig, sheath gas temperature 330 °C and flow rate 11 L/min, capillary voltage 2500 V, nozzle voltage 1000 V, and fragmentor voltage 30 V. Analytes were determined by their accurate mass (*m/z*) using extracted ion chromatograms (EIC) from the total ion count (TIC).

2.5. Extraction of human oxyHb from RBCs

Washed human RBCs were lysed by suspension in 1:4 (v/v) of a hypotonic solution containing 5 mM sodium phosphate (pH 8.0), followed by inversion of the suspension 5 times every 30 s for 5 min. The resulting mixture was cleared by centrifugation at 20,000 × *g* for 10 min at 4 °C. The supernatant was aspirated, and the pellets were washed with hypotonic sodium phosphate solution and centrifuged once more. Hb was then purified from lysate samples by passage through a G-25 size exclusion chromatography column with $HBSS^+$ as the eluent. OxyHb concentrations of the resulting extract were determined by measuring the absorbance at λ_{max} = 415 nm (oxyHb, ε = 125,000 cm^{-1} M^{-1}) [25]. Hb solutions were then diluted with

$HBSS^+$ to obtain a final heme concentration of 10 μM, kept on ice and used within the day.

2.6. Preparation of metHb-enriched human RBCs (metRBCs)

Fresh RBCs (Section 2.3) were treaded with 1.1 × molar equivalents of DEA/NO (relative to a heme concentration of 30 μM) for 30 min at 37 °C in $HBSS^+$. Final concentrations of metHb in metRBC were determined by measuring the absorbance at 408 nm (ε = 179,000 cm^{-1} M^{-1}).

2.7. Treatment of cell-free human Hb solution and intact human RBC suspension with Na_2S and UV-vis analysis

For reactions involving isolated human oxyHb and metHb, protein solutions were placed in wells of a 96-well plate at 37 °C to achieve a final heme concentration of ~10 μM and a final volume of 99 μL. For reactions involving whole human RBCs (both native RBCs and metRBCs), a 0.4% Ht suspension was placed in wells of a 96-well plate at 37 °C to achieve a final heme concentration of ~30 μM and a final volume of 99 μL (note: 0.4% Ht was chosen as this was the maximum amount of Ht that did not cause over-saturation of the Hb absorbance). Full UV-vis spectra were recorded for cell-free oxyHb and metHb solutions, as well as native RBC and metRBC suspensions in the absence of Na_2S (t = 0 min). Next, 1 μL of a Na_2S stock solution (10 mM in $HBSS^+$) was added to the desired wells using an automatic injector and UV-vis spectra were recorded every minute for up to 60 min (final [Na_2S] = 100 μM) in a FluoStarOmega spectrophotometer (BMG Labtech).

2.8. Treatment of cell-free human Hb solutions and intact RBCs with Na_2S and EPR spectroscopy

Na_2S (100 μM) was added to isolated human oxyHb or metHb (~10 μM heme) in $HBSS^+$. Samples were mixed and immediately transferred to EPR tubes and incubated at 37 °C for the specified times. About 300 μL of cell-free Hb solutions were filled into EPR quartz tubes (30 mm filling height in 5.0/3.8 mm o.d./i.d. tube). After the desired time of treatment, samples were snap frozen and stored in liquid nitrogen until the time of measurement. For anaerobic treatment of metHb (10 μM heme) with Na_2S (100 μM), all stock solutions and samples were prepared in a glovebox under an atmosphere consisting of < 0.001 ppm O_2. Similarly, Na_2S (100 μM) was added to RBCs (0.4 or 40% Ht) or metRBCs (0.4% Ht) in $HBSS^+$ and the samples were immediately transferred to EPR tubes. Again, samples were incubated at 37 °C for the specified times. After the desired treatment time, samples were frozen and stored in liquid nitrogen until the time of measurement. As controls, untreated oxyHb, metHb, RBCs and metRBCs were also analyzed. All CW EPR spectra were recorded at 15 K with a Bruker ELEXSYS E-500 CW X-band spectrometer, using the perpendicular mode of the ER 4116DM dual mode cavity. The sample temperature was controlled using a Helium gas controlled cryostat (ESR900, Oxford Instruments). The field modulation amplitude was generally set to 0.7 mT at 100 kHz. Two microwave power levels of 0.7 mW and 7 mW were used in order to exclude EPR signal saturation, and the external magnetic field was swept from 50 mT to 0.4 T.

2.9. Assessment of metHb-SH reactivity by UV-vis analysis

Stock solutions of Na_2S, CySH and GSH (all 10 mM) were prepared in $HBSS^+$ and used immediately. Isolated human Hb (~10 μM in heme) in $HBSS^+$ was added to wells of a 96-well plate at 37 °C to a final volume of 98 μL. Initial spectra were recorded for all samples (t = 0 min). At t = 1 min, 1 μL of a Na_2S stock solution (10 mM, final [Na_2S] = 100 μM) was added to metHb solutions without shaking and the UV-vis spectra were recorded every minute for 5 min. After 5 min (t = 6 min), 1 μL of Na_2S, CySH or GSH was added to the formed metHb-SH solution and the UV-vis spectra measured every minute for a further 9 min (final concentrations were either 200 μM Na_2S, 100 μM Na_2S + 100 μM CySH or 100 μM Na_2S + 100 μM GSH).

2.10. Statistical analysis

All data were analyzed by using GraphPad Prism software (version 6.01; Graph Pad, La Jolla, CA, USA), and are expressed as means ± S.E.M. of n individual samples as stated in Results and Figure legends. Statistical comparisons between groups were performed by two-ways ANOVA as required by experimental setting, followed by an appropriate multiple comparison post hoc test (Dunnet's or Sidak's) or *t*-test as indicated in the figure legend.

3. Results

3.1. Sulfide reacts with metHb in intact human RBCs

An initial characterization of the effects of sulfide on human RBCs was carried out by treating RBC suspensions in physiologic buffer at pH 7.4 containing Ca^{2+}/Mg^{2+} and glucose with Na_2S (100 μM). Changes in intracellular Hb redox state were monitored by EPR spectroscopy using suspensions of native RBCs at physiological 40% Ht. Fig. 1A and B are the resonance X-band CW EPR spectra for intact human RBC suspensions (blue line) and display a typical EPR signal at 115 mT (g = 5.83) corresponding to a high-spin Fe(III)Hb [26], which constitutes about 1–3% of Hb present in RBCs (or, 100–300 μM Fe(III)-heme) per cell. Upon the addition of 100 μM Na_2S, this high-spin signal instantly diminishes, resulting in a new low-spin EPR signal between 260 mT and 370 mT (Fig. 1A and B, green line). This spectrum is indicative of the formation of a metHb-SH species, which was observed before by treating isolated Hb with sulfide [14,18,27], implying that sulfide reacts with metHb to produce a metHb-SH complex in intact human RBCs.

3.2. Sulfide does not react with human oxyHb

To better understand the reactivity of sulfide in human RBCs, we wanted to determine if a reaction of sulfide with oxyHb is also possible, as Hb is primarily present as oxyHb (oxyHb is present at a concentration of about 10 mM) in human RBCs. To investigate the reaction of sulfide with isolated oxyHb (or "free" oxyHb), oxyHb (10 μM heme in $HBSS^+$) was treated with 100 μM Na_2S in a cell-free solution and the resulting UV-vis spectral changes were recorded. As shown in Fig. 1C and D, Na_2S does not react with free oxyHb to any significant degree, as no appreciable spectral changes were detected for up to 30 min following addition of Na_2S. This indicates that sulfide does not react with Hb in its reduced (Fe(II), ferrous) oxygenated state (oxyHb).

3.3. Reaction of Na_2S with metHb leads to formation of a metHb-SH adduct

Next, to confirm that sulfide reacts with metHb, we monitored this reaction using UV-vis spectrometry in cell-free solutions as well. We found that addition of 100 μM Na_2S to cell-free solutions of 10 μM metHb produced a peak with λ_{max} = 422 nm within 1 min of reaction (Fig. 2A, green line) and this species was stable for up to 30 min. (Fig. 2A, red line). Spectral changes were also observed with lower concentrations of Na_2S (Fig. 2B), albeit to a lesser magnitude. This peak has previously been attributed to a metHb-SH species [18]. To further characterize the reaction of metHb with Na_2S, EPR spectroscopy was also performed. The resonance X-band CW EPR spectra for metHb are shown in Fig. 2C and D (blue line). Again, a typical EPR signal at 115 mT (g = 5.83) corresponding to a high-spin Fe(III)Hb [26] is displayed, similar to that witnessed for intact human RBCs (Fig. 1A and B). Addition of 100 μM Na_2S diminishes this high-spin signal, producing a

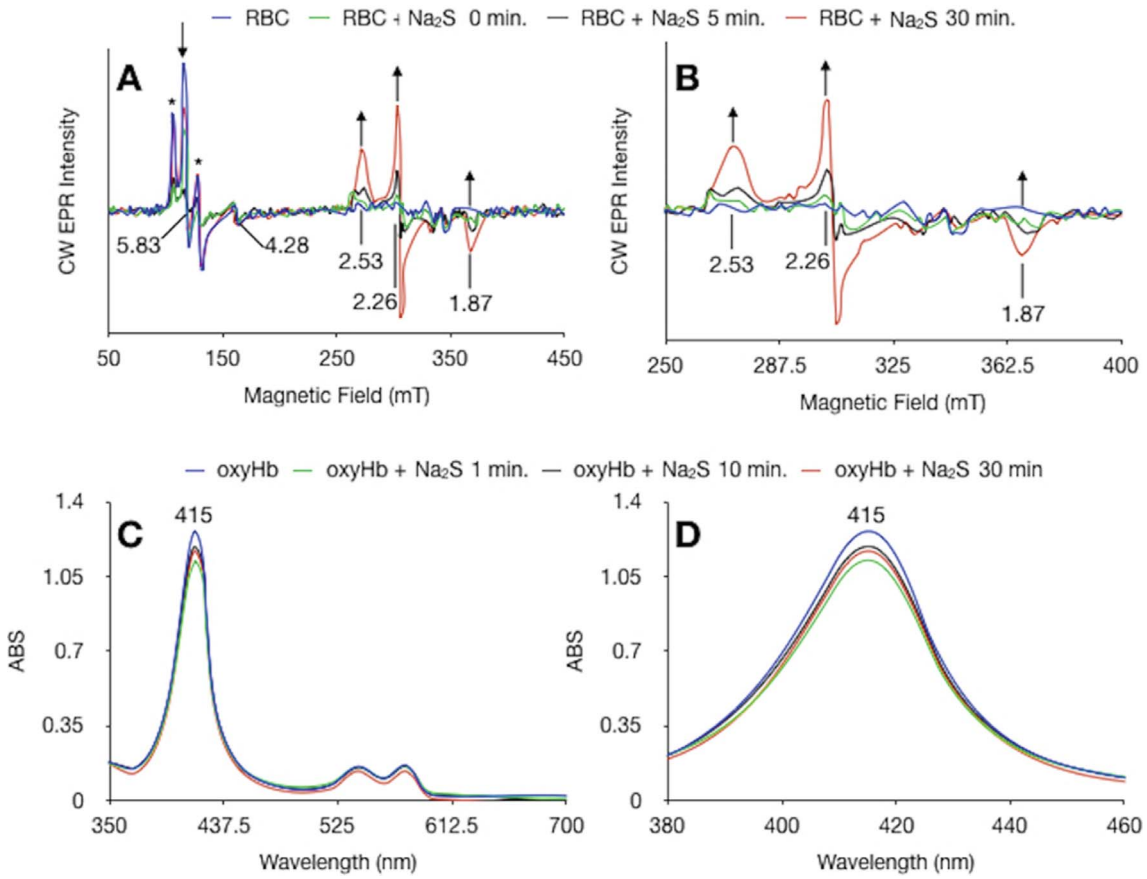

Fig. 1. Sulfide reacts with metHb and not oxyHb in intact human RBCs. X-band CW EPR spectra acquired at 15 K for (A) the treatment of native RBCs (40% Ht (100–300 μM metHb), blue line) with Na_2S (100 μM) for 0 (green line), 5 (black line) and 30 (red line) minutes. The numbers give g-values for the magnetic field positions corresponding to high-spin Fe(III)Hb (5.83); high-spin non-heme Fe(III) in rhombic coordination (4.28); and low-spin metHb-SH complexes with rhombic g-tensor (2.53, 2.26, 1.87). The asterisks mark the magnetic field positions corresponding to high-spin Fe(III) catalase g-values of 6.49 and 5.35 [46]; (B) selected low-spin metHb-SH region for A. (C/D) UV-vis spectra for (C) the addition of Na_2S (100 μM) to human oxyHb (10 μM heme). The peak at 415 nm corresponds to the absorbance for oxyHb and; (D) Soret's band for C. All experiments were performed in $HBSS^+$ at 37 °C (pH7.4). All tracings are representative of 3 independent observations.

new low-spin EPR signal between 260 mT and 370 mT as early as 5 min (Fig. 2C and D, black line) after addition of sulfide. Again, this spectrum is indicative of the formation of a metHb-SH species. Of note, the formed metHb-SH species is rather stable (Fig. 2C and D, red line) [28,29]. Taken together, these results show that sulfide reacts with metHb, forming a metastable metHb-SH adduct in both intact RBCs and cell-free solutions.

3.4. The metHb-SH adduct is detected in metHb-enriched RBCs

To this point, the reaction of sulfide with metHb in native human RBCs has only been examined using EPR spectroscopy. In order to further confirm formation of a metHb-SH species in RBCs, we sought to track this reaction by UV-vis spectrometry, similar to experiments using sulfide and cell-free metHb. A main complication with this is that in human RBCs, oxyHb is present at a concentration about 100 times that for metHb and thus, the absorbance for oxyHb saturates the UV-vis spectrum under conditions that would otherwise allow us to witness a reaction for sulfide with metHb. Therefore, to selectively increase the concentration of metHb in RBCs, we pretreated RBC suspensions at 0.4% Ht (corresponding to 30 μM heme) with equimolar concentrations of the NO donor DEA/NO (33 μM). As shown in Fig. 3A and B, this treatment induced near complete oxidation of oxyHb to metHb, as demonstrated by changes in the UV-vis spectrum (blue line, λ_{max} = 408 nm). Of interest, following the addition of 100 μM Na_2S, we observed rapid (< 1 min; Fig. 3A and B, green line) formation of the metHb-SH species (λ_{max} = 422 nm). Interestingly, we found that in metRBCs the formed metHb-SH species decays to oxyHb within 10–30 min (Fig. 3A and B, black line to red line). Thus, in intact RBCs, formed metHb-SH appears to be less stable than metHb-SH formed from isolated metHb and sulfide in cell-free solutions, the latter of which does not appear to decay over the same period of time.

Additionally, the reaction of Na_2S and metHb in metRBCs was monitored by EPR spectroscopy. The blue line in Fig. 3C and D depicts the EPR spectrum for metHb (0.4% Ht, 30 μM heme) resulting from the oxidation of RBCs by treatment with DEA/NO. The spectrum is dominated by the EPR signal of high-spin Fe(III)Hb. Subsequent addition of 100 μM Na_2S to this sample diminishes the high-spin Fe(III)Hb signal with concomitant formation of a new low spin metHb-SH signal within 5 min of reaction (Fig. 3C and D, black line). Importantly, the metHb-SH formed in metRBCs decays over time (from 5 to 30 min the metHb-SH EPR signal decreases by 20%; Fig. 3C and D, red line) to an EPR-silent species, likely being deoxyHb initially; under normal aerobic conditions the latter would immediately bind O_2 to form oxyHb, the product we indeed observed by UV-vis analysis (red line, Fig. 3A and B, λ_{max} = 415 nm). Thus, in intact human metRBCs, sulfide undergoes the same reaction with metHb as observed in cell-free solution, however, unlike in cell-free solutions where the formed metHb-SH is stable, formed metHb-SH in intact metRBCs is slowly reduced to oxyHb.

3.5. The metHb-SH complex is stable in the presence of biological thiols

In the next step, we considered it important to determine the stability of metHb-SH (λ_{max} = 422 nm) in the presence of biologically

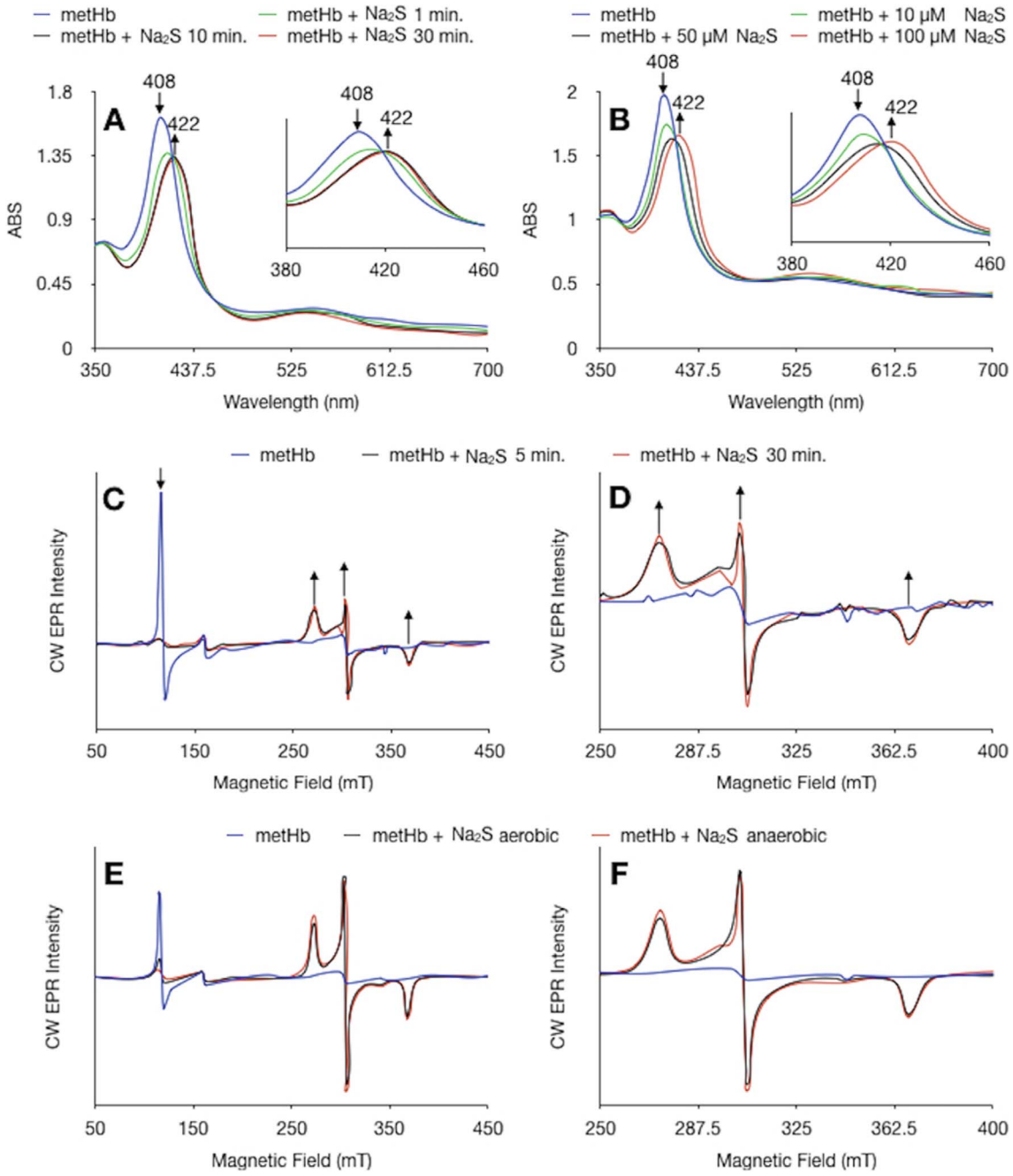

Fig. 2. Sulfide reacts with metHb leading to formation of a metastable metHb-SH complex. UV-vis spectra for (A) the addition of Na_2S (100 µM) to human metHb (10 µM heme, blue line) at 1 (green line), 10 (black line) and 30 (red line) minutes. The decrease in absorbance at 408 nm indicates a loss of metHb and the increased absorbance at 422 nm indicates formation of metHb-SH; (inset) Soret's band of A; (B) 30 min reaction of 10 (green line), 50 (black line) or 100 (red line) µM Na_2S with human metHb (10 µM heme, blue line) and; (inset) Soret's band of B. (C–F) X-band CW EPR spectra acquired at 15 K for (C) the treatment of human metHb (10 µM heme, blue line) with Na_2S (100 µM) for 5 (black line) and 30 (red line) minutes; (D) selected low-spin metHb-SH region for C; (E) 30 min treatment of metHb (10 µM heme, blue line) with Na_2S (100 µM) under aerobic (black line) and anaerobic (red line) conditions and; (F) selected low-spin metHb-SH region for E. All experiments were performed in $HBSS^+$ at 37 °C (pH7.4). For all spectra, the arrows indicate an increase (arrow up) or decrease (arrow down) in peak intensity. Spectra are representative of 3 independent observations.

relevant thiols, including GSH, CySH, as well as sulfide. As can be seen in Fig. 4, addition of 100 µM Na_2S to 10 µM human metHb results in rapid formation (within 1 min) of metHb-SH, as indicated by an increased absorbance at 422 nm. Subsequent addition of either 100 µM Na_2S (green line) or GSH (red line) 5 min later resulted in slightly enhanced formation of metHb-SH (indicated by a further increased absorbance at 422 nm), while addition of 100 µM CySH (black line) did not significantly affect metHb-SH levels, relative to the control not treated with a second equivalent of thiol (blue line). Increased formation of metHb-SH in the presence of additional Na_2S is likely due to a greater availability of free sulfide, maximizing the binding to metHb. Increased formation of metHb-SH in the presence of GSH is likely due to the reduction of formed and/or contaminating polysulfides, increasing the availability of free sulfide and also maximizing the binding to metHb. Neither CySH nor GSH caused spectral changes to metHb on their own. Taken together, this data implies that none of the thiols tested promote decomposition of formed metHb-SH, suggesting metHb-SH would be stable in an intracellular environment containing reduced thiols.

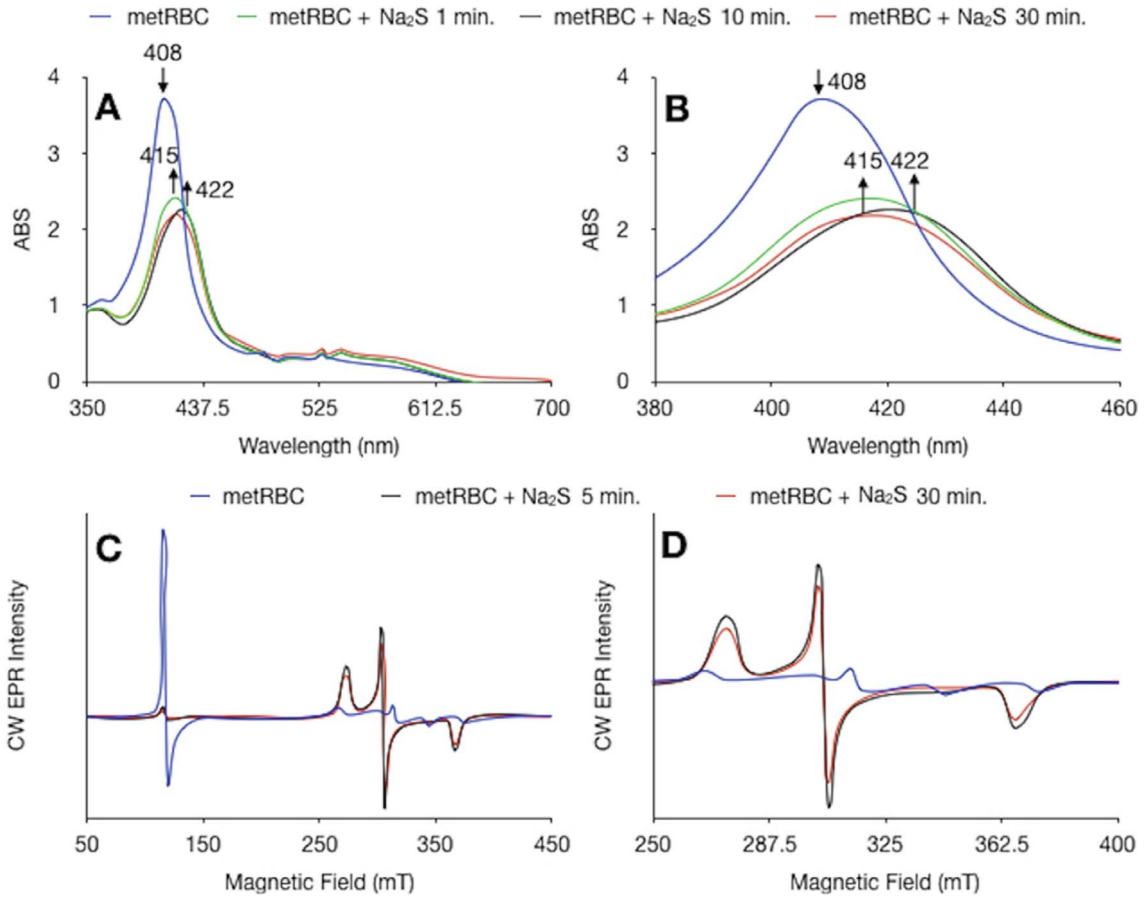

Fig. 3. Sulfide treatment leads to formation of the metHb-SH complex in intact metRBCs. (A/B) UV-vis spectra for (A) the addition of Na_2S (100 μM) to human metRBCs. (0.4% Ht (30 μM heme), blue line) after 1 (green line), 10 (black line) and 30 (red line) minutes. The decrease in absorbance at 408 nm indicates a loss of metHb and the increased absorbance at 422 nm indicates formation of metHb-SH. From 5 to 30 minuses the absorbance at 422 nm decreases with concomitant increase in absorbance at 415 nm, indicating loss of metHb-SH and formation of oxyHb, respectively and; (B) Soret's band for A. (C/D) X-band CW EPR spectra acquired at 15 K for (C) the addition of Na_2S (100 μM) to metRBCs (0.4% Ht (30 μM heme), blue line) at 5 (black line) and 30 (red line) minutes and; (D) selected low-spin metHb-SH region for C. The peaks corresponding to metHb-SH (see Fig. 2E and F) in C and D increase in intensity from 0 to 5 min, followed by a 20% decrease from 5 to 30 min, indicating formation and loss of the metHb-SH complex, respectively For all spectra, the arrows indicate an increase (arrow up) or decrease (arrow down) in peak intensity. All experiments were performed in $HBSS^+$ at 37 °C (pH7.4); representative of 3 independent observations.

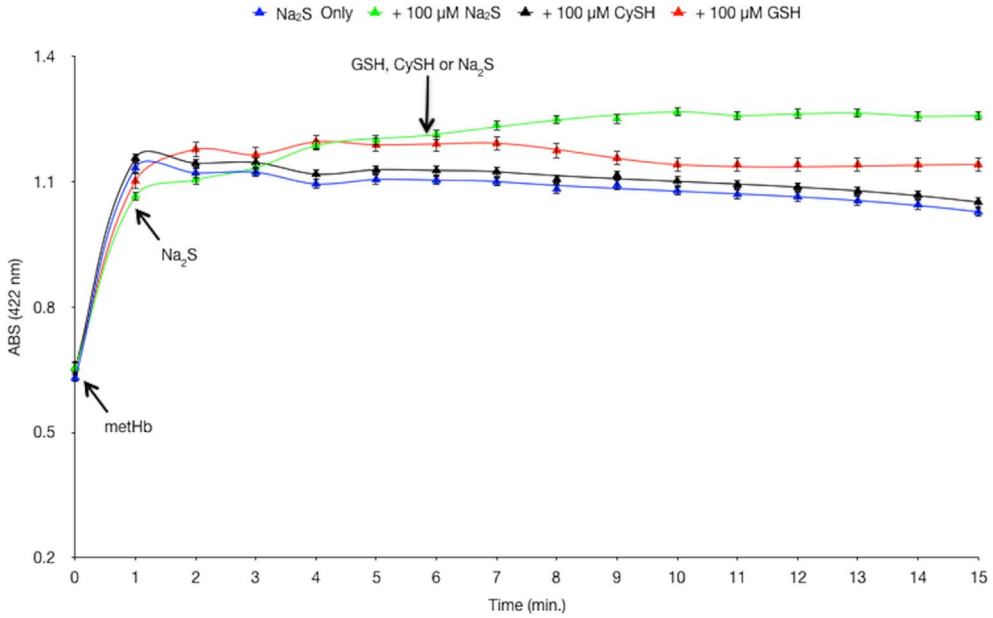

Fig. 4. The metHb-SH complex is stable in the presence of sulfide and biologically relevant thiols. Kinetic traces for the formation of metHb-SH (λ_{max} = 422 nm) in the presence of added thiol species. Isolated human metHb (10 μM heme) was treated with 100 μM Na_2S (blue line) for 5 min followed by the addition of additional 100 μM Na_2S (green line), CySH (black line) or GSH (red line). All experiments were performed in $HBSS^+$ at 37 °C (pH7.4). Spectra are representative of at least 3 independent observations.

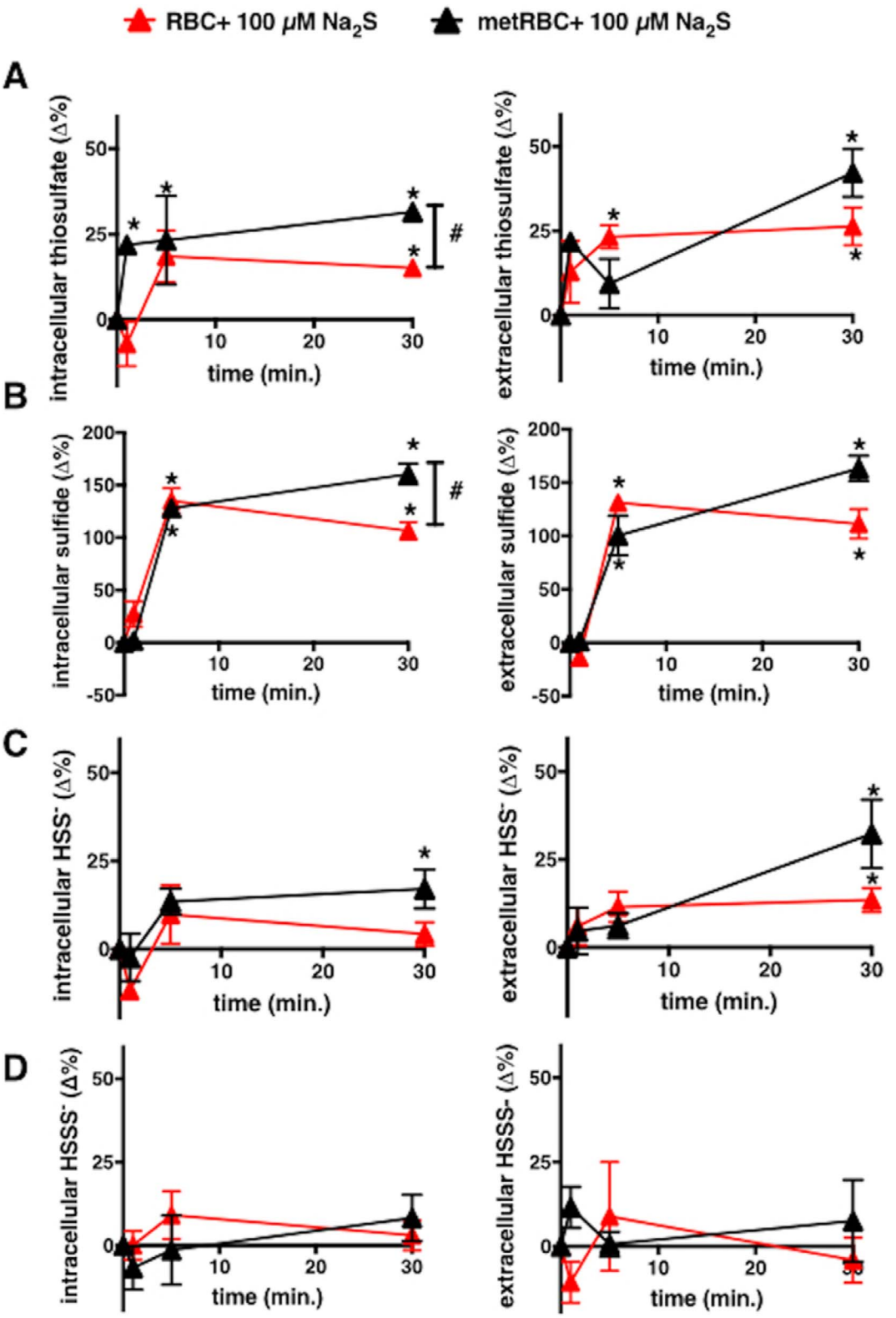

Fig. 5. Sulfide is metabolized into thiosulfate and polysulfides in intact human RBCs. Time course of relative changes in concentrations (delta%) of sulfide-related metabolites in human RBCs (red lines) and metRBCs (black lines) suspension at 40% Ht in physiological buffer exposed to 100 μM Na2S. The left panel represents the changes of metabolites within the cells, while the right panel represents the changes in the supernatant of the cells. Depicted results are representative of mean and SEM form n = 3 independent samples obtained form 3 different blood donors; data are expressed as percentage change in peak areas as compared to baseline assessed at (t = 0 min). 2-way-RM-ANOVA time vs. time = 0 min $p < 0.01$; *Dunnet's vs. time = 0 min $p < 0.05$; # T-test RBC vs. metRBC.

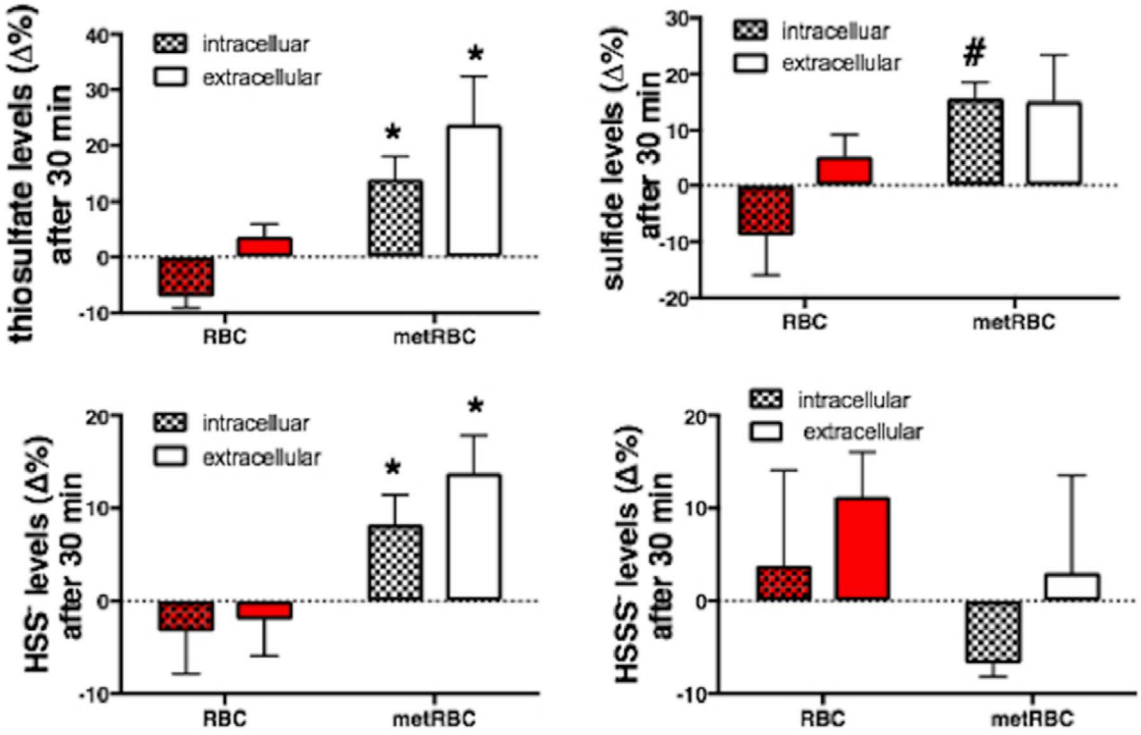

Fig. 6. Accumulation of thiosulfate and polysulfide in intact metHb-enriched RBCs and their supernatant. Levels of metabolites in cells and supernatants RBCs and metRBCs suspension at 40% Ht following 30 min incubation of in physiological buffer (in the absence of added sulfide). Changes for RBCs and metRBCs are relative to untreated RBCs and metRBCs, respectively. Depicted results are representative of mean and SEM form n = 3 independent samples obtained form 3 different blood donors; data are expressed as percentage change in peak areas as compared to baseline assessed at t = 0 min. 2-way-RM-ANOVA RBC vs. met RBC $p < 0.05$, * Sidak's vs. RBC $p < 0.05$. # T-Test vs. RBC $p < 0.05$.

3.6. RBCs metabolize sulfide to polysulfides and thiosulfate

To determine if treatment of RBC suspensions with sulfide leads to oxidation of sulfide and to characterize the final oxidation products, we examined sulfide-derived species present in RBCs and their supernatants by LC-MS analysis of the carboxyamidomethylated reaction products resulting from derivatization with IAM [30,31]. We found that treating suspensions of native RBCs at physiological 40% Ht (which contains 1–3% metHb, corresponding to 100–300 μM Fe(III)-heme per cell) with 100 μM Na_2S caused a rapid increase in the levels of intra- and extracellular $HS_2O_3^-$ (Fig. 5A, red line), compared to untreated cells (Fig. 5, baseline). Concomitant increases in intra- and extracellular levels of sulfide and polysulfides (Fig. 5B–D, red line) were also witnessed, and while the initial rate (0–5 min) for sulfide production was similar to that for production of $HS_2O_3^-$, polysulfide formation occurred more slowly. Extra- and intracellular levels of $HS_2O_3^-$ in treated RBCs were maintained for up to 30 min following treatment, while levels for sulfide and polysulfides decreased 5 min after treatment (See Fig. 6.).

Treatment of metRBCs at physiologic 40% Ht (containing almost 100% metHb, corresponding to 10 mM Fe(III)-heme per cell) with 100 μM Na_2S also caused an increase in intra- and extracellular $HS_2O_3^-$, sulfide and polysulfide levels (Fig. 5A–D, black line), compared to RBCs not treated with Na_2S (Fig. 5, baseline). Production of $HS_2O_3^-$ in metRBCs occurred more rapidly than sulfide production, which occurred more rapidly than polysulfide production. Importantly, initial (0–5 min) formation of intra- and extracellular $HS_2O_3^-$ occurred to a similar extent in both metRBCs and RBCs treated with Na_2S, however, this level of $HS_2O_3^-$ was attained more rapidly by metRBCs than by RBCs. Furthermore, after 5 min of treatment, metRBCs continued to produce $HS_2O_3^-$ for up to 30 min, while $HS_2O_3^-$ levels for native RBCs plateaued after 5 min of treatment. As for sulfide, initial (0–5 min) intra- and extracellular sulfide levels for metRBCs (Fig. 5B, black line) increased at a similar rate and to a similar extent as compared to native RBCs treated with Na_2S. However, while sulfide levels for native RBCs decreased after 5 min of treatment, sulfide levels for metRBCs continued to increase for up to 30 min after treatment. Inorganic polysulfide accumulation occurred at a slower initial rate (0–5 min) both inside and outside of metRBCs (Fig. 5C and D, black lines), compared to their accumulation inside and outside of treated RBCs. Importantly, accumulation of inorganic polysulfides by metRBCs continued for up to 30 min after sulfide treatment, while their levels either plateaued (as for H_2S_2) or decreased (as for H_2S_3) from 5 to 30 min following treatment of native RBCs with Na_2S. Taken together, these data indicate that native RBCs metabolize sulfide to $HS_2O_3^-$ and inorganic polysulfides, reaching maximum production of these species at 5 min, while full oxidation of RBCs to metRBCs allows for prolonged metabolism of sulfide to $HS_2O_3^-$ and inorganic polysulfides for up to 30 min.

4. Discussion

Hemoglobin has been proposed to play a central role in sulfide metabolism in RBCs, but the reaction of Hb and sulfide was never studied in intact cells. The aim of this work was to investigate the role of Hb in the metabolism of sulfide by human RBCs. We found that exposure of isolated cell-free metHb to sulfide led to a stable metHb-SH species while exposure of intact metRBC suspensions to sulfide led to formation of a metHb-SH intermediate that was slowly reduced to oxyHb. Moreover, treatment of native RBC suspensions (at physiologic Ht) with sulfide led to the formation of $HS_2O_3^-$ and polysulfides. These results show that under physiological conditions the reaction of H_2S with metHb is different in cell-free solutions and intact cells, and that RBCs metabolize sulfide via initial formation of a metHb-SH intermediate, the decomposition of which ultimately leads to reduction of metHb to oxyHb; the latter suggests a role for intra-erythrocytic sulfide to help maintain Hb in the reduced oxygen binding state.

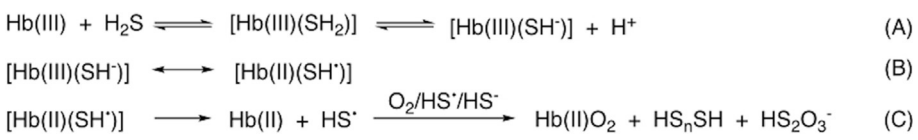

Fig. 7. Proposed reactions leading to the formation of thiosulfate, inorganic polysulfides and oxyHb from H_2S and metHb.

4.1. The chemical biology of the reaction of methemoglobin with sulfide

Data presented here show that reaction of H_2S and metHb leads to the formation of a metHb-SH species, not only in cell-free solutions, but also in intact RBCs; to the best of our knowledge, the latter has not been studied before. Previous work only utilized isolated human protein and/or human RBC lysates and indicated that H_2S reacts with metHb to afford a metHb-SH adduct, which was characterized by UV-vis and EPR spectroscopy [18–21,28,29]. These studies found that the formed metHb-SH was stable for extended periods of time, with slow decomposition leading to reduced Fe(II)Hb, $HS_2O_3^-$ and/or polysulfides. Here we confirm and extend these findings in cell-free solutions to show that metHb-SH, formed from isolated metHb and H_2S, is rather stable and resistant towards reaction with additional H_2S, GSH and CySH (Fig. 4). Conversely, we find that decomposition of metHb-SH, formed in metRBCs (and not in cell-free solutions) treated with H_2S, ultimately results in reduction of metHb to oxyHb (Fig. 3), which is paralleled by the production and release of $HS_2O_3^-$ and inorganic polysulfides (Fig. 5). As cell-free metHb is not reduced to oxyHb to any significance after treatment with sulfide, these findings appear to indicate that RBCs contain some type of machinery capable of facilitating the reduction of metHb-SH to oxyHb, and that this machinery is likely lost and/or destroyed upon the isolation of metHb or lysis of RBCs.

On the basis of our understanding of the reactivity of intermediates and products observed here, it is possible to propose a mechanism for the reaction of sulfide and metHb in RBCs. This is depicted in Fig. 7 and described in the following text. Consistent with all data presented here, sulfide preferentially reacts with metHb, but not oxyHb present in RBCs. This can be reasoned by the fact that sulfide is an electron-rich ligand and thus is expected to bind to electron-deficient metals (i.e. Fe(III)Hb) [32,33]. Binding of sulfide to metHb in RBCs results in a metHb-SH intermediate (Fig. 7A), which we identified as a low-spin Fe(III) adduct; this is also consistent with EPR data obtained from the analysis of the complex formed in solution with isolated Hb presented here and elsewhere [14,18,27]. Importantly, with regard to Hb, some reports have concluded that H_2S, rather than HS^-, initially binds to Fe(III)Hb [18,29,34], and it is believed that once bound, deprotonation of H_2S is facilitated by a distal histidine residue in the protein pocket [28,29]. Indeed, our finding that metHb-SH is resistant towards reactivity with biologically relevant nucleophilic thiols is consistent with a Fe(III)($^-$SH) type species (as two negatively charged species are not likely to react with one another), which can be formed under biologically relevant conditions. However, more studies analyzing pH dependence and structural information are needed to identify whether H_2S or HS^- is bound to the iron within intact RBCs and to confirm the mechanism we propose here. In any case, we witnessed that the binding of sulfide to metHb in RBCs results in a metHb-SH intermediate.

We found that in intact RBCs, the reaction of sulfide and metHb ultimately leads to formation of oxyHb, inorganic polysulfides and $HS_2O_3^-$ as final products. Thus, under the assumption that no other reaction partners contribute to this reaction, we propose that once the metHb-SH complex is formed, an inner-sphere electron transfer process takes place (Fig. 7B), facilitating the subsequent homolytic cleavage of the metHb-SH bond and resulting in deoxyHb initially, which is expected to quickly bind O_2, and thiyl radical ($HS^•$, Fig. 7C). If true, such a mechanism would be akin to the reaction of nitroxyl (HNO/NO^-) with metHb, resulting in the formation of nitrosylhemoglobin (Fe(II)Hb-NO). In analogy to this reaction (mechanistically termed a 'reductive nitrosylation'), we wish to term the process involved in the reaction of metHb with sulfide "reductive sulfhydration". Dissociation of $HS^•$ is likely followed by reaction with a second equivalent of $HS^•$ (producing H_2S_2), another molecule of hydrosulfide anion (HS^-, yielding hydrogen persulfide radical anion $HS_2^{•-}$) and/or O_2, ultimately leading to the formation of polysulfides and $HS_2O_3^-$ (a similar mechanism was proposed by us and others to explain formation of thiosulfate, sulfite and sulfate from the chemical interaction of NO with sulfide) [6,9,10,35]. Importantly, it has previously been determined that decay of metHb-SH occurs via first order kinetics, yielding Fe(II) and free $HS^•$ [28]. Furthermore, it has also been determined that O_2 consumption accompanies the reaction of H_2S and metHb [18,19], though these studies indicated that O_2 has no effect on the rate of metHb-SH decay itself, making its consumption/reactivity likely part of a secondary process following metHb-SH degradation. Alternatively, others have proposed a direct reaction between metHb-SH and a second equivalent of HS^- [18] to yield polysulfides and Fe(II) [29,34].

Taking all of the above into consideration, our results presented here are mostly consistent with metHb-SH decay being a first order process, as we found the addition of biological thiols (Fig. 4) and the presence of O_2 (Fig. 2E and F) to have little effect on the rate of metHb-SH decomposition. Importantly, our data does not support a direct reaction between sulfide and metHb-SH because the addition of Na_2S (as well as GSH or CySH) to metHb-SH had no effect on the rate of decay for metHb-SH, indicating no reaction with H_2S (or biologically relevant thiols for that matter). Thus, based on data presented here, it currently seems most reasonable to consider a mechanism involving homolytic cleavage of the metHb-SH bond, resulting in deoxyHb (and then oxyHb under aerobic conditions) and $HS^•$, followed by subsequent reaction of $HS^•$ with a second equivalent of $HS^•$, HS^- and/or O_2, ultimately leading to polysulfide and $HS_2O_3^-$ formation (Fig. 7).

4.2. Is the reaction of metHb with sulfide physiologically relevant?

The finding that sulfide preferentially binds to metHb rather than oxyHb in RBCs begs the question as to what the biological significance of such an interaction may be. In RBCs, Hb predominantly resides as oxyHb, with only about 1–3% present as metHb [36]. The concentration of oxyHb in RBCs is about 10 mM, corresponding to metHb concentrations of approximately 100–300 μM. At these concentrations, metHb could be expected to scavenge biological levels of sulfide, which are predicted to be in the nanomolar to low micromolar range [37,38]. Here we find that treatment of RBCs at physiologic cell concentrations (40% Ht in whole blood) with equimolar concentrations of sulfide (100 μM), as compared to expected metHb concentrations (100–300 μM), results in $HS_2O_3^-$ and inorganic polysulfide formation (Fig. 5). Furthermore, EPR analysis (Fig. 1A and B) of these same cells indicates that formation of those species succeeds formation of a metHb-SH intermediate. These findings suggest that naturally occurring metHb levels prevalent in RBCs are sufficient to scavenge and metabolize sulfide to $HS_2O_3^-$ and inorganic polysulfides. Interestingly, when RBCs were exposed to NO to selectively elevate levels of intracellular metHb (by virtue of the co-oxidation reaction of oxyHb with NO to form metHb and nitrate) prior to sulfide treatment, these oxidized cells produced more or less the same amounts of sulfide-derived products compared to native RBCs; this is likely due to the fact that 100 μM Na_2S was used for both metRBCs (10 mM Fe(III)-heme) and native RBCs (100–300 μM Fe(III)-heme), making Na_2S the limiting substrate. However, the formation of these sulfide-derived species occurred more rapidly in metRBCs due to the greater prevalence of metHb

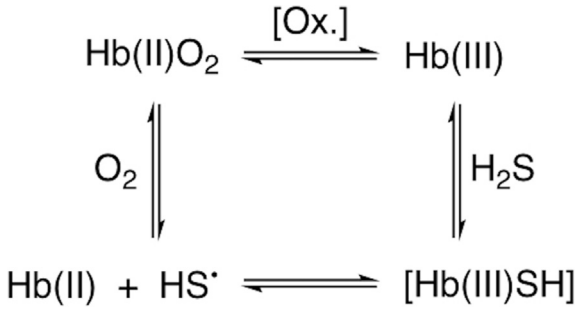

Fig. 8. Proposed redox cycle for the recycling of metHb to oxyHb by H_2S.

(Fig. 5). Additionally, metRBC treatment with sulfide also caused reduction of metHb back to oxyHb (Fig. 3), indicating a possible role for sulfide mediated regulation of metHb levels in RBCs (discussed below).

4.3. Sulfide and methemoglobinemia: toxicology and potential physiological role of sulfide reaction with metHb

The finding that H_2S only reacts with metHb and not oxyHb in RBCs may become relevant in conditions like methemoglobinemia, where RBCs have elevated levels of metHb, as compared to normal cells. In humans this can occur as a result of xenobiotics, cytochrome-b_5-metHb reductase deficiency and/or elevated levels of oxidizing species in the cell [40,41]. All cases lead to the accumulation of metHb in RBCs and prevent Hb from performing its canonical function of transporting O_2 and CO_2. Interestingly, nitrite-induced methemoglobinemia is still one of the best remedies proposed for treating sulfide poisoning [42]. Although other mechanisms were suggested to be involved in sulfide detoxification by nitrite administration (including the effects of nitrite on mitochondrial enzymes), a further mechanism may well be the enhanced oxidative inactivation of sulfide by increasing metHb levels in RBCs.

The fact that we find sulfide also reduces metHb to oxyHb in intact metRBCs makes it tempting to additionally consider H_2S-induced reduction as a cytochrome-b_5-metHb reductase-independent pathway for the reduction of both disease-induced, as well as naturally formed metHb in RBCs (Fig. 8). Surprisingly, patients with methemoglobinemia resulting from cytochrome-b_5-metHb reductase deficiency are able to maintain low levels of metHb, suggesting they are capable of regulating metHb levels in RBCs by some other mechanism(s). Whether this is mainly due to ascorbate and/or glutathione-dependent reducing enzymes, both of which are abundant in RBCs, or also involves endogenous sulfide needs to be investigated. It is important to also consider, however, that RBCs carry relatively high concentrations of a number of other enzymes that are likely to be involved in sulfide/polysulfide metabolism as well. These include 3-mercaptopyruvate sulfurtransferase (3-MST, a member of the transsulfuration pathway), and antioxidant enzymes like catalase, superoxide dismutase and thioredoxins [2]. All these enzymes have been described to play a central role in sulfide/polysulfide metabolism by us and others [39], and their actions on sulfide also result in the same sulfide-derived products characterized here. Therefore, it is tempting to speculate that sulfide metabolism by human RBCs may also occur independently of intracellular Hb, and in fact, that metHb-SH formation may simply be a biomarker (rather than a metabolic intermediate) for intracellular sulfide concentrations in RBCs, however, this remains speculative at this time.

4.4. Oxidation of sulfide results in thiosulfate formation in RBCs

In addition to metHb reduction in metRBCs, we find that sulfide is oxidized to inorganic polysulfides and $HS_2O_3^-$ in both native and oxidized RBCs. That RBCs produce $HS_2O_3^-$ via metHb mediated oxidation of sulfide is intriguing, as it has previously been determined that the enzyme 3-MST [18] utilizes $HS_2O_3^-$ as a substrate to produce sulfite (SO_3^{2-}) [43] and enzyme-bound sulfane sulfur. While sulfite is further metabolized to sulfate (SO_4^{2-}), the enzyme-bound sulfane sulfur can be transferred to other thiol substrates (i.e. GSH, CySH or other thiol-containing proteins) present in the cell, thus leading to the generation of hydropersulfides (RS_nSH, $n \geq 1$). This is particularly interesting because hydropersulfides have been described as enhanced antioxidants (relative to analogous low-molecular-weight thiol antioxidants), making them potentially critical for normal cellular function/defense [30,31,33,44]. Coupled with our current findings that H_2S/metHb-induced $HS_2O_3^-$ generation appears to occur to a greater extent under oxidized cellular conditions (conditions in which hydropersulfides are also speculated to be produced), this may imply a purpose for $HS_2O_3^-$ production in defending against cellular oxidative stress. In other words, H_2S may not only have a direct function in the reversal of oxidative stress in RBCs by reducing metHb back to deoxy/oxyHb, but it may also play an indirect role through its oxidation to $HS_2O_3^-$ and subsequent production of hydropersulfides (via 3-MST [45]) to further aid in cellular protection.

In summary, we conclude that RBCs metabolize sulfide via a metHb-dependent pathway. Importantly, when normal human RBCs are subjected to NO-induced methemoglobinemia, the resulting metHb is a potent scavenger of exogenous sulfide and results in formation of an intermediate metHb-SH complex that slowly decomposes to yield inorganic polysulfides, $HS_2O_3^-$ and oxyHb. These data suggest that endogenous sulfide may serve as a cytochrome-b_5-metHb reductase-like pathway to maintain low metHb levels, thereby preserving the O_2 carrying capacity of human RBCs, while enhancing the antioxidant capacity of RBCs via secondary sulfuration reactions potentially forming intracellular hydropersulfides.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

CB planned and executed experiments, analyzed data, and drafted the manuscript; AS planned and executed experiments, analyzed data, provided conceptual and intellectual input, and critically revised the manuscript; MF made substantial contributions to interpretation of the work, and wrote the manuscript; MCK designed and coordinated the work and wrote the manuscript. All authors have given their final approval of the version of the manuscript to be published.

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