ORIGINAL RESEARCH COMMUNICATION

Carbon Monoxide Signaling in Human Red Blood Cells: Evidence for Pentose Phosphate Pathway Activation and Protein Deglutathionylation

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Abstract

Aims: The biochemistry underlying the physiological, adaptive, and toxic effects of carbon monoxide (CO) is linked to its affinity for reduced transition metals. We investigated CO signaling in the vasculature, where hemoglobin (Hb), the CO most important metal-containing carrier is highly concentrated inside red blood cells (RBCs). **Results:** By combining NMR, MS, and spectrophotometric techniques, we found that CO treatment of whole blood increases the concentration of reduced glutathione (GSH) in RBC cytosol, which is linked to a significant Hb deglutathionylation. In addition, this process (i) does not activate glycolytic metabolism, (ii) boosts the pentose phosphate pathway (PPP), (iii) increases glutathione reductase activity, and (iv) decreases oxidized glutathione concentration. Moreover, GSH concentration was partially decreased in the presence of 2-deoxyglucose and the PPP antagonist dehydroepiandrosterone. Our MS results show for the first time that, besides Cys93, Hb glutathionylation occurs also at Cys112 of the β -chain, providing a new potential GSH source hitherto unknown. **Innovation:** This work provides new insights on the signaling and antioxidant-boosting properties of CO in human blood, identifying Hb as a major source of GSH release and the PPP as a metabolic mechanism supporting Hb deglutathionylation. **Conclusions:** CO-dependent GSH increase is a new RBC process linking a redox-inactive molecule, CO, to GSH redox signaling. This mechanism may be involved in the adaptive responses aimed to counteract stress conditions in mammalian tissues. *Antioxid. Redox Signal.* 20, 403–416.

Introduction

IN RECENT YEARS, it has become increasingly evident that exogenous and heme oxygenase (HO)-dependent exposure to carbon monoxide (CO) favors a pro-oxidant milieu in aerobic mammalian cells (35, 44). The resulting activation of intracellular signaling depends on reactive oxidizing species (ROS) and leads to the activation of redox-sensitive transcription factors, protein kinases, and antioxidant enzymes, including the CO-generating HO (35, 44). The bad reputation of CO, due to its well-known toxicity, is counterbalanced by its cytoprotective effects when regulated amounts of CO, biliverdin, and iron are produced by HO isoforms during heme catabolism (44). Low levels of CO, continuously pro-

duced in mammals by the constitutive enzyme HO-2, can markedly increase under stress conditions through HO-1 induction. Intracellularly generated CO acts as a signaling molecule and vasodilator, with anti-ischemic and anti-inflammatory effects (35, 44).

Recent findings emphasize the potential of using CO gas or CO-releasing compounds as therapeutic agents in pathophysiological events, such as hepatocyte cytotoxicity (3), cardiac myocyte hypertrophy (48), and ischemia-induced renal failure (52). It is interesting to note that CO shares its biological activities, including the regulation of vascular homeostasis and central nervous system functions, with the two other endogenous gases, nitric oxide (NO) and hydrogen sulfide (24, 37). With NO, in particular, CO shares reactivity

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Innovation

This work provides new insights on the signaling and antioxidant-boosting properties of carbon monoxide (CO) in human blood, where it reacts with hemoglobin (Hb), its most important and highly concentrated carrier. We show that reduced glutathione (GSH) concentration increases in CO-treated red blood cells (RBCs) through a mechanism involving Hb deglutathionylation. Our MS results show for the first time that GSH binds Hb β -chain at Cys112, in addition to the previously known Cys93. We hypothesize that CO-dependent GSH increase is a new pathway linking a redox inactive molecule, CO, to GSH antioxidant signaling in RBCs. This pathway might enhance the adaptive responses to stress conditions in mammalian tissues.

with metal centers; however NO, at variance with CO, can impose oxidative, nitrosative, and nitrative stresses in the presence of oxygen, transition metal-containing compounds, or ROS. Therefore, these two gases differ in their redox properties. In fact, CO is largely a redox-inert molecule, unreactive with most biological species, while NO is an effector of redox-regulated pathways. Both NO and CO bind to hemoproteins and are known to mutually regulate each other's production (12, 35, 37). However, while the mechanisms of NO-dependent signaling have been thoroughly studied, there is still a lack of consensus on the specific cellular target(s) of CO, with the exception of hemoglobin (Hb). In general, the biological effects generated by the formation of metal-CO complexes range from enzyme activation (as in the case of guanylate cyclase) or inhibition (e.g., mitochondrial electron transport cytochromes allowing ROS formation) (35). Understanding CO signaling is even more important in the vasculature where the most effective carrier of this gas, Hb, is highly concentrated. The elevated affinity of Fe^{+2} -Hb for CO, which is about 220-fold higher than the affinity for molecular oxygen, leads to the formation of carbonmonoxy-Hb (COHb) and compromises oxygen supply to tissues. Although CO can be oxidized to CO_2 by mitochondria, this is not the major pathway for elimination of excess CO, which is primarily eliminated *via* exhalation through the lungs. Our understanding of CO biology is centered on its high affinity for transition metals of hemoproteins. In contrast, many gaps exist mainly in the biochemical mechanisms of signaling, making it hard to discriminate between physiologic, adaptive, and toxic effects.

This work focuses on the signaling properties of CO, following its binding to Hb in human red blood cells (RBCs). We report that CO strongly increases intracellular reduced glutathione (GSH) levels mainly through Hb deglutathionylation. Moreover, we measured a CO-dependent activation of enzymes belonging to the pentose phosphate pathway (PPP). Interestingly, some of these enzymes can both reduce oxidized glutathione (GSSG) and favor Hb deglutathionylation, then we hypothesize that PPP could support the CO-dependent GSH increases. Since CO formation in tissues is a consequence of stress conditions, we suggest that CO-dependent GSH increase is a new cellular mechanism linking a redox-inactive molecule, CO, to RBC antioxidant signaling. This pathway may be involved in adaptive responses to stress conditions in mammalian tissues.

Results

CO increases GSH concentration in RBCs

We firstly investigated the possible effects of CO on whole blood thiol levels by using the reliable Ellman's colorimetric assay (11). As shown in Figure 1A, thiol concentration, measured by 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB) in air-equilibrated whole blood (2.1 ± 0.2 mM), significantly increased after CO treatment (2.9 ± 0.2 mM). Since GSH is largely the most abundant low-molecular weight thiol in blood, we hereafter refer to thiols as GSH. GSH concentration was significantly lower (1.56 ± 0.1 mM) in venous blood, that is, at lower O_2 tensions (34 ± 3 mm Hg) in accordance to Thom *et al.* (47). However, after CO treatment, GSH concentration in venous blood increased up to 2.141 ± 0.15 mM. These results indicate that O_2 tensions affect the basal levels of GSH, but the increase of GSH after CO treatment is similar to that measured in fully oxygenated samples (41% and 37% in fully oxygenated- and venous blood, respectively).

The source of CO-dependent GSH increase was then studied by analyzing separated blood components. GSH concentration in RBCs was similar to that found in whole blood and, in agreement, significantly increased after CO treatment (Fig. 1A). A possible contribution from plasma was ruled out because its low GSH concentration (3 ± 0.1 μM) (18, 56) was not modified by CO treatment (3 ± 0.09 μM). To identify the source of GSH in RBCs, its concentration was measured in RBC whole lysate, membrane-free lysate, or isolated membranes (Fig. 1B). In air-equilibrated whole lysate and membrane-free lysate, CO treatment increased GSH concentration to levels comparable with those of CO-treated whole blood. Membranes did not significantly contribute to thiol level modification (25 ± 1 μM and 24 ± 3 μM GSH in air and after CO treatment, respectively). Moreover, GSH concentrations in whole RBC lysate and membrane-free lysate were comparable to those measured in whole blood and intact RBCs, both before and after CO treatment (compare Fig. 1A, B). Taken together, these results prove that CO-dependent GSH increases in blood pertain to RBCs.

FIG. 1. Treatment with carbon monoxide (CO) increases reduced glutathione (GSH) concentration in red blood cells (RBCs). GSH concentration was measured in air-equilibrated or CO-treated (A) whole blood (WB), RBCs suspended at 50% hematocrit in 5 mM glucose (RBC), and plasma (PI) or (B) RBC whole lysate (W Lys), membrane-free RBC lysate (M-f Lys), RBC membranes suspended at 50% v/v in 5 mM glucose (M). After 30 min of treatment with CO, aliquots were acidified with trichloroacetic acid (TCA) (1:2 v/v) and GSH was measured in cleared supernatants, using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). Bars represent mean $\pm$ SD of three independent samples, each constituted by a pool of RBCs obtained from two different donors. * $p \leq 0.001$ versus air-equilibrated samples.

To verify that CO specifically increases GSH content in RBCs, we used NMR spectroscopy (5, 20, 38). Human RBCs, treated or not with CO in the presence of glucose, were extracted with ethanol and analyzed by NMR (Fig. 2A). Air-equilibrated RBCs showed signals assigned to lactate (Lac; 1.33 ppm), creatine (Cr; 3.04 ppm), and GSH (4.56 ppm); signals related to GSSG were undetectable, confirming its low concentration (33). After treatment with CO, we found a massive intracellular GSH increase (2.1 ± 0.15 mM and 3.6 ± 0.6 mM in air-equilibrated and CO-treated RBC, respectively; $p < 0.01$, $n = 7$), while the concentrations of Lac and Cr were not modified and GSSG remained undetectable (Fig. 2A, B). The role of glucose in CO-dependent GSH increases was further investigated by NMR in RBCs suspended in PBS in the absence of glucose. GSH concentration in air-equilibrated glucose-free cells was 1.2 ± 0.12 mM, and increased to 1.76 ± 0.13 mM after a 30-min CO treatment. These results suggest that glucose affects GSH basal levels and CO-dependent GSH increases.

FIG. 2. NMR evidence of GSH concentration increase in CO-treated RBCs. (A) Superimposed ^1H NMR spectra (9.4 T) of aqueous-phase extracts of air-equilibrated (black profile) and CO-treated (red profile) RBCs after a 30-min incubation at room temperature. Peak assignment: Ala (alanine); Lac (lactate); Glx (glutamate + glutamine + glutathione); GSH (glutathione); tCr (total creatine: creatine plus phosphocreatine); TSP, internal reference signal. (B) Relative quantification of aqueous metabolites (mean values $\pm$ SEM) in air-equilibrated and CO-treated RBCs ($n = 5$ with each sample being constituted by a pool of RBCs obtained from two different donors). Untreated, air-equilibrated RBCs are made equal to 100%. $*p \leq 0.001$ versus air-equilibrated samples. The relative quantification of aqueous metabolites has been performed as reported in "Materials and Methods." To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

The kinetics of GSH increase was then investigated in RBCs suspended in glucose-free or glucose-enriched PBS. GSH content (1.95 ± 0.2 mM), measured by DTNB in air-equilibrated, glucose-enriched RBCs, increased rapidly upon CO treatment, reaching 2.75 ± 0.1 mM after 30 min (Fig. 3). Interestingly, GSH continued to increase even after Hb became completely saturated, after 10 min of CO treatment (Fig. 3). A similar kinetics was observed with glucose-free PBS although, as expected, GSH concentrations were reduced both before and after CO-treatment (1.51 ± 0.10 mM and 2.18 ± 0.13 mM, respectively). To study the reversibility of GSH increase, CO was replaced with O_2 . This procedure induced a progressive decrease in GSH content (Fig. 3) in the presence of glucose, but not in its absence.

In summary, the initial NMR and spectrophotometric experiments yielded comparable results, showing that CO induces GSH increases through a mechanism marginally affected by glucose. However, reduction of GSH levels upon exposure to O_2 is significantly influenced by the presence of glucose.

The PPP participates in CO-dependent GSH increase

The unmodified Lac levels (Fig. 2) in CO-treated RBCs strongly suggest that glycolysis was not activated and the GSH increase was thus tentatively linked to PPP activation. This pathway indeed, maintain high GSH:GSSG ratios in RBCs, and requires NADPH and glucose metabolism to

FIG. 3. CO treatment of RBCs increases GSH in a time- and dose-dependent manner. After 10 min of equilibration in air, RBCs suspended at 50% hematocrit in 5 mM glucose-enriched or glucose-free PBS were treated with CO for 30 min at room temperature. The gas flow was then switched from CO to pure O_2 . Sample aliquots were collected at the indicated times to perform both carbonmonoxy-hemoglobin (COHb) determination by hemogas analysis and, after sample acidification with TCA (1:2 v/v), GSH measurement in the clear supernatants, using DTNB. Time-dependent COHb percentage is reported in the bar below. Data points represent mean $\pm$ SD of three independent samples, each constituted by a pool of RBCs obtained from two different donors.

regenerate this cofactor (40). To validate this hypothesis, we used two different approaches. First, we searched to confirm that glycolysis was not upregulated. We remember that, in RBCs, glycolysis activation may be triggered by the binding of Hb to the membrane protein Band 3 under oxidative or hypoxic conditions (2, 27, 50). The Hb binding to Band 3 dislodges the glycolytic enzymes (GE), which are physiologically anchored in their inactive forms to the cytoplasmic domain of Band 3 (2). Then GE, among which glyceraldehyde-3-phosphate dehydrogenase (GAPDH), become activated once released in the cytosol. In other words, the decrease of GAPDH level in the RBC membrane means that glycolysis is activated. We then assessed the abundance of GAPDH and the binding of Hb to membranes from air-equilibrated and CO-treated RBCs. Western blotting analysis of membranes from RBCs before and after CO treatment showed that GAPDH content was unaffected by CO treatment (Fig. 4A). Unexpectedly, when the nitrocellulose filter was stripped and blotted with an anti-Hb antibody, CO-treated RBCs showed membrane-bound Hb (Fig. 4A). Spectrophotometric measurements, performed in 0.1% sodium dodecyl sulfate (SDS)

to dissociate COHb from membranes, estimated about $30 \pm 1.2 \mu\text{M}$ ($n=4$) bound Hb. We conclude that CO-treatment promotes Hb binding to RBC membranes but does not induce GAPDH translocation.

The second approach was aimed to study in depth the PPP involvement. GSH content was measured in air-equilibrated and CO-treated RBCs suspended in glucose or 2-deoxy-glucose (2DG) (Fig. 4B). The latter cannot undergo glycolysis and directs cellular metabolism toward PPP. CO treatment induced comparable GSH increases in air-equilibrated glucose- and 2DG-enriched RBCs (+41% and +45%, respectively). These results suggest that any effects of glucose on GSH accumulation are mediated by the PPP. Hence, we further assessed the role of PPP by measuring GSH concentration in glucose- or 2DG-enriched RBC lysates incubated in the presence of dehydroepiandrosterone (DHEA). This compound is a noncompetitive androgen antagonist of glucose-6-phosphate-dehydrogenase (G6PDH) (17) and is expected to inhibit PPP. Figure 4B shows that DHEA did not affect the GSH content of air-equilibrated glucose-enriched RBC lysates in a statistically significant fashion. In contrast, in CO-treated samples, accumulation of the thiol was decreased by 12%. In 2DG-enriched RBC lysates, DHEA reduced GSH accumulation by about 15% in air-equilibrated samples and 33% after CO treatment (Fig. 4B). The inhibitory effects of DHEA on CO-dependent GSH increase were confirmed by NMR measurements of GSH concentrations in RBCs enriched in glucose (–25% GSH increase) or 2DG (GSH increase abolished) (Fig. 4C). The inhibitory effects of DHEA confirm PPP involvement in the CO-dependent increase in GSH concentration.

CO treatment of RBCs increases the activity of enzymes linked to GSH metabolism

The indication of PPP involvement obtained by DHEA led us to investigate the activities of key enzymes involved in the

FIG. 4. Pentose phosphate pathway (PPP) contributes to the CO-dependent increase of GSH concentration in RBCs. (A) CO treatment does not affect the expression of the glycolytic enzyme (GE) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in RBC membranes. RBC suspensions (50% hematocrit in 5 mM glucose) were treated with CO for 30 min at room temperature. Membranes from air-equilibrated and CO-treated RBCs were prepared as described in "Materials and Methods," loaded (10 μg protein/lane) on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, separated under reducing conditions, stained with Ponceau Red, and blotted with an anti-GAPDH antibody. Nitrocellulose filters were then stripped (see "Materials and Methods"), and incubated again with an anti-Hb antibody. The panel shows a typical result from five independent experiments (B) The CO-dependent increase in GSH concentration is canceled by a PPP inhibitor. GSH content was measured using DTNB in TCA-treated aliquots of air-equilibrated and CO-treated lysates of RBCs suspended at 50% hematocrit in PBS enriched with 5 mM glucose or 2-deoxy-glucose (2DG) in the absence or presence of 0.5 mM Dehydroepiandrosterone (DHEA) or solvent (1% DMSO). (C) NMR relative quantification of GSH content in samples shown in (B). The relative quantification of GSH has been performed as reported in "Materials and Methods." Bars represent mean ± SD of three independent samples, each constituted by lysates of a pool of RBCs from two different donors. * $p \leq 0.001$ and ** $p \leq 0.01$ versus the respective air-equilibrated or CO-treated samples.

formation of reducing equivalents (NADH or NADPH) necessary for the activity of enzymes linked to GSH homeostasis, that is, GAPDH, G6PDH, and 6-phosphogluconate dehydrogenase (6PGDH). As reported in Table 1, GAPDH activity in membranes of CO-treated RBCs was not significantly different from that measured in air-equilibrated RBCs. To assess that RBCs were able to respond to a stimulus, other than CO, able to activate intracellular metabolism, we measured GAPDH activity in hypoxic conditions ($pO_2 = 15 \pm 5$ mm Hg). This condition is known to favor translocation and activation of GE in the RBC cytosolic fraction (49). Table 1 shows the decrease of about 30% of GAPDH activity in membranes of hypoxic RBCs. Table 1 also shows that G6PDH and 6PGDH activities in cell lysates of CO-treated RBCs were increased by about 22% and 24%, respectively, *versus* air-equilibrated samples. Notably, in hypoxic conditions, the activities of these enzymes were significantly inhibited by about 21% and 26%, respectively, due to enhancement of glycolysis and reduction of PPP flux (23, 55). Collectively, these results suggest that CO does not trigger glycolysis, as previously reported (21, 23), but boosts PPP, presumably to generate NADPH.

Hb deglutathionylation as a source of CO-mediated GSH increase in RBCs

In general, increased GSH concentrations can be ascribed to (i) *de novo* synthesis, (ii) reduction of intracellular GSSG, or (iii) release of GSH from intracellular mixed disulfides formed with redox-sensitive Cys residues (deglutathionylation). No-

teworthy, both the GSSG reduction and protein deglutathionylation are processes correlated with the activation of PPP, which provide NADPH as a substrate for related specific enzymes, such as glutathione reductase (GR). The first possibility, *de novo* synthesis, was ruled out since incubation with 4 mM buthionine sulfoximine, an inhibitor of γ -glutamate-cysteine ligase, did not significantly affect GSH amounts after CO treatment ($2.61 \pm 0.02 \mu M$ and $2.67 \pm 0.03 \mu M$ in CO-treated RBCs in the absence or presence of buthionine sulfoximine, respectively; $n=3$). The second possibility was investigated by measuring GSSG amounts before and after CO treatment. As shown in Figure 5A, GSSG concentration decreased in a time-dependent manner as a function of CO treatment. After 30 min, GSSG was decreased by about 84%. Considering that two moles of GSH are generated per mole of GSSG reduced, after 30 min, GSH concentration may have been increased by up to $90 \mu M$. However, since the CO-dependent increase in GSH is in the millimolar range, the contribution of GSSG is modest.

The third scenario, that is, protein deglutathionylation, led us to investigate Hb, which is the only substrate present in RBCs at millimolar concentrations. The involvement of Hb deglutathionylation was investigated by separating on nonreducing gels membrane-free lysates of RBCs before and after CO treatment; western blotting was performed with an anti-GSH antibody. In air-equilibrated RBC lysates, we detected three major, well-separated bands decorated by the anti-GSH antibody, showing apparent molecular weights of about 20, 30, and 50 kDa (Fig. 5B). Interestingly, the intensities of the glutathionylated bands slightly decreased after 5 min and, more significantly, after 30 min of CO treatment (Fig. 5B, C). These results suggest that the GSH increase in CO-treated RBCs is controlled through the modulation of mixed disulfides between Hb and GSH. The molecular weight of the marked bands suggested that Hb, as a monomer (17 kDa), dimer (34 kDa), and trimer (51 kDa), is the major RBC protein involved in CO-dependent GSH increase. To verify that the three bands of Figure 5B were Hb, the nitrocellulose filter was stripped and blotted with an anti-Hb antibody. Figure 5B shows that the Hb bands matched the glutathionylated ones. These results indicate that Hb is the major glutathionylated protein of RBCs and that CO treatment decreases the amounts of GSH bound to this protein. As a final confirmation, the presumptive Hb monomer band (Fig. 5D) was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Hb α -, β -, δ - and γ - chains were identified using the Swiss-Prot human database (www.uniprot.org/downloads) (Fig. 5E).

MS analysis of Hb glutathionylation sites

We used MS analysis to investigate potentially modified Hb Cys residues. After removal of low-molecular weight compounds by dialysis, RBC lysates were incubated with iodoacetamide (IAM) to irreversibly block free thiols and minimize further thiol-disulfide exchanges. RBC proteins were digested with chymotrypsin, which preferentially cleaves proteins at the carboxyl terminus of Trp, Tyr, Phe, Leu, and Met, allowing extensive coverage by MS analysis. A search in the Swiss-Prot human protein database identified three proteins: Hb α - and β - chains and carbonic anhydrase. Hb α - and β - chains were by far the most abundant proteins,

TABLE 1. CO-DEPENDENT AND HYPOXIA-MEDIATED MODULATION OF THE ACTIVITIES OF ENZYMES LINKED TO GLYCOLYSIS AND PPP IN RBCs

	GAPDH ^a μmol NADH min/mg prot	G6PDH ^b μmol	6PGDH ^b NADPH min/g Hb	GR ^c μmol GSH/min
Air	8.44 ± 0.3	0.87 ± 0.03	1.07 ± 0.1	0.031 ± 0.002
CO	7.96 ± 0.5	$1.06 \pm 0.01^*$	$1.33 \pm 0.1^*$	$0.073 \pm 0.003^*$
Hypoxic	$5.99 \pm 0.8^*$	$0.68 \pm 0.02^*$	$0.80 \pm 0.1^*$	<i>Nd</i>

Washed RBCs (50% hematocrit in 5 mM glucose) were divided in three aliquots (1 ml each). The first was taken as air-equilibrated control, the second and the third were submitted to CO- (after 30 min: COHb 100%) and nitrogen treatment (after 15 min: $pO_2 = 15 \pm 5$ mm Hg). RBCs were then lysed and Hb-free membranes separated from cell lysates.

^aGAPDH activity was measured, as reported in (46), in membranes dissolved in Triton X-100 to allow the enzyme activation through its dissociation from band 3.

^bG6PDH and 6PGDH activities were simultaneously measured, as reported in (39), in cell lysates suspended in 100 mM triethanolamine containing 0.5 mM EDTA, pH 8.0, in the presence of their substrates (glucose 6-phosphate and 6-phosphogluconate).

^cGR activity was measured, as reported in (10), in RBC lysates by in the presence of GSSG and NADPH. TCA was added (1:2 v/v) for GSH determination.

* $p < 0.001$.

Nd, not done; CO, carbon monoxide; PPP, pentose phosphate pathway; Hb, hemoglobin; RBCs, red blood cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G6PDH, glucose-6-phosphate-dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; GSH, reduced glutathione; COHb, carbonmonoxy-Hb; GSSG, oxidized glutathione; GR, glutathione reductase; TCA, trichloroacetic acid.

FIG. 5. CO treatment of RBCs decreases oxidized glutathione (GSSG) content and Hb glutathionylation. (A) CO decreases GSSG concentration. RBCs (50% hematocrit in 5 mM glucose) were submitted to CO treatment. At the time reported, aliquots were acidified with TCA (1:2 v/v), and GSSG was measured in cleared supernatants, using DTNB-GSSG reductase recycling assay as reported in “Materials and Methods.” Symbols represent mean $\pm$ SD of three independent cleared supernatants, each constituted by a pool of RBCs from two different donors. (B) CO induces a time-dependent deglutathionylation of a cytosolic protein immunoreactive with an anti-Hb antibody. Lysates from RBCs (50% hematocrit in 5 mM glucose) equilibrated in air or treated with CO for 5–30 min were loaded (230 μ g protein/lane) on a 10% SDS-PAGE gel, separated under non reducing conditions, stained with Ponceau Red and blotted with an anti-GSH antibody as reported in “Materials and Methods.” With the exception of low expressed tetramer (TT), the anti-GSH antibody decorated the monomer (M), dimer (D), and trimer (T) Hb structures. The anti-GSH antibody was then removed and the nitrocellulose filter was again blotted with an anti-Hb antibody. Since no differences were found between the electrophoretic patterns of samples treated for 5 and 30 min, only Ponceau Red staining related to 30 min CO treatment was shown. The panel shows a typical result from five independent western blotting experiments. (C) Densitometric analysis of the anti-GSH immunoreactive bands reported in (B) after 5- and 30-min CO treatments. The effects of CO are reported as % of band intensities of Hb M, D, and T structures versus their respective air-equilibrated structures. All values were significant with $p < 0.001$. (D) MS identification of Hb as the major cytosolic protein immunoreactive with the anti-GSH antibody. Aliquots of air-equilibrated RBCs were loaded (230 μ g protein/lane) on a 4%–12% SDS-PAGE gel, separated under non reducing conditions, and stained with Coomassie Blue. (E) Hb α , β , δ , and γ chains were identified from the stained bands after tryptic digestion. Protein probability is calculated by Sequest as the $-10^* \log$ probability of casual identification; sequence coverage is the percentage of mapped amino acids in the protein; the number of individual (number of peptides) and redundant peptides (spectral counts) mapped for each identified protein are reported.

Scan 1: Full MS		Scan 2: CID MS ²		Scan 3: ETD MS ²		Scan 4: CID MS ³	
Disulfide	Cys	Precursor (charge)		Precursor (charge)	fragments (charge)	Precursor (charge)	
P1 → (86-104) P# → GSH	β 93	633.6 (z=4)		633.6 (z=4)	→ P1 = 1112.8 (z=2) → P# = 308.1	P1 = 1112.9 (z=2)	
P2 → (89-103) P# → GSH	β 93	696.9 (z=3)		696.9 (z=3)	→ P2 = 891.8 (z=2) → P# = 308.1	P2 = 891.8 (z=2)	
P3 → (105-114) P# → GSH	β 112	674.3 (z=2)		674.3 (z=2)	→ P3 = 1042.3 (z=1)	/	

FIG. 6. MS method used in data-dependent acquisition. Full MS (Scan 1) is followed by two consecutive MS² events, CID-MS² (Scan 2) and electron transfer dissociation (ETD)-MS² (Scan 3) of the most intense ion detected in Scan 1, and finally CID-MS³ (Scan 4) of the four most abundant ions detected in Scan 3. These scan events allowed the identification of disulfide bonds between P1, P2 (containing β-chain Cys 93), P3 (containing β-chain Cys 112), and GSH (first two columns from left). Precursor ions corresponding to peptides linked by a disulfide bridge and their charges are shown for each dependent scan event. ETD activation breaks disulfide bonds, producing fragment ions corresponding to GSH and the Hb peptides P1, P2, and P3; these ions are further fragmented by CID-MS³ and their sequence is reconstructed if their charge is at least 2.

as expected and as suggested by their relative spectral counts (42 and 122 respectively for Hb α- and β-chains, 5 for carbonic anhydrase).

The MS approach used to map disulfide bonds was based on a specific data-dependent mode acquisition, as reported by Wu et al. (57) and described in Figure 6. Briefly, the most intense ion detected in the full scan is automatically chosen for two consecutive fragmentation steps, the first one by collision-induced dissociation (CID) and the second by electron transfer dissociation (ETD) (57). Subsequently, the four most intense ions detected in the third step (ETD-MS²) are automatically selected for MS³, using CID fragmentation. During CID fragmentation, disulfide bonds are not cleaved, while b and y ions, produced after peptide bond breaking, are detected and allowed to reconstruct the amino acid sequence of the fragmented peptide. About 80% sequence coverage was obtained for both α and β Hb chains by CID fragmentation. The Cys residues (positions 93 and 112 in the β- and 104 in the α-chain) were detected in the alkylated form, indicating their originally reduced state (data not shown). Nevertheless, an oxidized state, that is, a disulfide bond, was also detected for the β-chain Cys residues. Indeed, if GSH is bound to a Cys-containing peptide, a CID-MS² spectrum of a peptide adduct is acquired: the precursor ion has a characteristic mass increase of 305 Da and its fragmentation pattern allows to recognize the modified site. Hb β chain peptides 86–104, 89–103, and 105–114, respectively, later indicated as P1, P2, and P3, were found to be involved in disulfide bridges with GSH, as reported in Figure 6. The schematic summary in Figure 6 shows that ETD activation causes the break of disulfide bonds and the formation of fragments corresponding to GSH and an Hb peptide, the latter being the precursor ion for the following MS³ event. In Figure 7, panels A, B, and G show CID-MS² spectra corresponding to P1, P2, and P3 bound to GSH molecules. We have found that each of these spectra contains a characteristic ion (circled in green), which is also the most abundant peak produced by the cleavage between the Glu and the Cys belonging to GSH. We indicated this peak as y₂[#], followed by the indication of its charge. We reasoned that this

fragmentation product might represent a flag for the presence of a GSH molecule bound to the peptide. The CID-MS² spectrum resulting from the fragmentation of precursor ion 674.3 (Fig. 6G) has been assigned to peptide P3 with GSH bound to Cys112 with a very high degree of confidence (peptide probability 7.6·10⁻⁷; Xcorr 2.8). Moreover, almost all predicted b and y ions deriving from P3 were observed (Fig. 7G, inner panel). It is generally accepted that Cys93 of the β-chain is the major site of Hb glutathionylation (28). To our knowledge, this is the first time that glutathionylation of β-chain Cys112 has been detected. This new observation has been obtained most likely thanks to the insertion of m/z 674.3 among the ions in the parent mass list in the acquiring method.

In the case of the peptides containing Cys93 (P1 and P2), the b and y ion patterns are not very rich (Fig. 7A, B), but the spectra have been manually assigned (inserts inside the panels). To further confirm these attributions, we employed ETD fragmentation, taking advantage of the peculiar disulfide bond split that occurs by this method. Indeed, ETD breaks the disulfide bond to produce a protonated (Cys-SH) and an odd electron (Cys-S[•]) species (4, 57, 60). Panels C, D, and H of Figure 7 show the ETD-MS² spectra of the same precursor ions that had been fragmented by CID in the previous scans. Ions corresponding to peptides P1, P2, and P3 in the protonated form, after disulfide bond break, are clearly detectable. Moreover, a peak at m/z 308, corresponding to the protonated GSH (P#), is also visible in panels C and D. In addition, some charge-reduced species of the precursor ions, such as some c and z ions, typical of ETD fragmentation, were clearly assigned. These data strongly support the identification of mixed disulfide bonds involving P1, P2, P3, and GSH. In addition, fragment ions detected in the ETD spectra, in particular m/z 1112.9 and 891.8, respectively assigned to P1 and P2, have been further fragmented by CID (Fig. 7E, F). The resulting MS³ spectra definitively allowed the identification of the expected peptide sequences. This approach provided conclusive evidence that both Cys93 and Cys112 of the Hb β-chain form disulfide bonds with GSH. No other

glutathionylated Cys residues, deriving from any other human protein, were identified in our sample. We did not detect glutathionylated Cys112 in RBCs kept under more physiological O₂ concentration (34 ± 3 mm Hg). This negative result does not necessarily imply that Cys112 is not modified in venous blood, as this cysteine is presumably modified only in a minor fraction of the Hb molecules and is thus difficult to detect.

CO increases the activity of GR in RBCs

The indication that Hb deglutathionylation was a likely mechanism explaining the CO-mediated GSH increase in RBCs led us to investigate the involvement of enzymes known to favor this reaction such as GR, glutaredoxin (25), Trx/TrxR, or sulfiredoxin (Srx) (13, 25, 32, 36) (Fig. 8A). As for Srx, the contribution on Trx/TrxR to deglutathionylation events in RBCs is not clear (26). The driving force of the deglutathionylation process in RBCs seems to be GR, even though the activity of Grx has been reported to be greater (9) or at least similar (32) to that of GR in these cells. Considering that CO induces increases in GSH content (Fig. 1) and reduction of GSSG (Fig. 5A), we hypothesized that GR might be involved in Hb deglutathionylation. In keeping with this hypothesis, GR activity was doubled in CO-treated RBCs, compared with air-equilibrated samples (39). In support of GR activation, we found that the 30 min-treatment of RBCs with CO, in the absence or in the presence of 1 mM NADPH, GSH concentration increased from 2.75 ± 0.1 mM to 3.11 ± 0.1 mM ($p \leq 0.001$; $n = 5$), respectively. Although a direct effect of CO on GR is not expected because this enzyme does not contain metal-center, we investigated a possible direct effect of CO on GR by using DTNB-GSSG reductase recycling assay (1). The GSSG-reducing activity of the purified enzyme (266 U/ml) was not affected by the treatment with CO (GSH formed from 20 μM GSSG: 46.5 ± 4.2 μM and 44.6 ± 3.9 μM, respectively).

Discussion

The ability of CO to stimulate GSH-linked antioxidant defenses in animal tissues has been previously reported (47, 54). In rat blood, Thom's group measured the twofold increase of GSH:GSSG levels after administering CO *in vivo* in a manner known to cause brain damage and induce oxidative stress biomarker formation (47). RBCs were identified as the source of glutathione increase through a mechanism involving NO-derived oxidants. Here, we present new insights on the mechanism underlying the ability of CO to induce GSH increases in RBCs (Fig. 8B). We hypothesize that Hb deglutathionylation is mediated mainly by conformational changes induced by CO binding. The following binding of

COHb to RBC membrane activates PPP, which contributes as a minor mechanism to GSH release. COHb, being in a relaxed conformation similar to the oxygenated form, does not trigger glycolysis, even when bound to the cell membrane. Indeed, GAPDH, and presumably the other GE (phosphofructokinase, aldolase, pyruvate kinase, and lactate dehydrogenase), are not released in their activated form into the RBC cytoplasm from Band 3, their membrane binding dock. Its metabolic consumption by the glycolytic pathway being prevented, glucose is then forced to enter PPP, similar to what occurs in RBCs under high oxygen saturation conditions (30). The increased NADPH production might support the activation of GR, which reduces GSSG and promotes Hb deglutathionylation (Fig. 8B).

The involvement of Hb in CO-dependent thiol increases was indicated by (i) GSH increases in whole blood, intact and lysed RBCs, but neither in plasma nor in separated RBC membranes, and (ii) decreased levels of glutathionylated Hb. In human RBCs, Hb glutathionylation has been previously reported to occur at Cys93 of the β-chain (6, 14, 28, 29, 45), to be low in physiological conditions (16, 29), and significantly increased in oxidizing conditions (10, 29) or in pathological states (15, 34). Our MS results show for the first time that, in blood from healthy donors, not only Cys93 but also Cys112 of the Hb β-chain form disulfide bonds with GSH. Human Hb has one Cys residue in each α-chain (Cys104) and two Cys residues in each β-chain (Cys93, Cys112). βCys93 was regarded as the only glutathionylated site, being five-fold more accessible than βCys112, whereas αCys104 is completely buried and thus inaccessible to GSH. We were able to detect glutathionylated βCys112 by inserting specific ions in the parent mass. Such ions corresponded to a number of theoretical peptides produced by chymotrypsin cleavage and covalently bound to GSH. This strategy allowed fragmentation of the selected ions, if present, independent of their relative abundance.

In general, protein glutathionylation plays a critical role in redox signal transduction by binding proteins/enzymes and reducing or increasing their functions. Importantly, glutathionylation can be reversed in a reducing environment, in an enzyme-dependent or independent manner (7, 13, 15, 36, 58). Notably, a previous report (9) showed that RBC protein glutathionylation (mainly Hb) in human blood decreased immediately after the oxidative challenge and increased again in parallel with the modification of the GSH-GSSG ratio. As reported by Kleinman *et al.* (22), protein glutathionylation of human blood accounted for 4%–27% of total GSH and, importantly, >98% of protein glutathionylation occurs in RBCs with a protein-mixed disulfide:Hb molar ratio ranging from 1 to 16. This means that 20 mM Hb in RBCs can be bound by 0.2 to 3.2 mM GSH. This estimated range of glutathionylated Hb

FIG. 7. MS² and MS³ analysis of peptides P1, P2, and P3 linked with GSH. Dialyzed RBC lysates, alkylated with iodoacetamide and digested with chymotrypsin, were processed for MS analysis as reported in "Materials and Methods." The panels show CID-MS² spectra of the m/z 633.6 (A) and m/z 696.9 (B) ions, ETD-MS² spectra of the m/z 633.6 (C) and m/z 696.9 (D) ions, CID-MS³ spectra of the m/z 1112.8 (E) and m/z 891.8 (F) ions, CID-MS² spectrum of the m/z 674.3 (G) ion, and ETD MS² spectrum of the m/z 674.3 (H) ion. The peptide sequences with the observed fragment ions are shown in the inset of each panel. The expected product ions are labeled in blue in the spectra. The P1 (86–104), P2 (89–103), P3 (105–114), and P# (GSH) peptides, followed by their charges, are included in red boxes; y2#, followed by its charge and circled in green, is the product of the cleavage between the Glu and Cys residues belonging to GSH. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

FIG. 8. Intracellular GSH sources and effects of CO treatment on RBC thiol homeostasis. (A) Sources of GSH. Besides *de novo* synthesis, GSH content can be regulated by the reduction of GSSG, dependent on glutathione reductase (GR) and mediated by the PPP oxidative branch or by protein de-glutathionylation, that is, the release of GSH from mixed disulfides formed with redox-sensitive Cys residues of proteins (P-S-SG). Deglutathionylation may be achieved by changes in the intracellular redox status (increase in reduced thiols, changes in GSH/GSSG ratio, and thiol-disulphide exchange with GSH) or by enzymatic reduction mediated by glutaredoxin (Grx), GR, the thioredoxin/thioredoxin reductase (Trx/TrxR) system, and/or sulfiredoxin (Srx). **(B)** Mechanisms hypothesized to contribute to the CO-dependent GSH increase in RBCs. The major mechanism hypothesized is Hb deglutathionylation mediated mainly by conformational changes induced by CO binding and, to a lesser extent, by the de-glutathionylating activity of GR. The latter contribution might be supported by a minor mechanism linked to the activation of PPP (gray pathway) and boosted by the binding of COHb to the RBC membrane. COHb, being in a relaxed conformation similar to the oxygenated form, does not trigger glycolysis (black pathway) even when bound to the cell membrane. Indeed, GAPDH, and presumably the other GE (phosphofructokinase, aldolase, pyruvate kinase, and lactate dehydrogenase), are not released in their activated form into the RBC cytoplasm from Band 3, their membrane binding dock. Its metabolic consumption by the glycolytic pathway being prevented, glucose is then forced to enter PPP, similar to what occurs in RBCs under high oxygen saturation conditions (30). The increased NADPH production might support the activation of GR, which reduces GSSG and promotes Hb deglutathionylation.

is in good agreement with our data showing a CO-dependent increase of about 0.8 mM GSH.

Hb glutathionylation has been reported to affect both functional (oxygen affinity, cooperativity, alkaline Bohr effect, and rate of autoxidation) and structural (tertiary/quaternary structure in the region close to β Cys93) hemoprotein properties (6, 14). We hypothesize that the CO-dependent Hb conformational changes could favor GSH release from Cys93 and/or Cys112 of the Hb β -chain. This hypothesis is supported by the existence of at least three COHb structures in the relaxed conformation, different from both the "classical" relaxed and tense Hb structures (8, 42, 43, 51). Structural analyses (42) suggested that these Hb structures could be either allosteric intermediates or liganded end states in equilibrium with each other or with the "classical" relaxed Hb conformation. It is interesting to note that these COHb structures show changes at the $\alpha\beta$ interface close to Cys93 and Cys112. The same structures might expose otherwise inaccessible thiols, which could reduce the GSH-Hb mixed disulfide (41).

The contribution of PPP to the CO-dependent GSH increase is supported by the findings that pretreatment of RBCs with 2DG decreased GSH concentration in the presence of a G6PDH antagonist. In addition, CO treatment (i) increased the activities of the PPP enzymes G6PDH and 6PDGH, (ii) increased the activity of GR, (iii) decreased GSSG concentration, and (iv) did not affect Lac and GAPDH content, but at the same time increased the binding of Hb to the RBC membrane. We hypothesize that PPP is activated by CO-induced Hb binding to the membrane (Figs. 4A and 8B), a mechanism similar to that previously proposed (2, 27, 50) for activation of GE by oxidized or deoxygenated Hb. In the case of COHb, however, only enzymes linked to PPP would be activated.

The CO-dependent increase in G6PDH and 6PDGH activities might contribute to the CO-induced GSH increase through GR-dependent GSSG reduction and Hb deglutathionylation (Fig. 8B). G6PDH and 6PDGH activities were increased by an apparently modest 24%. However, two moles of NADPH are produced per mole of glucose-6-phosphate entering PPP. These, added to NADPH basal amounts (about 40 μ M) (53), can efficiently increase GR activity. We cannot exclude that Grx, the other important enzyme devoted to Hb deglutathionylation (25, 32, 45), might also be involved. However, rapid and significant Hb deglutathionylation mediated by this enzyme should be of minor importance. Indeed, as calculated from the data reported in Table 1, the contribution of GR to the CO dependent GSH increase is only about 1.3 μ M in 30 min.

Finally, our results suggest that CO treatment and hypoxic conditions differently affect the RBC metabolism because CO does not affect the glycolytic pathway but favors PPP activation (21, 23), while hypoxia has opposite effects (2, 21). Glucose is, however, important in redox equilibrium restoration in CO-treated RBCs, as indicated by the reversibility experiments. Glycolysis activation in RBCs mainly serves the purpose of restoring the redox state altered by an oxidative challenge and maintaining Fe^{2+} -Hb in its reduced form by increasing the Fe^{3+} -Hb reductase cofactor NADH. Since in CO-treated RBCs metHb is not formed, the pathway competent for metHb reduction need not be activated.

We believe that the results of this work, though mostly obtained at high Hb-saturating concentrations to highlight CO effects, provide new insights on the signaling and

antioxidant-boosting properties of this gas in RBCs. The CO-dependent GSH increase linked to PPP-supported de-glutathionylation of a highly concentrated source (Hb) might represent a new cell pathway involved in the adaptive response to stress conditions in mammals.

Materials and Methods

Chemicals

All chemicals, unless otherwise indicated, were from Sigma.

Preparation of RBCs and related components

Heparinized fresh human blood was obtained from healthy donors following informed consent. After plasma and buffy coat removal by centrifugation at 1000g for 10 min, RBCs were washed thrice with PBS, pH 7.4, and pooled (RBCs from two different donors were pooled and used for each experimental point). RBCs were lysed in 10 volumes ice-cold 5 mM phosphate buffer, pH 8.0, containing 0.1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml of leupeptin, and 10 μ g/ml aprotinin (lysis buffer). Hb-free membranes were prepared by centrifuging lysates at 40,000g for 10 min at 4°C, removing hemolysate and washing several times with lysis buffer. RBCs were suspended at 50% hematocrit in PBS, 5 mM glucose or 2DG, for 2 h at 37°C. The preincubation with 0.1 mM DHEA (stock: 250 mM in dimethylsulfoxide) was performed in 5 mM glucose for 15 min, at 37°C. The same amount of dimethylsulfoxide was added to untreated samples. The presence of 2DG or DHEA did not significantly induce Hb oxidation. To avoid metal-catalyzed reactions, all buffers were extensively treated with Chelex 100 and contained 0.1 mM diethylenetriaminepenta-acetic acid.

Preparation of CO-saturated and hypoxic RBCs

Fully oxygenated ($pO_2 = 200 \pm 5$ mm Hg) whole blood or its derivatives (1 ml final volume) were treated with absolute CO by directing the gas flow to the samples through a peristaltic pump. CO treatment of venous blood was obtained by bubbling CO directly into the collection tubes. COHb formation was monitored spectrophotometrically ($\epsilon_{540} = 13.4 \text{ mM}^{-1} \text{ cm}^{-1}$) and by hemogas analysis (Blood Gas Analyzer GEM Premier 400; Instrumentation Laboratory Co). Hypoxic samples were obtained by submitting RBCs to nitrogen flux for 15 min. All samples were analyzed for pO_2 , pCO_2 , pH, and % of oxyHb, deoxyHb, carboxyHb, and metHb.

GSH and GSSG measurement

Sample aliquots were deproteinized by adding 1.22 M iced trichloroacetic acid (TCA) (1:2 v/v), kept 5 min in ice and centrifuged at 10,000 rpm, 5 min, 4°C. GSH was determined in the clear supernatants by adding 0.1 mM DTNB to 0.1 mM phosphate buffer/1 mM diethylenetriaminepenta-acetic acid, pH 7.4, containing aliquots of samples or GSH standard curve (11). After 5 min, samples were read at 412 nm and the GSH content was normalized to RBC Hb content. Likely artifacts linked to the contribution of heme release were ruled out by the lack of absorbance in the range 350–450 nm of TCA-treated supernatants of CO-treated RBCs. GSSG was mea-

sured in 25 μ l cleared acidified supernatants by using DTNB-GSSG reductase recycling assay (1).

Enzyme activities

The activities of G6PDH and 6PGDH were measured in RBC lysates as reported in (39). GAPDH activity was measured in RBC membranes as reported in (46). GR activity was measured in RBC lysates, as reported in (10).

Western blot analysis

Samples were dissolved in 4 $\times$ loading buffer under non-reducing or reducing conditions, resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (loaded proteins: 230 μ g and 10 μ g of cell lysates and membranes, respectively) and visualized with Ponceau Red or Coomassie blue staining. Proteins were then transferred to nitrocellulose paper at 35 V overnight. Blots were rinsed with washing buffer (Tris-saline, 0.05% Tween 20, pH 7.4), and blocked with 3% bovine serum albumin for 2 h. Washed nitrocellulose filters were incubated overnight at 4°C with polyclonal anti-GSH (Millipore), anti-GAPDH (Enzo Life sciences), and anti-Hb (Sigma) antibodies at a dilution of 1:1000, 1:2000, and 1:1000, respectively. Immunoreactive bands were detected by chemiluminescence coupled with peroxidase activity (ECL kit; Pierce). To remove antibodies, nitrocellulose filters were incubated with stripping buffer (Thermo Scientific) for 30 min, rinsed with washing buffer, blocked with 3% bovine serum albumin for 4 h, rinsed again, and blotted with another antibody. For protein MS identification Coomassie stained bands were reduced with Dithiothreitol, alkylated with IAM, digested with sequencing grade modified porcine trypsin (Promega) and analyzed by LC-MS/MS.

Preparation of RBC extracts in aqueous phase and NMR spectroscopy

RBCs were washed twice with ice-cold physiological saline solution and the pellets resuspended in 0.5 ml of ice-cold twice-distilled water. Cells extracts were prepared by adding to the packed RBCs four volumes of 70% (v/v) EtOH:H₂O according to an established protocol (19, 31). High-resolution NMR experiments (25°C) were performed at either 400 or 700 MHz (Bruker AVANCE spectrometers). Quantification of individual metabolites was obtained from peak areas using correction factors determined by experiments at the equilibrium of magnetization (90° pulses, 30.00 s interpulse delays).

MS/MS identification of disulfide bonds in the Hb chains

Dialyzed RBC lysates (cutoff 10,000) were incubated 30 min, room temperature, with IAM (1:100 molar ratio). Protein content was digested by chymotrypsin (enzyme/substrate 1/50 w/w) in 25 mM ammonium bicarbonate, 37°C over night. Peptide mixture was desalted by using μ C18 Zip Tip from Millipore, concentrated in speed vacuum to near dryness and finally reconstituted with 5% acetonitrile and 0.1% formic acid. Peptide mixture was analyzed by nanoflow-reversed-phase liquid chromatography-tandem mass spectrometry (RP-LC-MS/MS) using an HPLC Ultimate 3000 (DIONEX) connected on line with a linear Ion Trap (LTQ;

Thermo Fisher Scientific). Peptides were further desalted in an on-line trap column (AcclaimPepMap100 C18, LC Packings, DIONEX) and then separated in an in-house slurry-packed C18 column (a fused silica capillary, 75 μm i.d. $\times$ 10 cm from New Objective and a 5 μm , 200 Å pore size C18 resin from Michrom BioResources). Peptides were eluted using a linear gradient from 15% to 50% of buffer B containing 95% acetonitrile and 0.1% formic acid in 44 min, then increasing buffer B to 80% in 5 min and finally washing the column with 90% buffer B at 300 nl/min flow rate. MS analysis was performed in positive ion mode with high voltage potential around 1.7–1.8 kV. A data-dependent acquisition performed automatically in sequence a full MS (scan 1) in the mass range from m/z 400 to 2000, CID-MS² (48) of the most intense ion detected in the scan 1, ETD-MS² (scan 3) of the most intense ion detected in scan 1, and finally CID-MS³ of the four most abundant ions detected in the scan 3 (Fig. 6). CID fragmentation employed normalized collision energy of 35% while ETD required 100 ms activation time. Target ions already fragmented were dynamically excluded for 30 s. Parent ions, which correspond to a number of Cys-containing sequences in both α and β Hb chains, were inserted in the parent mass list to be selected for fragmentation, even if they are not the most abundant ions in the full MS scan. MS/MS spectra were matched against Swiss-Prot human protein database and through SEQUEST algorithm (59) incorporated in the Bioworks software (version 3.3, Thermo Fisher) using no enzyme constraints, variable cysteine modification by alkylation (Δm : +57 Da) or glutathionylation (Δm : +305 Da) and partial modification by oxidation on methionine, (Δm : +16 Da). Statistical filters for protein identification were cross correlation scores of 1.8 for $z=1$, 2.5 for $z=2$, 3 for $z=3$, and a probability cutoff for randomized identification of $p<0.001$. Individual peptide fragmentation to produce b and y ions (in CID spectra) or c and z ions (in ETD spectra) was studied to determine the amino acid sequence and to confirm and characterize disulfide bonds.

Statistical test used

Results have been evaluated by using Student's *t*-test.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

CID = collision-induced dissociation
 CO = carbon monoxide
 COHb = carbonmonoxy-hemoglobin
 2DG = 2-deoxy-glucose
 DHEA = dehydroepiandrosterone
 DTNB = 5,5'-dithiobis-(2-nitrobenzoic acid)
 ETD = electron transfer dissociation
 GAPDH = glyceraldehyde-3-phosphate dehydrogenase
 GE = glycolytic enzymes
 G6PDH = glucose-6-phosphate-dehydrogenase
 GR = glutathione reductase
 Grx = glutaredoxin
 GSH = reduced glutathione
 GSSG = oxidized glutathione
 Hb = hemoglobin
 IAM = iodoacetamide
 HO = heme oxygenase
 NO = nitric oxide
 6PGDH = 6-phosphogluconate dehydrogenase
 PPP = pentose phosphate pathway
 RP-LC-MS/MS = nanoflow-reversed-phase liquid chromatography-tandem mass spectrometry
 RBC = red blood cell
 ROS = reactive oxidizing species
 SDS = sodium dodecyl sulfate
 SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis
 Srx = sulfiredoxin
 TCA = trichloroacetic acid
 Trx/TrxR = thioredoxin/thioredoxin reductase