

Upregulation and Mitochondrial Sequestration of Hemoglobin Occur in Circulating Leukocytes during Critical Illness, Conferring a Cytoprotective Phenotype

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The classical role of hemoglobin in the erythrocytes is to carry oxygen from the lungs to the tissues via the circulation. However, hemoglobin also acts as a redox regulator and as a scavenger of the gaseous mediators nitric oxide (NO) and hydrogen sulfide (H₂S). Here we show that upregulation of hemoglobin (α , β and δ variants of globin proteins) occurs in human peripheral blood mononuclear cells (PBMCs) in critical illness (patients with severe third-degree burn injury and patients with sepsis). The increase in intracellular hemoglobin concentration is a result of a combination of enhanced protein expression and uptake from the extracellular space via a CD163-dependent mechanism. Intracellular hemoglobin preferentially localizes to the mitochondria, where it interacts with complex I and, on the one hand, increases mitochondrial respiratory rate and mitochondrial membrane potential, and on the other hand, protects from H₂O₂-induced cytotoxicity and mitochondrial DNA damage. Both burn injury and sepsis were associated with increased plasma levels of H₂S. Incubation of mononuclear cells with H₂S induced hemoglobin mRNA upregulation in PBMCs *in vitro*. Intracellular hemoglobin upregulation conferred a protective effect against cell dysfunction elicited by H₂S. Hemoglobin uptake also was associated with a protection from, and induced the upregulation of, HIF-1 α and Nrf2 mRNA. In conclusion, PBMCs in critical illness upregulate their intracellular hemoglobin levels by a combination of active synthesis and uptake from the extracellular medium. We propose that this process serves as a defense mechanism protecting the cell against cytotoxic concentrations of H₂S and other gaseous transmitters, oxidants and free radicals produced in critically ill patients.

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INTRODUCTION

Although hemoglobin is best known for its role as an oxygen carrier in erythrocytes, many biological functions of hemoglobin predate the evolution of the circulatory system and are used to modulate various redox functions and interactions with gaseous transmitters such as nitric oxide (NO) and hydrogen sulfide (H₂S) (1–5). For instance, clams living in H₂S-rich waters upregulate intracellular

hemoglobin levels as a protective mechanism against H₂S toxicity (5).

The current report describes a significant upregulation of hemoglobin in human peripheral blood mononuclear cells (PBMCs) in two distinct forms of critical illness (sepsis and severe burn injury) and highlights the consequences of this phenomenon in the context of oxidant-, free radical- and gasotransmitter-associated cytotoxicity.

MATERIALS AND METHODS

Materials

All chemicals were obtained from Sigma-Aldrich, unless stated otherwise.

Cell Culture

Human monocyte histiocytic lymphoma cells (U937) and human embryonic kidney epithelial cells (HEK239T17) were obtained from ATCC and maintained in RPMI1640 with 10% fetal bovine serum (Life Technologies).

Isolation of Human PBMCs

Blood samples from critically ill patients and from healthy volunteers were collected with the permission of the local institutional review boards (IRBs). For the component of the clinical study involving burn patients, adult patients

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Table 1. List of primers used in the present study.

Name	Forward	Reverse
Hemoglobin α	5-CAG AGA GAA CCC ACC ATG GTG-3	5-GGT GGT GGT TCT GGA TGA AGG-3
Hemoglobin β	5-CAG ACA CCA TGG TGC ATC TG-3	5-CTC CAA GAA ACT CAG GAA ACC C-3
Hemoglobin γ	5-GGA AGG CTC CTG GTT GTC TAC C-3	5-CGT TCT TCC ACG ACT GAA GGG-3
Hemoglobin δ	5-CAA ACA GAC ACC ATG GTG CAT C-3	5-GTC CAA GGG TAG ACC ACC AG-3
CD10	5- CTG GAA TCT ATA AAG AGG CTT GTA CAG C-3	5-GGT TTA GCC GTA GCA TTG GC-3
CD19	5-CCT CAG CCA GGA CCT CAC-3	5-CGT CTC CAT TAC CCA CAT ATC TC-3
CD34	5-CCA ACA GAA CAG AAA TTT CCA GC-3	5- GGT GAC CAG TGC AAT CAG G-3
CD38	5-CCT GAG ATG AGA CAT GTA GAC TGC-3	5- GAG AAC TGA TGG GCC AGA TC-3
CD40	5-GGT CAA GCA GAT TGC TAC AGG-3	5- GTC TCA CAG CTT GTC CAA GG-3
CD45	5-CCA CAG GTG TTT CAT CAG TAC AGA C-3	5- CCT CTC TCC TGG GAC ATC TGA G-3
CD117	5-CCA GAC AGG CTC TTC TCA ACC-3	5- GAT CAG TGC ATA ACA GCC TAA TCT C-3
CD127	5-GAC GCA TGT GAA TTT ATC CAG C-3	5-GGA CTC CAT TCA CTC CAG AAG C-3
Integrin 1b	5-CCC ATT GAC CTC TAC TAC CTT ATG G-3	5-GCC AAA TCC AAT TCT GAA GTC C-3
Integrin 4a	5-GGA TCC ATC GTG ACT TGT GG-3	5-GGA TAT TCC AGC TTG ACA TGA TGC-3

with burns covering $\geq 30\%$ of the total body surface area (TBSA) were enrolled. Patients received standard burn care (6) and blood samples were obtained at the time of admission (0 wk) and 3 wks later (3 wks). For the component of the clinical study involving septic patients, adult patients with freshly diagnosed sepsis according to the established sepsis criteria (7) were enrolled at the time of diagnosis (0 wk) and 1 wk later (1 wk). Age-matched healthy human subjects served as healthy controls. Leukocytes and plasma were isolated using Histopaque (Sigma-Aldrich) (8).

Protein Identification by Mass Spectrometry

Protein extract of isolated PBMCs were separated on SDS-PAGE, stained with Coomassie Brilliant Blue (Bio-Rad), and ~15-kDa bands were excised from gel and subjected for identification by ESI-LC/MS/MS (4000 QTRAP with LC Packings capillary LC system, Applied Biosystems) (9).

Western Blotting

Western blotting was performed as before (9–11) using: anti-hemoglobin α (Santa Cruz Biotechnology), anti-hemoglobin- β (Santa Cruz Biotechnology) and anti-hemoglobin $\beta/\gamma/\delta$ (Santa Cruz Biotechnology). Loading was normalized with anti- β -actin-HRP (Santa Cruz Biotechnology). Blots were devel-

oped with Supersignal West Pico Chemiluminescent (Thermo Scientific).

RT-qPCR

Total RNA was extracted with TriReagent (MRC), cDNA was synthesized by high-capacity cDNA reverse transcriptase kit (Life Technologies) and expression of the following genes was determined: hemoglobin α , β , γ , δ , CD10, CD19, CD34, CD38, CD40, CD45, CD117, CD127, Integrin 1 β , Integrin 4 α , HIF1- α , Nrf2. Actin, 36B4, 18S was used as an internal control. Sequences of the primers are listed in Table 1.

Measurement of Hemoglobin Concentration

Concentration of hemoglobin in plasma and RPMI was determined using the Hemoglobin Assay Kit (Sigma-Aldrich).

Flow Cytometric Analysis

PMBCs were fixed in 3.7% paraformaldehyde for 30 min at 4°C followed by three washes with PBS and permeabilized with 0.1% Triton-X containing PBS for 30 min, followed by three washes with PBS. CD45 (Santa Cruz Biotechnology) and primary antibody against hemoglobin- β was applied at 500 \times dilution in blocking buffer (1% BSA in PBS) for 1 h, followed by washing with PBS and subsequent incubation with Alexa Fluor-conjugated secondary antibodies (Life Technologies) for 30 min. Samples were analyzed using a FACSArray Bioanalyzer (BD Biosciences).

Immunocytochemistry

U937 cells were treated for 24 h with 2, 20 or 200 mg/dL of purified hemoglobin, isolated from the blood of healthy volunteers, then washed two times with RPMI and fixed with 4% paraformaldehyde in slide chambers (Lab-Tek). Cells were triple stained with: DAPI (Life Technologies), Alexa Fluor 594 conjugated (Mix-n-Stain, Sigma-Aldrich) to anti-CD163 antibody and Alexa Fluor 488 conjugated (Mix-n-Stain, Sigma-Aldrich) to anti-hemoglobin α . Images were visualized using Nikon Eclipse 80i fluorescent microscope with CoolSNAP HQ camera and analyzed with NIS Elements BR3.10 software.

Proximity Ligation Assay (PLA)

In situ protein:protein proximity/interaction studies were performed with Duolink *in situ* (Sigma-Aldrich) as before (10) using the following antibodies: anti-CD163 (Santa Cruz Biotechnology), anti-hemoglobin α (Abcam), anti-cadherin (Santa Cruz Biotechnology), anti-Na⁺K⁺ATPase (Santa Cruz Biotechnology), anti-lamin (Cell Signaling Technology), anti-PDI (Cell Signaling Technology), anti-histone (Cell Signaling Technology), anti-LDH (Cell Signaling Technology), anti-Nduf53 (mitochondrial complex I subunit, Abcam), anti-mitochondrial complex II 70 kDa subunit (Life Technologies), anti-mitochondrial complex IV subunit (cytochrome c oxidase subunit II, Life Tech-

nologies), anti-mitochondrial complex V 56 kDa subunit (Life Technologies) and anti-TFAM (mitochondrial transcription factor A, Genetex). Images were visualized using Nikon Eclipse 80i fluorescent microscope with CoolSNAP HQ camera and analyzed with NIS Elements BR3.10 software.

Quantification of Cell Death using LDH Release

U937 cells (5×10^5 cells) were incubated with hemoglobin (2-20-200 mg/dL) for 24 h and washed two times with RPMI media and challenged with 5 mmol/L NaSH, 500 μ mol/L H_2O_2 , 100 μ mol/L SIN or 500 μ mol/L SNAP for 24 h. LDH activity in the culture medium, an indicator of cell necrosis, was measured as described (10).

Measurement of Mitochondrial and Total Cellular Oxidant Production

Detection of mitochondrial superoxide was achieved with MitoSOX (Life Technologies) and total ROS levels were quantified with H_2DCF (Life Technologies) as described (11).

Citrate Synthase Activity Measurement

Specific activity of citrate synthase was detected in total cell lysate of 5×10^5 U937 cells previously incubated with hemoglobin (2-20-200 mg/dl) for 24 h using Citrate Synthase Assay Kit (Sigma-Aldrich) as described (11).

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential was measured using TMRE (Life Technologies) in 5×10^5 of U937 cells as described (9).

Measurement of Intracellular ATP Content

ATP content was measured using CellTiter-Glo Luminescent Cell Viability Assay (Promega) as described (11).

Analysis of Bioenergetic Parameters by Extracellular Flux Analysis

U937 cells (2×10^5) were plated in 24-well Seahorse culture plates (BD Bio-

sciences) followed by 24 h incubation with hemoglobin (2-20-200 mg/dl). Bioenergetic parameters were measured with XF24 Extracellular Flux Analyzer (Seahorse Bioscience) as described (12).

Measurement of Plasma H_2S Content

Concentration of H_2S in human plasma samples or in samples of animals subjected to burn injury according to a model previously described (13) were detected with 7-azido-4-methylcoumarine (AzMC) H_2S -specific fluorescent probe as described (14).

Transient Transfection of HEK293T17 Cells with Hemoglobin

HEK293T17 cells were transfected on 24-well plates with full-length human hemoglobin cDNA inserted into pCMV6-XL4 vector purchased from Origene Technologies. β -galactosidase-inserted plasmid was used as a control (Origene). Transfection of HEK293T17 cells was performed using Lipofectamine 2000 (Life Technologies) with 0.5 μ g of plasmid DNA per well. After 48 h HEK293T17 cells were challenged with 7.5 mmol/L NaSH for 1 h. LDH release into the media was quantified and hemoglobin expression was confirmed by Western blotting.

Measurement of Mitochondrial DNA Damage

Damage to mitochondrial DNA was determined as described (10).

Statistical Analysis

All data are presented as means \pm SEM and were analyzed using GraphPad Prism software. Statistical analysis was performed by ANOVA followed by Bonferroni's multiple comparisons.

RESULTS

Hemoglobin Protein and mRNA Levels Are Increased in PBMCs during Critical Illness

SDS-PAGE electrophoresis gels prepared from homogenates of PBMCs from patients with severe third-degree burns

(Figure 1A) and patients with sepsis (Figure 1B) showed a marked increase in the amount of a protein in the 15-20 kDa range. Mass spectroscopic analysis identified this protein as hemoglobin. Western blotting confirmed abundant levels of hemoglobin, primarily the α isoform, in the same set of samples (Figures 1A, B). Subsequent mRNA analysis of the PBMCs isolated from the blood of patients demonstrated an upregulation of the Hb- α subcluster genes, and, to a lesser extent, Hb- β and Hb- δ mRNA levels (Figure 1C).

Potential Mechanisms of the Upregulation of Hemoglobin in Critical Illness

One possibility for the expression of hemoglobin is related to a mobilization of stem cells into the circulation during critical illness, as early hematopoietic progenitor cells (as opposed to mature leukocytes) express significant levels of hemoglobin, including the fetal variants (15). qPCR analysis of the PBMCs from burn patients showed an increase in various hematopoietic cell markers for common myeloid progenitors (D34, FLT3) and lymphoid progenitors (CD34, CD38, CD45, CD117, CD127, CD10) (Figure 2A), indicating that there is an increase in hematopoietic stem cell/immature leukocyte content in PBMCs during critical illness.

Another possibility that may contribute to the observed increase in the hemoglobin content of PBMCs may be the uptake of hemoglobin from the circulation, since burn and sepsis is known to induce significant hemolysis, which can markedly increase plasma-free hemoglobin concentrations (16). A significant hemolysis was also confirmed in the present samples (Figure 2B). Flow cytometric analysis showed double staining of PBMCs isolated from burn patients for the presence of hemoglobin and CD45⁺ (leukocyte common antigen) in the PBMCs of burn patients (Figure 2C), thereby localizing the hemoglobin to the leukocytes. In subsequent studies, we have confirmed the ability of PBMCs and U937 cells to take up hemoglobin from the culture medium *in vitro* (Figure 2D).

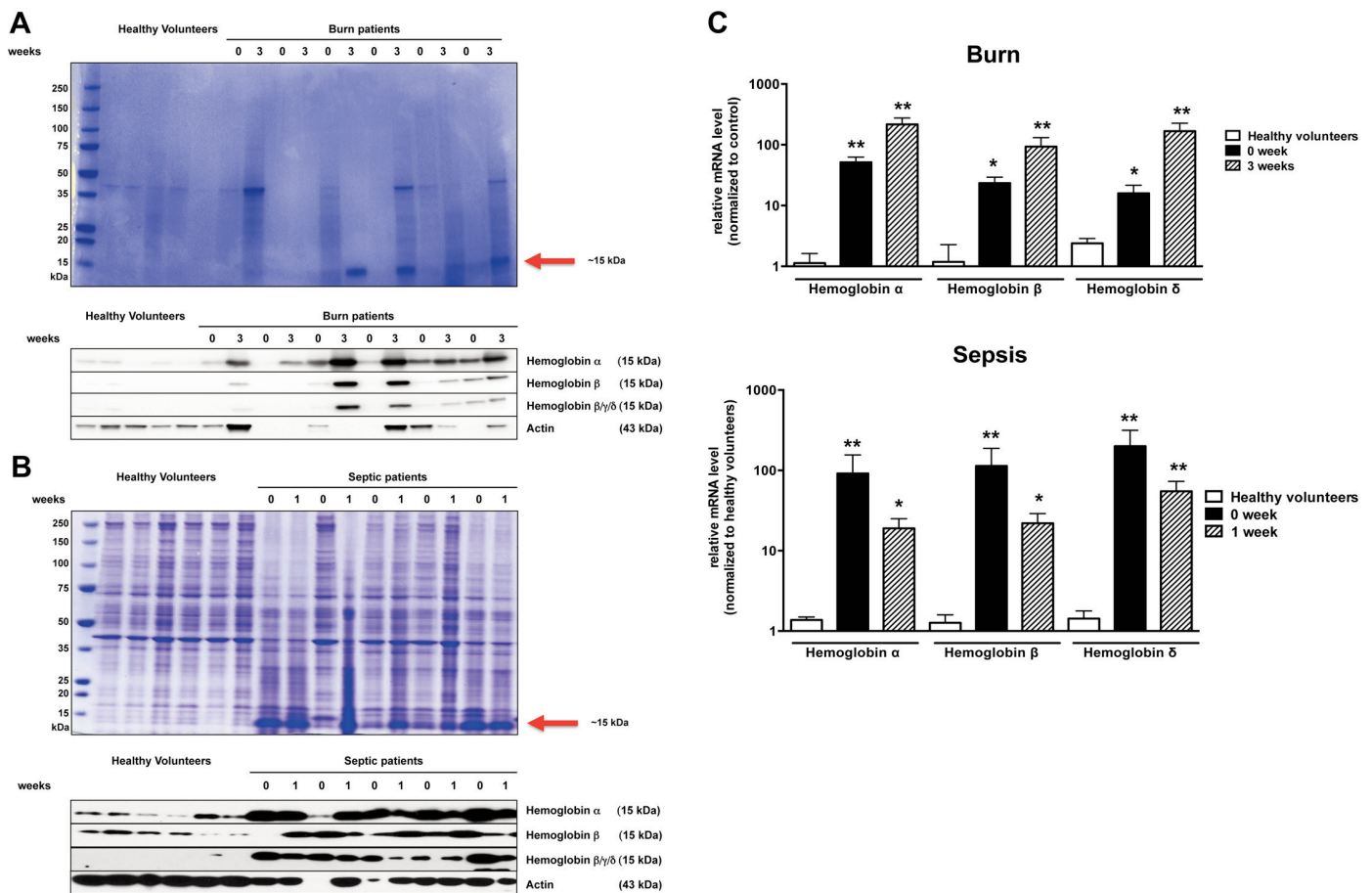


Figure 1. PBMCs of burn and septic patients accumulate hemoglobin. Coomassie-stained and Western analysis of protein extract isolated from PBMCs of patients with burn injury (A) and septic shock (B) showed accumulation of a 15 kDa protein (in comparison to healthy volunteers), identified as hemoglobin. Western blot analysis of PBMCs extract confirmed accumulation of hemoglobin α , β and γ/δ . (C) Expression of hemoglobin α , β and γ/δ was markedly elevated in patients with burn or sepsis at early and later times post admission, in comparison to healthy volunteers. Week 0 represents patient admission day to the hospital (in burn patients) and diagnosis of sepsis (in patients with sepsis). Blood was collected and analyzed 3 wks after admission (burn patients) or 1 wk after diagnosis of sepsis (septic patients). Even though equal protein loading was performed, actin bands were markedly different because in critical illness many proteins (including proteins normally used for loading control, such as actin) can be up- or downregulated. For lanes where no detectable actin lanes were observed, we have utilized an average of the densitometry values of all lanes evaluated for the particular patient population for the densitometry analysis. Data represent mean with \pm S.E.M.; $n = 6$; * $p < 0.05$; ** $p < 0.01$.

CD163-Mediated Uptake and Mitochondrial Sequestration of Hemoglobin

Cellular hemoglobin uptake is mediated by CD163 (scavenger receptor cysteine-rich type 1 protein M130), a membrane transporter protein (17). CD163 exhibits a significant upregulation on the surface of PBMCs in sepsis and other forms of critical illness (18). Upon incubation of U937 cells with hemoglobin, there was a significant colocalization of hemoglobin with CD163 (Figure 3A); inhibition of the interaction of

CD163 with hemoglobin by preincubating the cells with a CD163-blocking peptide attenuated hemoglobin uptake (Figure 3B). PLA studies localized the intracellular hemoglobin to close proximity with the mitochondrial complex I, but not with LDH (an abundant cytosolic protein), nor with various nuclear or cell membrane proteins (Figure 3C). Further investigation of the potential interaction of hemoglobin with several additional mitochondrial proteins revealed that hemoglobin does not show any significant interaction with complexes

II, IV or V, nor with TFAM (a mitochondrial DNA-associated protein) (Figure 3D), suggesting the specificity of the hemoglobin-complex I interaction.

Uptake of hemoglobin into U937 cells did not adversely affect cell viability (Figure 4A), failed to induce reactive oxygen species (ROS) production (Figures 4B, C) and did not affect mitochondrial volume (Figure 4D), but did cause a slight increase in mitochondrial membrane potential and a slight increase of mitochondrial basal and maximal respiratory rates (Fig-

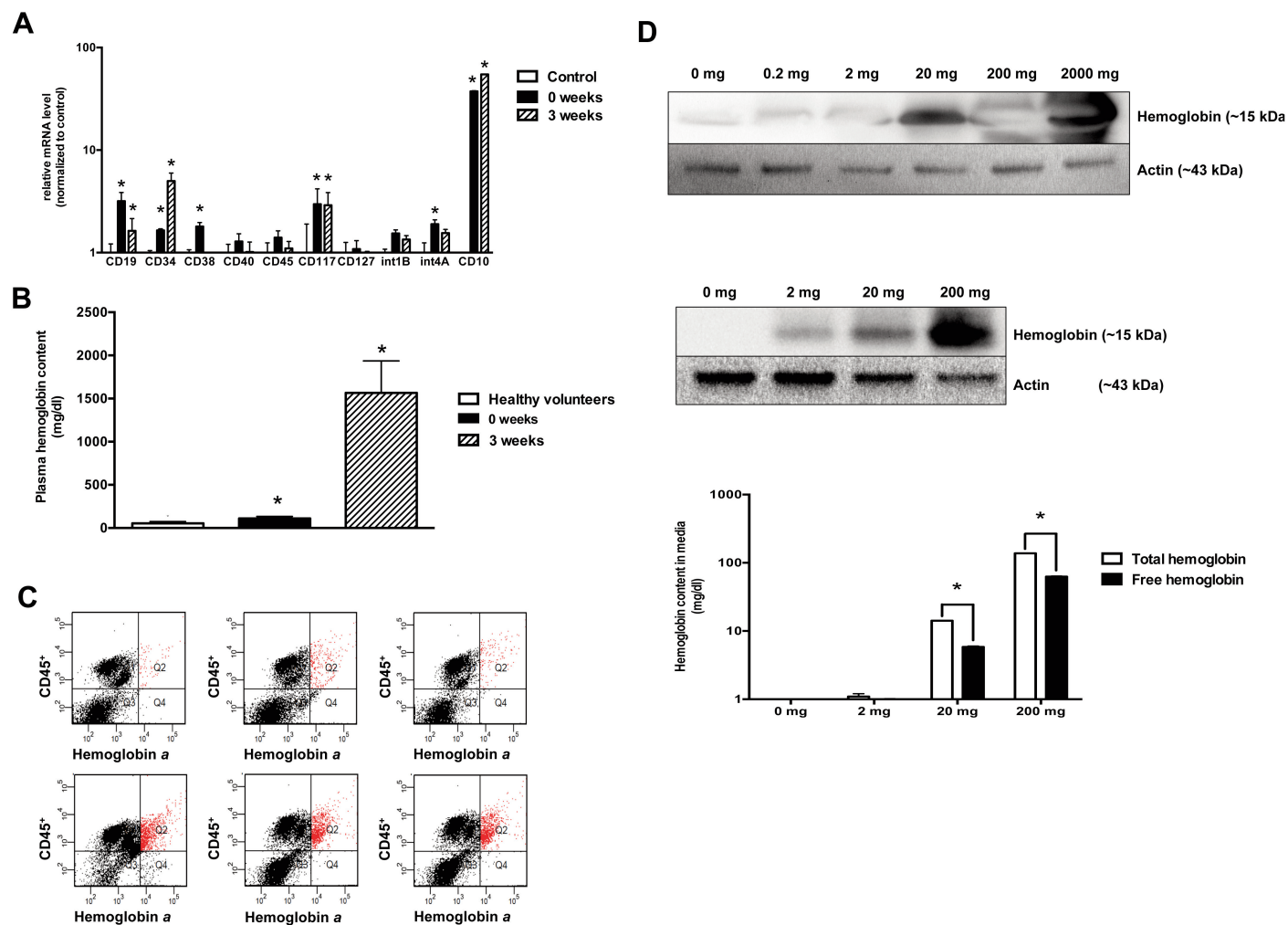


Figure 2. Immature leukocyte populations are enriched after burn injury, and hemoglobin is associated with CD45⁺ positive PBMCs. (A) The expression levels of various bone marrow derived progenitor cell CD markers are significantly elevated in PBMCs of burn patients. Data represent mean ± S.E.M.; n = 6; *p < 0.05. (B) An increased amount of free hemoglobin detected in serum of burn patients. (C) Flow cytometric analysis shows increased CD45⁺/hemoglobin α⁺ double positive cells in total PBMC population (from 2% ± 1% in healthy volunteer samples to 16% ± 4% in burn patient samples; n = 3; **p < 0.01.) (D) PBMCs (top subpanel) and U937 cells (middle and bottom subpanel) bind hemoglobin onto the cell surface in a dose-dependent manner. This causes a decrease free hemoglobin content of media in proportion to hemoglobin uptake by U937 cells (bottom subpanel, showing the decrease of total and free hemoglobin from the cell culture media after incubation of the hemoglobin-containing culture medium with U937 cells). Data represent mean with ± S.D.; n = 3; *p < 0.05

ures 4E–G), without significantly affecting total cellular ATP content (Figure 4H). Hemoglobin also induced an upregulation of HIF-1α and Nrf-2 mRNA, two significant cellular regulators of redox homeostasis (Figures 4I, J).

H₂S Induces Hemoglobin Upregulation; Intracellular Hemoglobin Protects from H₂S Cytotoxicity

We hypothesized that the increased Hb mRNA expression of PBMCs constitutes

an active response to various oxidants, free radicals and gaseous mediators that PBMCs may be exposed to in critical illness. H₂S is a novel gaseous biological mediator with multiple pathogenetic roles in critical illness (19), which has been shown to cause an upregulation of hemoglobin in lower organisms (5). We have detected marked elevations in the plasma H₂S in burn and septic patients, as well as in a murine model of burn (Figure 5A). Furthermore, incubation of

PBMCs or U937 cells with H₂S resulted in the upregulation of the Hb-α subunit mRNA (Figure 5B). Increased intracellular hemoglobin content (either as a result of active upregulation by the cells, or after uptake from the extracellular space) attenuated the cellular damage elicited by incubation of the leukocytes with high concentrations of H₂S (Figure 5C).

The protection afforded by hemoglobin extended to a protection against the loss of cellular viability by SIN-1 (a simultane-

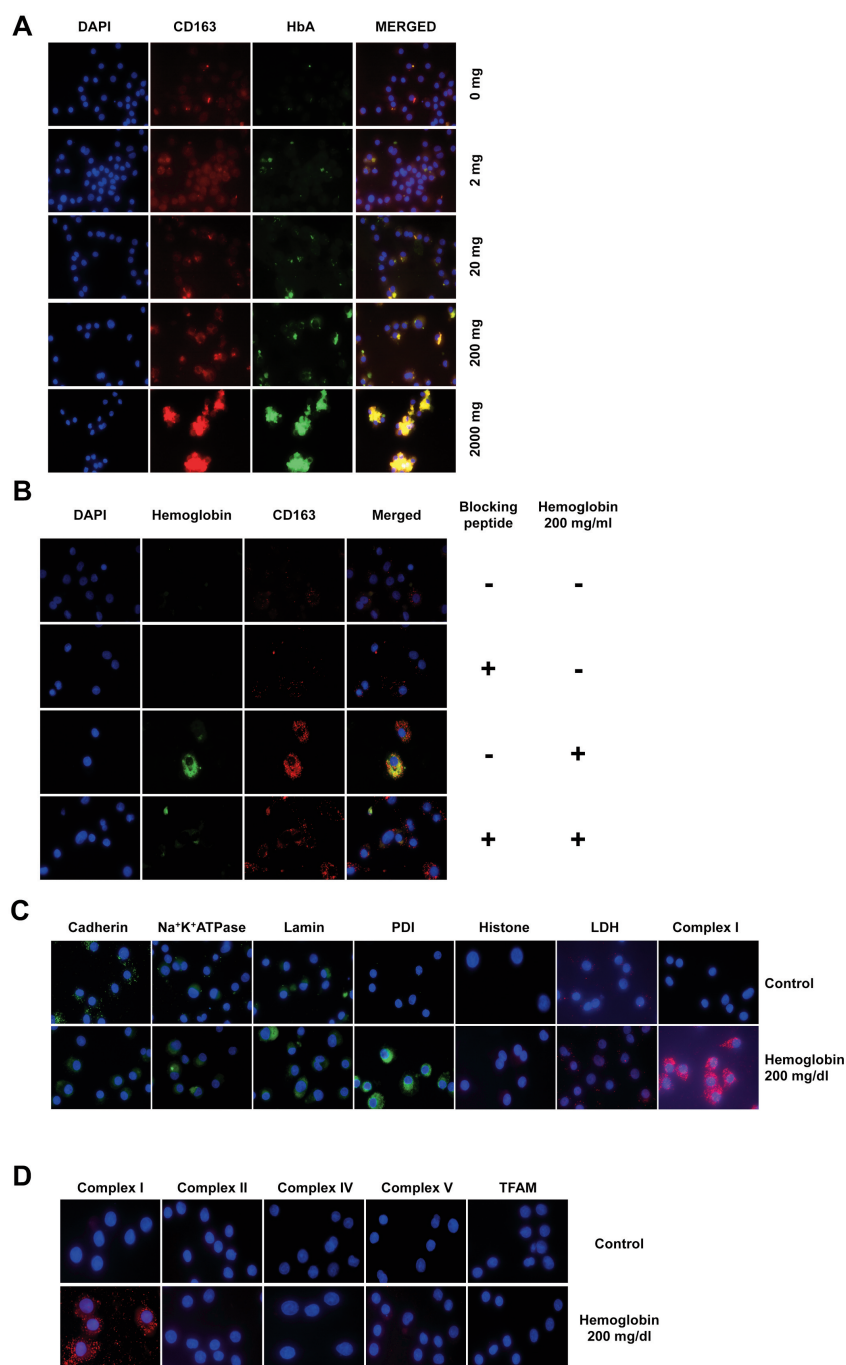


Figure 3. Hemoglobin is taken up by U937 via the CD163 receptor and localizes to mitochondria where it interacts with complex I. (A) Immunocytochemistry analysis of U937 cells incubated with hemoglobin indicates the colocalization of hemoglobin with the CD163 receptor in a dose-dependent manner. (B) Proximity ligation assay confirms the interaction between CD163 receptor and hemoglobin; this interaction is inhibited by preincubation of U937 cells with a blocking peptide of the CD163 receptor. (C) Hemoglobin interacts with mitochondrial complex I, (D) but not with other mitochondrial oxidative phosphorylation complexes or the mitochondrial transcription factor A (TFAM), as assessed by *in situ* PLA analysis. Figures show representative images of at least three independent determinations conducted on different experimental days (n = 3).

ous donor of superoxide and NO, that is, a generator of peroxynitrite (Figure 6A); hemoglobin also attenuated the cell injury induced by SNAP (a NO donor) (Figure 6B) or by hydrogen peroxide (H_2O_2) (Figure 6C). H_2O_2 -induced damage to the mitochondrial DNA was also attenuated by hemoglobin (Figure 6D).

DISCUSSION

Circulating hemoglobin, or cell-free hemoglobin, as a result of excessive intravascular hemolysis (for instance, as a consequence of infusion of stored blood products, ABO incompatibility or various forms of critical illness) (15,16,20) is generally considered a deleterious molecule, which exerts its toxic effects via several distinct mechanisms including pro-oxidant redox cycles catalyzed by heme (4), scavenging physiologically essential NO from the vascular space, leading to endothelial dysfunction and vasoconstriction (15), activation of proinflammatory pathways via TLR4 receptors (21) and other mechanisms. In this context, uptake of hemoglobin into mononuclear cells from the circulation via CD163 is viewed as a protective pathway that serves to reduce circulating free hemoglobin levels (17).

Several lines of independent studies, however, implicate hemoglobin as a cytoprotective protein. For instance, hypoxia upregulates hemoglobin expression in alveolar epithelial cells *in vitro* (22) and bacterial lipopolysaccharide upregulates the β hemoglobin subunit in murine macrophages (23). H_2O_2 increases Hb- $\alpha 1$ and Hb- β expression in HepG2 and HEK293 cells, which, in turn, protects the cells from oxidative stress (24). Hemoglobin has been localized in kidney mesangial cells, where it confers oxidative stress resistance (25). The expression of Hb- $\alpha 1$ and Hb- β also has been demonstrated in mesencephalic dopaminergic neurons and glial cells, and has been linked to the modulation of oxygen homeostasis, oxidative phosphorylation, oxidative stress and NO biosynthesis (26). Finally, red blood cell hemolysate and hemoglobin both reduce the effect of oxidative stress on peripheral blood mononuclear cell DNA damage in-

duced by H₂O₂ (27). These studies, when taken together with the results of the current study, indicate that upregulation of intracellular hemoglobin (either via uptake from the extracellular space, and/or through *de novo* biosynthesis) can be a reactive response of the cell to adverse conditions such as oxidative stress, hypoxia or high concentrations of gaseous mediators, which, in turn, serves to protect the cell from the damage.

We conclude that in the current study, the upregulation of hemoglobin in the PBMCs of critically ill patients probably results from a combination of uptake from the extracellular space and an active biosynthesis of hemoglobin, at least in part by immature leukocytes mobilized from the bone marrow. The *in vitro* data (upregulation of Hb mRNA in U937 cells after incubation with H₂S) coupled with the findings showing that (a) circulating H₂S levels are increased in critical illness and (b) cells with elevated intracellular hemoglobin levels are protected from H₂S-mediated cytotoxicity are consistent with the existence of a reactive process whereby upregulation of hemoglobin serves as a protective mechanism in PBMCs against H₂S toxicity in critical illness. This model is not in disagreement with prior work showing that extracellular circulating hemoglobin can have multiple deleterious effects (20); in fact, uptake and sequestration of hemoglobin from the plasma into the PBMCs via CD163 may contribute to a reduction of circulating free hemoglobin levels.

We do not propose that the sole purpose of intracellular hemoglobin in PBMCs is the modulation of H₂S homeostasis or protection from H₂S-mediated injury. It is more likely that hemoglobin exerts pleiotropic effects in these cells; it modulates the cellular responses to various oxidants and free radicals (NO, superoxide, H₂O₂, peroxynitrite, and so on); hemoglobin, either through acting on membrane receptors or via modulation of intracellular pathways and signal transduction processes, may also regulate the expression of various oxidant-responsive elements (as evidenced, in our study, by

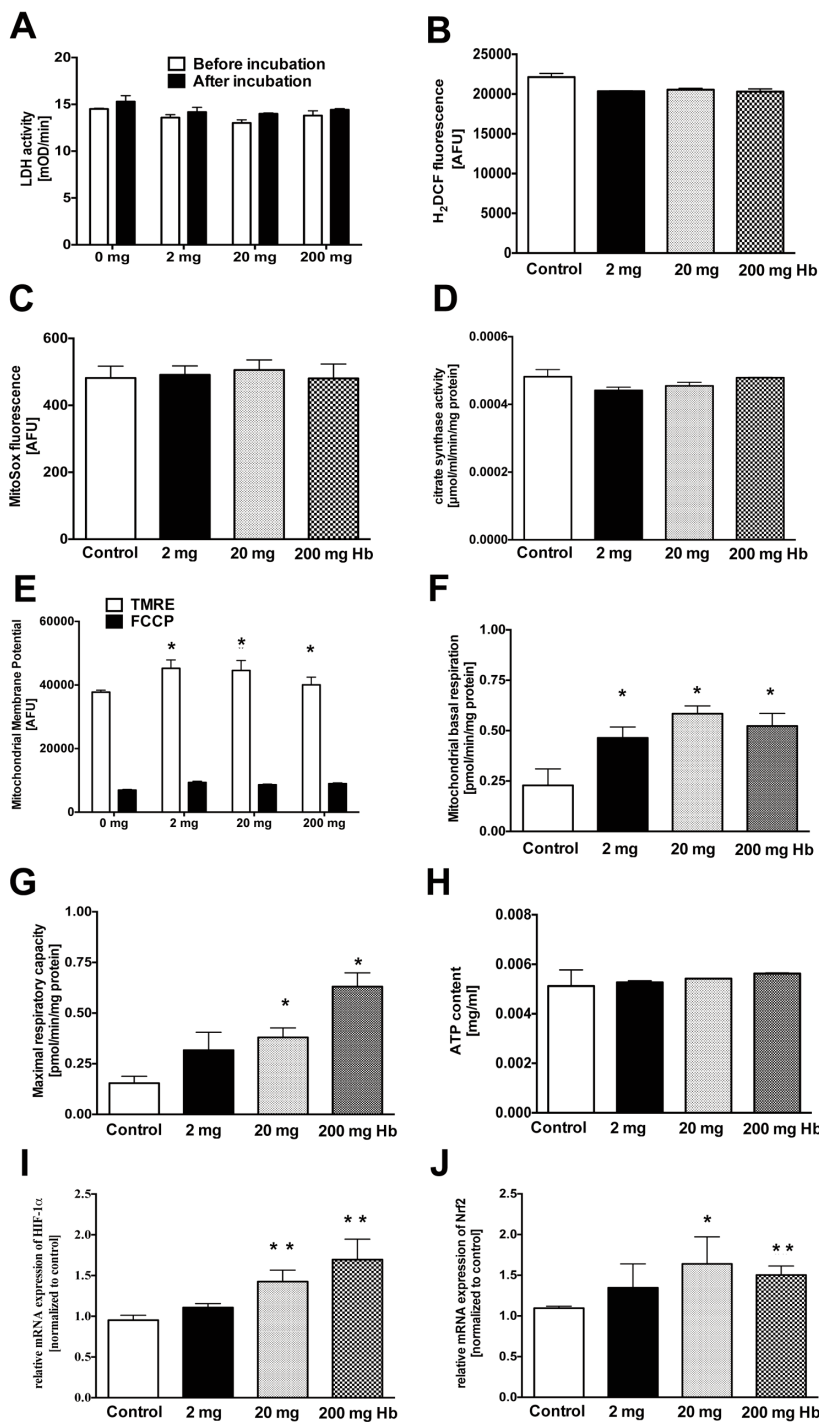


Figure 4. Increased intracellular level of hemoglobin affects mitochondrial parameters. The effect of various concentrations of hemoglobin on U937 cells on (A) cytotoxicity measured by LDH release, (B) levels of intracellular reactive oxygen species, (C) levels of mitochondrial-specific reactive oxygen species, (D) mitochondrial content, (E) mitochondrial membrane potential, (F) basal mitochondrial respiratory capacity, (G) maximal mitochondrial respiratory capacity, (H) cellular ATP content and expression of (I) HIF1- α and (J) Nrf2 mRNA. Incubation of U937 cells with hemoglobin stimulates mitochondrial membrane potential, basal and maximal respiratory capacity. Data represent mean \pm S.E.M.; n = 3; *p < 0.05.

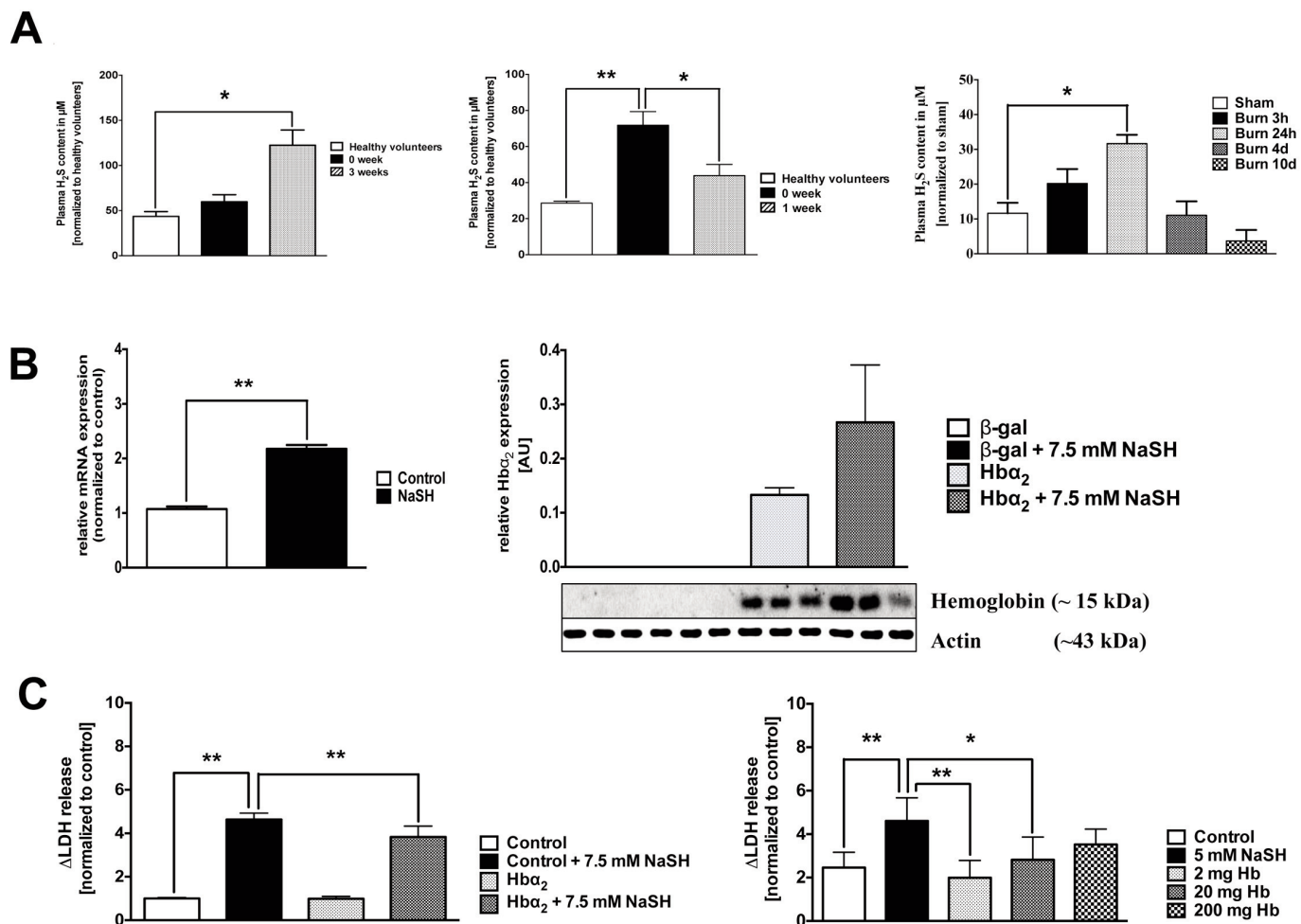


Figure 5. Cellular upregulation of hemoglobin protects against H₂S-induced cytotoxicity. (A) H₂S levels are elevated in the plasma of severe burn and septic patients and in the plasma of mice subjected to burn injury. Data represent mean \pm S.E.M.; $n = 6-8$; * $p < 0.05$; ** $p < 0.01$. (B) Left panel: Treatment of PBMCs isolated from healthy volunteers with 1 mmol/L NaSH for 1 h induced the expression of hemoglobin mRNA. HEK293T17 cells transfected with a hemoglobin α overexpression construct showed a further increase in the expression of intracellular hemoglobin when the transfection was combined with H₂S treatment (7.5 mmol/L). Hb- α expression was quantified by Western blotting at 48 h. Data represent mean \pm S.E.M.; $n = 3$; ** $p < 0.01$. (C) Expression of hemoglobin in HEK293T17 cells and uptake of hemoglobin into U937 cells protected against the cytotoxic effect of a high concentration (7.5 mmol/L) of NaSH, as measured by LDH release into the medium. Data represent mean \pm S.E.M.; $n = 3$; * $p < 0.05$; ** $p < 0.01$.

the hemoglobin-induced upregulation of HIF-1 α and NRF2). Upregulation of both of these factors is known to confer cytoprotection and ischemic/hypoxic preconditioning via a variety of interacting downstream pathways (28,29).

Intracellular hemoglobin shows remarkable compartmentalization; it concentrates to the mitochondria, where it shows a particularly close association with mitochondrial complex I. It is interesting to note that a recent proteomic analysis of control brains and brains from multiple sclerosis

patients has identified the mitochondrial localization of the hemoglobin β chain (30). The functional consequence of mitochondrial hemoglobin remains to be further elucidated; based on the current results, we speculate that it may either contribute to a specific mitochondrial protection (for example, against mitochondrial oxidative stress), or it may play a more specific role in the regulation of mitochondrial electron transport; the latter hypothesis is supported by our extracellular flux analysis findings indicating an increased

basal and FCCP-uncoupled maximal rate of mitochondrial respiration in cells with increased mitochondrial hemoglobin content. Our results also indicate that mitochondrial hemoglobin may serve to protect the integrity of mitochondrial DNA from oxidative damage.

The conditions and factors determining the deleterious versus protective roles of hemoglobin in various pathophysiological conditions are likely to depend on the localization of hemoglobin (intracellular versus extracellular), the degree of oxidative

stress, the rate of the gaseous transmitters produced, as well as many additional factors. Based on several sets of independent investigations, it appears that when oxidative stress is relatively low, hemoglobin (and its degradation products, for example, heme) increases oxidative stress, and scavenges physiologically necessary amounts of NO, but when the degree of oxidative stress is high, hemoglobin tends to protect from cellular damage (22–27,31). One also has to keep in mind that hemoglobin decomposes into multiple products, including heme (which increases oxidative stress, but also acts as a substrate of the gasotransmitter carbon monoxide, produced by heme oxygenase), while other degradation products (for example, bilirubin) have significant antioxidant effects (31). These factors remain to be carefully evaluated to dissect the multiple roles of hemoglobin in the regulation of various pathophysiological conditions.

There are a number of limitations of the current study, as well as a number of follow-up issues that remain to be elucidated in future experiments. (a) In addition to the upregulation of Hbs, there are a number of additional protein bands that show enrichment in the PBMCs of critically ill patients (Figure 1); changes in the entire PBMC proteome, as well as additional individual proteins that are up- or downregulated remain to be studied in future experiments. (b) What is the relative contribution of Hb mRNA/protein upregulation and uptake of Hbs from the extracellular space to the observed enrichment of PBMC Hbs in burn and in sepsis? Furthermore, with respect to the upregulation of Hb mRNA, what is the contribution of upregulation in mature PBMCs versus in immature/stem forms that are mobilized into the circulation during the critical illness insult? These issues may be more directly addressed by future preclinical studies (for example, rodent models of sepsis or burns). (c) We have only studied the reactive upregulation of Hbs in response to H₂S in the current project. It remains to be elucidated if similar reactive responses can also be elicited by other stimuli (for example, hypoxia, oxidative stress, NO, CO and oth-

ers) in PBMCs. In this context, it is worth mentioning that, in other cell types, Hb upregulation has been demonstrated in response to bacterial lipopolysaccharide stimulation in murine monocytemacrophages (23), in response to hypoxia in epithelial cells (22) or in response to oxidative stress in hepatocytes (24). (d) What is the exact functional role of Hbs in the mitochondria, and how does Hbs translocation occur into the mitochondria? Since Hbs are large molecules, the process is likely to involve active transport mechanisms. It also remains to be determined whether Hbs incorporate into respiratory complex I and, if so, do they directly participate in mitochondrial electron transport? One limitation of the results is that PLA studies show close vicinity to the two proteins studied (in this case complex I and Hb) but direct binding requires confirmation by additional methods (for example, confocal microscopy, immunoprecipitation and/or pull-down techniques). In this context it is important to keep in mind that mitochondrial complex I consists of a large number of subunits, including the NADH dehydrogenase module (N module), the electron transfer module (Q module) and the proton translocation module (P module), which are made up of 14 core subunits (32). Specific interaction(s) of Hbs with these subunits remain to be studied in further experiments. (e) The exact kinetics and reactions of Hbs with NO, H₂O₂ and H₂S remain to be further studied in the cytoprotective context. With respect to the reaction of NO with Hbs, the literature is substantial and goes back to a century; it has been put into the biological (vascular) context by the original work of Murad, Ignarro and Furchgott (33–36), who have identified Hbs as scavengers of NO (as well as of endothelium-derived relaxing factor [EDRF], which later has been demonstrated to be identical with NO). Similarly, with respect to the interactions of Hbs with oxyradicals, substantial work has been conducted already, which can be reviewed in the literature (37–39). With respect to the interactions of Hbs with H₂S, several lines of studies have demonstrated the formation of sulfhemoglobins (40,41),

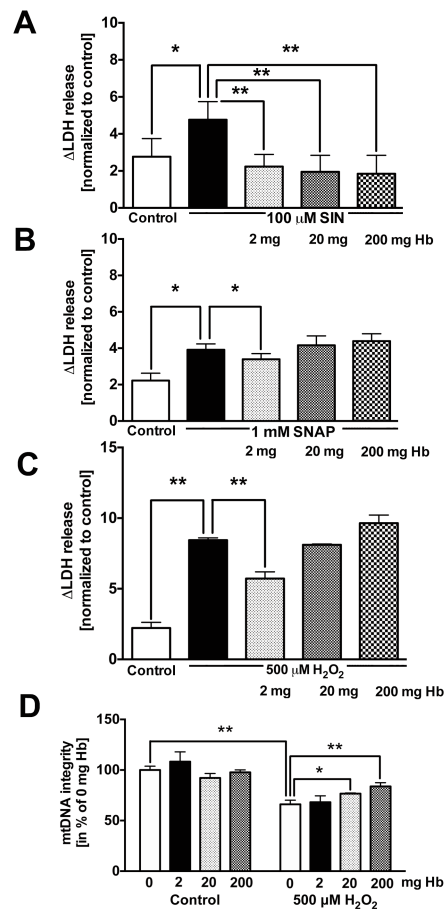


Figure 6. Hemoglobin protects U937 cells against ROS/RNS induced cytotoxicity. Incubation of U937 cells with hemoglobin protects against cell death initiated by (A) SIN-1 (100 μmol/L), (B) SNAP (500 μmol/L) and (C) H₂O₂ (500 μmol/L). (D) Hemoglobin protects against H₂O₂-induced mitochondrial and nuclear DNA damage. Data represent mean ± S.E.M; n = 4–6. **p* < 0.05; ***p* < 0.01.

and Banerjee and colleagues have recently outlined several novel aspects of the interaction, especially with respect to the interactions of H₂S with methemoglobin (42).

CONCLUSION

Although much additional work remains to be conducted to explore the above issues, in summary, at least under the conditions studied in the current report, we can conclude that cellular uptake of hemoglobin does not adversely affect the viability of leukocytes, and protects them from various gaseous and

oxidative insults. Therefore, we hypothesize that the upregulation of hemoglobin in PBMCs during critical illness constitutes a cytoprotective mechanism.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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