Oxidized Ferric and Ferryl Forms of Hemoglobin Trigger Mitochondrial Dysfunction and Injury in Alveolar Type I Cells

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Abstract

Lung alveoli are lined by alveolar type (AT) 1 cells and cuboidal AT2 cells. The AT1 cells are likely to be exposed to cell-free hemoglobin (Hb) in multiple lung diseases; however, the role of Hb redox (reduction–oxidation) reactions and their precise contributions to AT1 cell injury are not well understood. Using mouse lung epithelial cells (E10) as an AT1 cell model, we demonstrate here that higher Hb oxidation states, ferric Hb (HbFe³⁺) and ferryl Hb (HbFe⁴⁺) and subsequent heme loss play a central role in the genesis of injury. Exposures to HbFe²⁺ and HbFe³⁺ for 24 hours induced expression of heme oxygenase (HO)-1 protein in E10 cells and HO-1 translocation in the purified mitochondrial fractions. Both of these effects were intensified with increasing oxidation states of Hb. Next, we examined the effects of Hb oxidation and free heme on mitochondrial bioenergetic function by measuring changes in the mitochondrial transmembrane potential and oxygen consumption rate. In contrast to HbFe²⁺, HbFe³⁺ reduced basal oxygen consumption rate, indicating compromised mitochondrial activity. However, HbFe⁴⁺ exposure not only induced early expression of HO-1 but also caused mitochondrial dysfunction within 12 hours when compared with HbFe²⁺ and HbFe³⁺. Exposure to HbFe⁴⁺ for 24 hours also

caused mitochondrial depolarization in E10 cells. The deleterious effects of HbFe³⁺ and HbFe⁴⁺ were reversed by the addition of scavenger proteins, haptoglobin and hemopexin. Collectively, these data establish, for the first time, a central role for cell-free Hb in lung epithelial injury, and that these effects are mediated through the redox transition of Hb to higher oxidation states.

Keywords: hemoglobin; lung epithelium; mitochondria; heme oxygenase; hemolysis

Clinical Relevance

We describe a novel oxidative pathway by which ferric and ferryl hemoglobin (Hb) oxidation products induce cellular injury and mitochondrial dysfunction, which may contribute to inflammatory responses and vascular toxicity. Intervention with endogenous plasma-derived antioxidants may control Hb oxidative side reactions and offer potential therapeutic remedies in hemolytic anemias and when free Hb is used as oxygen therapeutics.

Red blood cells are highly enriched in hemoglobin (Hb), a tetrameric protein consisting of two α and two β subunits ($\alpha_2\beta_2$). Each subunit has a single, iron-containing protoporphyrin (heme) moiety.

The highly reducing environment within red blood cells (RBCs) prevent the oxidation of Hb and maintains the functional ferrous form (HbFe²⁺) (1). Small amounts of Hb are released to

circulation due to aging and during the process of RBC renewal. The released Hb tetramers dissociate rapidly into $\alpha\beta$ dimers, which can be cleared by binding to haptoglobin (Hp) to form a complex of

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Hp-Hb (2, 3). The complex then binds to CD163 receptor for endocytosis and degradation in the lysosomes (4). Heme oxygenases (HOs) catalyze the degradation of heme to generate carbon monoxide, free heme iron, and biliverdin. Finally, biliverdin reductase further catalyzes the conversion of biliverdin to bilirubin. Both ferritin, which sequesters the free iron, and HO-1 are induced by multiple stimuli, including the presence of acellular Hb and heme (5).

Acellular Hb concentration increases after excessive hemolysis in malaria, sickle cell disease, blood transfusion, and infusion of Hb-based oxygen carriers. Severe vasoconstriction due to nitric oxide sequestration by Hb is thought to be a cause of Hb-induced toxicity in hemolytic anemias and in therapeutic uses of Hb (6, 7). It is increasingly becoming evident that heme loss after Hb oxidation may also play a significant role in the toxicity associated with acellular Hb. During auto-oxidation, pro-oxidants, such as the superoxide ion (O_2^-) and hydrogen peroxide (H_2O_2) drive a catalytic pseudoperoxidase cycle that oxidize $HbFe^{2+}$ into $HbFe^{3+}$, $HbFe^{4+}$, and the ferryl radical ($\cdot HbFe^{4+}$). These oxidatively unstable Hb intermediates oxidize residues within the Hb globin chains (and other proteins within proximity), ultimately leading to Hb degradation and heme loss (8). Our recent study indicated that heme derived from the hemolysis of sickle RBCs acts as a damage-associated molecular pattern molecule (DAMP) (9). A number of highly efficient endogenous scavenging mechanisms exist to sequester acellular Hb and heme, including Hp, hemopexin (Hpx), albumin, and α -Hb stabilizing protein (10). Hp, an acute-phase protein is predominantly expressed in the liver, lung, skin, and spleen (11). Hp attenuated Hb-induced vascular, cardiac, and renal injury after infusion of free Hb and aged RBCs in dogs, guinea pigs, and in a mouse model of sickle cell disease, mainly by preventing extravasation of Hb through the kidney. Moreover, Hp-Hb complex was also shown to diffuse the damaging ferryl radicals associated with the vicious ferric/ferryl cycle (12–15).

Diffuse intra-alveolar hemorrhage, a life-threatening condition, occurs due to accumulation of RBCs in the lung alveoli (16). Extravasation of blood into air spaces is also common in multiple other lung diseases, such as chronic bronchitis, bronchiectasis, cystic fibrosis, lung

cancer, pulmonary embolism, pneumonia, tuberculosis, and traumatic injury (17). Subsequent RBC lysis leads to release of acellular Hb, which, in turn, damages the alveolar epithelial cells. Pulmonary complication of hemolytic diseases, such as sickle cell disease, have been reported (18, 19). Lung alveoli are the functional units of respiration. Lung alveoli are lined by squamous alveolar type (AT) 1 and cuboidal AT2 cells. The AT1 cells are also likely to be exposed to very high concentrations of acellular Hb, due to their anatomical predisposition (occupy >95% of lung alveolar surface area and close apposition to endothelium) and inherent sensitivity. However, the mechanisms of acellular Hb-induced effects of lung epithelial cells, particularly on AT1 cells, remain unknown.

Mitochondria are considered to be the most critical regulators of cell survival and death, due to their roles in the maintenance of cellular energy homeostasis and regulation of redox-dependent signaling pathways (20). Functional mitochondrial bioenergetics have recently been advocated for use as a bioenergetic health index in humans (21). However, mitochondrial bioenergetic analyses have not been explored extensively in the context of Hb cytotoxicity. Recently, however, heme and cell-free Hb have been shown to disrupt mitochondrial bioenergetic function in different cell types (22–24). Using lung epithelial cells (E10) as an AT1 cell model, we investigated the differential toxicity of acellular Hb and its oxidized species on the mitochondrial transmembrane potential and also on oxygen consumption rate (OCR). Furthermore, the roles of Hp (Hb scavenger) and Hpx (heme scavenger) were also investigated in attenuating Hb-induced injury.

Materials and Methods

Preparation of Oxidized Hb

Adult $HbFe^{2+}$ and $HbFe^{3+}$ were prepared as previously described (25). $HbFe^{4+}$ was produced by addition of 10-fold molar excess of H_2O_2 to endotoxin-free $HbFe^{3+}$ in potassium phosphate buffer (pH 7.4). Spectral identification and quantification of $HbFe^{2+}$ and $HbFe^{3+}$ were performed based on published extinction coefficients (25). The $HbFe^{4+}$ was quantified using sodium sulfide (Na_2S) derivatization, as previously described (26). The concentrations of $HbFe^{2+}$, $HbFe^{3+}$, and $HbFe^{4+}$ were expressed as heme equivalents.

Cell Culture

Mouse E10 cells were cultured in CMRL-1066 supplemented with 10% FBS and antibiotics. The cells were used between passages 8 and 20. The media were changed every 48 hours (for detailed protocols, see the online supplement).

Exposure of E10 Cells to Different Hb Oxidation States

E10 cells were grown to 80–90% confluency in complete media. Before exposures, the cells were serum starved overnight. The cells were then exposed to $HbFe^{2+}$, $HbFe^{3+}$, or $HbFe^{4+}$ for varying periods of time (12 or 24 h). For studying the role of Hp, human plasma-derived unfractionated Hp (50 μ M) was added to the media before the addition of Hb. $HbFe^{2+}$, $HbFe^{3+}$, and $HbFe^{4+}$ (in equimolar ratio to Hp) were then added immediately. After exposure to Hb proteins for specified time periods, cells were washed extensively in ice-cold PBS and cell lysates were prepared for further studies.

Isolation of Mitochondria

Mitochondria were isolated from cultured E10 cells using a mitochondria isolation kit for cultured cells (Pierce Biotechnology, Rockford, IL).

Western Blotting

Immunoblotting was done as previously reported (27). The primary antibodies used were in 1:2,500 dilutions. The proteins were visualized using enhanced chemiluminescence kit (GE Healthcare, Piscataway, NJ). The expression of HO-1 and ferritin, α , and β subunits of Hb was not detectable in unexposed cells. For quantification, the density of HO-1 was normalized with density of β -actin. Fold expression was obtained by comparing the normalized expression in unexposed cells.

Microscopy

The cells were exposed to the indicated concentrations of $HbFe^{2+}$, $HbFe^{3+}$, and $HbFe^{4+}$. Immunocytochemistry was performed as described previously (27). The colocalization of the HO-1 protein with Mito Tracker Red CMXRos (Thermo Fisher Scientific, Waltham, MA) was visualized using an LSM710 Meta Laser-scanning confocal microscope (Zeiss, Thornwood, NY).

Mitochondrial Membrane Potential

Loss of mitochondrial transmembrane potential was assessed in E10 cells using a cationic lipophilic dye, tetraethylbenzimidazolyl carbocyanine iodide (JC-1). The cells were exposed to the Hb proteins, as indicated earlier for 24 hours. The cells were thoroughly washed four to five times in prewarmed PBS to remove excess unbound Hb, then loaded with JC-1 dye (8 μ M) for 30 minutes as described previously (24). After removal of excess dye, cells were detached using 0.025% trypsin-EDTA and washed in PBS. Cell suspensions were analyzed for red fluorescence (λ_{ex} 530 nm, λ_{em} 590 nm) for J-aggregates (indicative of hyperpolarization) and green fluorescence (λ_{ex} 490 nm, λ_{em} 530 nm) from JC-1 monomer (indicative of low mitochondrial transmembrane potential or depolarization) in a Synergy HTX multi-mode plate reader (Biotek Instruments, Inc. Winooski, VT). The ratios were then plotted on a percentage scale in which the ratio from oligomycin (1 μ M)-treated cells indicates 100% hyperpolarization (maximum mitochondrial transmembrane potential) and ratio from uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP 1 μ M)-treated cells indicates 0% or complete depolarization (22). The percentage values thus obtained from Hb-treated cells were represented as the percent of hyperpolarized cells. The emission fluorescence ratio of 590 nm versus 530 nm from Hb-treated cells that were not incubated with JC-1 was also monitored to eliminate any Hb interference (see Figure E2 in the online supplement).

Mitochondrial Bioenergetic and Glycolytic Flux Measurements

Mitochondrial bioenergetic function and the glycolytic flux were simultaneously monitored in intact E10 cells using an XF24 extracellular flux analyzer (Seahorse Bioscience, Billerica, MA). Briefly, E10 cells were seeded (20,000 cells/well) and cultured for 24 hours in a specialized 24-well XF cell culture plate (V7) obtained from Seahorse Bioscience. Before exposure, the cells were cultured in serum-free media overnight. They were then exposed to either HbFe²⁺ or HbFe³⁺ or HbFe⁴⁺ for various time periods up to 24 hours. The cells were washed thoroughly in unbuffered XF-assay medium (pH 7.4). Mitochondrial OCR and extracellular acidification rate (ECAR) were assessed as described previously (24). The

OCR values from individual wells were recorded and plotted using XF24 software, version 1.8. The values were normalized with total protein in individual wells after the completion of the assay. Blank wells with Hb variants were also run to eliminate any background OCR. Different Hb species were also injected directly to the cells grown on V7 plates before the addition of oligomycin to assess Hb interference in the XF assay. However, in our experimental setup, Hb species showed no noticeable interference on OCR or ECAR. Various cellular bioenergetic parameters, such as basal respiration, ATP-linked respiration, proton leak, maximal OCR, and reserve respiratory capacity, were calculated as described previously (28).

Statistical Analysis

All values are expressed as mean ($\pm$ SEM). A value of *P* less than 0.05 was considered significant. The different treatments were compared using unpaired Student's *t* test.

Results

Hb Preparation Purification and Handling

Highly purified human HbFe²⁺ and HbFe³⁺ were used in this study. The

catalase activity was reduced by over 98%. The levels of HbFe²⁺ and HbFe³⁺ in these preparations contained greater than 95% ferrous or ferric heme, respectively. These preparations were further cleared of contaminating endotoxin using polymyxin B immobilized agarose gel columns. Endotoxin levels were confirmed to be lower than 5 EU/ml in all stock solutions. Similarly, final HbFe⁴⁺ solutions contained over 95% ferryl heme. Typical ultraviolet-visible absorbance spectroscopy of the main redox states of Hb is shown in Figure 1. Ferrous/oxy Hb (red) is characterized by two major absorption peaks at 541 and 576 nm, respectively. The oxidized form of Hb (ferric; brown) absorbs strongly at 500 and 630 nm, respectively. The spectrum of ferryl Hb (HbFe⁴⁺; green) in the visible region is characterized by two peaks at 544 and 585 nm, and a flattened region between 600 and 700 nm. To confirm the redox identity of the ferryl state intermediate, sodium sulfite was added to convert it to a sulfhemoglobin (purple), which absorbs very strongly at 620 nm.

Effect of Oxidized Hb Exposure on E10 Cells

Mouse E10 cells were exposed to increasing concentration of HbFe²⁺ and HbFe³⁺ for

Figure 1. Representative spectra of ferrous hemoglobin (HbFe²⁺), ferric Hb (HbFe³⁺), and ferryl Hb (HbFe⁴⁺): Hb was isolated from human blood. HbFe³⁺ was prepared as described in MATERIALS AND METHODS. HbFe⁴⁺ was prepared by adding 10-molar excess of hydrogen peroxide (H₂O₂) to HbFe³⁺. The HbFe⁴⁺ was derivatized by addition of Na₂S to produce sulfHb (absorption peak at 620 nm). A spectral scan was performed from 450 to 700 nm to confirm oxidation status and quantify the heme content. Shown are representative spectra (*n* > 6). The spectra of different Hb oxidation states are identified by characteristic peaks in the visible region (HbFe²⁺, 541 and 576 nm; HbFe³⁺, 500 and 630 nm; HbFe⁴⁺, 544 nm, 585 nm, and a flattened region between 600 and 700 nm). AU, arbitrary units.

24 hours. Hb is known to dimerize at lower concentration, while it remains a tetramer at higher concentration (29). We observed a dose-dependent increase in HO-1 after exposure to HbFe²⁺ and HbFe³⁺, whereas the untreated control cells did not express HO-1 protein (Figure 2A). We monitored the expression of P₂X₇ receptor protein, a phenotypic marker of AT1 cells, after exposure to HbFe²⁺ and HbFe³⁺ and, as can be seen in Figure 2A, there were no alterations in the expression of P₂X₇ receptor protein. Exposure to Hb and its oxidized species did not result in cell death. The cell viability was over 90% after exposure to different exposure regimens, as shown by trypan blue and

propidium iodide/annexin V staining. We may therefore rule out the induction of apoptosis as described previously (30). Similarly, exposure to HbFe²⁺ and HbFe³⁺ did not induce or alter the expression of heat shock protein of 70 kD (Hsp70) (a stress marker protein) or proliferation cell nuclear antigen (proliferation marker) in E10 cells. Thus, our exposure regimens did not adversely affect the phenotype, cell viability, or differentiation in E10 cells. We also found that HbFe²⁺ and HbFe³⁺ activate NF-κB and mitogen-activated protein kinase pathways, as shown by phosphorylation of NF-κB p65 subunit and p44/42, respectively (Figure E3) as noted previously (31).

Hp Attenuates the Effects of HbFe²⁺ and HbFe³⁺ on E10 Cells

Hp is a potent scavenger of acellular Hb, and is known to reduce Hb-induced injury. Hp attenuated HbFe²⁺- and HbFe³⁺-induced up-regulation of HO-1 and H-ferritin proteins (Figure 2B). Exposure to ferric Hb (HbFe³⁺) induced a significant expression in HO-1 protein (15.17 ± 1.04-fold) when compared with HbFe²⁺ (9.32 ± 0.76-fold)-induced expression (Figure 2C). Similarly, HbFe³⁺ induced a significant expression H-ferritin protein (60.40 ± 2.76-fold) when compared with a 25.25 (±1.91)-fold induction by HbFe²⁺ (Figure 2D). Western blotting of cell lysates indicated the presence of significant amounts of Hb α and β

Figure 2. Haptoglobin (Hp) attenuates HbFe²⁺- and HbFe³⁺-induced effects in mouse lung epithelial (E10) cells: E10 cells were exposed to HbFe²⁺ and HbFe³⁺ at the indicated concentration for 24 hours. The cell lysates were immunoblotted to confirm the expression of heme oxygenase (HO)-1 and P₂X₇ receptor proteins. Equal loading was confirmed by reprobing the blots against β-actin. A dose-dependent increase was observed after exposure to HbFe²⁺ and HbFe³⁺. (A) A representative Western blot image after exposure to increasing doses of HbFe²⁺ and HbFe³⁺. The E10 cells were exposed to human HbFe²⁺ (50 μM) and HbFe³⁺ (50 μM) in the presence and absence of Hp (50 μM) for 24 hours. The cell lysates were probed for expression of HO-1 and H-ferritin proteins. Equal loading was confirmed by reprobing the blots with β-actin. (B) Representative images of HO-1, H-ferritin, and P₂X₇ receptor (n = 5). The expression of HO-1 and H-ferritin was normalized against β-actin. Fold expressions are shown for (C) HO-1 and (D) H-ferritin. The cell lysates were probed for detection of human Hb α and β subunits in the cell lysates. The Western blot images are shown in E (n = 4). The treatment groups were compared using unpaired Student's *t* test. **P* < 0.05 versus control; \$*P* < 0.05 versus HbFe²⁺; #*P* < 0.05 versus HbFe²⁺; and &*P* < 0.05 versus HbFe³⁺. Data presented are means (±SEM) of fold expression.

subunits. Furthermore, mass spectrometric analysis of lysates unequivocally confirmed the identity of human Hb α and β subunits in the cell lysates (data not shown). Hp reduced the uptake of Hb, as indicated by reduced Hb α and β subunits in the cell lysates (Figure 2E).

HbFe³⁺ Up-Regulates the Expression of HO-1 in the Mitochondria and Alters Mitochondrial Function

Previous studies have shown hemin, cigarette smoke, and indomethacin induced the translocation of HO-1 into the mitochondria (32–34). We reasoned that the increased HO-1 expression in E10 cells might also be associated with its translocation into the mitochondria. The mitochondrial and cytosolic fractions were isolated, purified, and then probed for the expression of HO-1 protein. We found a significant enrichment of HO-1 in the mitochondrial, but not in the cytosolic fractions after exposure to HbFe²⁺ and HbFe³⁺ (Figures 3A and 3B). Isolation and equal loading of the mitochondrial proteins were confirmed by probing the fractions with cytochrome c oxidase IV, a mitochondrial respiratory chain complex protein. Exposure to HbFe²⁺ and HbFe³⁺ did not alter the expression of cytochrome c oxidase IV protein (Figure 3A). Interestingly, Hp attenuated the HbFe²⁺- and HbFe³⁺-induced HO-1 expression in the mitochondria (Figures 3A and 3B).

Extracellular flux analyzer was used to assess the mitochondrial bioenergetics and glycolytic proton flux in real time. The OCR and ECAR serve as indicators of mitochondrial respiration and cellular glycolytic flux, respectively. E10 cells were exposed to HbFe²⁺ and HbFe³⁺ for 24 hours, as described earlier. The changes in OCR and ECAR were then examined. Exposure to HbFe³⁺, but not HbFe²⁺, caused a significant decrease in basal OCR in the E10 cells. This effect indicates a loss of mitochondrial bioenergetic function after exposure to HbFe³⁺ (Figures 3C and 3D). However, coincubation with Hp completely restored the HbFe³⁺-induced decrease in OCR (Figure 3C). No significant changes in the ECAR after exposure to HbFe²⁺ and HbFe³⁺ for 24 hours were observed in E10 cells (data not shown).

Ferrylhemoglobin Causes Loss of Mitochondrial Function in E10 Cells after Short Incubation

HbFe⁴⁺ is a highly reactive and proinflammatory species produced during

the oxidation of HbFe²⁺ or HbFe³⁺ with H₂O₂. E10 cells were exposed to freshly prepared HbFe⁴⁺ for 12–24 hours. Exposure to HbFe⁴⁺ caused a significant up-regulation of HO-1 within 12 hours when compared with HbFe²⁺ and HbFe³⁺ (Figures 4A and 4B). We found significant enrichment of HO-1 in mitochondrial fraction after exposure to HbFe³⁺ and HbFe⁴⁺ (Figure 4C). To support this finding, we also employed immunocytochemical staining using laser confocal microscopy to confirm colocalization of HO-1 protein within the mitochondria in Hb-treated cells. Mitochondria were stained with a highly selective fluorescent probe, Mito Tracker Red CMXRos (Figure 4D). We found a significant colocalization of HO-1 protein within the mitochondria, as indicated by the appearance of strong merged (yellow) fluorescence after exposures to either HbFe³⁺ or HbFe⁴⁺ (Figure 4D).

Mitochondrial activity is routinely assessed by monitoring mitochondrial transmembrane potential ($\Delta\psi_m$) using JC-1, a potentiometric dye (35, 36). The dye preferentially accumulates in the mitochondria, as indicated by the high 590:530 nm emission fluorescence ratio. The ratio decreases drastically with a loss of mitochondrial bioenergetic function (23). We monitored the changes in the red: green ratio and expressed them on a percent scale drawn from the ratio obtained from oligomycin (100% hyperpolarized cells) and from FCCP (0% or completely depolarized cells). Interestingly, incubation of E10 cells with HbFe²⁺ for 24 hours resulted in a significant rise in the $\Delta\psi_m$. However, incubation with HbFe³⁺ showed no hyperpolarization over untreated control (Figure 5A). Exposure to HbFe⁴⁺ resulted in a drop in hyperpolarized cell percentage over untreated control, thus indicating a significant mitochondrial depolarization or compromised mitochondrial respiration. We assumed that this HbFe⁴⁺-mediated loss of $\Delta\psi_m$ is due to its oxidative reactivity. We confirmed heme-mediated loss of $\Delta\psi_m$ after exposure of E10 cells to hemin (10 μ M) for 24 hours. Hemin drastically reduced $\Delta\psi_m$, as indicated by a greater fall of 590:530 nm emission ratio, thus explaining the HbFe⁴⁺-mediated loss of $\Delta\psi_m$. Hpx, an endogenous scavenger of heme restored heme-induced decrease in $\Delta\psi_m$ (Figure 5A). The Hb-treated cells that were

not incubated with JC-1 showed no significant fluorescence at 590 nm compared with the JC-1-incubated cells (Figure E2).

Interestingly, HbFe⁴⁺ also caused a significant loss in basal cellular oxygen consumption, indicating an impaired mitochondrial respiration (Figures 5B and 5C). ATP linked respiration, an important bioenergetic parameter, was also significantly altered by HbFe⁴⁺ without affecting maximal OCR and reserve respiratory capacity. Exposure to HbFe²⁺ and HbFe³⁺ did not affect mitochondrial respiration within short-time exposure (12 h). However, we have seen a potentiation of reserve respiratory capacity by HbFe²⁺, probably due to an additional nonspecific uncoupling effect by HbFe²⁺. Hp, when incubated with HbFe⁴⁺, partially reversed the inhibitory effects of HbFe⁴⁺ on OCR (Figures 5B and 5C). However, HbFe⁴⁺ did not cause any alteration in the ECAR (data not shown).

E10 cells, where exposed to 25 μ M (heme) HbFe²⁺, HbFe³⁺, or HbFe⁴⁺ separately in the presence and absence of equimolar amount of Hpx. We found that Hpx effectively attenuated HO-1 induction by all redox forms of Hb. Slightly less inhibition was seen in the presence of HbFe⁴⁺, however, possibly due to a slower rate of heme release from the ferryl protein (unpublished observations; Figure E1).

Discussion

Several recent cell culture experiments in which Hb toxicity were investigated have shown conflicting outcomes as to the precise contribution of Hb oxidation states. For example, HbFe⁴⁺ (20 μ M), but not HbFe³⁺ or HbFe²⁺, induced cytoskeletal reorganization, inflammation and disrupted barrier integrity in endothelial cells after 8 hours of incubation with the proteins. Furthermore, the toxicity of HbFe³⁺ was attributed to its contamination with LPS (37). In the same study, it was reported that HbFe⁴⁺ did not elicit any inflammatory response in macrophages/monocytes (37). Exposure to a higher concentration of HbFe⁴⁺ (100 μ M) increased endothelial cell permeability and, in the case of a cross-linked Hb (α -bis[3,5-dibromosalicyl] fumarate), HbFe⁴⁺ caused cell death

Figure 3. HbFe³⁺ up-regulates mitochondrial HO-1 expression and alters basal mitochondrial respiration: mouse E10 cells were exposed to HbFe²⁺ and HbFe³⁺ with and without Hp for 24 hours. The mitochondria were isolated and probed for expression of HO-1 by Western blotting. The blots were reprobed with cytochrome c oxidase IV (COX-IV), a mitochondrial marker protein. The expression of glyceraldehyde phosphate dehydrogenase (GAPDH) and extracellular signal-regulated kinase (ERK) (as possible cytosolic markers) was also used to confirm the purity of the mitochondria. (A) Shown are representative images ($n = 3$). Mitochondrial HO-1 levels were quantified as fold expression over control after normalization with COX IV ($n = 3$). (B) Shown are means ($\pm$ SEM) of fold expression. Mitochondrial function was analyzed by extracellular flux analysis. Various bioenergetics parameters were calculated from the shown oxygen consumption rate (OCR) plots. Shown is (C) OCR plot and (D) bioenergetic profile of E10 cells after exposure to HbFe²⁺ and HbFe³⁺ in the presence and absence of Hp. OCR plot shown is from one representative experiment repeated three times. * $P < 0.05$ versus control; # $P < 0.05$ versus HbFe³⁺, t test, unpaired. AA, antimycin; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; Oligo, oligomycin; Rot, rotenone.

Figure 4. HO-1 translocates into mitochondria after exposure to oxidized Hbs: E10 cells were exposed to freshly prepared HbFe⁴⁺ for 12 hours in the presence and absence of equimolar concentration of Hp. (A) Representative immunoblot images are shown. The expression of HO-1 was quantified by comparison with β-actin. (B) Shown is fold expression of HO-1. Mitochondria (and cytosolic fractions) were isolated and probed for the expression of HO-1 and cytosolic markers. **P* < 0.05 versus Hp; \$*P* < 0.05 versus HbFe²⁺; #*P* < 0.05 versus HbFe⁴⁺. (C) Shown are the representative immunoblot images. The expression and localization of HO-1 was also confirmed by confocal microscopy. (D) Shown are representative confocal images indicating expression of HO-1 and Mitotracker Red. Data presented are means (±SEM). Con, control.

(38, 39). A recent study ruled out any significant contribution of methHb-iron and redox-mediated reactions in human lung AT2 cells (31). It is thus evident that Hb toxicity depends on the cell type, duration of exposure, and, more importantly, on the nature of heme/iron redox states used in these experiments. Because Hb undergoes auto-oxidation (Fe²⁺ → Fe³⁺) and autoreduction

(Fe⁴⁺ → Fe³⁺) spontaneously, controlling Hb redox transition becomes an important element in the validity of these studies.

Highly purified and well characterized redox forms of human Hb (50 μM, heme) were used in this study. Although the concentrations of Hb or its oxidized species in lung air spaces are not known, AT1 cells might be exposed to chronically low levels of acellular Hb in patients with acute lung

injury, hemolytic disorders, and very high acute concentrations after massive blood and Hb-based oxygen carrier transfusion (>500 μM plasma heme [40]).

The exposure of AT1 (E10) cells to HbFe²⁺ and HbFe³⁺ for 24 hours under our experimental conditions did not affect proliferation, fibrosis, apoptosis, and loss of AT1 cell phenotype. Hb α and β subunits, however, were detected in the cell lysates

Figure 5. Oxidized Hbs alter mitochondrial membrane potential and cause dysfunction: E10 cells were exposed to HbFe²⁺, HbFe³⁺, and freshly prepared HbFe⁴⁺ for a period of 12 hours (for XF24 assay) or 24 hours (for membrane potential assay) in the presence and absence of equimolar concentration of Hp. The mitochondrial membrane potential was measured after incubation using tetraethyl-benzimidazolyl carbocyanine iodide (JC-1) dye. (A) Shown is

Figure 6. Proposed mechanism of Hb (and its oxidized species)-induced toxicity: Hb undergoes oxidation to higher oxidation states, such as HbFe^{3+} and HbFe^{4+} . These products activate the NF- κ B pathway, leading to release of chemokines. The oxidized species lose heme-iron easily, leading to increased HO-1 and ferritin synthesis. HO-1 translocates into the mitochondria, leading to possible dysfunction. Free heme and CO generated by HO-1 can also inhibit mitochondrial respiratory chain function (electron transport chain [ETC]/mitochondrial transmembrane potential [$\Delta\Psi_m$]). Hp attenuates the Hb-induced effects, possibly by diffusing Hb radicals. Caveoli and some unknown pathways may also be involved in endocytosis of Hb. BV, biliverdin; HBOC, Hb-based oxygen carrier; Hpx, hemopexin; TLR4, Toll-like receptor 4.

after exposures to HbFe^{2+} and HbFe^{3+} . Earlier studies have shown that endothelial cells endocytose Hb even when present in high-molecular weight complexes (41, 42). Although it is possible that some Hb is bound to cell membrane, up-regulation of HO-1 is a strong indication of the internalization of the protein in our experiment.

Oxidized Hbs are generally less stable than the ferrous forms, due to unfolding of the proteins and subsequent heme loss (25). Exposure of E10 cells under our experimental conditions to HbFe^{3+} resulted in a higher expression of HO-1 and H-ferritin when compared with HbFe^{2+} ,

which may be due to the reported differences in the rates of heme release from the two proteins. Exposure of these cells to HbFe^{4+} , on the other hand, elicited a robust increase in HO-1 expression when compared with HbFe^{2+} , but these expressions were lower in intensity than HO-1 content induced by the ferric form. The persistence of the ferryl iron in solutions and its radical protein together are, however, more damaging to biological molecules and tissues than the HbFe^{3+} (24). Regardless of differences in the rates of heme release from these proteins, confocal microscopy images and immunoblotting of purified mitochondrial

fractions confirmed that a portion of expressed HO-1 translocated into the mitochondria. HOs are predominantly localized in the microsomes, but translocation into the mitochondria under certain stressful conditions has been reported (32, 34). Mitochondrial translocation of HO-1 might enable detoxification of accumulated heme as a result of oxidation and unfolding of Hb. In the absence of such a phenomenon, the bioenergetics might be more severely compromised. A recent study indicated that expression of HO-1 targeted to mitochondria attenuated oxidative stress (43). These observations, taken together, suggest that mitochondrial translocation of HO-1 could serve as a cytoprotective mechanism against possible mitochondrial dysfunction induced by oxidative or metabolic insults (32). However, hemin or LPS-induced mitochondrial accumulation of HO-1 was associated with decreased mitochondrial heme content and reduced expression of heme-sensitive subunit I of complex IV with loss of activity (33). Moreover, mitochondrial translocation of HO-1 can also lead to localized CO production as a result of heme degradation, thus inhibiting electron flow through mitochondrial electron transport chain complex IV (44).

HbFe²⁺ did not have any significant effect on the mitochondrial OCR, even after 24-hour exposure. However, a significant rise in cellular respiration was observed when E10 cells were exposed to HbFe²⁺ for a shorter incubation period (12 h). This could be also due to its slower rate of auto-oxidation that maintained higher oxygen supply to cells, apart from a possible uncoupling effect. In contrast, HbFe³⁺ produced significant inhibition in basal OCR respiration within 24 hours. We also compared the effects of all three redox forms of Hb on mitochondrial dysfunction for a shorter time period (12 h). HbFe⁴⁺ at an equivalent heme concentration produced significant changes in the basal, ATP-linked respiration in E10 cells within a shorter exposure. However, respiratory reserve capacity was not affected by HbFe⁴⁺. Interestingly, we did

Figure 5. (Continued). the JC-1 590:530-nm emission ratio expressed as a percent of oligomycin-treated hyperpolarized cells. The cells were exposed to hemin (5 and 10 μM), HbFe^{2+} , and HbFe^{3+} as described earlier. The cell lysates were probed for expression of HO-1. Equal loading was confirmed by reprobing the immunoblots against β -actin. Shown in the *upper panel* is a representative immunoblot. The mitochondrial oxygen consumption was measured using XF24 extracellular flux analyzer. (B) OCR plot after exposure to HbFe^{4+} ($n = 4$). (C) Bioenergetic profile was calculated from the representative OCR plot repeated three times. $*P < 0.05$ versus control; $^{\#}P < 0.05$ versus HbFe^{4+} , t test, unpaired. Data presented are means ($\pm$ SEM).

not observe any significant effect by HbFe^{2+} or HbFe^{3+} on these bioenergetic parameters of cells when compared with the ferryl species. It is thus clear that the higher oxidation state of Hb and duration of exposure determined the toxicity in our cell culture system. The inhibitory effect of cell-free Hb on human platelet mitochondrial function and cellular bioenergetics in sickle cell disease has recently been shown (22). Reduction in complex V activity was proposed to be a possible mechanism behind the compromised bioenergetic profile. Previous reports from our laboratory and others also found that cell-free Hb reduced (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (MTT) reduction capacity, indicating a loss of mitochondrial activity (45, 46). It has also been shown that heme induces significant mitochondrial dysfunction in endothelial cells (23). Similarly, our results showing a loss of mitochondrial function with a concomitant increase in mitochondrial HO-1 colocalization can be correlated with a possible loss mitochondrial respiratory chain activity (35).

Hp attenuated the HbFe^{2+} -, HbFe^{3+} -, and HbFe^{4+} -induced increase in HO-1 and H-ferritin. Moreover, Hp also prevented heme loss from HbFe^{3+} and

attenuated the HbFe^{3+} - and HbFe^{4+} -induced mitochondrial dysfunction (14, 25). Our results also indicate that Hp reduced Hb uptake, as shown by the absence of Hb α and β subunits in the cell lysates. Thus, Hp not only reduces heme overload externally, but also internally by reducing endocytosis. We have in recent years outlined the mechanistic basis of Hp-mediated Hb protection (14, 25, 47). The binding of Hp to Hb (known to be one of the strongest in biological systems) effectively diffuses Hb-mediated radicals, even when challenged with excess H_2O_2 . Intriguingly, although H_2O_2 and other ligands were found to have direct access to the heme pocket and unhindered pseudoperoxidase activity, this complex prevented heme loss, even at longer time period of incubation (25). In our current investigation, Hp might have also prevented the transport of Hb across E10 cell monolayer, possibly due to the large size of the Hb-Hp complex and/or altered surface charge (48).

In summary, we propose a likely mechanism that can explain Hb-induced injury reported here (see Figure 6). Exposure to Hb and its oxidized products

increases heme overload on the AT1 cells. Heme overload induces the expression of HO-1 and iron-sequestering proteins, such as ferritin. HO-1 translocates to the mitochondria, which leads to mitochondrial dysfunction. Alternatively, increased HO-1 activity in the cytosol might lead to excess production of CO, which could also reduce mitochondrial function. Hp effectively attenuates mitochondrial dysfunction. Our results also indicate that the higher oxidation species, HbFe^{3+} and HbFe^{4+} , elicit more pronounced effects when compared with HbFe^{2+} , due to their higher redox activities and rates of heme release. ■

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