Effect of Excess α-Hemoglobin Chains on Cellular and Membrane Oxidation in Model β-Thalassemic Erythrocytes

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Abstract

While red cells from individuals with β thalassemias are characterized by evidence of elevated in vivo oxidation, it has not been possible to directly examine the relationship between excess α-hemoglobin chains and the observed oxidant damage. To investigate the oxidative effects of unpaired α-hemoglobin chains, purified α-hemoglobin chains were entrapped within normal erythrocytes. These "model" β-thalassemic cells generated significantly ($P < 0.001$) greater amounts of methemoglobin and intracellular hydrogen peroxide than did control cells. This resulted in significant time-dependent decreases in the protein concentrations and reduced thiol content of spectrin and ankyrin. These abnormalities correlated with the rate of α-hemoglobin chain autoxidation and appearance of membrane-bound globin. In addition, α-hemoglobin chain loading resulted in a direct decrease (38.5%) in catalase activity. In the absence of exogenous oxidants, membrane peroxidation and vitamin E levels were unaltered. However, when challenged with an external oxidant, lipid peroxidation and vitamin E oxidation were significantly ($P < 0.001$) enhanced in the α-hemoglobin chain-loaded cells. Membrane bound heme and iron were also significantly elevated ($P < 0.001$) in the α-hemoglobin chain-loaded cells and lipid peroxidation could be partially inhibited by entrapment of an iron chelator. In contrast, chemical inhibition of cellular catalase activity enhanced the detrimental effects of entrapped α-hemoglobin chains. In summary, entrapment of purified α-hemoglobin chains within normal erythrocytes significantly enhanced cellular oxidant stress and resulted in pathological changes characteristic of thalassemic cells in vivo. This model provides a means by which the pathophysiological effects of excess α-hemoglobin chains can be examined. (*J. Clin. Invest.* 1993. 91:1706–1712.) Key words: catalase • vitamin E • glutathione • iron • heme

Introduction

The β thalassemias arise as a consequence of decreased or absent synthesis of the β-hemoglobin chains (1, 2). As a result of the altered β-hemoglobin synthesis, the concentration of the $\alpha_2\beta_2$-hemoglobin tetramer (Hb A) is substantially reduced, or absent, and a pool of unpaired α-hemoglobin chains is observed in erythroid precursors and, to a lesser extent, peripheral blood erythrocytes. It is believed that as a consequence of these excess α-hemoglobin chains, ineffective erythropoiesis and shortened erythrocyte survival is observed (3, 4).

While no single mechanism is likely to account for the complex pathophysiology of the β-thalassemic erythrocyte, oxidation of cellular components has been implicated as an important factor. This hypothesis is supported by the observation that isolated α-hemoglobin chains directly generate reactive oxygen species (5, 6). In addition, because of the inherent instability of α-hemoglobin chains, damage to the red cell by oxidative means may be further potentiated by the heme, or heme-derived iron, released from the excess α-hemoglobin chains (7–9). Indeed, elevated oxidant generation and heme release in thalassemic erythrocytes may be directly responsible for the decreased reactive sulfhydryl content of membrane proteins (10–13). Previous studies have demonstrated that the in vitro oxidant sensitivity of hemoglobin E erythrocytes (14) (a β-thalassemia–like disease) and α-hemoglobin chain loaded (15) red blood cells to exogenous oxidants correlated with the amount of membrane-bound hemoglobin or heme/iron and was amplified by increasing the amount of membrane-bound hemoglobin. Similarly, in vivo β-thalassemic red cells show evidence of elevated oxidative damage, as well as enhanced sensitivity to xenobiotic oxidants (16–18).

However, while many erythrocyte abnormalities in β thalassemia have been related to the presence of increased α-hemoglobin chains, it has not been possible to determine whether these changes arise directly from the presence of these chains or to other factors present during erythropoiesis. Since the most damaged cells are destroyed within the bone marrow, and are therefore not available for analysis, we have directly determined the effects of unbound α-hemoglobin chains on erythrocyte oxidation by entrapping purified α-hemoglobin chains within normal erythrocytes by reversible osmotic lysis (19–21). As previously shown, osmotic lysis and resealing results in resealed erythrocytes exhibiting normal morphology, hemoglobin concentration, volume, ATP concentration, oxidant sensitivity, and deformability while allowing for the efficient entrapment of exogenous compounds. The effect of entrapped α-hemoglobin chains on oxidant generation, antioxidant status, hemoglobin oxidation, and membrane peroxidation in normal erythrocytes was then assessed.

Methods

After informed consent, red blood cells (RBC)[1] were collected in heparinized tubes from laboratory volunteers at Children's Hospital (Oak-

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1. *Abbreviations used in this paper:* DFO-Dex, dextran derivative of deferoxamine; IOMs, inside-out membranes; NEM, *N*-ethylmalemide; RBC, red blood cells.

land, CA). Blood samples were processed immediately or stored at 4°C for a maximum of 24 h before utilization. All biochemicals, unless otherwise noted, were obtained from Sigma Chemical Co. (St. Louis, MO). Hemoglobin A ($\alpha_2\beta_2$) and the heme-containing α and β subunits were prepared as previously described (22–24). In some experiments, radiolabeled α-hemoglobin chains were used to quantitate their intracellular entrapment (21).

Osmotic lysis and resealing was done by the method of Scott et al. (19–21). After resealing, cells were washed at 4°C with isotonic saline until the supernatant was clear (five to seven times). Unless otherwise indicated, the results presented in this study used RBC resealed in the presence of 10 mg α-hemoglobin chains/ml packed red cells. The control and resealed erythrocytes were resuspended at pH 7.4 in either NCTC109 media or HBSS as indicated and supplemented with 5 mM glucose and antibiotics (100 IU penicillin and 0.1 mg streptomycin/ml) for experimental analysis. The samples were incubated in a shaking water bath at 37°C for $\leq$ 20 h. Aliquots were removed at the indicated time points for analysis. Statistical significance was determined by the Student's t test.

Hydrogen peroxide (H_2O_2) generation in the control, control-resealed, and α-hemoglobin chain–loaded cells was determined by the H_2O_2-dependent inactivation of catalase-compound I by 3-amino-1,2,4-triazole (aminotriazole) (25–27). Using this method, an inverse correlation between H_2O_2 generation and residual catalase activity is observed. RBC hemolysates were assayed for catalase activity by the spectrophotometric disappearance of H_2O_2 and expressed as units per gram hemoglobin (IU/g Hb) (27). GSH concentration in the control, control-resealed and α-hemoglobin chain–loaded red cells was determined as previously described and expressed as μmol/g Hb (28, 29). Erythrocyte hemoglobin concentration was determined by ferricyanide-cyanide (Drabkin's) reagent (30).

The effect of excess α-hemoglobin chains on erythrocyte membrane antioxidant capacity was assessed by determination of the α-tocopherol (vitamin E) concentration (31–33). In addition to steady-state vitamin E levels, the loss of vitamin E in response to an exogenous oxidant (50 μM cumene hydroperoxide) was assessed after 0 and 20 h incubation at 37°C in HBSS (supplemented with 5 mM glucose and 10% hematocrit, pH 7.4). Briefly, after incubation for 0–20 h, the cells were resuspended to a 1% hematocrit in HBSS (no glucose) and challenged with cumene hydroperoxide (final concentration of 50 μM). Aliquots were removed at the indicated time points, extracted, and analyzed for vitamin E by HPLC. The values are expressed as nmol α-tocopherol/ml packed RBC. HBSS was used in these studies since NCTC109 contains vitamin E.

Hemoglobin oxidation was determined by spectrophotometric analysis (500–700 nm); the concentration of oxy-, met-, and hemichrome hemoglobin was calculated according to the method of Winterbourn (34). Hemoglobin degradation was determined by following the loss of heme absorption (35). Changes in cellular deformability were determined as previously described (21) using the Cell Transit Analyzer from ABX (Levallois, France), which measures the time required for individual erythrocytes to pass through a membrane pore.

Lipid peroxidation was determined by parinaric acid oxidation (36–39). Parinaric acid is a fluorescent polyunsaturated fatty acid that is rapidly incorporated into membranes (36–39). Exposure of intact RBCs labeled with parinaric acid to oxidants results in the loss of fluorescence as a result of parinaric acid peroxidation. This loss of fluorescence has been shown to correlate with the oxidation of other membrane lipids. Parinaric acid fluorescence was measured continuously (excitation wavelength, 312 nm; emission wavelength, 455 nm) in a fluorometer (LS-5B; Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England) in response to H_2O_2, cumene hydroperoxide, and tert-butyl hydroperoxide challenge.

Membrane-bound heme and iron concentrations in the control, control-resealed, and α-hemoglobin chain–loaded cells were determined in membrane ghosts and in inside-out membranes (IOMs) as described by Kuross et al. (40). The use of IOMs has the advantage over standard ghosts or inside-out vesicles in that they allow for the

removal, by washing, of heme/Fe simply entrapped within the membrane ghosts. Furthermore, studies in sickle cells have demonstrated a close correlation between IOM-bound heme and Fe and susceptibility to membrane oxidation (40). To further investigate the effects of membrane bound heme and Fe on the membrane's susceptibility to iron-mediated oxidants, a high molecular mass (70 kD) dextran derivative of deferoxamine (DFO-Dex) (41, 42) was entrapped within the control and α-hemoglobin chain–loaded cells. As previously shown, DFO-Dex has virtually identical iron-binding characteristics to deferoxamine but, unlike DFO, is unable to cross the membrane and does not mediate methemoglobin generation (42). Intracellular DFO-dextran concentration was determined using the molar extinction coefficient of 2,300 $M^{-1} \cdot cm^{-1}$ for iron saturated DFO (43) (ferrioxamine) and expressed as mol DFO/g Hb as previously described (42).

Alterations in membrane skeletal proteins and reduced thiol groups in the normal, control-resealed, α-hemoglobin chain–loaded RBC were examined by urea-triton PAGE of membrane ghosts as previously described (12, 21, 44). Labeling of the membrane ghosts with [^{3}H] N-ethylmalemide ([^{3}H]NEM) (Dupont-New England Nuclear, Boston, MA), specific activity 44.6 Ci/mmol NEM, was done to visualize and quantitate the loss of membrane reactive thiol groups. Erythrocyte membranes for electrophoretic analysis were prepared from the incubated cells by the method of Dodge et al. (45), and, after extensive washing to remove any trapped hemoglobin chains, the protein concentrations of the membrane ghosts were determined by the method of Lowry et al. (46). The relative protein and reduced thiol group concentrations of the urea-triton gels were determined by densitometry at 570 nm on a densitometer (Sebia Preference, Paris, France), and are expressed as percentage of the total membrane extractable protein (membrane specific and skeletal proteins) excluding globin. Membrane-bound α-hemoglobin chain concentration is expressed as percent of normal membrane protein; i.e., excluding globin.

Results

Analysis of the purified α-hemoglobin chains by mass spectroscopy demonstrated the expected mass value for the α chains. In addition, the purified α-hemoglobin chains used in this study were soluble and completely bound added β-hemoglobin chains to form the normal hemoglobin tetramer. These data indicated that no structural alterations had occurred during the preparation of the purified α-hemoglobin chains.

Previous studies (21) with α-hemoglobin chains labeled with [^{3}H]NEM showed that under the conditions used in the current study, 10 mg α-hemoglobin chains/ml packed RBC, the entrapped α-hemoglobin chain concentration is $3.6\pm0.6\%$ of the total hemoglobin in erythrocytes. However, because of the increased instability of [^{3}H]NEM α-hemoglobin chains (21), they were used only to quantitate the efficacy of entrapment and all subsequent experiments used nonlabeled α-hemoglobin chains. Additionally, as has been previously reported, osmotic lysis and resealing alone had no substantial effects on normal red cell characteristics (19–21, 27, 29, 42, 47).

Entrapment of the α-hemoglobin chains resulted in a time-dependent increase in membrane-bound chains (Fig. 1). As previously shown (21), the membrane associated α-hemoglobin chains arose entirely from the entrapped α-hemoglobin chain pool, since no β-hemoglobin chain band was detected to indicate dissociation of normal hemoglobin. The increase in membrane-bound α-hemoglobin chains was closely correlated with decreases in the absolute protein ($r = 0.966$; $P < 0.001$) and reduced thiol ($r = 0.998$; $P < 0.001$) concentrations of ankyrin (Fig. 1). Decreases in spectrin protein and thiol concentrations were similarly correlated ($r = 0.894$ and 0.939, re-
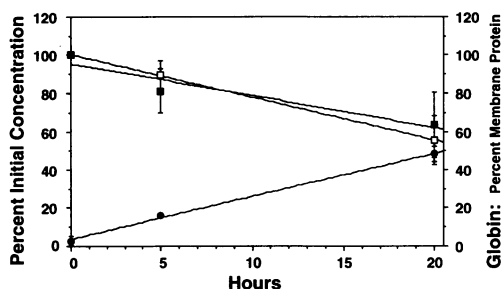
Figure 1. The increase in membrane-bound globin (●, *right axis*) is directly associated with the decrease in the protein (■) and reduced thiol (□) concentrations of ankyrin (*left axis*) in the α-hemoglobin chain loaded red cells. The samples were incubated for ≤ 20 h at a 5% hematocrit in NCTC109 media and aliquots were removed at the indicated time points. No membrane-bound globin was observed in either the control or control-resealed cells. Similarly, no significant alterations in membrane protein constituents were observed in either control group. As described in the Results, similar results were observed with spectrin. The results shown are mean±SD for a minimum of three independent experiments.

spectively; $P < 0.01$) with the increase in membrane-bound α-hemoglobin chains, while other membrane proteins (e.g., lane *3*) were unaffected in the α-hemoglobin chain loaded cells (21, 47). In contrast, no membrane-bound globin was observed in either the control or control-resealed cells and, consequently, no significant changes in the protein and reactive thiol concentrations of spectrin or ankyrin were observed over the time course of the experiment (21, 47).

In addition to the alteration of membrane proteins, cytoplasmic proteins were also significantly altered by the entrapped α-hemoglobin chains. As shown in Fig. 2, α-hemoglobin chain loading resulted in significant hemoglobin oxidation. Interestingly, while the entrapped α-hemoglobin chains accounted for only about 4% of the total hemoglobin after 20 h incubation 26.4±0.8% of the hemoglobin ($P < 0.001$; 5% hematocrit in NCTC109 buffer) was oxidized to methemoglobin in the α-hemoglobin chain–loaded cells. In contrast, methemoglobin accounted for only 4.2±0.4 and 10.1±0.2% of the total hemoglobin in the control and control-resealed red cells respectively. Hemichrome concentration was also increased significantly ($P < 0.01$) in the α-hemoglobin chain loaded–erythrocytes and accounted for 6.4±0.8% of the total hemoglobin after 20 h incubation. No significant increases in hemichrome were observed in the normal or control resealed cells. The elevated
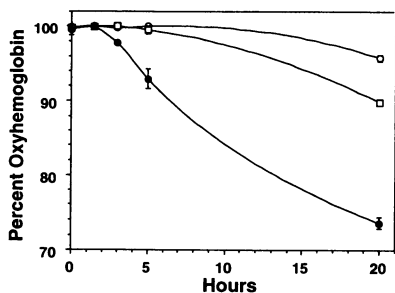
Figure 2. During incubation at 37°C, the α-hemoglobin chain loaded cells exhibited a significant ($P < 0.001$) decrease in oxyhemoglobin. Shown is the response of normal (○), control-resealed (□), and α-hemoglobin chain–loaded (●) erythrocytes during 20 h. Sample aliquots were removed at the indicated time points and oxyhemoglobin was determined as described in the Methods. The results presented are the mean±SD of three independent experiments.

levels of oxidized hemoglobin in the α-hemoglobin chain–loaded cells were thought to be indicative of an enhanced rate of generation of reactive oxygen species.

To directly establish whether the entrapped α-hemoglobin chains resulted in elevated intracellular levels of reactive oxygen species, the generation of hydrogen peroxide (H_2O_2) was determined by the H_2O_2-dependent inactivation of catalase by aminotriazole (25–27). As shown in Fig. 3, α-hemoglobin chain–loaded cells generated significantly ($P < 0.01$) greater amounts of H_2O_2 than the control-resealed cells. Further analysis of the rates of H_2O_2 generation and methemoglobin concentration in the α-hemoglobin chain loaded cells demonstrated a significant relationship ($r = 0.843$) between these two parameters. Thus, the elevated methemoglobin levels and H_2O_2 generation in the α-hemoglobin chain–loaded cells clearly demonstrate that the entrapped chains resulted in enhanced oxidative stress on the erythrocyte.

Interestingly, inclusion of aminotriazole in the incubation media during the determination of H_2O_2 generation significantly ($P < 0.01$) exacerbated the effects of α-hemoglobin chain loading on the loss of cellular deformability. In the current study, the addition of 10 mM aminotriazole to the cell populations resulted in mean cell transit times, as measured by the Cell Transit Analyzer (ABX), of 1.01±0.03, 1.06±0.4, 1.34±0.01, and 1.49±0.04 ms for the control, control-resealed, α-hemoglobin chain, and α-hemoglobin chain/aminotriazole red cells, respectively, after 20 h incubation at 37°C. In contrast, control-resealed erythrocytes treated with aminotriazole had a mean cell transit time of 1.11±0.02 ms. This data strongly implicated an important role for reactive oxygen species in mediating the loss of cellular deformability in the model β-thalassemic cells.

To determine whether the elevated generation of reactive oxygen species directly altered the antioxidant status of the erythrocyte, GSH concentration, membrane vitamin E levels, and catalase activity were determined. As shown in Fig. 4, GSH levels decreased significantly ($P < 0.001$ at 20 h) in the α-hemoglobin chain loaded cells and the extent of the reduction was dependent on the amount of α-hemoglobin chains entrapped
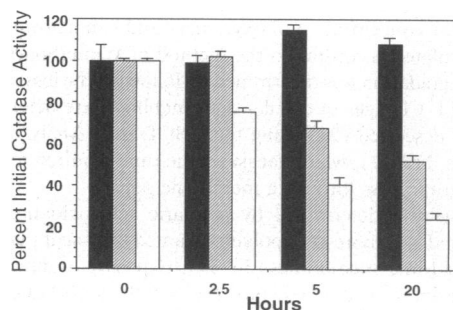

Figure 3. Hydrogen peroxide generation in the control-resealed and α-hemoglobin chain loaded cells. Hydrogen peroxide was indirectly measured by the H_2O_2-dependent inactivation of catalase by aminotriazole. In the presence of aminotriazole, entrapment of α-hemoglobin chains (□) resulted in a significant ($P < 0.01$) increase in intracellular H_2O_2 generation in comparison to the control-resealed (▨) cells. As has been previously demonstrated, a direct inverse relationship between H_2O_2 generation and aminotriazole-mediated catalase inactivation exists (25–27). Also shown is the catalase activity of the control resealed (■) cells over time in the absence of aminotriazole. Shown are the mean±SD of three experiments.
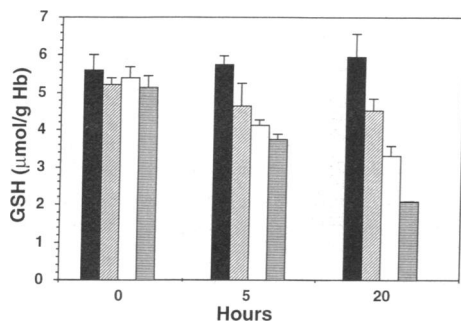
Figure 4. Indicative of increased metabolic stress and oxidant generation, intracellular GSH concentration was diminished in the α-hemoglobin chain–loaded cells ($\square$ 5 mg α-hemoglobin chain/ml pRBC; $\blacksquare$ 10 mg α-hemoglobin chain/ml pRBC) in relation to the control ($\blacksquare$) and control-resealed ($\blacksquare$) cells. As shown, the decrease in GSH status was dependent on the amount of entrapped α-hemoglobin chains. GSH levels were determined as described in the methods. Given are the mean±SD of a minimum of three experiments for all samples and time points.

within the erythrocytes. In contrast, in the absence of an exogenous oxidant stress, membrane vitamin E levels were unaffected by incubation at 37°C for $\leq$ 20 h (Fig. 5 *A*). However, when the α-hemoglobin chain loaded cells were exposed to a mild oxidative stress (50 μM cumene hydroperoxide at a 1% hematocrit), their vitamin E levels decreased significantly ($P < 0.01$), faster relative to either the control or control-resealed cells (Fig. 5 *B*). Perhaps of greater physiologic importance,
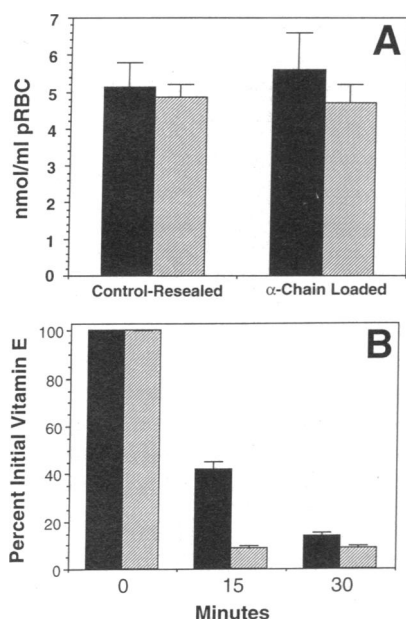
Figure 5. Entrapment of α-hemoglobin chains in the (*A*) absence of an exogenous oxidant had no effect on membrane vitamin E concentrations when compared to control-resealed cells. Cells were incubated for 0 ($\blacksquare$) and 20 ($\blacksquare$) h in HBSS (supplemented with 5 mM glucose and antibiotics) at a 10% hematocrit. In addition, no change in vitamin E levels between normal and control-resealed cells was noted (data not shown). (*B*) In contrast, exposure of the cells to an exogenous oxidant, 50 μM cumene hydroperoxide, as described in Methods, resulted in a more rapid depletion of vitamin E in the α-hemoglobin chain–loaded cells ($\blacksquare$) than in control-resealed ($\blacksquare$) cells. The cells were suspended to a hematocrit of 1% in HBSS and treated as described. Shown are the results of a typical experiment after incubation in HBSS (pH 7.4, supplemented with 5 mM glucose). Vitamin E levels were determined by HPLC as described in the methods.

however, was the finding that even in the absence of aminotriazole, α-hemoglobin chain incorporation significantly ($P < 0.001$) inhibited catalase activity. After 20 h incubation at 37°C only 61.5±2.9% of the initial catalase activity remained in the α-hemoglobin chain–loaded cells vs 104.6±4.5 and 108.2±3.2% in the control and control-resealed cells, respectively (the effect of incubation time on catalase activity, in the absence of aminotriazole, of the control-resealed cells is also shown in Fig. 3).

As a consequence of the altered antioxidant status of the α-hemoglobin chain–loaded cells, membrane susceptibility to lipid peroxidation was assessed. As demonstrated in Fig. 6, oxidation of parinaric acid (a fluorescent polyunsaturated fatty acid) was significantly enhanced in the α-hemoglobin chain–loaded cells in response to cumene hydroperoxide. Similar findings were obtained with H_2O_2 and *tert*-butyl hydroperoxide (data not shown). Interestingly, the enhanced oxidant susceptibility of the α-hemoglobin chain–loaded cells was observed before any significant globin binding to the membrane. These data supports the previous findings (9, 21) that the isolated α-hemoglobin chains themselves are more susceptible to oxidative denaturation. As has been previously reported, no differences between the oxidant sensitivity of the control and control-resealed cells were observed (19–21, 27, 29).

To further determine whether membrane specific factors might account for the enhanced vulnerability of these cells to exogenous oxidants, extensively washed membrane ghosts were prepared and their susceptibility to H_2O_2 (an iron dependent reaction) was assessed. As indicated in Fig. 7, membranes prepared from α-hemoglobin chain–loaded erythrocytes demonstrated a significantly ($P < 0.001$) enhanced rate of parinaric acid oxidation. Similar to the alterations noted in membrane proteins and reduced thiol groups, cellular and membrane oxidant sensitivity in the parinaric acid degradation assay was directly correlated ($r = 0.961$ and 0.991, respectively; $P < 0.001$) with membrane α-hemoglobin chain deposition.

To determine whether heme and iron release from the entrapped α-hemoglobin chains correlated with the observed functional and structural changes and increased oxidant susceptibility in the model β thalassemic cells, heme and iron levels were determined in control, control-resealed and α-hemoglobin chain loaded cells. After entrapment and storage at 4°C for $\leq$ 20 h, no significant concentrations of heme were detected in membrane ghosts (2.72, 3.75, and 4.87 nmol heme/mg ghost protein) or IOM (all values were $>$ 1 nmol heme/mg IOM
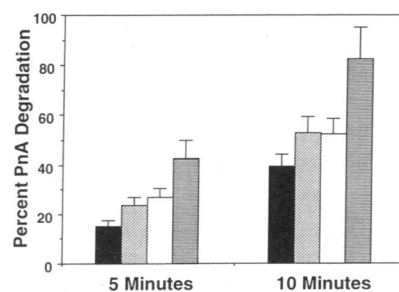
Figure 6. Intracellular entrapment of α-globin chains enhanced membrane susceptibility to iron-mediated oxidation. Shown is the percent degradation of parinaric acid (*PnA*), a fluorescent polyunsaturated fatty acid, after 0 and 20 h incubation, respectively, at 37°C in the control-resealed ($\blacksquare$, $\blacksquare$) and α-hemoglobin chain–loaded ($\square$, $\blacksquare$) erythrocytes. Cells were suspended to a hematocrit of 1% in HBSS and challenged with 200 μM cumene hydroperoxide. The values given are the mean±SD of three independent experiments.
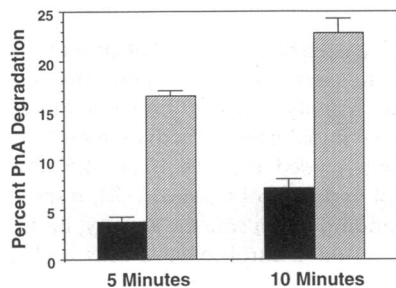
Figure 7. Indicative of heme/iron deposition, membrane ghosts prepared from α-hemoglobin chain loaded cells (▨) showed greatly enhanced lipid peroxidation upon exposure to hydrogen peroxide. In contrast, the control-resealed (■) membranes exhibited only minor membrane peroxidation. Lipid peroxidation was determined by the degradation of PnA, a fluorescent fatty acid, in ghosts prepared from normal and α-hemoglobin chain loaded erythrocytes after 20 h incubation at 37°C. Ghost membranes containing ~ 400 nmol phospholipid were suspended in HBSS and challenged with hydrogen peroxide (400 μM) in the absence of any exogenous iron. The values given are the mean±SD of three independent experiments.

protein) prepared from control, control-resealed, or α-hemoglobin chain–loaded erythrocytes, respectively. However, as shown in Fig. 8, after 24 h incubation at 37°C, the α-hemoglobin chain–loaded cells exhibited a very large and significant ($P < 0.001$) increase in membrane associated heme in both the Dodge ghost and IOM preparations. In contrast, membrane ghosts and IOMs prepared from control and control-resealed cells exhibited substantially lower amounts membrane associated heme iron after 24 h incubation at 37°C.

Nonheme iron levels were also significantly increased ($P < 0.001$) in the Dodge ghosts and IOMs of the α-hemoglobin chain loaded cells when compared to control and control-resealed cells after 24 h incubation at 37°C (Fig. 9). Nonheme iron in the α-hemoglobin chain loaded cells was found to be greater than eightfold than observed in ghosts prepared from either the control or control-resealed cells. Similarly, in the IOMs prepared from the α-hemoglobin chain–loaded cells, a significant amount ($P < 0.001$) of reactive iron was detected. In contrast, no detectable iron was observed in IOMs prepared from the control and control-resealed erythrocytes. The observed levels of heme and nonheme iron detected in the α-hemoglobin chain loaded cells are far in excess of those previously observed in sickle cells; e.g., mean heme and nonheme iron values of 2.0 and 4.3 nmol/mg protein, respectively, in sickle IOMs (40).

The enhanced susceptibility of the α-hemoglobin chain loaded cells to iron-mediated oxidants (cumene hydroperoxide and H_2O_2), was further investigated using a high molecular mass dextran derivative of deferoxamine (DFO-Dex, 70 kD)
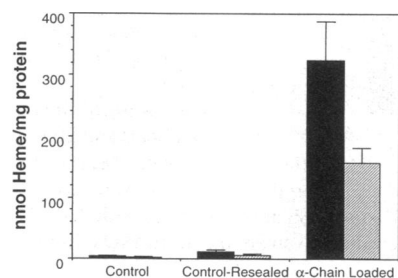
Figure 8. Effect of α-hemoglobin chain loading on membrane bound heme in Dodge ghosts (■) and IOMs (▨). Heme concentration and the preparation of IOMs were done according to the procedure of Kuross et al. (40). The results shown are the mean±SD of 10 samples from two independent experiments.
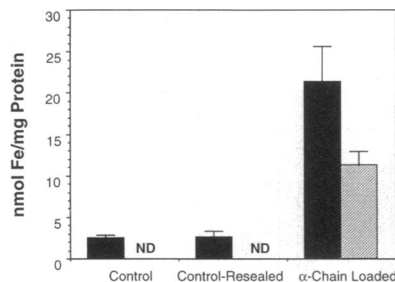
Figure 9. Membrane bound iron concentration in Dodge ghosts (■) and IOMs (▨). Iron concentrations and the preparation of inside-out membranes (IOMs) were done according to the procedure of Kuross et al. (40). The results shown are the mean±SD of 10 samples from two independent experiments. ND, not detectable.

(42), which was entrapped within normal and α-hemoglobin chain loaded erythrocytes. Spectrophotometric analysis of the entrapped DFO-dextran, in the absence of exogenously added iron, indicated that immediately after resealing > 8% of the entrapped DFO-dextran was present in the ferrioxamine (iron binding) state. Hence, DFO-dextran loading resulted in the presence of a potent (491±46 nmol DFO-equivalents/g Hb) intraerythrocytic iron chelating capacity. Challenge of these chelated cells with cumene hydroperoxide (as per Fig. 6) before incubation at 37°C resulted in a significant decrease (24.9±4.1 percent; $n = 4$; $P < 0.01$) in the rate of parinaric acid degradation. After incubation for 20 h at 37°C, the effect of the DFO-Dex on lipid peroxidation (11.7±2.7 percent inhibition; $n = 4$; $P > 0.05$) was almost completely lost. The loss of DFO-Dex inhibition of parinaric acid oxidation was associated with the finding that the entrapped DFO-Dex was primarily present in the ferrioxamine state. However, regardless of incubation time, entrapment of DFO-Dex did not totally abolish the prooxidant effects of the α-hemoglobin chains. It is hypothesized that the inability of DFO-Dex to completely inhibit iron-mediated lipid oxidation was caused by the intercalation of the heme and iron into the cell membrane (15) and the inability of the DFO-Dex to remove the heme and nonheme iron from this cellular compartment.

Discussion

The potentially dangerous combination of oxygen and iron (hemoglobin or hemoglobin derived) within the erythrocyte is normally kept in check by a number of endogenous mechanisms (19, 27, 29, 48). In normal cells the integrity of the hemoglobin tetramer itself is a very important mechanism by which iron and oxygen are kept in redox-inert states. The thalassemias (both α and β) circumvent this protective mechanism by altering tetramer formation/stability, as well as by affecting the globin-heme interaction of the monomers. Previous studies have demonstrated that excess α-hemoglobin chains autoxidize, release heme, and generate superoxide (O_2^-), at a rate eight times that of normal Hb (5, 6, 9, 21). The released heme has been shown to directly inhibit a number of cytoplasmic enzymes, further disrupting normal cellular homeostasis and predisposing the cell to additional injury (49). In addition to the released heme, the α-hemoglobin chain mediated production of reactive oxygen species (O_2^-, H_2O_2) is of importance. Interaction between these reactive oxygen species and the heme or heme-derived iron (15, 50) has been shown to result in a "biologic Fenton reaction" (7) that catalyzes the production of the hydroxyl radical (·OH) and reactive lipid radicals. These species are believed to mediate much of the damage seen in thalassemic erythrocytes.

In point of fact, in vivo β-thalassemic erythrocytes exhibit numerous lesions characteristic of oxidative damage (3, 4, 9–12, 14–18). However, it has not been previously possible to directly correlate this damage and the subsequent changes in cell function and structure with the presence of excess α-hemoglobin chains and the generation of reactive oxygen species. This is in large part caused by two factors: (a) the pathophysiologic changes of interest have already occurred in vivo, and thus the underlying mechanisms of the cellular injury can not be readily studied; and (b) peripheral blood erythrocytes in β-thalassemic patients represent the small fraction of the erythroid precursors that survive extrusion from the bone marrow and rapid removal from the peripheral blood by the spleen and reticuloendothelial system. Consequently, the surviving peripheral blood erythrocytes may not fully demonstrate the pathophysiologic effect of excess α-hemoglobin chains on cell function and structure and they most certainly can not be used to study the biogenesis of the red cell damage.

To directly examine the oxidant-mediated effects of excess α-hemoglobin chains, we have used normal erythrocytes in which purified α-hemoglobin chains had been entrapped. This model of the β-thalassemic erythrocyte has been previously used by us to determine the effects of excess α-hemoglobin chains on the structural (skeletal) and functional (deformability) characteristics of normal cells and to compare these alterations to those observed in patient-derived β-thalassemic cells (21, 47). These studies demonstrated that the model β-thalassemic red cells exhibited structural and functional changes very similar to those observed in patient samples. In addition, this model provides a means by which the mechanisms underlying the α-hemoglobin chain induced erythrocyte pathophysiology can be investigated.

As reported in this study, the structural and functional changes seen in the model β thalassemic cells are closely correlated with α-hemoglobin chain autoxidation, membrane globin binding, membrane iron (heme and nonheme) deposition, and the generation of reactive oxygen species. The release of heme and heme-derived iron and the generation of the reactive oxygen species are further correlated with direct decreases in cytoplasmic, but not membrane, antioxidant activity (decreases of > 50% and 38% for GSH and catalase, respectively). The increase in redoxactive iron and the loss of cytoplasmic antioxidants further enhances the susceptibility of α-hemoglobin chain loaded erythrocytes to both endogenous and exogenous oxidants. Indeed, exposure of the α-hemoglobin chain-loaded cells to even low levels of exogenous oxidants (H_2O_2 and cumene hydroperoxide) resulted in the very rapid oxidation of membrane lipids, depletion of membrane vitamin E, and the increased oxidation of cytoplasmic proteins. In addition, the α-hemoglobin chain dependent generation of H_2O_2, in the presence of aminotriazole, further exacerbated the loss of cellular deformability in the α-hemoglobin chain loaded cells. In vivo, this loss of cellular deformability could result in the impaired extrusion of reticulocytes from the marrow and shortened survival of the peripheral blood erythrocytes.

Hence, while the reducing capacity of the normal erythrocyte is > 250 times its oxidizing potential (51), excess α-hemoglobin chains can circumvent (i.e., loss of the hemoglobin tetramer) and overwhelm (decreased catalase activity and GSH concentration) the normal protective mechanisms of the cell. This effect could, in part, be reversed by augmentation of the antioxidant capacity of the model thalassemic cell. As shown in this study, intracellular entrapment of an iron chelator diminished the susceptibility of the α-hemoglobin chain loaded cells to exogenous oxidants. These results support the hypothesis that oxidative mechanisms contribute to the cellular pathophysiology of thalassemic cells (6, 9–12, 14–18, 47, 50, 52).

Finally, a finding in this study of particular interest is the rapidity with which the α-hemoglobin chain mediated damage occurs. Within 20 h, which represents only ~ 0.7% of the expected life span of the red cell, the model thalassemic erythrocytes exhibited structural and functional changes very similar to those observed in β-thalassemic cells. The rapidity of these changes indicates that any therapeutic intervention aimed at preventing erythrocyte damage in β thalassemia must be targeted at the stage in erythroid maturation when α-hemoglobin chains synthesis is initiated. This study also suggests that agents capable of decreasing and/or stabilizing the unpaired α-hemoglobin chains or, alternatively, enhancing the intracellular antioxidant status (e.g., oxidant scavengers or iron chelators) might be capable of improving the effective erythropoiesis of β-thalassemic patients. In contrast, agents that simply stimulate erythrocyte production without altering globin chain imbalance, would be of very limited value. A recent study in homozygous β-thalassemic mice (53) has shown that the improved hematologic parameters following erythropoietin treatment were associated with increased synthesis of the β-minor chain and an improved β-/α-hemoglobin chain synthesis ratio and not with an absolute increase in erythrocyte production.

In conclusion, the pathologic defects that characterize the red cell in β thalassemia can be generated within normal cells by the intracellular entrapment of purified, heme-containing, α-hemoglobin chains. The results of this study strongly implicate oxidative mechanisms as an important pathway in the premature destruction of the thalassemic cell. The results of our study clearly demonstrate a close interrelationship between α-hemoglobin chain autoxidation and the appearance of membrane bound globin, membrane deposition of heme and iron, increased intracellular oxidant generation, and the subsequent pathophysiology of the model β-thalassemic cells. In addition, α-hemoglobin chain mediated reductions in cytoplasmic antioxidants (GSH and catalase) were observed and may be of importance in further exacerbating the oxidant damage arising from the elevated intracellular generation of H_2O_2. The decreased levels of intracellular antioxidants combined with the presence of high concentrations of membrane associated globin (both heme containing and heme free), heme, and iron were also correlated with a significantly enhanced sensitivity to exogenous iron-driven oxidants (H_2O_2 and cumene hydroperoxide). Chemical inactivation of endogenous catalase activity was found to strongly exacerbate α-hemoglobin induced injury while the presence of an intracellular iron-chelator resulted in a decreased susceptibility to oxidant mediated damage. This study gives further evidence that the model β-thalassemic cell provides an excellent means by which the mechanisms and ontogeny of damage by excess α-hemoglobin chains can be investigated. Furthermore, this study suggests that this model may be useful in evaluating possible therapeutic interventions that may be capable of inhibiting α-hemoglobin chain induced damage to the thalassemic red cell and its progenitors.

Acknowledgments

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