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Role of the membrane in the formation of heme degradation products in red blood cells

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Abstract

Aims—Red blood cells (RBCs) have an extensive antioxidant system designed to eliminate the formation of reactive oxygen species (ROS). Nevertheless, RBC oxidant stress has been demonstrated by the formation of a fluorescent heme degradation product (ex.321 nm, em 465 nm) both in vitro and in vivo. We investigated the possibility that the observed heme degradation results from ROS generated on the membrane surface that are relatively inaccessible to the cellular antioxidants.

Main Methods—Membrane and cytosol were separated by centrifugation and the fluorescence intensity and emission maximum was measured. The effect on the maximum emission of adding oxidized and reduced hemoglobin to the fluorescent product formed when hemin is degraded by H₂O₂ was studied.

Key findings—90% of the fluorescent heme degradation products in hemolysates are found on the membrane. Furthermore, these products are not transferred from the cytosol to the membrane and must, therefore, be formed on the membrane. We also showed that the elevated level of heme degradation in HbCC cells that is attributed to increased oxidative stress was found on the membrane.

Significance—These results suggest that, although ROS generated in the cytosol are neutralized by antioxidant enzymes, H₂O₂ generated by the membrane bound hemoglobin is not accessible to the cytosolic antioxidants and reacts to generate fluorescent heme degradation products. The formation of H₂O₂ on the membrane surface can explain the release of ROS from the RBC to other tissues and ROS damage to the membrane that can alter red cell function and lead to the removal of RBCs from circulation by macrophages.

Keywords

Red blood cells; Hemoglobin; membrane; Heme degradation; Fluorescence; Oxidative stress; Autoxidation; Hydrogen peroxide

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Introduction

An increase in red blood cell (RBC) oxidative stress has been reported in many pathological conditions associated with various diseases including renal failure (Caimi 2002), Alzheimer's disease (Delibas et al. 2002; Baldeiras et al. 2008), Behcet's disease (Kose et al. 2002) idiopathic thrombocytopenic purpura (Polat et al. 2002), Buerger disease (Arslan et al. 2009), sickle cell, thalassemia and hemolytic anemia (Fibach and Rachmilewitz 2008; Hebbel 1990; Shinar and Rachmilewitz 1990; Winterbourn 1990), in addition to cellular aging (Piccinini et al. 1995) and organism aging (Kasapoglu and Ozben 2001).

Hydrogen peroxide (H_2O_2) produced by autoxidation of Hb is a predominant reactive oxygen species (ROS) in RBCs. If this H_2O_2 is not scavenged immediately by antioxidant defense enzymes, it oxidizes hemoglobin (Hb) to the higher oxidation state of ferrylhemoglobin and oxoferrylhemoglobin, which can cause oxidative stress to RBCs (Alayash et al. 2001). This oxidative stress is usually assessed by measuring lipid oxidation products such as malondialdehyde and 4 hydroxynonenol (Gil et al. 2006). Spectroscopic methods to measure these products have been shown to be complicated due to Hb interference.

We have instead used an improved sensitive direct measure of RBC oxidative stress based on the reaction of Hb with H_2O_2 in vitro that results in the degradation of hemes to produce fluorescent heme degradation products (Nagababu and Rifkind 1998; Nagababu et al. 2000a; Nagababu and Rifkind 2000b). We have also shown that one of the same heme degradation products is produced in vivo in RBCs of humans and animals (Nagababu and Rifkind 2004; Nagababu et al. 2008a; Nagababu et al. 2008b).

RBCs have an extensive antioxidant system including catalase, glutathione peroxidase (GPX) (Nagababu et al. 2003) and peroxiredoxin (Lee et al. 2003) that would be expected to neutralize any H_2O_2 generated. Studies have shown that even 1% of the total catalase in RBCs can neutralize a significant amount of H_2O_2 (Mueller et al. 1997). The efficiency of the catalase system was confirmed by the observation that the addition of H_2O_2 to intact RBCs does not produce any heme degradation unless catalase is completely inhibited by azide (Nagababu et al. 2000a). It is, therefore, necessary to explain how fluorescent degradation products form in RBCs.

The present study shows that the heme degradation products formed in RBCs are associated with the RBC membrane. This implies that the degradation products are formed when Hb is associated with the membrane and the ROS formed at membrane site are less accessible to the cytoplasmic antioxidants.

Materials and methods

Preparation of human RBCs and Hb

Blood was collected from healthy volunteers in EDTA tubes and used to prepare RBCs as described earlier (Nagababu et al. 2003). Hb, free of superoxide dismutase and catalase was prepared from fresh RBCs as previously described (Nagababu and Rifkind 1998) and stored at $-150^{\circ}C$.

Measurement of basal RBC fluorescence

Washed RBCs were used to adjust the Hb concentration to $50 \mu M$ as previously described (Nagababu et al. 2008a). This hemolysate was then divided into two aliquots. One aliquot was used to measure the fluorescence spectrum of the total hemolysate from 400 nm to 600 nm at an excitation wavelength (ex) of 321 nm using a Perkin Elmer model LS 50B spectrofluorimeter (Nagababu and Rifkind 1998). The ex and emission wavelength (em) slit

widths were kept at 10 nm. The second aliquot was centrifuged at 35,000 g for 40 min to pellet the membranes. The supernatant (referred to as cytosol) was separated and the fluorescence emission spectrum was measured as described above. The membrane pellet was washed two more times by suspending the pellet in 5 mM phosphate buffer to remove the bound Hb. The final pellet was resuspended in 5 ml of 50 μ M purified Hb or 5 ml of water and the fluorescence emission spectrum was determined, as described above.

Hydrogen peroxide induced fluorescence in cytosol and membranes

Catalase, GPX and peroxiredoxin in the cytosolic preparations (above) were inactivated by incubating with 0.1 mM sodium azide and 1 mM iodoacetamide at room temperature for 15 min in subdued light. The cytosolic fraction was then incubated with 1 to 5 μ M H_2O_2 at room temperature for 60 min. The fluorescent emission spectra were recorded at an ex of 321 nm. Similarly, membranes that are free of Hb or membranes that are associated with Hb were treated with the same concentration of inhibitors and then reacted with 8 to 40 μ M H_2O_2 for 60 min. The fluorescence emission spectra were recorded at an ex of 321 nm.

Shift of the maximum emission when the fluorescent product interacts with hemoglobin or heme

Hb (1 mM) was reacted with 5 mM H_2O_2 for 30 min and the emission spectrum was measured from 400 nm to 600 nm at an ex 321 nm. This reaction mixture was then filtered using 10,000 molecular weight cut off microfilter tubes (Rainin cat. No.7016-027). The emission spectrum of the Hb free filtrate was then measured at an ex of 321 nm.

Hemin (ferriprotoporphyrin) was dissolved in 20 mM sodium hydroxide and then centrifuged to remove undissolved crystals. The hemin concentration was determined from the absorbance at 385 nm using a mM extinction coefficient of 58.4. 50 μ M hemin in 50 mM phosphate buffer, pH 7.4 was reacted with 5 mM H_2O_2 [§] for 30 min at room temperature. The unreacted H_2O_2 was inactivated by adding 50 units of catalase and then filtered through a Whatman No.44 filter to remove any precipitation. The emission spectrum of the filtrate was measured from 400 nm to 600 nm at an ex of 321 nm. 50 μ l of 2 mM methemoglobin (metHb) or oxyhemoglobin (oxyHb) were added to 2 ml of the heme filtrate resulting in a Hb concentration of 50 μ M Hb and then the fluorescence emission spectrum was measured.

Transfer of heme degradation products from cytosol to membranes

RBCs (20% hct) in phosphate buffered saline (PBS) were incubated with 1 mM H_2O_2 for 1 hr in the presence of sodium azide (catalase inhibitor) at room temperature in the dark and the fluorescence in the hemolysate was measured at an ex of 321 nm. 1 ml aliquots of the RBC, azide and H_2O_2 reaction mixture were centrifuged at 1620 g after 1 h, 12 h, 24 h and 36 h to remove the PBS. The cell pellet was lysed in 1 ml of water and the membrane and cytosol were separated by centrifugation and the fluorescence spectra of the cytosol and membranes were measured as described above.

Heme degradation during in vitro aging of red cells

RBCs (20% hct) in PBS pH 7.4 were incubated at 37°C for 24 h. Cells were separated, lysed in water and the total fluorescence was measured as described above. The fluorescence was again measured in the same samples after removing the membranes by centrifugation at 35000 g. Membrane fluorescence was obtained by subtraction of the cytosol fluorescence from the total fluorescence.

[§]The heme degradation reaction is most efficient with Fe(II)-heme. However, we have shown that the same products can be formed with Fe (III)-hemin when a very large excess of H_2O_2 is used.

Heme degradation in human hemoglobin CC RBCs

Blood samples from homozygous HbCC and HbAA subjects were obtained and processed as previously described (Fairhurst et al. 2005). Blood collection was approved by the Institutional Review Boards of the National Institute of Allergy and Infectious Diseases, Bethesda, USA, and the University of Bamako, Bamako, Mali. Cytosol and membrane fluorescence was measured as described for heme degradation during in vitro aging of cells (see above).

Results

Comparison of RBC fluorescence intensity in the cytosol and on the membrane

The Hb concentrations of all samples were adjusted to 50 μ M before measuring the fluorescence, to maintain the same level of fluorescence quenching by Hb. Figure 1 compares the fluorescence emission spectra of hemolysate, cytosol with the membranes removed by centrifugation and the pelleted membrane to which is added 50 μ M pure Hb with no degradation products. As shown in Fig. 1, the cytosolic fraction has only 10% of the hemolysate fluorescence. The 90% fluorescent material removed by centrifugation was recovered in the membrane pellet when 50 μ M Hb was added to maintain the same level of Hb quenching present in the total hemolysate.

Membrane fluorescence associated with the reaction between hydrogen peroxide and hemoglobin

Our previous reports (Nagababu and Rifkind 1998) indicate that the reaction of Hb with H_2O_2 generates a fluorescent band with an ex of 321 nm. In RBCs, fluorescent products can also be generated by reactions of membrane lipids with ROS (Bidlack and Tappel 1973). To determine whether the fluorescence observed in the membrane fraction (Fig 1) is also attributed to the reaction of H_2O_2 with Hb, and not lipids, the fluorescence intensity was measured following the incubation with H_2O_2 of (1) cytosol (Hb fraction), (2) membranes where all the Hb was not removed, and (3) white ghosts where essentially all of the Hb has been removed from the membrane. As seen in figure 2A, the fluorescence readily increased in the cytosolic fraction with increasing concentrations of H_2O_2 . For Hb bound ghosts, the fluorescence also increased with increasing concentrations of H_2O_2 (Fig. 2B). However, incubation of white ghosts free of Hb with H_2O_2 resulted in only a slight increase in fluorescence for the initial dose of H_2O_2 , probably due to the presence of a small amount Hb still associated with the membranes. The addition of subsequent doses of H_2O_2 did not result in any further increase in fluorescence (Fig 2C). These results indicate that the fluorescence requires Hb and H_2O_2 and/or hydroperoxides. Support for the H_2O_2 reaction with Hb previously described (Nagababu and Rifkind 2004) taking place in RBCs is, thus, provided although the possibility of a Hb catalyzed lipid reaction cannot be ruled out.

Shifting of the maximum emission wavelength by methHb and oxyHb

The white ghosts shown in figure 2C had a basal fluorescent signal prior to the addition of H_2O_2 . A comparison of the maximum emission in figure 2C with that obtained in figures 1 and 2A where Hb was present indicates an appreciable shift of the maximum emission to 442 nm. To understand this, we studied the fluorescent emission spectrum of hemin (ferriprotoporphyrin) degraded by H_2O_2 (Fig. 3A). The maximum emission at 442 nm for an ex of 321 nm was similar to the emission band obtained for the membrane suspension (Fig. 2C). The maximum emission of the hemin degradation product at 442 nm was, however, shifted to 465 nm when methHb was added (Fig. 3A). Similarly, when oxyHb was added to the hemin degradation product, the maximum emission was shifted from 442 nm to 480 nm (Fig. 3A). These results demonstrate that Hb interactions with the fluorescent heme degradation product formed when hemin is degraded by H_2O_2 shift the emission to longer wavelengths.

We next wanted to see if the same Hb induced shift observed with the fluorescent product formed when hemin reacts with H_2O_2 is observed with the fluorescent product formed when Hb reacts with H_2O_2 . For this purpose 50 μ M oxyHb was reacted with 5 mM H_2O_2 for 30 min. The excess H_2O_2 in this reaction mixture completely oxidizes the xyHb to metHb. As reported earlier (Nagababu and Rifkind 1998), the maximum emission of this reaction mixture was observed at 465 nm (Fig. 3B). This em maximum was similar to that obtained when metHb was added to the hemin degradation product (Fig. 3A). The metHb was then removed from the reaction mixture by ultrafiltration, and the fluorescence of the filtrate (low molecular weight degradation products) determined. The filtrate maximum em was 442 nm (Fig. 3B), which is similar to the fluorescence maximum obtained for the degradation of hemin (Fig. 3A). These results imply that the same fluorescent product is produced when either hemin or Hb reacts with H_2O_2 . Furthermore, Hb not only quenches the fluorescence, but also shifts the emission wavelength.

Formation of RBC heme degradation products on the membrane

Figure 1 demonstrates that most of the degradation products generated in vivo are associated with the membrane. This accumulation of degradation products on the membrane can reflect heme degradation occurring at the surface of the membrane. However, heme degradation products can also be formed in the cytosol and then slowly transferred to the membrane. To determine whether heme degradation products formed in the cytosol are transferred to the membrane, a pool of heme degradation products in the cytosol was prepared.

Treatment of cells with H_2O_2 in the presence of azide, a catalase inhibitor, results in elevated levels of fluorescent products (Fig. 4). This pool of heme degradation was confirmed as being predominantly in the cytosol by separating the membranes from the cytosol. By centrifuging a fraction of this pool of RBCs with elevated heme degradation products in the cytosol at various times, it was shown that no heme degradation products are transferred from the cytosol to the membrane even after 36 h (Fig. 4). This experiment implies that the fluorescence found on the membrane in vivo (Fig. 1) was formed on the membrane.

Increase in membrane fluorescence during in vitro aging of red cells

Incubation of RBCs in PBS at 37°C for 24 h resulted in a 2 fold increase in the basal hemolysate fluorescence. All of the increased fluorescence during this incubation was located on the membrane fraction, but not in the cytosol (Fig. 5). These results support our previous studies (Nagababu et al. 2003) where we suggested that the role of GPX in preventing the accumulation of heme degradation products during in vitro incubation involved the reaction with H_2O_2 generated predominantly at membrane sites.

Elevated levels of heme degradation in hemoglobin CC RBCs more susceptible to oxidative stress are found on the membrane

We have reported that there is an increase in fluorescence in vivo for RBCs with less stable Hbs (Nagababu et al. 2008). To determine if the observed increase in basal fluorescence involves heme degradation products produced on the membrane, we compared the fluorescence in HbCC cells with normal HbAA cells. HbCC cells had a 40% higher fluorescence ($p < 0.013$) than HbAA cells (Fig. 6). When the cytosol was separated from the membranes, it was found that there was no significant difference between the cytosolic fluorescence of HbCC cells and HbAA cells. The entire 40% increase was found on the membrane.

Discussion

We have shown in our previous studies that the reaction of H_2O_2 with Hb produces a fluorescent band with an ex of 321 nm. A similar band was also observed in human RBC hemolysates that

increased in intensity under oxidative conditions. We have now demonstrated that 90% of the fluorescence found in the hemolysate is present in the membrane fraction (Fig. 1). The increase in fluorescence during in vitro aging of cells and for pathological RBCs was also found in the membrane fraction (Figs. 5 & 6).

The fluorescence found in the membrane can come from the oxidation of membrane lipids, the transfer of heme degradation products from cytosol to membrane or heme degradation taking place on Hb associated with the membrane. The involvement of membrane lipid oxidation that does not involve Hb was ruled out by the fact that no fluorescence was found when Hb deprived membranes were incubated with H_2O_2 (Fig. 2C). The involvement of Hb oxidation by H_2O_2 was evident by finding that the same fluorescent band was observed when cytosol (hemolysate without membranes) or Hb bound ghosts were incubated with H_2O_2 (Figs. 2A&B).

The possibility of fluorescent products being formed in the cytosol and then transferred to the membrane was ruled out by the results shown in Fig. 4. Our results, therefore, imply that the fluorescence found on the membrane is formed by the reaction of Hb bound to membranes with H_2O_2 or hydroperoxide. The formation of the degradation products on the membrane must involve H_2O_2 produced near the surface of the membrane that are, therefore, not effectively neutralized by the cytoplasmic antioxidant enzymes.

The role of the membrane in the formation of H_2O_2 and the resultant heme degradation products can be explained by the known binding of Hb to the cytoplasmic end of band 3 on the RBC membrane (Evans and Fung 1972; Shaklai et al. 1977). Since deoxyhemoglobin has a higher affinity for band 3 (Shaklai et al. 1977) than oxyHb, hypoxic conditions found in the microcirculation will favor binding to the membrane. Partial oxygenation of Hb that enhance binding to the membrane also dramatically increases the rate of Hb autooxidation (Rifkind et al. 1991). Autooxidation of Hb bound to the membrane produces a pool of H_2O_2 that is relatively inaccessible to catalase, a cytoplasmic enzyme. This pool of H_2O_2 will increase under hypoxic conditions with the increased binding of Hb to the membrane. Although H_2O_2 in the region of the membrane can be removed by peroxiredoxin and GPX (Low et al. 2008), the activity of these enzymes is dependent on the reducing power of NADPH/NADH, which declines with age of cells and for various pathological conditions. Under these conditions, the H_2O_2 reacts with nearby Hb producing heme degradation products, before it diffuses into the cytoplasm to react with catalase. This contention is further supported by our recent experiments measuring heme degradation after RBCs were perfused through lung venules. In this experiment higher levels of heme degradation were found for hypoxic RBCs than normoxic RBCs (Kiefmann et al. 2008).

During the course of this investigation, we observed that with an ex of 321 nm the fluorescence maximum emission of the membrane was 442 nm, while the hemolysate had a maximum emission of 485 nm (Fig. 2A). Furthermore, in our initial studies on the oxidation of Hb with H_2O_2 we reported a maximum fluorescence emission of 465 nm. It was necessary to determine if these fluorescent signals with different emission maxima could be attributed to the same species. This discrepancy in maximum emission was resolved by degrading the heme moiety of Hb with H_2O_2 . Interestingly, degradation of the heme moiety of Hb with H_2O_2 produced the maximum fluorescence emission at 442 nm (Fig. 3A) that is very similar to the fluorescence maximum found with membranes (Fig. 2B & C). The addition of methemoglobin or oxyHb to the heme fluorescent product shifted the maximum fluorescence emission to 465 nm and 485 nm, respectively (Fig. 3A). These results indicate that the original heme degradation product has a maximum emission at 442nm, but that this emission maximum is red shifted dependent on the conformation and/or oxidation state of Hb. A similar change in fluorescence has been reported when proteins are associated with fluorescence material. (Lakowicz 2000; Razem and Hill 2007)

Conclusion

The fluorescent heme degradation products found in the RBC are formed when Hb is associated with the membrane. This results from ROS that are not quenched by the predominantly cytosolic cellular antioxidant system, when they are in close proximity to the membrane. The formation of these heme degradation products on the membrane was confirmed by in vitro incubation studies and by in vivo studies with HbCC. In both cases the increase in heme degradation products were found exclusively on the membrane.

These studies establish a pathway for oxidative stress associated with hemoglobin autoxidation, despite the extensive antioxidant system in RBCs. These ROS formed at the membrane surface can be released from the RBC to affect nearby tissues and/or react with the RBC membrane altering RBC function and possibly contributing to the removal of RBCs from circulation.

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Abbreviations

CumOOH	Cumene hydroperoxide
ex	excitation
em	emission
GPX	glutathione peroxidase
H ₂ O ₂	hydrogen peroxide
hemin	Ferriprotoporphyrin
Hb	hemoglobin
metHb	methemoglobin; oxyhemoglobin (oxyHb)
PBS	Phosphate buffered saline
RBCs	red blood cells
ROS	reactive oxygen species

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Figure 1. Basal fluorescence emission spectra of RBCs

(Straight line) RBCs were lysed in water and the fluorescent emission spectrum of the hemolysate was measured with an ex of 321 nm after the hemoglobin concentration was adjusted to 50 μ M. (Dotted line) The hemolysate was then centrifuged and the fluorescent emission spectrum of the supernatant (cytosol) was measured. (Dashed line) The membrane ghosts were resuspended in the original volume with freshly purified 50 μ M hemoglobin and the fluorescence spectrum measured.

Figure 2. Fluorescence of cytosol and membranes treated with hydrogen peroxide

A) The cytosol was pre-incubated with 0.1 mM sodium azide and 1 mM iodoacetamide for 15 min at room temperature to inhibit antioxidant enzymes. Cytosol diluted to 50 μM Hb was then incubated with 0, 1, 2, 3, 4, and 5 μM H_2O_2 for 1 hr at 21°C followed by a determination of the fluorescent em spectrum at an ex of 321 nm. **B)** Membranes with residual hemoglobin bound or **C)** white membranes were incubated with 0, 8, 16, 24, 32, and 40 μM H_2O_2 for 1 hr at 21°C before measuring the fluorescent em spectrum. For all 3 conditions the inset shows the intensity of fluorescence versus the concentration of H_2O_2 as the mean of 3 independent experiments.

Fig. 3a

Fig. 3b

Figure 3. Shift in the fluorescent spectra of heme degradation products by metHb and oxyHb
A) 10 μ M hemin was reacted with 5 mM H_2O_2 for 30 min at 21°C and then filtered. The filtrate was diluted 4 fold with water (solid line), metHb (dashed line) or oxyHb (dotted line) and the emission spectrum was recorded at an ex of 321 nm. **B)** The em spectrum at an ex of 321nm was determined for 50 μ M Hb reacted with 5 mM H_2O_2 for 30 min at room temperature (solid line) and for the filtrate of the reaction mixture (Dotted line).

Figure 4. Fluorescence intensity of cytosol and membranes following the reaction of RBCs with hydrogen peroxide

RBCs (20% hct) in PBS were reacted with 1 mM azide and 1 mM H_2O_2 at $21^\circ C$ for 60 min. Total fluorescence was determined in hemolysate. After 1 hr, 12 hrs, 24 hrs and 36 hrs the membranes and cytosol were separated. The fluorescence of the cytosol was measured with ex 321 nm and em 465 nm. The membrane fraction was adjusted to the original volume and its fluorescence was measured with ex 321nm and em 442 nm.

Figure 5. Increase in fluorescence of red cells during in vitro aging

RBCs (20% hct) in PBS were incubated at 37°C for 24 hours. Cells were separated by centrifugation and the fluorescence of the hemolysate and cytosol was measured as mentioned in the legend to Fig. 1. Membrane fluorescence was determined by subtraction of the fluorescence in the cytosol from the hemolysate.

Figure 6. Increase in heme degradation of RBC of hemoglobin CC disease

The fluorescence intensity (ex 321 nm; em 480nm) was measured in membrane and cytosol components as mentioned in the Methods section. Values are mean $\pm$ SD of 5 HbCC subjects and 3 HbAA subjects. * Significantly different from HbAA determined by Students *t*-test ($p < 0.01$).