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Research article

The influence of ferrylhemoglobin and methemoglobin on the human erythrocyte membrane

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The aim of the study was to examine and compare the effects of methemoglobin (metHb) and ferrylhemoglobin (ferrylHb) on the erythrocyte membrane. Kinetic studies of the decay of ferrylhemoglobin (*HbFe(IV)=O denotes ferryl derivative of hemoglobin present 5 min after initiation of the reaction of metHb with H_2O_2; ferrylHb) showed that autoredecay of this derivative is slower than its decay in the presence of whole erythrocytes and erythrocyte membranes. It provides evidence for interactions between ferrylHb and the erythrocyte membrane. Both hemoglobin derivatives induced small changes in the structure and function of the erythrocyte membrane which were more pronounced for ferrylHb. The amount of ferrylHb bound to erythrocyte membranes increased with incubation time and, after 2 h, was twice that of membrane-bound metHb. The incubation of erythrocytes with metHb or ferrylHb did not influence osmotic fragility and did not initiate peroxidation of membrane lipids in whole erythrocytes as well as in isolated erythrocyte membranes. Membrane acetylcholinesterase activity increased by about 10% after treatment of whole erythrocytes with both metHb and ferrylHb. ESR spectra of membrane-bound maleimide spin label demonstrated minor changes in the conformation of label-binding proteins in ferrylHb-treated erythrocyte membranes. The fluidity of the membrane surface layer decreased slightly after incubation of erythrocytes and isolated erythrocyte membranes with ferrylHb and metHb. In whole erythrocytes, these changes were not stable and disappeared during longer incubation.

Keywords: Hemoglobin, methemoglobin, ferrylhemoglobin, erythrocyte membrane

INTRODUCTION

Hemoglobin (Hb) is a heme-containing protein which binds oxygen reversibly and transports it to tissues. About 3% of Hb in the human body autoxidizes to methemoglobin (metHb) every day.[1] MetHb can react with H_2O_2, which is formed in the circulation by dismutation of the superoxide radical anion liberated during autoxidation of oxyhemoglobin (oxyHb),[2,3] activation of phagocytes,[4] and during metabolism of some xenobiotics.[5] This reaction generates two oxidizing species: ferrylhemoglobin which contains the oxoferryl complex (iron in the +4 oxidation state with an oxo-group as the sixth ligand) and the protein radical centered probably on the αTyr-42 residue.[6–11] The protein radical decays rapidly by intramolecular or intermolecular electron transfer and/or reacts rapidly with molecular oxygen generating a peroxyl radical.[9,12] Peroxyl radicals may, in turn, form more stable protein peroxides.[13]

The protein-based free radical in frozen blood has been shown to have the same spectroscopic properties as the signal of the globin-based free radical formed in the reaction of purified methemoglobin with H_2O_2 (•HbFe(IV)=O).[6] It indicates that, in spite of the presence of enzymes (catalase and glutathione peroxidase), the physiological steady-state concentration of hydrogen peroxide in erythrocytes reported[14] to be 5×10^{-11} M is high enough to bring about detectable oxidation of metHb to ferrylHb.

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In this study, we investigated the influence of *HbFe(IV)=O and metHb on the erythrocyte membrane in a whole-cell system and in preparations of isolated erythrocyte membranes. The ferryl derivatives of hemoglobin have been chosen for this study since their interaction with the erythrocyte membrane has been suggested to occur in a variety of physiological and pathophysiological states, including blast-pressure injury, ischemia-reperfusion and cerebral hemorrhage.[15–17] Moreover, the interaction of ferryl derivatives of hemoglobin with cells and their constituents has been rarely studied and is much more poorly understood than that of myoglobin derivatives.[18–21]

Materials and Methods

Materials

Erythrocytes, erythrocyte membranes and hemoglobin were obtained from blood of healthy donors. 4-Maleimido-tempo spin label (MSL) and 4′-trimethylammonio-1,6-diphenyl–1,3,5-hexatriene (TMA-DPH) fluorescence probe were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). All other chemicals were of analytical grade and were purchased from POCh (Gliwice, Poland). All solution were made with water purified by the Milli-Q system.

Preparation of erythrocyte suspension

Blood was centrifuged at 600 *g* for 10 min. After removal of the plasma and buffy coat, the erythrocytes were washed three times in 0.1 M phosphate buffer, pH 7.4, and suspended in the same medium.

Membrane preparation

Erythrocyte membranes were obtained by hypotonic lysis in 20 mM Na-phosphate buffer, pH 7.4. The lysed erythrocytes were washed successively by centrifugation at 13,000 *g* with 20 mM, 10 mM, and 5 mM phosphate buffer. All solutions used were cooled to 4°C.[22] Protein concentration was estimated by the method of Lowry *et al*.[23] using bovine serum albumin as the standard.

Hemoglobin and methemoglobin

Hemoglobin was isolated according to Drabkin[24] and purified by ion-exchange chromatography on carboxymethylcellulose CM-32 (Pharmacia) as described earlier.[25] Methemoglobin was prepared by oxidizing Hb with a 1.5-fold excess of $K_3Fe(CN)_6$ and purified on a Sephadex G-25 column using a 0.1 M phosphate buffer (pH 7.4) as the eluent. Concentrations of Hb and metHb were determined spectrophotometrically after conversion into the cyanmet form, assuming the absorption coefficient of 44,000 $dm^3mol^{-1}cm^{-1}$ (with respect to the tetramer). Absorption spectra of metHb and ferrylHb were recorded in the range of 490–650 nm. All spectrophotometric measurements were carried out at 25°C in a CARY-1 apparatus (Varian, Melbourne, Australia).

Formation of ferrylhemoglobin

FerrylHb was prepared by incubating metHb (2 mg/ml) with an equimolar amount of H_2O_2, in relation to the quantity of Fe, in 0.1 M phosphate buffer (pH 7.4), at 25°C for 5 min (a sample containing metHb (2 mg/ml) was always used in parallel).

Incubation of erythrocytes and erythrocyte membranes with ferrylhemoglobin

Erythrocytes (final hematocrit of 1%) or erythrocyte membranes (final membrane protein concentration equal to 0.1 mg/ml) were added to the metHb and ferrylHb and placed in a water bath (temperature of 25°C) for 10, 20, 30, 60, 120 and 180 min. After incubation, the erythrocytes or erythrocyte membranes, respectively, were centrifuged and washed 3 times with 0.1M phosphate buffer, pH 7.4, to remove ferrylHb or metHb and used for analyses.

The results are expressed as percentages of the values obtained for intact erythrocytes or erythrocyte membranes incubated in 0.1 M phosphate buffer, pH 7.4 in the absence of metHb and ferrylHb.

Rate constant of ferrylHb reduction

The decay of ferrylHb in the absence and in the presence of intact erythrocytes and erythrocyte membranes was monitored by the measurement of absorbance at 575 nm (maximum of the absorption band characteristic for ferrylHb), at 25°C for 20 min.

Measurement of the amount of membrane-bound Hb derivatives

Erythrocyte membranes, incubated with ferrylHb or metHb and washed with buffer (as described above) were dissolved in 3% solution of sodium dodecyl sulfate (SDS). After 10-min incubation at ambient temperature, absorption spectra were taken and absorbance at 405 nm was read from the spectra.

Acetylcholinesterase activity

Acetylcholinesterase activity was determined according to the colorimetric procedure of Ellman *et al*.[26]

Lipid peroxidation

Lipid peroxidation was estimated by measurement of absorbance of thiobarbituric acid-reactive materials at 532 nm.[27]

Erythrocyte membrane fluidity

Erythrocyte membrane fluidity was estimated using the fluorescent probe TMA-DPH, which localizes near the membrane surface. Fluorescence measurements were performed in a Perkin-Elmer Luminescence Spectrometer LS50B at λ_{ex} 358 nm and λ_{em} 428 nm.

Changes in membrane protein conformation

Changes in the conformation of membrane proteins were examined by an ESR method using a maleimide spin label (MSL), which binds covalently to sulfhydryl groups of erythrocyte membrane proteins. The h_w/h_s ratio (ratio of heights of signals of ESR spectra of the bound label, corresponding to weakly and strongly immobilized spin label residues, respectively) gives information about the environment of –SH groups of proteins binding MSL Fig. 4A).

Erythrocyte membranes, incubated with ferrylHb or metHb and washed with buffer (as described above) were labelled by MSL (1 h, 4°C, with shaking), and washed three times with 0.1 M phosphate buffer, pH 7.4, to remove unbound MSL. ESR spectra were measured at room temperature using a Bruker 300E ESR spectrometer. Typical operating conditions were: microwave frequency, 9.74 GHz; modulation amplitude, 2.0 G; gain 2×10^4; time constant, 20.5 ms; and scan range 3733–3812 G.

Statistical analysis

Data were analysed by the paired Student's *t*-test. Statistical significance was assumed for *P*-values < 0.05.

Results and Discussion

Hemoprotein-mediated oxidative stress is thought to play a major role in the pathophysiology of cerebral hemorrhage, blast-pressure injury, myocardial ischemia-reperfusion injury and rhabdomyolysis.[15–17] These conditions are generally characterized by the release of Hb or myoglobin by red blood cell hemolysis or muscle destruction, respectively. Red blood cells themselves are important targets for vascular damage resulting from the

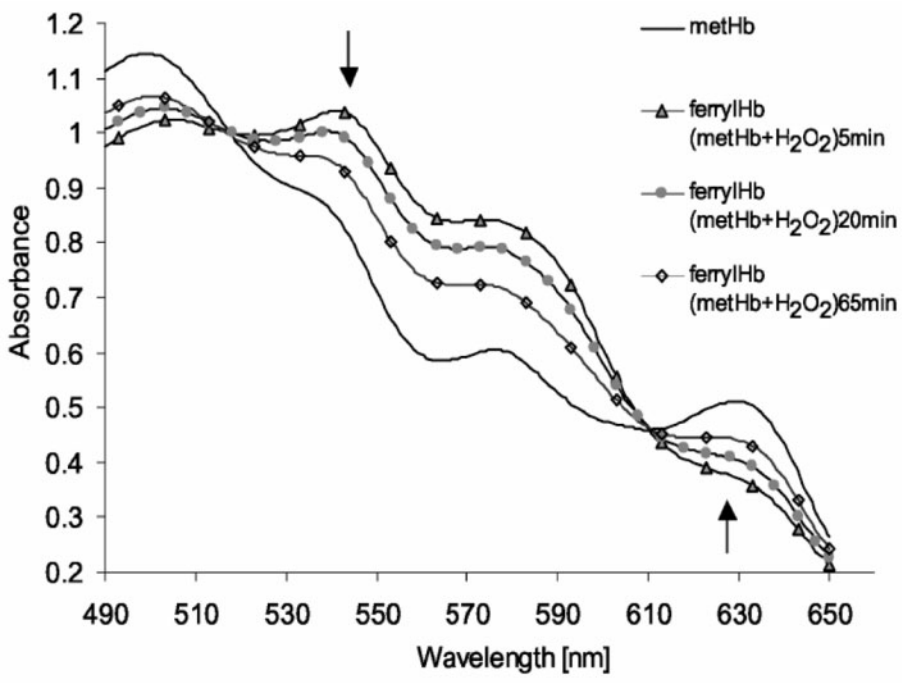


Fig. 1. Absorption spectra of metHb and spectra of ferrylHb after different incubation times.

Table 1. Rate constants for ferrylHb reduction in the absence and in the presence of whole erythrocytes or erythrocyte membranes

	Rate constant [s^{-1}]	*P*-value
Autoreduction (s^{-1})	$2.42 \pm 0.12 \times 10^{-4}$	
Reduction in the presence of intact erythrocytes (s^{-1})	$3.67 \pm 0.21) \times 10^{-4}$	< 0.001
Reduction in the presence of erythrocyte membranes (s^{-1})	$3.43 \pm 0.18 \times 10^{-4}$	< 0.002

P, statistical significance of differences between the rate constant for autoreduction and of reduction in the presence of whole erythrocytes or erythrocyte membranes.
Reaction rate constants for reduction in the presence of whole erythrocytes and erythrocyte membranes, respectively, are not significantly different ($P > 0.05$).

presence of hemoproteins inside erythrocytes and in the extracellular environment.

The ferryl derivative of hemoglobin is a product of reaction of methemoglobin with H_2O_2. Two-electron oxidation of metHb results in a transition of the heme iron from the III to IV oxidation state and formation of a radical within the heme group, most probably within the system of conjugated bonds yielding a cation radical of the ferryl derivative of hemoglobin ($^{\bullet}HbFe(IV)^{+}=O$). In the next step, a ferryl derivative with the free radical localized on the protein part of the molecule ($^{\bullet}HbFe(IV)=O$) is formed via intramolecular radical transfer from the heme moiety.[6–10] The radical located on the protein moiety decays due to intra- and intermolecular transfer leaving a 'longer-living' ferrylhemoglobin form with a modified protein moiety (*HbFe(IV)=O).[6–9]

Treatment of metHb with an equimolar (with respect to the quantity of Fe) amount of H_2O_2 is associated with spectral changes in the range of 490–650 nm (Fig. 1). The maximal concentration of ferrylHb was observed 4–5 min after the addition of H_2O_2. At this point, about 95–97% metHb was oxidized to the *HbFe(IV)=O form, and the majority of radicals localized on the protein moiety decayed. It has been estimated that the half-life of protein radicals formed by reaction of metHb with H_2O_2 is 50 s.[9] Svistunenko[28] showed that, under similar conditions, a considerable fraction of these radicals is subject to rapid decay and only a small fraction of peroxyl radicals localized on Trp-37 of the β-subunit persists 5 min after initiation of the reaction. So, 5 min after the addition of H_2O_2 to metHb, the main reactive products of the reaction are ferryl derivatives of hemoglobin, with modified protein moiety (*HbFe(IV)=O), protein hydroperoxides and a small amount of the above-mentioned peroxyl radicals. In the present study, we evaluated the effect of these products on some properties of whole erythrocytes and of isolated erythrocyte membranes. Erythrocytes or erythrocyte membranes were added to the metHb + H_2O_2 system 5 min after initiation of the reaction. Such a procedure excluded the effect of H_2O_2

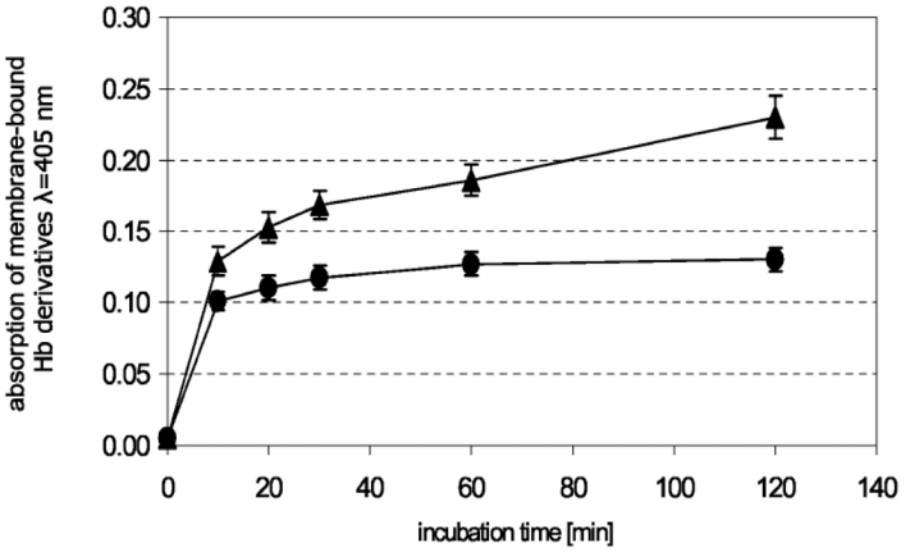


Fig. 2. Dependence of the amount of membrane-bound hemoglobin derivatives on the time of incubation of erythrocyte membranes (0.1 mg protein/ml) with metHb or ferrylHb for 10–120 min. Filled circles, metHb; filled triangles ferrylHb.

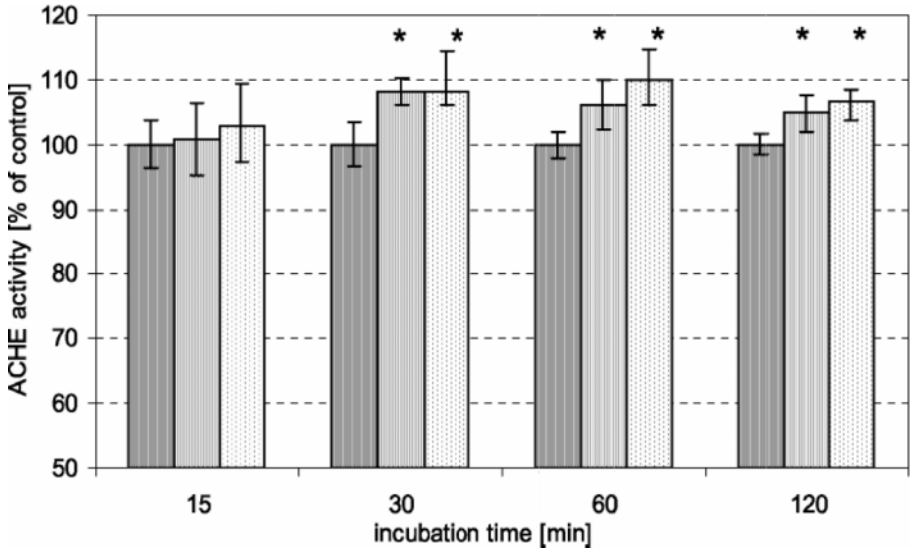
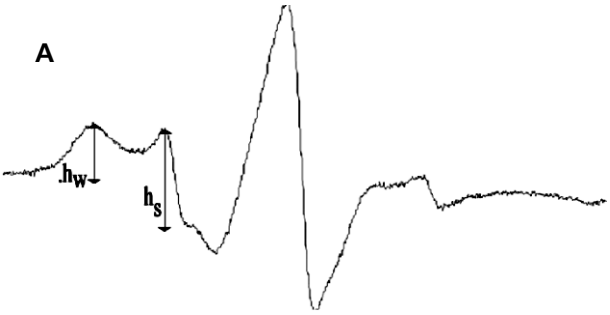


Fig. 3. Dependence of acetylcholinesterase activity of erythrocytes (hematocrit of 1%) on the time of incubation with hemoglobin derivatives. Results represent the mean value ± SD from 5–7 separate measurements. Statistical significance was analysed by the paired Student's *t*-test. The asterisks indicate differences significant at $P < 0.05$. For each time point: left, control; centre, metHb; and right, ferrylHb.



on the erythrocyte membranes (hydrogen peroxide was completely decomposed in the reaction with metHb after 5 min).

The ferryl derivatives of Hb are subject to spontaneous autoreduction to metHb, which is reflected in the absorption spectra (Fig. 1). Addition of either intact erythrocytes or erythrocyte membranes to ferrylHb led to an increase of the decay rate of ferrylHb. The rate of ferrylHb reduction in the presence of whole erythrocytes and erythrocyte membranes was 1.5 times greater than in

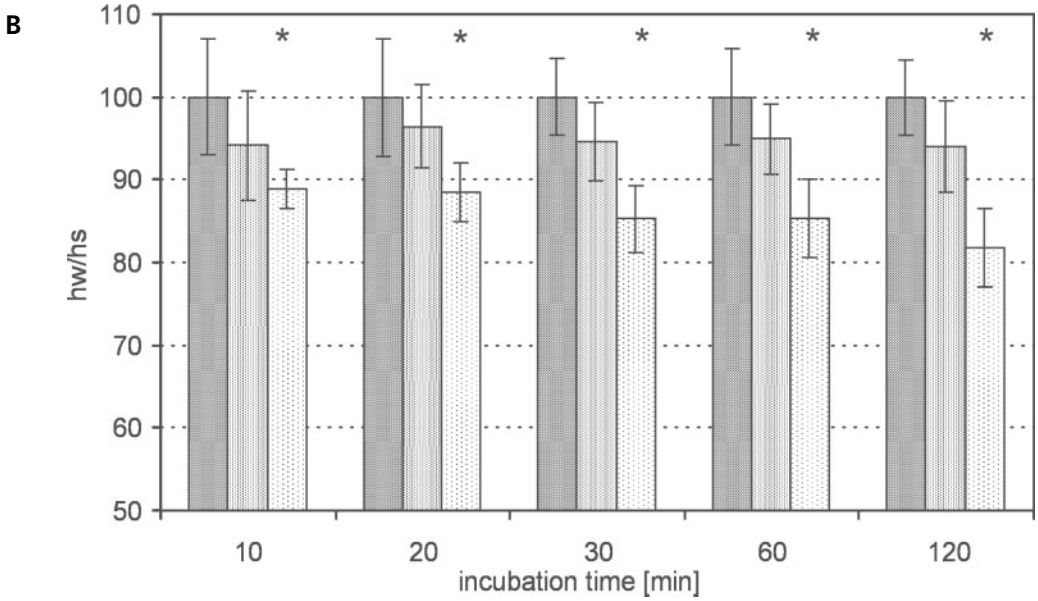


Fig. 4 (A) Typical ESP spectrum of maleimide-labelled erthyrocyte membranes. (B) Dependence of the h_w/h_s ratio of MSL on the time of incubation of erythrocyte membranes (0.1 mg protein/ml) with hemoglobin derivatives. Statistical analysis performed as in caption to Figure 3. For each time point: left, control; center, metHb; and right, ferrylHb.

the absence of these additives (Table 1). This result demonstrates interactions between ferrylHb and the erythrocyte membrane.

In order to check whether this interaction results in binding of the ferrylHb to the membrane, the amount of Hb bound to the membranes after the incubation was estimated. The amount of ferrylHb bound to the membranes increased with the incubation time and, after 2 h, was 2 times higher compared to the met-derivatives (Fig. 2). A fraction of hemoglobin might have been trapped in vesicles resealing during the incubation; however, in such a case, the amounts of ferrylHb and metHb trapped should not differ. Since significantly more ferrylHb was found to be membrane-bound, this must be due to increased binding of the derivative to the membrane, rather than trapping.

Acetylcholinesterase is a protein localized at the outer surface of the erythrocyte and is, therefore, especially prone to the action of exogenous oxidizing agents, and may be a sensitive index of changes in membrane properties. A slight increase in acetylcholinesterase activity was found in erythrocytes treated with both metHb and ferrylHb (Fig. 3). After 60- and 120-min incubations, a slightly higher activity increment was observed for preparations treated with ferrylHb (as compared with metHb). This increase in acetylcholinesterase activity might

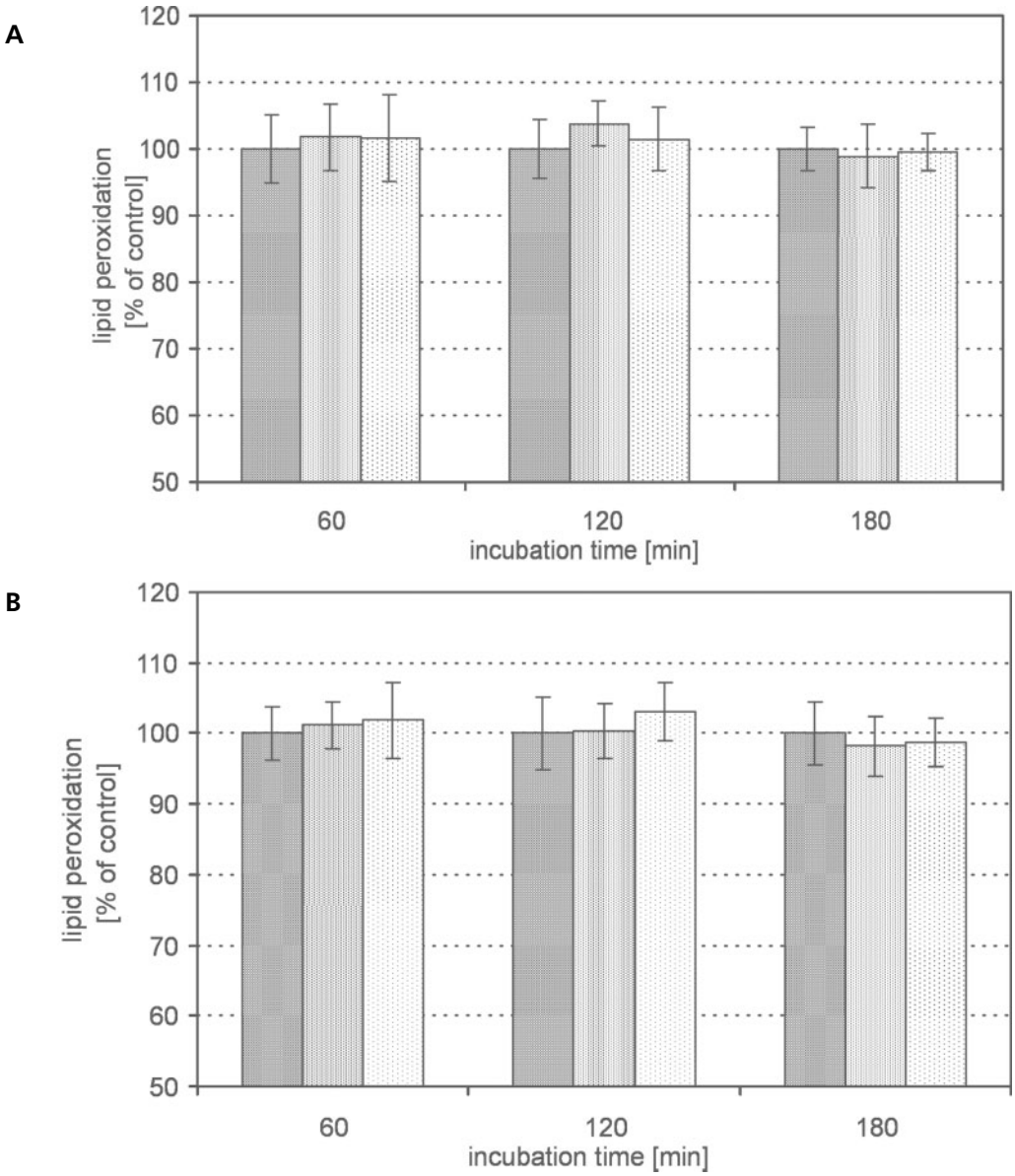


Fig. 5. Dependence of lipid peroxidation on the time of incubation of (A) erythrocytes (hematocrit of 1%) and (B) erythrocyte membranes (0.1 mg of protein/ml) with hemoglobin derivatives. Statistical analysis performed as in caption to Figure 3. For each time point: left, control; center, metHb; and right, ferrylHb.

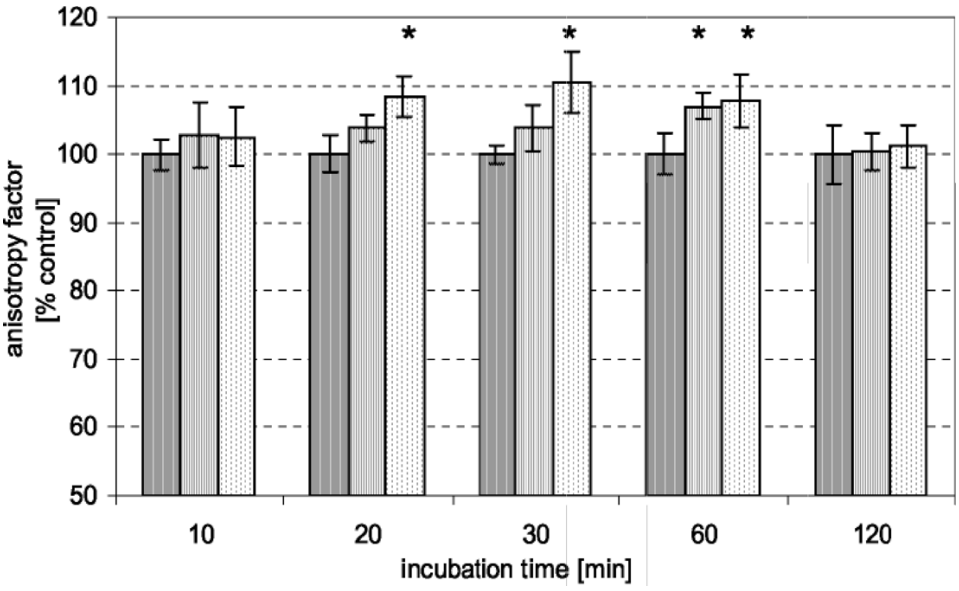


Fig. 6. Dependence of fluorescence anisotropy of TMA-DPH on the time of incubation of erythrocytes (hematocrit of 1%) with hemoglobin derivatives. Statistical analysis performed as in caption to Figure 3. For each time point: left, control; center, metHb; and right, ferrylHb.

be due to a change in membrane surface charge, caused by binding of the hemoglobin derivatives to the membrane.[29]

In order to examine changes in the structure of membrane proteins, a maleimide spin label binding covalently to –SH groups of membrane proteins was employed. Changes in the h_w/h_s ratio of ESR spectra of the protein-bound spin label (corresponding to the ratio of weakly and strongly immobilized, respectively, spin label residues) reflect alterations in the micro-environment of the label.

The h_w/h_s ratio decreased in the course of incubation of ferrylHb with erythrocyte membranes by up to 20% after 120-min incubation (Fig. 4B). This observation is consistent with our previous finding that the h_w/h_s ratio is an index of oxidative modification of proteins.[30] The decrease of the h_w/h_s ratio was much lower in preparations incubated with metHb under identical experimental conditions, and did not change during incubation.

Many studies document that both ferrylHb and ferrylMb may initiate lipid peroxidation in model membranes and lipid emulsions.[8,31,32] Our previous study showed that ferrylmyoglobin initiates lipid peroxidation in isolated erythrocyte membranes but not in whole erythrocytes.[33] The present results indicate that ferrylHb and metHb do not cause any peroxidation of membrane lipids in intact erythrocytes as well as in isolated erythrocyte membranes (Fig. 5). This indicates that ferrylMb is a stronger pro-oxidant species than ferrylHb with respect to erythrocyte membrane lipids.

We studied also the effect of oxidized hemoglobin derivatives on the membrane lipid fluidity using the TMA-DPH fluorescent probe. This probe intercalates membrane phospholipids and changes in its fluorescence anisotropy reflect modifications of the fluidity of membrane lipid bilayer. Incubation of erythrocytes with ferrylHb resulted in a slight increase of TMA-DPH anisotropy, which indicated a decrease of membrane fluidity (Fig. 6). The highest value of the anisotropy was observed after 30 min of incubation. Membranes treated with metHb showed a somewhat lower increase in anisotropy. Longer incubation times (120 min) restored the control value of fluorescence anisotropy. The same studies performed with isolated erythrocyte membranes showed that both ferrylHb and metHb caused a similar rise of anisotropy factor which was not reversed during prolonged incubation (Fig. 7). Changes in membrane fluidity may be due to association of ferrylHb and metHb molecules with membrane. Antioxidants present in whole erythrocytes (*e.g.* ascorbate, glutathione, α-tocopherol) could reduce the oxidized hemoglobin derivatives studied and thus cause their dissociation from membranes; this phenomenon could be responsible for the reversal of changes in fluorescence anisotropy of TMA-DPH upon prolonged incubation.

Incubation of erythrocytes with ferrylHb and metHb did not affect their osmotic fragility (not shown).

FerrylMb have been shown to oxidize various substrates, *e.g.* linoleate, low-density lipoproteins, styrene, glutathione and isolated erythrocytes membranes[8,31–33] and inactivate several enzymes. The latter effect has been attributed to oxidation of –SH groups of enzymatic proteins.[18,19]

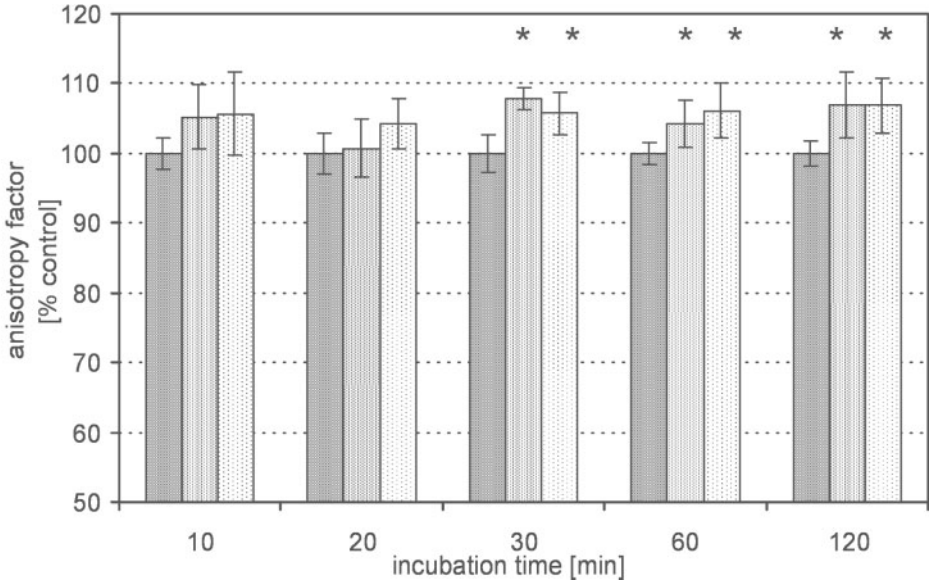


Fig. 7. Dependence of fluorescence anisotropy of TMA-DPH on the time of incubation of erythrocyte membranes (0.1 mg of protein/ml) with hemoglobin derivatives. Statistical analysis performed as in caption to Figure 3. For each time point: left, control; center, metHb; and right, ferrylHb.

CONCLUSIONS

Results presented in this paper demonstrate that oxidized forms of hemoglobin induce small, but detectable, changes in the structure and property of the membrane of erythrocytes and of isolated erythrocyte membranes. The ferryl derivative of hemoglobin is more active in initiation of these changes than metHb. This difference can be attributed, at least partly, to the stronger association of ferrylHb, than metHb, with the erythrocyte membrane.

Our study shows, moreover, that with respect to the erythrocyte membrane, ferrylHb is a weaker pro-oxidant species than ferrylMb, which can initiate high lipid peroxidation in isolated erythrocyte membranes. These differences in the reactivity of the ferryl forms of both proteins result apparently from the different size and the quaternary structure of hemoglobin.

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