Short Communication

The Influence of Oxidative Stress on Microviscosity of Hemoglobin-Containing Liposomes

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Abstract. Encapsulation of hemoglobin (Hb) within a liposome is one of the strategies in the development of artificial oxygen carriers. In this study the effects of oxygen radical generating system (xantine/xantine oxidase) on the internal microviscosity and protein degradation of hemoglobin-containing liposomes ('hemosomes') prepared from dipalmitoylphosphatidylcholine (DPPC) and different amounts of cholesterol (Ch) (0–0.5 mol/mol) were investigated. The results demonstrated a direct relationship between increasing oxidant stress and microviscosity of Hb vesicles and also showed clearly that the increase in internal viscosity was caused mainly by globin degradation. It was shown that the higher content of Ch, the lower Hb degradation and smaller increase in internal viscosity were observed. The significant protection effect against oxygen radicals was observed only for liposomes with the addition of 0.3 mol/mol or more of Ch. It seems that Ch concentration in liposomes is of prime importance for stabilizing of Hb in 'hemosomes'.

Key words: Hemoglobin oxidation — Protein denaturation — Internal viscosity — Liposome

Encapsulation of hemoglobin (Hb) within a liposome is one of the strategies in the development of artificial oxygen carriers. It maintains the oxygen transporting properties of Hb and eliminates the side effects of free Hb. Some authors have shown that encapsulated Hb ('hemosomes') have many properties of the red cells and they proved to be non-immunogenic and capable of transporting oxygen (Djordjevich and Miller 1980; Izumi et al. 1997).

After intravenous administration, circulating 'hemosomes' are constantly exposed to both intracellular and extracellular sources of oxygen radicals. Within 'hemosomes' the autoxidation of oxyhemoglobin to methemoglobin leads to concomitant production of superoxide radical ($^{\bullet}O_2^-$) (Gaber et al. 1983). The extracellular sources of oxygen radicals include granulocytes, macrophages and other

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metabolically active cells which generate hydrogen peroxide (H_2O_2) and $^{\bullet}O_2^-$ (Weiss 1980). Another problem in clinical trials with encapsulated Hb is reperfusion injury due to oxygen radicals generated by xantine-xantine oxidase (X-XO) system (Chang 2000). Therefore, as 'hemosomes' may play a role of blood substitute, the maintaining of Hb inside them in functional form is of prime importance.

A simple, well-controlled parameter describing Hb stability inside the red cell or liposomes is internal viscosity or microviscosity. Intracellular viscosity is one of the major factors determining erythrocyte deformability. Decreased deformability and increased internal viscosity of the red blood cell contribute to abnormal flow in the microcirculation.

The aim of this study was to determine the microviscosity of Hb solutions encapsulated in liposomes of different lipid composition exposed to oxidative stress induced by X-XO system.

All chemicals used in experiments were purchased from Sigma Chemicals Co. (St. Louis, MO, USA).

Concentrated erythrocyte hemolysate ($30 \pm 2\%$) was prepared by lysis and membrane extraction of the washed red cells using equal volume of tetrachloroethylene (Szebeni et al. 1984). Hb concentration was measured by cyanomethemoglobin method. Multilamellar vesicles were prepared from dipalmitoylphosphatidylcholine (DPPC) (30 mmol) with various amounts of cholesterol (Ch) (0–0.5 mol/mol) by the film method. Briefly, the desired amount of membrane components was dissolved in chloroform and dried by a rotary evaporator in a round-bottom flask to obtain a thin film on the wall. The solvent was removed completely under reduced pressure. A solution of erythrocyte hemolysate (2 ml) was incorporated into the liposomes during hydration at 42℃ with subsequent vigorous shaking (30 min) to obtain 'hemosomes'. As has been shown by Szebeni et al. (1984), using freeze-fracture electron micrographs, this kind of 'hemosomes' showed mainly multilamellar structures, irrespective of their lipid composition.

Mixtures of 1.6 ml 'hemosomes' suspension (10% v/v) and 1.6 ml phosphate buffer (PBS) were incubated (with vigorous shaking) with or without the oxygen radical generating system: 2.5 ml xantine and 0.2 units of xantine oxidase (grade III from buttermilk), extensively dialysed against PBS prior to use. To terminate the reaction, the samples were placed in ice for 5 min.

Alanine content, as an index of protein degradation, was measured enzymatically using a procedure described by Davies and Goldberg (1987).

The viscosity of Hb solution encapsulated in 'hemosomes' (internal viscosity) was determined by the use of electron paramagnetic resonance (EPR) spectroscopy, according to the method of Morse (1977), based on the rotational mobility of the spin probe 2-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPAMINE). After incubation in X-XO system, 'hemosome' samples were washed three-times and resuspended in aqueous solution of TEMPAMINE (1 mmol/l). After 10 min incubation and subsequent centrifugation at $2000 \times g$ the cell sediment was suspended in potassium ferricyanide (120 mmol/l), to quench the extracellular signal of TEMPAMINE. The EPR spectra of labeled 'hemosomes' were recorded at 37℃ on EPR

X-band (9.4 GHz) Radiopan spectrometer (modulation frequency of 100 kHz, scan time was 4 min, time constant was 0.3 s). All spectra showed narrow equally-spaced lines, indicative of a highly mobile species in a low viscosity regime, and eliminating the possibility of label binding to the cell membrane lipids, or to Hb molecules. From the EPR spectra rotational correlation time τ_c of the spin probe was calculated according to the standard formula for isotropic high mobility regime measurements at X-band (Morse 1977):

$$\tau_c = 6.5 \cdot 10^{-10} \cdot \Delta H \left[\sqrt{\frac{h_0}{h_{-1}}} - 1 \right]$$

where ΔH, h_0 and h_{-1} are mid-field line width, mid-field line height and high-field line height, respectively. The rotational correlation time is closely related to microviscosity of surrounding by the well-known Debay formula. The rotational correlation time of TEMPAMINE was measured as a function of viscosity in water/glycerol mixtures at 20°C. Over the whole range of viscosity investigated (up to 15 cP) a straight line was obtained (not shown).

Statistical comparisons were analysed by Student's paired t-test; correlation coefficients were computed by linear regression analysis. Values are expressed as mean ± S.D.

The addition of either XO or X alone did not change the internal viscosity and failed to enhance alanine production in the course of observations (5 hours). Exposure of 'hemosomes' prepared only from DPPC to X-XO has caused a marked time-dependent increase in internal viscosity of vesicles (Fig. 1) and dramatic increase in alanine production (7.08 nmol·ml^{-1}·h^{-1}) (Fig. 2). The addition of Ch at concentrations 0.3 and 0.5 mol/mol to DPPC liposomes had caused statistically significant decrease in microviscosity (Fig. 1) and rate of alanine production (4.73 nmol·ml^{-1}·h^{-1}, $p < 0.05$; 3.63 nmol·ml^{-1}·h^{-1}, $p < 0.05$, respectively) compared to DPPC vesicles. The addition of Ch at lower concentrations (0.1 and 0.2 mol/mol) had no influence on internal microviscosity and rate of alanine production (data not shown). A strong correlation between the rate of alanine production and microviscosity was observed (for pure DPPC liposoms: $r = 0.91$, $p < 0.05$; for DPPC/Ch 0.3 mol/mol: $r = 0.92$, $p < 0.05$ and for DPPC/Ch 0.5 mol/mol: $r = 0.95$, $p < 0.05$).

In the present study oxygen radical generating system (X-XO) has been employed in order to check the effects of free radicals on internal viscosity of hemoglobine-containing liposomes ('hemosomes'). It was shown that the exposure of 'hemosomes' to X-XO led to time-dependent increase in internal microviscosity of vesicles, which was connected with degradation of Hb molecules. A strong agreement between the increase in microviscosity and protein degradation indicates that Hb damage is the most important reason of observed increase in internal viscosity of 'hemosomes' exposed to oxidative stress. Using EPR spin labelling technique, Gennaro et al. (1996) have shown that the internal viscosity of normal erythrocyte depends only on Hb concentration inside the cell. Present results show that also

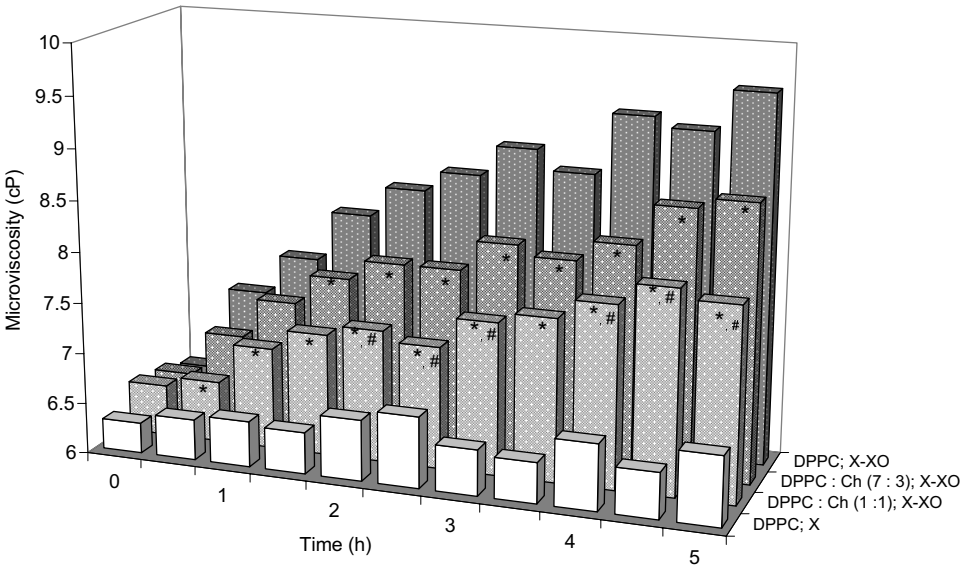

Figure 1. Time course of internal microviscosity of 'hemosomes' incubated in the presence of xantine oxidase (XO). Suspensions (5% v/v) of 'hemosomes' composed of DPPC with various amounts of Ch were incubated for varying periods of time in the presence of 2.5 mmol/l xantine (X). Where indicated, 0.2 units of XO were added. The final volume of each reaction tube was 3.2 ml. In the absence of XO, mean microviscosity value observed in the course of experiment (5 hours) was 6.5 ± 0.13 cP. Values are the means of five independent determinations; * significantly different from values for 'hemosomes' composed of pure DPPC, * $p < 0.05$; # significantly different from values for 'hemosomes' composed of DPPC and Ch (7 : 3), $^{\#}$ $p < 0.05$.

stability of Hb molecules inside the cell is a very important factor determining internal microviscosity.

X-XO generates mainly $^{\bullet}O_2^-$ and H_2O_2. Hydrogen peroxide can cross the lipid membrane and reacts rapidly with Hb generating a very reactive free radical species, e.g., hydroxyl radical ($^{\bullet}OH$) leading to degradation of Hb molecules (Van Den Berg et al. 1992). Gross structural changes of oxidatively modified protein results in increased proteolytic susceptibility. Therefore, in cells subjected to oxidative stress an increase in protein degradation by intracellular ATP-independent proteolytic system occurs and leads to releasing of free amino acids, that can be used as markers. Alanine production was shown to be a sensitive and convenient indicator of the overall rate of proteolysis (Davies and Goldberg 1987). The present study demonstrated that X-XO-treated 'hemosomes' induced time-dependent alanine production, indicating increased Hb degradation.

The increase in microviscosity of Hb solution exposed to oxygen radicals can be explained by the fact that concomitant protein degradation results in formation of

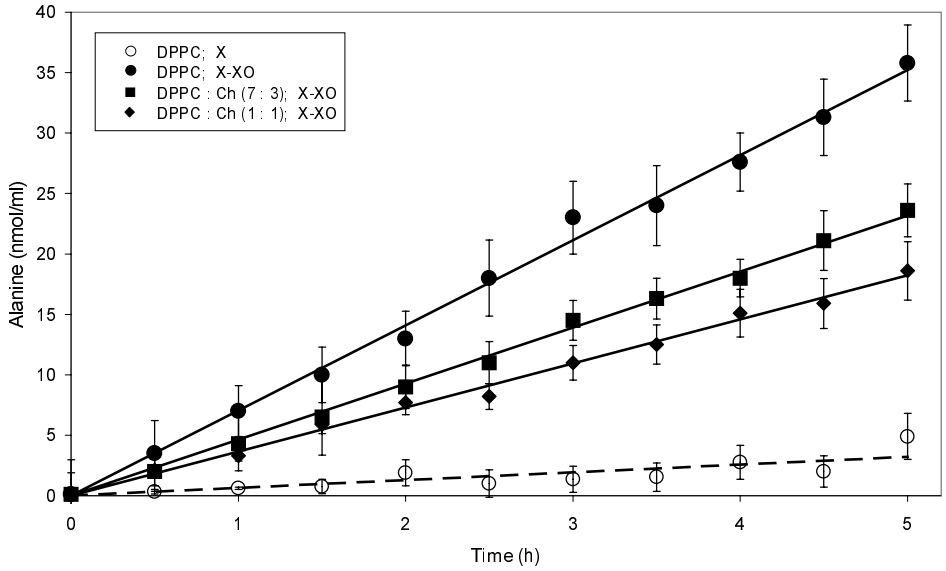

Figure 2. Time course of protein degradation induced by xantine oxidase (XO). Suspensions (5% v/v) of 'hemosomes' composed of DPPC with various amounts of Ch were incubated for varying periods of time in the presence of 2.5 mmol/l xantine (X). Where indicated, 0.2 units of XO were added. The final volume of each reaction tube was 3.2 ml. In the absence of XO, alanine production was 0.65 ± 0.08 nmol·ml^{-1}·h^{-1}. Values are the means ± S.D. of five independent determinations.

globin degradation products. Lavalette et al. (1999) considered the microviscosity of globular proteins in macromolecular environment (cosolvent) and showed that for a given protein the lower molecular weight (MW) of cosolvent, the higher microscopic viscosity was observed. In non-treated erythrocytes or 'hemosomes', Hb can be considered as its own cosolvent (Lavalette et al. 1999). Whereas oxidative damage to protein and its subsequent fragmentation leads to the decrease in cosolvent's MW and the increase in cell internal microviscosity.

In this study liposomes were made from saturated DPPC and Ch, prepared by the hand-shaking method which gives no hemoglobin denaturation (Szebeni et al. 1984). Present findings have shown that Ch incorporated in 'hemosomes' membrane exposed to oxidative stress strongly influenced the degree of Hb damage and the internal microviscosity of vesicles. The higher content of Ch, the lower Hb degradation and smaller increase in internal viscosity were observed. It seems that the concentration of Ch in liposomes is of prime importance for stabilizing of Hb in 'hemosomes'. Szebeni et al. (1985) have also shown that the presence of equimolar amounts of cholesterol in the phospholipid bilayer has a stabilizing effect on encapsulated hemoglobin, especially in liposomes made from negatively charged phospholipids. The instability of Hb is due to the protein interacting with the neg-

atively charged lipid bilayer, what leads in turn to hemoglobin denaturation. As was suggested by Szebeni at al. (1985), the presence of Ch possibly interferes with secondary interactions following the binding of Hb to the negatively charged lipids. This effect is pronounced with saturated phospholipids, but it is also observed, though to a lesser extent, with unsaturated ones, indicating that the bilayer fluidity has a modulating effect. The results obtained in this paper, concerning 'hemosome' samples subjected to oxidative stress, suggest that stabilizing role of Ch results from its influence on membrane fluidity and lipid bilayer structure. The more rigid membrane would better protect cell from deleterious active oxygen species, because penetration of these reactive species into hydrophobic membrane interior is a diffusion-limited process. The results obtained by Smondyrev and Berkowitz (1999) showed that addition of 0.5 mol/mol Ch to DPPC membrane results in a large reduction of the membrane area, what makes lipid bilayer more compact. Lower Ch contents also decreased the area of the membrane, but much less than in the case of large Ch concentrations. Compression of lipid bilayer was accompanied by the increasing order in hydrocarbon tails. Therefore, cholesterol-induced rigidification of lipid membrane would slow down propagation of oxygen radical species, thus minimizing their harmful effect to encapsulated Hb.

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