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Glutathione peroxidase and oxidative hemolysis in trout red blood cells

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Red blood cells from the trout *Salmo irideus* contain several hemoglobin components that are prone to oxidation with production of oxygen radicals. The rate of hemolysis has been correlated to the extent of methemoglobin formation. A difference in the rate of hemolysis between red blood cells saturated with either CO or O_2 was evident only when diminished glutathione peroxidase activity was observed. These results confirm the important role of this enzyme in providing protection against or repair of oxidative damage to the red cell membrane.

Methemoglobin; Oxygen radical; Hemolysis

1. INTRODUCTION

Red blood cells from the trout *Salmo irideus* contain several hemoglobin components characterized by functional differences which have been correlated to a different physiological role [1].

These hemoglobins are prone to oxidation, either as purified proteins or in the whole cell, the rate of auto-oxidation depending on oxygen saturation, pH and temperature (to be published). The possibility of controlling auto-oxidation rate allows investigation of the relationship between oxidation of hemoglobin and membrane components in erythrocytes.

Hemoglobin auto-oxidation results in the liberation of superoxide anion (O_2^-), and thereby of products such as H_2O_2 or hydroxyl radicals, which can be derived from O_2^- itself [2,3]. The cell membrane represents an important physiological target of oxygen radicals, which cause damage to membrane components and may impair function. Superoxide dismutase (SOD), catalase and glutathione peroxidase are the enzymes involved in the defence of the cell against this injury (reviews [4,5]).

Here, we present data on the correlation between hemoglobin oxidation and lysis of red blood cells from trout under different conditions of pH and temperature; we also describe data on the protective biochemical components, which have the function of a repair system for oxidative damage of the red blood cell membrane [4–6].

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2. MATERIALS AND METHODS

The cells used in this work were obtained from *S. irideus*, an inbred strain of trout. Blood was extracted by puncturing the lateral vein. Experiments involving carbonmonoxyhemoglobin were carried out after exposure of the red blood cell suspension to a weak vacuum and then to pure CO gas. Hemoglobin concentration was determined by the pyridine-hemocromogen method [7].

Percent hemolysis is expressed as $(100 \times A/10 \times A^*100\%)$, where A is the hemoglobin concentration present in the supernatant of the red cell suspension after centrifugation, and $A^*100\%$ is

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the hemoglobin concentration obtained after complete lysis with 10 vols distilled water at zero time of incubation. Methemoglobin formation was determined spectrophotometrically in the visible region after hypotonic hemolysis and exposure to pure CO gas. Reference values (i.e. complete reduction and oxidation) were estimated by addition of, respectively, sodium dithionite and ferricyanide. SOD activity was determined by the adrenaline method after chloroform-ethanol extraction [8]. Glutathione peroxidase activity was measured according to Paglia and Valentines [9], catalase activity following Lück [10] and glutathione (GSH) content as described by Beutler [11].

All reagents were of analytical grade. Glutathione reductase and nicotinamide adenine dinucleotide phosphate (reduced form) were obtained from Sigma; reduced glutathione was from Merck.

3. RESULTS AND DISCUSSION

Fig.1 shows the time course of hemoglobin auto-oxidation when red blood cells were suspended in isotonic medium (0.1 M phosphate buffer, 0.1 M NaCl, 0.2% citrate, 10^{-3} M EDTA) at pH 6.3 and incubated at 27°C, in air. The half-time ($t_{½}$) of this process corresponds to about 7 h incubation. Parallel experiments carried out in the presence of CO led to insignificant auto-oxidation over the same time range, even after 20 h (see fig.1). Fig.1 also shows the dependence of hemolysis for either CO-saturated erythrocytes or cells exposed to air under identical conditions. 50% hemolysis is obtained after ~14 h of incubation, including a long lag time (~8 h); the extent of the process is the same in the presence and absence of CO. These data appear to indicate that, under these conditions, lysis is not correlated to hemoglobin oxidation but obviously is related to the onset of other biochemical damages.

On the other hand, similar experiments, but carried out at a different temperature (37°C) (see fig.2), show a considerably faster rate of hemoglobin auto-oxidation ($t_{½}$ ~1 h) and hemolysis ($t_{½}$ ~2 h) when the red blood cells were incubated in air. Inhibition of hemolysis is apparent when erythrocytes were saturated with CO; for example after 140 min, hemolysis is ~90% in

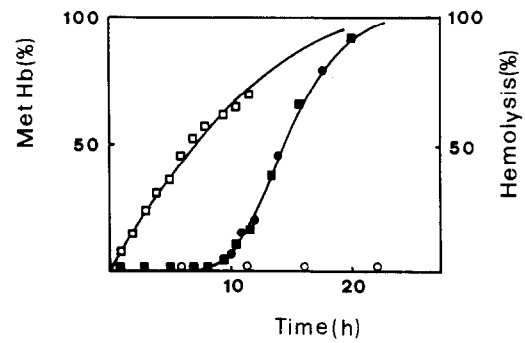

Fig.1. Time course of hemoglobin oxidation and hemolysis in erythrocyte suspensions (containing 2×10^6 red blood cells/ml in isotonic medium at pH 6.3), at 27°C. (□) Met-Hb formation (%) in cells exposed to air, (○) met-Hb formation (%) in cells exposed to CO, (■) hemolysis (%) in cells exposed to air, (●) hemolysis (%) in cells exposed to CO.

air, and only ~10% in the presence of CO. It may be noted that at 37°C some hemoglobin is eventually oxidized to methemoglobin, even under CO, which is likely to be related to oxygen leakage during the experiment. Comparison of the results obtained at the two temperatures is consistent with the hypothesis that oxygen radicals produced during oxidation of hemoglobin may react with red cell components, thereby inducing oxidative damage, eventually involved in the onset of hemolysis. Therefore, we undertook a series of ex-

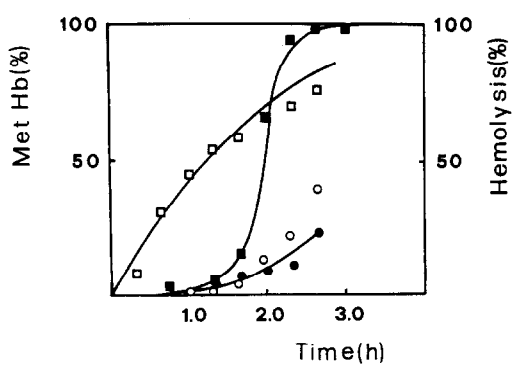

Fig.2. Time course of hemoglobin oxidation and hemolysis. Same conditions as in fig.1, but with incubation at 37°C. (□) Met-Hb formation (%) in cells exposed to air, (○) met-Hb formation (%) in cells exposed to CO, (■) hemolysis (%) in cells exposed to air, (●) hemolysis (%) in cells exposed to CO.

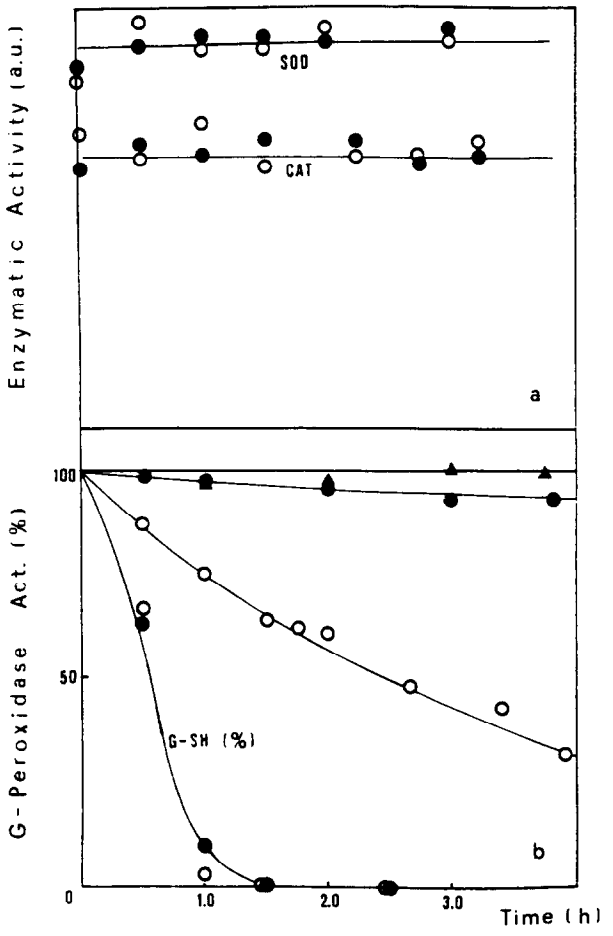

Fig.3. Enzymatic activity of SOD, catalase (a) and glutathione peroxidase (b) as a function of incubation time of trout erythrocytes. Panel b also shows the GSH content. (▲) Conditions as in fig.1 with cells exposed to air, (○) conditions as in fig.2 with cells exposed to air, (●) conditions as in fig.2 with cells exposed to CO.

periments in order to assess possible modifications of the activity of the enzymes which are known to play a defensive role against oxidative damage. The data reported in fig.3 (top panel) show that the activity of catalase and SOD is unaffected by incubation of trout erythrocytes at 37°C, over a time span of 3–4 h in both the presence and absence of CO. On the other hand, the activity of glutathione peroxidase during incubation at 37°C in the absence of CO decreases with time, while it remains constant at 27°C (as in fig.1). Moreover, it may be seen that the rate of loss of glutathione peroxidase activity in the presence of HbCO is significantly slower (especially during the first 2 h) than that in air, a finding which correlates qualitatively with the faster rate of auto-oxidation of hemoglobin reported in fig.2. Under the same conditions, we found that the GSH content of trout erythrocytes decreased rapidly and reached a very low value after only 1 h of incubation, for either CO-saturated erythrocytes or cells exposed to air (see fig.3b).

When the experiments were carried out at pH 7, the rate of hemolysis was the same in the presence and absence of CO, at both 27 and 37°C. At this pH, glutathione peroxidase activity was found to be time-independent (not shown), indicating that the loss of activity observed at 37°C and pH 6.3 (see fig.3) is not due simply to temperature inactivation.

Moreover, we observed that when experiments were carried out in the presence of 10 mM glucose in the incubation medium, the overall picture was essentially unaltered; in particular, the decrease of glutathione peroxidase activity, the onset of hemolysis and the increase in methemoglobin during incubation at 37.0°C and pH 6.3 is observed in air even in the presence of glucose, and the protective effect of CO is maintained (not shown).

It is understood that the hemolytic event, defined operationally by the release of hemoglobin in the supernatant of red blood cell suspensions, is a complex phenomenon dictated by the convergence of more than one type of biochemical damage. Nevertheless, these experiments taken together are fully consistent with the hypothesis that glutathione peroxidase, which can metabolize either H_2O_2 or lipid peroxides [12], is a key enzyme in providing protection against oxidative damage to red cell membranes, which may be a crucial event in the onset of hemolysis.

The fact that the rapid decrease in GSH content of erythrocytes is the same when incubation is in air or under CO, while hemolysis proceeds at a different rate under these two conditions (see fig.2), indicates that the disappearance of GSH by itself cannot account for hemolysis (via a decrease of glutathione peroxidase activity in the cell). On the other hand, over the initial 1–2 h only the activity of glutathione peroxidase is significantly protected by the presence of CO, and then correlated with the rate of methemoglobin formation and hemolysis, seen after incubation in air. Thus these

results support the model that formation of oxygen radicals associated with auto-oxidation of intraerythrocytic hemoglobins leads to membrane damage, which (in the absence of an efficient repair system) is a cause (among others) of the hemolytic event.

These results are in accordance with the data of Nichols [13] and Chiu et al. [14], as well as with the hemosome model of Szebeni et al. [15] and Aebi and Suter [16]; the latter authors support the idea that the glutathione system, as well as catalase, provide a mutual back-up in this role. Finally, these data have to be considered with reference to the work of Scarpa et al. [17], who reported evidence that the flux of O_2^- radicals in human erythrocytes is not significantly increased by the auto-oxidation of hemoglobin. In our view, it is possible that a limited flux of oxidants close to the cellular domain preferentially occupied by glutathione peroxidase (i.e. close to the cell membrane, see [18]) is more significant to the onset of the hemolytic event than the total flux of oxygen radicals produced within the bulk of the red blood cell.

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