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SUPEROXIDE ANION HANDLING BY ERYTHROCYTES LOADED WITH ALFA AND BETA HEMOGLOBIN CHAINS. A CHEMILUMINESCENCE STUDY

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Introduction

Reactive oxygen species (ROS) are continuously produced in vivo promoting tissue damage and disease. Many pathological situations are due to the alterated steady-state of these reactive species. In red blood cells (RBCs), an increased generation of reactive oxygen species is considered as a potential cause of oxidative injury that alters physiopathology of RBCs and their life span.

The thalassemia, that is characterized by an unbalanced production of the hemoglobin chains (alfa and beta) in the red cells, represents one of these cases. It is known that the free hemoglobin chains are less stable with respect to the tetrameric hemoglobin and then they can easily oxidize and subsequently form precipitates known as Heinz bodies according to the following overall scheme (1):

oxy-chains ------- met-chains ------- hemicromes ------ precipitate (Heinz bodies).

During the process that leads to the formation of Heinz bodies, the ROS production is altered due to autoxidation of hemoglobin and the release of O_2^- (1).

Moreover, the antioxidative system of the RBCs can be damaged. We have recently demostrated an inactivation of glutathione peroxidase (an enzymes which can metabolize either H_2O_2 or lipid peroxides) following hemoglobin oxidation. The loss of the enzymatic activity has been observed when hemicromes are formed (2,3). As reported (4) the entrapment of alfa or beta chains in human erythrocytes leads to the formation of an "in vitro" model of thalassemia that permits to follow, from a kinetic point of the view, the RBC alterations determined by the presence of free alfa or beta chains. Obviously, this information could not be obtained by studying erythrocytes from thalassemic patients where the damage has already occurred in vivo.

In the present study we report data regarding the superoxide generation and scavenging in human RBCs after the entrapment of alfa or beta chains. The loaded cells were obtained by a dialysis technique and the effect of superoxide anion has been investigated by using chemiluminescence assay. Superoxide anion was obtained by the xanthine/xanthine oxidase system.

Material and Methods

Instrumentation We used a Autolumat LB 953 (Berthold Co, Wildbad, Germany)

Reagents Xanthine, xanthine oxidase and lucigenin were purchased from Sigma Chem. Co., St. Louis MO (USA).

Methods Preparation of isolated hemoglobin chains was performed by using p-hydroxymercuribenzoate (5).

The purified chains, with regenerated sulphydryl groups, were encapsulated in human erythrocytes by a dialysis technique (6,7).Erythrocyte membranes were prepared by hypotonic hemolysis (8).

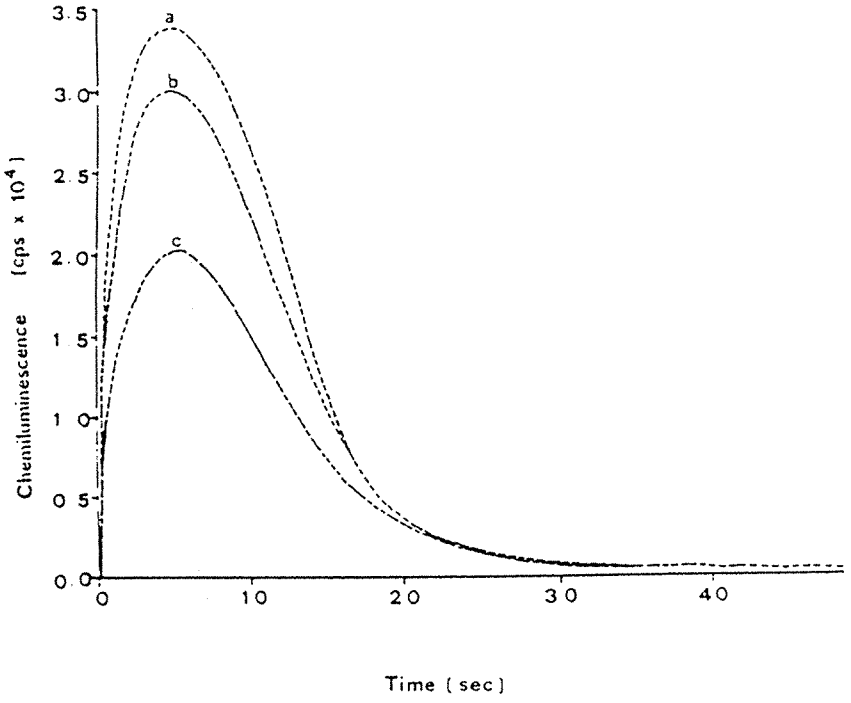
Chemiluminescence (CL) was measured at 37°C in presence of 0.9 U/ml xanthine oxidase, 150 μmol/L lucigenin, hemolysate containing Hb = 40 μg/ml or ghosts with a concentration of protein of 25 μg/ml and brought to a final volume of 1 ml with physiological

solution. The reaction was initiated by injecting xanthine at a final concentration of 50 µmol/L. The measurement was followed for 60 s as described (9). Values were expressed as counts per second (cps).
The significance of the values obtained was calculated using Student's t test.

Result and Discussion

The effective presence in the cells of free subunits was monitored by ion-focusing. The free chain concentration was about 1% of the total hemoglobin present in the hemolysate obtained by lysis of the loaded cell.

Lucigenin-amplified CL produced by superoxide generated from xanthine-xanthine oxidase reaction, shows a peack CL value 6-8 s after injection of xanthine, and the reaction is complete after about 20 s; the addition of superoxide dismutase is associated to a chemiluminescence inhibition.



Time-course of lucigenin-amplified CL of different hemolysates (see test)

Fig.1 shows the kinetics of the lucigenin-amplified chemiluminescence measured on the hemolysates obtained from the alfa, beta and unloaded RBCs.

The incubation at 37°C of chain loaded RBCs suspended in isotonic medium (tris 10 mmol/L pH 7.4 containing 100 mmol/L NaCl and 120 mmol/L glucose) is associated (fi.1) with a decrease of chemiluminescence with respect to unloaded RBCs.
Moreover, in the same figure we can see that the loss in chemiluminescence (counts per second), after seven hours incubation at 37°C, is significantly more marked in presence of alfa chains compared to the beta chains. No loss of chemiluminescence (counts per second) was detect with the hemolysates obtained from the control.
The peak appeared after about 6 s from beginning of the reaction. The maximum mean value of CL on the hemolysates obtained from alfa and beta chains at 0', control resealed at 0' and control at 0' and after 7 h of incubation was 3.402×10^4 (a). As it is possible to observe in the same figure, the peak after 7 h of incubation at 37°C is lower for hemolysate obtained from alfa chains (mean value = 2.024×10^4) (c) than beta chains (mean value = 3.007×10^4) (b).

Chemiluminescence measurements performed on RBCs membrane ghosts, obtained from the same samples, did not demostrate a significant difference among the alfa or beta loaded cells after seven hours incubation (data not shown).

The CL assay represents a sensitive method of assessing the overall oxidative state of the cell. Different information may be obtained on the type of chemiluminegenic probe utilized (10). Lucigenin is sensitive to the level of superoxide radical (O_2^-) and allows evaluation of cellular superoxide radical scavengers (11). The specific effect of lucigenin to measure, particularly, the level of superoxide anion was confirmed by inhibition of superoxide chemiluminescence by superoxide dismutase.

In our study, the level of CL indicates the presence of O_2^- in the medium. A higher level of oxidation in the hemolysate obtained from alfa-loaded cells with respect to that from the beta-loaded cells could be the cause of a lower level of CL. Probably, superoxide anion may have more effect on damaged structures than on unchanged ones.

In conclusion our experiments support the idea that oxygen radicals can cause damage to the intraerythrocytic proteins also in absence of detectable lipid peroxidation. Thus, protein oxidation is a more sensitive indicator of oxygen radical damage than lipid peroxidation (12).

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