The Interaction of Copper Chloride with Erythrocyte Membrane as a Source of Activated Oxygen Species. A Chemiluminescent Study

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Abstract. The possibility for the generation of activated oxygen species during the interaction between copper chloride and erythrocyte membranes was investigated. A chemiluminescent method for detecting superoxide radicals and hydrogen peroxide was used. It was found that the interaction of $CuCl_2$ with erythrocyte membrane is accompanied with O_2^- and H_2O_2 generation. On the base of this result it is proposed that the activated oxygen species generated by $CuCl_2$-membrane interaction may be able to initiate peroxidative breakdown processes in erythrocytes eventually leading to haemolysis.

Key words: Copper toxicity — Copper haemolytic action — Generation of activated oxygen species

Introduction

Haemolytic anemia is characteristic of both acute and chronic copper poisoning (Evans 1973; Ishmael et al. 1972; Owen and Hazelrig 1968), as well as Wilson's disease (Willms et al. 1972). It seams that the premature destruction of erythrocytes caused by free serum copper is intravascular in nature (Hochstein et al. 1978). But the mechanism of the haemolytic action of copper is essentially unknown. Some previous investigations (Hochstein et al. 1978; Ribarov and Benov 1981) show that oxygen radical mediated processes are possibly involved in this mechanism. There are many reactions between copper and cellular constituents that may result in the generation of the superoxide radical (O_2^-) and/or H_2O_2. The first is the copper catalysed haemoglobin oxidation to methaemoglobin (Carrell et al. 1978; Rifkind 1974) with O_2^- release (Carrell et al. 1978; Winterbourn and Carrell 1977; Bochev and Ribarov 1983). Another is the reaction between copper and sulphydryl compounds. The copper catalysed oxidation of sulphydryl groups may lead to a reduction of Cu (II) (Ivancheva and Russanov 1977; Carrell et al. 1978). Thus produced, Cu (I) may be reoxidized by molecular oxygen, with generation of O_2^-. Since the erythrocyte membrane

contains a large number of sulphydryl groups it is of considerable interest to study the possibility of O_2^- and/or H_2O_2 generation by the copper-erythrocyte membrane interaction.

The aim of the present work was to test this possibility in the hope that the results obtained will contribute to our understanding the mechanism of copper induced haemolysis.

Materials and Methods

Erythrocyte membranes were obtained from Guinea pig erythrocytes as previously described (Dodge et al. 1963). The packed erythrocyte membranes were suspended in distilled water to obtain a protein concentration of 0.12 mg/ml measured by the method of Lowry et al. (1951). The pH of the suspension was adjusted to 7.4 with 0.01 mol/l NaOH.

For the registration of activated oxygen species a previously described chemiluminescent method was used (Ribarov and Bochev 1982, 1983). Chemiluminescent assays were carried out in a two-compartment optical cuvette, containing in the lower compartment a luminol solution which consists of: luminol, 10^{-4} mol/l; horseradish peroxidase, 3.3×10^{-6} g/ml; EDTA, 10^{-4} mol/l in phoshate buffer, 10^{-1} mol/l (pH 7.8). The sample investigated is placed in the upper compartment, separated from the lower compartment by a dialysis membrane. Activated oxygen species (O_2^- and H_2O_2), produced in the upper compartment diffuse rapidly through the dialysis membrane into the luminol solution. Superoxide radicals can oxidize the luminol directly (Hodgson and Fridovich 1973) and H_2O_2 — catalyzed by peroxidase (Freeman and Seltz 1978). The oxidation of luminol leads to chemiluminescence. As a criterion for the quantity of the activated oxygen species the integral area under the kinetic chemiluminescent curve was used. All experiments were carried out with 3 ml samples at 37°C.

Superoxide dismutase (SOD) (EC 1.15.1.1) with a specific activity of 2300 IU/ml, estimated according to Maral et al. (1977) was prepared from bovine erythrocytes by the method of McCord and Fridovich (1969). Catalase (EC 1.11.1.6) — 150 000 IU/ml was obtained from Böehringer (FRG). Prior to use the catalase was passed through a short Sephadex G-50 column to remove the thymol added in commercial preparations of catalase as a stabilizing agent. Horseradish peroxidase type I (EC 1.11.1.7.) with a specific activity of approximately 80 IU/mg was purchased from Sigma. $CuCl_2$ was a product of Merck and luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) obtained from Fluka. All other reagents used were at least of reagent grade and the water was glass distilled.

Results and Discussion

Luminol chemiluminescence observed during the interaction of $CuCl_2$ with erythrocyte membranes

When the samples investigated consist only of erythrocyte membranes, the intensity of the luminol chemiluminescence is very low, with a slight increase over time (Fig. 1, curve A). This effect is possibly due to the spontaneous peroxidation of the erythrocyte membrane lipids. The process of peroxidation is known to be accompanied by the generation of superoxide radicals and/or other activated oxygen species.

The addition of $CuCl_2$ to the sample of erythrocyte membranes caused marked

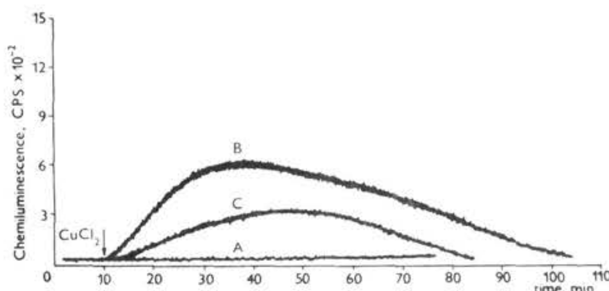

Fig. 1. Typical chemiluminescent kinetic curves recorded under experimental conditions as follows: 0.05 ml H_2O added to 3 ml erythrocyte membrane suspension with a protein concentration of 0.12 mg/ml (curve A); 0.05 ml $CuCl_2$, 6×10^{-3} mol/l added to 3 ml erythrocyte membrane suspension with the above concentration (curve B); 0.05 ml $CuCl_2$, 6×10^{-3} mol/l added to 3 ml H_2O (curve C).

increase of chemiluminescence (curve B). The chemiluminescent response commenced immediately after $CuCl_2$ addition. The intensity of the chemiluminescence increased rapidly and reached a maximal value in approximately 30 min. After that, the chemiluminescence gradually diminished.

In control experiments, when $CuCl_2$ in the same concentration was added to the sample of water instead of the membrane suspension, a significantly smaller chemiluminescent response was observed (curve C). It is most likely that the luminol chemiluminescence in this case is due to O_2^-, produced in the course of oxidation of Cu (I) by molecular oxygen. A small amout of Cu (I) may be generated through the reduction of Cu (II) by some components of the luminol solution diffusing accross the dialysis membrane. As is seen in Fig. 1 the integral area under curve B is significantly larger than the area under curve C. This fact indicates that the interaction between $CuCl_2$ and erythrocyte membrane causes the generation of activated oxygen species (O_2^- and/or H_2O_2).

Inhibitory effect of SOD and catalase on the chemiluminescent response

As mentioned above there are many sorts of activated oxygen species able to cause luminol oxidation with light emission. In order to clarify what is the initial activated oxygen form generated during the $CuCl_2$-erythrocyte membrane interaction additional experiments were carried out. To the samples of erythrocyte membranes were added separately or simultaneously superoxide dismutase and catalase and the $CuCl_2$—induced chemiluminescent response was registrated. The results of these experiments are shown in Fig. 2. When SOD (11 IU/ml) was added to the sample of erythrocyte membranes the copper-induced chemiluminescent response

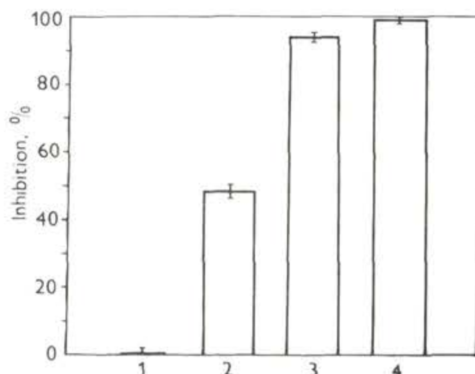

Fig. 2. Inhibitory effect of SOD and catalase on the chemiluminescent response. To 3 ml samples of erythrocyte membrane suspension with a protein concentration 0.12 mg/ml with or without SOD and/or catalase were added 0.05 ml $CuCl_2$ 6×10^{-3} mol/l. The final concentration of SOD and catalase in the reaction mixture were 11 IU/ml and 1000 IU/ml respectively. Each result is the mean value from six experiments and the bars represent the standard errors of the mean. Additions: 1 : H_2O ; 2 : SOD ; 3 : catalase ; 4 : SOD + catalase.

was diminished by 48 %. The addition of catalase (1000 IU/ml) to the membrane suspension led to a more expressed inhibition of the chemiluminescence. When both enzymes were used simultaneously the chemiluminescent response was negligible. SOD inhibits reactions which involve superoxide radicals. Moreover, in the presence of catalase, the chemiluminescent response is due only to generated O_2^-. Taking into account these facts and the results presented it may be concluded that superoxide radicals are the initial activated oxygen species produced during the $CuCl_2$-erythrocyte membrane interaction. The inhibitory effect of catalase on the copper-induced chemiluminescent response shows that H_2O_2 is also generated but possibly by O_2^-. These results strongly support the conclusion of Kumar et al. (1978) that copper-erythrocyte membrane interaction is accompanied with superoxide radical production.

The generation of O_2^- and H_2O_2 in the course of $CuCl_2$-membrane interaction may have very important consequences for the cell integrity. These oxygen species are easily converted to other more reactive oxygen species as singlet oxygen and $OH^{\cdot}$, able to initiate the breakdown processes of lipid peroxidation in the erythrocyte membrane leading eventually to haemolysis.

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