銅による溶血と Superoxide dismutase との関連性について

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Hemolysis of Bovine Erythrocytes induced by Copper and its Relationship to Superoxide Dismutase Activity

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ABSTRACT. Superoxide dismutase (SOD) activity and the percentage of hemolysis were measured with bovine erythrocytes incubated for 24 hrs at 37°C in 0.1 and 0.5 mM copper sulfate solutions. The SOD activity decreased rapidly, as only 50% and 15% of the initial erythrocyte content (2.8×10^3 units/g Hb) were found to be intact after 12 hrs incubation in 0.1 and 0.5 mM copper sulfate solutions, respectively. Prior to the development of hemolysis and during the hemolytic phase, we detected a decrease in SOD activity. The percentage of hemolysis reached 30% and 80% after incubation for 24 hrs with 0.1 and 0.5 mM copper sulfate solutions, respectively.—KEY WORDS: bovine erythrocyte, copper, hemolysis, superoxide dismutase (SOD).

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Copper poisoning involves alterations in the structure, function and metabolism of erythrocytes [6, 7, 12], but the cause of copper-induced hemolysis is not well understood. The interaction of copper with the erythrocyte membrane proteins was investigated in our laboratory. We found that copper does not bind to the erythrocyte membranes but can directly oxidize the sulfhydryls of the membrane proteins to form disulfide bonds [2, 3]. With the formation of these disulfide bonds, superoxide anions are formed within human erythrocyte membranes [11]. The superoxide anion, which has a deleterious effect on the erythrocytes, can be transformed in a catalyzation reaction with the metalloenzyme superoxide dismutase (SOD, EC 1.15.1.1) to less toxic hydrogen peroxide and molecular oxygen [16].

In this paper, the effect of copper on SOD activity in bovine erythrocytes was studied *in vitro,* and a direct relationship between the copper-induced hemolysis and SOD activity was examined.

MATERIALS AND METHODS

Preparation of the erythrocytes: Blood samples were collected from normal, adult cows (Holstein Friesian) into Alsever's solution. These specimens were used generally within 4 hrs of bleeding. After centrifugation ($1,200 \times g$, 10 min), the buffy layer and the plasma were separated and carefully discarded. The remaining erythrocytes were washed three times with NaCl-Tris buffer (280 OsM, pH 7.4) containing 5 mM KCl, either with or without 250 mg/100 m*l* glucose. Each of these solutions was also used as an incubation medium. The washed erythrocytes were then suspended in the two solutions described above and incubated for varying periods of time in a water-bath at 37°C with gentle agitation. Concentrated $CuSO_4$ was added to the erythrocyte suspensions to the final concentration of 0.1 or 0.5 mM/*l*. The copper concentrations in the cell suspensions were set according to the previous study [1]. The final hematocrit was adjusted to 27 to 30% and the glucose concentration was set at 125 mg/100 m*l*.

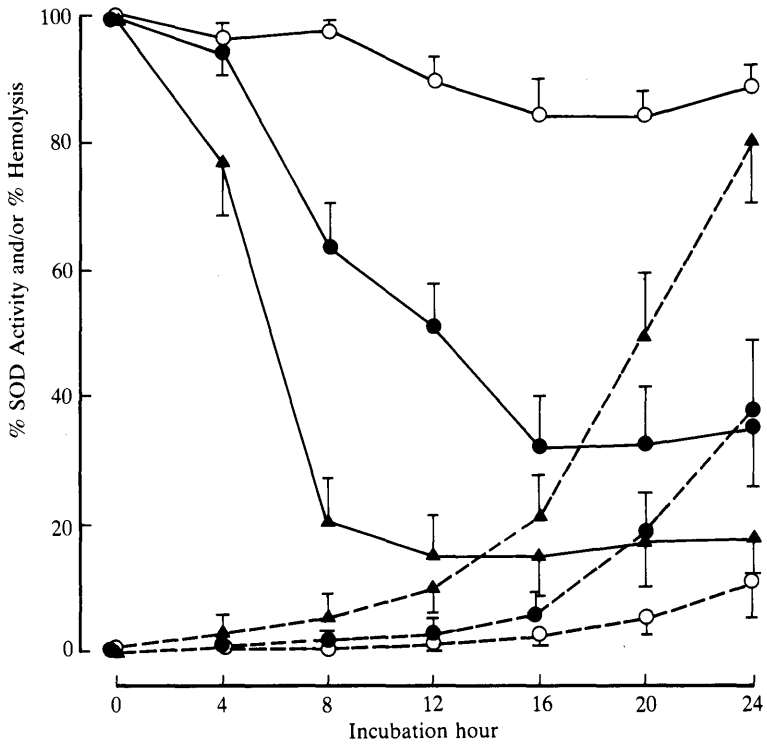

Fig. 1. Superoxide dismutase (SOD) activity (——) and percentage of hemolysis (----) of bovine erythrocytes incubated at 37°C for 24 hrs in 280 OsM NaCl-Tris buffer (pH 7.4) with 0 (○), 0.1 (●) and 0.5 mM (▲) $CuSO_4$. The SOD activity is expressed as percentage of the initial concentration of SOD in the erythrocytes. Each point represents the mean±SDM of eight experiments.

Superoxide dismutase (SOD) activity: The cell suspension was centrifugated at 1,200× g for 10 minutes, then the cells were washed three times with NaCl-Tris buffer and hemolyzed by the addition of 1.5 volumes of distilled water. The hemoglobin concentrations were determined and adjusted to 10 g per 100 m*l*. A chloroform-ethanol extract was prepared by adding 0.5 m*l* of the hemolysate to 3.5 m*l* of ice-cold water, followed by 1.0 m*l* of ethanol and, then, by 0.6 m*l* of chloroform [4]. This solution was shaken for 2 minutes and centrifuged at 1,200×g for 15 minutes. The enzyme activity was verified as being in the supernatant. The supernatant in 0.05 m*l* was mixed with 0.2 m*l* of 0.1 M EDTA, 0.1 m*l* of 1.5 mM nitro blue tetrazolium (NBT), 0.1 m*l* of 0.2% bovine serum albumin, 0.05 m*l* of 0.12 mM riboflavin and 1/15 M phosphate buffer (pH 7.8). This mixture was then placed in a box containing an 8 W fluorescent tube and illuminated uniformly for 7 minutes. The optical density of this mixture was measured at 560 nm. The results were expressed as units of SOD per gram of hemoglobin and one unit was defined as the amount of enzyme capable of causing half of the maximum inhibition of NBT reduction [18].

Hemolysis: Before determination, the cell suspensions were centrifuged at 1,000×g for 5 minutes. Hemolysis was quantified by measuring the concentration of free hemoglobin in the supernatant by the standard human blood cyanmethemoglobin method.

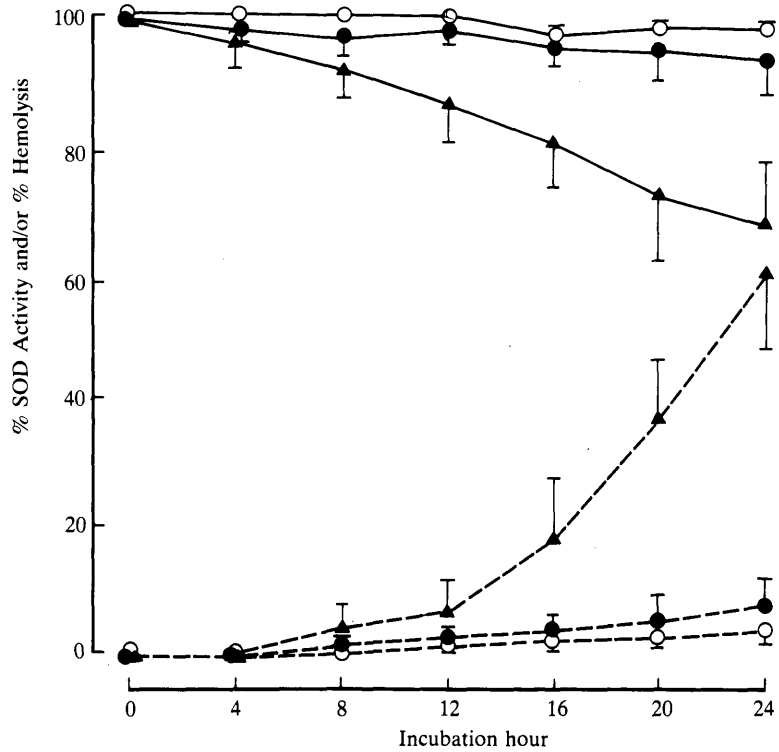

Fig. 2. SOD activity and percentage hemolysis of bovine erythrocytes incubated in the NaCl-Tris buffer containing glucose (125 mg/100 m*l*) under identical conditions as described in Fig. 1. The mean±SDM of eight experiments.

RESULTS

SOD activity: Immediately after incubation, the SOD content of the washed erythrocytes was 2.8×10^3 units/g Hb. The SOD activity was expressed as a percentage of the initial SOD content of the erythrocytes. Without glucose, the SOD activity repidly decreased, reaching 50% and 15% of the initial activity after 12 hrs of incubation in the copper solutions at concentrations of 0.1 and 0.5 mM, respectively (Fig. 1). When glucose was present, only 7% and 32% of the initial SOD activity of the erythrocytes were lost when incubated with 0.1 and 0.5 mM copper solutions, respectively (Fig. 2).

Hemolysis: The percentage of hemolysis increased relatively slowly during the first 12 hrs of incubation (Fig. 1). After incubation, hemolysis increased very rapidly, reaching 30% and 80% after 24 hrs of incubation with 0.1 and 0.5 mM copper solution, respectively. The increase in hemolysis was significantly ($P<0.05$) depressed by the presence of glucose in the incubation medium (Fig. 2).

DISCUSSION

Copper and the cupric ion are believed to cause cytotoxicity owing to their oxidant actions [8], but the precise mechanism by which copper induces hemolysis under certain pathological conditions is not well understood. Metz and Sagone [13] observed that the incubation of erythrocytes with copper *in vitro* caused the depletion of glutathione as well as the oxidation of oxyhemoglobin to methemoglobin prior to

hemolysis. Furthermore, the superoxide anion was produced in the erythrocytes during the oxidation of hemoglobin. Salhany *et al.* [15] suggested that the direct oxidation of sulfhydryls in the human erythrocyte membrane by the cupric ion lead to the formation of disulfide links among the membrane proteins. Kumar *et al.* [11] also demonstrated that the cupric ion reacted with the erythrocyte membrane sulfhydryls to cause the generation of superoxide anions. The superoxide anion may be important in the general phenomenon of lipid peroxidation and particularly in the process of peroxidative hemolysis of erythrocytes [9]. Superoxide dismutase catalyzes the dismutation reaction, to which two superoxide anion radicals are transformed into H_2O_2 and O_2 [16]. Deleterious effects of the radical can thus be avoided. There are few papers which have examined the exact relationship between copper and SOD activity in erythrocytes. Inadequate levels of copper in the diet were associated with the reduction of SOD activity in the erythrocytes of swine [17] and rats [5]. Rigo *et al.* [14] also reported that SOD activity in erythrocytes of copper-deficient cows increased when they were given adequate copper in their diet, and it became maximal when they were fed a diet containing 10 to 14 mg copper/kg. These results, however, are mainly concerned with the effect of dietary copper on SOD activity in erythrocytes. It remains undetermined whether high levels of copper in the erythrocytes may induce changes in the SOD activity.

High levels of copper induced a significant decrease in SOD activity in the erythrocytes when glucose was absent in the incubation medium. On the contrary, a slight decrease in SOD activity was observed with the presence of glucose. It has been demonstrated that SOD can be inactivated by hydrogen peroxide which is produced by the catalytic action of SOD in the erythrocytes [10]. Mets and Sagone [13] noted that copper had an accelerating effect on the production of hydrogen peroxide in erythrocytes. Erythrocytes have glutathione peroxidase and catalase activities, both of which protect the erythrocytes from damage due to the hydrogen peroxide [8]. To increase the activity of these enzymes, glucose is required by the erythrocytes. The decrease in SOD activity in the erythrocytes incubated with copper may be due to the increase in the production of hydrogen peroxide, and glucose may have a significant role in the inhibition of copper-induced decrease in SOD activity. In the present study, the development of hemolysis induced by copper was preceded and accompanied by a decrease in SOD activity. These observations suggest that copper-induced hemolysis is perhaps related to the increase in the intracellular production of hydrogen peroxide. In conclusion, evidence has been presented to indicate that the copper-induced hemolysis is, to a certain extent, dependent upon the intracellular level of SOD.

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要　約

銅による溶血と Superoxide dismutase との関連性について：浅野隆司・保刈成男（日本大学農獣医学部獣医薬理学教室）——銅による溶血と Superoxide dismutase（SOD）との関連性を検討するため，*in vitro* でウシ赤血球に硫酸銅（0.1及び0.5mM）を作用させ，経時的に SOD 活性及び溶血度の測定を行った。溶血に先行して SOD 活性は著明に低下し，硫酸銅処理開始12時間後には 0.1 及び 0.5 mM 硫酸銅処理赤血球において，それぞれ処理開始時の活性（2.8×10^3 units/gHb）の50％及び15％に減少した。溶血は SOD 活性の減少に伴って進行し，24時間後における溶血度は 0.1 及び 0.5 mM 硫酸銅処理赤血球でそれぞれ30％及び80％であった。これら SOD 活性の低下，溶血の進行はグルコースの添加によって著明に抑制された。