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# RELATIONSHIP BETWEEN THE HEMOLYTIC ACTION OF HEAVY METALS AND LIPID PEROXIDATION

STEFAN R. RIBAROV and LUDMIL C. BENOV

*Department of Biophysics, University School of Medicine, Pleven, 5800 (Bulgaria)*

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## Summary

It is well known that some of the heavy metals have a hemolytic action, but the mechanisms responsible for this effect are not well established. In order to elucidate whether or not the hemolytic action of heavy metal ions is associated with the peroxidation of membrane lipids, the relationship between metal-induced hemolysis and the generation of malonaldehyde has been studied.

The results obtained show that metal-induced hemolysis is associated with the development of peroxidative processes in erythrocyte membranes. The peroxidation is caused by metals with and without pro-oxidant catalytic action. The level of the malonaldehyde products rises before the appearance of hemolysis which proves that the development of peroxidative processes precedes but does not result from hemolysis.

The suggestion has been made that the peroxidation of membrane lipids is a possible mechanism of damage to the red cell membrane in metal-induced hemolysis.

## Introduction

In the course of extensive studies on the effects of heavy metals on erythrocytes in vitro it has been observed that some of these metals have a hemolytic action [1,2]. It has also been found that $Ag^+$, $Hg^{2+}$ and $Pb^{2+}$ increase the cation permeability of the erythrocyte membranes [3]. In other experiments, it has been demonstrated that some of the heavy metals decrease the deformability of red blood cells [4] and are capable of inducing conformational changes in proteins of the erythrocyte membrane [5,6].

These studies, however, do not provide a satisfactory explanation for the mechanisms responsible for the hemolytic action of heavy metals. The hypoth-

esis put forward by some authors [1] that the effect is purely osmotic and entirely connected with the changes in the permeability of the erythrocytes membrane cannot explain a number of phenomena observed.

Our experiments show that substances with antioxidant action (vitamin E, butylated hydroxytoluene) strongly reduce the level of lead-induced hemolysis, while $H_2O_2$, even in concentrations lower than $10^{-5}$ M, drastically potentiates the hemolytic action of the metal ions [7].

Others authors [4] have found a decrease in deformability of erythrocytes from vitamin E-deficient lead-poisoned rats. The authors suggest that the lipid peroxidation of unsaturated fatty acids in the membrane is directly related to the observed phenomenon. In addition, it was found that oxidative stress also decreases the deformability of erythrocytes and that the decrease is much greater for vitamin E-deficient rats [8]. On the other hand, there is evidence that $Cu^{2+}$ may react with plasma membranes to cause the generation of superoxide radicals and hence to initiate peroxidation of membrane phospholipids [9]. All these findings suggest a possible association between metal-induced hemolysis and peroxidation of membrane lipids.

The aim of the present work was to study the relationship between the hemolytic action of heavy metals and lipid peroxidation of erythrocyte membranes in vitro.

## Materials and Methods

All experiments were carried out using bovine and guinea-pig erythrocytes. No qualitative difference was found in the behavior of the two types of erythrocyte. The results presented below concern bovine erythrocytes.

The level of hemolysis and the degree of peroxidation were studied in the presence of $CoCl_2$, $PbCl_2$, $CuCl_2$, $FeCl_3$, $FeCl_2$, $HgCl_2$ and $AgNO_3$. Each metal was used at a final concentration of $10^{-3}$ M. The erythrocytes were washed three times with cold 0.15 M NaCl (for $Ag^+$ with 0.15 M $NaNO_3$) and were then suspended in an equal volume of 0.15 M NaCl (for $Ag^+$ in 0.15 M $NaNO_3$) containing the respective metal ion. All samples were incubated at 37°C. The degree of hemolysis and lipid peroxidation were determined at definite time intervals.

For the measurement of hemolysis, the red cells remaining undamaged were removed by centrifugation and the hemoglobin was then estimated in the clear supernatant by using the cyanmethemoglobin method [10].

The degree of peroxidation was measured by estimation of malonaldehyde, a secondary breakdown product of lipid peroxides. A modification of the method described by Benisovich and Idelson [11] was used. To 6 ml of erythrocyte suspension, 0.1 ml of 10% Triton X-100 was added and immediately after vigorous stirring, 6 ml of 30% trichloroacetic acid and 2 ml of 0.6% thiobarbituric acid were added. After filtering twice, 3 ml of the clear filtrate were mixed with 2 ml of 0.6% thiobarbituric acid and the samples incubated for 15 min in a boiling water bath. After cooling, 2 ml of $CHCl_3$ were added followed by vigorous shaking and centrifugation. The absorbance of the clear pink supernatant was determined at 532 nm in a 2 cm light-path cuvette.

Since some authors [12] consider the possibility of accumulation of both

polar and non-polar peroxidation products, we determined malonaldehyde in lipids extracted from erythrocytes incubated with metal ions by using the method of Folch et al. [13] with slight modifications. For this purpose, 2 ml of erythrocytes were homogenized in 10 ml $CH_3OH$ containing 1 mg% butylated hydroxytoluene as antioxidant. 2 vol. of $CHCl_3$ were added and the samples were stored for 2 h at 0°C. After centrifugation the precipitate was discarded, and the supernatant washed twice with 0.2 vol. of distilled water. After centrifugation the upper phase was removed by suction and the residue was evaporated in a vacuum evaporator. 5 ml of glacial acetic acid and 3 ml of 0.6% thiobarbituric acid were added, and the samples were heated at 90°C for 30 min. After cooling, 3 ml of $CHCl_3$ were added and the mixture vigorously shaken to remove dispersed lipids. The samples were then centrifuged and the absorbance of the clear supernatant was determined at 532 nm in a 2 cm light-path cuvette.

The experiments described were repeated at least three times with three to five replicates.

## Results

Table I presents the results for the level of hemolysis and malonaldehyde production in erythrocytes incubated with $Co^{2+}$, $Pb^{2+}$, $Fe^{2+}$, $Fe^{3+}$, $Cu^{2+}$, $Hg^{2+}$ and $Ag^{+}$. It is seen that the extent of hemolysis is greatest in erythrocytes incubated with $Ag^{+}$ and $Hg^{2+}$ (second column). With the exception of $Co^{2+}$, under our experimental conditions, all other tested metals exert a hemolytic action which decreases in the following order: $Cu^{2+} > Pb^{2+} > Fe^{3+} > Fe^{2+}$. In the third and fourth columns of the table are presented the levels of malonaldehyde production over the same incubation period, estimated from both methods described. The results obtained by measuring the amount of polar malonaldehyde products (third column) show that the degree of peroxidation is highest in erythrocytes treated with metal ions possessing pro-oxidant properties ($Cu^{2+}$, $Fe^{3+}$ and $Fe^{2+}$). Some different results were obtained for the malonaldehyde products in lipid extracts (fourth column). In these cases, the highest degree of peroxidation was found in the presence of the strong hemolytic agents: $Ag^{+}$,

TABLE I

THE LEVEL OF HEMOLYSIS AND MALONALDEHYDE PRODUCTION IN ERYTHROCYTES AFTER INCUBATION FOR 60 min WITH HEAVY METALS IONS

Values are mean ± S.E. Details are given in Materials and Methods

| Tested metal | Hemolysis ($A_{540}$) | Malonaldehyde in total incubation mixture ($A_{532}$) | Malonaldehyde in lipid extracts ($A_{532}$) |
|---|---|---|---|
| Control | 0.050 ± 0.009 | 0.090 ± 0.001 | 0.090 ± 0.002 |
| $Co^{2+}$ | 0.044 ± 0.010 | 0.085 ± 0.001 | 0.086 ± 0.006 |
| $Cu^{2+}$ | 0.403 ± 0.019 | 0.160 ± 0.002 | 0.132 ± 0.010 |
| $Fe^{3+}$ | 0.206 ± 0.016 | 0.130 ± 0.003 | 0.128 ± 0.007 |
| $Fe^{2+}$ | 0.156 ± 0.012 | 0.142 ± 0.002 | 0.104 ± 0.007 |
| $Pb^{2+}$ | 0.216 ± 0.011 | 0.103 ± 0.002 | 0.148 ± 0.005 |
| $Hg^{2+}$ | 0.634 ± 0.019 | 0.103 ± 0.006 | 0.132 ± 0.004 |
| $Ag^{+}$ | 0.666 ± 0.020 | 0.099 ± 0.004 | 0.154 ± 0.006 |

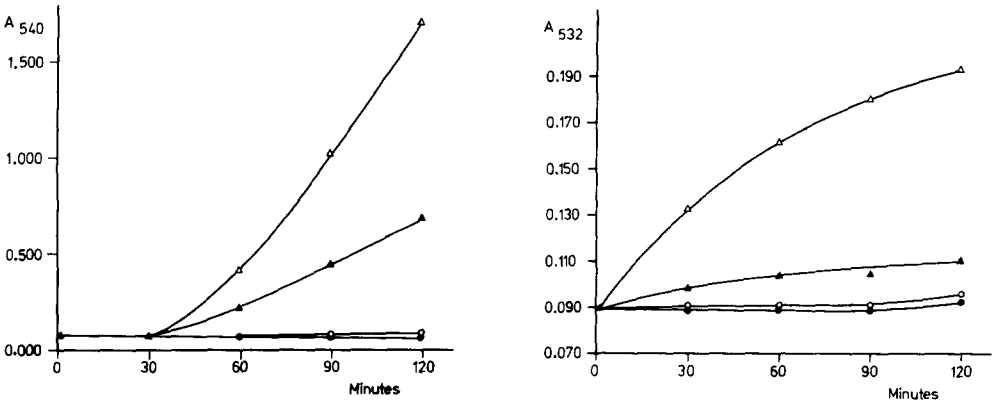


Fig. 1. Time course of metal-induced hemolysis, measured by the cyanmethemoglobin method, with respect to the absorbance at 540 nm. Erythrocytes were incubated in the presence of $Cu^{2+}$ (△), $Pb^{2+}$ (▲) and $Co^{2+}$ (●) at final concentrations of $10^{-3}$ M. In the control samples (○) heavy metal ions were not added.

Fig. 2. Kinetics of malonaldehyde generation, measured as the absorbance at 532 nm, in erythrocytes incubated with heavy metals. The reactions were stopped with trichloroacetic acid and malonaldehyde was estimated in the clear filtrate by the thiobarbituric acid test. Erythrocytes were incubated in the presence of $Cu^{2+}$ (△), $Pb^{2+}$ (▲), $Co^{2+}$ (●) and without heavy metal ions (○).

$Hg^{2+}$, $Cu^{2+}$ and $Pb^{2+}$, but there was no quantitative correlation between the extent of hemolysis and the degree of peroxidation. Despite the discrepancy between the values obtained from both methods, it is clear that the extent of peroxidation is higher in erythrocytes incubated with metals exerting hemolytic action than in the controls. Furthermore, $Co^{2+}$, which does not induce hemolysis, does not increase the malonaldehyde level measured by both methods.

In order to elucidate whether the peroxidation precedes the hemolysis, we studied the kinetics of the processes. The action of two typical hemolytic agents ($Pb^{2+}$ and $Cu^{2+}$) was compared with that of $Co^{2+}$ and a control. The two chosen metals differ considerably: $Cu^{2+}$ is a strong catalyst of lipid peroxidation [12,14] but does not lead to leakage of $K^+$ [15], while $Pb^{2+}$ does not catalyse the peroxidation of unsaturated fatty acids [14] but causes a rapid $K^+$ leakage [3,15]. From the results presented in Fig. 1, it is seen that at the 30th minute of incubation none of the metals tested causes hemolysis. Later, hemolysis occurs in the $Pb^{2+}$- and $Cu^{2+}$-treated erythrocytes, and the hemolytic action of $Cu^{2+}$ is considerably stronger as shown by the steeper curve. In observing the development of peroxidative processes in erythrocytes, it becomes evident (Fig. 2) that as early as the 30th minute during incubation, the malonaldehyde level in erythrocytes incubated with hemolytic agents ($Cu^{2+}$ and $Pb^{2+}$) is considerably higher compared with the controls. $Co^{2+}$, which does not induce hemolysis throughout the entire period of incubation, also does not lead to generation of malondialdehyde in the erythrocytes.

## Discussion

Our experiments show that the hemolytic action of the heavy metals tested is connected with the development of peroxidative processes in erythrocyte membranes. The fact that the level of malonaldehyde products is raised in erythrocytes treated with hemolytic metals even before the appearance of hemolysis, i.e., before the occurrence of any noticeable destruction of the membranes, suggests that the development of peroxidative processes precedes but does not result from hemolysis.

The data obtained show that lipid peroxidation of erythrocyte membranes is caused by hemolytic metals, irrespective of whether or not they possess pro-oxidant catalytic action. Different results on the degree of peroxidation were obtained from both methods used. Thus, when malonaldehyde was measured in the total incubation mixture, we found the highest degree of peroxidation in the presence of metals with strong pro-oxidant activity ($Cu^{2+}$, $Fe^{2+}$ and $Fe^{3+}$). When lipid extracts were used, the degree of peroxidation was highest in erythrocytes incubated with strong hemolutics agents ($Ag^{+}$, $Pb^{2+}$, $Hg^{2+}$ and $Cu^{2+}$). These discrepancies cannot been explained easily. The observed higher level of polar malondialdehyde products in erythrocytes incubated with $Cu^{2+}$, $Fe^{2+}$ and $Fe^{3+}$ than in erythrocytes incubated with $Ag^{+}$, $Hg^{2+}$ and $Pb^{2+}$ may be due to the catalytic breakdown of accumulated lipid peroxides to final, water-soluble products. The high level of peroxidation in the presence of $Pb^{2+}$, $Hg^{2+}$, $Ag^{+}$ and $Cu^{2+}$, estimated by measuring the peroxides in lipid extracts, suggests that an indirect mechanism for the initiation of lipid peroxidation may be involved. Recently, it has been demonstrated by Carrel et al. [16] that $Cu^{2+}$ greatly stimulates hemoglobin auto-oxidation by a mechanism involving superoxide production. Our investigation demonstrates that $Pb^{2+}$ is also able to produce superoxide radicals by a direct interaction with hemoglobin (Ribarov, S.R., Benov, L.C. and Benchev, I.C., unpublished data). It is possible that the other metals with hemolytic action have the same effect. The activated oxygen forms generated by the metal-induced auto-oxidation of hemoglobin are capable of initiating peroxidative processes of membrane lipids. Thus, it is probable that metals with hemolytic action, irrespective of their pro-oxidant catalytic properties, create opportunities for peroxidation of the membrane lipids.

In the present work, we have not found a correlation between the extent of hemolysis and the degree of peroxidation. It is probably due to significant differences in the properties of hemolytic heavy metals. As mentioned, some of these metals increase the cation permeability of erythrocyte membranes [1], thus leading to increased osmotic pressure accelerating the hemolysis. Furthermore, some of the tested metals inhibit to different degrees the enzymic systems playing an important role in the functional and structural integrity of red blood cells. Nevertheless, it is clear from the data presented that the degree of peroxidation of erythrocytes membranes is higher in the presence of hemolytic metals than in the controls.

Thus, it is tempting to speculate that lipid peroxidation is a possible mechanism of damage to the red cell membrane caused by hemolytic heavy metals. It is likely that this process is initiated in different ways by the various metals. The final effect is that the hemolysis would also depend on the ability of the

heavy metal ions to interact with membrane proteins and to cause changes in membrane conformation and permeability.

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