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MECHANISMS OF HEMOLYSIS INDUCED BY COPPER

AFONSO FERNANDES, M. LOURDES MIRA, M. SILVA AZEVEDO and CARLOS MANSO

Institute of Physiological Chemistry, Faculty of Medicine, 1600 Lisbon, Portugal

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An excess of copper is the cause of hemolysis in a number of clinical conditions. Incubation of human erythrocyte (RBC) suspensions with copper (II) causes the formation of methemoglobin, lipid peroxidation and hemolysis.

A new variant of the thiobarbituric acid (TBA) method, which minimizes the formation of interfering chromophores, was used to detect lipid peroxidation. Lipid peroxidation precedes hemolysis and the antioxidant vitamins C and E, which inhibit lipid peroxidation, also inhibit hemolysis. Consequently lipid peroxidation appears to be the cause of RBC destruction. Lipid peroxidation arises mostly from the oxidation of oxyhemoglobin by copper as it is inhibited in RBCs with carbon monoxyhemoglobin or methemoglobin. A direct interaction of copper with the red cell membrane seems to play only a minor role. Copper effects depend on the presence of free SH groups. Lipid peroxidation is probably initiated by activated forms of oxygen as it is increased by an inhibitor of catalase and reduced by hydroxyl radical scavengers. With higher copper concentrations hemolysis is greater: its mechanism appears different as lipid peroxidation is smaller but hemoglobin alterations, namely precipitation, are more pronounced.

KEY WORDS: Copper, lipid peroxidation, hemolysis, methemoglobin.

INTRODUCTION

Copper has been shown to be responsible for serious intoxications both acute and chronic in a variety of animals. In sheep, intravascular hemolysis, hepatic and tubular necrosis were described.[1] In humans, acute intoxication is followed by methemoglobin formation and intravascular hemolysis.[2–4] In hepatolenticular degeneration episodes of hemolysis follow sudden necrosis of the liver with release of large amounts of copper into the circulation.[5–7]

Copper toxicity was also described as a complication of hemodialysis[8] and after treatment of skin burns with copper sulphate.[9] The severity is related with the copper concentration in whole blood or in the RBC, but not in serum[2,8] since the plasma copper enters into the red cells,[10] where it affects metabolic pathways, hemoglobin and membranes.

Metabolic effects result from inhibition of glycolytic kinases.[11,12] As a consequence, both ATP and NADH levels are reduced. The decrease in ATP concentration impairs the activity of the calcium and sodium/potassium pumps. Decrease of NADH levels reduces the activity of methemoglobin reductase.

Reduced glutathione (GSH) may also undergo a significant decrease in concentration due either to direct oxidation by copper[13] or to its use as a cofactor of glutathione peroxidase which scavenges the H_2O_2 formed.[14] A consequence of this is the decrease in NADPH available for glutathione reductase.

Copper affects hemoglobin (Hb) oxidizing it to methemoglobin (metHb) with

formation of hemichromes, precipitation of Hb and generation of superoxide radicals.[15,16] It induces the oxidation of cysteine (β^{93}) residue which is located near the haem group. The oxidation may be rapid if the copper is present in elevated concentrations or slow if its concentration is low[17,18] In this case a continuous transfer of electrons from the metal to dioxygen with radical generation occurs. Once the superoxide radical is formed, superoxide dismutase and oxyhemoglobin generate H_2O_2 which in the presence of redox metals such as iron and copper may induce the formation of hydroxyl radicals through a Fenton catalysed Haber–Weiss reaction.

Copper may act also directly on cell membranes, either by oxidizing SH groups and generating active forms of oxygen [19] or by inducing the peroxidation of lipids, followed by their fragmentation, with formation of reactive aldehydes and protein polymerization. As a consequence there is RBC agglutination,[20] spherocytosis, increased permeability and rigidity of membranes.[21]

In the present study an evaluation of the effect of different concentrations of copper on red cells and the relationship between Hb oxidation and membrane alteration was carried out.

MATERIAL AND METHODS

Reagents of the highest purity were purchased and used without further purification.

Cupric chloride ($CuCl_2$, $2H_2O$) was obtained from May and Baker, sodium formate from Carlo Erba and sodium benzoate from Bush. Organic solvents, 2-thiobarbituric acid and phosphotungstic acid from Merck, sodium nitrite from BDH. All other reagents were from Sigma.

Blood was collected in ACD and obtained daily from healthy volunteers. Red blood cells were washed 3 times with saline. Cell suspensions were prepared in saline, in a final concentration of 5% v/v. The pH during the assays remained within 6.9–7.0. RBC suspensions with carbon monoxyhemoglobin (HbCO) were obtained by bubbling CO in the suspensions for 20 min, followed by collection of the cells by centrifugation and resuspension in saline. For preparation of metHb, the cells were washed with saline and an equal volume of 1% sodium nitrite in saline was added. This was followed by incubation for 30 min at 25°C. Cells were then washed 3 times with saline.

In all the assays, a preincubation of the red cell suspensions with 2 mM sodium azide was carried out for 1 hour at 37° in a shaking water bath. The copper and/or other compounds were added to a final volume of 7 ml.

At the end of the incubation period, 0.5 ml of RBC suspension were placed into 4.5 ml saline. After centrifugation the supernatant was used for determination of the degree of hemolysis and the cells for the determination of metHb. Percent hemolysis was calculated spectrophotometrically at 409 nm on the basis of heme released. The value corresponding to 100% hemolysis was calculated by adding 0.1 ml of suspension to 9.9 ml distilled water. For determination of metHb concentration the RBCs were hemolyzed with distilled water, centrifuged and the supernatant diluted to give an absorbance at 525 nm of 0.180. The absorbance at 630 nm was also recorded. The amount of hemoglobin precipitated was calculated from the relative decrease of the absorbance of the 100% hemolysed RBC suspensions incubated with copper and/or other compounds when compared to RBC suspensions incubated only with azide.

The determination of the degree of lipid peroxidation, was carried out using a modification of the TBA method avoiding the formation of chromophores which

interfere with the absorbance readings at 532 nm. To 25 ml of red cell suspension 25 μl of 10% Triton X-100 were added. After shaking, deproteinization with 1 ml of 5% phosphotungstic acid is carried out. The supernatant was separated by centrifugation and filtered. To 1.5 ml of the filtrate 1 ml 0.8% TBA were added and the mixture incubated in a boiling water bath for 30 min. After cooling, 2 ml butanol were added and the solution was vigorously shaken for 15 seconds and centrifuged. The absorbance of the supernatant was measured at 532 nm. All determinations were carried out in duplicate.

RESULTS

The incubation of a 5% RBC suspension up to 150 min, preceded by a preincubation of 1 hour in 2 mM sodium azide, does not induce hemolysis, metHb formation, Hb precipitation or lipid peroxidation. In the presence of 0.05 to 2 mM copper changes dependent on the copper concentration were observed. With 0.05 mM, the alterations are minimal and consist in a slight increase in metHb formation. For concentrations of copper from 0.1 to 0.25 mM there is a partial hemolysis, moderate metHb formation and definitive induction of lipid peroxidation. For copper concentrations of 0.5 to 2 mM, hemolysis increases until it becomes complete, metHb formation goes up to 58% and Hb precipitation becomes severe. Lipid peroxidation is maximal at 0.175 mM copper and then decreases (Fig. 1).

Effect of ascorbate and of tocopherol

Ascorbate or tocopherol were added before preincubation to a final concentration of 0.2 mM and 8 mM respectively. In the presence of 0.1 mM copper both compounds

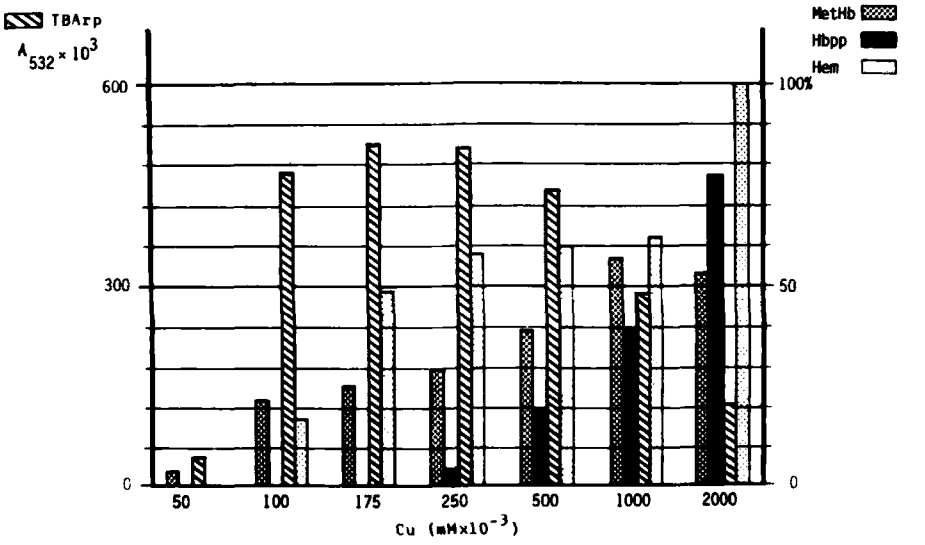

FIGURE 1 MetHb formation, Hb precipitation, TBA reactive products formation and hemolysis after exposure of RBC suspensions to different copper concentrations. Incubation time = 75 min.

were found to inhibit lipid peroxidation and hemolysis. Ascorbate had no effect on metHb formation which was 35% inhibited by tocopherol. Ascorbate in the presence of 1 mM copper does not alter metHb formation; lipid peroxidation was 96% inhibited, Hb precipitation 56% and hemolysis 44% inhibited respectively.

Lipid peroxidation and hemolysis as a function of time

Fig. 1 shows that for concentrations of copper between 0.100 and 0.175 mM, lipid peroxidation is similar, although hemolysis almost triplicates. Since lipid peroxidation must precede hemolysis it was decided to investigate both parameters as a function of time at copper concentrations of 0.1 and 0.175 mM. In Fig. 2 it can be observed that lipid peroxidation precedes hemolysis in both cases. For the highest concentration of copper, lipid peroxidation commences earlier as well as hemolysis.

Influence of hemoglobin

Lipid peroxidation induced by 0.175 mM copper in red cells containing HbCO starts later and progresses more slowly than in the presence of oxyHb (Fig. 3). After 90 min, lipid peroxidation in the presence of oxyHb is 3 times higher than in the presence of HbCO. In the presence of 100% metHb there is no lipid peroxidation nor hemolysis after 90 min incubation. However, the percentage of metHb goes down from 100% to 48%.

Influence of hydroxyl radical scavengers

In order to clarify the influence of hydroxyl radicals on lipid peroxidation incubations were carried out in the presence of several scavengers (Table I). Inhibition of both

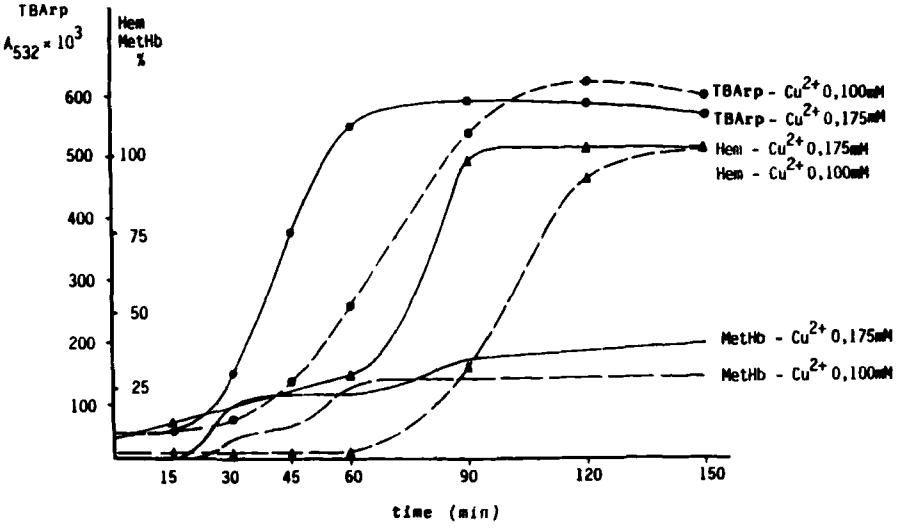

FIGURE 2 Evolution with time of metHb and TBA reactive products formation and of hemolysis induced by $CuCl_2$ — 0.100 and 0.175 mM — in RBC suspensions.

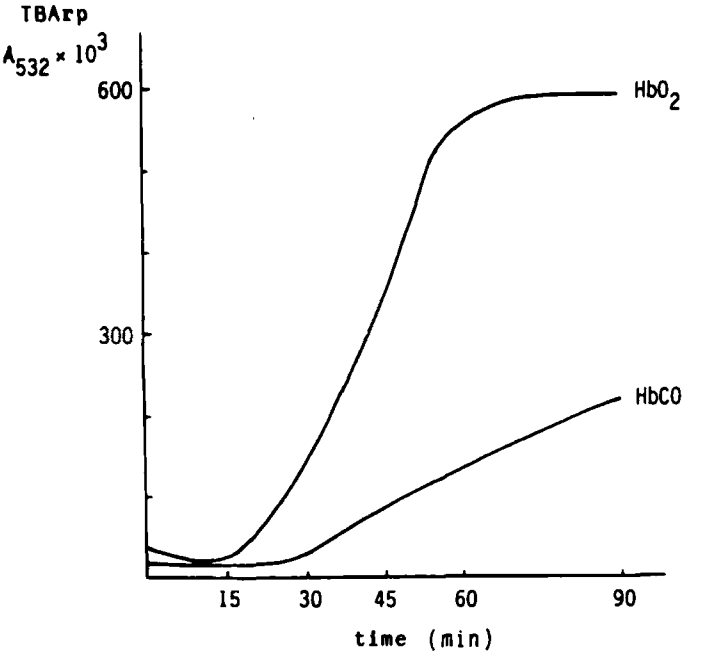

FIGURE 3 TBA reactive products formation in RBC suspensions with oxyHb or with COHb, incubated with 0.175 mM $CuCl_2$.

TABLE I
Effect of hydroxyl radical scavengers on TBA reactive product formation (TBArp) and on hemolysis ($[Cu^{2+}] = 0.1$ mM).

Scavenger	Concentration (mM)	% inhibition	
		TBArp	hemolysis
Formate	100	100	97
Benzoate	100	100	96
Isopropanol	100	98	96
Mannitol	100	29	71
	200	100	98
Ethanol	100	14	37
	200	44	46

lipid peroxidation and hemolysis was observed. Controls show that with the scavengers, hemolysis was always lower than 2.6%.

Effect of N-ethylmaleimide (NEM)

Copper affects the sulphydryl groups of glutathione, Hb and glycolytic enzymes. NEM penetrates the red cells and binds to SH groups.[22]

Three experiments were carried out using NEM to a final concentration of 4 mM (0.08 mmoles/ml RBC):

a) NEM added either in the preincubation step with azide or during incubation in the absence of copper. There was a minimal degree of hemolysis (< 2%) and no lipid peroxidation or metHb formation in 90 min incubation.

b) NEM added before the addition of 0.1 mM copper, followed by 90 min incubation. A complete inhibition of metHb formation, doubling of lipid peroxidation and total hemolysis was observed.

c) NEM present in the preincubation step, followed by a 90 min incubation in the presence of 0.1 mM copper. Lipid peroxidation was found to be totally inhibited and hemolysis was reduced to 11%.

DISCUSSION

Ribarov and Benov[23] exposed bovine and guinea pig RBC to 1 mM copper. For human cells we were unable to observe the absorbance peak at 532 nm with the TBA method as previously reported by Ribarov and Benov for the measurement of malonyldialdehyde (MDA) in the "whole incubation mixture". Interferences from hemoglobin and TCA have been described in the determination of lipid peroxidation by the TBA method[24,25]. Phosphotunsgtic acid was used by Yagi[26] as a deproteinizing agent for determination of lipoperoxides in serum and plasma. However although the determinations was modified as described in the material and methods section, human RBC did not exhibit MDA production. Human RBC have been reported to be more resistant to oxidative stress[27] with their susceptibility varying[28] with differences in the efficiency of antioxidant defence, namely levels of vitamins C and E.

Since copper and other metals are known to induce the production of hydrogen peroxide in RBC,[14] we utilized the technique described by Stocks and Dormandy[29] to amplify the effect of copper including a preincubation of the cells with sodium azide which is an inhibitor of catalase and prevents degradation of MDA by H_2O_2. Even using this technique we found some variability in the results with RBC from different donors.

The effects produced by copper depend on its concentration. There is a progressive aggravation in the Hb levels in agreement with the results of Winterbourn and Carrel for oxidation, denaturation and precipitation by copper.[18] We did not evaluate the amount of copper which penetrates into the RBC nor the fraction bound to other molecules but the relationship between the number of cupric ions and the number of hemoglobin tetramers associated to certain effects are similar to those referred by Winterbourn and Carrel[18] using oxyHb solutions. The fact that percentages of metHb higher than 50% are not obtained may be due to the copper oxidising only the β-chain.

Lipid peroxidation induced by elevated concentrations of copper is smaller. This may be due to the speed of hemolysis, disrupting the spatial relationship between Hb and cell membrane or to the protective effect of metHb.[30]

The demonstration that the peroxidation precedes hemolysis and that prevention of the peroxidation by antioxidants prevents the cell damage led us to conclude that hemolysis by copper in the lowest range of concentrations is a consequence of lipid peroxidations of red cell membranes. Such demonstration is needed to prove that lipid peroxidation is not the result of cell injury.[31]

Accepting the cause-effect relationship between lipid peroxidation and hemolysis in

the presence of 0.1 mM copper, the next step was to evaluate if lipid peroxidation results from direct interaction of copper with membrane lipids or with Hb. The experiments with HbCO and with metHb favour the second possibility. Only the small degree of lipid peroxidation detected in RBC with HbCO may result from the interaction of copper with membrane lipids, since no metHb formation is detected.

In RBC with 100% metHb, lipid peroxidation could depend from radicals produced from copper reduced by membrane protein SH groups but this reaction is unlikely to take place because metHb could act as a scavenger of superoxide radicals. This is confirmed by the decrease in metHb and increase in oxyHb concentrations found at the end of the incubation period.

The next step was therefore the identification of the active forms of oxygen which act as intermediates. Hydroxyl radical was found to be the most likely candidate. It can initiate lipid peroxidation either by hydrogen abstraction or through an addition reaction to a double bond with aldehyde formation. The results obtained with hydroxyl radicals scavengers confirm our hypothesis.

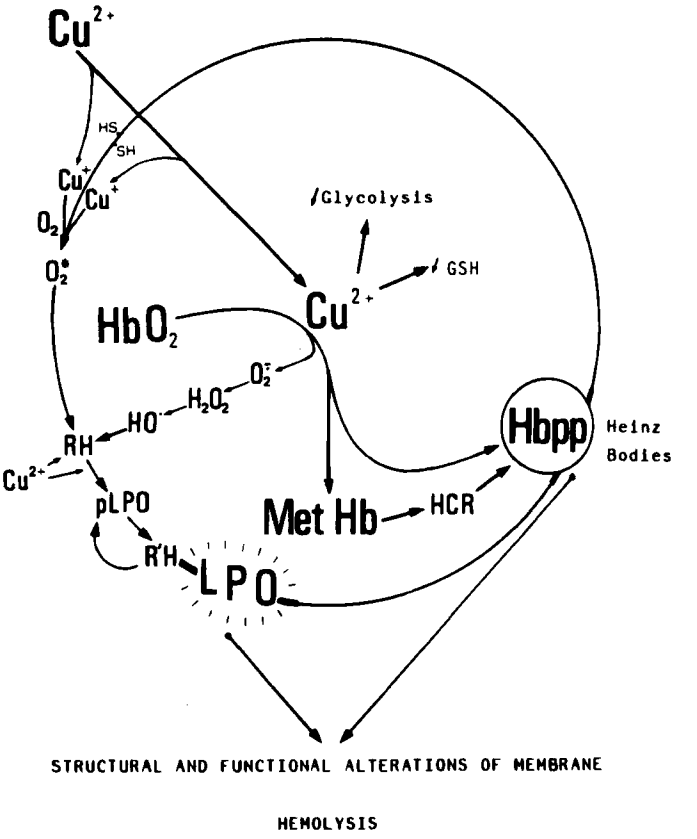

FIGURE 4 Mechanisms suggested for the hemolysis induced by copper (SH = membrane SH groups, RH, R'H = membrane polyunsaturated fatty acids, pLPO = lipid peroxidation products, HCR = hemichromes).

The experiments with N-ethylmaleimide (NEM) confirm the importance of free SH groups for the effect of copper. When NEM is added just before copper, it may block GSH. The observed increase in lipid peroxidation and hemolysis may consequently result from inhibition of GSH peroxidase resulting in raised levels of H_2O_2. But when NEM is present in the preincubation period blocking all the SH groups, including those of the membrane and of hemoglobin lipid peroxidation and hemolysis are not observed following addition of copper.

The mechanisms suggested for the hemolysis induced by copper are summarized in Fig. 4. Lipid peroxidation probably induces a severe lesion of the membrane inducing hemolysis. The sudden crises of intravascular hemolysis probably result from a similar mechanism. Our studies justify the therapeutic use of antioxidant agents to prevent or to minimize the hemolysis caused by copper *in vivo*.

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