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ENHANCEMENT OF THE COPPER-INDUCED HEMOLYSIS BY PROGESTERONE

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SUMMARY

Incubation of human erythrocytes with copper sulfate results in hemolysis. Progesterone enhances substantially the copper-induced hemolysis under conditions in which progesterone alone does not cause hemolysis. Addition of progesterone, after preincubation with copper sulfate, affects the induction period that occurs before hemolysis; increasing the time of preincubation decreases the induction period. In contrast, the length of time needed for detecting hemolysis is not affected by the length of the preincubation period with progesterone. The time period required for hemolysis to commence after copper sulfate is added, following a preincubation with progesterone, is similar to that time period which is observed when copper sulfate and progesterone are added together at the beginning of the incubation. Calcium chloride inhibits the copper-progesterone hemolysis. Copper sulfate alone causes the erythrocytes to have an increased osmotic fragility. The results suggest that progesterone initiates hemolysis by binding to sites exposed after copper ions cause alterations in the membrane.

INTRODUCTION

Progesterone is taken up by intact human erythrocytes and can bind to isolated erythrocyte membranes[1]. This steroid protects red cells from storage lesions, namely spontaneous hemolysis and increases in osmotic fragility[2]. At low concentrations of progesterone, erythrocytes are protected against hypotonic hemolysis, whereas at higher concentrations it increases hypotonic hemolysis[3]. Progesterone also causes lysis in isotonic medium[4, 5]. We have found that under conditions where progesterone alone does not cause hemolysis, the steroid enhances the hemolysis which occurs when red cells are incubated together with cupric ions.

Studies have been carried out to delineate which alterations in the erythrocyte influence the copper-induced hemolysis. The intracellular effects produced on incubation with copper ions include the loss of reduced glutathione, conversion of oxyhemoglobin to methemoglobin, formation of Heinz bodies, formation of hydrogen peroxide[6], stimulation of the hexose monophosphate shunt[7], and inhibition of some of the glycolytic enzymes[8]. Cupric ions also influence membrane phenomena including sodium-potassium permeability[9], glycerol permeability[10, 11] membrane ATPases[12] and red cell morphology[13]. In the present paper it is postulated that copper affects the erythrocyte membrane to permit the initiation of destructive changes by progesterone.

Abbreviation—Hepes, N-2-hydroxyethylpiperazine-N'2 ethanesulfonic acid.

MATERIAL AND METHODS

Progesterone was obtained from Sigma Chemical Co. The steroid was dissolved in ethanol. Bovine serum albumin, fatty acid poor, was purchased from Schwarz–Mann. The source of cupric ions was copper sulfate. Solutions of this salt were prepared in 0·154 M sodium chloride as were the solutions of calcium chloride and magnesium chloride. Hepes buffer was obtained from Calbiochem.

Heparinized blood from normal male volunteers was used. The plasma and buffy coat were removed within 15 min after collection. The red cells were washed 4 times with Hepes medium consisting of 0·01 M Hepes buffer, 149 mM NaCl, 5 mM KCl and 50 mM glucose (adjusted to pH 7·4). Hepes buffer, unlike phosphate buffer or Tris–buffer, does not complex copper[14].

The cells were incubated in Hepes medium, containing bovine serum albumin at a final albumin concentration of 1·6%. Copper ions cause erythrocytes to agglutinate[15] and albumin prevents this phenomenon.

Unless otherwise stated, the cupric sulfate concentration is 1 mM. Cupric sulfate at this concentration causes the incubation solution to decrease in pH from 7·4 to 7·2. Hepes-albumin media in which copper sulfate was absent were adjusted to pH 7·2. The progesterone concentration was 570 μM unless otherwise stated. The steroid was routinely added in a volume of 0·04 ml ethanol per 10 ml of incubation suspension to give a final ethanol concentration of 0·086 M. This ethanol concentration was also maintained in the incubations without progesterone.

The incubations were carried out at 37 C in a water bath with gentle agitation. The hematocrit of the incubation suspensions was adjusted to values ranging between 5 and 7%. The incubations were initiated by adding the erythrocytes to Hepes–albumin media containing the prerequisite ingredients.

Periodically, samples were withdrawn, the cells removed by centrifugation, and the per cent hemolysis determined by measuring the optical density of the supernatant at 590 nm, using a Cary-14 spectrophotometer. Cupric ions convert oxyhemoglobin to methemoglobin[16] with an isobestic point at 590 nm. Complete hemolysis is measured by lysing the erythrocyte suspension with 0·01 M Hepes buffer at pH 7·4.

Osmotic fragility studies were performed on cell suspensions which were incubated for various time periods. The cells were washed once with Hepes medium, resuspended in this medium, and added to 0·01 M Hepes, pH 7·4, containing various concentrations of NaCl. After a 5 min incubation at 37 C, the amount of hemolysis was determined as described above. Osmotic fragility is a standard method of measuring erythrocyte membrane integrity.

The copper content of the erythrocytes was determined with diethyldithiocarbamate by the method of Gubler *et al.*[17] after an aliquot of the incubating suspension was removed and the cells were washed twice with Hepes medium. The reduced glutathione level of the washed cells was determined by the procedure of Beutler *et al.*[18].

Lipid peroxidation was determined by measuring the formation of malonaldehyde, by the method of Stocks and Dormandy[19], with slight modifications. The cells were treated with 10% trichloroacetic acid containing disodium EDTA and the pink chromogen was developed in the cell free supernatant with a 0·4% thiobarbituric acid solution prepared in 0·05 M NaOH. EDTA was added to the cell free supernatant before addition of thiobarbituric acid. The determination of malonaldehyde in cells treated with copper sulfate is complicated by formation of some turbidity rendering the method only semiquantitative.

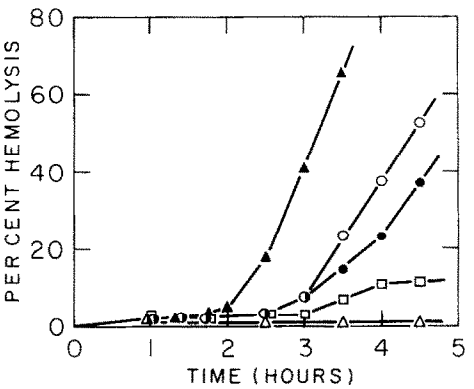

Fig. 1. The effect of progesterone concentration on hemolysis in the presence of 1 mM $CuSO_4$. Progesterone concentrations: (▲) 570 μM, (○) 380 μM, (●) 190 μM, (□) no progesterone, (△) 570 μM but no $CuSO_4$. Ethanol was routinely added when progesterone was omitted from the incubation.

RESULTS

Hemolysis of human erythrocytes occurs upon incubation with cupric ions. As seen in Fig. 1, hemolysis becomes appreciable after 3 h of incubation with 1 mM $CuSO_4$. In the absence of $CuSO_4$, no detectible hemolysis occurs during the incubation period. The addition of progesterone significantly increases the hemolysis detected in the presence of cupric sulfate. This effect, which we refer to as the copper-progesterone-hemolysis, is dependent on progesterone concentration. The induction period for hemolysis decreases with increasing progesterone concentration. With extensive hemolysis, progesterone causes turbidity, which limits the measurement of hemolysis. Incubation with progesterone alone does not cause hemolysis.

Increasing copper sulfate concentration causes the rate of hemolysis to increase (compare 2 mM $CuSO_4$ alone in Fig. 2 with 1 mM $CuSO_4$ in Fig. 1). The enhancement of hemolysis by progesterone is highly dependent on the cupric sulfate concentration, as shown in Fig. 2. Decreasing the cupric sulfate concentration from 1 mM to 0·5 mM, considerably reduces the rate of hemolysis, whereas increasing the copper concentration to 2 mM increases the rate of hemolysis.

Cell copper measurements, depicted in Fig. 3, show that the level in the erythrocytes reflects the $CuSO_4$ concentration in the incubation medium; the cell copper levels are lower at 0·5 mM than at 1 mM $CuSO_4$. At both concentrations the rate of uptake of cupric ions is biphasic; a rapid phase is followed by a slow phase which commences at about 2·5 h. Progesterone has no effect on the uptake of copper through the first

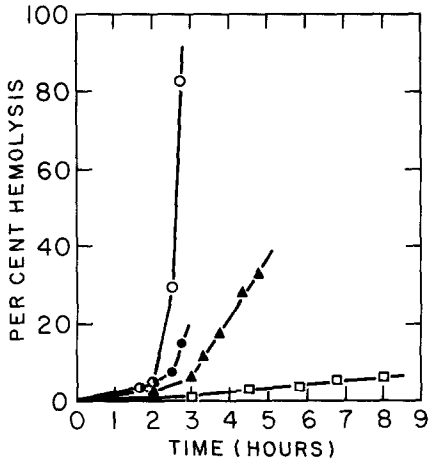

Fig. 2. The effect of $CuSO_4$ concentration on hemolysis in the presence of 570 μM progesterone. $CuSO_4$ concentrations; (○) 2 mM, (△) 1 mM, (□) 0·5 mM, (●) 2 mM—no progesterone. Hemolysis in the absence of progesterone with either 1 mM or 0·5 mM $CuSO_4$ are not shown but after 4 h hemolysis was less than 5%.

3 h of incubation in the presence of 1 mM $CuSO_4$. It is not known whether red cell copper increases after progesterone initiates the hemolytic process.

The hemolysis in the presence of 1 mM $CuSO_4$ is affected by the albumin concentration. Lowering the albumin concentration to 0·8% increases the hemolysis both in the absence and presence of progesterone. This albumin effect and the $CuSO_4$ concentration dependency (Fig. 2) can be explained by considering the high and low affinity sites for cupric ions on albumin[20]. Increasing the cupric ion concentration when the albumin concentration is 1·6% (equivalent to 0·23 mM) should result in the binding of cupric ions at lower affinity sites. This will enable the erythrocytes to take up more cupric ions. Decreasing albumin concentration causes a reduction in the effective amount of cupric ions bound to high affinity sites on the albumin. Thus, more cupric ions become available to the erythrocyte. In this context, it has been shown that the presence of plasma proteins decreases the uptake of radioactive copper by sheep erythrocytes[21].

Preincubation with either progesterone or with copper sulfate affects the time course of hemolysis. Figure 4 shows how preincubation with 1 mM $CuSO_4$ influences the hemolysis. After addition of progesterone, following preincubation with cupric ions for 1·5 h, substantial hemolysis occurs after a lag period of about 2·5 h. On preincubation for longer periods of time, the lag period is reduced. Preincubating for 4 h causes substantial hemolysis to occur 15 min after progesterone is added. Cells were also preincubated with progesterone for 1·5 to 3 h. In all cases, after addition of $CuSO_4$, a lag period of at least 3 h is observed before hemolysis begins. A similar period of time precedes hemolysis when copper and progesterone are added at the start of the incubation.

The substitution of choline chloride for the sodium chloride and potassium chloride components of the buffered media does not change the copper–progesterone hemolysis or the hemolysis by copper sulfate

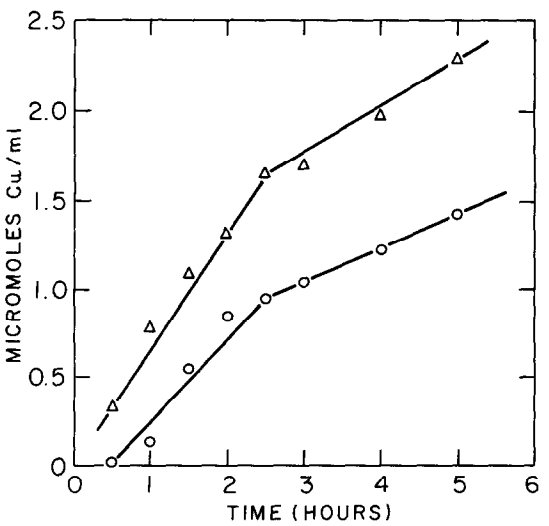

Fig. 3. Uptake of copper ions by erythrocytes expressed in micromoles of copper per ml packed cells (i.e. approximately 10^{10} cells). $CuSO_4$ concentrations in the incubation solutions; (○) 0·5 mM, (△) 1 mM.

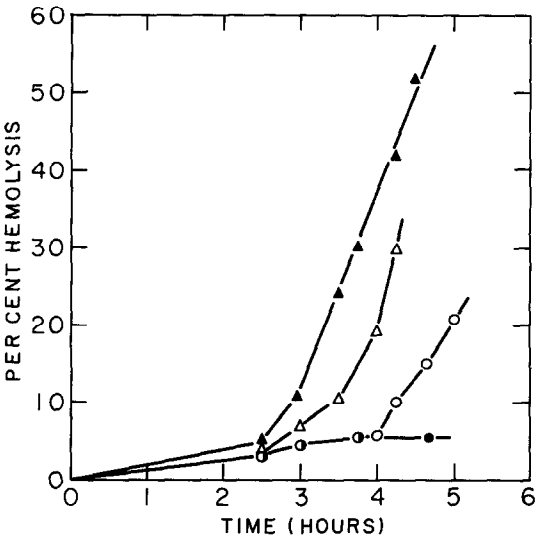

Fig. 4. The effect of preincubation on hemolysis with 1 mM $CuSO_4$. Progesterone at a final concentration of 570 μM was added at the following times; (▲) 0 h, (△) 1·5 h, (○) 4 h. No progesterone (●).

alone. Complete conversion of oxyhemoglobin to methemoglobin, by preincubation with sodium nitrite (followed by washing the cells to remove the sodium nitrite) also does not change the rate of hemolysis.

Since the steroid was added as an ethanolic solution, the effect of ethanol on hemolysis was investigated. Hemolysis by 1 mM $CuSO_4$ generates the same hemolytic pattern in the absence of ethanol as in the presence of 0·086 and 0·172 M ethanol. On the other hand, 0·172 M ethanol increases the copper progesterone hemolysis. The final ethanol concentration, arising from the addition of progesterone, is routinely 0·086 M. These results indicate a specific interaction of ethanol with the erythrocytes in the presence of progesterone.

The presence of 1 mM calcium chloride inhibits the copper–progesterone hemolysis, whereas 1 mM magnesium chloride does not (Fig. 5). Higher concentrations of magnesium chloride (2·5 and 5·0 mM) do, however, inhibit the hemolysis. The degree of inhibition by 5 mM $MgCl_2$ is similar to that obtained with 1 mM $CaCl_2$. Hemolysis in the presence of 1 mM $CuSO_4$ alone is not significantly affected by concentrations of $CaCl_2$ or $MgCl_2$ that inhibit the copper–progesterone hemolysis. Furthermore, the uptake of cupric ions is not affected by 1 mM $CaCl_2$, at least during the first 3 h of incubation.

During our studies, different blood samples gave variations in the length of the induction period and the rate of hemolysis. As exemplified by the 570 μM progesterone and 1 mM $CuSO_4$ curves of Figs. 1 and 2, a longer induction period is followed by a slower rate of hemolysis. For all the blood samples progesterone enhances substantially the copper-induced hemolysis.

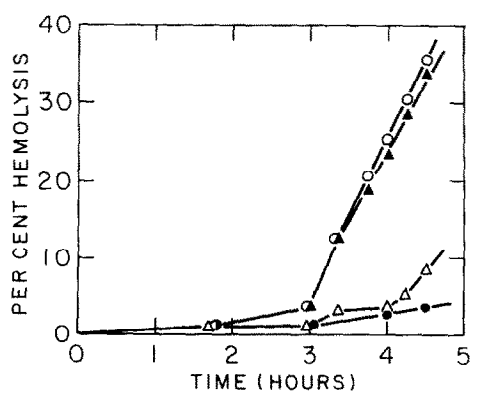

Fig. 5. The effect of $CaCl_2$ and $MgCl_2$ on hemolysis with 1 mM $CuSO_4$ and 570 μM progesterone. Incubations contained: (▲) no $CaCl_2$ or $MgCl_2$, (○) 1 mM $MgCl_2$, (△) 1 mM $CaCl_2$, (●) 1 mM $CaCl_2$ or 1 mM $MgCl_2$ but no progesterone. The data for $CuSO_4$ alone is not shown in this figure but the rate of hemolysis coincides with the curve for $CaCl_2$, progesterone and $CuSO_4$ (△).

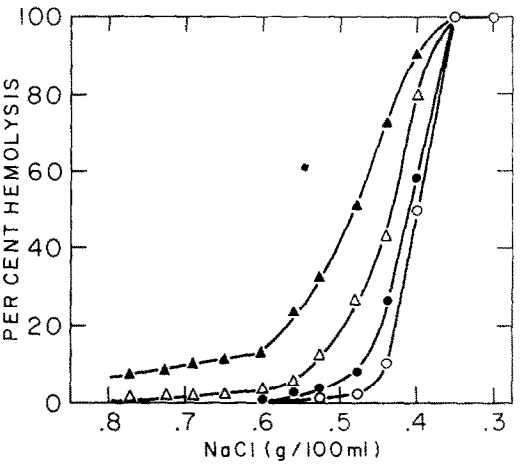

Fig. 6. The effect of incubation time on the osmotic fragility of erythrocytes with and without 1 mM $CuSO_4$. Incubation conditions: 1 mM $CuSO_4$, (△) 2 h, (▲) 3 h; no $CuSO_4$, (○) 0 h, (●) 3 h.

Realizing that small alterations in the concentrations of either progesterone or cupric sulfate could affect the characteristics of hemolysis, we concluded after extensive experimentation that the variations were due to differences in the erythrocyte samples.

Cupric ions increase the osmotic fragility of human erythrocytes (Fig. 6). Significant increases occur during the first 3 h of incubation. This time period coincides with the induction period of the copper progesterone hemolysis. In the absence of $CuSO_4$, the osmotic fragility of the cells changes minimally. Calcium chloride (1 mM) does not affect the copper-induced changes in osmotic fragility.

Some peroxidation of membrane fatty acids, as measured by malonaldehyde formation, occurs on incubation of erythrocytes with copper sulfate. The pink chromogen formed, after the cells are incubated for 3 h with 1 mM $CuSO_4$, does not appear in the absence of copper.

Copper ions decrease the level of erythrocyte reduced glutathione[7]. In our system, 1 mM $CuSO_4$ decreases the reduced glutathione to less than 10% of its original value after 2 h of incubation. During this time, minimal hemolysis occurs in the presence of $CuSO_4$ or with $CuSO_4$ and progesterone. In the absence of copper sulfate, on the other hand, the reduced glutathione level is about 90% of its original value, after 5 h of incubation.

DISCUSSION

The present studies show that on incubation with copper sulfate, hemolysis of human erythrocytes is

considerably enhanced by progesterone, under conditions in which progesterone alone does not cause the erythrocytes to hemolyze. To explain this observation, we postulate that cupric ions induce an alteration in the membrane exposing new sites to which progesterone can bind. This hypothesis is based on the following observations.

(a) Incubation of erythrocytes with copper sulfate markedly increases the osmotic fragility of the erythrocytes without causing substantial hemolysis.

(b) Substantial hemolysis occurs after a short period of time, on addition of progesterone when erythrocytes are preincubated for 3–4 h with 1 mM $CuSO_4$. On the other hand, after preincubation with progesterone, the length of time required to cause substantial hemolysis, after copper sulfate addition, is similar to the length of the time required to cause hemolysis when there is no preincubation.

(c) Decreasing the copper sulfate concentration (from 1 to 0·5 mM) markedly decreases the copper–progesterone hemolysis. This indicates that critical concentrations of copper ions are needed to cause an alteration in the membrane.

Our hypothesis is supported by the observation that progesterone does not increase the rate of copper uptake by erythrocytes during the period of minimal hemolysis. Also, lipid peroxidation, occurring on incubation with cupric ions, suggests a modification of normal membrane interactions. Such changes may, in part, be responsible for increases in osmotic fragility in the presence of $CuSO_4$. In lipid bilayers peroxidation has been associated with a decrease in membrane stability[22].

Copper sulfate could cause alterations in the membrane by two mechanisms. Cupric ions can produce intracellular changes[6, 7, 8] which may have a subsequent detrimental effect on the membrane. The decrease in reduced glutathione occurs during the early stages of incubation. Loss of reduced glutathione cannot, however, be correlated directly with hemolysis, but the absence of reduced glutathione can indirectly cause membrane damage[23]. Since copper sulfate causes the formation of Heinz bodies[6, 24], it is conceivable that membrane changes induced by copper involve the attachment of Heinz bodies to the membrane. Jacob[25] postulated that hemolysis caused by certain unstable hemoglobins is related to the attachment of Heinz bodies to the membrane.

The alterations in the membrane could also arise from the binding of cupric ions directly to membrane components. It has been shown that 30% of endogenous erythrocyte copper is bound to the membrane[26]. In sheep erythrocytes incubated with cupric ions the membrane contains approximately 10% of the total cell copper[21]. Since cupric ions are known to bind to proteins and to alter their configuration[27, 28], it is conceivable that they could bind directly to membrane proteins causing alterations. Conformational changes in membrane proteins could also result from the copper-induced intracellular changes. Changes in protein conformation could alter the membrane so as to permit the binding of progesterone at sites which result in the leakage of hemoglobin from the red cells. Hubbell *et al.*[29] found that perturbation of isolated erythrocyte membranes with lytic concentrations of benzyl alcohol leads to exposure of protein binding sites for a spin labelled steroid which are not available in the intact membrane.

Progesterone is known to bind to erythrocyte membranes[1, 30]. Evidence was obtained which shows that progesterone reacts with a soluble protein fraction of isolated erythrocyte membranes, but not with the lipid components[31]. It is possible that the enhancement of hemolysis in copper-treated cells involves the binding of progesterone to modified membrane proteins.

Alcohols have also been shown to effect hemolysis[3]. The role of ethanol in the copper–progesterone hemolysis is unknown. It cannot be ruled out that, after progesterone initiates the processes which cause leakage of hemoglobin, ethanol acts to augment the hemolytic processes.

The inhibition of the copper–progesterone hemolysis by calcium may be related to its ability to stabilize the membrane. Calcium chloride has been shown to reduce hemolysis of erythrocytes by hypotonic solutions[32], virus particles[33], stilbestrol[5], a hemolysin-prymnesin[34] and butanol[35]. The fact that calcium chloride does not affect the increase in osmotic fragility induced by cupric sulfate may indicate that it influences parameters affected by progesterone. Calcium binds to erythrocyte membrane proteins and phospholipids[36, 37] and it has been suggested that it forms a bridge between these two components, thereby stabilizing the membrane[36].

Calcium has been shown to prevent fragmentation under a variety of conditions. Loss of protein and lipid during preparation of bovine erythrocyte membranes[38] can be prevented by calcium. In human red cell ghosts fragmentation at low ionic strength is inhibited if calcium is present[39]. Fragmentation also precedes many types of hemolysis[40]. It is possible that the copper–progesterone hemolysis is preceded by fragmentation.

Copper can cause *in vivo* hemolysis; indeed during the course of Wilson's disease, a metabolic disorder of copper-homeostasis, hemolysis occurs[24, 41–44]. In cases of Wilson's disease where hemolysis was

detected, the concentration of copper in the red cells is increased[24, 42]. Hemolysis also occurs after accidental ingestion of copper sulfate[45–47]. Copper arising from certain equipment that has been used for hemodialysis has caused hemolytic anemia[48]. Although the progesterone and copper levels used in this study are greater by some magnitudes than physiological levels, the progesterone enhancement of copper-induced hemolysis might be relevant to *in vivo* situations.

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