0014-4754/95/060572-05$1.50 + 0.20

Antioxidant and prooxidant roles of copper in Tween 20-induced hemolysis of hamster and pig erythrocytes containing marginal vitamin E

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Received 15 August 1994; received after revision 5 December 1994; accepted 19 December 1994

Abstract. The concentration-dependent effects of copper acting either as an antioxidant or as a prooxidant were examined in vitro using Tween 20-induced hemolysis. When cupric ion concentration was more than 10 μM, free copper(II) acted as a prooxidant; both extensive hemolysis and production of unknown thiobarbituric acid-reactive substance occurred in hamster and pig erythrocytes irrespective of vitamin E status. However, when cupric ion concentration was 2–4 μM in the incubation medium, copper showed a clear antioxidant activity, reducing both hemolysis and malondialdehyde production induced either by diluted peroxide-containing Tween 20 with ascorbic acid and sodium azide in vitamin E-deficient hamster erythrocytes, or by peroxide-containing Tween 20 in pig erythrocytes containing marginal amounts of vitamin E. Copper(II) is taken up by the erythrocytes, where copper(I)-complexes may contribute to the protection of cells with membrane vitamin E against oxidative radical attack.
Key words. Copper; vitamin E; Tween 20; hemolysis; malondialdehyde; prooxidant; antioxidant.

Normal human plasma and erythrocytes contain 17.0 μmol/l and 10.0 μmol/kg of copper, respectively[1]. Over 90% of human plasma copper is associated with the protein ceruloplasmin and the remaining 5–10% of plasma copper is believed to be fairly loosely attached to amino acids, to small peptides or to serum albumin[2]. Copper is a constituent of superoxide dismutase, and superoxide dismutase activity in erythrocytes becomes an index of copper nutrition[3]. In erythrocytes a protective factor that is different from vitamin E or catalase is supposed to exist[4]. Some copper-chelates employing simple amino acids and peptides have proved to be capable of reacting with superoxide[5,6]. In copper deficiency selenium-dependent glutathione peroxidase activity also decreases[7]. Copper-deficient animals show increased susceptibility of subcellular organelles to in vitro oxidation[8,9]. Increased susceptibility to infectious illnesses and immune dysfunction have been noted in copper-deficient animals[10]. Scottish Blackface lambs are genetically predisposed to low copper status and to increased mortality resulting from bacterial infections, and copper supplementation increases survival[11]. In the swine industry a large amount of copper (125–250 ppm) as copper sulfate has been included in the diets to promote growth and feed efficiency[12]. Injections (i.p.) of various copper compounds, including copper sulfate, prevent paracetamol-induced lipid peroxidation in vivo[13]. Although cupric ions at more than 10 μM cause hemolysis or other peroxidative damage, copper may catalyze several biological processes at a concentration of less than 10 μM[14].

We have established two procedures bringing about hemolysis, specific to vitamin E deficiency in various animals, using peroxide-containing Tween 20[15–17]. The objective of the present work was to examine the concentration-dependent effects of copper(II) acting either as an antioxidant or as a prooxidant in vitro, using Tween 20-induced hemolysis in hamster and pig erythrocytes of different vitamin E status as a test system.

Materials and methods

Male golden hamsters were fed with purified diets devoid of vitamin E or supplemented with 100 ppm of vitamin E (dl-α-tocopheryl acetate) for two months. Percentage composition of the basal diet was: casein 19.0, starch 66.7, soybean oil 5.0, cellulose 5.5 and vitamin and mineral mixture 3.8%[18]. Weaned cross-bred pigs of 5–6 weeks of age were fed vitamin E-unsupplemented and vitamin E-supplemented diets for 6 weeks. Percentage composition of the basal diet was: wheat flour 34.3, dried skimmed milk 40.0, glucose 12.0, fish meal 5.0, soybean meal 3.0, tallow 4.0, tricalcium phosphate 1.4 and vitamin and mineral mixture 0.3[19]. The basal diet contained 13.6 ppm of vitamin E. In the first trial the vitamin E supplementation level was set to 150 ppm. In the second trial linseed oil containing more than 50% of α-linolenic acid was added at 1% to the above basal diet and the vitamin E supplementation level was set at 100 ppm.

The hemolytic test for hamster erythrocytes was conducted as reported before[16]. Heparinized blood samples were washed, and erythrocytes corresponding to 0.2 ml blood were suspended in 5.0 ml of Hepes-saline buffer (pH 7.4) containing 25 mM Hepes (2-[4-(2-hydroxy-

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ethyl)-1-piperazyl]ethanesulfonic acid). An erythrocyte suspension containing 4.8 µl of packed red blood cells was incubated for 30 min in 1.9 ml of incubation mixture. The final concentrations of Tween 20, ascorbic acid and sodium azide were 0.001%, 1.2 mM and 5.3 mM, respectively. An aqueous $CuCl_2$ solution was added to the incubation mixture just before the start of incubation. The hemolytic test using pig erythrocytes was conducted as reported before[17]. The washed erythrocyte portion was incubated with 0.8% (final concentration) Tween 20 in Hepes-saline buffer (pH 7.4) for 15 min at 37.5 °C. An aqueous $CuCl_2$ solution was added to the incubation mixture just before the start of incubation. Plasma α-tocopherol was determined by the fluorometric method[20].

Hydrogen peroxide derived from peroxides in commercial Tween 20 reagents was estimated by the following method. One milliliter of the reagent solution (pH 7.14) containing 20 mM Hepes, 0.75 mM N-ethyl-N-sulfopropyl-m-toluidine and 0.30 mM 4-aminoantipyrine, 0.5 ml of 0.8% Tween 20 solution and 20 µl of horseradish peroxidase (Boehringer Mannheim) were mixed. After warming the mixture for 10 min at 37.5 °C, the optical density of purplish red quinoid pigment at 550 nm was determined. A Tween 20 reagent used for inducing hemolysis contained peroxides equal to 1.2 mM hydrogen peroxide equivalent. Thiobarbituric acid-reactive substance (TBARS) was measured by a modification of the method described by Yasuda et al.[21]. After 15 or 30 min incubation of the hemolytic solution (1.0 or 1.9 ml) at 37.5 °C, twice as much by volume of the reagent solution (2.0 or 3.8 ml) containing 0.375 g thiobarbituric acid, 15 g trichloroacetic acid and 0.025 moles HCl per 100 ml was added, and the mixture was centrifuged. Two ml portions of the supernatant were heated for 15 min in a boiling water-bath. After cooling with water, 4.0 ml of n-butanol was added, shaken vigorously and centrifuged. TBARS in butanol was measured with an Aminco-Bowman Spectrophotofluorometer. A fluorescence intensity of 1 mg of quinine sulfate per liter of 0.1 N H_2SO_4 was calibrated to 100 at 360 nm excitation and 440 nm emission. RFI-1 corresponding to malondialdehyde ap- peared at 515 nm excitation and 553 nm emission and another unknown RFI-2 appeared at 435 nm excitation and 468 nm emission. 1,1,3,3-Tetraethoxypropane (Wako Pure Chemical Ind., Osaka) was used as a standard for malondialdehyde.

Data was expressed as the means ± SE and was compared by analysis of variance (ANOVA) using a statistical computer program (StatView ANOVA, Abacus Concepts, Berkeley, CA, USA, 1987). The levels of significance were determined at $p < 0.05$.

Results

Plasma α-tocopherol contents were 2.2 ± 0.1 and 14.5 ± 1.0 µg/ml in vitamin E-deficient and vitamin E-sufficient hamsters, respectively ($p < 0.05$). At 0–3.9 µM copper, vitamin E-deficient erythrocytes showed significantly greater hemolysis than vitamin E-sufficient ones (fig. 1). Addition of copper to give 2.0 and 3.9 µM significantly decreased the hemolysis induced by perox-

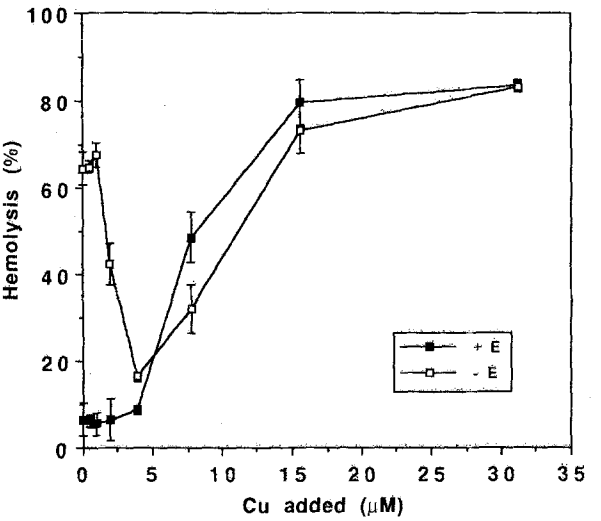

Figure 1. Effects of cupric ions on Tween 20, ascorbic acid and azide-induced hemolysis (%) of vitamin E-deficient and vitamin E-sufficient hamster erythrocytes. The mean hemolysis for 3 animals with SE (bar) is shown. There are significant differences ($p < 0.05$) between the hemolysis of vitamin E-deficient erythrocytes (−E) and that of vitamin E-sufficient ones (+E) at 0 to 3.9 µM copper concentrations.

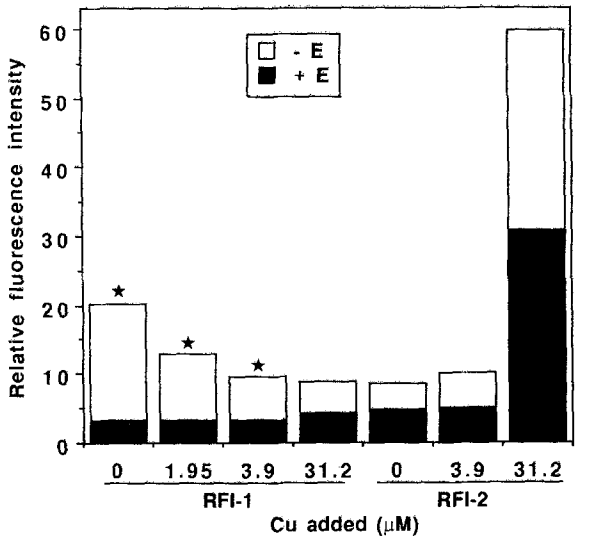

Figure 2. Effects of cupric ions on thiobarbituric acid-reactive substance (TBARS) production in vitamin E-deficient and vitamin E-sufficient hamster erythrocytes. Relative fluorescence intensity means the fluorescence intensity of TBARS corresponding to 5.7 ml of total reaction mixture (1.9 ml of hemolytic solution plus 3.8 ml of reagent). RFI-1 (malondialdehyde) is shown at 0, 1.95, 3.9 and 31.2 µM of copper concentrations and RFI-2 (unknown TBARS) at 0, 3.9 and 31.2 µM copper concentrations. Each white and black column is an average of 3 animals. An asterisk signifies a significant difference between vitamin E-deficient and vitamin E-sufficient erythrocytes ($p < 0.05$).

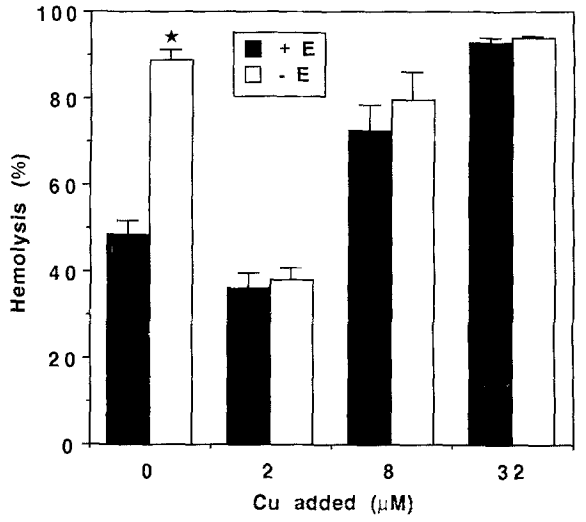

Figure 3. Effects of cupric ions on Tween 20-induced hemolysis (%) of vitamin E-unsupplemented and vitamin E-supplemented erythrocytes in the first pig trial. Numbers of animals used for the blood sampling in each treatment group were 5. The mean hemolysis with SE (bar) is shown. An asterisk signifies a significant difference ($p < 0.01$) between the hemolysis of vitamin E-unsupplemented erythrocytes (white column) and that of vitamin E-supplemented ones (black column).

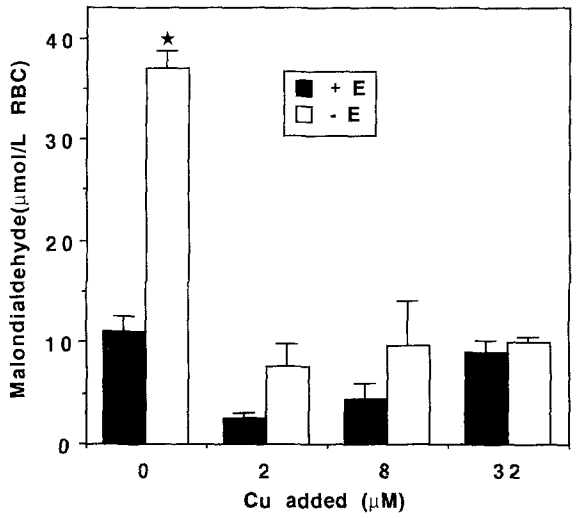

Figure 4. Effects of cupric ions on malondialdehyde production (μmol/L red blood cells) in vitamin E-unsupplemented (white column) and vitamin E-supplemented (black column) erythrocytes in the first pig trail. Blood sampling was the same as in figure 3. An asterisk signifies a significant difference in malondialdehyde production between vitamin E-unsupplemented and vitamin E-supplemented erythrocytes ($p < 0.01$).

ide-containing Tween 20 with ascorbic acid and azide in vitamin E-deficient erythrocytes (fig. 1). At 0–3.9 μM copper addition, vitamin E-deficient erythrocytes produced thiobarbituric acid-reactive substance (TBARS) with a fluorescence peak at 515 nm excitation and 553 nm emission corresponding to malondialdehyde, in significantly greater amounts than vitamin E-sufficient erythrocytes (fig. 2). Addition of copper at more than

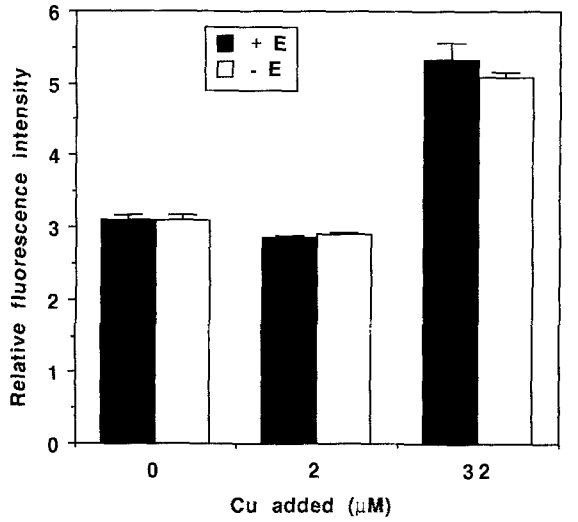

Figure 5. Effects of cupric ions on thiobarbituric acid-reactive, unknown substance in vitamin E-unsupplemented (white column) and vitamin E-supplemented (black column) erythrocytes in the first pig trial. Blood sampling was the same as in figure 3. Relative fluorescence intensity was calculated per 5.8 μl of red blood cells used for each hemolytic solution.

10 μM increased both hemolysis and production of another unknown TBARS with a fluorescence peak at 435 nm excitation and 468 nm emission, irrespective of the vitamin E status (figs 1 and 2).

In the first pig trial, plasma α-tocopherol contents were 2.09 ± 0.16 and 4.52 ± 0.34 μg/ml in vitamin E-unsupplemented and vitamin E-supplemented animals, respectively ($p < 0.05$). Without copper addition vitamin E-unsupplemented erythrocytes showed a significant increase in both hemolysis and malondialdehyde production in comparison to vitamin E-supplemented ones (figs 3 and 4). Addition of 2 μM $CuCl_2$ protected vitamin E-unsupplemented erythrocytes against hemolysis and lipid peroxidation induced by peroxide-containing Tween 20 (figs 3 and 4). When EDTA-2Na at 0–50 μM was present in the incubation medium of vitamin E-unsupplemented erythrocytes containing 2 μM $CuCl_2$, the protection against hemolysis caused by copper addition was nullified at the level of about 25 μM EDTA. Addition of copper at 32 μM caused extensive hemolysis, but malondialdehyde production did not increase (figs 3 and 4). Instead, another unknown TBARS with a fluorescence peak at 435 nm excitation and 468 nm emission increased, irrespective of the vitamin E status (fig. 5).

In the second pig trial, when linseed oil was added at 1% to the basal diet, the plasma α-tocopherol content of vitamin E-unsupplemented and that of vitamin E-supplemented pigs was 0.89 ± 0.07 and 2.74 ± 0.20 μg/ml, respectively ($p < 0.05$). Feeding linseed oil caused further decreases in plasma vitamin E levels. At 0–13.6 μM copper addition there were significant differences in hemolysis induced by peroxide-containing Tween 20

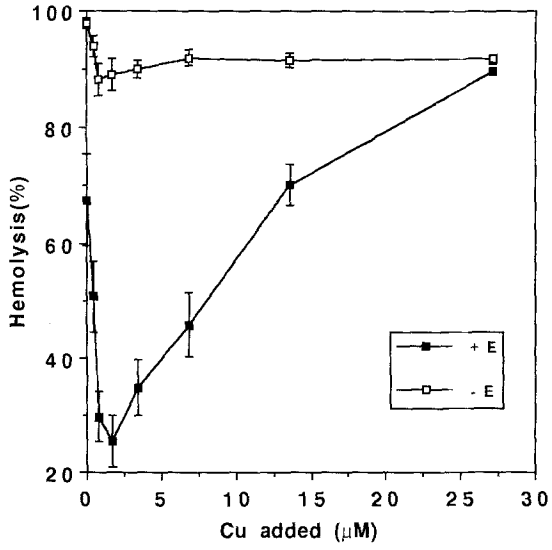

Figure 6. Effects of cupric ions on Tween 20-induced hemolysis of vitamin E-unsupplemented and vitamin E-supplemented erytrocytes in the second pig trial. Numbers of animals used for the blood sampling in each treatment group were 6. The mean hemolysis with SE (bar) is shown. There are significant differences ($p < 0.05$) between the hemolysis of vitamin E-unsupplemented erythrocytes (−E) and that of vitamin E-supplemented ones (+E) at 0 to 13.6 μM copper concentrations.

between vitamin E-unsupplemented and vitamin E-supplemented erythrocytes (fig. 6). In contrast to the previous result (fig. 3), the addition of $CuCl_2$ at 2 μM gave significant protection against hemolysis only in the vitamin E-supplemented erythrocytes (fig. 6). When either $CuCl_2$ (2 μM) or catalase (50 μg/ml) was added to the incubation medium of vitamin E-unsupplemented erythrocytes, the hemolytic rate did not decrease significantly. However, when both $CuCl_2$ and catalase were added simultaneously, the hemolysis of vitamin E-unsupplemented erythrocytes significantly decreased from 98 to 40%, which suggested that copper and catalase had a synergistic effect in protecting the vitamin E-depleted erythrocytes.

Discussion

Tween 20 (polyoxyethylene sorbitan monolaurate), a non-ionic detergent, causes hemolysis of vitamin E-deficient erythrocytes during incubation at 37 °C for 15 min[15]. Commercial Tween 20 reagents contain organic peroxide impurities[22]. Aqueous solutions of Tween 20 undergo autoxidation[23]. Tween 20 itself is thought to interact with the cholesterol moiety of membrane lipid[24]. The specific hemolysis of vitamin E-deficient chick, kid, pig, human and cow erythrocytes can be induced by 0.2–2% peroxide-containing Tween 20 only[17], whereas that of vitamin E-deficient rat and hamster erythrocytes can be brought about by an extremely diluted peroxide-containing Tween 20 with ascorbic acid and azide[16]. Catalase prevents Tween 20-induced hemolysis but superoxide dismutase does not[17]. Exogenous superoxide dismutase may not remove intracellular superoxide anion. Dithiothreitol and manganese ion additions also prevent Tween 20-induced hemolysis at millimolar concentrations[17].

In the experiments described here, copper(II) became a prooxidant when its concentration was more than 10 μM. When copper concentration is more than 10 μM, hydroxyl radicals may be generated by a Harber-Weiss type reaction, which further produces the Hepes radical as reported before[18]. Hepes can form a complex with copper[25]. Incubation of a human erythrocyte suspension with excessive copper(II) causes the formation of methemoglobin, lipid peroxidation and hemolysis[26]. The reduced glutathione (GSH) level declines in erythrocytes at a rate proportional to the rate of copper ion entry, but hemolysis does not seem to be causally related to the level of GSH in the erythrocytes[27]. Under the hemolytic stress of Tween 20, potassium loss precedes the cell lysis, suggesting earlier peroxidative damage of membrane Na^+, K^+-ATPase[16]. 21-Aminosteroid inhibits copper-induced lipid peroxidation at a concentration far below the added copper concentration[28], which suggests that 21-aminosteroid is effective in preventing site-specific damage by copper(II).

Copper ions at 2–4 μM concentration exhibited a clear antioxidant activity against hemolysis and lipid peroxidation in the erythrocytes taken from the animals possessing about 2–3 μg/ml of plasma α-tocopherol (figs 1–4, 6). However, hemolysis of vitamin E-unsupplemented erythrocytes from linseed oil-fed pigs possessing 0.9 μg/ml of plasma α-tocopherol was not prevented by copper addition alone (fig. 6). Instead, addition of both copper and catalase protected against hemolysis. Either severe depletion of vitamin E or increased deposition of polyunsaturated fatty acids or both may weaken the protective power. The antioxidative effect of copper was observed not only in Hepes-saline buffer but also in other buffers such as Tris-saline and phosphate-saline. However, in the latter buffers the copper effect occurred weakly and irregularly.

The hypothetical mechanism of the antioxidative effect of copper in Tween 20-induced hemolysis is as follows. There is a labile copper pool in the erythrocytes, shown by the fact that when radioactive copper is incubated with normal human erythrocytes for 15 to 30 min, it moves rapidly into and out of the cells[29]. Cu(II) added to the incubation medium is taken up by erythrocytes. Copper coordinates with N which can be derived from histidine-imidazoles, α-amino groups of amino acids, or N of peptide bonds such as GSH, and following binding, the Cu(II) can be reduced to Cu(I) by intracellular GSH[30]. Cu(I)-complexes can reductively cleave organic peroxides[31] as well as remove superoxide anion[5,6]. GSH contributes to stabilizing copper in the +1 oxidation

state[32]. The Cu(I)-GSH complex can replenish the copper in Cu, Zn-superoxide dismutase[33]. However, since the diets used in this work were not deficient in copper, a metal-free (apo) form of superoxide dismutase did not exist to a significant extent.

When erythrocytes are incubated in Hepes-saline buffer, copper from a labile copper pool could move from the cells into the incubation medium and be complexed to Hepes molecules, which might elicit a transient deficiency of copper in the labile copper pool and weaken the cellular defense system. If the cellular vitamin E content is deficient or marginal, the hemolysis will occur more rapidly and more extensively. When exogenous Cu(II) at 2–4 μM is added to the incubation medium, copper in the pool will be replenished and Cu(I)-complexes reductively cleave the organic peroxide derived from Tween 20.

Although there are many hemolytic agents such as hydrogen peroxide, dialuric acid and *t*-butyl hydroperoxide, the antioxidative effect of copper has not been reported in these hemolyses. Compared with these hemolytic agents, Tween 20 is unique because the hemolysis occurs in a short incubation time of 15–30 min, owing to the interactive attacks of organic radicals derived from Tween 20 and the surface-active action of Tween 20, in which the lauryl residue is important[15]. The effective antioxidant copper concentration needed to protect against Tween 20-induced hemolysis was 2–4 μM, which is similar to the copper concentration of synovial fluid in healthy humans[34]. The increase in plasma copper associated with inflammatory disease is regarded as a natural defense mechanism against oxygen radical-mediated tissue damage[34,35]. To cope with emergencies Cu(I)-complexes of the labile copper pools in cells could play an antioxidant role together with vitamin E.

Acknowledgment. The author expresses his sincere thanks to Ms. E. Nakayama for her assistance.

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