Free Rad. Res., Vol. 24, No. 4, pp. 291–298
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Red Cell Vitamin E and Oxidative Damage: A Dual Role of Reducing Agents

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Accepted by Professor M.T. Smith

(Received June 19th, 1995; in revised form, September 18th, 1995)

The purpose of this study was to determine the role of reducing agents in maintaining the integrity of vitamin E-deficient red cells. Three groups of one-month-old male Wistar rats were fed a basal vitamin E-deficient diet supplemented with either 0, 10 or 100 mg d, 1-α-tocopheryl acetate per kg diet for up to 12 weeks. Washed red blood cells (5%) were resuspended in saline-phosphate buffer, pH 7.4, and were incubated at 37°C with or without containing 12.5 mM 2,2'-azobis (2amino- propane) dihydrochloride (AAPH), 2.8 mM glucose, 1 mM ascorbic acid, 10 mM hydrogen peroxide (H_2O_2), 250 μM dimethylsulfoxide (DMSO) or 2.8 mM deoxyribose (DR) for up to 20 hours. Addition of either glucose, AAPH, ascorbic acid or H_2O_2 markedly accelerated the rates of hemolysis and lipid peroxidation in the red cells of vitamin E-deficient rats. On the contrary, both glucose and ascorbic acid were protective against oxidative damage to the red cells of vitamin E-supplemented rats in a dose-dependent manner. Also, vitamin E-supplemented red cells were more resistant to AAPH and H_2O_2 than the deficient cells. DMSO or DR had no significant effects on the rates of hemolysis or lipid peroxidation. Glucose, but not others, maintained or slowed down the loss of glutathione (GSH) during incubation. The results obtained suggest a dual role of ascorbic acid and GSH in the function of vitamin E in maintaining red cell integrity: these reducing agents may exert antioxidant function by participating in vitamin E regeneration when certain levels of vitamin E is maintained, but promote oxidative damage by enhancing free radical generation when vitamin E is low or depleted.

Key words: Red blood cells, vitamin E, ascorbic acid, glutathione, glucose, oxidative damage

INTRODUCTION

Vitamin E is the most important lipid-soluble antioxidant and free radical scavenger in biological systems.[1-3] Deprivation of dietary vitamin E results in species-dependent and tissue-specific pathological lesions. However, the mechanisms by which vitamin E protects or prevents various deficient symptoms remain to be elucidated. It has long been recognized that the antioxidant function of vitamin E is interrelated with other antioxidant systems,[1,3,4] including ascorbic acid and reduced glutathione (GSH). Both ascorbic acid and GSH have been shown to involve in vitamin E regeneration.[5-8] On the other hand, these two reducing

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agents are capable of initiating/promoting hemolysis and lipid peroxidation under certain conditions,[9–11] presumably by maintaining transition metals in a reduced or catalytic state. Also, we have previously shown that glucose, while increasing or maintaining higher levels of GSH, accelerated the rates of hemolysis and lipid peroxidation in the red cells of vitamin E- deficient rats.[12] The reason for these paradoxical findings is not clear. In the present study, we examined the effect of oxidizing and reducing agents on the rates of hemolysis and lipid peroxidation and the levels of GSH in the red cells of vitamin E-deficient and supplemented rats. Results obtained from this research suggest that GSH and ascorbic acid, depending upon the status of vitamin E, play a dual role in maintaining the integrity of the red cells.

MATERIALS AND METHODS

Experimental animals and diets

Thirty weanling male Wistar rats, weighing 59.0 ± 5.4 g, were purchased from the Laboratory of Animal Center, College of Medicine, National Taiwan University (Taipei, Taiwan). They were housed individually in stainless-steel wire cages in a room maintained at 25 ± 2°C with a controlled 12-hour light-dark cycle. Food and water were provided *ad libitum*. The animals were initially fed the Laboratory Rodent Chow (Ralston Purina Co., MO) for 3 days. They were then randomly divided into three groups and were fed the basal vitamin E-deficient diet supplemented with either 0, 10 or 100 ppm vitamin E (as d, l-alpha-tocopheryl acetate). The basal diet is similar to the AIN-76A diet[13] that contained 20% casein (Sigma Co., St. Louis, MO), 32.5% corn starch (Roquatte, France), 32.5% dextrose (Taitung Co., Taoyuan, Taiwan), 5% cellulose (Arbocel^{R}, Type 600/300, J. Bettenmaier & Sohne, Germany), 1% vitamin E-free AIN-76 vitamin mixture, 0.3% DL-methionine, 0.2% choline bitartrate, 3.5% AIN-76 mineral mixture and 5% tocopherol-stripped corn oil (United States Biochemical Corp., Cleveland, OH). The status of vitamin E was monitored during the feeding period. Rats were sacrificed by carbon monoxide exposure at the rate of 2 per group per week following 56 to 84 days on the respective diets. Heparinized blood was drawn via the superior vena cava.

Antioxidant and oxidative status

The status of vitamin E was monitored by measuring the degree of spontaneous hemolysis and activity of plasma pyruvate kinase. Beginning from the sixth week of feeding, approximately 0.2 ml of blood was drawn from the tail of 3–5 animals from each group weekly to measure the rate of red cell hemolysis[14] and activity of plasma pyruvate kinase[15] Increased activity of plasma pyruvate kinase resulting from myodegeneration is a specific and sensitive indicator of vitamin E deficiency in rats.[16,17] The red cells obtained at sacrifice were analyzed for the levels of alpha-tocopherol by a high performance liquid chromatographic procedure.[18]

Fresh whole blood samples obtained following sacrifice were also analyzed for the levels of GSH and ascorbic acid following deproteinization with trichloroacetic acid. The levels of ascorbic acid was measured following reaction with 2,4-dinitrophenylhydrazine according to the procedure of Omaye *et al.*,[19] and of GSH was measured at 415 nm following reaction with 5,5′-dithiobis-(2-nitrobenzoic acid).[20] Red cell lysates were measured for the activities of antioxidant enzymes, GSH peroxidase, catalase and superoxide dismutase. GSH peroxidase activity was assayed by coupling with GSSG reductase according to the method of Lawrence and Burk[21] using hydrogen peroxide as substrate, and sodium azide was added to inactivate catalase. Superoxide dismutase activity was assayed according to the method of McCord and Fridovich[22] by measuring the inhibition of cytochrome c reduction by superoxide radicals. Catalase activity was assayed by measuring the absorbance change at 240 nm.[23] The content of hemoglobin was measured at 540 nm using Drabkin's reagent.[24]

Additionally, the levels of lipid peroxidation products, thiobarbituric acid (TBA) reactive substances, in the freshly obtained plasma and red cells were measured by the modified procedure of Tatum *et al.*[25] One ml deproteinized sample was added to 1 ml of 0.4% TBA 4% in 0.4N HCl and 0.1 ml of 0.2% butylated hydroxytoulene in ethanol, vortex-mixed and incubated at 50°C for 60 minutes. The mixture was then extracted with 2 ml of isobutanol and the absorbency at 532 nm of the isobutanol layer was measured. 1,1,3,3-Tetramethoxy-propane was used as a standard.

Treatment of the red cells

Freshly obtained heparinized blood samples were centrifuged at 500 × g for 10 min to separate plasma. The red cells were then washed three times with 5 volumes of phosphate-buffered saline (PBS), pH 7.4, resuspended in PBS and adjusted to 5% (v/v). The red cell suspension was incubated at 37°C under constant shaking for various time periods with or without the addition of deoxyribose (DR), 2.8 mM (final concentration), dimethylsulfoxide (DMSO), 250 μM, glucose, 2.8 mM, ascorbic acid, 1 mM, hydrogen peroxide, 10 mM, and/or 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), 12.5 mM. AAPH, which decomposes spontaneously and generates peroxy radicals at 37°C, can induce red cell membrane damage and hemolysis.[26] Both DR and DMSO have been shown to be capable of trapping hydroxyl radicals.[27]

Aliquots of the incubated red cell suspension were taken and analyzed for the extent of hemolysis,[14] and levels of GSH[20] and TBA reactive substances[25] at various time intervals.

Analysis of data

The significance of difference among groups were analyzed statistically by ANOVA and Duncan's multiple range test at 95% confidence interval ($p<0.05$).

RESULTS

Antioxidant and oxidative status

As expected, plasma vitamin E content was markedly influenced by the status of dietary vitamin E. At sacrifice, vitamin E concentrations in the red cells averaged 0.3, 1.7 and 3.6 μg/ml, respectively, for animals receiving 0, 10 and 100 ppm vitamin E in the diet. No significant differences were observed in the activities of GSH peroxidase, catalase and superoxide dismutase and levels of GSH and ascorbic acid in the blood of the three animal groups (data not shown). The activity of plasma pyruvate kinase of the animals in E0 group was significantly higher than that of the E10 and E100 groups after 6 weeks on the respective diets, and was approximately 5-fold higher at the sacrifice time. The activity of plasma pyruvate kinase was not significantly different between E10 and E100 groups at various time periods measured. The levels of TBA reactive substances in the fresh plasma and red cells were not significantly different among the 3 groups (data not shown).

Hemolysis

As also expected, the rate of hemolysis was faster in the red cells of E0 group than the other two groups. After 6 weeks on the respective diets, the rate of spontaneous hemolysis (red cells in buffer only) was significantly higher in the red cells of animals fed the vitamin E-deficient diet (E0) as compared to those of 10 ppm (E10) and 100 ppm (E100) vitamin E-supplemented groups. At the time of sacrifice, the extent of spontaneous hemolysis in the red cells of the E0 group was significantly higher than that of the E100 group (26.6 ± 7.0% vs. 18.1 ± 4.0%) 6 hours after incubation. At the end of 20 hours, the spontaneous hemolysis increased to 88 ± 4% in the E0 group as compared to 46 ± 21% in the E10 group and 37 ± 19 in the E100 group. The rate of spontaneous hemolysis was not significantly different between

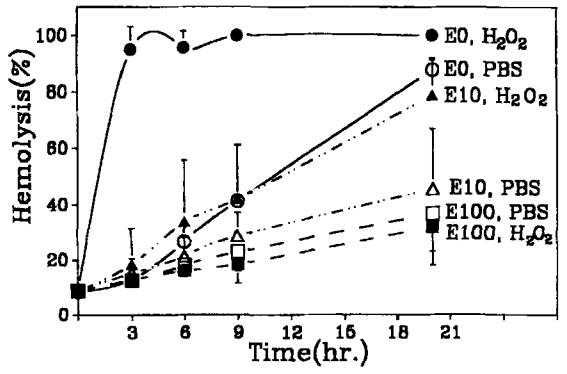

FIGURE 1 Effect of hydrogen peroxide (H_2O_2, 10 mM) on the rate of hemolysis. Red cell suspension (5%) was incubated at 37°C with constant shaking. E0 denotes the red cells of vitamin E-deficient rats; E10, the red cells of rats receiving 10 ppm vitamin E in the diet; E100, the red cells of rats receiving 100 ppm vitamin E in the diet; PBS, and phosphate-buffered saline. Each value represents the mean and standard deviation of ten animals.

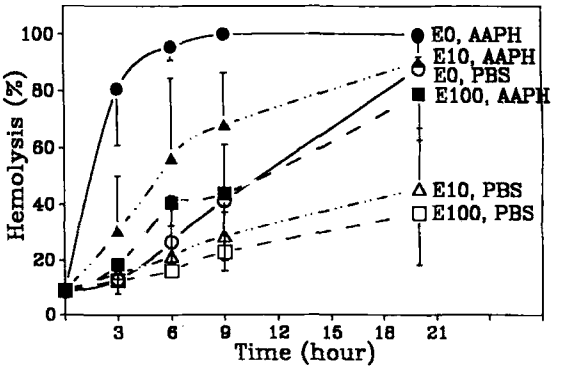

FIGURE 2 Effect of 2,2′-azobis (2-aminopropane) dihydrochloride (AAPH, 12.5 mM) on the rate of hemolysis. See the figure legend of Figure 1 for more detail.

the E10 and the E100 groups, although the values were larger in the E10 group than the E100 group.

The effects of 10 mM H_2O_2 on the rate of hemolysis are shown in Figure 1. Relative to the red cells incubated in PBS only, H_2O_2 markedly accelerated the hemolysis rate of E0 red cells, and the red cells were completely hemolyzed within 3 hours of incubation. On the other hand, the rate of hemolysis of E100 red cells was not significantly altered by the presence or absence of H_2O_2. Hydrogen peroxide had an significant acceleration effect on the % of hemolysis of E10 red cells only after incubation for 20 hours.

Relative to the red cells incubated in PBS, 12.5 mM AAPH accelerated the rate of hemolysis in all three animal groups (Figure 2). Approximately 80% of the E0 red cells were hemolyzed after 3 hours of incubation as compared to less than 30% of the other two groups. At the end of 9 hours incubation with AAPH, averaged 43, 68 and 100% hemolysis were found in the red cells E100, E10 and E0 groups, respectively.

The effect of glucose on the rate of hemolysis is shown in Figure 3. Addition of 2.8 mM of glucose to the incubation medium accelerated the rate of hemolysis of E0 red cells. After incubation for 9 hours, the % hemolysis were 42% and 84%, respectively, for the E0 red cells without and with adding glucose. On the contrary, glucose significantly slowed down the rates of hemolysis in the red cells of E10 and E100 groups. Similar to glucose, ascorbic acid accelerated the rate of hemolysis of E0 red cells, and significantly retarded the rate of hemolysis in the red cells of E10 and E100 groups (Figure 4). Ascorbic acid at a lower concentration (1 mM) exerted a more profound hemolytic effect than glucose (2.8 mM) on the E0 red cells.

Incubation of the red cells with hydroxyl radical scavenger, DR (2.8 mM) or DMSO (250 μM), had no significant effects on the rate of

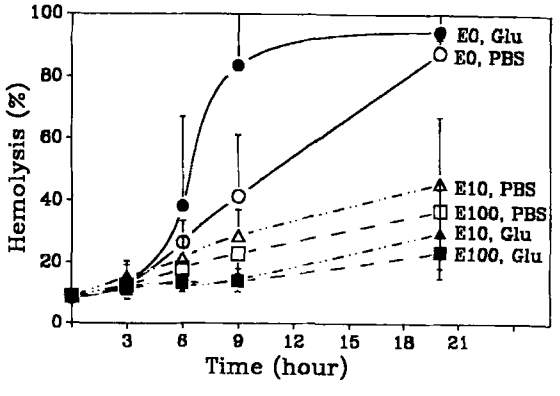

FIGURE 3 Effect of glucose (GLU, 2.8 mM) on the rate of hemolysis. See the figure legend of Figure 1 for more detail.

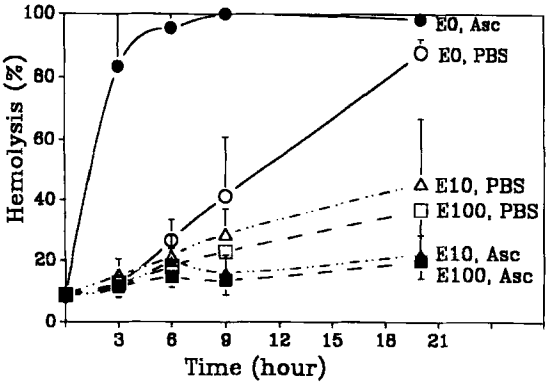

FIGURE 4 Effect of ascorbic acid (ASC, 1 mM) on the rate of hemolysis. See the figure legend of Figure 1 for more detail.

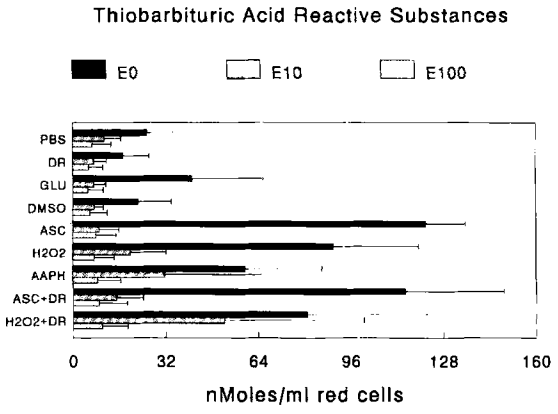

FIGURE 5 Effect of glucose (2.8 mM), AAPH (12.5 mM), ascorbic acid (1 mM), H_2O_2 (10 mM), dimethylsulfoxide (DMSO, 250 μM) and deoxyribose (DR, 2.8 mM) on the production of TBA reactive substances. Red cell suspension (5%) was incubated at 37°C for 6 hours. See the figure legend of Figure 1 for more detail.

spontaneous hemolysis in the red cells of all three groups of animals. Also, DR and DMSO had no significant effect on the rate of hemolysis with or without the presence of ascorbic acid or hydrogen peroxide (data not shown).

Lipid peroxidation

Similar to the rate of hemolysis, incubation of E10 or E100 red cells with glucose, DMSO, ascorbic acid, H_2O_2, AAPH, ascorbic acid plus DR or H_2O_2 plus DR for 3 hours produced lower levels of TBA reactive substances than with E0 red cells (data not shown). Relative to PBS, incubation of E10 red cells with AAPH or H_2O_2, but not with DR, glucose or ascorbic acid, also resulted in a significant increase in TBA reactive substances. None of those compound had any significant effect on the levels of TBA reactive substances formed in E100 red cells following 3 hour incubation. After 6-hour incubation, the levels of TBA reactive substances in E0 red cells were all significantly higher than those of E10 and E100 red cells, with or without the presence of those compounds (Figure 5). Glucose, ascorbic acid, AAPH and H_2O_2 all increased the production of TBA reactive substances in E0 red cells, but had no effect on E100 red cells. Similar to hemolysis, AAPH and H_2O_2, but not glucose and ascorbic acid, had a moderate effect on E10 red cells after 6 hour incubation. The extent of hemolysis and levels of TBA reactive substances are highly correlated [% Hemolysis = 8.218 + TBA reactive substances (nmoles/ml red cells)/1.1344; $r = 0.93$; $P<0.001$].

Glutathione

The levels of GSH were decreased gradually during incubation, and the rate was accelerated by the presence of H_2O_2 or AAPH. On the contrary, addition of glucose resulted in a higher level or slowed down the loss of GSH during incubation (Figure 6). While the levels of GSH were decreased in the red cells of all 3 groups following incubation for 6 hours or longer, the E100 red cells retained higher GSH levels than that of E10 and E0 red cells.

DISCUSSION

While the essentiality of vitamin E in protection against hemolytic stress to the red cells has long been recognized,[2,3,28–30] the mechanism by which vitamin E exerts its protective effect remains to be elucidated. As expected, the red cells of vitamin E-deficient rats are more susceptible to oxidative

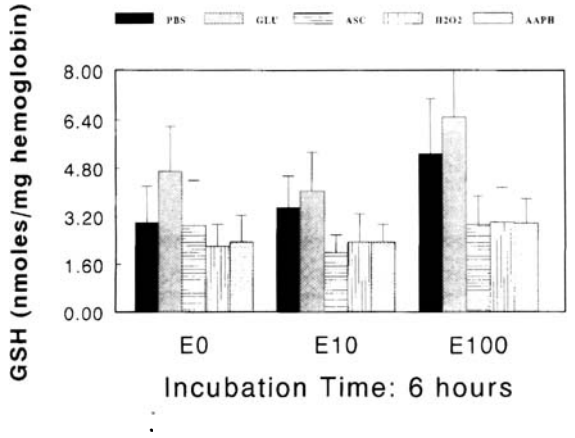

FIGURE 6 Effect of glucose (2.8 mM), AAPH (12.5 mM), ascorbic acid (1 mM) and H_2O_2 (10 mM) on the levels of reduced glutathione (GSH). Red cell suspension (5%) was incubated at 37°C for 6 hours. See the figure legend of Figure 1 for more detail.

and hemolytic stress than that of the supplemented animals, and both H_2O_2 and AAPH accelerated the rates of hemolysis and lipid peroxidation. Also, the extent of hemolysis and lipid peroxidation was generally negatively correlated to the status of vitamin E (E0 >> E10 > E100). Peroxyl radicals generated by AAPH when incubated at 37°C appear to be responsible for its hemolytic action.[26] Since red cell catalase activity was not altered by dietary vitamin E, the hemolytic action of H_2O_2 may be due to its involvement in free radical generation. The presence of vitamin E in the red cells (e.g., E10 or E100 group) may serve to prevent the detrimental effects of AAPH and H_2O_2 by scavenging free radicals generated via these two agents.

In agreement with previous reports,[9–12] both ascorbic acid and glucose were found to accelerate the rates of hemolysis and lipid peroxidation in the red cells of vitamin E-deficient rats. However, these two compounds were found to slow down the rates of hemolysis and lipid peroxidation in the vitamin E-supplemented red cells, and the effect was more profound in the red cells of E100 group than E10 group. Higher levels of GSH, coupled with faster rates of hemolysis and lipid peroxidation, found in the red cells of vitamin E-deficient groups incubated with glucose suggest that GSH may be involved in free radical generation when vitamin E is absent. Therefore, the paradoxical effects of glucose on the red cell integrity observed may be due to a) glucose is converted to NADPH, NADH and ATP, b) NADPH is needed for the regeneration of GSH by GSSG reductase, and ATP for GSH biosynthesis, and c) both NADH and GSH are involved in ascorbic acid regeneration.[31,32] The protective effect of glucose (and ascorbic acid) on the vitamin E-supplemented red cells observed can be partly attributed to its involvement in vitamin E regeneration. On the other hand, the aggravating effect of glucose (and ascorbic acid) on the vitamin E-depleted red cells may be due to its involvement in promoting free radical generation, possibly by maintaining transition metal ions in a reduced state.[9–11,33,34] Thus, when vitamin E is low or absent, reducing agents may promote/initiate free radical generation and hemolytic events in the red cells. However, this study did not establish the amount of vitamin E required to prevent these reducing agents from participating free radical generation. Mino *et al.*[35] have shown that a minimum of 40 μg/dl packed red cells or 180 μg/dl plasma is needed to prevent rat blood cells from hemolysis induced by dialuric acid.

Due to the high reactivity, hydroxyl radicals have been implicated to be the likely damaging species responsible for inducing oxidative damage in the biological system.[33,34] In the present study, hydroxyl radical scavengers, DR and DMSO, had no significant effect on the rate of hemolysis or lipid peroxidation in the red cells of any animal groups. The finding, however, does not rule out the possibility that hydroxyl radicals are the damaging species responsible for hemolytic event. The lack of effect by DR or DMSO observed may be due to the failure of these compounds to reach the specific site of hydroxyl radical generation at a concentration needed. DR degradation also generates TBA reactive substances.

Except for vitamin E status, other antioxidant systems in the red cells measured were not

significantly altered by dietary vitamin E. Therefore, the markedly accelerated rates of hemolysis and lipid peroxidation observed in E0 red cells, but not E10 or E100 red cells, when incubated with H_2O_2, glucose, ascorbic acid or AAPH, clearly indicate an indispensable role of vitamin E in maintaining the integrity of the red cells. While the rates of spontaneous hemolysis and lipid peroxidation, and activity of plasma pyruvate kinase of the E10 group were not significantly different from those of the E100 group, the rates of hemolysis and lipid peroxidation were significantly higher in the E10 red cells than those of the E100 group after incubation with hydrogen peroxide or AAPH for 6 hours or longer. The findings suggest that 10 ppm vitamin E in the diet may be insufficient to prevent oxidative damage to the red cells under stressed conditions.

The present study provides experimental evidence for the essential role of vitamin E in maintaining red cell integrity, and offers a better understanding of the mechanism by which reducing agents, ascorbic acid and GSH, initiate hemolysis in the vitamin E-deficient red cells. The results obtained also suggest that the status of vitamin E is critical in determining whether these reducing agents act as antioxidant or prooxidants. When sufficient vitamin E is present, those reducing agents may act to complement the antioxidant/free radical scavenging function of vitamin E by participating in its regeneration. On the other hand, when vitamin E is low or absent, ascorbic acid and GSH may promote free radical generation.

Acknowledgements

Supported by the National Science Council, Taipei, Taiwan (#80-0409-B002-65), and University of Kentucky Agricultural Experiment Station (KY010001).

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