***tert*-Butyl Hydroperoxide-Induced Hemolysis of
 α -Tocopherol-Decreased Erythrocytes from
Selenium-Deficient and Selenium-
Adequate Rats**

Cheon Ho KIM,¹ Kyoden YASUMOTO,^{1,*} Tetsuya SUZUKI,¹
and Munehiro YOSHIDA²

¹ *The Research Institute for Food Science, Kyoto University,
Uji, Kyoto 611, Japan, and*

² *Department of Public Health, Kansai Medical College,
Moriguchi, Osaka 570, Japan*

(Received June 6, 1988)

Summary The protective function of α -tocopherol, glutathione (GSH), and glutathione peroxidase (GSH-Px) from *tert*-butyl hydroperoxide (*t*-BuOOH)-induced hemolysis was studied with the erythrocytes from male Wistar rats fed selenium (Se)-adequate or -deficient diet for 3 months. By the preincubation with a water-soluble radical initiator, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), at 10 mM for 6 h at 37°C, α -tocopherol levels of the erythrocytes were decreased to 40% of the original level, that is, to the level insufficient for supporting the normal functions of the erythrocytes. With the Se-deficient cells, the hemolysis proceeded rapidly irrespective of the presence or absence of GSH in the incubation medium, and irrespective of the presence or absence of AAPH in the preincubation medium. Contrarily, GSH suppressed the hemolysis of Se-adequate cells which were preincubated with and without AAPH. These results are consistent with the notion that Se serves as the prime, important defense mechanism in the *t*-BuOOH-induced hemolysis through the activity of GSH-Px. Either α -tocopherol or GSH by itself, or both by themselves, may not play so significant a role as Se does in suppressing the hemolysis.

Key Words α -tocopherol, selenium, glutathione peroxidase, hemolysis, erythrocytes, 2,2'-azobis(2-amidinopropane) dihydrochloride

Selenium (Se) has been accepted as an essential trace element for animals. It plays a protective role against oxidative damage in the animal cells mainly through the activity of glutathione peroxidase (GSH-Px)(1-4). Long-term feeding of

¹ 金 天浩, 安本教傳, 鈴木鐵也, ² 吉田宗弘

* To whom all correspondence should be addressed.

animals on a Se-depleted diet causes a magnitude of disorders including increased sensitivity to *in vitro* hemolysis of erythrocytes. Simultaneous induction of vitamin E deficiency exacerbates most of these Se-deficiency disorders (5,6). These two nutrients have sparing effects upon the dietary requirement for both (7,8), and hence the two nutrients are considered to have overlapping, complementary functions in the antioxidative defense mechanisms in the cells of different mammalian organs.

The quantitative significance remains yet unclear, however, regarding the protection afforded by GSH-Px relative to that afforded by vitamin E. In light of the potent chain-breaking antioxidant function of vitamin E, the hypothesis is put forward that it functions as the main protector against lipid peroxidation in the membrane and the dietary Se functions in a secondary antioxidant role through the activity of cytosolic GSH-Px (9). This hypothesis is supported by the findings well documented in the literature that dietary vitamin E can protect the erythrocytes from hemolysis induced by exposure to dialuric acid (10), ascorbic acid (11), Tween 20 (12), and hydrogen peroxide (13).

A hypothesis different from that mentioned above is also possible (14); GSH-Px may be of primary importance in antioxidant defense mechanisms because the enzyme acts on peroxides before they can attack the cellular membranes, while vitamin E acts within the membrane itself in preventing the chain-reactive autoxidation of the membrane lipids. In the previous report (15), we demonstrated that glutathione (GSH) afforded a substantial protection against *t*-BuOOH-induced hemolysis of the erythrocytes from the Se-adequate rats, but not from Se-deficient rats, and thereby suggested that the GSH-Px system might play a dominant function in protecting erythrocytes from oxidative damages.

In the present study, we investigated the *t*-BuOOH-induced hemolysis of erythrocytes after preincubation with 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). This azo compound was used for generating a water-soluble alkylperoxy radical in the incubation medium and for selectively decomposing α -tocopherol in erythrocytes (16). The obtained results confirmed the protective function of GSH-Px system against the *t*-BuOOH-induced hemolysis in the AAPH-treated erythrocytes.

MATERIALS AND METHODS

Animals and diets. Male weanling rats of the Wistar strain were housed individually and fed their respective diets and water *ad libitum* for 3 months. The Se-deficient basal diet was prepared as described previously (15). It was composed of 30% torula yeast, 44% sucrose, 13% soybean oil, 4% mineral mix, 1% vitamin mix, and 2% cellulose powder and was supplemented with 0.3% DL-methionine. The vitamin mix provided 50 ppm *dl*- α -tocopheryl acetate. The basal diet, which contained less than 0.01 ppm Se on analysis, was supplemented with 0.1 ppm of Se as sodium selenite. In the present report, the basal diet and the Se-supplemented diet

are referred to as Se-deficient diet and Se-adequate diet, respectively.

Reagents. Torula yeast was purchased from Sanyo Kokusaku Pulp Co. (Goutsu, Japan). GSH and *t*-BuOOH were obtained from Nakarai Co., Ltd. (Kyoto, Japan). NADPH was purchased from Kohjin Co., Ltd. (Tokyo, Japan). AAPH was obtained from Wako Pure Chemical Co., Ltd. (Osaka, Japan). GSH reductase was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). All other dietary constituents and chemicals used were of reagent grade commercially available.

Blood sampling. Blood was withdrawn from the coccygeal vein of ether-anesthetized rats using a heparinized syringe. The plasma and buffy coat were removed by centrifugation at $750 \times g$ for 15 min at 0 to 4°C. The obtained erythrocytes were washed twice with an equal volume of saline. For the assay of GSH-Px activity, the washed erythrocytes were lysed with distilled water, and the lysates were cleared by centrifugation at $15,600 \times g$ for 1 min in an Eppendorf centrifuge.

Preincubation with AAPH. The washed erythrocytes were preincubated with freshly prepared 10 mM AAPH or its suspending basal medium for 6 h at 37°C with occasional, gentle shaking. The basal medium contained 1 mM NaN_3 and 1 mM KCN in an isotonic saline-phosphate buffer (pH 7.4). In preliminary studies, this concentration of AAPH was found to be optimal and there was no appreciable autohemolysis. Preincubation with 20 mM AAPH decreased α -tocopherol level to less than 10% of the original level, but also caused the hemolysis to proceed at variable, but no negligible, rates. This prevented us from conducting the hemolysis test at 20 mM AAPH.

***t*-BuOOH-induced hemolysis.** The preincubated erythrocytes were collected by centrifugation at $750 \times g$, and resuspended at a 10% packed volume in the basal medium. The hemolysis test was started by the addition of 0.6 mM freshly prepared *t*-BuOOH to the erythrocytes suspension. GSH and glucose were added to the basal medium at a final concentration of 2 and 16 mM, respectively, in the experiments designed to evaluate the effect of these additions. After indicated periods of incubation, the suspensions were centrifuged at $15,600 \times g$ for 30 s and the absorbance of the supernatants was measured at 540 nm. The hemolysis rate was expressed in the percentages of maximal hemolysis.

Assays. GSH-Px activity was determined by the method of Little *et al.* (17) using 0.29 mM *t*-BuOOH as the peroxide substrate. α -Tocopherol in the erythrocytes was extracted by the method of Ishibashi *et al.* (18) and determined by reversed phase high-performance liquid chromatography using electrochemical detection as described by Yamada *et al.* (19). Se and hemoglobin contents were determined in the erythrocytes by the fluorometric technique (20) and by the azide-methemoglobin method (21), respectively. Remaining *t*-BuOOH in the incubation mixtures was determined by the iodometric method of Wills (22).

Statistical analysis. The data were evaluated by analysis of variance or by Student's *t*-test.

RESULTS

Se content, GSH-Px activity, and α -Tocopherol level

Table 1 shows Se contents and GSH-Px activities in freshly prepared erythrocytes from the rats fed the experimental diets for 3 months. Se contents in the erythrocytes from the rats fed the Se-deficient diet decreased to about 5% of those observed in the Se-adequate counterparts. Similarly, GSH-Px activities decreased to a barely detectable level in the Se-deficient erythrocytes. As also shown in Table 1, the dietary Se contents had no significant effect on α -tocopherol level of the freshly prepared erythrocytes.

Depletion of α -tocopherol by preincubation with AAPH

Figure 1 shows the effect of preincubation with 10 mM AAPH on α -tocopherol levels of the erythrocytes. Irrespective of Se status, AAPH decreased the α -tocopherol levels to about 40% of the native levels after 6 h of incubation. Hemolysis occurred by less than 5% after 6 h of preincubation with AAPH. Preincubation with 20 mM AAPH decreased the α -tocopherol level to less than 10% of the native level (data not shown), but resulted in no negligible, and highly variable, degrees of hemolysis. By limiting the AAPH concentration to 10 mM and the preincubation period to 6 h, we prepared " α -tocopherol-decreased erythrocytes," which were used in the hemolysis test in the present investigation.

**t*-BuOOH-induced hemolysis*

Figure 2 shows the time course of the *t*-BuOOH-induced hemolysis in the basal medium, which did not contain added GSH. Irrespective of the Se status and of the AAPH pretreatment, over 90% of erythrocytes hemolyzed within 2 h after the addition of 0.6 mM *t*-BuOOH.

The effect of GSH addition to the incubation medium is shown in Fig. 3. GSH suppressed remarkably the hemolysis of the Se-adequate erythrocytes pretreated with and without AAPH. Less than 20% of hemolysis occurred even after 6 h of

Table 1. Selenium and α -tocopherol contents and glutathione peroxidase activities in erythrocytes of rats fed selenium-adequate or selenium-deficient diet for 3 months.

	Se-adequate	Se-deficient
Selenium ($\mu\text{g/g}$ Hb)	1.56 ± 0.10	$0.07 \pm 0.01^{***}$
α -Tocopherol ($\mu\text{g/ml}$ packed cells)	4.15 ± 0.35	4.43 ± 0.57
Glutathione peroxidase (mmol NADPH oxidized/min/g Hb)	303 ± 9	$5 \pm 1^{***}$

Values are means $\pm$ SEM for 4 rats. ***Significantly different from Se-adequate at $p < 0.001$.

Fig. 1. The decrease of α -tocopherol in the Se-adequate (●) and Se-deficient (○) rat erythrocyte during the preincubation with (—) or without (-----) 10 mM AAPH for 6 h. Vertical bars represent SEM ($n=4$).

Fig. 2. t -BuOOH-induced hemolysis of Se-adequate (●) and Se-deficient (○) rat erythrocytes in the basal medium after 6 h of preincubation with (a) or without (b) AAPH. Vertical bars represent SEM ($n=4$).

incubation with t -BuOOH. On iodometry, t -BuOOH levels in the incubation medium were found to decrease to 50% and 5% of the originally added level after 1 and 2 h of incubation of Se-adequate erythrocytes with GSH. On the other hand, t -BuOOH-induced hemolysis proceeded rapidly with Se-deficient erythrocytes even in the presence of added GSH.

In Fig. 4 is shown the effect of glucose on the hemolysis of the Se-deficient and (or) α -tocopherol-decreased erythrocytes. We observed in a previous study that added glucose prevented the hemolysis regardless of the Se status of the cells (15). In the present study, we observed the contrary; glucose showed no preventive effect for

Fig. 3. Effect of GSH on *t*-BuOOH-induced hemolysis of Se-adequate (●) and Se-deficient (○) rat erythrocytes after 6 h of preincubation with (a) or without (b) AAPH. Vertical bars represent SEM ($n=4$).

Fig. 4. Effect of glucose on *t*-BuOOH-induced hemolysis of Se-adequate (●) and Se-deficient (○) rat erythrocytes after 6 h of pre-incubation with (a) or without (b) AAPH. Vertical bars represent SEM ($n=4$).

the hemolysis of erythrocytes incubated with or without AAPH for 6 h.

DISCUSSION

The close nutritional and biochemical interrelationship between Se and vitamin E has long been recognized in terms of their antioxidant functions (23). However, some uncertainty arises as to the function of dietary Se and the activity of GSH-Px for the normal function of animals *in vivo*, because all Se-deficiency diseases of

animals are claimed to respond to vitamin E at some level (24).

Dietary vitamin E is found to decrease the requirement of Se to prevent most of its deficiency diseases and disorders as well as hemolysis induced by oxidant stresses in many species of animals (25). Siddons and Mills (26) found that an adequate dietary level (60 ppm) of vitamin E lowers the susceptibility of erythrocytes to the ascorbate- or the hydrogen peroxide-induced hemolysis in calves fed a Se-deficient diet for 36 wk. The protective effect of dietary Se against erythrocytes lysis was observed only when erythrocytes were studied *in vitro* and were prepared from the rats deficient in both Se and vitamin E (11, 27). Hematological parameters *in vivo* in the Se-deficient rats remained normal and no evidence of a compensated hemolytic anemia was found. It is therefore possible to venture the hypothesis that vitamin E serves as the prime important defense in the biological systems by preventing free radical attack of unsaturated fatty acids in the membrane, and Se functions only as an extra defense system through the activity of GSH-Px to metabolize hydrogen peroxide and lipid hydroperoxides in the cytosol.

To test this hypothesis in this study, we used the erythrocytes in which α -tocopherol was decreased to approximately 40% of the original level by preincubating the cells with a water-soluble radical initiator, AAPH (Fig. 1), which is known to decrease very selectively the vitamin E levels in membrane systems (16). The decreased level of α -tocopherol can be regarded as significantly lower than that normally required for rat erythrocytes. Bieri and Poukka (28) have shown that the α -tocopherol level required to prevent significant hemolysis induced by dialuric acid ranges from 122 to 196 $\mu\text{g}/100\text{ ml}$ packed cells. Tanaka and Mino (29) reported that α -tocopherol levels of more than 80 $\mu\text{g}/100\text{ ml}$ packed cells is needed to completely inhibit hemolysis induced by dialuric acid. Based on these reported values, we considered that the levels of α -tocopherol in the erythrocytes we used in this study are marginal at least, or sufficiently low enough so as to allow us to assess the contribution of α -tocopherol in protecting erythrocytes from hemolysis.

The above-ventured hypothesis allows the speculation that the native erythrocytes containing about 4 μg α -tocopherol/ml packed cells will show lower susceptibility to the *t*-BuOOH-induced hemolysis than the α -tocopherol-decreased cells. As shown in Fig. 2, however, *t*-BuOOH-induced hemolysis proceeded similarly for both of the erythrocytes preincubated with or without AAPH. Added GSH could effectively prevent similarly the *t*-BuOOH-induced hemolysis of both AAPH-treated and AAPH-untreated erythrocytes obtained from Se-adequate rats (Fig. 3). These results agree closely with those previously reported from this laboratory: GSH prevented the *t*-BuOOH-induced hemolysis in Se-adequate erythrocytes, but did not in Se-deficient erythrocytes (15).

In the previous report (15), we demonstrated that glucose suppressed *t*-BuOOH-induced hemolysis independently of Se status. The prevention of the oxidant-induced hemolysis by glucose is mediated through the ability of the cells to regenerate NADPH *via* glucose-6-phosphate dehydrogenase and to regenerate GSH with NADPH *via* GSH reductase (11). In the present study, the glucose effect was

not observed for the preincubated erythrocytes with or without AAPH (Fig. 3). The observed absence of glucose effect could be explained by a rapid decomposition of adenosine nucleotides during the course of prolonged incubation (30). These nucleotides are involved in the phosphorylation of glucose. It thus appears that glucose can exhibit its protection against *t*-BuOOH-induced hemolysis only after being phosphorylated to serve as the substrate for glucose-6-phosphate dehydrogenase. That added GSH could protect from hemolysis those erythrocytes which showed no glucose effect appears to reinforce our previous inference that externally added GSH can serve indirectly as hydrogen donor to GSH-Px localizing within the cells (15).

It is well established that vitamin E is a potent radical scavenger acting as the "final line of defense" in the biomembrane. However, the antioxidant function of α -tocopherol appears to be hitherto overestimated. Johnson *et al.* (31) reported that vitamin E does not decrease the rate of chronic hemolysis in glucose-6-phosphate dehydrogenase deficiency. We propose that a reasonable working hypothesis, at the present stage of study, is to picture GSH-Px as one of the most dominant defenses against peroxides insulting the erythrocytes. Further experimentation is, however, necessary to understand more precisely the function exerted by GSH-Px with its localization in the cytosol of erythrocytes.

This study was supported by a Grant-in-Aid for Scientific Research (No. 60480054) from the Ministry of Education, Science and Culture of Japan.

REFERENCES

- 1) Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., Hafeman, D. G., and Hoekstra, W. G. (1973): Selenium: biochemical role as a component of glutathione peroxidase. *Science*, **179**, 588-590.
- 2) Flohe, L., Gunzler, W. A., and Schock, H. H. (1973): Glutathione peroxidase: A selenoenzyme. *FEBS Lett.*, **32**, 132-134.
- 3) Chow, C. K., and Tappel, A. L. (1974): Response of glutathione peroxidase to dietary selenium in rats. *J. Nutr.*, **104**, 444-451.
- 4) Sunde, R. A., and Hoekstra, W. G. (1980): Structure, synthesis and function of glutathione peroxidase. *Nutr. Rev.*, **38**, 265-273.
- 5) Thompson, J. N., and Scott, M. L. (1969): Role of selenium in the nutrition of the chick. *J. Nutr.*, **97**, 335-342.
- 6) Thompson, J. N., and Scott, M. L. (1970): Impaired lipid and vitamin E absorption related to atrophy of the pancreas in selenium-deficient chicks. *J. Nutr.*, **100**, 797-809.
- 7) Bieri, J. G. (1961): The nature of the action of selenium in replacing vitamin E. *Am. J. Clin. Nutr.*, **9**, 89-96.
- 8) Hamilton, J. W., and Tappel, A. L. (1963): Lipid antioxidant activity in tissues and proteins of selenium-fed animals. *J. Nutr.*, **79**, 493-502.
- 9) Tappel, A. L. (1980): Vitamin E and selenium protection from *in vivo* lipid peroxidation, in *Micronutrient Interactions: Vitamins, Minerals, and Hazardous Elements*, ed. by Levander, O. A., and Cheng, L., N. Y. Acad. Sci., New York, pp. 18-31.

- 10) Bunyan, J., Green, J., Edwin, E. E., and Diplock, A. T. (1960): Studies on vitamin E: 3. The relative activities of tocopherols and some other substances *in vivo* and *in vitro* against dialuric acid-induced haemolysis of erythrocytes. *Biochem. J.*, **75**, 460–467.
- 11) Rotruck, J. T., Pope, A. L., Ganther, H. E., and Hoekstra, W. G. (1972): Prevention of oxidative damage to rat erythrocytes by dietary selenium. *J. Nutr.*, **102**, 689–696.
- 12) Hamada, T., and Matsumoto, M. (1980): The role of vitamin E in preventing the hemolysis of kid and chick erythrocytes with Tween 20. *Experimentia*, **36**, 978–979.
- 13) Horn, L. R., Barker, M. O., Reed, G., and Brin, M. (1974): Studies on peroxidative hemolysis and erythrocyte fatty acids in the rabbit: effect of dietary PUFA and vitamin E. *J. Nutr.*, **104**, 192–201.
- 14) Levander, O. A. (1986): Selenium, in *Trace Elements in Human and Animal Nutrition*, 5th edition, ed. by Mertz, W., Academic Press, New York, pp. 209–279.
- 15) Kim, C. H., Yasumoto, K., Suzuki, T., and Yoshida, M. (1988): Failure of glutathione in suppressing *tert*-butyl hydroperoxide-induced hemolysis *in vitro* of selenium-deficient rat erythrocytes. *Nutr. Res.*, **8**, 767–775.
- 16) Yamamoto, Y., Niki, E., Eguchi, J., Kamiya, Y., and Shimasaki, H. (1985): Oxidation of biological membranes and its inhibition. Free radical chain oxidation of erythrocyte ghost membranes by oxygen. *Biochim. Biophys. Acta*, **819**, 29–36.
- 17) Little, C., Olinescu, R., Reid, K. G., and O'Brien, P. J. (1970): Properties and regulation of glutathione peroxidase. *J. Biol. Chem.*, **245**, 3632–3636.
- 18) Ishibashi, K., Abe, K., Ohame, M., Kawabe, K., and Katsui, G. (1977): Determination of tocopherols in red blood cells by high-speed liquid chromatography. *Vitamins*, **51**, 415–422.
- 19) Yamada, K., Terao, J., and Matsushita, S. (1987): Electrochemical detection of phospholipid hydroperoxides in reverse-phase high performance liquid chromatography. *Lipids*, **22**, 125–128.
- 20) Watkinson, J. H. (1966): Fluorometric determination of selenium in biological material with 2,3-diaminonaphthalene. *Anal. Chem.*, **38**, 92–97.
- 21) Vanzetti, G. (1966): An azide-methemoglobin method for hemoglobin determination in blood. *J. Lab. Clin. Med.*, **67**, 116–126.
- 22) Wills, E. D. (1971): Effects of lipid peroxidation on membrane-bound enzymes of the endoplasmic reticulum. *Biochem. J.*, **123**, 983–991.
- 23) Hoekstra, W. G. (1975): Biochemical function of selenium and its relation to vitamin E. *Fed. Proc.*, **34**, 2083–2089.
- 24) Combs, G. F., Jr., and Combs, S. B. (1984): The nutritional biochemistry of selenium. *Annu. Rev. Nutr.*, **4**, 257–280.
- 25) Whitacre, M. E., Combs, G. F., Jr., Combs, S. B., and Parker, R. S. (1987): Influence of dietary vitamin E on nutritional pancreatic atrophy in selenium-deficient chicks. *J. Nutr.*, **117**, 460–467.
- 26) Siddons, R. C., and Mills, C. F. (1981): Glutathione peroxidase activity and erythrocyte stability in calves differing in selenium and vitamin E status. *Br. J. Nutr.*, **46**, 345–355.
- 27) Rotruck, J. T., Hoekstra, W. G., and Pope, A. L. (1971): Glucose-dependent protection by dietary selenium against haemolysis of rat erythrocytes *in vitro*. *Nature*, **213**, 223–224.
- 28) Bieri, J. G., and Poukka, R. K. H. (1970): *In vitro* hemolysis as related to rat erythrocyte content of α -tocopherol and polyunsaturated fatty acids. *J. Nutr.*, **100**, 557–564.
- 29) Tanaka, H., and Mino, M. (1986): Membrane-to-membrane transfer of tocopherol in red blood cells. *J. Nutr. Sci. Vitaminol.*, **32**, 463–474.

- 30) Weed, R. I., LaCelle, P. L., and Merrill, E. W. (1969): Metabolic dependence of red cell deformability. *J. Clin. Invest.*, **48**, 795-809.
- 31) Johnson, G. J., Vatassery, G. T., Finkel, B., and Allen, D. W. (1983): High-dose vitamin E does not decrease the rate of chronic hemolysis in glucose-6-phosphate dehydrogenase deficiency. *New Eng. J. Med.*, **308**, 1014-1017.