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Vitamin E inhibits hemolysis induced by hemin as a membrane stabilizer

Fang Wang [a,1], *Tinghua Wang* [b], *Jianhua Lai* [a], *Ming Li* [a], *Chenggang Zou* [a,*]

[a] *School of Life Sciences, Yunnan University, Kunming, Yunnan 650091, China*
[b] *Institute of Neuroscience, Kunming Medical College, Kunming, Yunnan 650031, China*

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ABSTRACT

Hemin is a potential cytolytic agent. To test the effect of vitamin E on hemin-mediated permeability in cell membranes, sheep erythrocytes were chosen as an appropriate model to study hemolysis induced by hemin. Hemin-induced hemolysis but did not elicit lipid peroxidation in sheep erythrocytes. Vitamin E was effective in inhibiting hemin-mediated hemolysis. Both chromanol ring and the isoprenoid side chain of tocopherols were essential for inhibition of hemin-induced hemolysis. There was a strong correlation between the inhibitory effects of tocopherols on hemin-induced erythrocyte hemolysis and their effects on fluorescence anisotropy of cell membranes. Our results suggested that, in contrast to its antioxidant activity, vitamin E inhibits hemolysis induced by hemin as a membrane stabilizing agent.

1. Introduction

Heme (iron protoporphyrin IX) is the functional group of various proteins, including hemoglobin, myoglobin, cytochromes, and nitric oxide synthases [1]. Under some pathological conditions or oxidative stress, heme may be released and exert various noxious actions. Hemin is quite hydrophobic, readily intercalates into lipid membranes of cells, and greatly disturbs the bilayer structure of cells, thus leading to cellular damage in erythrocytes [2,3] and endothelial cells [4]. The toxic effects of hemin on erythrocytes include the induction of potassium leakage [2], the dissociation of erythrocyte membrane skeletal proteins [5] and inhibition of a number of erythrocyte enzymes [6,7]. Hemin is a potent hemolytic agent [2]. It has been shown that hemin causes hemolysis by a colloid-osmotic mechanism, i.e. hemolysis is preceded by the loss of potassium from erythrocytes. In the process, no malonyldialdehyde is detected [8]. Various free radical scavengers (mannitol, formate, histidine, benzoate, dimethyl sulfoxide and hypoxanthine) have no effect on the hemolysis [8].

Vitamin E is a fat-soluble vitamin widely distributed in the membranes of cells. Vitamin E, especially α-tocopherol, has generally been considered as an excellent antioxidant. α-Tocopherol inhibits free radical-mediated hemolysis in erythrocytes [9,10]. Vitamin E administration results in decreased chronic hemolysis as evidenced by the improved erythrocyte life span in glucose-6-phosphate dehydrogenase deficiency

* *Corresponding author*. Tel.: +86 871 5030633; fax: +86 871 5033724.
E-mail address: chgzou@ynu.edu.cn (C. Zou).
[1] Present address: Department of Biochemistry, Kunming Medical College, Kunming, Yunnan 650031, China.
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[11]. Besides its well-known effect as an antioxidant, vitamin E can function as a membrane stabilizer by forming complexes with products of membrane lipid hydrolysis and counteracting their disruptive effects [12]. It has been shown that vitamin E inhibits hemolysis induced by retinol and tamoxifen by virtue of its action as a membrane stabilizer [13,14].

Although the level of α-tocopherol is markedly decreased in sickle-cell disease [15], thalassemia [16] and glucose-6-phosphate dehydrogenase deficiency [17], no direct effect of vitamin E on hemin-mediated permeability in cell membranes has been reported to date. In the current study, sheep erythrocytes were used to investigate whether α-tocopherol could influence cellular permeability induced by hemin. Our results suggested that vitamin E inhibits hemolysis induced by hemin as a membrane stabilizing agent.

2. Materials and methods

2.1. Chemicals

Hemin (ferric protoporphyrin IX) was purchased from Biobasic Inc. (Canada). α-Tocopheryl acetate, α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, α-tocopheryl acetate, trolox, phytol, 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma Chemical (St. Louis, USA). Thiobarbituric acid (TBA) was from the Second Shanghai Chemical (Shanghai, China). All other reagents were of analytic grade.

2.2. Materials

Blood was obtained from healthy adult sheep by veinpuncture into heparinised tubes and centrifuged at 2000 × *g* for 10 min at 4 °C. The plasma was removed and packed erythrocytes were washed three times with PBS (10 mM sodium phosphate, 135 mM NaCl, pH 7.4). The buffy coat of white cells was removed. Erythrocyte membranes were prepared from the reaction mixtures according to the method reported by Hanahan and Ekholm [18].

In these experiments, tocopherols were dissolved in ethanol. Hemin was prepared fresh at the beginning of each experiment as a stock solution of about 1–2 mM in 5 mM NaOH and was kept on ice in the dark. Hemin concentration was determined using a millimolar extinction coefficient of 58.4 at 385 mM [19].

2.3. Hemolysis assay

One percentage of erythrocyte suspension was incubated with various reagents. One milliliter aliquots were removed and centrifuged for 3 min at 3000 × *g*. The degree of hemolysis was determined from the absorbance of hemoglobin at 540 nm in the supernatant. Absorbance at 100% hemolysis was determined by adding 10 μl of Triton X-100 (10% (v/v)) to 1 ml of the erythrocyte suspension.

2.4. Oxygen consumption

Oxygen consumption was determined at 37 °C using air-saturated erythrocyte suspension with or without 30 μM of hemin and/or tested compounds in a Clark-type oxygen electrode (Hansatech Instruments, UK).

2.5. Fluorescence polarization

Erythrocyte membranes were suspended with PBS in a final concentration of 0.1 mg membrane protein/ml. Subsequently, the suspension of erythrocyte membranes was preincubated with tocopherols dissolved with ethanol for 30 min at 25 °C. Then the membrane suspension was incubated in 2 nmol/ml of DPH (final concentration) dissolved in tetrahydrofurane for another 30 min at 25 °C.

Membrane fluidity was measured by a fluorescence polarization method which determines the fluorescence anisotropy (*r*) of a probe (DPH) incorporated in the membrane [20]. The fluorescence anisotropy at 25 °C was measured using a spectrofluorometer (RF-540, Shimadzu) with polarizing filter for monitoring I_p and I_s. I_p and I_s are the parallel and perpendicular emission intensities, respectively. The excitation wavelength was 360 nm and the emission wavelength 430 nm. The fluorescence anisotropy *r* was calculated according to the formula: $r = (I_p - I_s)/(I_p + 2I_s)$. The background noise of light diffusion was corrected by a control membrane suspension. Results are expressed as percentages of the anisotropy value measured on the blank control (without tocopherols).

2.6. Measurement of thiobarbituric acid-reactive substances (TBARS)

TBARS were assayed as described by Stock and Dormandy [21]. The TBARS was determined as absorbance at 532 nm with quantification based upon a molar extinction coefficient of 1.56×10^5 M.

2.7. Measurement of hemin content in cell membranes

To determine hemin content in erythrocyte membranes, the ghosts were dissolved by adding SDS (1% (w/v) final concentration). Hemin content was determined as absorbance at 399 nm and calculated using a millimolar extinction coefficient of 83.5 [19].

2.8. Statistics

Data represent mean ± S.D. of five separate experiments. Paired *t*-test was used to compare mean values of two groups (with and without tocopherols).

3. Results

3.1. α-Tocopherol inhibited hemin-induced hemolysis by a dose-dependent manner

When a 1% suspension of sheep erythrocytes was incubated with hemin, the erythrocytes were hemolyzed. The degree of hemolysis induced by 20 μM of hemin reached a maximal value of about 37% after 60 min incubation of sheep erythrocytes (Fig. 1a). The same concentration of hemin

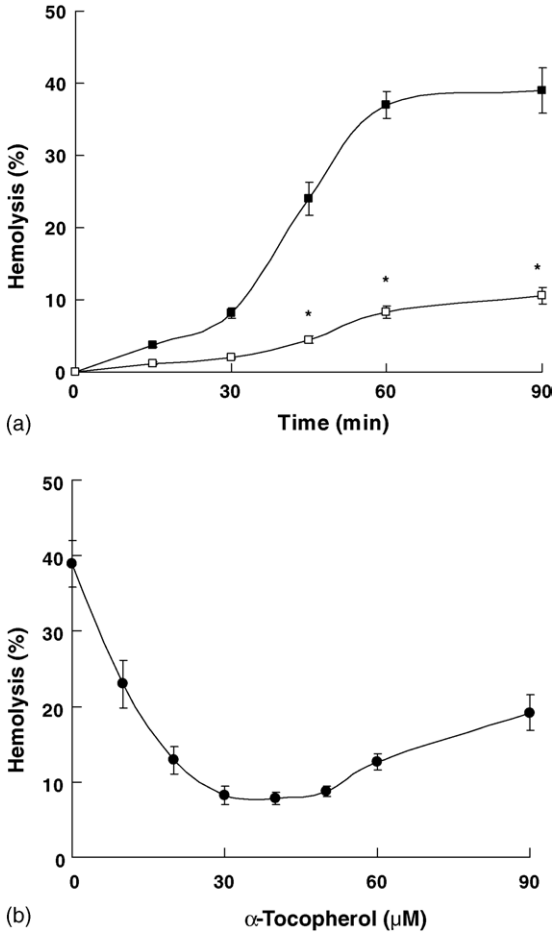


Fig. 1 – α-Tocopherol inhibits hemolysis induced by hemin. Erythrocyte suspensions (1%) were first incubated with α-tocopherol at 37 °C for 30 min, and then with 20 μM hemin for 60 min. (a) Hemolysis induced by hemin in the presence of 40 μM α-tocopherol (empty square) and in the absence of α-tocopherol (solid square); (b) hemolysis induced by hemin in the presence of α-tocopherol. *$P < 0.05$, compared with hemin alone (Student's paired *t*-test). Data represent mean ± S.D. of five separate experiments.

induced only 8% hemolysis if erythrocytes were preincubated with 40 μM α-tocopherol for 60 min. Thus, α-tocopherol significantly inhibited hemin-induced hemolysis. The effect of α-tocopherol on hemin-mediated hemolysis was dose-dependent and 30 μM α-tocopherol was sufficient to achieve a maximal effect (Fig. 1b). However, the inhibitory effect of α-tocopherol on hemolysis was decreased if the concentrations of α-tocopherol exceeded 60 μM. Ethanol (2% (v/v)) itself did not induce hemolysis (data not shown).

When erythrocytes were washed after preincubation with α-tocopherol, the protection against hemin-induced hemolysis was still maintained and α-tocopherol exhibits nearly the same inhibitory effects as in unwashed erythrocytes (data not shown). These data suggest that the protective effect of α-tocopherol against hemin-induced hemolysis probably results from interaction of α-tocopherol in the membrane core, excluding any surface action.

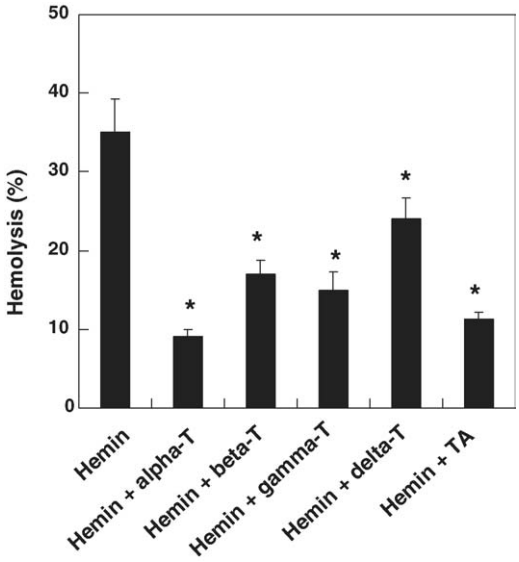
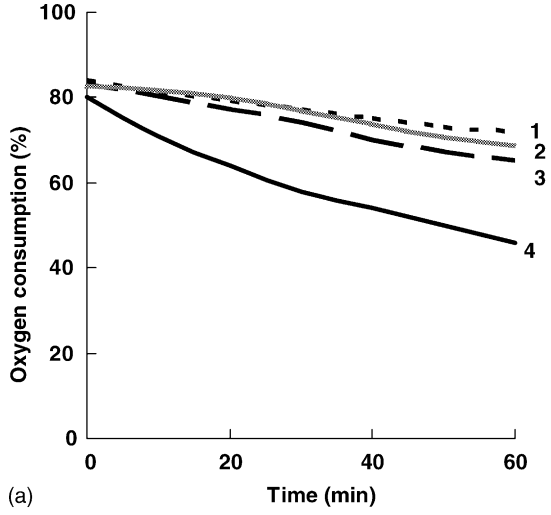


Fig. 2 – Effect of tocopherols on hemin-induced hemolysis. Erythrocyte suspensions (1%) were first incubated with 40 μM tocopherols (Ts) or α-tocopheryl acetate (TA) at 37 °C for 30 min, and then with 20 μM hemin for 60 min. *$P < 0.05$, compared with hemin alone (Student's paired *t*-test). Data represent mean ± S.D. of five separate experiments.

3.2. *The protective effect of vitamin E is not due to its antioxidant properties*

In the next experiment, we investigated the inhibitory effect of α-tocopherol and related compounds on hemin-induced hemolysis (Fig. 2). Of these compounds tested, α-tocopherol was most effective in suppressing hemolysis. β-Tocopherol, γ-tocopherol and δ-tocopherol also inhibited hemolysis induced by hemin although they were less effective than α-tocopherol. Interestingly, α-tocopheryl acetate, with no hydroxyl group, was almost as effective as α-tocopherol. The potential effects of hemolysis inhibition by tocopherols were the following order: α-tocopherol ≈ α-tocopheryl acetate > β-tocopherol ≈ γ-tocopherol > δ-tocopherol. These results indicate that the hydroxyl group in the chromanol ring is not critical for protecting erythrocytes against hemolysis induced by hemin.

Oxygen consumption has often been used to estimate oxidative stress of cells [22]. To clarify the role of oxidative stress on hemin-mediated hemolysis, oxygen consumption during hemolysis and its inhibition by α-tocopherol was investigated. As shown in Fig. 3, hemolysis induced by 20 μM of hemin was not significantly accompanied by oxygen consumption. Although hemolysis was inhibited by α-tocopherol, there was no significant change in oxygen consumption during 60 min incubation. In contrast, hemolysis was inhibited by 1 mM glutathione concurrently with an abrupt oxygen consumption (Fig. 3a). It has been shown that glutathione mediates hemin degradation by an oxygen-dependent manner [19,23]. When erythrocytes were incubated with hemin, no apparent formation of TBARS was observed in



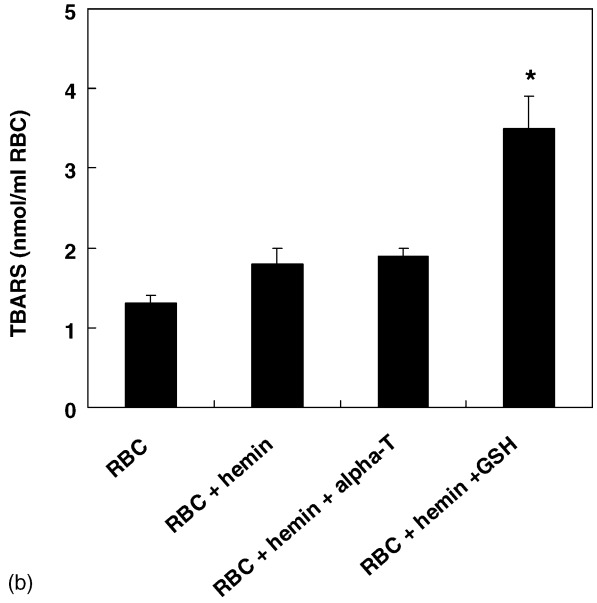


Fig. 3 – α-Tocopherols has no effect on the rate of (a) oxygen consumption, and (b) the formation of TBARS. Erythrocyte (RBC) suspensions (1%) were incubated with 20 μM hemin in the presence or absence of 40 μM α-tocopherol (T) or 1 mM glutathione (GSH) at 37 °C for 60 min. (a) Oxygen consumption was determined using an oxygen electrode. (1) erythrocytes alone; (2) erythrocytes + hemin + α-tocopherol; (3) erythrocytes + hemin; (4) erythrocytes + hemin + glutathione. (b) TBARS was determined as absorbance at 532 nm with quantification. $^{*}P < 0.05$, compared with hemin alone (Student's paired *t*-test). Data represent mean ± S.D. of three separate experiments.

our experiments (Fig. 3b). α-Tocopherol did not induce the formation of TBARS in erythrocytes in the presence of hemin. In contrast, glutathione stimulated an increase in formation of TBARS in erythrocytes in the presence of hemin (Fig. 3b). Since hemin easily penetrates the erythrocyte membranes [3], our data implicate that hemin mediates hemolysis probably by

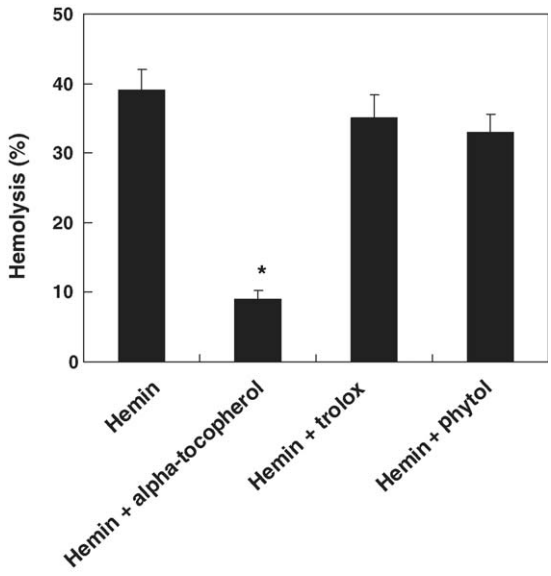


Fig. 4 – Both the chromanol ring and the isoprenoid side chain of tocopherols are important for the inhibition of hemolysis by hemin. Erythrocyte suspensions (1%) were first incubated with 40 μM tocopherols at 37 °C for 30 min, and then with 20 μM hemin for 60 min. Neither trolox nor phytol was effective in inhibiting hemin-induced hemolysis. However, α-tocopherol, which has both the chromanol ring and the isoprenoid side chain, effectively inhibited hemolysis. $^{*}P < 0.05$, compared with hemin alone (Student's paired *t*-test). Data represent mean ± S.D. of five separate experiments.

inducing the disorder of the membrane micelle, rather than eliciting oxidative stress.

3.3. *Both the chromanol ring and the isoprenoid side chain of tocopherols are essential for inhibition of hemolysis*

Since the effect of α-tocopherol on hemolysis induced by hemin is not due to its antioxidant properties, α-tocopherol is presumed to physically stabilize the cell membrane. To clarify the mechanism underlying the inhibitory effect of vitamin E, the effects of trolox and phytol on hemin-induced hemolysis were investigated. As shown in Fig. 4, trolox, which has the chromanol ring and hydroxyl group but no isoprenoid side chain, had no effect on hemolysis by hemin. Phytol, a model of the isoprenoid side chain, had no effect on hemolysis by hemin, either (Fig. 4).

3.4. *Tocopherols decreases membrane fluidity in erythrocytes*

The effects of α-tocopherols, β-tocopherol, γ-tocopherol, δ-tocopherol, trolox and phytol on membrane fluidity in erythrocytes were then investigated. As shown in Fig. 5, tocopherols resulted in an increase in fluorescence anisotropy, indicating that a decrease in membrane fluidity in erythrocytes after incubation with tocopherols. The effects of tocopherols on membrane fluidity were the following order:

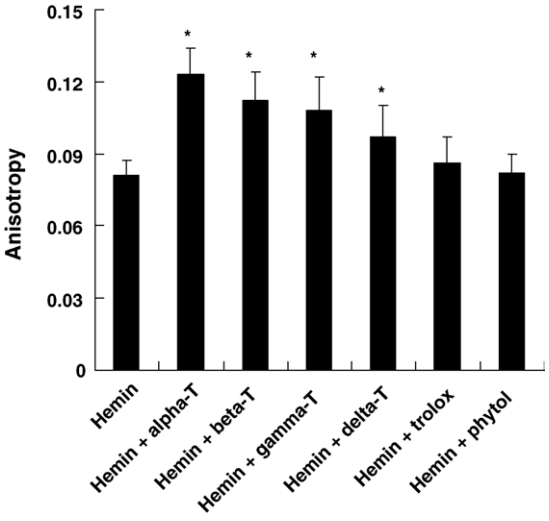


Fig. 5 – Anisotropy of erythrocyte membrane in the presence of tocopherols. Erythrocyte suspensions (1%) were first incubated with 40 μM tocopherols (Ts) or trolox or phytol at 25 °C for 30 min, and then with 2 nmol/ml of DPH (final concentration) dissolved with tetrahydrofurane for another 30 min. $^{*}P < 0.05$, compared with control (Student's paired t-test). Data represent mean ± S.D. of five separate experiments.

α-tocopherol > β-tocopherol ≈ γ-tocopherol > δ-tocopherol > phytol > trolox.

4. Discussion

Hemin is a potent hemolytic agent. In this study, we have demonstrated for the first time that vitamin E inhibits hemolysis induced by hemin. Although it has been shown that hemin induces lipid peroxidation in erythrocytes in the presence of H_2O_2 [24], whether hemin itself promotes lipid peroxidation has not been clearly established [25,26]. Schmitt et al. suggested that hemin-induced decrease of bilayer order is responsible for an increase in cellular permeability [27]. And the cellular permeability induced by hemin also depends on the state of hemin [27]. At lower concentration (<1 μM), the main state of hemin is monomers. Hemin aggregates with increases in concentration. Monomers are more effective in promoting lipid peroxidation whereas aggregates are responsible for increases in permeability and membrane disorder. In the current study, hemin itself caused hemolysis but was unable to induce apparent formation of TBARS (Fig. 3b). α-Tocopherol inhibited hemolysis induced by hemin and did not significantly affect the oxygen consumption (Fig. 3a). The hydroxyl group in the chromanol ring of vitamin E was dispensable for protecting erythrocytes against hemolysis by hemin (Fig. 2). Thus, the oxidative stress is not involved in the hemolysis induced by hemin and the protective effect of α-tocopherol is not due to its antioxidant properties.

Two small molecule antioxidants, glutathione and desferrioxamine, have been shown to inhibit hemin-induced hemolysis. Binding of glutathione to hemin leads to hemin degradation [19]. Desferrioxamine, an iron chelator, can also bind to hemin, thus removing hemin from cell membranes [8]. To elucidate the mechanism of hemolysis inhibition by vitamin E, we investigated the effect of Vitamin E on hemin degradation and the content of hemin in cell membranes. In our experiments, α-tocopherol did not mediate hemin degradation and was not capable of removing hemin previously bound to erythrocyte membrane (data not shown). Moreover, we found that α-tocopherol did not change the hemin spectrum in the Soret and visible bands (data not shown). These data suggest that, unlike glutathione and desferrioxamine, vitamin E inhibits hemin-induced hemolysis in a different manner.

There are many studies to focus on the effect of α-tocopherol on the membrane lipid assemblies by using model membranes consisting of aqueous dispersions of phospholipids [12,13,28]. In these structures, the chromanol ring of α-tocopherol extends towards the aqueous–lipid interface and interacts with phosphatidylcholine in cell membranes. Existence of the chromanol moiety causes a decrease in membrane fluidity. On the other hand, the isoprenoid side chain of α-tocopherol is located to the phospholipids acyl residues. Since the isoprenoid side chain is embedded in the membrane interior, it would retain the α-tocopherol molecule in the lipid core of membrane as an anchor [13]. In the current study, both chromanol ring and the isoprenoid side chain of tocopherols were essential for inhibition of hemin-induced hemolysis (Fig. 4). The inhibitory effects of tocopherols on hemin-induced erythrocyte hemolysis were highly correlated with their effects on fluorescence anisotropy of cell membranes. The interaction of the chromanol moiety of α-tocopherol with phospholipids in cell membranes can lead to decreases in membrane fluidity and permeability [12,13]. On the basis of these data, it is concluded that vitamin E inhibits hemin-induced hemolysis by acting as a membrane stabilizer.

Mammalian cells have several antioxidation systems, including heme oxygenase-1, albumin, and hemopexin, responsible for protection against hemin-mediated cell injury [29]. However, during hemolytic events under some pathological conditions, a rapid local increase in hemin may overwhelm hemin scavengers, leaving them unable to neutralize toxicity of hemin.

Erythrocytes lysis and hemin overload result in the accumulation of hemin in blood plasma, with further import to various organs and tissues [30,31]. The toxic effects of hemin are associated with a number of pathological states, including not only acute conditions such as intravascular hemolysis, but also chronic processes such as atherogenesis [32].

One may speculate that vitamin E may provide the protective effects on lesions of endothelium associated with hemin overload. Intravascular hemolysis is involved in endothelial cell injury in diseases such as thrombotic thrombocytopenic purpura and disseminated intravascular coagulation [33]. Moreover, intravascular hemolysis also potentiates the atherogenicity of moderate hypercholesterolemia [34]. Simon et al. have reported that hypercholesterolemic asymptomatic men have lower vitamin E content in erythrocytes [35]. In this study, we have shown that vitamin E inhibits hemin-mediated hemolysis. Therefore, vitamin E may prevent further hemolysis induced by hemin released from intravascular hemolysis. Indeed, high-dose vitamin E admin-

istration has been reported to reduce markedly chronic hemolysis in patients with glucose-6-phosphate dehydrogenase deficiency [11]. Since hemin can associate with the phospholipid membranes, altering the bilayer structure which leads to cell disruption, it is reasonable to presume that vitamin E can function as a membrane stabilizer to inhibit endothelial cytolysis induced by hemin.

On the other hand, vitamin E is a critical antioxidant protector of endothelial cell membranes against the consequences of oxidative stress, particularly lipid peroxidation. Thus, the membrane stabilizing effects and the antioxidant properties of vitamin E are thought to exist cooperatively one another with cell membranes.

In conclusion, in contrast to its antioxidant activity, vitamin E has a physicochemical membrane stabilizing effect in suppressing hemolysis induced by hemin. Clearly, the mechanism underlying the protective effect of vitamin E is very important for the maintenance of membrane function during the pathologies associated with hemin overload.

Acknowledgements

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