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Inhibition of Monosodium Urate Monohydrate-mediated Hemolysis by Vitamin E

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Abstract Microcrystals of monosodium urate monohydrate (MSUM) induce cytolysis and hemolysis in erythrocytes. In this report, we studied the effect of vitamin E on MSUM-mediated hemolysis in human erythrocytes. Vitamin E significantly inhibited hemolysis induced by MSUM. The hydroxyl group in the chromanol ring of vitamin E is dispensable for protecting erythrocytes against hemolysis induced by MSUM, indicating that the inhibitory effect of vitamin E is not due to its antioxidant properties. However, both the chromanol ring and the isoprenoid side chain are important for vitamin E to suppress MSUM-induced hemolysis. Our current study suggests that vitamin E inhibits hemolysis induced by MSUM as a membrane stabilizer.

Key words monosodium urate monohydrate; erythrocyte; hemolysis; vitamin E; membrane stabilizer

Microcrystals of monosodium urate monohydrate (MSUM) produce an inflammatory reaction by its precipitation within joints [1,2]. The crystal-induced inflammation is the root cause of gouty arthritis. MSUM can bind to the phagolysosomal membrane, leading to membrane lysing, neutrophil cytolysis and inflammation [3,4]. MSUM also induces hemolysis in erythrocytes [4–6]. The mechanism of hemolysis by MSUM has been suggested to be colloid-osmotic in nature [6]. MSUM crystals initially cause a small lesion in membranes by binding to erythrocyte membranes and inducing an alteration of membrane proteins. The formation of pores or aqueous leaks occurs and colloid-osmotic lysis of the cell then follows.

Vitamin E，especially α-tocopherol, is an excellent antioxidant [7]. Vitamin E treatment has a beneficial effect in rheumatoid arthritis [8–10]. For instance, in a mouse model, vitamin E prevents joint destruction [8] and inhibits the expression of interleukin (IL)-1, IL-6, IL-10, IL-12 and tumor necrosis factor-α [9]. In addition, clinical trials have shown significant pain reduction in patients with rheumatoid arthritis treated with vitamin E [8,10]. Besides its action as an antioxidant, vitamin E serves as a membrane stabilizer by interacting with membrane lipids [11–13]. It has been shown that vitamin E inhibits hemolysis induced by retinol [11], tamoxifen [12] and hemin [13] by virtue of its action as a membrane stabilizer.

In this study, we investigated the possible influence of α-tocopherol on MSUM-mediated hemolysis.

Materials and Methods

Materials

Uric acid, α-tocopheryl acetate, α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, trolox and phytol were purchased from Sigma Chemical (St. Louis, USA). Thiobarbituric acid was from Shanghai Hengxin Chemical Reagent Company (Shanghai, China). All other reagents were of analytical grade.

Preparation of erythrocytes

Blood was obtained from healthy human volunteers by venepuncture, collected in heparinised tubes and centrifuged at 3000 *g* for 10 min at 4 ºC. The plasma was removed. Erythrocytes were washed three times with

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phosphate-buffered saline (10 mM sodium phosphate, 135 mM NaCl, pH 7.4), then resuspended in phosphate-buffered saline in a final hematocrit of 1%.

Preparation of MSUM crystals

MSUM crystals were prepared as previously described [14]. Briefly, uric acid was initially treated for 2 h at 200 ºC. Then uric acid solution (0.03 M, pH 7.5) was prepared by dissolution of equimolar quantities of the uric acid and sodium hydroxide, with sodium chloride (0.1 M final concentration) added to accelerate the formation of MSUM crystals. Levels of endotoxin in MSUM crystals were determined by Limulus Amebocyte Lysate assay using Pyrotell&reg Gel Clot Assay kit (Association of Cape Cocl Company, Falmouth, USA). In all samples, bacterium contamination was not detected.

Measurement of potassium (K^+) efflux and hemolysis

After 1% erythrocyte suspension was incubated with various reagents, the mixture was centrifuged for 5 min at 3000 *g*. K^+ concentration in the supernatant was measured by flame photometry. The degree of hemolysis was measured from the absorbance of hemoglobin at 540 nm in the supernatant. The total value of K^+ concentration in erythrocytes and the percentage of hemolysis were determined by disrupting cells with distilled water.

Measurement of thiobarbituric acid-reactive substances

Thiobarbituric acid-reactive substances (TBARS) in erythrocytes were measured as described by Stocks and Dormandy [15]. The TBARS were determined as absorbance at 532 nm with quantification based on a molar extinction coefficient of 1.56×10^5 M.

Statistics

Data represent the mean±SD of five separate experiments. Statistical analysis was performed using Student's paired *t* test.

Results

MSUM induced hemolysis by a colloid-dependent mechanism

When a 1% suspension of human erythrocytes was incubated with 10 mg/ml MSUM, hemolysis occurred. **Fig. 1** shows the time-courses of K^+ efflux and hemolysis mediated by MSUM (10 mg/ml). MSUM induced a rapid efflux of K^+, preceding the slower hemolytic process. The time to reach 50% of K^+ efflux was about 10 min, but there was no significant hemolysis at this point. It took about 45 min to reach 50% hemolysis in the presence of MSUM. However, only 23% hemolysis occurred in 120 min in the presence of 5 mg/ml MSUM.

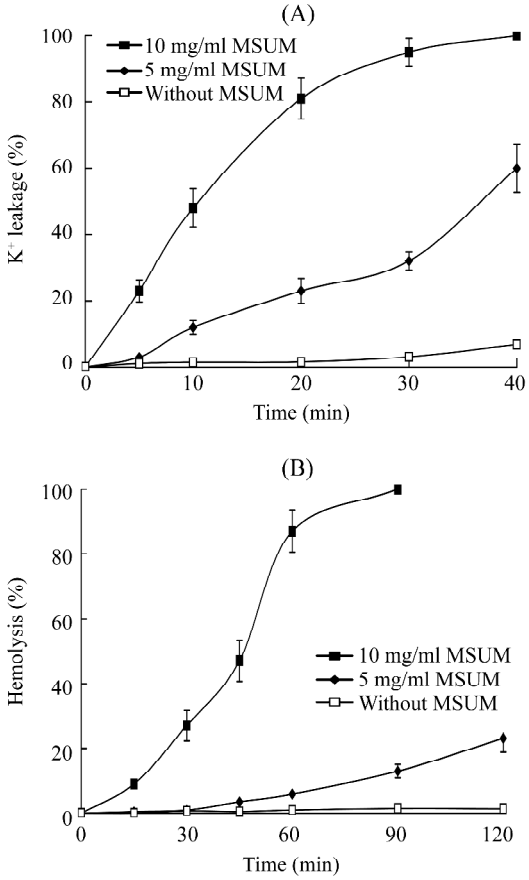

Fig. 1 Time-courses of potassium (K^+) efflux (A) and hemolysis (B) induced by monosodium urate monohydrate
A 1% suspension of human erythrocytes was incubated with monosodium urate monohydrate at 37 ºC. Data represent the mean±SD of five separate experiments.

Vitamin E inhibited hemin-induced hemolysis

α-Tocopherol is the main biologically active form of vitamin E. We found that α-tocopherol significantly inhibited MSUM-induced hemolysis in a dose-dependent manner with the concentration range 10–30 μM (**Fig. 2**). However, the inhibitory effect of α-tocopherol on hemolysis decreased if the concentration of α-tocopherol was more than 40 μM. Interestingly, we found that α-tocopherol alone induced hemolysis if its concentration exceeded 40 μM (**Fig. 3**).

As α-tocopherol is effective in inhibiting MSUM-mediated hemolysis, we investigated the effects of other toco-

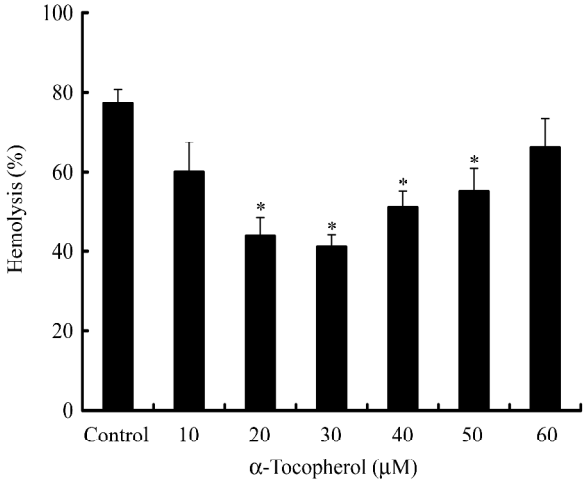

Fig. 2 α-Tocopherol inhibits monosodium urate monohydrate (MSUM)-induced hemolysis in a dose-dependent manner

A 1% suspension of human erythrocytes was incubated with serial concentrations of α-tocopherol at 37 ºC for 30 min, then with 10 mg/ml MSUM for 60 min. Control, MSUM only. * $P<0.05$, compared with MSUM alone (Student's paired *t*-test). Data represent the mean±SD of five separate experiments.

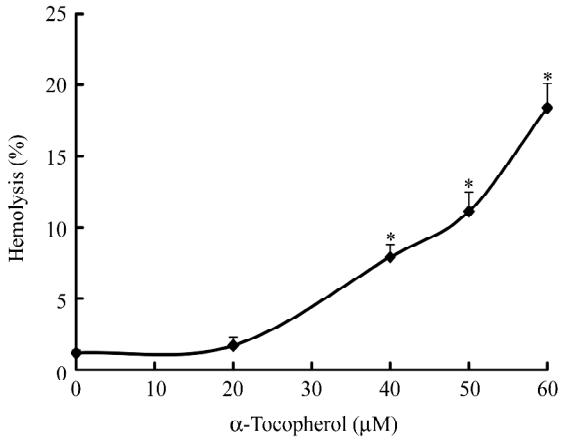

Fig. 3 α-Tocopherol elicits hemolysis at high concentrations

A 1% suspension of human erythrocytes was incubated with serial concentrations of α-tocopherol at 37 ºC for 90 min. * $P<0.05$, compared with MSUM alone (Student's paired *t* test). Data represent means±SD of five separate experiments.

pherols on hemolysis. In the current study, β-tocopherol, γ-tocopherol and δ-tocopherol also inhibited MSUM-induced hemolysis, although they were less effective than α-tocopherol (**Fig. 4**). The potential hemolysis inhibition by tocopherols was found to be: α-tocopherol>β-tocopherol≈γ-tocopherol>δ-tocopherol.

Inhibitory effect of vitamin E is not due to its antioxidant properties

MSUM did not induce apparent formation of TBARS in

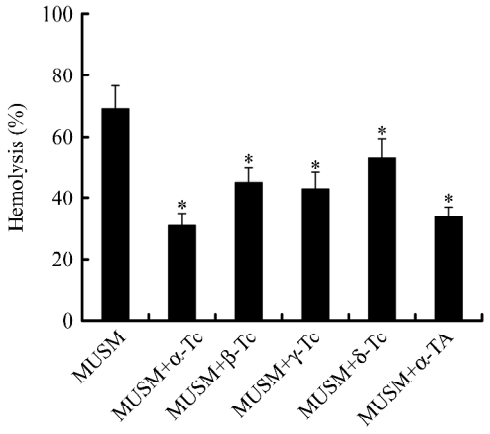

Fig. 4 Effect of tocopherols on monosodium urate monohydrate (MSUM)-induced hemolysis

Erythrocyte suspensions (1%) were incubated with 30 μM tocopherols (Tc) or α-tocopheryl acetate (α-TA) at 37 ºC for 30 min, then with 10 mg/ml MSUM for 60 min. * $P<0.05$, compared with MSUM alone (Student's paired *t*-test). Data represent the mean±SD of five separate experiments.

erythrocytes (data not shown). As MSUM interacts with the erythrocyte membranes [4,5], these data imply that MSUM mediates hemolysis by inducing disorder of the membrane micelle. We then determined the effect of α-tocopheryl acetate on MSUM-mediated hemolysis. As shown in **Fig. 4**, α-tocopheryl acetate, which has no hydroxyl group, was almost as effective as α-tocopherol. These results indicate that the hydroxyl group in the chromanol ring of vitamin E is not critical for protecting erythrocytes against hemolysis induced by MSUM. Thus, the inhibitory effect of vitamin E is not due to its antioxidant properties.

Vitamin E inhibits functions as a membrane stabilizer in inhibiting MSUM-induced hemolysis

The protective effect of vitamin E on MSUM-induced hemolysis is not due to its antioxidant properties, therefore vitamin E is presumed to physically stabilize the cell membrane. We found that protection against MSUM-induced hemolysis was maintained when erythrocytes were washed after preincubation with α-tocopherol (**Fig. 5**). To gain insight into the inhibitory effect of vitamin E, we next investigated the effects of trolox and phytol on MSUM-induced hemolysis. As shown in **Fig. 5**, trolox, which has the chromanol ring but no isoprenoid side chain, had no effect on hemolysis by MSUM. In addition, phytol, which has the isoprenoid side chain but no chromanol ring, also had no effect on hemolysis by MSUM (**Fig. 5**). These data indicated that either the chromanol ring or the isoprenoid side chain of tocopherols is indispensable for

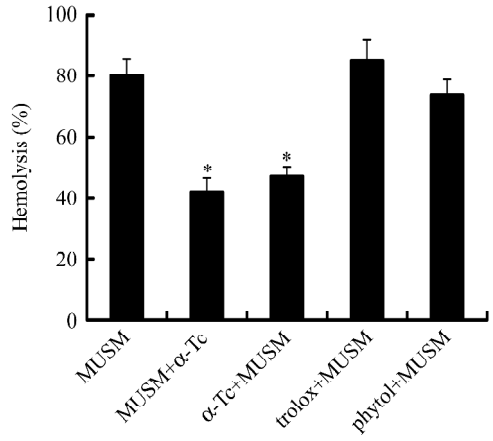

Fig. 5 Chromanol ring and isoprenoid side chain of tocopherols essential for inhibition of hemolysis by monosodium urate monohydrate (MSUM)

Erythrocyte suspensions (1%) were incubated with 30 μM of various reagents at 37 ºC for 30 min, then with 10 mg/ml MSUM for 60 min (α-tocopherol+MSUM, trolox+MSUM, and phytol+MSUM). In another group, erythrocytes were washed after preincubation with 30 μM α-tocopherol. These erythrocytes were resuspended to a final hematocrit of 1%. These erythrocyte suspensions were then incubated with 10 mg/ml MSUM for 60 min (MSUM+α-tocopherol). * $P<0.05$, compared with MSUM alone (Student's paired *t*-test). Data represent the mean±SD of five separate experiments.

hemolysis inhibition.

Discussion

One of the toxic actions of MSUM is to interact with the cell membrane, leading to membrane lysing and neutrophil cytolysis [3–5]. MSUM is also a potent hemolytic agent [6]. Erythrocytes were used as a good model to study cytolysis induced by MSUM. MSUM induces hemolysis by a colloid-dependent mechanism [6]. First, MSUM crystals interact with cell membrane proteins, inducing the redistribution of transmembrane proteins. Second, pore formation results from protein aggregation. Third, water enters, and the cell swells. Finally, hemolysis occurs. In this report, we have shown that vitamin E inhibits MSUM-induced hemolysis.

In the current study, MSUM caused hemolysis but failed to induce formation of TBARS in erythrocytes. Although vitamin E is a well-known antioxidant, our data indicate that the hydroxyl group in the chromanol ring of vitamin E is not essential for protecting erythrocytes against MSUM-mediated hemolysis (**Fig. 3**). These results suggest that oxidative stress is not involved in hemolysis induced by MSUM and the inhibitory effect of vitamin E is not due to its antioxidant properties.

When erythrocytes were washed after preincubation with vitamin E, vitamin E showed similar protective effects as in unwashed erythrocytes (**Fig. 5**). These data suggest that the inhibitory effect of vitamin E against MSUM-induced hemolysis probably results from interaction of vitamin E with the erythrocyte membrane core, excluding any surface action. In this study, neither trolox nor phytol inhibited MSUM-mediated hemolysis (**Fig. 5**). Unlike tocopherols, trolox has only the chromanol ring, without the isoprenoid side chain, whereas phytol is a model of the isoprenoid side chain without the chromanol ring. Thus, both the chromanol ring and isoprenoid side chain of vitamin E play critical roles in inhibiting MSUM-induced hemolysis. According to the model suggested by Quinn [16], the chromanol ring of vitamin E can interact with phosphatidylcholine of erythrocyte membrane and extend into the aqueous-lipid interface. The interaction of the chromanol ring with membrane lipid results in an increase in membrane stabilization and a decrease in membrane permeability [13,16]. In contrast, the function of the isoprenoid side chain is to keep molecules of the vitamin E in the lipid bilayer of erythrocyte membrane by inserting into the inner leaflet of the membrane [16,17]. Thus, our results suggest that vitamin E inhibits MSUM-induced hemolysis by functioning as a membrane stabilizer.

There are no published studies on treatment of gout with vitamin E. As MSUM not only produces an inflammatory reaction [1,2], but also causes cytolysis [3–6], one might speculate that vitamin E treatment is of great benefit to the improvement of gouty arthritis. As the major lipid soluble antioxidant in plasma [7], vitamin E exerts an anti-inflammatory effect in rheumatoid arthritis [9,18]. This is associated with the inhibition of the arachidonic acid pathway or scavenging of free radicals [19,20]. However, neutrophil cytolysis is one of the factors in the development of inflammation induced by MSUM [3]. Because vitamin E can function as a membrane stabilizer, it is reasonable to presume that vitamin E can inhibit neutrophil cytolysis.

In this study, we made the unexpected observation that α-tocopherol at high concentrations (>50 μM) induces hemolysis (**Fig. 3**). It is reasonable to believe that α-tocopherol at high concentrations should enhance hemolysis induced by MSUM. Both MSUM and α-tocopherol can interact with erythrocyte membrane. However, these two components probably compete for similar sites located around the cell membrane. Thus, we did not observe an additive effect of MSUM crystals and α-tocopherol on hemolysis (**Figs. 2** and **3**). In our experiment, α-toco-

pherol was dissolved in 100% ethanol concentration. In every reaction system, the ethanol concentration was 2% (*V*/*V*). Although ethanol has been reported to induce hemolysis, no apparent hemolysis was observed in the presence of 2% ethanol alone. Thus, high concentrations of α-tocopherol *per se* induced hemolysis. However, further investigation is needed in light of our current results.

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