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Mechanism of hemolysis of red blood cell mediated by ethanol

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The effects of ethanol on hemolysis of human red blood cells (RBCs) were studied at 21 ± 1°C in the saline buffer (138 mM NaCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 5 mM glucose and pH 7.4). The hemolysis process for ethanol-treated RBCs was preceded by the leakage of the small cation K^+ from the cells indicating the colloid-osmotic nature of lysis. Since the extent of membrane lesion increased with an increasing ethanol concentration, osmotic protection experiments by using solutes varying in size were carried out to estimate the diameter of the pore. Quantitative analysis of the data by considering the effect of molecular sieving of the protectants with different sizes indicated that ethanol induced formation of membrane pores with a diameter of approximately 13 Å. There was no detectable release of membrane fragments as assayed by the acetylcholinesterase activity, but the membrane structures were significantly perturbed, presumably at the membrane cytoskeletal protein, as evidenced by the altered rheological properties of RBC in the presence of ethanol. It is suggested that the creation of membrane pores might involve in the deranged cytoskeletal network of ethanol-treated RBC.

Introduction

The molecular processes involved in the phenomenon of hemolysis induced by chlorpromazine [1], lysophosphatidylcholine [2,3], or amphipathic peptides such as gramicidin S or melittin [4,5] have been studied in the past decade. It has been suggested that the interaction of these molecules with cells resulted in the formation of membrane pores. Such pores can lead to hemolysis of red blood cells (RBCs) by a colloid-osmotic mechanism; i.e., they allow solutes to equilibrate but retain hemoglobin and its counterions within the cells as an unbalanced load [1,6]. The cells finally swell under this force until they burst. This mechanism is evidenced by the loss of membrane permeability barriers for ions such as K^+, preceding the release of hemoglobin [3,4].

The interaction of ethanol with human RBC membranes has received particular attention for its well-known anesthetic effect and for the problem of alcoholism in the society. At low concentration, ethanol protects RBC from hypotonic hemolysis by partitioning into the cell membrane to increase its surface area [7]. At high concentration, ethanol induces time-dependent shape transformation of human RBCs from the echinocytic to the stomatocytic shape [8,9]. The mechanisms of the hemolysis of RBC mediated by ethanol, however, have not been studied. Since the interactions of ethanol with RBC membranes [10,11] are known to be different from previously studied molecules such as lysophosphatidylcholine [12] which partitions mainly into the outer monolayer and chlorpromazine [13] which penetrates preferentially into the inner monolayer of membrane bilayers, it seemed of interest to investigate the details of the lysis of RBC mediated by ethanol. In this study, we performed osmotic protection experiments by using solutes varying in size to estimate the number and diameter of the membrane pores. The molecular processes involved in the formation of such pores are discussed.

Materials and Methods

Blood was obtained from healthy adult volunteers by venipuncture and collected into 3.2% sodium citrate with a blood : sodium citrate ratio of 9 : 1 (v/v). RBCs were separated from plasma and buffy coat by centrifugation at 3000 rpm (IEC, Centra-4B) for 5 min, and washed with saline buffer (138 mM NaCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 5 mM glucose, and pH 7.4) four times as described previously [10]. Cell morphology was followed by light microscopic method.

Abbreviations: AChE, acetylcholinesterase; PEG, poly(ethylene glycol); RBC, red blood cell.

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Briefly, approx. 0.5% hematocrit of RBCs were fixed by 0.5% glutaraldehyde in 150 mM NaCl at room temperature for 20 min. Cell morphology was graded on a scale of +1 to +5 for echinocytes and −1 to −4 for stomatocytes [10]. The average score of 100 RBCs in several fields was taken as the morphological index (MI).

Hemolysis detection

For the assessment of hemolysis, the RBC suspensions were centrifuged at $14000 \times g$ for 1 min and the optical density of the supernatant was determined at 540 nm. The hemolysis studies were performed at RBCs concentration of 0.5% (v/v). Reference values were obtained from the same amount of RBCs in distilled water (100% hemolysis) and in reagent-free buffer (0% hemolysis).

Measurements of K^+ efflux

The efflux of K^+ from RBCs was measured with a K^+ ion-selective electrode as reported by Katsu et al. [14]. In brief, RBCs were suspended in 25 ml of saline buffer with or without ethanol at the final RBCs concentration of 0.5% (v/v). The percentage of K^+ efflux was calibrated as follows. The K^+ efflux of RBCs without agents was defined as 0%, and lyzed completely by detergent (1% Triton X-100) as 100%. Continuous monitoring of the K^+ leakage process was done by a pH meter (microprocessor pH meter 2000, Suntex) interfaced with a paper recorder (2210 recorder, LKB).

Osmotic protection experiments

RBCs (0.5%, v/v) were suspended in saline buffer with or without 30 mM of one of the following substances: D-mannitol, sucrose, raffinose or poly(ethylene glycol) (PEG) 1000. Then ethanol was added and hemolysis was determined at the time specified in the figure. The above chosen protectants have been checked independently not to interact with RBC membranes by monitoring the morphology and hemolysis of RBC upon incubation with these protectants. The following molecular diameters of substances were used: mannitol, 8.4 Å; sucrose, 10.4 Å; raffinose, 12.4 Å [1], and PEG 1000, 20 Å [4], respectively.

Membrane fragmentation measurements

Following incubation, samples were centrifuged at $14000 \times g$ for 1 min and the cell-free supernatant was monitored for acetylcholinesterase (AChE) activity according to the method of Ellman [15]. AChE activity in the supernatant was expressed as percentage of total activity in the RBCs suspension. After solubilized by Triton X-100 (1%), the total AChE activity in the RBCs suspension was determined and expressed as 100%. Without any reagent treatment, the AChE activity in the supernatant is taken as 0%.

Micropipette aspiration experiments

To study the effect of ethanol on the rheological properties of RBC, the recovery of RBC membrane from the micropipette tip was monitored and recorded with the use of a video camera and tape recorder system. Micropipettes with internal radius of approx. 0.5 μm were prepared with the use of micropipette puller and its tip was manipulated for positioning at the membrane surface of RBC in its dimple area. A step-negative pressure was applied to induce the deformation by aspiration for 20 s and then the aspiration was removed to allow the time dependent recovery. The video image recorded on the tape was then played back through a dimension analyzer. Time-dependent traces of the recovery process obtained from video dimension analyzer were then recorded to indicate the rheological properties of RBC membranes.

Results

Fig. 1 shows the representative studies of the time-course of K^+ efflux and hemolysis induced by ethanol. Ethanol caused a rapid efflux of K^+ preceding the slower hemolysis process. For instance, at 4.1 M concentration of ethanol the leakage process of RBC for small K^+ cation occurred immediately after treated with ethanol, but there were no significant hemolysis within the first 10 min period. The lag time for the detection of hemolysis increased significantly when the concentration of ethanol decreased. It took about 50 min to reach 50% hemolysis at 3.4 M ethanol, whereas less than 15 min was needed at 4.1 M ethanol. This result suggests the colloid-osmotic nature of hemolysis of ethanol-treated RBC. To confirm this idea, the pro-

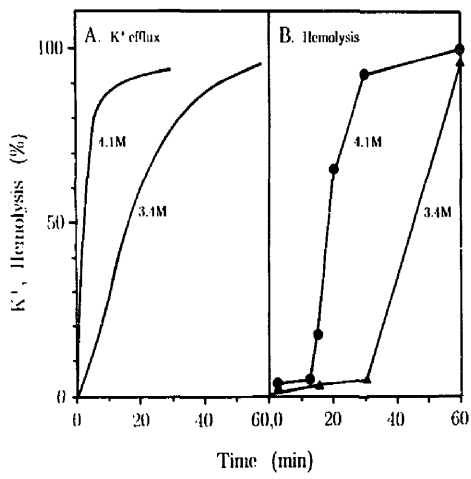

Fig. 1. Time-courses of (A) K^+ efflux and (B) hemolysis of the ethanol-treated RBCs. RBCs at 0.5% hematocrit were incubated with 3.4 M and 4.1 M ethanol at room temperature (ca. 21°C).

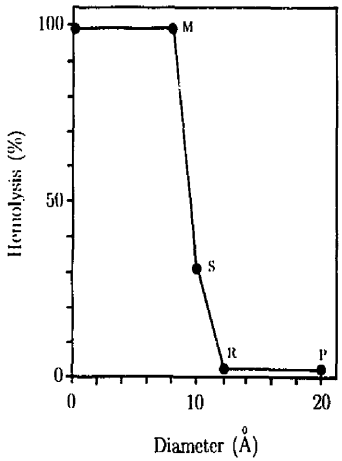

Fig. 2. Ethanol-induced hemolysis in the presence of various colloid-osmotic protectants. RBCs (final 0.5%, v/v) were treated with 4.1 M ethanol in saline buffer with or without 30 mM protectant. After 1 h incubation, hemolysis was measured at 540 nm. M, mannitol; S, sucrose; R, raffinose; P, PEG 1000.

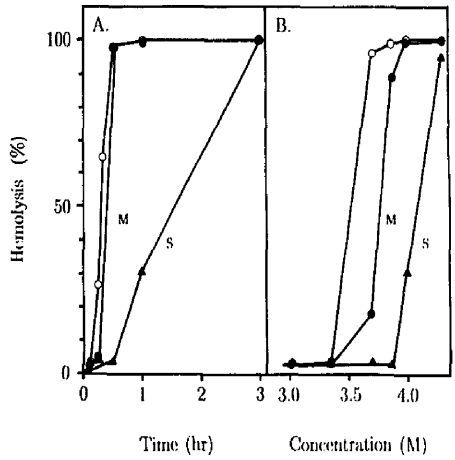

Fig. 3. Time-courses (A) and concentration dependence (B) of hemolysis of ethanol-treated RBCs. The alcohol concentration used in panel A was 4.1 M ethanol. In panel B, protection of hemolysis was determined by treating known concentrations of ethanol for 1 h. Hemolysis of ethanol-treated RBCs was then measured in saline buffer with (closed symbol) or without (open symbol) 30 mM mannitol (M) or sucrose (S).

tective effect on hemolysis of solutes varying in size was checked.

Fig. 2 demonstrates the effect of solutes on the hemolysis determined in the ethanol-treated RBCs. In this figure, the degree of hemolysis was plotted as a function of the diameters of the protectants. From the observation that PEG 1000 fully protects against hemolysis, a value of 20 Å for the diameter of the ethanol-induced pore may be derived as a first approximation. To get a more precise estimation of the pore diameter, the efficiency of protection against time-dependent hemolysis of solutes varying in size was measured. The results indicate that the larger the solutes the longer the time is needed for RBC to reach 50% hemolysis (Fig. 3A). This is consistent with the idea that colloid-osmotic hemolysis might induce a time dependent membrane lesion resulting from molecular sieving effect [1,16]. In other words, the diameter of membrane pores, although large enough to allow small solutes to pass, is small enough to sieve the aforementioned solutes. In Fig. 3B, protection against hemolysis at various concentrations of ethanol was also studied. At higher concentration of ethanol, only larger solute can protect cell from hemolysis. The quantitative analysis of this effect showed that ethanol induced formation of pores with diameter of approximately 13 Å (Table I). We will explore the details of this calculation in the Discussion section.

To understand the action mechanism leading to the creation of pores in RBCs that finally results in the lysis of the cells, we monitored the extent of (A) release of acetylcholinesterase and (B) morphological change as a

TABLE I

Determination of membrane pore size and number from hemolysis times

Ethanol (M)	Time for half-lysis (min)		Apparent pore area (Å²)		Pores per membrane	Pore radius (Å)
	M	S	M	S		
3.86	40.8	180	30.6	9.1	2.41	6.4
4.1	20	84	62.7	19.4	4.94	6.45

M, mannitol; S, sucrose.

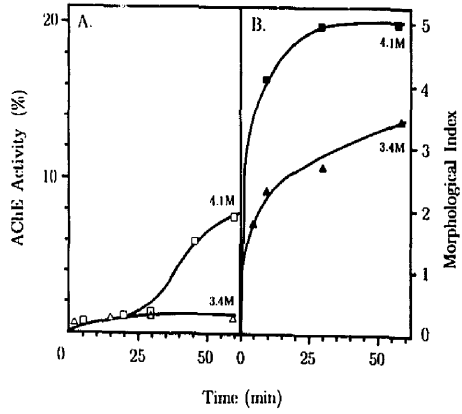

Fig. 4. Kinetics of morphological change and membrane fragmentation for ethanol-treated RBCs. AChE activity (panel A) and morphological index (panel B) were measured at 3.4 and 4.1 M ethanol.

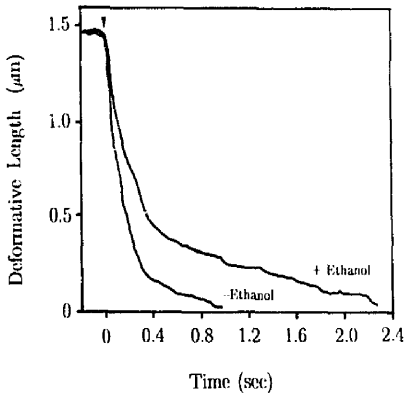

Fig. 5. Records obtained from video dimension analyzer showing the time-courses of the relaxation of a portion of RBC from a micropipette after removal of the aspiration pressure (2.5 mm H_2O). The vertical scale (μm) indicates the extent of RBC membrane remained inside a micropipette before and during the recovery experiments. The arrow shows the time when the aspiration pressure is removed.

function of time (Fig. 4). We used the release of acetylcholinesterase as an indicator for the process of membrane fragmentation. By comparing Fig. 4 with Fig. 1, the time spans for the four investigated processes were in the following order: morphological changes $\cong K^+$ efflux < hemolysis $\cong$ membrane fragmentation. The morphological change and K^+ efflux became detectable for ethanol-treated RBC within the first several mintues, but it took a long time to observe significant hemolysis and membrane fragmentation. At the studied ethanol concentration, however, the lipid packing and membrane rheological properties of RBC membranes have already been significantly perturbed. Fig. 5 shows the representative traces of the micropipette aspiration experiment for the recovery of RBC membranes after the removal of an aspiration pressure in the presence and absence of ethanol (0.7 M). At this concentration, ethanol did not produce hemolysis and the morphology of most RBC (ca. 70%) remained to be normal biconcave shape. Since the rheological property of the ethanol-treated RBC was different from that of the RBC itself, the membrane structures must be significantly perturbed.

Discussion

In the present study, the colloid-osmotic nature of ethanol-induced hemolysis is demonstrated. This is based on our observations that first, the hemolysis of RBC mediated by ethanol is preceded by a lag phase and second, the extent of protection against hemolysis by non-permeable solutes depends on their size. In the following discussion, we will give our quantitative account on the details of the molecular processes.

As shown in Fig. 3, the larger the solutes, the longer the time for ethanol-treated RBCs to become hemolytic. This is mainly due to the presence of the limited number of pores, which are also small enough to sieve the protectants [1,6]. A quantitative account on the mechanism has been described as follows: if r is the radius of the pores and a is the Stokes radius of the probe because of sieving, the apparent pore area is $A = n\pi(r - a)^2$. According to Lieber and Steck [16], this equation could be further converted to the following equation:

$$A = \Delta X[(V^*)^2 - (V_0)^2]/2DV_0t^* \quad (1)$$

where ΔX (cm) is the membrane thickness, t^* (s) is the interval time required for 50% hemolysis, V^* (cm^3) is the average RBC aqueous volume at t^*, and V_0 (cm^3) is the cellular aqueous space present before the entry of the permeable osmotic solute. D (cm^2/s) is the diffusion coefficient of the probe. They are $6.13 \cdot 10^{-6}$ and $4.7 \cdot 10^{-6}$ cm^2/s for mannitol and sucrose at 21°C, respectively. For our estimation of the pore size, the following numerical values were used for the calculations: $\Delta X = 6 \cdot 10^{-7}$ cm, $V^* = 1.5 \cdot 10^{-10}$ cm^3, and $V_0 = 7.2 \cdot 10^{-11}$ cm^3 [1]. From the Stokes radius and apparent pore areas (A_a and A_b) for mannitol and sucrose, the true mean radius (r) and number (n) of the pores can be calculated according to Eqns. 2 and 3

$$r = \frac{b\sqrt{A_a} - a\sqrt{A_b}}{\sqrt{A_a} - \sqrt{A_b}} \quad (2)$$

$$n = \frac{A_a}{\pi(r-a)^2} = \frac{A_b}{\pi(r-b)^2} \quad (3)$$

This result is summarized in Table I and suggests that ethanol induces pores with a radius of about 6.4 Å, but variable number (2.41 and 4.94 for 3.86 M and 4.1 M ethanol, respectively). If circular, the pores would have a diameter of approx. 13 Å.

The ethanol-induced increase of membrane permeability may be related to a number of membrane modifications: (1) Membrane fusion during the shedding of exovesicles [21] might produce a transient decrease of the permeability barrier. (2) Increase of lipid dynamics by the alcohol [22] could decrease the packing of the bilayer [10]. The membrane barrier behaves like a soft polymer, which can sieve solutes [23]. The meshes in the polymer might become larger if its packing density is reduced. (3) Lateral phase separation of lipids could induce packing defects in the lipid domain. This has been observed for long chain alcohols [19] and postulated to be responsible for the increase of membrane permeability by amphiphiles [20]. (4) Increase of the dielectric constant of the membrane by the alcohol would also increase the partition of hydrophilic solutes into the membranes. Such an increase has been pos-

tulated to be responsible for the increase of the permeability by aliphatic alcohols [22]. (5) Modification of the intrinsic membrane domain might follow modification of the membrane skeleton by the alcohol. Accordingly, aggregation of intrinsic proteins might cause membrane modification mentioned under point 2 to 4.

Although it is not possible to decide between the various possibilities from the present data, we showed (Fig. 4) that the release of membrane fragments from ethanol-treated RBC was not a requirement for the creation of membrane pores since it occurred at a time much later than the detection of K^+ leakage. In addition, we found that changes of membane rheological properties preceded the permeability increase (Fig. 5). These properties have been related to the membrane skeletal protein spectrin [24]. Moreover, ethanol has been shown to affect the skeleton [25,26]. The processes leading to the formation of pores in ethanol-treated RBC may thus relate to a deranged cytoskeletal network, followed by the aforementioned alteration of membrane properties.

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