Does ethanol metabolism affect erythrocyte hemolysis?

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Abstract

The effects of ethanol and acetaldehyde on the hemolytic stability of rabbit erythrocytes have been compared. Incubation of normal erythrocytes with ethanol facilitated both acidic and oxidative hemolysis and increased the percentages of cells that were hemolyzed at maximal rate. Acetaldehyde exerted a similar destabilizing effect on erythrocytes only in the case of oxidative hemolysis. The destabilizing effect of ethanol was observed in catalase-inactivated erythrocytes under acidic, but not oxidative, hemolysis conditions. It is concluded that the destabilizing effect of unmetabolized ethanol occurs under conditions of acidic hemolysis, whereas the destabilizing effect of the oxidation of ethanol to acetaldehyde takes place only under the conditions of oxidative hemolysis. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Erythrocyte stability depends on many factors [1], and it has been shown that increased levels of erythrocyte hemolysis (Zieve’s syndrome) and chronic anemia accompany alcoholism [2,3]. These phenomena may be related to the effects of ethanol on the erythrocyte membrane [4–7], and it has been reported that unmetabolized ethanol can have direct effects on membrane properties, generally causing an increase in fluidity [8–10].

Ethanol metabolites can induce oxidative stress in cell membranes [11–14], and as erythrocytes do not express alcohol dehydrogenase or ethanol-inducible cytochrome P-450 (CYP2E1), catalase is regarded as the sole erythrocyte enzyme capable of oxidatively metabolizing ethanol to acetaldehyde [15–17]. The rate of erythrocyte catalase activity is about 600 \textmu mol/min per ml of blood [18], which would be sufficient to transform up to 85 mM ethanol into acetaldehyde in 60 min, with the intracellular acetaldehyde formed being oxidized to acetate by erythrocyte acetaldehyde dehydrogenase [16,19]. These figures suggest that ethanol could readily be converted to acetaldehyde inside the erythrocyte following its penetration of the erythrocyte membrane, and that the
effects of ethanol may be a consequence of the generation of cytotoxic acetaldehyde [20,21]. The cytotoxicity of intracellular acetaldehyde has been explained by its ability to generate free radical species [22], and its involvement in Amadori product formation, a non-enzymatic protein modification by aldehydes known to have deleterious effects [23,24].

Because the contributions of ethanol and products of its metabolism to alcoholic damage of erythrocytes have not previously been clearly distinguished, we have performed experiments to determine if the effects of ethanol on erythrocytes are a consequence of its oxidative metabolism to acetaldehyde. In this study, we have examined the effects of ethanol and acetaldehyde on the resistance of rabbit erythrocytes to acidic and oxidative hemolysis in order to determine if ethanol has a direct effect on erythrocyte stability. Both acidic and oxidative hemolysis were analyzed as these reflect different mechanisms of cell stress, with acidic hemolysis being related to actin precipitation at low pH induced by changes in the ionic permeability of the membrane [25], and oxidative hemolysis reflecting vulnerability of the membrane lipids to peroxidation [26]. Hemolysis was analyzed using both erythrocytes in diluted whole blood in order to examine erythrocytes under conditions closest to their normal physiological status, and isolated erythrocytes with either active or inactivated catalase, as catalase is the only erythrocyte enzyme capable of oxidizing ethanol to acetaldehyde.

2. Materials and methods

2.1. Materials

Adult male rabbits (Shinshilla breed) were obtained from Manihino Nurseries, Moscow Region. Purified acetaldehyde (99%) was a generous donation from Dr. E.E. Kulikov (Institute of Molecular Biology of Russian Academy of Sciences, Moscow); all other reagents were purchased from Sigma.

2.2. Erythrocyte preparations

Blood samples were collected from rabbits by ear vein puncture, and sodium citrate (final concentration 0.38% w/v) was added to prevent clotting. Samples were diluted 10-fold with unbuffered 0.85% NaCl and then, without any other additions to the samples, incubated for 2 h at 37°C for restitution. For experiments in which catalase activity of erythrocytes was inactivated, collected blood (0.5 ml) was washed twice in Hanks’ buffered salt solution (4.2 mM NaHCO$_3$, 5 mM KCl, 0.4 mM KH$_2$PO$_4$, 138 mM NaCl, 0.34 mM Na$_3$HPO$_4$, 5 mM glucose, pH 7.0) to remove plasma solutes, leukocytes and platelets, and the washed cells were then suspended in 0.75 ml of the same medium.

Inhibition of catalase activity was carried out by preincubation of washed erythrocytes in buffer containing 3-aminotriazole (25 mM) and methylene blue (0.01 mg/ml) for 60 min at 37°C [18], and verification of the loss of catalase activity was determined by the procedure of Aebl [27] as described below. Control samples containing the same concentration of methylene blue and with no additions were also included in these experiments. After treatment, all samples were diluted with 50 ml of unbuffered 0.85% NaCl, incubated for 15 min at 37°C and centrifuged at 3000 × g for 5 min, with the washing procedure being repeated five times to remove 3-aminotriazole and methylene blue. The washed, isolated erythrocytes were then suspended in unbuffered 0.85% NaCl and used in hemolysis experiments.

2.3. Catalase activity measurement

For this procedure, 0.05 ml of each washed blood sample was mixed with 2.5 ml of double-distilled water to lyse the erythrocytes and 0.1 ml of this solution was added to the reaction medium (50 mM phosphate buffer at pH 7.0 and 10 mM H$_2$O$_2$). The decomposition of H$_2$O$_2$ was measured for 1 min spectrophotometrically as the decrease in absorbance at 240 nm [27]. Catalase activity in control samples was $207 \pm 36$ μmol/min per 10$^6$ cells, and was only slightly decreased to $182 \pm 29$ μmol/min per 10$^6$ cells in methylene blue-treated samples. In the samples treated with both 3-aminotriazole and methylene blue, catalase activity was inhibited by 95–100%.

2.4. Incubation of erythrocytes with ethanol and acetaldehyde

Erythrocyte preparations at approximate cell den-
sities of $2 \times 10^8$ cells/ml were treated with ethanol or acetaldehyde in concentration ranges between 0.1 and 0.5% and 0.05 and 0.25% respectively, and hemolysis assays were performed either immediately following the addition or after a preincubation for 60 min at 37°C. These ranges of ethanol and acetaldehyde concentrations were chosen based on the physiological levels of ethanol attained in severely alcohol-intoxicated individuals and the prior use of both compounds at these or even higher concentrations in previous studies [28–31]. Control experiments demonstrated that the concentrations of ethanol or acetaldehyde used in the experiments had no hemolytic effects on the cells in the absence of added HCl or NaOCl.

2.5. Hemolysis assays

Hemolysis assays on diluted blood or washed erythrocyte samples were performed in exactly the same manner. Following ethanol or acetaldehyde treatment, 0.05 ml samples of diluted blood or washed erythrocytes were then added to 2 ml of unbuffered 0.85% NaCl solution in order to minimize the osmotic effect of ethanol or acetaldehyde during the hemolysis assay. Hemolysis experiments were performed within 4 h of blood collection as described by Chi and Wu [28], and each 2 ml sample contained about $1 \pm 0.2 \times 10^7$ cells which corresponded to 0.7 units of absorbance at 630 nm. Hemolysis at 23 ± 0.5°C was initiated by addition of either 2 mM HCl (acidic hemolysis [32]) or 0.2 mM NaOCl (oxidative hemolysis [33]). The time course of hemolysis was monitored at 630 nm following the addition of HCl or NaOCl (pH values after HCl or NaOCl addition were 2.3 and 8.4 respectively), and the process was measured until no further changes in absorbance were registered. Verification that total hemolysis had occurred was seen as there were no further absorbance changes on addition of HCl to a 1 M final concentration, a condition known to cause total hemolysis [25].

2.6. Analysis of hemolysis

For data analysis, the difference between initial and final absorbance values was designated as 100% hemolysis. Data are presented as differential curves of hemolysis (erythrograms) in which the rate of change of absorbance rather than absolute absorbance is measured [32]. This enabled two parameters to be determined from the time course of hemolysis, namely $T_{\text{max}}$ (the time when the maximal number of cells is being hemolysed), and $\%_{\text{max}}$ (the percentage of erythrocytes hemolysing at $T_{\text{max}}$). Decreased values of $T_{\text{max}}$ reflect decreased erythrocyte stability and $\%_{\text{max}}$ shows the relative homogeneity of the response of the cell population to the hemolysis
condition, and is a parameter which increases as erythrocytes become destabilized [32].

2.7. Statistical analysis

Experiments were performed on 14 different erythrocyte preparations in studies on unwashed erythrocytes and on seven different preparations in experiments involving washed erythrocytes. Duplicate analyses of hemolysis curves for each sample were performed. The data in the tables are presented as means ± S.D., and one-way ANOVA using the Dunnett method was performed to determine significant differences between data sets. Data sets with $P < 0.05$ were considered to be significantly different.

3. Results

3.1. Hemolysis studies on diluted whole blood

Fig. 1 shows erythrograms of typical experiments illustrating the effects of a 60 min preincubation in the presence of 0.1%−0.5% (17−85 mM) ethanol on the stability of erythrocytes in diluted whole blood to acidic (Fig. 1A) and oxidative (Fig. 1B) hemolysis respectively. In both hemolysis assays, ethanol preincubation was found to cause a reduction of $T_{\text{max}}$ and an increase in $\%_{\text{max}}$, both of which are indicative of decreased erythrocyte stability. The graphs also show that these effects of ethanol were concentration-dependent.

Table 1 shows statistical data for these experiments, demonstrating that preincubation with ethanol significantly decreased $T_{\text{max}}$ and increased $\%_{\text{max}}$ in both types of hemolysis. When 0.5% ethanol was added immediately before acidic hemolysis, $T_{\text{max}}$ was unaffected and $\%_{\text{max}}$ was only slightly increased, but in oxidative hemolysis, it was found that zero time ethanol treatment decreased $T_{\text{max}}$ and increased $\%_{\text{max}}$, with a more pronounced effect on $\%_{\text{max}}$ than in acidic hemolysis.

Fig. 2 shows that acetaldehyde decreased $T_{\text{max}}$ and increased $\%_{\text{max}}$ for oxidative hemolysis, and this figure and the data in Table 1 indicate that the 60 min preincubation with acetaldehyde caused a much greater increase in $\%_{\text{max}}$ compared to no preincubation, although no significant difference was found between the changes in $T_{\text{max}}$ with and without preincubation. In contrast, when resistance to acidic hemolysis conditions was measured, acetaldehyde in a concentration range from 0.05% to 0.25% (11−55 mM), and for zero time or after a 60 min preincubation, did not have any significant effect on the erythrogram parameters (data for 0.25% acetaldehyde are shown in Table 1).

3.2. Hemolysis studies on isolated erythrocytes

The data in the footnotes of Tables 1 and 2 dem-

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td>Effects of ethanol (EtOH) and acetaldehyde (ACH) on hemolysis parameters of rabbit erythrocytes in diluted blood</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Hemolysis assay</th>
<th>Incubation conditions</th>
<th>$T_{\text{max}}$ (% of control)</th>
<th>$%_{\text{max}}$ (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic</td>
<td>0.10% EtOH, 60 min</td>
<td>93.6 ± 2.2*</td>
<td>111.9 ± 4.4*</td>
</tr>
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<td></td>
<td>0.25% EtOH, 60 min</td>
<td>93.7 ± 5.8*</td>
<td>126.7 ± 6.3*</td>
</tr>
<tr>
<td></td>
<td>0.50% EtOH, 60 min</td>
<td>86.7 ± 11.5*</td>
<td>118.6 ± 8.2*</td>
</tr>
<tr>
<td></td>
<td>0.50% EtOH, 0 min</td>
<td>99.0 ± 1.1</td>
<td>108.3 ± 4.7*</td>
</tr>
<tr>
<td></td>
<td>0.25% ACH, 60 min</td>
<td>98.2 ± 0.3</td>
<td>104.9 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>0.25% ACH, 0 min</td>
<td>96.1 ± 2.3</td>
<td>114.4 ± 11.7</td>
</tr>
<tr>
<td>Oxidative</td>
<td>0.10% EtOH, 60 min</td>
<td>68.5 ± 1.4</td>
<td>178.8 ± 67.4</td>
</tr>
<tr>
<td></td>
<td>0.25% EtOH, 60 min</td>
<td>73.6 ± 7.9</td>
<td>164.6 ± 12.3</td>
</tr>
<tr>
<td></td>
<td>0.50% EtOH, 60 min</td>
<td>63.5 ± 6.4</td>
<td>163.7 ± 36.4</td>
</tr>
<tr>
<td></td>
<td>0.50% EtOH, 0 min</td>
<td>75.0 ± 0.7</td>
<td>164.8 ± 35.3</td>
</tr>
<tr>
<td></td>
<td>0.25% ACH, 60 min</td>
<td>80.4 ± 2.9</td>
<td>145.5 ± 10.3</td>
</tr>
<tr>
<td></td>
<td>0.25% ACH, 0 min</td>
<td>83.3 ± 2.0</td>
<td>119.0 ± 9.2</td>
</tr>
</tbody>
</table>

The respective control (no ethanol, no acetaldehyde) values for $T_{\text{max}}$ and $\%_{\text{max}}$ under acidic hemolysis conditions were 5.5 ± 0.5 min and 21 ± 4%, and under oxidative hemolysis conditions were 2.4 ± 1.2 min and 32 ± 10%. Data are expressed as percentages relative to these control values. *$^*P < 0.05$ and $^*P < 0.01$, respectively, compared to the control value. Data were obtained from 14 different experiments.
onstrate that $T_{\text{max}}$ and $\%_{\text{max}}$ values for isolated erythrocyte hemolysis were significantly different from the values for hemolysis of erythrocytes in diluted blood samples, reflecting the destabilizing effect on erythrocytes that the isolation and washing procedures cause. As shown in Table 2, catalase inactivation in isolated erythrocytes did not cause further destabilization of the erythrocytes, as the $T_{\text{max}}$ and $\%_{\text{max}}$ values were statistically indistinguishable for cells with active or inactivated catalase activity.

Fig. 3 shows representative erythrograms for acidic hemolysis of the effect of a 60 min preincubation with 0.5% ethanol on isolated erythrocytes which had either active catalase (Fig. 3A) or inactivated catalase (Fig. 3B), and Fig. 4 shows representative erythrograms for the same experiments performed under oxidative conditions of hemolysis. Table 2 shows statistical data for both the acidic and oxidative hemolysis studies, and these data demonstrate that $T_{\text{max}}$ and $\%_{\text{max}}$ values were altered differently by ethanol in cells with active catalase and catalase-inactivated cells for acidic and oxidative hemolysis. Thus, when assayed by acidic hemolysis, ethanol treatment of both types of cells resulted in significant decreases in $T_{\text{max}}$ although $\%_{\text{max}}$ was unaffected. In contrast, when measured by oxidative hemolysis, ethanol treatment had little or no effect on $T_{\text{max}}$ but increased $\%_{\text{max}}$ for cells with active catalase, whereas for catalase-inactivated cells, a significant decrease in $\%_{\text{max}}$ occurred.

4. Discussion

The present data show that ethanol and acetaldehyde both had deleterious effects on unwashed erythrocyte stability, causing decreases in $T_{\text{max}}$ and increases in $\%_{\text{max}}$ values. However, there were significant differences in the way these compounds affected stability, as ethanol manifested its destabilizing effect under both acidic and oxidative hemolysis conditions whereas acetaldehyde destabilized cells only under oxidative hemolysis conditions. The destabilizing effect of ethanol under acidic hemolysis conditions might be related to its action on membrane permeability for ions which influence membrane integrity and fluidity [28]. More recent work [25] has also shown that the rate of HCl-induced lysis of erythrocytes is related to the rate of transfer of acidic equivalents into the cytosol, and it is possible that ethanol could specifically influence this process.

Table 2
The effect of ethanol on hemolysis parameters of isolated rabbit erythrocytes with active and inactivated catalase

<table>
<thead>
<tr>
<th>Hemolysis assay</th>
<th>Catalase activity</th>
<th>$T_{\text{max}}$ (% of control)</th>
<th>$%_{\text{max}}$ (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic</td>
<td>Active</td>
<td>89.8 ± 1.4*</td>
<td>95.3 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>Inactive</td>
<td>82.7 ± 7.7*</td>
<td>114.3 ± 12.7</td>
</tr>
<tr>
<td>Oxidative</td>
<td>Active</td>
<td>94.5 ± 3.4*</td>
<td>168.9 ± 33.0*</td>
</tr>
<tr>
<td></td>
<td>Inactive</td>
<td>112.0 ± 11.5</td>
<td>75.2 ± 5.9*</td>
</tr>
</tbody>
</table>

The respective control (no ethanol) values of $T_{\text{max}}$ and $\%_{\text{max}}$ for cells with active catalase and no ethanol added were 4.5 ± 0.25 min and 27 ± 5% under acidic hemolysis conditions, and 27 ± 5 s and 20 ± 4% under oxidative hemolysis conditions. The corresponding control values for catalase-inactivated cells were 4.0 ± 0.25 min, 25 ± 3%, 25 ± 5 s and 27 ± 5%, which were not significantly different from those for cells with active catalase. Cells were incubated in 0.5% ethanol for 60 min before hemolysis assay and the data for these experiments are expressed as percentages relative to the control values. *$P<0.05$ compared to the control value. Data were obtained from seven different experiments.
However, as ethanol also destabilizes cells under oxidative hemolysis conditions, the ion permeability changes may occur under these conditions and the ethanol effect may be a combination of such changes and the conversion of ethanol to acetaldehyde which could subsequently modify membrane components. Under oxidative conditions, such modifications involving acetaldehyde promote hemolysis by facilitating peroxidation by NaOCl, but under acidic conditions, acetaldehyde does not affect hemolysis despite the membrane component modification. These results therefore appear to be a reflection of differences in the mechanisms of acidic and oxidative hemolysis [25,26].

Our results also show that when erythrocyte catalase was inactivated, the destabilizing effect of ethanol on the cells still occurred under acidic conditions. This suggests that the destabilization may not be related to the oxidation of ethanol by erythrocyte catalase, and that part of the destabilizing effect of ethanol on erythrocytes may be a direct interaction.

Fig. 3. Acidic hemolysis of isolated erythrocytes in the absence or presence of ethanol. The graphs are representative erythrograms of cells with active catalase (A) and inactivated catalase (B). Curve 1 in each graph is for cells not treated with ethanol, and curve 2 for cells after 60 min preincubation in the presence of 0.5% ethanol.

Fig. 4. Oxidative hemolysis of isolated erythrocytes in the absence or presence of ethanol. The graphs are representative erythrograms of cells with active catalase (A) and inactivated catalase (B). Curve 1 in each graph is for cells not treated with ethanol, and curve 2 for cells after 60 min preincubation in the presence of 0.5% ethanol.
of unmetabolized ethanol with the erythrocyte membrane or other cell components. This interaction appeared to be a relatively slow process as ethanol only affected acidic hemolysis after the 1 h preincubation, suggesting that the interaction of unmetabolized ethanol with membrane components to cause changes in the membrane may not be a fast process, although it is known that ethanol permeates the erythrocyte membrane very rapidly [34]. Evidence already exists that unmetabolized ethanol can permeate cell membranes of erythrocytes and other cells and interact with membrane components, modifying membrane structure and properties [8–10], but other studies have also shown that ethanol may affect membrane structure and stability following metabolic modification [21,35,36]. Our results do not completely exclude the possibility of such latter effects, and further studies will be needed to clarify whether the effects of ethanol on the erythrocyte membrane are solely due to unmetabolized ethanol or if ethanol metabolites or other compounds produced as a consequence of ethanol exposure play a role in erythrocyte membrane destabilization.

In the case of oxidative hemolysis, our results show that the destabilizing effect of ethanol after its preincubation with unwashed erythrocytes was not solely due to acetaldehyde formation itself, as ethanol preincubation had a more pronounced effect than the effect of acetaldehyde preincubation at the same concentration (Table 1). It therefore appears that the destabilizing effect of ethanol under conditions of oxidative hemolysis is a combination of a direct effect of unmetabolized ethanol and the result of ethanol metabolism by erythrocyte catalase. In the experiments on the effects of ethanol on oxidative hemolysis, $T_{\text{max}}$ for catalase-inactivated cells was significantly higher and $\%_{\text{max}}$ was significantly lower in comparison to the values for the control cells, indicating that the inhibition of acetaldehyde production through catalase inactivation decreased the susceptibility of the cells to hemolysis.

The data on the susceptibility of isolated erythrocytes to acid hemolysis suggest that acetaldehyde production is not involved in destabilization under these conditions as there was no difference between hemolysis parameters for control and catalase-inactivated cells. However, with oxidative hemolysis, where the conditions of pH and oxidative stress more closely resemble in vivo circumstances than those of acid hemolysis, acetaldehyde production by catalase did affect hemolysis as the cells with active catalase had a lower $T_{\text{max}}$ and higher $\%_{\text{max}}$ than the catalase-inactivated cells. It is possible that this effect may result, in part, from free radical generation during the oxidation of ethanol and the subsequent peroxidation of membrane lipids and proteins [22,37,38], but acetaldehyde produced by catalase must be another factor causing decreased cell stability under oxidative conditions, because a direct destabilizing effect of acetaldehyde on unwashed cells occurred. The latter effect suggests that acetaldehyde increases the vulnerability of the membrane lipids and/or proteins to peroxidation by sodium hypochlorite, although aldehyde-mediated damage to the cells does not make the cells more susceptible to acidic hemolysis. Although the mechanisms of action of acetaldehyde and hypochlorite in this process are not established, protein cross-linking can be induced by both acetaldehyde [39] and hypochlorite [24], and amino acid oxidation by hypochlorite has been shown to involve aldehyde formation [40,41]. Thus it seems likely that there may be a common mechanism of cell damage induced by both acetaldehyde and hypochlorite which involves protein cross-linking. Further studies in this area are currently in progress in our laboratories.

In conclusion, ethanol-induced erythrocyte instability appears to be a combination of the effects of unmetabolized ethanol directly on the cell and the oxidation of ethanol to acetaldehyde by erythrocyte catalase. Further studies will be necessary to determine the exact mechanisms by which these processes occur, and to determine if similar changes are seen in human erythrocytes in order to evaluate their possible significance in alcoholic anemia.

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[32] V.D. Prokopieva, N.A. Bohan, P. Johnson, A.A. Boldyrev,


