Ethanol Raises Prostacyclin In Vivo and In Vitro

Moderate doses of ethanol were shown to induce a significant rise in prostacyclin (PGI₂) concentration in cultures of endothelial cells derived from umbilical veins. Administration of 32 g of ethanol to six volunteers elevated plasma levels of PGI₂ in parallel with those of blood alcohol. Although not specific for ethanol, this alcohol induced the largest change in PGI₂. Withdrawal of the stimulant alcohol caused prompt reduction of the elevated prostacyclin to baseline values. The activity of ethanol appears to be due to a direct stimulation of cyclooxygenase. The release of [³²P]arachidonic acid from prelabeled endothelial cells was decreased by ethanol. PGE₂ production was also enhanced by exposure of endothelial cells to ethanol. The physiologic significance of these alcohol-induced changes in PGI₂ levels remains to be established.

ONE OF THE MOST striking and best-known effects of ethanol is its vasodilatory action, the mechanism of which has not been elucidated. Recently, it has been recognized that ethanol may influence hemostasis,1,2 and inhibition of platelet aggregation has been found in vitro1,3–5 and in vivo.1,4 Inhibition of platelet function in vitro seems to require higher levels of ethanol than in vivo inhibition.1 This disparity suggests that ethanol may modify platelet function not only by a direct action on platelets, but also via other mechanisms that are nonplatelet in origin. Prostacyclin (PGI₂), the potent vasodilator and antiaggregating agent synthesized by endothelial cells, is a natural target for investigation. We have studied the effect of ethanol on levels of PGI₂, or rather, its oxidation product 6-keto-PGF₁α, both in endothelial cells grown in culture and in plasma of individuals given ethanol. Both in vitro and in vivo results give firm evidence for a stimulant effect of ethanol on prostacyclin levels. This probably explains its heightened effect on platelet function in vivo and could be related to the ethanol-induced vasodilatation.

MATERIALS AND METHODS

Cell Cultures

Human endothelial cells obtained from umbilical cords, as described by Jaffe et al.,5 were cultured in T-25 flasks (Corning Glass Works, Corning, NY). The cells were grown in culture medium 199 (GIBCO, Grand Island, NY) containing 20% fetal calf serum (GIBCO). The flasks were incubated at 37 °C under 5% CO₂ and fed every three days with a complete change of culture medium. After reaching confluency, the cells were harvested by trypsinization and subcultured in 24-well cluster plates (Costar, Cambridge, Mass). Endothelial cells were used for experimental purposes at confluency stage after the first subculture, which was always within 20 days of collection.

To measure the effect of ethanol on PGI₂ production, endothelial cell monolayers were washed twice with Dulbecco’s buffered saline (DBS) (GIBCO) and incubated for 30 minutes at 37 °C with 0.5 mL of DBS containing different concentrations of ethanol (0% to 1.2%). The supernatants were then rapidly frozen and kept at − 70 °C until the assay could be performed. After incubation, the endothelial cells from each well were trypsinized and counted by Coulter counter. Concentrations of 6-keto-PGF₁α and PGE₂ in the supernatants were assayed by radioimmunoassay using [³H]6-keto-PGF₁α and [¹²⁵I]PGE₂ kits, respectively. Detection limits were 8 pg/0.1 mL for the 6-keto-PGF₁α assay and 0.3 pg/0.1 mL for the PGE₂ assay.

Possible cell injury induced by ethanol was checked using trypan blue.1 At the highest concentration of ethanol that was commonly used (160 mg/100 mL), no evidence of staining with this dye was noted.

In Vivo Studies

Six healthy volunteers (two men and four women) ranging in age from 21 to 50 years, who denied any drug intake during the preceding two weeks, were given 32 g of ethanol. Pure ethyl alcohol (Industrial Chemical Co, New York) was diluted in 200 mL of water and ingested over a period of ten minutes. Blood levels4 were determined before and at hourly intervals after administration of ethanol.

Peripheral venous blood was collected with minimal stasis, ie, without application of a tourniquet, through a 19-gauge butterfly needle. After discarding the first 2 mL, the blood was collected into a syringe containing 0.1 vol of 3.8% sodium citrate and indomethacin, 5 μg/mL blood. Blood was withdrawn by gentle pull on the syringe plunger, was thoroughly mixed and transferred into glass tubes kept on ice. Plasma was obtained by centrifugation for 15 minutes at 2,200 g at 4 °C. Each blood collection during the experiment was performed through a separate venipuncture, taking the above precautions.

The plasma was extracted by the following method. For assay of 6-keto-PGF₁α, plasma fractions, 1 mL in volume, were acidified to pH 3 with 0.1 mL of 2 mol/L citric acid centrifuged at 1,000 g for ten minutes at 4 °C. The supernatants were applied to octadecyl (C₁₈) columns (J.T. Baker, Phillipsburg, NJ) and then eluted directly onto Bond Elut Si columns (Analytichem Int, Harbor City, Calif) by sequential washes with 2-mL portions of H₂O, 10% methanol, and benzene. The Si columns were then washed with 1 mL each of the following solvent mixtures: (1) benzene:ethyl acetate (60:40), (2) benzene:ethyl acetate:methanol (60:40:2), (3) benzene:ethyl acetate:methanol (60:40:10), and benzene:ethyl acetate:methanol (60:40:30), and the extract dried under nitrogen. The dried extract was dissolved in 0.3 mL of 0.050 mol/L potassium phosphate buffer, pH 6.8, containing 0.9% NaCl, 0.01 mol/L EDTA, 0.4% bovine γ-globulin, 0.005% Triton X-100.
and 0.05% sodium azide (assay buffer) and filtered through a 0.2-μm filter (Millipore, Boston). The recovery of 6-Keto-PGF₁α was estimated by the use of [³H]-6-keto-PGF₁α (New England Nuclear, Boston) as an internal standard. Generally, the recovery was >90%. 6-Keto-PGF₁α was determined by radioimmunoassay using [¹²⁵I]-6-keto-PGF₁α RIA kit (New England Nuclear). Detection limit was 2 pg/0.1 mL. Comparison between the [³H]- and the [¹²⁵I]-6-keto-PGF₁α RIA kits showed that there was good agreement in the measurement of standard samples containing between 15 and 250 pg of 6-keto-PGF₁α. Individual results did not differ by more than 8% with the two different RIA kits.

On each subject, two plasma samples were extracted for each experimental point. The duplicate determinations agreed within 7%. They were averaged, and their means are presented throughout this article.

Repeated sampling of an individual who had not ingested ethanol over a five-hour time period produced variations that ranged from 5% to 12% of their mean value.

For other experiments, endothelial cells grown in monolayer culture were incubated with [¹⁴C]arachidonic acid. The usual culture medium was first removed by gentle aspiration and replaced by plain medium 199. The concentration of cells varied between 3 and 5 x 10⁶ cells/mL. The cells were incubated with 10 μmol/L indomethacin and 2 μmol/L [¹⁴C]arachidonic acid (specific activity 56.9 mCi/mmol) (New England Nuclear). After 30 minutes at 37 °C, the cells were gently removed with a rubber policeman, centrifuged, and the supernatant removed. Resuspended in fresh medium 199 (concentration: 5 x 10⁶ cells/mL), the cells were incubated with ethanol, ranging in concentration from 0% to 0.2%, for 30 minutes at 37 °C, while being gently agitated. They were then extracted with chloroform and processed for analysis by high performance liquid chromatography (HPLC).

Insoluble phospholipase A₂ from bee venom (Sigma Chemical Co., St Louis) was incubated with phosphatidyl choline in the presence of a series of ethanol concentrations ranging from 0% to 2%. Phospholipase A₂ absorbed onto Sepharose beads (1 U/mL) was suspended in 0.05 mol/L Tris/HCl buffer, pH 8.5, containing 0.1% (wt/vol) fatty acid-free bovine serum albumin and 10 mmol/L Ca²⁺. After addition of ethanol, 3 mmol/L dipalmitoyl-DL-α-glyceryl-phosphoryl choline was added and a ten-minute incubation at 37 °C begun. The incubation mixture was extracted with chloroform and prepared for HPLC analysis of free fatty acids released from the phospholipid. Retention times were established with authentic fatty acid standards. The lower limit of detection of free fatty acids by this method was approximately 3 nmol.

Bleeding times were measured twice in the course of the experiment by the Simplate II method.¹⁰

RESULTS

In Vitro Studies

Human endothelial cells in monolayer cultures were exposed to ethanol concentrations ranging from 0.2% to 1.2%. The response of prostacyclin production is shown in Fig 1. The stable degradation product of PGI₂, 6-keto-PGF₁α, increased in parallel with the ethanol level up to 80 mg%. Higher concentrations did not yield any further increase in 6-keto-PGF₁α. Although statistically not significant, ethanol doses as low as 0.02% raised 6-keto-PGF₁α. Other alcohols, such as methanol and isopropanol, also increased the oxidized conversion product of PGI₂, but higher concentrations of the respective alcohol were needed to raise the level to those observed with ethanol (Table 1). The stimulant effect of ethanol was short-lived. Removal of the alcohol from the medium by washing the cells caused a return of 6-keto-PGF₁α to baseline values within 30 minutes. The maximal stimulation was 1.9 times the basal production rate of PGI₂. The average 6-keto-PGF₁α of control preparations was 418 pg/30 min/10⁵ cells, while ethanol-stimulated cells had an average production of 816 pg/30 min/10⁵ cells.

PGE₂ was also measured. Control cultures produced 184 ± 16 pg/30 min/10⁵ (mean ± SEM) cells, while alcohol-exposed cells showed 383 ± 40 pg/30 min/10⁵ cells.

The site of ethanol action was investigated in intact endothelial cells and in a cell-free system. Endothelial cells were prelabeled with [¹⁴C]arachidonic acid. After removal of unbound fatty acid, the cells were treated with indomethacin and exposed to varying concentrations of ethanol ranging from 0% to 0.2%. As Table 2

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**Table 1. Effect of Different Alcohols on Production of 6-Keto-PGF₁α by Endothelial Cells**

<table>
<thead>
<tr>
<th>Alcohol</th>
<th>6-Keto-PGF₁α (pg/30 min/10⁵ cells)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>80 mg%</td>
</tr>
<tr>
<td></td>
<td>160 mg%</td>
</tr>
<tr>
<td>Control (buffer)</td>
<td>418 ± 20</td>
</tr>
<tr>
<td>Methanol</td>
<td>666 ± 26, P &lt; .005</td>
</tr>
<tr>
<td>Ethanol</td>
<td>833 ± 34, P &lt; .005</td>
</tr>
<tr>
<td>2-Propanol</td>
<td>685 ± 30, P &lt; .005</td>
</tr>
</tbody>
</table>

Production of 6-keto-PGF₁α was measured at two different concentrations of alcohol. The means ± 1 SEM of three experiments each are shown. The differences between control and alcohol-incubated samples were evaluated by Student’s t test.
shows, ethanol reduced the release of $[^{14}C]$arachidonic acid from phospholipids. A similar result was obtained using immobilized phospholipase A$_2$ and phosphatidyl choline. In this cell-free system, ethanol also produced a marked reduction of the release of fatty acyl residues.

**In Vivo Studies**

Administration of 32 g of pure ethanol to six volunteers changed alcohol levels, as shown in Fig 2. The maximal ethanol concentration varied from 69 to 99 mg/100 mL. All curves peaked one hour after alcohol ingestion. Plasma levels of 6-keto-PGF$_{1a}$ measured at the same time intervals showed a rise that varied between 1.8 and 3.9 times the baseline values. Response curves of the PGI$_2$ oxidation product peaked between two and three hours after alcohol administration. The results in each of the six individuals tested are presented in Fig 2. Bleeding times were measured at or near the maximal response of 6-keto-PGF$_{1a}$ (Table 3). No significant differences from baseline values were observed. Levels of 6-keto-PGF$_{1a}$, however, showed a highly significant rise.

**DISCUSSION**

Our studies give clear evidence of an ethanol-induced rise in 6-keto-PGF$_{1a}$ of equal magnitude both in vitro and in vivo. Of special note is that relatively low concentrations of ethanol were required. Our results do not allow us to conclude whether increased production or decreased degradation of prostacyclin is the responsible mechanism. Enhanced release of arachidonic acid from phospholipids of endothelial cells in the presence of ethanol could be ruled out as a possible cause of the stimulation, as cyclooxygenase-inhibited cells, even those that were incubated with small concentrations of ethanol, released markedly smaller amounts of $[^{14}C]$arachidonic acid than did their controls. An ethanol-induced inhibition of phospholipase A$_2$ activity was also recognizable in the cell-free system we used. PGI$_2$ and PGE$_2$ showed parallel increases. We believe that cyclooxygenase is enhanced by ethanol in low concentrations. Previous investigators have shown that arachidonic acid-induced platelet aggregation is not decreased by ethanol, as is collagen-stimulated and thrombin-stimulated aggregation, but in fact, is increased. This may be due to an effect of alcohol on the uptake by, or the binding of, arachidonic acid to platelets. Although PGI$_2$ stimulation by ethanol was greater than that of other alcohols, there was sufficient enhancement by methanol or isopropanol to suggest that a physicochemical alteration of membrane structure may be responsible for our findings.

Although peak values of 6-keto-PGF$_{1a}$ generally trailed ethanol maxima by about one hour, this does not necessarily invalidate the hypothesis that the alcohol itself is the stimulant, rather than one of its metabolic degradation products. Several reasons can be listed: (1) alcohol dehydrogenase is principally a liver enzyme$^{11}$ that is not present in endothelial cells, (2) all alcohols tested gave similar results, yet isopro-

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### Table 2. Effect of Ethanol on the Release of Free Fatty Acids From Phospholipids

<table>
<thead>
<tr>
<th>Ethanol Concentration (mg/100 mL)</th>
<th>0</th>
<th>40</th>
<th>80</th>
<th>160</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Endothelial cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$[^{14}C]$arachidonic acid released (cpm)</td>
<td>738</td>
<td>490</td>
<td>320</td>
<td>298</td>
</tr>
<tr>
<td><strong>Cell-free system</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitic acid released (number of area slices computed)</td>
<td>236,290</td>
<td>159,640</td>
<td>92,315</td>
<td>80,287</td>
</tr>
</tbody>
</table>

Results of two individual experiments are listed.

### Table 3. Response of Bleeding Time and Prostaglandin 6-Keto-PGF$_{1a}$ to Ethanol Ingestion (32 g)

<table>
<thead>
<tr>
<th>Hours After Ingestion</th>
<th>6-Keto-PGF$_{1a}$ (pg/mL)</th>
<th>Bleeding Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20.58 ± 3.53</td>
<td>6.17 ± 1.66</td>
</tr>
<tr>
<td>1</td>
<td>27.33 ± 6.59, p &lt; .25</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>41.60 ± 19.38, p &lt; .25</td>
<td>6.25 ± 2.07</td>
</tr>
<tr>
<td>3</td>
<td>33.65 ± 6.15, p &lt; .001</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>25.21 ± 4.05, p &lt; .5</td>
<td>—</td>
</tr>
</tbody>
</table>

Means ± SD of values in six individuals are shown. The differences between baseline (0 h after ingestion) and postethanol measurements of 6-keto-PGF$_{1a}$ were evaluated by Student's t test.

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![Fig 2](image-url)
panol is degraded to acetone rather than the aldehyde, (3) the peak blood concentration may not correspond to the peak value in the areas of maximal prostacyclin production, and (4) the maximal levels of 6-keto-PGF\(_{1\alpha}\) may have occurred closer to the ethanol peaks than apparent from the hourly measurements.

The failure of the bleeding time to show any prolongation after ethanol ingestion can be explained by the modest rise in the level of PG\(_I_2\). Although not measured in our experiments, there may have been no significant reduction in platelet thromboxane synthesis. We can infer that the latter is essential for a prolonged bleeding time based on the observations with aspirin. The inhibitory effects of alcohol on platelet function have usually been measured at levels that substantially exceeded our own values. We must stress that the ethanol administered to the volunteers in this study did not raise plasma concentrations to a level considered intoxicating, normally > 100 mg/100 mL. There is no question that our in vivo results were indeed due to ethanol or possibly one of its metabolites, as only chemically pure (95%) alcohol was used.

A study of the effect of acute ethanol intake on plasma concentrations of thromboxane and prostacyclin in humans has recently been reported. No change in PG\(_I_2\), but a slight decrease in thromboxane levels, was noted. This investigation differs from ours in the quantity of alcohol administered. Ingestion of ethanol was two to three times that used in our study.

Whether or not our results provide an explanation for the well-known vasodilatory action of alcohol cannot be answered. Direct measurements of vasodilation were not made, yet prostacyclin is one of the most potent vasodilators. It might be expected, however, that the increase in PG\(_I_2\) will also affect the sensitivity of platelets to aggregating agents. Although we did not test platelet aggregation in this group of individuals, it is unlikely that PG\(_I_2\) is directly involved in the inhibitory effect of ethanol on platelet aggregation, as the alcohol appears to have a greater effect on aggregation induced by collagen, thrombin, and Ca\(^{2+}\)-ionophore. PGI\(_2\), on the other hand, also strongly inhibits adenosine diphosphate-induced and epinephrine-induced aggregation.

Our findings that prostacyclin levels increase after ethanol ingestion may provide a rationale for the protective effect of alcohol against cardiovascular disease.

REFERENCES
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Ethanol raises prostacyclin in vivo and in vitro

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