Interaction of Ethanol, Prostacyclin, and Aspirin in Determining Human Platelet Reactivity in Vitro

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Ethanol partitions into cellular membranes and alters membrane-associated phenomena in numerous cell types. Since platelet aggregation and its inhibition by prostacyclin are mediated by membrane-associated receptors and enzymes, we examined the interaction of ethanol, prostacyclin, and aspirin on human platelet reactivity. Using platelet-rich plasma, we examined the effect of increasing concentrations of ethanol (0.05% to 1.0%) on the platelet-inhibitory effects of a submaximal dose (5 x 10^-10 M) of prostacyclin, the concomitant production of cyclic 3',5'-adenosine monophosphate (AMP), and the release of thromboxane A2. Ethanol alone had little effect on platelet aggregation induced by 5 μg/ml collagen; however, it potentiated the inhibitory effect of prostacyclin on platelet aggregation in a dose-dependent manner in the range of 0.05% to 1.0% ethanol. Whereas prostacyclin increased platelet cyclic AMP levels, ethanol had no further effect on cyclic AMP levels. Ethanol alone reduced thromboxane A2 generation, but this effect could not totally account for the observed interaction of ethanol and prostacyclin on aggregation, since aspirin did not totally abolish the interaction. The dose range in which the ethanol/prostacyclin/aspirin interactions occur encompasses the plasma levels of ethanol that may be achieved by the consumption of alcoholic beverages. The results may, in part, explain the dose-related physiological and pathological consequences of chronic alcohol consumption on the cardiovascular system.

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Vascular prostacyclin biosynthesis and release may constitute an endogenous mechanism for the local control of platelet aggregation and thrombus formation. Prostacyclin's potent inhibition of platelet reactivity is primarily mediated by the activation of adenylate cyclase and the consequent elevation of intraplatelet cyclic 3',5'-adenosine monophosphate (AMP) levels. Adenylate cyclase is a membrane-bound enzyme and one that is affected by the state of its lipid environment in several cell types including human platelets. In addition to adenylate cyclase, several other enzymes that control platelet activity are membrane-bound, including those enzymes responsible for the generation of the potent proaggregatory compound, thromboxane (TX) A2. Agents that tend to stabilize membranes, such as cholesterol, increase the release of TXA2 and the aggregation response, while agents that tend to destabilize (fluidize) platelet membranes, such as ethanol, reportedly decrease platelet responsiveness. However, much contradiction on the effects of ethanol on human platelet function exists.

In addition to reports of a direct effect of ethanol on platelets, Landolfi and Steiner have recently reported that ethanol stimulates the generation of prostacyclin both in vitro and in vivo. Ethanol may, therefore, inhibit platelet reactivity indirectly by stimulating the generation of prostacyclin. This report describes studies on the interaction of ethanol, prostacyclin, and aspirin in vitro human platelet reactivity.

Methods

Subjects and Blood Collection

The studies described herein had prior approval of the Institutional Review Board, and the experiments were fully explained to the subjects before obtaining their informed consent. The subjects in this study were healthy hospital and laboratory personnel who did not use medications for at least 14 days before blood donation. Blood was drawn via a 21-gauge butterfly cannula, and the initial 3 ml was discarded. Collected blood was anticoagulated with either a 1/8th volume of acid-citrate-dextrose (ACD) or a 1/10th volume of 3.8% trisodium citrate (citrated). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by differential centrifugation as described. Each individual's platelet preparation was treated with the entire range of ethanol concentrations (0 to 1.0%) on the same day and the n values given reflect the total number of subjects studied.

Aggregation Studies

Platelet aggregation in citrated PRP was monitored as increasing light transmission by use of standard turbido-
metric techniques in a four-channel Platelet Aggregation Profiler (Biodata Corporation, Hatboro, PA).

Platelet concentrations in PRP were monitored, but not adjusted [mean ± SD = 3.16 ± 0.18 x 10^11/ml, range 2.9 to 3.4)]. Platelet aggregation was induced by the addition of 5 μg/ml collagen (Hormon Chemie, Munich, West Germany). Prostacyclin or buffer (50 μl) was added directly into the stirred PRP (400 μl) in the aggregation cuvette at 37°C at 1 min before collagen (50 μl) was added. On each day the concentration of prostacyclin was adjusted so that, in the absence of ethanol, collagen-induced platelet aggregation was reduced by approximately 30%. This submaximal level of inhibition allowed the study of the effects of ethanol, aspirin, or both on prostacyclin-mediated inhibitory effects. The mean concentration of prostacyclin required to effect such inhibition in the absence of aspirin was 5.1 x 10^{-10} M (range 2 to 8 x 10^{-10} M). Prostacyclin was stored at −70°C as a stock solution of 10^{-7} M in 1 mM sodium bicarbonate buffer (pH 10.2). On study days, the appropriate dilutions were made with the same buffer at 4°C, and working solutions were stored on ice during use. Control aggregations included the appropriate volume of 1 mM bicarbonate buffer. Ethanoldi (USP) or its diluent (0.15 M NaCl) was added to 9 vol of PRP 10 min before study of platelet aggregation. Preliminary experiments indicated that the effects of ethanol were complete within 5 min of addition to PRP. All ethanol concentrations are given on a wt/vol basis.

Thromboxane A2 Generation

The generation of thromboxane A2 by platelets in PRP stimulated with collagen was determined as immunoreactive TXB2. Five min after the addition of collagen, indomethacin was added directly into the cuvette to a final concentration of 10 μM. Platelets were sedimend by centrifugation (12 000 g for 2 min) and the plasma was aspirated and stored at −20°C before radioimmunoassay. After dilution to 1:100 or greater with assay buffer (0.15 M Tris-HCl, 0.14 M NaCl, 0.5 mg/ml gelatin) a radioimmunoassay of TXB2 was performed as previously described. The TXB2 antiserum was a gift from Lawrene Levine (Brandeis University, Waltham, MA) and it exhibited less than 2% crossreactivity with other common metabolites of arachidonic acid. Prostacyclin, ethanol, or both at the concentrations used in the experiments described did not affect the radioimmunoassay. The lower limit of sensitivity of this assay system was 1 ng/10^9 platelets.

Platelet Cyclic AMP Levels

Platelets in PRP (ACD anticoagulated) were sedimented (2000 g for 15 min), the plasma was aspirated, and the platelets were resuspended in an equal volume of autologous citrated platelet-free plasma (PFP) that had been previously treated with charcoal to remove cyclic AMP. The pretreatment consisted of adding charcoal (Norit A, Sigma) to a final concentration of 10 mg/ml and incubating this for 15 min at room temperature. The charcoal and adsorbed cyclic AMP were removed by centrifugation (3000 g for 15 min) and the supernatant plasma was filtered through a 0.22 μm filter (Millex GS, Millipore) before use. Preliminary experiments indicated that this procedure stripped the plasma of more than 99% of endogenous or added (3H- or 125I-labeled) cyclic AMP. This procedure markedly increased the sensitivity of the assay for intraplatelet cyclic AMP. Platelets resuspended in charcoal-stripped plasma and treated with prostacyclin and ethanol gave aggregation profiles identical to platelets in citrated plasma prepared by conventional methods.

To determine the intracellular level of cyclic AMP in platelets, the following procedure was employed. Platelets in autologous charcoal-stripped plasma were solubilized by the addition of a 1/10th final volume of 10% Triton X-100 in 100 mM EDTA at the appropriate time after the addition of ethanol, prostacyclin, or both to the PRP. We previously used 1% Triton X-100 to solubilize platelets and the removal of Ca^{2+} and Mg^{2+} by EDTA prevented adenylate cyclase and cyclic nucleotide phosphodiesterase activities from altering cyclic AMP levels. The stability of cyclic AMP levels under these conditions was confirmed by incubating solubilized PRP samples for various time periods up to 4 hours before assay. Further, solubilized PRP cyclic AMP levels were stable for at least 1 month at −20°C. Cyclic AMP levels in solubilized samples were determined by radioimmunoassay by using a commercially available plasma cyclic AMP radioimmunoassay kit (Immunonuclear Corp., Stillwater, MN). We used the acetylation procedure described by the manufacturers and adjusted the concentration of the individual components so that in the absence of cyclic AMP the antibody bound 40% of the 10,000 cpm of the 125I-cyclic AMP added and we found that the assay had a lower level of sensitivity of 10 fmol/tube. All solubilized samples were diluted by 1:50 or greater before acetylation. Preliminary experiments indicated that this procedure obviated nonspecific competition between factors in the solubilized samples and the cyclic AMP antiserum. Lower dilutions did not completely remove components that interfered with the assay. For the majority of samples, dilution factors of 1:100 and 1:1000 were routinely used and yielded excellent agreement of corrected values. All dilutions were performed with 0.05 M sodium acetate buffer (pH 5.0). Representative graphs of the dose response and time course of prostacyclin-induced platelet cyclic AMP generation are illustrated in Figure 1.

Results

Platelet Aggregation

Representative tracings illustrating the interaction of ethanol and prostacyclin on platelet aggregation are presented in Figure 2. The experiments were designed so that, in the absence of ethanol, pre-incubation of platelets with prostacyclin for 1 min gave submaximal inhibition of collagen-induced aggregation. The collagen concentration was 5 μg/ml, which represents a medium to strong stimulus, but one that allowed aggregation in the presence of prostacyclin. Initial experiments for each individual identified a concentration of prostacyclin that inhibited aggregation by approximately 30%. This concentration was then used throughout subsequent experiments on each individual. Pre-incubation of the platelets with etha-
Figure 1. Time course and dose response of prostacyclin-induced cyclic AMP generation in platelet-rich plasma. Platelets (2.5 \times 10^8/ml) in charcoal-stripped, autologous, citrated plasma were equilibrated in aggregation cuvettes at 37°C for 2 min before the addition of prostacyclin. In time-course experiments, the final concentration of prostacyclin was 1 \times 10^{-10} M. Dose-response experiments were terminated 1 min after the addition of prostacyclin. In all experiments, the reaction was terminated by the addition of Triton X-100 and EDTA to final concentrations of 1% and 10 mM, respectively. After dilution and acetylation, cyclic AMP was quantitated by radioimmunoassay. In the absence of platelets, cyclic AMP in the charcoal-stripped plasma was less than 1 pmol/ml (0.3 pmol/10^8 platelets). Data are means ± range of duplicate experiments, and are representative of those obtained with platelets and plasma from five persons.

Figure 2. Effect of increasing ethanol concentrations on platelet aggregation in the presence of a submaximal inhibitory dose of prostacyclin (PGI_2, 4 \times 10^{-10} M). Results are typical of those obtained in nine individuals. Ethanol (+) or 0.15 M NaCl (-) was added to platelet-rich plasma at least 10 min before placement in the aggregometer. After 2 min equilibration at 37°C in the aggregometer, PGI_2 (+) or vehicle (-) (1 mM bicarbonate buffer, pH 10.2) was added. Aggregation was induced 1 min later by the addition of 5 \mu g/ml collagen (COLL). 0.1% ethanol (EtOH) = 21.7 mM.

Figure 3. Interaction of ethanol and prostacyclin on human platelet aggregation in platelet-rich plasma. The indicated concentration of ethanol was added to platelet-rich plasma at least 10 min before equilibration at 37°C in the aggregometer. Prostacyclin (+ PGI_2, approximately 5 \times 10^{-10} M) (•) or vehicle (− PGI_2, 1 mM bicarbonate buffer, pH 10.2) (○) was added, and 1 min later aggregation was induced by the addition of 5 \mu g/ml collagen. Aggregation was allowed to proceed for 5 min. The parameters were: top panel, lag time = the time between addition of collagen and the initiation of aggregation, middle panel, rate = the maximum velocity of aggregation, lower panel, aggregation = the extent of aggregation measured 5 min after the addition of collagen. Data are means ± SD of the nine subjects tested. 0.1% ethanol = 21.7 mM.

Figure 4. In contrast to ethanol's general lack of effect in the absence of aspirin and prostacyclin, when it was added to aspirin-treated platelets in concentrations greater than 0.1%, a dose-dependent inhibition of aggregation was evident. In this series of experiments on aspirin-treated platelets, the concentration of prostacyclin required to give 30% inhibition was reduced from 4.7 ± 0.4 to 1.4 ± 0.5 \times 10^{-10} M. This increased sensitivity of aspirin-treated platelets agrees with previous findings.18,19 In the
Figure 4. The effect of ethanol on platelet aggregation in the presence (+) and absence (−) of aspirin (ASA) and prostacyclin (PGI2). Platelet aggregation was studied in eight subjects before (−ASA) and 2 hours after (+ASA) ingestion of 650 mg aspirin. Aggregation studies in citrated, platelet-rich plasma were performed as described in the Methods section and in the legends to Figures 2 and 3. Data are means ± SD. 0.1% ethanol = 21.7 mM.

Figure 5. The effect of ethanol and prostacyclin on platelet cyclic AMP levels. Platelets in charcoal-stripped, citrated plasma were incubated with ethanol or diluent for at least 10 min before placement in the aggregometer at 37°C. After equilibration for 2 min, prostacyclin (+PGI2) or buffer (−PGI2) were added for 1 min. The reaction was terminated and platelets were solubilized by the addition of 1% Triton X-100 and 10 mM EDTA. After dilution and acetylation, cyclic AMP was measured by radioimmunoassay. The results are means ± SD, n = 4. 0.1% ethanol = 21.7 mM.

Figure 6. The effect of ethanol and prostacyclin on collagen-induced thromboxane A2 generation. Platelets in citrated plasma were incubated with the indicated concentrations of ethanol and equilibrated at 37°C. Prostacyclin (+PGI2) (●) or buffer (−PGI2) (○) were added, followed 1 min later by collagen (5 μg/ml). Five min after collagen addition, indomethacin was added to a final concentration of 10 μM, and platelets were immediately sedimented (12 000 g for 2 min). After dilution, thromboxane B2 was measured by radioimmunoassay. Results are means ± SD, n = 8. Platelets from subjects pretreated with 650 mg of aspirin released less than 1 ng TXB2/10⁸ platelets in response to 5 μg/ml collagen. 0.1% ethanol = 21.7 mM.

presence of this lower concentration of prostacyclin, the aspirin-treated platelets were further inhibited by ethanol. Unlike the inhibition induced by ethanol alone, the presence of prostacyclin rendered the platelet sensitive to ethanol in the 0.05 to 0.1% range. At concentrations greater than 0.1%, the curve generally paralleled that obtained with ethanol alone.

Cyclic AMP
In separate experiments, the intraplatelet concentration of cyclic AMP was measured 1 min after prostacyclin or buffer addition in the presence of increasing ethanol concentrations. The data are presented in Figure 5. Using the modified plasma cyclic AMP assay, we found that the basal levels of cyclic AMP in platelets (1.78 ± 0.1 pmol/10⁸ platelets) agreed with values previously obtained with alternative techniques.¹⁴,²⁰,²¹ In the presence of ethanol, the addition of prostacyclin (5 × 10⁻¹⁰ M) raised the level of cyclic AMP from basal levels of 1.78 ± 0.1 to 6.13 ± 0.62 pmol/10⁸ platelets (p < 0.001). In the absence of prostacyclin, cyclic AMP levels were not altered by increasing the concentration of ethanol by 0.05% to 1.0%. Similarly, in the presence of prostacyclin, cyclic AMP levels did not demonstrate a significant response in the range of 0.05% to 0.8% ethanol. Between 0.8% and 1.0% ethanol, platelet cyclic AMP showed a modest increase from 7.59 ± 1.07 to 10.21 ± 1.73 pmol/10⁸ platelets; however, this difference was not statistically different (p > 0.05 by paired t-test).

Thromboxane A₂ Generation
The levels of TXB₂ in plasma 5 min after the addition of collagen to PRP pretreated with ethanol, prostacyclin, or both are shown in Figure 6. In contrast to ethanol's lack of effect on platelet aggregation, ethanol alone induced a dose-dependent decrease in TXA₂ generation by collagen-stimulated platelets. In the presence of prostacyclin, TXA₂ generation was reduced by approximately 50% and, apart from a small early (nonsignificant by paired t-test) increase, the dose-dependent decrease in TXA₂ induced by ethanol was parallel to that obtained in the absence of prostacyclin. These experiments also confirmed the efficacy of the aspirin treatment, which reduced collagen-induced TXA₂ generation (in the absence of prostacyclin) from 111 ± 11 ng TXB₂/10⁸ platelets to less than 1 ng/10⁸ platelets (lower limit of detection).

Discussion
Studies on the effects of ethanol on platelet activities have been widely reported. However, contradictory data and the use of unrealistically high concentrations of eth-
anol have made a physiologically relevant interpretation of the data difficult. The present investigation studied a wide range of ethanol levels that include those achieved by moderate and excessive alcohol consumption. We examined the direct effect of ethanol on in vitro human platelet aggregation, TXA₂, and cyclic AMP generation, and we probed for a potential interaction between ethanol, prostacyclin, and aspirin. It is widely believed that both aspirin and prostacyclin inhibit in vivo platelet activities and thereby limit thrombus formation.

We found that in vitro platelet aggregation in citrated PRP, induced by a moderately high dose of collagen, was not affected by ethanol in the range of 0.05% to 1.0% (10.9 to 217 mM). However, when the same collagen stimulus and ethanol concentrations were used in the presence of a submaximal inhibitory concentration of prostacyclin, a clear dose-dependent decrease in platelet aggregation was observed with increasing ethanol concentrations. This effect was evident with ethanol concentrations as low as 0.05%. We also performed experiments in which platelet-activating factor replaced collagen as the aggregating agent, and we obtained similar results (data not shown).

Our initial hypothesis was that ethanol potentiated the inhibitory effect of prostacyclin by promoting the response of adenylate cyclase to prostacyclin. In neuronal tissue, it has been shown that ethanol stimulates adenylate cyclase activity by virtue of its membrane active properties.\(^ {22,23}\) To examine the possibility of increased platelet cyclic AMP levels, preliminary experiments were performed in a plasma-free system in which gel-filtered platelets were incubated with ethanol and prostacyclin. However, with gel-filtered platelets, in the presence of prostacyclin, ethanol in the 0.05% to 0.2% range actually stimulated platelet aggregation and TXA₂ release (data not shown). Hence, we devised a cyclic AMP assay system that could be used in the presence of plasma. This was achieved by using a plasma cyclic AMP radioimmunoassay and releasing intraplatelet cyclic AMP into plasma by disruption of the platelets with the detergent Triton X-100. EDTA was present to chelate Mg\(^{++}\) and Ca\(^{++}\), thereby inhibiting further generation or degradation of cyclic AMP. The sensitivity of the assay system was increased by suspending the platelets in plasma that had been stripped of platelet membranes.\(^ {21}\) This distribution makes small changes in platelet cyclic AMP difficult to detect. Using a modified assay system, we found that ethanol alone had no effect on platelet cyclic AMP levels, nor did increasing ethanol after prostacyclin-stimulated levels of cyclic AMP. A slight elevation of prostacyclin-stimulated cyclic AMP levels was noted at 1.0% ethanol. Atkinson et al.\(^ {24}\) also reported that, while 1-butyl and amyl alcohols stimulated human platelet cyclic AMP accumulation, ethanol had no significant effect. In contrast, Noe et al.\(^ {25}\) reported that basal and PGE₁-sensitive platelet membrane adenylate cyclase activity was inhibited by ethanol, while smooth muscle adenylate cyclase was stimulated in a dose-dependent fashion, as has been reported for adenylate cyclase of neuronal origin.\(^ {22,23}\) Cowan et al.\(^ {26}\) also reported that basal platelet cyclic AMP levels were depressed by ethanol, while PGE₁-stimulated levels were unaffected.\(^ {26}\) In contrast, data from Hwang et al.\(^ {27}\) suggest that the levels of cyclic AMP in rat PRP plasma were increased by ethanol.

Since ethanol reportedly influences eicosanoid metabolism,\(^ {11,28}\) we also examined the possibility that altered platelet arachidonate metabolism mediated the ethanol effect. While ethanol alone did, indeed, inhibit TXA₂ generation in a dose-dependent manner, this was not reflected in altered platelet aggregation. These data are consonant with a reported lack of effect of ethanol on the bleeding time.\(^ {29}\) In the presence of prostacyclin, while the overall levels of TX generation were lower, the ethanol-induced attenuation was parallel. The fact that the ethanol-induced decrease in platelet TXA₂ production did not influence platelet aggregation is consistent with the observation that collagen-induced aggregation at the medium to high concentration of 5 μg/ml is only partially dependent on TXA₂ generation. This is evident from the data in Figure 4 in which postaspirin platelet aggregation in the absence of ethanol is only partially inhibited compared to pre-aspirin aggregation, despite a greater than 99% inhibition of TXA₂ generation. Further studies are required to determine the mechanism by which ethanol inhibits platelet TXA₂ generation.

The possibility that the ethanol/prostacyclin interaction reflects an amplification of the mild inhibition of TXA₂ production by ethanol is suggested by these data. However, since, in the presence of aspirin, we observed a dose-dependent inhibition of aggregation both in the presence and in the absence of prostacyclin, clear interpretation is difficult. The interaction of aspirin and ethanol, while only evident at ethanol concentrations greater than 0.1% ethanol, is consistent with the ability of ethanol to potentiate the aspirin-induced prolongation of the bleeding time\(^ {30}\) and to increase fecal blood loss.\(^ {31}\)

Overall, our data indicate that ethanol is a mild inhibitor of collagen-induced platelet TXA₂ generation. The level of inhibition is not sufficient to reduce in vitro platelet aggregation. However, in the face of diminished platelet aggregation induced by a submaximal inhibitory dose of prostacyclin, the mild inhibitory effect of ethanol manifests as a further decrease in platelet aggregation. Delineation of the relationship between the above in vitro observations and the reduced incidence of thrombotic cardiovascular disease\(^ {32,34}\) and increased incidence of hemorrhagic stroke\(^ {25,36}\) associated with moderate alcohol consumption warrants further investigation.

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