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Effects of Ethanol on Pathways of Platelet Aggregation In Vitro

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Key words

Platelet aggregation – Platelet secretion – Platelet inhibition – Ethanol

Summary

Ethanol, at physiologically tolerable concentrations, did not affect the primary phase of ADP-induced aggregation of human or rabbit platelets, which is not associated with the secretion of granule contents. Potentiation by epinephrine of the primary phase of ADP-induced aggregation of rabbit platelets was also not inhibited by ethanol. However, ethanol did inhibit the secondary phase of ADP-induced aggregation which occurs with human platelets in citrated platelet-rich plasma and is dependent on the formation of thromboxane A_2. Inhibition by ethanol of thromboxane production by stimulated platelets is likely due to inhibition of the mobilization of arachidonic acid from membrane phospholipids, as ethanol had little or no effect on aggregation and secretion induced by arachidonic acid or the thromboxane mimetic U46619. Rabbit platelet aggregation and secretion in response to low concentrations of collagen, thrombin, or PAF were inhibited by ethanol. Inhibition of the effects of thrombin and PAF was also observed with aspirin-treated platelets. Thus, in addition to inhibiting the mobilization of arachidonate for thromboxane formation that occurs with most agonists, ethanol can also inhibit aggregation and secretion through other effects on platelet responses.

Introduction

There is evidence from epidemiological studies indicating that moderate consumption of alcoholic beverages is inversely related to the incidence of the clinical complications of coronary heart disease (1–5). Several reasons have been proposed for this protective effect of alcohol, including personality characteristics of drinkers and non-drinkers, increased plasma levels of high density lipoproteins or prostacyclin that are associated with ingestion of alcohol, and direct inhibitory effects of alcohol on platelet function (6, 7). Ethanol has been shown to inhibit aggregation and thromboxane production by platelets (8–14), and platelets are known to be involved in both the initiation of atherosclerotic lesions and in the thromboembolic complications of coronary heart disease (15). However, the mechanism (or mechanisms) by which ethanol inhibits platelet responses is not known. Ethanol inhibits production of arachidonate metabolites by platelets stimulated with collagen or thrombin (12–14), and it has been suggested that ethanol inhibits Ca^{2+}-activated phospholipase A_2 in platelets (16). In addition, membrane fluidity of cells is increased by ethanol (17), and since increased platelet membrane fluidity has been reported to inhibit some platelet responses (18, 19), it may be that an alteration in membrane fluidity by ethanol is involved in its inhibitory effects on platelet function.

Most aggregating agents also induce secretion of granule contents of platelets and activate the arachidonate pathway, leading to thromboxane production (20, 21). To determine the effect of ethanol on various pathways of platelet responses, we have examined the action of ethanol at physiologically tolerable concentrations on aggregation and secretion of granule contents by platelets in response to a variety of agonists, to several of which platelets can be exposed in vivo. Platelets in citrated platelet-rich plasma were used to study the effect of ethanol on ADP-induced primary aggregation (rabbit platelets) and primary and secondary aggregation (human platelets). Washed platelets were used in the remaining experiments so that platelet responses could be studied in media containing concentrations of Ca^{2+} in the physiological range. Rabbit platelets were used in the majority of these experiments, as we wished to establish the effects of ethanol on platelets from animals that we are using for other studies of the effects of ethanol on thrombosis and atherosclerosis (22).

Materials and Methods

Materials

Materials were obtained from the following suppliers: ADP, epinephrine, arachidonic acid, and acetylsalicylic acid (aspirin), from Sigma Chemical Co., St. Louis, MO; synthetic 1-0-alkyl-2-acetyl-sn-glyceryl-3-phosphoryl choline (PAF, platelet activating factor) from Calbiochem, La Jolla, CA. Solutions of arachidonic acid were prepared as previously described (23). Imipramine was from Geigy Canada, Dorval, Que.; bovine thrombin (topical) from Parke-Davis Inc., Scarborough, Ont.; human fibrinogen, grade L, from AB Kabi, Stockholm (treated before use with diisopropylfluorophosphate, Sigma) (24) and partially purified by the method of Lawrie et al. (25); bovine albumin (fraction V) from Boehringer Mannheim, Dorval, Que.; absolute ethanol from Consolidated Alcohols, Toronto; the thromboxane mimetic U46619 from the Upjohn Co., Kalamazoo, MI. Apyrase was prepared from potatoes (26, 27), dissolved in 0.15 M NaCl and stored at −20° C; acid soluble collagen was prepared from bovine tendon collagen (Sigma) (28). 5-Hydroxy-3'-^{14}C-tryptamine creatinine sulfate (^{14}C-serotonin, 60 mCi/mmol) was from Amersham Corp., Oakville, Ont.; $Na_2\ ^{51}CrO_4$ (200–500 Ci/g of Cr) and a radioimmunoassay kit (NEK-007) for thromboxane B_2 (TxB_2) was from NEN Canada, Lachine, Que. Unless indicated otherwise, reagents were dissolved and diluted in modified Tyrode solution (no added calcium salt). All concentrations are expressed as final concentrations after all additions.

Methods

Suspensions of washed platelets from humans and rabbits were prepared as described elsewhere (26, 29, 30). In the first washing fluid, the platelets were labelled with ^{14}C-serotonin (2 μCi per 10 ml of washing fluid), and for some experiments with ^{51}Cr (0.5 μCi/2 × 10^9 platelets). For the preparation of aspirin-treated platelets, aspirin (500 μM) was included in the first washing fluid. Platelet suspensions (0.5 × 10^9/ml in Tyrode solution containing 0.35% albumin, 5 μM imipramine and apyrase, pH 7.35) were incubated for at least 20 min at 37° C before testing.

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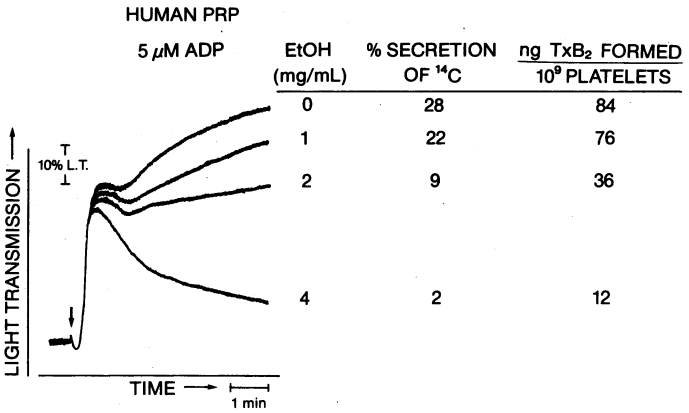

Fig. 1 Effects of ethanol on ADP-induced responses of human platelets in citrated platelet-rich plasma (PRP). The PRP was stirred with or without ethanol for 2 min prior to addition of ADP (5 μM), indicated by the arrow. The extent of secretion of ^{14}C-serotonin from prelabelled platelets and TxB_2 formation were determined 5 min after addition of ADP and representative values are given (3 experiments)

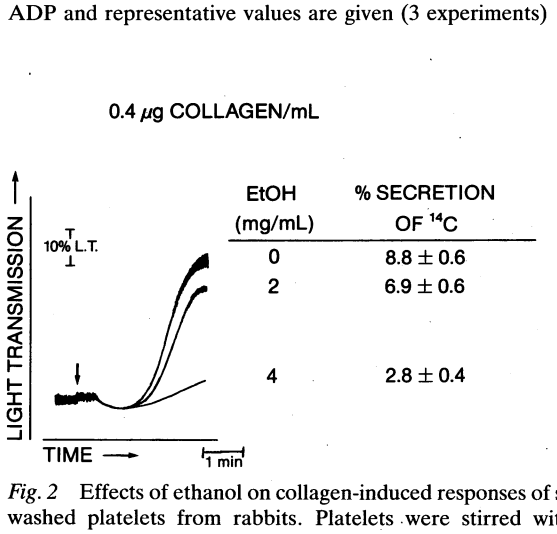

Fig. 2 Effects of ethanol on collagen-induced responses of suspensions of washed platelets from rabbits. Platelets were stirred with or without ethanol for 2 min prior to addition of collagen (0.4 μg/ml), indicated by the arrow. The extent of secretion of ^{14}C-serotonin from the prelabelled platelets was determined in samples taken 3 min after addition of collagen. Aggregation curves are representative and mean values for secretion are shown for 4 experiments

Human or rabbit platelet-rich plasma was prepared from blood taken into sodium citrate (1 part 3.8% to 9 parts of blood) and centrifuged at 2,000 × g for 2 min at 37° C for human platelets and at room temperature for rabbit platelets. During the previous 14 days, the human donors had not ingested drugs that affect platelet function. The platelet count was adjusted with platelet-poor plasma to 0.3×10^9/ml (human platelets) and 0.5×10^9/ml (rabbit platelets) and the platelet-rich plasma was kept at 37° C. Platelets were labelled by incubation of platelet-rich plasma with ^{14}C-serotonin for at least 10 min.

Platelet aggregation was studied at 37° C in an aggregation module (Payton Associates, Scarborough, Ont.). Fibrinogen (0.3 mg/ml) was added to the suspensions of washed human platelets. Ethanol or diluent (Tyrode solution with no added calcium or magnesium salts) was added 2 min before the aggregating agent. Three or five min after addition of agonist, supernatant samples were prepared by centrifugation of platelet-rich plasma or suspension for 1 min at 12,000 × g in an Eppendorf centrifuge (Brinkmann, Rexdale, Ont.). These samples were used to measure loss of ^{51}Cr and secretion of ^{14}C-serotonin (31, 32) and for radioimmunoassays of TxB_2. Values for extent of secretion are given as means ± S.E.M., and paired t-tests were used to test for significance of differences.

Results

Addition of ethanol (1–9 mg/ml) to stirred suspensions of human or rabbit platelets did not cause a change in either the oscillations of light transmission or light transmission itself, indicating that ethanol did not induce platelet shape change. No significant lysis of platelets (<1% loss of ^{51}Cr) was observed with concentrations of ethanol up to 13 mg/ml.

Unless otherwise stated, the effect of ethanol on platelet responses to agonists was tested at 1, 2, and 4 mg/ml. When an effect was observed, it was consistent and most marked at 4 mg/ml. At 2 mg/ml, effects were marginal, and at 1 mg/ml, rarely different from control.

ADP

In accord with our earlier observations (29, 33), ADP induced only the primary phase of aggregation of rabbit platelets in citrated platelet-rich plasma or of rabbit or human platelets in suspensions of washed platelets in Tyrode-albumin solution containing 2 mM Ca^{2+}. Secretion of ^{14}C-serotonin was less than 1%, even with high concentrations of ADP. Ethanol (1-9 mg/ml) had no effect on shape change or primary aggregation of washed human or rabbit platelets, or of rabbit platelets in citrated platelet-rich plasma stimulated with ADP (0.25–10 μM). Human platelets in citrated platelet-rich plasma underwent both primary and secondary aggregation in response to ADP at concentrations above 2–3 μM; the secondary phase of aggregation was accompanied by secretion of granule contents and production of TxB_2. Ethanol (1–4 mg/ml) inhibited the secondary phase of aggregation, and also inhibited secretion of ^{14}C-serotonin from prelabelled platelets and production of TxB_2 (Fig. 1). The extent of inhibition was directly related to the concentration of ethanol.

Experiments were done to examine the effect of ethanol on the synergistic effect of epinephrine on ADP-induced aggregation of rabbit platelets. By itself, epinephrine (0.5–50 μM) in the absence or presence of ethanol, caused no discernible change in the oscillations or extent of light transmission through stirred suspensions of rabbit platelets. The small aggregation response induced by ADP (0.1–0.5 μM) was potentiated by epinephrine, but ethanol (1–4 mg/ml) did not affect this potentiation. Secretion of dense granule contents was not detectable.

Collagen

Ethanol (4 mg/ml) inhibited aggregation of suspensions of washed rabbit platelets in response to low concentrations of collagen (0.4 μg/ml) (Fig. 2). The extent of secretion of ^{14}C-serotonin induced by 0.4 μg collagen/ml was significantly inhibited by 4 mg ethanol/ml (Fig. 2, $p < 0.005$). At higher concentrations of collagen (1.25 μg/ml) when maximum aggregation was achieved, ethanol had little or no effect on platelet aggregation and secretion.

Thrombin

Ethanol (2 and 4 mg/ml) inhibited aggregation of suspensions of washed platelets from rabbits in response to low concentrations of thrombin (<0.010 U/ml) (Fig. 3). The extent of secretion of ^{14}C-serotonin in response to thrombin (0.008 U/ml) was significantly inhibited in the presence of ethanol (Fig. 3, $p < 0.001$). At higher concentrations of thrombin when maximum aggregation was achieved, ethanol had little or no effect on platelet aggregation and secretion. An inhibitory effect of ethanol on thrombin-induced aggregation and secretion of granule contents was also evident when the platelets had been pre-treated with aspirin at a

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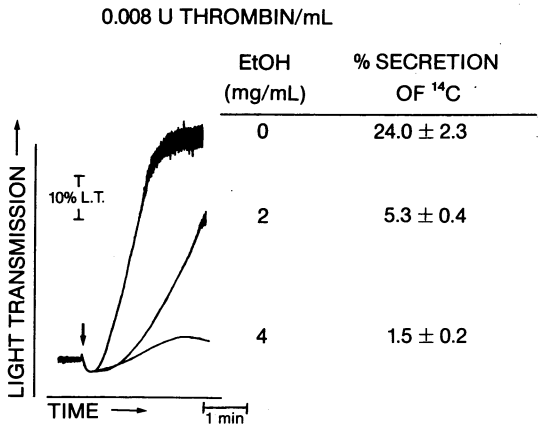

Fig. 3 Effects of ethanol on responses of suspensions of washed platelets from rabbits to thrombin (0.008 U/ml). Aggregation curves are representative and mean values for secretion are shown for 5 experiments. (See legend of Fig. 2 for details)

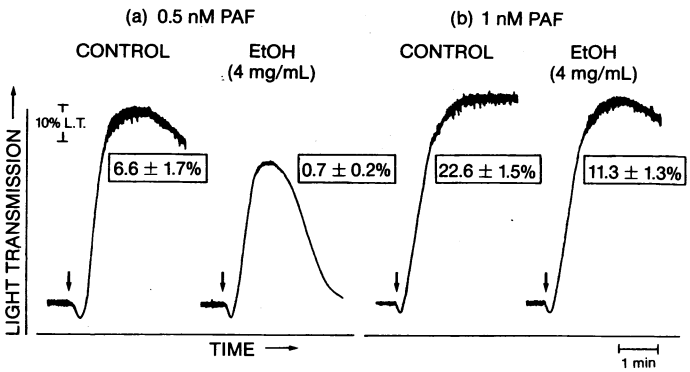

Fig. 4 Effects of ethanol on PAF-induced responses of suspensions of washed platelets from rabbits. Aggregation curves are representative and mean values for secretion are shown for 7 experiments. (See legend of Fig. 2 for details)

concentration that blocks thromboxane formation (33). The extent of secretion of ^{14}C-serotonin by aspirin-treated platelets stimulated with thrombin (0.008 U/ml) was inhibited by ethanol (0.2 ± 0.2% compared with 19.3 ± 2.2% in the absence of ethanol, n = 6, $p < 0.001$).

PAF

Ethanol (4 mg/ml) inhibited aggregation by suspensions of washed rabbit platelets in response to a low concentration of PAF (0.5 nM) (Fig. 4a). The extent of secretion of ^{14}C-serotonin induced by PAF was significantly inhibited by ethanol (Fig. 4a, $p < 0.01$). Secretion of granule contents induced by a higher concentration of PAF (1 nM) was also inhibited by ethanol ($p < 0.001$), however, ethanol had no major effect on the extent of aggregation induced by PAF at this concentration although deaggregation occurred more readily in the presence of ethanol (Fig. 4b). At concentrations of PAF greater than 1 nM, ethanol had little or no effect on platelet aggregation and secretion. An inhibitory effect of ethanol on PAF-induced aggregation was also evident when the platelets had been pretreated with aspirin at a concentration that blocks thromboxane formation (33). The extent of secretion of ^{14}C-serotonin by aspirin-treated platelets stimulated with PAF (1 nM) was significantly inhibited by ethanol (5.3 ± 2.5% compared with 16.3 ± 3.1% in the absence of ethanol, n = 5, $p < 0.05$).

Arachidonic Acid and U46619

Ethanol (1–4 mg/ml) had no detectable effect on aggregation and secretion induced by a range of concentrations of arachidonic acid (15–250 μM) or the thromboxane mimetic U46619 (0.35–5 μM) in 4 experiments with suspensions of washed platelets from rabbits.

Discussion

We have found that ethanol (4 mg/ml) inhibits aggregation of platelets in response to some, but not all agonists and that the inhibition of platelet aggregation is associated with inhibition of secretion of granule contents, thus extending findings by others who have reported inhibition of platelet aggregation by ethanol (8–11).

Platelet aggregation can be caused by a wide variety of agonists, most of which also cause the secretion of granule contents and the formation of TxA_2. Secreted ADP, and the TxA_2 that is generated amplify the platelet responses. The effects of ethanol on the various reactions that cause and/or amplify aggregation and secretion have not previously been considered in detail, and so in the present studies, investigations have been done to determine the effects of ethanol on the response to ADP, the mobilization of arachidonic acid, the response to TxA_2, and the response to thrombin or PAF when TxA_2 formation has been inhibited.

Human and rabbit platelets suspended in media containing albumin, fibrinogen, and physiological concentrations of Ca^{2+}, and rabbit platelets in citrated platelet-rich plasma, aggregate in response to stimulation with ADP, but do not undergo secretion of their amine storage granule contents to an appreciable extent, even with high concentrations of ADP (20, 29, 33, 34). In the present experiments, we have established that ethanol does not inhibit primary ADP-induced aggregation of human and rabbit platelets. Epinephrine, which interacts with α_2-receptors on the platelet surface, increases the sensitivity of rabbit platelets to ADP, although epinephrine by itself does not aggregate rabbit platelets, as shown by others (35). Ethanol had no effect on the potentiation of ADP-induced aggregation of rabbit platelets by epinephrine, indicating that it does not interfere with the interaction of epinephrine with its receptor, or with subsequent events involved in its potentiation of aggregation by another agonist.

When human platelets in citrated platelet-rich plasma (or in artificial medium with a concentration of Ca^{2+} in the micromolar range) are stimulated with ADP at concentrations greater than 2–3 μM, they undergo not only primary aggregation, but also a secondary aggregation response that is associated with activation of the arachidonate pathway, resulting in thromboxane production and secretion of granule contents (33). Ethanol inhibited this second phase of aggregation, and inhibited the secretion of granule contents and production of thromboxane. However, this effect of ethanol must be on the mobilization of arachidonic acid from membrane phospholipids rather than on the conversion of arachidonic acid to TxA_2 because we and others (11) have found that ethanol does not inhibit arachidonic acid-induced platelet aggregation and ethanol had no discernible effect on platelet aggregation and secretion in response to the thromboxane mimetic U46619. Thus, ethanol must inhibit, either directly and/or indirectly, the (phospho)lipases responsible for arachidonic acid mobilization (16).

Collagen-induced platelet aggregation is mediated largely through TxA_2 that is formed and ADP that is secreted when

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platelets are stimulated with collagen (20, 36). Since ethanol does not inhibit the ADP pathway of platelet aggregation, it is likely that the inhibitory effect of ethanol on collagen-induced aggregation and secretion by rabbit platelets is due to inhibition of the mobilization of arachidonic acid for the generation of TxA_2. Hwang and associates (13, 14) have shown that high concentrations of ethanol inhibit thromboxane production by rat and human platelets stimulated with collagen.

Since ethanol inhibited thrombin-induced responses of both untreated and aspirin-treated rabbit platelets (which cannot form thromboxane), ethanol must also inhibit the aspect of thrombin-induced aggregation that occurs independently of TxA_2 and ADP. We have recently found that the inhibitory effect of ethanol on thrombin-induced platelet aggregation and secretion in the absence of thromboxane production is associated with an inhibition of inositol trisphosphate (IP_3) formation (37); IP_3 is a second messenger involved in Ca^{2+}-mobilization in many cells including platelets (38, 39) and it seems likely that inhibition of IP_3 formation and hence Ca^{2+}-mobilization may be responsible for the inhibitory effect of ethanol on thrombin-induced aggregation.

PAF is another aggregating agent of platelets that exerts its action in at least three ways, by stimulating secretion of ADP, production of TxA_2, and a pathway that is independent of these (20, 40). Since ethanol inhibited PAF-induced responses of both untreated and aspirin-treated rabbit platelets, it is apparent that ethanol can also inhibit the PAF-induced pathway of aggregation that is not dependent on the release of ADP or the formation of TxA_2.

The concentration of ethanol used in these studies is physiologically tolerable; blood alcohol levels as high as 5.3 mg/ml have been achieved in experiments with humans (9). Rabbits with blood alcohol levels of approximately 4 mg/ml (22) are unconscious, but they recover without any long-term ill effects.

Thus, we have found that ethanol, at physiologically tolerable concentrations, did not inhibit ADP-induced platelet aggregation, but did inhibit thromboxane production, apparently by inhibiting the activation or activity of the (phospho)lipases that are involved in arachidonic acid mobilization. Ethanol also inhibited the pathways of thrombin- and PAF-induced aggregation that are independent of the formation of TxA_2, possibly through inhibition of IP_3 formation (37). Thus, inhibition by ethanol can be attributed to at least two, or possibly three, effects.

From these studies, however, we can only speculate as to the mechanism(s) by which ethanol exerts its inhibitory effects on platelet aggregation and secretion of granule contents. Tandon and his associates (18) have shown that platelets with increased platelet membrane fluidity brought about by depletion of cholesterol from the membranes, have a reduced number of thrombin receptors and a decreased aggregation response to thrombin. It may be that ethanol, by virtue of its fluidizing effect of membranes (17), alters exposure of specific receptors (41) (e.g. receptors for thrombin and PAF, but not for ADP, epinephrine or thromboxane) on the platelet plasma membrane, thereby inhibiting stimulus-response coupling in platelets. A direct effect of ethanol on the enzymes involved in stimulus-response coupling cannot be ruled out, but seems unlikely because of the failure of ethanol to inhibit the responses to arachidonic acid and to the thromboxane mimetic U46619.

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