Effects of Chronic Administration of Ethanol on Platelets From Rabbits With Diet-Induced Hypercholesterolemia

Unchanged Characteristics and Responses to ADP but Reduction of Enhanced Thrombin-Induced, TxA2-Independent Platelet Responses

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Abstract To investigate the effect on platelet function of the interaction between dietary cholesterol and moderate, chronic doses of ethanol, hypercholesterolemia was induced in rabbits by 8 weeks of administration of a chow diet with added (0.25% wt/wt) cholesterol; during the eighth week, a moderate amount of ethanol (6% in drinking water) was given. Blood alcohol levels were not detectable in ethanol-treated rabbits at the time of exsanguination. Ethanol did not affect plasma cholesterol levels or the cholesterol to phospholipid molar ratio in platelets. Platelet membrane fluidity, which decreased with cholesterol feeding, was not altered further by administration of ethanol. The overall fatty acid composition of platelet phospholipids was not affected by either cholesterol feeding or chronic ethanol intake. Responses of washed platelets stimulated with either ADP or thrombin were studied to determine whether ethanol administration modified platelet functions in hypercholesterolemia. Primary ADP-induced aggregation was not affected by cholesterol feeding or chronic ethanol intake, but thrombin-induced aggregation and secretion of [14C]serotonin from prelabeled platelets, which were enhanced by cholesterol feeding, were diminished by administration of ethanol to hypercholesterolemic rabbits. This reduction in thrombin-induced responses was also observed with aspirin-treated platelets, which cannot form thromboxane A2. Thus, chronic short-term administration of a moderate amount of ethanol inhibited the enhanced responses of platelets from rabbits with diet-induced hypercholesterolemia, via a thrombin-induced, thromboxane A2-independent pathway. (Arterioscler Thromb. 1994;14:1372-1377.)

Key Words • platelet function • dietary cholesterol • hypercholesterolemia • ethanol • aspirin

There is epidemiological evidence that moderate, daily consumption of alcoholic beverages is associated with a reduced risk of the thromboembolic complications of coronary artery disease.1-3 Although ethanol elevates levels of high-density lipoprotein (HDL),1 this effect of HDL appears to account for only half of the protection.4-5 Another proposed mechanism of action of ethanol is its antithrombotic effect,1 because several studies indicate that ethanol may directly or indirectly inhibit platelet functions (see references below).

When given acutely at high but physiologically tolerated concentrations, ethanol reduces experimentally induced thrombosis in rabbits6,7 and inhibits platelet responses ex vivo in blood from humans and experimental animals.8,9 In vitro, ethanol inhibits responses of platelets to certain agonists by inhibiting specific pathways of signal transduction.10-15

The effects of chronic consumption of moderate amounts of alcohol on platelet functions have not been thoroughly investigated. In a population study of the effects of moderate, lifelong consumption of alcoholic beverages on platelet aggregation, Renaud and associates16 found that in subjects who drank moderately, the odds ratios of high platelet aggregation responses to ADP and collagen but not to thrombin were significantly reduced compared with those of nondrinkers. In addition, there appears to be an interaction between alcohol and dietary fat in the prediction of the risk of mortality due to coronary artery disease.17

Hypercholesterolemia is associated with enhanced platelet responses.18-21 Using cholesterol-enriched platelets from rabbits fed a chow diet with added cholesterol, we have recently shown that aggregation in response to ADP is not affected but that platelets become hypersensitive via a thromboxane A2 (TxA2)-dependent pathway and a thrombin-induced, TxA2-independent pathway.22 The extent of the inhibitory effect of ethanol, added acutely in vitro, on thrombin-induced aggregation of cholesterol-enriched platelets is attenuated compared with that on thrombin-stimulated platelets from normocholesterolemic rabbits.23

In the present study, we have focused on the effects of chronic administration of moderate amounts of ethanol to cholesterol-fed rabbits. A variety of platelet characteristics, including those affected by cholesterol feeding and those reported to be altered by chronic exposure of platelets (and other cells) to ethanol, were determined. Additionally, the effects on platelet aggregation induced
Methods

Animals and Diets
Male New Zealand White rabbits (2.7±0.1 kg, N=36) were fed a regular chow diet (150 g/d; B-W Feed & Seed Ltd) for a run-in period of 1 week. This chow diet contained <0.0005% [wt/wt] cholesterol. Then the rabbits were switched to 150 g/d of a chow diet enriched with 0.25% [wt/wt] cholesterol (Sigma grade 99%+, Sigma Chemical Co) for 8 weeks. The cholesterol-enriched diet was prepared as described previously.22 The rabbits were given water ad libitum for 7 weeks, and then 18 rabbits were given 6% (vol/vol) ethanol (700 mL/d) to drink instead of water (700 mL/d) for the eighth, final week of the dietary period. (Six percent ethanol is approximately the maximum concentration of ethanol that rabbits will drink.)

The intake of ethanol by the ethanol-treated, cholesterol-fed rabbits was approximately 16% of total calories and averaged 4.8 mL of absolute ethanol per kilogram of body weight per day.

After the run-in period, the rabbits that served as normo-cholesterolemic controls were fed the regular chow diet (150 g/d) for 8 weeks and were given water ad libitum. The care and use of the experimental animals conformed to the guidelines of the Canadian Council on Animal Care.

Analyses of Plasma Cholesterol and Platelet Characteristics
At the end of the 8-week dietary period, 8.5-mL samples of blood were taken from rabbit ear arteries into syringes containing the anticoagulant acid-citrate-dextrose.24 Plasma cholesterol; whole-blood platelet count; and platelet size, protein, and free cholesterol to phospholipid molar ratio were determined as described.22

Fluorescence polarization was used to measure membrane fluidity of intact platelets labeled with the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (DPH; Molecular Probes Inc)25-27 Steady-state fluorescence polarization measurements were done at 37°C using an Aminco SPF-500 spectrophotometer equipped with polarizers in the excitation and emission beams. Each series of measurements was done using platelets from one animal from each dietary group. Lipid fluidity has been defined as the reciprocal of the lipid structural-order parameter, and lower values of r\textsubscript{s}, the steady-state fluorescence anisotropy, indicate decreased structural-order parameters and increased membrane fluidity.22 Use of an achromatic scrambler eliminated the need for a grating correction factor.25,26

For analysis of phospholipid fatty acids, platelet lipids were extracted with solvents containing 0.01 mg/mL butylated hydroxytoluene.22 Total phospholipids were separated from other lipids by thin-layer chromatography22 and were eluted with methanol, which was then evaporated under a stream of N\textsubscript{2}. Methyl esters of fatty acids from the phospholipids were prepared by transmethylation with 6% H\textsubscript{2}SO\textsubscript{4} in methanol.31 Methyl esters were separated by gas-liquid chromatography.32

Platelet Function Studies
At the end of week 8, the rabbits were anesthetized by intravenous injection of a solution of 6.5% (wt/vol) sodium pentobarbital. Blood was withdrawn via a polyethylene catheter inserted into a carotid artery and was collected into acid-citrate-dextrose.24 Alcohol levels, as measured by an enzymatic assay,33 were undetectable in the blood from cholesterol-fed rabbits given ethanol to drink during the last week of the dietary period; the detection limit of the assay was approximately 10 μmol/L. Suspensions of washed platelets were prepared as described elsewhere.34,35 Suspensions of washed platelets were used instead of platelet-rich plasma, since the opacity of hypercholesterolemic plasma confounds turbidimetric aggregation studies. In the first washing fluid, [\textsuperscript{14}C] serotonin (as 5-hydroxy-\textsuperscript{3}H\textsubscript{3}tryptamine creatinine sulfate, 60 mCi/mmol, American Radio Corp; 0.05 μCi/mL, platelet suspension) was used to label the contents of the amine-storage granules of platelets. The percent uptake of radiolabeled serotonin was similar in all groups of animals. In some studies, 500 μmol/L aspirin (Sigma) was added to the platelets in the first washing solution. The aspirin-treated platelets did not aggregate when stimulated with collagen (1 μg/mL). Suspensions of platelets (0.5×10\textsuperscript{5} platelet/mL in Tyrode's solution containing 2 mmol/L Ca\textsuperscript{2+}, pH 7.35, with added 0.35% albumin and apyrase) were incubated at 37°C for at least 20 minutes before use. Platelets were stimulated by a range of concentrations of ADP (Sigma) or thrombin (topical bovine thrombin, Parke-Davis). Aggregation of rapidly stirred (1100 rpm) 1-mL samples of platelet suspensions at 37°C was recorded with an aggregometer (Payton Associates). The extent of aggregation was expressed as described,28 setting 100% aggregation with the platelet-suspending medium. Three minutes after addition of ADP or 5 minutes after addition of thrombin, supernatant samples were prepared by centrifugation of the stimulated platelet suspensions for 1 minute at 12,000g in an Eppendorf centrifuge. These samples were used to determine secretion of [\textsuperscript{14}C] serotonin from prelabeled platelets29 and formation of TxB\textsubscript{2} (by radioimmunoassay; NEK-007, NEN Canada).

Statistical Analyses
Values are reported as mean±SEM, with the number of experiments indicated. Student's t tests were used to analyze differences between values of plasma cholesterol and characteristics of platelets from cholesterol-fed rabbits and cholesterol-fed rabbits given ethanol. Because there were slight day-to-day variations in the absolute values of fluorescence anisotropy, ie, r\textsubscript{s}, paired t tests were used to analyze differences between r\textsubscript{s} values of DPH-labeled platelets from chow-fed rabbits, cholesterol-fed rabbits, and cholesterol-fed rabbits given ethanol.27,40 ANOVAs were used to analyze differences in the fatty acid composition of phospholipids of platelets from rabbits in the three dietary groups. Nonorthogonal two-way ANOVAs were used to determine differences in responses of platelets from cholesterol-fed rabbits and cholesterol-fed rabbits given ethanol. Differences were deemed to be statistically significant when P<.05.

Results
Administration of 6% ethanol to cholesterol-fed rabbits during the final week of the dietary period did not appear to affect their general health. Those given ethanol drank less fluid than those given water (304±26 mL/d, n=18, versus 404±25 mL/d, n=18) and also consumed less of the cholesterol-enriched diet during the final week of the dietary period (130±6 g/d, n=18, versus 144±2 g/d, n=18). At the end of the dietary period, there was no difference in body weight of the cholesterol-fed rabbits given ethanol (3.81±0.05 kg, n=18) and the cholesterol-fed rabbits given water (3.72±0.06 kg, n=18). Plasma cholesterol levels reached steady-state values by 6 weeks and were not different between the two groups at the end of 8 weeks (see below).

As observed previously,22 plasma cholesterol levels were significantly higher in cholesterol-fed rabbits compared with chow-fed controls (14.15±0.69 mmol/L, n=18, versus 12.0±0.07 mmol/L, n=13). Administration of ethanol to cholesterol-fed rabbits did not affect their elevated plasma cholesterol levels (15.24±1.31 mmol/L, n=18), nor did it affect the extent to which the
ethanol was administered to the cholesterol-fed rabbits olemic, chow-fed controls was not further altered when (data not shown). As shown previously with platelets

**Platelet Responses**

In preliminary studies, it was found that administration of 6% ethanol in the drinking water to normocholesterolemic, chow-fed rabbits for 1 week did not significantly affect responses of platelets to ADP or thrombin (data not shown). As shown previously with platelets from cholesterol-fed rabbits and chow-fed controls, aggregation induced by a range of concentrations of ADP was essentially identical in normocholesterolemic and diet-induced hypercholesterolemia (Fig 1). ADP-stimulated aggregation of platelets from cholesterol-fed rabbits was not altered by administration of ethanol (Fig 1). In accordance with earlier observations, ADP did not induce appreciable secretion of [14C]serotonin or the formation of TXB2.

Hypercholesterolemic platelets stimulated with a range of concentrations of thrombin aggregated more extensively (Fig 2, left) and secreted a higher percentage of [14C]serotonin (Fig 2, right) than did platelets from normocholesterolemic chow-fed rabbits. Administration of ethanol to cholesterol-fed rabbits was associated with a significant diminution of these enhanced platelet responses to low concentrations of thrombin (Fig 2, left and right). The mean extent of aggregation was reduced to within the range of thrombin-stimulated aggregation of platelets from normocholesterolemic rabbits, but secretion was not reduced as far. Ethanol administration also significantly reduced thrombin-induced responses (aggregation, secretion of [14C]serotonin) of aspirin-treated platelets from cholesterol-fed rabbits (Fig 3, left and right).

**Discussion**

Administration of 6% ethanol in the drinking water for 1 week to normocholesterolemic, chow-fed rabbits did not affect plasma cholesterol levels, platelet characteristics, or platelet responses to ADP or thrombin. The plasma cholesterol levels of chow-fed rabbits are approximately 1 to 2 mmol/L. We have previously shown and now confirmed that feeding a cholesterol-enriched diet to rabbits to achieve plasma cholesterol levels of 14 mmol/L results in enrichment of platelets in cholesterol and in enhancement of platelet responses to thrombin but not to ADP. We have now found that administration of 6% ethanol to hypercholesterolemic rabbits for 1 week is associated with a reduction in enhanced thrombin-induced platelet responses. ADP-induced aggregation, which was not affected by cholesterol feeding, was also not altered by administration of ethanol to cholesterol-fed rabbits.
The alcohol intake by the rabbits was approximately 16% of total calories, which is within the range (6% to 18%) of the amount of alcohol consumed by moderate drinkers. The alcohol was administered for 1 week to ensure a complete turnover of platelets in the animals’ circulation. The mean life span of rabbit platelets ranges from 60 to 77 hours, so in 1 week the platelets in the rabbits’ circulation have undergone at least two complete turnovers. The hypercholesterolemic rabbits given ethanol consumed less chow than those given water to drink for the final week of the dietary period. It is possible that the rabbits were compensating for the extra calories obtained by metabolism of ethanol by reducing their chow intake. We did not explore the effects of lower concentrations of ethanol in this study, so we do not know to what concentration ethanol could be reduced before less attenuation of thrombin-induced responses would be apparent.

Neither elevated plasma cholesterol levels of the cholesterol-fed rabbits nor the cholesterol enrichment of their platelets was affected by administration of ethanol. Other platelet characteristics (listed in the Table) were also not changed significantly with ethanol treatment. Heavy, long-term consumption of alcohol by alcoholics can be associated with thrombocytopenia, and there are reports that administration of moderate amounts of alcohol to guinea pigs or mice is associated with small but significant reductions in platelet count. However, in accord with studies in squirrel monkeys, we have also given in the diet, which could have affected the size, ie, measuring the diameter of discoid platelets from light-microscopic video images, was used by Smith et al to show that administration of moderate doses of alcohol to guinea pigs is indeed associated with a slight but significant 13% reduction in platelet size.

In other cell types, chronic exposure to ethanol results in an adaptive response to the continuing presence of ethanol; in some cases, this is associated with decreased fluidity of cell membranes. We were unable to detect such an effect on membrane fluidity by fluorescence polarization using platelets labeled with DPH. Cholesterol feeding was associated with decreased platelet membrane fluidity, as reported by others, but this was not affected further by administration of ethanol. These findings were concordant with measurements of platelet cholesterol content, a determinant of membrane fluidity. Cholesterol is well known to decrease membrane fluidity; the cholesterol content of platelets, as expressed by the free cholesterol to phospholipid molar ratio, was increased with cholesterol feeding but was not further altered by administration of ethanol. The degree of unsaturation of phospholipid acyl chains was not a major affector of membrane fluidity in the present study, since neither cholesterol feeding nor administration of ethanol to cholesterol-fed rabbits was associated with an altered fatty acid composition of total phospholipids or an altered unsaturation index of the fatty acids in platelet phospholipids. Dalal and associates have reported that platelets from cholesterol-fed rabbits with plasma cholesterol levels of 18 to 26 mmol/L contain 90% more arachidonic acid in phosphatidylinositol than do platelets from normocholesterolemic rabbits. In their study, 2% to 4% peanut oil was also given in the diet, which could have affected the
fatty acid composition of phosphatidylsphingositol. However, in the present study, the fatty acid composition of total phospholipids was measured, and therefore, a significant alteration in the fatty acid composition of individual phospholipid species cannot be precluded. On the other hand, administration of ethanol chronically to achieve continuously measurable concentrations of blood ethanol has been well described to result in decreases in arachidonic acid levels in many cell types, including platelets. By comparison, in the present study, ethanol treatment was more moderate (eg, blood alcohol content was not detectable at the time of exsanguination), and this may account for the lack of effect on arachidonic acid levels in platelet phospholipids.

Primary ADP-induced aggregation of platelets from normal subjects and from normocholesterolemic and hypercholesterolemic rabbits is not affected by acute in vitro ethanol, nor does such aggregation appear to be altered by ethanol administration to chow-fed and cholesterol-fed rabbits (present study). It should be noted that cholesterol feeding of rabbits does not alter platelet aggregation stimulated by ADP. However, it may be that prolonged treatment with ethanol does lead to decreased platelet aggregation to ADP, since Littleton and associates found that ADP-induced platelet aggregation in rats given ethanol by inhalation to produce blood alcohol levels of about 50 mmol/L was not affected after treatment for 5 to 7 days but was markedly impaired after treatment for 30 days. Primary ADP-induced aggregation of platelets from alcohols shortly after withdrawal of alcohol (when blood alcohol levels are negligible) is significantly reduced even when blood alcohol levels are undetectable.

In keeping with the findings of Renaud's group, who gave ethanol chronically to rats and rabbits fed diets high in saturated fats, we observed that ethanol administration reduced the enhanced thrombin-induced responses of platelets from cholesterol-fed rabbits. Because there was no effect of ethanol administration on ADP-induced aggregation of platelets from cholesterol-fed rabbits, the reduction in enhanced thrombin-induced aggregation and secretion of granule contents may be due to inhibition of TXA2-dependent responses and/or thrombin-induced, TXA2-independent responses. An effect of ethanol administration on the latter is more likely, because thromboxane formation is very slight by platelets that have been stimulated with thrombin at low concentrations (up to 0.01 U/mL), at which levels the effect of ethanol is most evident. To investigate the effect of ethanol on thrombin-dependent, TXA2-independent responses further, platelets were treated with aspirin to block formation of TXA2. We had previously found that thrombin-induced aggregation of aspirin-treated platelets is enhanced with cholesterol feeding; in the present study, enhanced aggregation and secretion of granule contents by cholesterol-enriched platelets were reduced by ethanol administration. This inhibition may be mediated via decreased thrombin binding (decreased binding affinity and/or decreased number of receptors) to membrane receptors and/or impairment of thrombin-specific signaling pathways that are not shared with ADP.

It appears that acute and chronic ethanol treatments have similar effects on platelets from hypercholesterolemic rabbits: ADP-induced aggregation is not affected, whereas the enhanced thrombin-induced responses, including TXA2-independent responses, are reduced. Although the acute effects of ethanol were manifested only at concentrations of 2 mg/mL ethanol or higher, chronic effects occurred in the absence of detectable levels of alcohol in the blood, at least at the time of exsanguination and in the absence of ethanol during platelet function testing, since washed platelets were used. Thus, chronic exposure to low levels of ethanol in vivo is associated with alterations in platelets that result in inhibition of cholesterol-enhanced platelet responses. These alterations do not involve any of the platelet characteristics measured in the present study. It may well be that the alterations occur at the level of the megakaryocyte, since there is evidence for effects (eg, reductions in megakaryocyte number and size, protein synthesis in maturing megakaryocytes, and deformability of mature megakaryocytes) of moderate ethanol intake on megakaryocytes in guinea pigs and mice. Several studies have indicated that ethanol has a greater inhibitory effect on responses of platelets enriched in saturated versus unsaturated fats. We have shown in the present study that administration of moderate amounts of ethanol to hypercholesterolemic rabbits inhibits the enhanced thrombin-induced responses of the cholesterol-enriched platelets. Our results thus support the possibility that an inhibitory effect of ethanol on platelet responses to thrombin plays a role in the protection offered by moderate ethanol consumption against the thromboembolic complications of coronary artery disease.

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