Enhanced Collagen-Induced Responses of Platelets From Rabbits With Diet-Induced Hypercholesterolemia Are Due to Increased Sensitivity to TxA₂
Response Inhibition by Chronic Ethanol Administration in Hypercholesterolemia Is Due to Reduced TxA₂ Formation

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Abstract
The effects of dietary cholesterol and chronic administration of moderate amounts of ethanol on collagen-induced platelet responses were investigated. Three groups of rabbits were fed the following diets for 8 weeks: a normal chow diet, a cholesterol-enriched (0.25% wt/wt) chow diet, and a cholesterol-enriched chow diet plus 6% ethanol in the drinking water for the final week of the dietary period. Cholesterol feeding enhanced collagen-induced responses—aggregation, secretion of [³⁵S] serotonin from prelabeled platelets, and thromboxane formation—of suspensions of washed platelets, and chronic ethanol treatment significantly reduced these enhanced responses. These effects are mediated by thromboxane A₂ (TxA₂) rather than ADP. Experiments with collagen-stimulated platelets in which feedback amplification of TxA₂ was blocked with the prostaglandin H₂/TxA₂ receptor blocker BM 13.177 and experiments with aspirin-treated platelets stimulated with the stable TxA₂ mimetic U46619 showed that cholesterol feeding enhanced platelet sensitivity to TxA₂ rather than formation of TxA₂ by platelets that had interacted with collagen. Without BM 13.177 or aspirin, TxA₂ increased the amount of TxA₂ formed by feedback amplification. In contrast, decreased responsiveness to collagen by platelets from cholesterol-fed rabbits given ethanol was due to inhibition of TxA₂ formation rather than reduced sensitivity to TxA₂. Platelets from cholesterol-fed rabbits given ethanol did not develop tolerance to the acute inhibitory effects of ethanol. Our results indicate that administration of moderate amounts of ethanol to cholesterol-fed rabbits inhibits enhanced collagen-induced responses of platelets by a TxA₂-dependent pathway that involves reduction of TxA₂ formation rather than reduction of platelet responses to TxA₂. (Arterioscler Thromb. 1994;14:1379-1385.)

Key Words • platelet function • dietary cholesterol • ethanol • collagen • thromboxane

Reduction in the incidence of thromboembolic complications of coronary artery disease by moderate consumption of ethanol¹⁻³ may be partially attributable to the inhibition of platelet functions.¹ Hypercholesterolemia is a risk factor for these complications and is associated with enhanced platelet responses,⁴⁻⁷ including thrombin-induced collagen-A₂ (TxA₂)-independent aggregation.⁸ In the preceding article, we have shown that administration of ethanol to rabbits with diet-induced hypercholesterolemia diminishes the enhanced responses of platelets to thrombin.⁹ However, ADP-induced aggregation is not affected by cholesterol feeding or ethanol administration to hypercholesterolemic rabbits.⁸⁻⁹

Other studies from our laboratory have also indicated that platelets from rabbits with diet-induced hypercholesterolemia are hypersensitive to collagen.⁸ Platelet aggregation in response to collagen is mediated by synergism between TxA₂ and released ADP.¹⁰,¹¹ On adherence to collagen, platelets are stimulated to secrete the contents of their storage granules, including ADP from amine-storage granules, and to activate phospholipase A₂, which leads to TxA₂ formation from the arachidonic acid that is hydrolyzed from platelet membrane phospholipids. Removal of released ADP by a scavenging system partially inhibits collagen-induced aggregation, as does inhibition of TxA₂ formation by nonsteroidal anti-inflammatory drugs. In the presence of both an ADP-scavenging system and an inhibitor of TxA₂ formation, aggregation in response to all but very high concentrations of collagen is essentially abolished. Not only do platelets from cholesterol-fed rabbits form more TxA₂ when they aggregate in response to collagen, but also they aggregate more extensively when stimulated by the stable TxA₂ mimetic U46619.⁸ Whether they form more TxA₂ when they initially interact with collagen was investigated in the present study, which employed a prostaglandin H₂ (PGH₂)/TxA₂ receptor blocker to prevent feedback amplification by newly formed TxA₂.

In the present study, we have also investigated whether ethanol consumption can affect enhanced TxA₂-dependent, collagen-induced responses in platelets from rabbits with diet-induced hypercholesterolemia. The effects of chronic ethanol administration on

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reactions associated with platelet responses to collagen—TxA2 formation by platelets that interact with collagen and stimulation of platelet aggregation by the TxA2 mimetic U46619—were determined to gain a better understanding of the mechanisms by which exposure of hypercholesterolemic platelets to ethanol in vivo can affect platelet responses. Chronic exposure to ethanol has been reported to cause adaptive cellular changes that involve resistance to the effects of acute ethanol, so we determined whether the acute inhibitory effect of ethanol in vitro on collagen-induced aggregation was affected by chronic ethanol administration to hypercholesterolemic rabbits.

## Methods

### Animals and Diets

Male New Zealand White rabbits (2.7±0.1 kg, N=50) were fed a regular chow diet (150 g/d, B-W Feed & Seed Ltd) for a 1-week run-in period. This chow diet contained <0.005% (wt/wt) cholesterol. Ten of the rabbits were maintained on the regular chow diet for 8 weeks while the other 40 rabbits were switched to an 8-week diet of 150 g/d of chow enriched with 0.25% (wt/wt) cholesterol (Sigma grade 99%+, Sigma Chemical Co). The cholesterol-enriched diet was prepared as described. The rabbits received water ad libitum for 7 weeks, and then for the eighth, final week of the dietary period, the levels in cholesterol-fed rabbits (n=20) were 1.19±0.09 mmol/L, whereas the levels in cholesterol-fed rabbits given ethanol (n=20; 14.96±1.28 mmol/L) were greater and not significantly different from those of cholesterol-fed rabbits given ethanol. P<.05 was deemed statistically significant.

### Platelet Function Studies

Suspensions of washed platelets were prepared by previously described methods. Suspensions of platelets (0.5 x 10^8/mL in Tyrode's solution containing 2 mmol/L CaCl2, pH 7.35, with added 0.35% albumin and apyrase) with their amines-storage granules prelabeled with [14C]serotonin were incubated at 37°C for at least 20 minutes before use. Platelets were stimulated by a range of concentrations of acid-soluble collagen prepared from bovine tendon collagen (Sigma), and platelet responses were assessed in the absence and presence of the PGH2/TxA2 receptor blocker BM 13.177 (4-[2-(benzene-sulfonamido)-ethyl]-phenoxyacetic acid, 100 μmol/L; a generous gift from Dr K. Stegmeier, Boehringer Mannheim), which was added 30 seconds before the aggregating agent. In some experiments with collagen (2.5 μg/mL) and BM 13.177, creatine phosphate/creatine phosphokinase (5 mmol/L:10 U/mL, Sigma) which converts the released ADP to ATP, was added 15 seconds before the agonist. Aspirin-treated platelets were stimulated with a range of concentrations of the stable TxA2 mimetic U46619 (9,11-dideoxy-11a,9a-epoxymethanoprostaglandin F1α, Sigma). In studies on the effects of in vitro ethanol addition on collagen-induced platelet responses, ethanol (final concentration, 4 mg/mL) or saline was added 2 minutes before the aggregating agent. This concentration of ethanol is physiologically tolerated, and previous experiments have shown that when platelet aggregation is inhibited by ethanol, inhibition is consistent at 4 mg/mL.

### Statistical Analyses

Values are reported as mean±SEM, with the number of experiments indicated. Nonorthogonal two-way ANOVAs were used to determine differences in the responses of platelets from normocholesterolemic versus cholesterol-fed rabbits or cholesterol-fed rabbits versus cholesterol-fed rabbits given ethanol. P<.05 was deemed statistically significant.

### Results

Neither cholesterol feeding nor administration of 6% ethanol to the cholesterol-fed rabbits during the eighth, final week of the dietary period appeared to affect weight gain or the general health of the rabbits. At the end of the dietary period, plasma cholesterol levels of chow-fed rabbits (n=10) were 1.9±0.09 mmol/L, whereas the levels in cholesterol-fed rabbits given water (n=20; 13.97±0.66 mmol/L) were greater and not significantly different from those of cholesterol-fed rabbits given ethanol (n=20; 14.96±1.28 mmol/L).

### Responses of Platelets in Hypercholesterolemia and Normocholesterolemia

Platelet responses (aggregation, secretion of [14C]serotonin, and formation of TxB2) induced by a range of concentrations of collagen were enhanced in hypercholesterolemia (Fig 1A through 1C). Collagen-induced responses of platelets from both normocholesterolemic and hypercholesterolemic rabbits were lowered in the presence of the PGI2/TxA2 receptor blocker BM 13.177 (Figs 1A through 1C and 2A through 2C; note difference in the scales for collagen concentration). However, there were no longer any significant differences in extent of aggregation, release of [14C]serotonin, and formation of TxB2 by collagen-stimulated platelets from the two groups of animals (Fig 2A through 2C).
Responses of Platelets From Ethanol-Treated Normocholesterolemic and Hypercholesterolemic Rabbits

In preliminary studies, we found that administration of 6% ethanol in the drinking water to normocholesterolemic, chow-fed rabbits for 1 week did not significantly affect platelet responses to collagen (data not shown). However, administration of ethanol to cholesterol-fed rabbits was associated with a significant reduction of enhanced aggregation, secretion of [14C]serotonin, and TxB2 formation by platelets that had been stimulated by a range of concentrations of collagen (Fig 1A through 1C). Aggregation was reduced to within the range of that observed for normocholesterolemic platelets, whereas secretion and thromboxane formation remained somewhat higher.

Ethanol administration to hypercholesterolemic rabbits had no apparent effect on the aggregation of aspirin-treated platelets induced by a range of concentrations of U46619 (Fig 3). In the presence of BM 13.177, platelet responses—aggregation, secretion of [14C]serotonin, and TxB2 formation—induced by a range of concentrations of collagen were significantly lowered by ethanol administration to cholesterol-fed rabbits (Fig 4A through 4C). In two experiments, platelets were stimulated with 2.5 µg/mL collagen in the presence of 100 µmol/L BM 13.177 and added creatine phosphate/creatine phosphokinase (5 mmol/L:10 U/mL) to convert the released ADP to ATP. There was a large reduction in platelet aggregation and secretion of [14C]serotonin by platelets from hypercholesterolemic rabbits given either water or ethanol, but platelet responses were lower in hypercholesterolemic rabbits given ethanol (Fig 5).

As shown previously, addition of ethanol (4 mg/mL) in vitro inhibited collagen-induced aggregation and secretion (Fig 6A and 6B) by platelets from cholesterol-fed rabbits; this was also observed with platelets from hypercholesterolemic rabbits given ethanol (Fig 6A and 6B). To determine whether the extent of inhibition by ethanol added in vitro was different between the two groups of rabbits, inhibition was expressed as a percentage at equal extents of aggregation. Inhibition by in vitro ethanol of collagen-induced aggregation expressed in this way showed no difference between platelets from cholesterol-fed rabbits and those from cholesterol-fed rabbits given ethanol in their drinking water (Fig 6C).

Discussion

We found previously that platelets from cholesterol-fed rabbits were enriched in cholesterol, as shown by an increased cholesterol to phospholipid molar ratio, compared with platelets from chow-fed rabbits. Furthermore, administration of ethanol to cholesterol-fed rabbits did not alter the extent to which platelets were enriched in cholesterol.

As shown previously, cholesterol feeding of rabbits results in enhancement of collagen-induced platelet aggregation, secretion of [14C]serotonin from prelabeled amine-storage granules, and formation of thromboxane. We now show that these enhanced collagen-induced responses are diminished on the administration of 6% ethanol in the drinking water for 1 week to cholesterol-fed rabbits.

Aggregation of platelets exposed to collagen is almost entirely dependent on the ADP that is released from platelet storage granules and the TxA2 that is formed when platelets adhere to collagen. We have recently found that chronic administration of ethanol to hypercholesterolemic rabbits does not affect ADP-induced aggregation of platelets, which is also not altered by cholesterol feeding. The observed reduction of en-
Enhanced collagen-induced responses by chronic ethanol intake must thus be mediated by TxA₂. These effects of chronic ethanol administration on collagen-induced platelet responses are not evident in chow-fed rabbits with plasma cholesterol levels of 1 to 2 mmol/L.

To gain an understanding of the mechanisms involved in the inhibition by chronic ethanol administration of collagen-induced responses of platelets from hypercholesterolemic rabbits, it was necessary to establish the ways in which cholesterol enrichment of platelets results in hypersensitivity (including increased thromboxane formation) to collagen. We have previously found that cholesterol feeding does not affect primary ADP-induced aggregation, so the hypersensitivity to collagen must be mediated via TxA₂ rather than ADP. To determine the effect of platelet cholesterol enrichment on thromboxane formation by platelets that interacted with collagen, we investigated collagen-induced responses—aggregation, secretion of granule contents, and thromboxane formation—of platelets in the presence of BM 13.177. This compound, a PGH₂/TxA₂ receptor blocker, prevents any effects on platelets of the TxA₂ that forms when platelets interact with collagen. Collagen-induced responses of platelets from normocholesterolemic and hypercholesterolemic rabbits were greatly reduced on addition of BM 13.177 but were not significantly different from each other with respect to the amount of TxB₂ formed, thereby indicating that cholesterol feeding does not affect thromboxane formation by platelets that have reacted with collagen, nor does such feeding affect the secretion response or aggregation due to the ADP released from platelets that have interacted with collagen. That thromboxane formation was unaffected was an unexpected finding, since previous studies showed enhanced TxA₂ formation in cholesterol-enriched platelets stimulated with collagen or thrombin. However, these earlier studies did not examine TxA₂ mobilization in collagen-stimulated platelets in the presence of a PGH₂/TxA₂ receptor blocker to prevent feedback amplification by TxA₂.

We have previously shown that aspirin-treated platelets from rabbits with diet-induced hypercholesterolemia have an enhanced aggregation response to the stable TxA₂ mimetic U46619. Others have found that human platelets, enriched in cholesterol in vitro, are hypersensitive to stable TxA₂ analogues compared with control platelets and that the hypersensitivity is mediated by an increased number of binding sites for the agonists rather than an increased affinity.

Because thromboxane formation by collagen-stimulated rabbit platelets (in the absence of BM 13.177) is enhanced in diet-induced hypercholesterolemia (Reference 8 and the present study), the TxA₂ formed on the initial interaction of platelets with collagen must then increase the amount of thromboxane formed by feedback amplification. Our findings indicate that thromboxane formation by platelets that interact directly with collagen is not affected by cholesterol enrichment of the platelets, whereas aggregation induced by the TxA₂ that is formed is enhanced by cholesterol enrichment. The inhibitory effect of ethanol observed in the present study did not occur by a reversal of the way in which cholesterol-enriched platelets manifest an en-
enhanced response to collagen (ie, enhanced response to TxA₂), because responsiveness to TxA₂, as shown by the use of aspirin-treated platelets and U46619, was not affected by chronic ethanol intake. Rather, the amount of thromboxane formed by collagen-stimulated, cholesterol-enriched platelets in the presence of BM 13.177 was reduced by ethanol. In addition, secretion of granule contents as well as the aggregation response was diminished; the latter is due in large part to the ADP that is released from amine-storage granules. When the released ADP was converted to ATP by creatine phosphate/creatine phosphokinase, aggregation and secretion of granule contents stimulated by a high concentration of collagen were reduced in cholesterol-enriched platelets and were completely inhibited in platelets from cholesterol-fed rabbits given ethanol. The residual aggregation response by cholesterol-enriched platelets was likely due to serotonin secreted from the stimulated platelets.¹¹

It has been shown that cells undergo an adaptive response to chronic exposure to ethanol; development of this so-called tolerance is associated with a resistance to the effects of acute ethanol and involves changes in the anionic phospholipids of cell membranes.¹² ³⁸ When ethanol is added acutely at high but physiologically tolerated concentrations, it inhibits
collagen-induced responses of platelets from human subjects and experimental animals (including normocholesterolemic and hypercholesterolemic rabbits). A-C. Acutely added ethanol in vitro reduces thromboxane formation by stimulated platelets, likely by inhibition of phospholipase A2. Recently Stubbs and Rubin reported a direct inhibitory effect of ethanol on cytosolic phospholipase A2 in rat platelets. In a previous study, we found that the inhibitory effect of acutely added ethanol is the same on collagen-induced responses of platelets from normocholesterolemic and hypercholesterolemic rabbits. In the present study, we have shown that ethanol added in vitro also inhibits collagen-induced responses of platelets from cholesterol-fed rabbits given ethanol. However, the extent of inhibition of collagen-induced aggregation was not different between platelets from hypercholesterolemic rabbits given water or ethanol, indicating a lack of development of functional tolerance by hypercholesterolemic rabbit platelets exposed to chronic ethanol in vivo for 7 days.

In the accompanying article, we have reported that aggregation and secretion induced by low concentrations of thrombin (0.0075 to 0.02 U/mL), which were enhanced by cholesterol feeding, were diminished by administration of ethanol to hypercholesterolemic rabbits. Although thrombin does stimulate TXA2 production (which, in view of our findings with collagen, would be diminished on ethanol administration), the amount of TXA2 formed by platelets stimulated with low concentrations of thrombin is slight. These are the concentrations of thrombin with which the effect of ethanol is most evident. Because the reduced responses to thrombin due to ethanol were also observed with aspirin-treated platelets, we concluded that the main effect of ethanol administration on thrombin-induced aggregation and secretion by platelets from hypercholesterolemic rabbits was part of a TXA2-independent pathway. This observation contrasts sharply with the effect of ethanol on collagen-induced responses described in the present study.

The chronic inhibitory effects of ethanol that we observed are independent of the acute effects of ethanol because there was no detectable alcohol in the blood of rabbits at the time of exsanguination and no ethanol in the platelet suspensions at the time of function testing. The alterations that occur to platelets as a result of chronic exposure to low levels of alcohol in vivo may occur at the level of the megakaryocyte and do not involve changes in the cholesterol content of the platelets, platelet membrane fluidity, or fatty acid composition of platelet phospholipids. However, it is clear that administration of moderate amounts of ethanol to cholesterol-fed rabbits inhibits enhanced collagen-induced responses of cholesterol-enriched platelets by a TXA2-dependent pathway that involves reduction of TXA2 formation rather than reduction of platelet responses to TXA2.

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