Relationship between Fatty-Acid Composition of Platelets and Platelet Aggregation in Rat and Man

RELATION TO THROMBOSIS

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ABSTRACT

Male rats fed a diet rich in butter or stearic acid presented a marked predisposition to endotoxin-initiated thrombosis. This was preceded by hypercholesterolemia, hypercoagulability, and an increased susceptibility of platelets to thrombin-induced aggregation. In contrast to this, feeding of corn oil or linoleic or oleic acids did not result in such marked changes in the blood or in severe thrombosis.

Gas-liquid chromatographic analysis of total lipids of platelet and plasma indicated that the thrombogenic fat or fatty acid resulted in a highly significant increase, mostly in the platelets, of the ratio of saturated + monounsaturated to polyunsaturated fatty acids (S + M)/P.

In patients who had suffered a myocardial infarction as compared with men without risk factors for coronary heart disease, an increase in the (S + M)/P in plasma and platelets was also observed.

Among 17 active middle-aged businessmen, the five who presented signs of coronary heart disease also showed the highest susceptibility to thrombin-induced aggregation, but not to ADP or collagen. In these 17 subjects, the results of the thrombin aggregation could be correlated with the platelet (S + M)/P, but not with the plasma or platelet cholesterol.

In both the rat and man, a high thrombotic tendency may be associated with a platelet hypersusceptibility to thrombin which, in turn, is related to changes in fatty-acid composition.

ADDITIONAL KEY WORDS

hyperlipemia dietary fat

Earlier studies in rats (1, 2) revealed that the thrombogenic factor in the dietary fats fed was the long-chain, saturated fatty acids. Such fats as cocoa butter and butter, which are rich in these acids, were the most thrombogenic of the commonly used fats (2). In more recent experiments, it was shown that these thrombogenic fats or fatty acids predispose to thrombosis, partly by accelerating coagulation (3) but mostly by increasing the platelet susceptibility to thrombin-induced aggregation (4, 5). The purpose of the present study was to determine in man whether an increased susceptibility of platelets to aggregation could be detected in coronary heart disease, a disease in which thrombosis and especially blood platelets could play an essential role. We also wished to verify whether fatty acids in the platelets could be implicated in the response of these cells to aggregation. Coronary heart disease is related to the ingestion of animal fats (6, 7), among which butter and dairy products account for a large part.

We compared the changes found in the
fatty-acid composition of platelet and plasma of patients with myocardial infarction with those observed in rats fed highly thrombogenic fats or fatty acids. In addition, the fatty-acid composition of the plasma and platelets was also determined in a group of middle-aged men and related to the susceptibility of their platelets to aggregation, as in the rat. Results obtained are reported here.

**Materials and Methods**

**Patients**

The subjects of experiment 3 were 10 hospitalized patients with a mean age of 43 years, who had had a myocardial infarction 10 days earlier as confirmed by ECG and enzyme changes. The 12 controls of this experiment, considered as normals, were physicians or hospital employees with a mean age of 39 years, who presented no risk factors for coronary disease, such as hypercholesterolemia, diabetes, hypertension, obesity, or familial history of the disease.

In experiment 4, the determinations were performed on a group of 17 businessmen with a mean age of 45 years, who had come to the hospital for a medical examination before undertaking a program of physical exercise.

**Animals**

A total of 215 male Holtzman rats with an initial body weight of 160 to 180 g were used. The rats were housed three per cage in a constant-temperature environment and given tap water and food ad libitum. Group 1 of experiment 1 was fed laboratory chow (Purina laboratory chow, Ralston Purina Co. of Canada) exclusively. All the other animals received the following diet, considered as normals, were physicians or hospital employees with a mean age of 39 years, who presented no risk factors for coronary disease, such as hypercholesterolemia, diabetes, hypertension, obesity, or familial history of the disease.

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**Fatty Acid Composition**

Fatty acid composition was determined in plasma and platelets from patients with myocardial infarction and controls. The fatty acid composition of the plasma and platelets was also determined in a group of middle-aged men and related to the susceptibility of their platelets to aggregation, as in the rat. Results obtained are reported here.

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**Platelet Aggregation**

Platelet aggregation studies were performed on platelet-rich plasma. The process was the same for platelet studies, whether for platelet aggregation in the rat and to 500,000 for humans by suitable dilution of platelet-rich plasma, as described elsewhere (10). To determine the platelet survival of the individual samples in the human subjects. The platelet concentration was determined on a Coulter Counter and decreased to 550,000/mm³ with plasma stored at this temperature, highly reproducible results can be obtained for at least 2 hours.

Platelet aggregation at 37°C was studied by a turbidimetric device (Bryton Manufacturing, Rexdale, Ontario, Canada) utilizing 0.5 ml of plasma for each determination. The aggregating
agents were added in a 0.1 ml volume of complete Tyrode's solution, pH 7.4. Thrombin (Thrombin Topical, bovine origin, Parke Davis Co.) (experiment 1, 2, 4); adenosine diphosphate disodium salt (ADP) ( Sigma Chemical Co., St. Louis, Missouri); and collagen (Sigma Chemical Co.) (experiment 4) were diluted in Tyrode's solution to the required concentrations. A standard suspension of collagen that can be kept frozen indefinitely with highly reproducible results was prepared as follows: 500 mg of collagen was homogenized in 50 ml of complete Tyrode's solution (pH 7.4) at 4°C for 6 minutes in a Virtis homogenizer (45,000 rpm). The suspension was centrifuged for 4 minutes at 100 g to remove the largest particles. The supernatant suspension with the finest sedimented particles (approximately 1 mm of the upper layer) was utilized as the standard suspension to be diluted as required.

The extent of platelet aggregation in experiments 1 and 2 was calculated by triangulating the area under the curves as already described (5). The curves in experiment 2 did not show a peak, as the test was performed on washed platelets; the base of the triangle was therefore arbitrarily determined at 5 cm from the origin.

In human studies, even on platelet-rich plasma, peaks were not observed in very high tracings (Fig. 7) obtained with thrombin or ADP as the aggregating agent, and were never observed in collagen-induced aggregation. When a peak was present, the maximum height (in centimeters) of collagen-induced aggregation. When a peak was present, the maximum height (in centimeters) of the tracing was taken to express the extent of platelet aggregation; in the absence of a peak, the height at 5 cm from the origin was used for that purpose.

**BLOOD PLATELET SUSPENSION**

Platelet suspensions were prepared for determination of cholesterol (experiments 3 and 4) and fatty acids (experiment 1-4), and for the platelet-aggregation study in experiment 2. In experiment 2, most of the plasma of animals fed oleic acid (group 3) were milky and the results of platelet aggregation in platelet-rich plasma were not reliable. For this reason, platelet-aggregation studies in that experiment were performed on washed platelets.

Only minor modifications were made in the technique previously reported (5) for platelet suspension. Unlike the studies on platelet-rich plasma, platelets were kept at room temperature during the entire process of collection and washing, and only a few minutes before starting aggregation was the platelet suspension stored at 7 to 8°C.

For platelet-aggregation studies (experiment 2), platelets of three rats were pooled and the two washings were performed with incomplete Tyrode's solution containing no Ca or Mg (pH 6.6). The platelets were resuspended in complete Tyrode's solution (pH 7.4) with added gelatin (2.5 g/liter), as described by others (11). The platelet count was then adjusted to 550,000/mm.³. For determination of cholesterol and fatty acids as well as the final suspension was done with incomplete Tyrode's solution (pH 6.6) containing Na₂-EDTA (0.4 g/liter). The total platelet count for each sample was 1 X 10⁹ platelets (in 1 ml of Tyrode's solution) for cholesterol and 1.3 X 10⁹ (in 4 ml of Tyrode's solution) for fatty-acid analysis.

**GAS CHROMATOGRAPHY**

Fatty-acid analyses in platelets and plasma were performed on individual samples by gas-liquid chromatography on a Micromerk 220 instrument with flame ionization detector. The peaks were calculated with a disc integrator. The 6-foot columns contained 15% ethylene glycol succinate on 60-80 mesh chromosorb W (Chromatographic Specialties Ltd., Brockville, Ontario) maintained isothermally at 180°C. For methylation of the fatty acids, different techniques were utilized in preliminary experiments and conducted to comparable results in both plasma and platelets. The entire technique was carried out under nitrogen atmosphere. In the present study, saponification was performed by adding 4 ml of 2.5% NaOH-methanol to 1 ml of plasma or 4 ml of the platelet suspension, and heating for 1 hour at 75°C. After overnight refrigeration, the nonmethylable material was extracted twice with 20 ml of petroleum ether and the supernatant fluid discarded after shaking by hand for 2 minutes. After cooling at 4°C and acidification to pH 0 to 3, the fatty acids were extracted three times with 6 ml petroleum ether and dried down with nitrogen. The fatty acids were methylated by heating for 2 minutes in a boiling-water bath with 3 ml of boron trifluoride methanol (Applied Science Laboratories Inc., State College, Pa.), according to the technique of Metcalfe and Schmitz (12). The resulting esters were extracted with 4 ml of water and 6 ml of petroleum ether.

**CHOLESTEROL ANALYSIS**

Total cholesterol in plasma and platelets was determined on individual samples by a manual adaptation of the Technicon Auto Analyzer automated ferric chloride technique (2). In the platelets, this was performed after extracting the cholesterol with isopropanol (4 ml) for 1 minute, under strong agitation.

**THROMBOSIS IN RAT**

To induce formation of thrombosis in the
hepatic veins, a S. typhosa (0901, Boivin type) lipopolysaccharide (0.3 mg/kg, experiment 1; 0.6 mg/kg, experiment 2) in saline was injected intravenously after removing blood and through the same needle, as described previously (8, 9). Autopsy was performed in all animals, the survivors being killed with chloroform 24 hours after the injection. At autopsy, there were red hepatic infarcts due to occlusion of hepatic veins by large thrombi (8). A quantitative evaluation of the thrombotic phenomenon could, therefore, be obtained in such animals by weighing the liver before and after removing the infarcted parts with scissors. The severity of thrombosis could then be expressed as the percent in weight of infarcted liver, as previously reported (2).

Results

Experiment 1

Feeding either laboratory chow (group 1) or the potentially thrombogenic diet containing corn oil (group 2) or butter (group 3) for 10 weeks to groups of 21 rats induced markedly different results in the various groups. Plasma cholesterol was the smallest in group 1 and the highest in group 3. In group 2, the cholesterol response was intermediate as was the response of the plasma-clotting time, which was roughly inversely related to the cholesterol values in the three groups. The findings for thrombin-induced aggregation were similar, group for group, to those noted for plasma cholesterol. Only in the group fed butter could severe lesions of thrombosis be noted after the endotoxin injection.

Figure 2 shows the results of the gas-liquid chromatographic analysis of the fatty-acid composition of the total lipids of plasma and platelets of three groups of rats (14 in group 1 and 18 in each of the other two groups) treated similarly to those of the above experiment (Fig. 1). Since it was necessary to remove 4 ml of blood to determine the fatty-acid composition individually, these rats were not used for other analyses or studies of thrombosis.

In the plasma, variations were noted between group 1 and 2, but there was no significant difference between these two groups in the ratio of saturated + monounsaturated to polyunsaturated fatty acids (S + M)/P. In the platelets, the fatty-acid composition of group 2 was only slightly different from that of group 1, as was the (S + M)/P. However, group 3 showed drastic differences in both the plasma and platelets as compared with the other two groups. These differences

Fatty-acid pattern of the total lipids of plasma and platelets determined by gas-liquid chromatography of rats fed the same diets as those used in the experiment reported in Figure 1. Number of determinations: group 1, 14; group 2, 18; group 3, 18. Values are means ± SE as shown. 

\[ \frac{S + M}{P} \] = ratio of saturated + monounsaturated to polyunsaturated fatty acids.

Influence of the type of dietary fatty acid on the production of endotoxin-initiated thrombosis in rats. As in Figure 1, before removing blood and S. typhosa lipopolysaccharide (0.8 mg/kg) injection, the animals were fed the following diets for 6 weeks: group 1, linoleic acid; group 2, oleic acid; and group 3, stearic acid (5%) added to the potentially thrombogenic base as in Figure 1 but also containing butter (35% + water 1%). In the aggregation test performed on platelet suspension the final thrombin dilution was 0.04 U. Number of determinations = 3 per group. Values are means ± SE. PCT = plasma clotting time.

Consisted mostly in an increase in the monounsaturated (palmitoleic and oleic acids) and a decrease in the polyunsaturated fatty acids (linoleic and arachidonic acids). As a result, the (S + M)/P was markedly higher in group 3 than in groups 1 and 2.
Fatty acid pattern of the total lipids of plasma and platelet of rats fed the same diets as those used in experiment reported in Figure 3.

Experiment 2

Addition to a butter-rich thrombogenic diet of linoleic (group 1), oleic (group 2), or stearic acid (group 3), with 24 rats per group, induced noticeably different results, depending on the fatty acid used (Fig. 3). Plasma cholesterol was the lowest in rats fed linoleic acid, while no significant difference was observed between groups 2 and 3 fed oleic and stearic acid, respectively. The plasma clotting time and the thrombin-induced aggregation, on the other hand, presented quite...
PLATELET FATTY ACIDS AND AGGREGATION

Comparison in platelets between the change in fatty-acid pattern in infarcted patients and in rats (fed stearic acid) highly susceptible to thrombosis.

Another picture. Here, the values in groups 1 and 2 did not differ significantly, but those in group 3 were markedly lower (plasma clotting time) or higher (thrombin aggregation). Severe thrombotic lesions were recorded only in group 3.

In Figure 4 can be seen the results of individual gas-liquid chromatographic analyses of the total lipids of platelet and plasma in 10 rats per group fed the butter-rich diet containing either linoleic (group 1), oleic (group 2), or stearic acid (group 3). As in experiment 1, these rats were not utilized for any other study, since 4 ml of blood was taken. Depending on the fatty acid used, various differences were noted in the plasma and platelets. In the plasma, the lowest (S + M)/P was observed in the rats fed linoleic acid, but it did not differ significantly in the groups fed oleic acid or stearic acid. In contrast to this, the ratio in the platelets was significantly higher in group 3 than in group 2, and in group 2 than in group 1. Group 2, fed oleic acid, did not show a higher percent of oleic acid in the platelets than group 3, fed stearic acid. However, group 3 showed a higher percent of stearic acid in the platelets and a lower amount of linoleic and arachidonic acid.

Experiment 3

The fatty-acid pattern in the 10 men with infarcts as compared with that of the 12 controls indicated a somewhat similar trend in both plasma and platelets. In the men with infarcts, the percent of saturated and monounsaturated fatty acids was higher than in the normal men, at the expense of the polyunsaturated fatty acids. As a result, elevation of (S + M)/P was highly significant in the former group. In contrast to this, at the time of the determination, there was no difference in the plasma cholesterol between the two groups, and no significant difference in the platelet cholesterol (Fig. 5). For purposes of comparison, Figure 6 shows the results obtained with gas-liquid chromatography in platelets of men with infarcts and normal men, as reported above, and those obtained in experiment 2 in rats fed stearic or oleic acid. Except that there is much less arachidonic and much more palmitic acid in rat platelets, the changes induced in these platelets by feeding stearic acid compared to those with oleic acid are similar in type to those noted between men with infarcts and normal men.

Experiment 4

Blood was taken to determine plasma and platelet cholesterol, platelet aggregation in
Examples of platelet-aggregation reproducibility on platelet-rich plasma, in four subjects (A to D) of experiment 4. In each case, tracing 2 was performed from 1 to 6 months after tracing 1. The final dilution of thrombin was 0.17 U, and of ADP, 0.83 μg.

Relationship between thrombin, ADP, and collagen-induced aggregation, and the ratio of saturated + monounsaturated to polyunsaturated fatty acids in platelet total lipids (S+M/F) in 17 middle-aged men. The final dilution of thrombin in the platelet-rich plasma was 0.17 U, and of ADP, 0.83 μg.
platelet-rich plasma, and fatty-acid composition of plasma and platelets in 17 active businessmen. At the same time, these men were given a general medical examination, including ECG at rest and after exercise. The laboratory tests were carried out by a team unaware of the results of the medical examination and, conversely, the physicians were unaware of the laboratory results when they examined the patients. For verification of the results obtained, the same series of tests was performed a second time on 8 of these 17 men, from 1 to 6 months after the first determinations. In Figure 7 are illustrated the results of the platelet-aggregation study on four of the men, showing the individual responses to ADP (patients A to C) and to thrombin (patients A to D). The lower tracings were obtained from the platelet-rich plasma of the same men, but from 1 to 6 months after the initial tracings. As can be seen from this figure, the reproducibility was excellent, similar results being obtained in seven out of eight patients.

In the present platelet-aggregation study, it was thought desirable to utilize 500,000/mm³ platelets, since only in this way could we obtain sizeable tracings in healthy young men (A in Fig. 7) under the conditions used here. The platelet count was between 480,000 and 500,000 in 11 samples, but below 400,000 in three. In preliminary experiments, by using different dilutions of platelet-rich with platelet-poor plasma, a correction factor was established for tracings obtained with platelet counts below 460,000, according to the equation $h = h_0 + (d \times K \times h_1)$. In this equation, $h$ is the expected height of the tracing, $h_0$ the actual height obtained, $d$ the difference in the platelet count, and $K$ a correction coefficient. The K value has been found to be $1.3 \times 10^{-8}$ for thrombin, $0.9 \times 10^{-8}$ for collagen, and $0.4 \times 10^{-8}$ for ADP-induced aggregation.

Figure 8 shows the results of thrombin, ADP, and collagen-induced aggregation in relation to the $(S + M)/P$ in the platelets. It can be seen that a positive correlation was found ($r = 0.96$) between the thrombin aggregation and the ratio of fatty acids in platelets, while none was noted with ADP. With collagen, a negative correlation was observed ($r = -0.58$).
In Figure 9, the results of thrombin-induced aggregation were plotted on the ordinate, and those of cholesterol in plasma and platelets and of the \((S + M)/P\) in plasma, on the abscissa. In all three graphs, the resulting points were scattered over a large area and none of the correlations appeared to be significant.

In this study, five subjects presented thrombin-induced aggregation tracings higher than 3.5 cm. Among these five, four had typical or atypical chest pain and four also had ECG abnormalities, suggesting that all five subjects could have had coronary disease. None of the other 11 subjects presented either chest pain or ECG changes. As regards ADP-induced aggregation, six subjects exhibited curves equal to or higher than 6 cm, but only two of the six had chest pain or ECG abnormalities.

With collagen, eight subjects had curves of 8 cm or more, but only one of these eight subjects could have been suspected of having coronary heart disease.

Discussion

The present study in rats confirms our previous results (2) in that:

1. Butter and stearic acid are highly thrombogenic.
2. Corn oil and linoleic acid appear to inhibit the potentially thrombogenic effect of cholesterol and a bile salt, and even of butter.
3. Oleic acid, despite an effect on cholesterolemia similar to that produced by stearic acid, did not induce the hypercoagulability or the increase in susceptibility of platelets to aggregation observed with stearic acid. As a result, thrombosis was almost as low in rats fed oleic acid as in those fed linoleic acid.

With regard to platelet aggregation, we showed previously that the severity of thrombosis in rats was related to thrombin but not to ADP or collagen-induced aggregation (4, 5). The present study in rats confirms these earlier results, at least as far as thrombin aggregation is concerned.

In experiment 1, in rats fed a diet that was potentially thrombogenic but contained corn oil, the fatty-acid composition, particularly in the platelets, did not differ markedly from that of the rats fed laboratory chow. The severity of thrombosis was also comparable in these two groups. In contrast, feeding butter induced drastic changes that were mostly characterized by an increase in the monounsaturated at the expense of the polyunsaturated fatty acids. Also in these butter-fed rats, the platelets were highly susceptible to thrombin and severe thrombotic lesions could be induced.

This increase of monounsaturated fatty acids in platelets in connection with thrombosis appears to contradict our previous results (1) in that dietary oleic acid is not thrombogenic. On the other hand, experiment 2 of the present study confirmed our earlier findings inasmuch as it indicates that feeding oleic acid compared with feeding stearic acid does not increase platelet oleate. In addition, polyunsaturated fatty acids in the platelets did not decrease as much as with stearic acid, since the \((S + M)/P\) was significantly lower with oleic than it was with stearic acid, and this could explain the beneficial effect of dietary oleic acid.

These results are in good agreement with those obtained by others in the rat (13), although these workers examined the liver instead of the platelets. They concluded that tissue oleic acid is not related to dietary oleic acid but is directly proportional to the amount of saturated fatty acid and inversely proportional to the polyunsaturated acids in the diet. In contrast to this, increasing the amount of dietary linoleic acid resulted in a corresponding increase of this fatty acid in tissue lipid.

Similarly, in men with myocardial infarction as compared with normal subjects, a decrease was noted in polyunsaturated fatty acids and an increase of all the saturated and monounsaturated fatty acids. In atherosclerotic patients, some workers have reported similar results on different serum lipids fractions (14, 15) and even in the aorta (16), while others (17) have failed to demonstrate such changes. However, as recognized by Bang et al. (17), their controls were not optimal while, in the
present study, the controls were selected because they presented no risk factors for coronary disease. Differences in the technique used could also have accounted for the discrepant findings.

The fact that the changes noted in the fatty-acid composition of the platelets of men with myocardial infarction can be reproduced in rats by feeding certain fats or fatty acids suggests that the changes noted in coronary patients could be due to dietary habits.

In the first five patients with myocardial infarction studied, platelet-aggregation tests were performed at the same time as the fatty-acid composition of platelets was determined. However, the results did not differ significantly from those noted in the controls and, for this reason, the study was not completed. This disappointing result was attributed to the intensive treatment to which these patients had been subjected, particularly to heparin, a drug well known to markedly reduce platelet aggregation. In contrast to this, when the tests were performed in active middle-aged businessmen, a population group known to be highly susceptible to coronary disease, a certain percent presented a tremendous increase in the susceptibility of their platelets to thrombin-, ADP-, and collagen-induced aggregation. Nevertheless, only those presenting a high susceptibility to thrombin exhibited chest pain, ECG abnormalities, or both. Here, we should mention a previous study (5) in which it was observed that in rats with a high thrombotic tendency the platelets were more susceptible to thrombin, but they were less susceptible to ADP and collagen than those of rats fed laboratory chow (5). In the group of businessmen in the present study, the susceptibility of the platelets to thrombin, but not to ADP or collagen, was correlated with the (S + M)/P in platelets. Since this ratio is considerably increased in patients with myocardial infarction as well as in rats that are highly susceptible to thrombosis, these results suggest a relationship between the thrombotic tendency, the susceptibility of platelets to thrombin-induced aggregation, and the fatty-acid composition of the platelets. Although further experiments are needed to verify this relationship, it seems appropriate to mention briefly in this connection the preliminary results of tests we are presently conducting in patients with angina pectoris. Depending on the severity of the disease, the platelets of these patients as compared with those of normal subjects appear to have an increased (from 5 to 100 times) susceptibility to thrombin-induced aggregation, but not to that induced by ADP or collagen.

In one study of patients with various thrombotic phenomena, no significantly increased rate of aggregation was observed, although the mean results were slightly higher than the corresponding values for healthy subjects (18). These results tend to confirm the present observation that there was no increased rate of aggregation 10 days after myocardial infarction. But more recently (19), it was reported that the time of maximum aggregation induced by ADP was significantly greater in patients with myocardial infarction than in the controls. However, since the platelet-aggregation tests are influenced by many endogenous or exogenous substances, such as corticoids, anti-inflammatory drugs, and heparin as well as, to a great extent, by small technical details, such discrepancies are not surprising. Nevertheless, probably owing to the substantial improvements made in the method originally devised by Mustard et al. (20), the platelet-aggregation technique used in the present studies was highly reproducible in the human subjects, and the results could be compared from day to day. In the rats however, for reasons that are not clear as yet, adequate controls still had to be run each time with the experimental groups, for the sake of exact comparison.

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