Platelets, Fatty Acids and Thrombosis

By John C. Hoak, M.D., Emory D. Warner, M.D., and William E. Connor, M.D.

ABSTRACT

The sodium salts of stearic, oleic, linoleic and linolenic acids were added to human washed platelet suspensions and platelet-rich citrated plasma. Aggregation of the platelets was measured microscopically and with a turbidimetric method. All of the fatty acids had the ability to produce aggregation when added to washed platelets, but stearic acid, a long-chain saturated fatty acid, was more potent than were the unsaturated acids when added to platelet-rich plasma. Aggregation of platelets by fatty acids required the presence of calcium ions and the aggregation was irreversible. The addition of albumin diminished the aggregating effects of fatty acids, but microscopic aggregates still formed in most instances. Subnormal aggregation was noted when sodium stearate was added to platelet-rich plasma from a patient with a severe deficiency of factor XII (Hageman factor). Thus, fatty acids are now known to have two potential thrombogenic effects: platelet aggregation and the activation of clotting factors involved in the early stages of blood coagulation.

ADDITIONAL KEY WORDS

lipids blood coagulation Hageman factor platelet aggregation stearic acid oleic acid linoleic acid linolenic acid calcium adenosine diphosphate

In recent years the effects of lipids upon blood coagulation and their potential to cause thrombosis have been studied extensively. Our interest has been centered upon the free fatty acid (FFA) fraction of the blood. The results of this work indicate that fatty acids can exert a striking effect upon blood clotting and can produce hypercoagulability of the blood which results in thrombosis (1-5). It is pertinent that an elevated concentration of FFA in the plasma has been found in certain pathologic or physiologic conditions associated with thromboembolic complications (6-10).

In an earlier study it was shown that the thrombi which formed following injections of sodium stearate into animals had the morphological features of natural thrombi rather than of blood clots. Electron microscopy revealed that platelet aggregates formed conspicuous components of these thrombi (11). The present report is concerned with additional observations concerning the effects of fatty acids upon platelets. Platelet aggregation was studied in detail with turbidimetric methods.

Material and Methods

Blood was collected for the in-vitro studies from normal human volunteers with disposable 18-gauge stainless steel needles and was allowed to run through plastic tubing into silicone-coated glass centrifuge tubes. Washed platelet suspensions were prepared by the method of Haslam (12). In the studies with washed platelets, 0.02 ml of 0.11 M calcium chloride was mixed with the platelet suspension before the fatty acid was added. Platelet-rich plasma was prepared from blood which had been collected in sodium citrate, 9 parts blood to 1 part 3.13% sodium citrate. In one part of the study, the blood was collected in 0.077 M disodium ethylenediaminetetraacetic acid (EDTA) to test whether the aggregation of platelets by fatty acids required calcium ions; 0.75

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ml of the EDTA solution was added to 9.25 ml of blood. Platelet aggregation was also studied in a patient with a severe defect in Hageman factor (deficiency of Factor XII).

Platelet aggregation was studied with turbidimetric methods similar to those described by others (13, 14). Light from a lamp passed through the tube containing 1 ml of platelet-rich plasma or platelet suspension to a photoelectric cell. A magnetic stirrer beneath the tube provided continuous stirring of the sample when a steel bar, coated with Monocote, was placed in the tube. The holder for the tube was made of a thermostatically controlled, electrically heated block. The rate of stirring was monitored continuously. The photoelectric cell was coupled directly to a recorder.

Aggregation of platelets produced changes in light transmission which were traced with the recorder. As the platelets aggregated, more light was transmitted through to the photoelectric cell, and produced a deflection of the recorder pen upward from the base line. As the platelet aggregates increased in size, they interrupted the light beam periodically, and produced an oscillation along the track of the recorder pen.

Samples of the platelet preparations were examined by phase contrast microscopy before and after the addition of platelet-aggregating agents as a check on the turbidimetric results.

The fatty acids used in the study were of high purity. The methods for preparing the suspensions of the salts of the fatty acids, 3.5 \( \text{mM}, \) pH 8, have been described previously (1). In some instances sodium stearate or sodium oleate was added to a 5% solution of fatty acid-poor fraction V bovine albumin\(^3\) in an amount sufficient to bring the final FFA concentration of the resulting solution to approximately 2 mEq/liter. Human serum albumin was also used in one part of the study.\(^4\)

### Results

In these studies, fatty acids caused aggregation of platelets in most instances. The results are shown in Table 1. In all instances when aggregates were seen by gross visual examination, changes indicative of platelet aggregation were recorded by the turbidimetric method. It was not uncommon to find microscopic aggregates of platelets, produced by the addition of unsaturated fatty acids to platelet-rich plasma, which were not detected by gross visual examination or by the turbidimetric method. The addition of 3.5 \( \text{mM} \) sodium stearate to washed platelets resuspended in buffered saline caused prompt aggregation (Fig. 1). This effect was partially inhibited by the addition of albumin prior to the stearate, but aggregation still occurred. When stearate was combined with albumin, as shown in the lower curve, less of an effect was produced, but microscopic aggregates formed in many instances.

The addition of stearate to citrated platelet-rich plasma also caused prompt macroscopic aggregation of platelets. This effect was not inhibited by heparin (Fig. 2) in concentrations as high as 6 units of heparin/ml of plasma. When stearate was added to platelet-rich plasma, prepared from blood collected in EDTA, no aggregation resulted.
### Table 1

Effects Produced by the Addition of Different Fatty Acids to Washed Platelets or Platelet-Rich Plasma

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Amount of fatty acid added (μEq)</th>
<th>Number of observations</th>
<th>Aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Washed Platelets</td>
</tr>
<tr>
<td>Stearic (C18:0)</td>
<td>0.35</td>
<td>5</td>
<td>5 0 0</td>
</tr>
<tr>
<td></td>
<td>0.70</td>
<td>10</td>
<td>10 0 0</td>
</tr>
<tr>
<td>Oleic (C18:1)</td>
<td>0.14</td>
<td>1</td>
<td>0 0 1</td>
</tr>
<tr>
<td></td>
<td>0.35</td>
<td>4</td>
<td>4 0 0</td>
</tr>
<tr>
<td>Linoleic (C18:2)</td>
<td>0.35</td>
<td>2</td>
<td>0 2 0</td>
</tr>
<tr>
<td></td>
<td>0.70</td>
<td>2</td>
<td>0 2 0</td>
</tr>
<tr>
<td>Linolenic (C18:3)</td>
<td>0.35</td>
<td>1</td>
<td>1 0 0</td>
</tr>
<tr>
<td></td>
<td>0.70</td>
<td>5</td>
<td>1 4 0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
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</tbody>
</table>

Thus, aggregation of platelets by fatty acids depends on the presence of calcium ions. Long-chain unsaturated fatty acids, including linolenic acid, also produced prompt aggregation of platelets when added to washed platelets or platelet-rich plasma (Figs. 3 and 4). Unsaturated fatty acids produced less aggregation when added to platelet-rich plasma than when they were added to washed platelets. This may represent the effect of binding of the fatty acids by plasma albumin; the latter was not present in the washed platelet suspension. Figure 5 shows the influence of albumin upon platelet aggregation by sodium oleate. When unbound oleate was added to washed platelets, prompt aggregation resulted. When albumin was added after oleate had been added, little inhibition was observed. When albumin was added before oleate or was combined with...
Platelet aggregation produced by sodium oleate.

Effect of sodium linolenate on washed platelets.

albin and then added as shown in curve D, much less aggregation occurred than after oleate alone.

The aggregation of both washed platelets and platelet-rich plasma by fatty acids was irreversible. Incubation and stirring of the platelet aggregates that were produced by the addition of saturated or unsaturated acids, caused the aggregates to increase in size.

When sodium stearate was added to the platelet-rich plasma from a patient with a deficiency of Hageman factor, the response was considerably less than was seen with normal plasma (Fig. 6). When larger amounts of stearate were added to plasma from this patient, macroscopic aggregates formed. This, however, required four times the amount of stearate necessary to form macroscopic aggregates in normal plasma. It is of interest that washed platelets from this patient aggregated normally in response to the usual amount of stearate (Fig. 7). Likewise, when 0.1 µmole of adenosine diphosphate (ADP) was added to 1 ml of the patient's platelet-rich plasma, macroscopic platelet aggregates formed promptly.

Discussion

Several other studies have been performed in which fatty acids have been observed to cause platelet aggregation. Haslam reported that the long-chain saturated fatty acids caused rapid aggregation of washed human platelets in the presence of calcium ions when suspended in tris-buffer, and suggested that fatty acids mediated these effects by the release of ADP from platelets (12). Shore and Alpers found the addition of stearic and other long-chain saturated fatty acids to rabbit platelet-rich plasma caused clumping of platelets with the release of serotonin and histamine (15). Kerr et al. found that most

The influence of albumin on aggregation of washed human platelets by sodium oleate. In each test 3.5 mm sodium oleate or oleate and 5% human albumin were added to 1 ml of a washed platelet suspension containing an added 2.2 µm of calcium chloride. In part A, the total FFA concentration was 0.35 µEq while in parts B and C, it was 0.44 µEq. In part D, the final FFA concentration was 0.25 µEq. The albumin had a FFA concentration of 900 µEq/L.
sodium salts of free fatty acids, dissolved in lecithin, caused aggregation of platelets in platelet-rich plasma (16). These workers found that linoleate and linolenate did not cause platelet aggregation, and linolenate inhibited the aggregation of platelets induced by saturated fatty acids. More recently, Mahadevan et al. also found that long-chain saturated fatty acids and oleic acid produced platelet aggregation in platelet suspensions, but linoleic and linolenic acids were ineffective (17). In their studies, linolenic acid also inhibited the effects of behenic acid. Ardlie et al., using low concentrations of stearic acid, found that it enhanced platelet aggregation in platelet-rich plasma by epinephrine and adenosine triphosphate, but had no effect by itself (18).

Much of the variability of results with fatty acids and platelet aggregation in the literature probably reflects the different concentrations used and the influence of binding by plasma albumin. As demonstrated in this study, the presence of albumin tends to decrease the aggregating effects of the fatty acids. This is particularly true when unsaturated fatty acids are studied, since these acids are bound more rapidly by albumin than are long-chain saturated acids (unpublished work).

In addition, in any discussion of platelet aggregation studies, it is pertinent to mention the limitations of turbidimetric methods. In general, the turbidimetric method is a satisfactory and easy procedure, but small aggregates may be present and cause no change in optical density. Therefore, in all of our studies, the platelet suspensions were examined by phase contrast microscopy before and after the addition of the agents being tested for aggregating properties. This procedure was particularly important in evaluating the effects of the salts of linoleic and linolenic acids when these fatty acids were added to platelet-rich plasma. As a rule there was little if any deflection of the base line when either linoleate or linolenate was added to platelet-rich plasma, but, in most instances, there were microscopic aggregates of platelets. The failure of other workers to observe platelet aggregation with linoleic or linolenic acids may represent their failure to examine
the platelet suspensions microscopically. Such microscopic aggregates may explain why in earlier studies linoleate and linolenate, injected intravenously in dogs, produced thrombocytopenia to as great an extent as did oleate and saturated fatty acids (4). Zbinden obtained similar results when he injected fatty acids intravenously into rabbits (19).

Although there might be alternative explanations for the difference in effects of stearate upon washed platelets and platelet-rich plasma from the patient with the Hageman defect, these results are consistent with the presence of an inhibitor in the plasma of individuals with Hageman trait. The platelets of this patient aggregated normally when ADP was added to his platelet-rich plasma. If fatty acids act via the release of ADP, these results would not be expected unless there is inhibition of ADP release from platelets in the patient with Hageman trait. If this is the case, the normal response with washed platelets to stearate may indicate a direct detergent effect upon the platelet membrane, in the absence of an inhibitor, which led to rapid release of ADP.

Although these in-vitro studies leave no doubt that fatty acids can aggregate platelets, the relevance of these results to clinical thrombosis remains speculative. The important question is, do the plasma free fatty acids which circulate bound to albumin ever produce platelet aggregation and thrombosis in the body?

The experimental evidence, from the studies performed with fatty acids to date, suggest that thrombosis does not result from the effect of "bound" fatty acids until the concentration is such that the two "tight" binding sites on the albumin molecule are no longer available. Goodman has found that each albumin molecule bears two sites with very high affinity for fatty acids, five with a lesser affinity, and a large number with much less ability to bind fatty acids (20). If the plasma albumin concentration were 4g/100 ml, saturation of the tight binding sites would occur with a plasma free fatty acid (FFA) concentration of 1150 to 1200 μEq/liter. Plasma FFA concentrations of this level or greater have been found in a number of physiological and pathological conditions in which there is an increased frequency of thromboembolic complications (6-10).

Evidence in support of the hypothesis that fatty acids can produce hypercoagulability and thrombosis under more physiological conditions has been provided by additional studies. When subcutaneous injections of adrenocorticotropin (ACTH) were given to normal rabbits, hypercoagulability and thrombosis occurred in most instances (21). These events were associated with high plasma FFA concentrations due to mobilization of endogenous lipid by the ACTH. The dose of ACTH used in the study was large, 50 units/kg of body weight. In later studies of a similar nature, thrombosis and high plasma FFA concentrations still resulted frequently when a dose of ACTH as low as 0.5 unit/kg was used (4). Rabbits, given this small dose of ACTH, exhibited high plasma FFA concentrations, hypercoagulability and thrombosis at 1 hour after the injection of ACTH. Two hours after the ACTH injection, the plasma FFA concentration and silicone clotting times had returned to normal, but thrombi were found in the lungs. These findings suggest the possibility that a transient period of hypercoagulability existed, which culminated in pulmonary thrombosis.

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References

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