Release of Arachidonic Acid From Human Platelets.  
A Key Role for the Potentiation of Platelet Aggregability in Normal Subjects as Well as in Those With Nephrotic Syndrome

By Nobuhiko Yoshida and Nobuo Aoki

Low (nonaggregating) concentrations of collagen that potentiate platelet aggregation did not induce the formation of measurable amount of malondialdehyde (MDA) but released small but significant amounts of radioactivity from 14C-arachidonic acid–labeled platelets. A major portion of the radioactive compounds released by nonaggregating concentrations of collagen existed as arachidonic acid and a minor part as thromboxane B2.

The nephrotic syndrome enhances platelet aggregability, and this effect is abolished by correcting hypoalbuminemia in vitro and in vivo by the addition of albumin, which is the main carrier for free fatty acids, including arachidonic acid. Human albumin (fatty acid free) inhibited collagen-induced aggregation, MDA formation, and release of the radioactivity from 14C-arachidonic acid–labeled platelets in normals as well as in those with nephrotic syndrome. These data support our hypothesis that the main mechanism responsible for the potentiation of platelet aggregation is the release of arachidonic acid from platelet membrane phospholipids via the activation of phospholipase A2. Furthermore, enhanced platelet aggregation in the nephrotic syndrome was at least partly attributable to an increased availability of arachidonic acid released secondary to hypoalbuminemia. Albumin inhibits aggregation probably by binding to released arachidonic acid preventing arachidonic acid from being metabolized to potent aggregating substances, endoperoxides and thromboxane A2. The mechanism of release of arachidonic acid may play a key role in the potentiation of platelet aggregability in normals as well as in pathologic conditions such as the nephrotic syndrome.

Low (nonaggregating) concentrations of stimulants such as collagen, thrombin, arachidonic acid, or epinephrine potentiate platelet responsiveness, and the potentiated platelets aggregate with a subsequent addition of low (nonaggregating) concentrations of stimulants.1,3 On the other hand, enhanced platelet aggregation has been seen in some disorders such as the nephrotic syndrome4 or diabetes mellitus,5 and platelets in these disease states readily aggregate with low concentrations of stimulant. The enhanced platelet aggregation might be involved in the production and perpetuation of thrombosis and other various disorders.

We previously reported that collagen-treated platelet membranes release an appreciable amount of the precursors of prostaglandins and potentiate platelet...
aggregation. We then suggested that the material responsible for the potentiation might be arachidonic acid or its derivatives. In this report we present some evidence to support further our hypothesis that the main mechanism responsible for the potentiation is the release of arachidonic acid from platelet membranes. Albumin may inhibit platelet aggregation by binding to released arachidonic acid and thus preventing it from being metabolized to potent aggregating materials, endoperoxides and thromboxane A2.

MATERIALS AND METHODS

Platelet samples. Human blood was collected from healthy donors who had not taken any drug within the previous week and from a patient with nephrotic syndrome. The patient was a 16-yr-old male, and the diagnosis was made from massive edema, proteinuria (>20 g/day), hypoalbuminemia (<1 g/dl), and hypercholesterolemia. Histologic examination of a renal biopsy specimen showed minimal changes. Blood was collected from antecubital veins into 0.1 vol 3.8% trisodium citrate and centrifuged at 650 g for 4 min at room temperature to obtain platelet-rich plasma (PRP). Platelet-poor plasma (PPP) was obtained by centrifugation of citrated blood at 2000 g for 20 min.

Preparation of 14C-arachidonic acid-labeled platelet suspensions. This was prepared according to the method of Bills et al.\(^5\) with a minor modification. PRP (10 ml) was incubated for 60 min at 37°C with 10 \(\mu\)l of 5 \(\mu\)Ci/ml 14C-arachidonic acid (Radiochemical Centre, Amersham, England) dissolved in ethanol. At the end of the incorporation procedure 0.1 ml of 0.1 M EDTA (pH 7.4) was added to the incubation mixture. The mixture was centrifuged at 2000 g for 10 min at room temperature. The supernatant was removed and the platelet pellet was resuspended in 10 ml of 0.015 M Tris-HCl pH 7.4, 0.134 M NaCl, 1 mM EDTA, and 5 mM glucose (washing solution) and recentrifuged. Washing procedures were performed twice. Platelets were finally resuspended in modified Tyrode solution containing 1 mM EDTA and 0.1% human fibrinogen but no CaCl\(_2\) to obtain about 4 \times 10^8 platelets/ml and stored at room temperature.

Uptake of radioactivity by platelets varied between 20\% and 45\%. The distribution of incorporated radioactivity was studied by extracting lipids from the platelets according to the methods described by Bills et al.,\(^5\) and the results were essentially the same as those reported. Greater than 90\% of the incorporated radioactivity was located in the platelet phospholipids: 50\% in phosphatidylcholine, 25.9\% in phosphatidylserine and phosphatidylinositol, 16.4\% in phosphatidylethanolamine, and the remainder in neutral lipids. Free or loosely bound arachidonic acid was removed during washing procedures. The platelet membrane fraction was prepared by the method of Barber and Jamieson\(^7\) from 14C-arachidonic acid-labeled platelets, and approximately 95\% of radioactivity was found in membrane phospholipids.

Platelet aggregation was studied turbidometrically at 37°C with continuous recording of light transmission\(^6\) (aggregometer: Bryston, Rexdale, Ontario). Platelets were incubated at 37°C with stirring for 3 min in the aggregometer in the presence or absence of 20 \(\mu\)M indomethacin or various concentrations of human albumin before addition of an aggregating agent. When washed platelets were used, CaCl\(_2\) was added to obtain 3 mM CaCl\(_2\) immediately before each experiment. The difference in light transmission caused by aggregation at 3 min (otherwise indicated) after the addition of aggregating agent was divided by the difference of light transmission between PRP and PPP, and multiplied by 100. This percentage value was used as an indicator of aggregation. Indomethacin (Japan Merck Banyu, Tokyo, Japan) was dissolved in ethanol, and the volume added to platelets did not exceed 1\%. Human albumin (Cohn fraction V, fatty acid free) was purchased from Miles Laboratories, Kankakee, Ill., and dissolved in 0.85\% NaCl. Collagen (Sigma Chemical, St. Louis, Mo.), arachidonic acid (Sigma), and epinephrine (Sankyo Pharmaceutical, Tokyo, Japan) were prepared as described previously.\(^3\)

Release of radioactivity during aggregation of 14C-arachidonic acid-labeled platelets. Reactions of 14C-arachidonic acid-labeled platelets with collagen or saline as a control (total volume 0.415 ml) were terminated by adding 0.1 ml of 0.1 M ice-cold EDTA according to the method of Rittenhouse-Simmons and Deykin.\(^9\) The total radioactivity of the platelet suspension was determined by liquid scintillation counting. EDTA-containing samples were immediately centrifuged at 2000 g for 20 min at 4°C, and the radioactivity of the supernatant solution was
determined. The data from the saline control was subtracted and the release of radioactivity was calculated. The radioactive compounds in the supernatant were analyzed according to the method of Isakson et al.\textsuperscript{10} using 20 ml platelet suspension for each analysis. The supernatant was acidified with formic acid and extracted twice with ice-cold ethylacetate. The combined extracts were analyzed by thin-layer chromatography on thin-layer aluminum sheets (TLC aluminum sheets, silica gel F254, E. Merck, Darmstadt, W. Germany) in solvent system of chloroform:methanol:acetic acid:water (90:8:1:0.8). Lipids were visualized with iodine vapor, and each spot was determined using arachidonic acid and thromboxane B\textsubscript{2} (kindly supplied by Dr. T. Miyatake, Dept. of Neurology) as standard. Silica gel in the areas corresponding to arachidonic acid and thromboxane B\textsubscript{2} was scraped off, and radioactivity of each sample was determined by liquid scintillation counting.

Malondialdehyde (MDA) formation during platelet aggregation. MDA formed in washed platelet suspensions during platelet aggregation was assayed by the spectrophotometric method of Smith et al.\textsuperscript{11} Reactions of platelet suspension by collagen or saline as a control (total volume 0.415 ml) were terminated by adding 0.4 ml 20% trichloroacetic acid in 0.6 N HCl with mixing. Samples were centrifuged, and 0.5 ml of the supernatant was added to 0.1 ml 2.4% thiobarbiturate in water and heated in a closed vessel in a boiling-water bath for 30 min. After cooling and clarification by centrifugation, the absorbance of each sample was read at 532 nm. Concentration of MDA was determined from a standard curve constructed with known amounts of MDA (>99% pure, Tokyo Kasei Chemical, Tokyo, Japan), and MDA formed was expressed as the amount from 1 ml platelet suspension (\(4 \times 10^{10}\) platelets).

Determination of protein concentration. Protein was determined by the method of Lowry et al.\textsuperscript{12} with crystallized albumin as a standard or by absorbance at 280 nm.

RESULTS

Release of radioactivity from \(^{14}\)C-arachidonic acid–labeled platelets. When collagen was added to \(^{14}\)C-arachidonic acid–labeled platelets, the radioactivity was released in proportion to the amount of collagen added and to the time elapsed after the addition of collagen. The amount released during the initial 30 sec after addition of 50 \(\mu\)g/ml collagen (60% aggregation) was about the same as that 2 min after addition of 25 \(\mu\)g/ml collagen (90% aggregation). Therefore the extent of aggregation was not proportional to the release. Release by various amounts of collagen during the initial 3 min of aggregation was investigated (Fig. 1). The amount of release was proportional to the amount of collagen added. No measurable aggregation was elicited at the concentrations of collagen less than 4 \(\mu\)g/ml, whereas the release mounted up to about 30% of the amount released by 24 \(\mu\)g/ml collagen, which was the smallest dose tested that was able to cause nearly full aggregation. Three independent sets of analysis of the radioactive compounds released in the milieu showed that 55%-65% of the radioactivity released by less than 4 \(\mu\)g/ml collagen that caused no aggregation existed as arachidonic acid, and 5%-9% was converted to thromboxane B\textsubscript{2}. Even in the presence of 20 \(\mu\)M indomethacin, which completely inhibited aggregation, low concentrations (0.8, 1.6, 4.0 \(\mu\)g/ml) of collagen were still able to release the radioactivity (9%, 10%, 17% of the amount released by 24 \(\mu\)g/ml collagen in the absence of indomethacin, respectively).

MDA formation. Very little or no measurable aggregation was elicited at concentrations of collagen less than 4 \(\mu\)g/ml, and MDA formation was not detected (Fig. 2). The amounts of MDA formed were fairly proportional to the concentrations of collagen in the range 7.5–120 \(\mu\)g/ml. However, 20 \(\mu\)M indomethacin completely prevented MDA formation in response to up to 60 \(\mu\)g/ml collagen, with \(^{14}\)C-arachidonic acid–labeled or unlabeled platelets.
Enhanced platelet aggregation in the nephrotic syndrome. Platelets from a patient with nephrotic syndrome aggregated upon stirring without any addition of a stimulant or with an addition of very low concentrations of stimulant such as 0.1 μM epinephrine or 25 μM arachidonic acid, which are unable to induce platelet aggregation in normal PRP (Table 1). This hyperaggregability was corrected to normal by the addition of albumin in vitro; enhanced aggregation was suppressed by increasing concentrations of albumin, and finally no aggregation was induced by 0.1 μM epinephrine or 25 μM arachidonic acid in the presence of 4.0 g/dl albumin (Table 1). In the presence of 4.0 g/dl albumin, which is usually found in normal plasma, a more concentrated stimulant such as 1 μM epinephrine or 500 μM arachidonic acid was necessary to induce normal aggregation (Table 1), as in normal PRP. ADP-induced secondary aggregation

Fig. 1. Radioactivity released by various concentrations of collagen from 14C-arachidonic acid–labeled platelets. 14C-arachidonic acid–labeled platelets (0.4 ml) in modified Tyrode's solution was incubated with 5 μl of 0.25 M CaCl₂ for 3 min in an aggregometer tube with stirring followed by addition of collagen or saline (5 μl). Reactions were terminated by adding 0.1 ml of 0.1 M ice-cold EDTA 3 min after collagen. Samples were immediately centrifuged and radioactivity of supernatant determined by liquid scintillation counting. Data from saline control were subtracted. Amount of radioactivity released from platelets by 480 μg/ml collagen was 29% of radioactivity of whole platelets. Amount of radioactivity released by 24 μg/ml collagen, which caused nearly full aggregation, was arbitrarily defined as 1, and extent of release was expressed relative to this unit. Radioactivity and amounts of collagen presented on logarithmic scales. Means of duplicate determinations —, Radioactivity released, (left-hand scale); ---, aggregation (%) (right-hand scale).

Fig. 2. MDA formation by various concentrations of collagen during aggregation of washed platelets. Experimental conditions same as in Fig. 1, but nonradioactive washed platelet suspension was used and reactions were terminated by adding 0.4 ml 20% trichloroacetic acid in 0.6 N HCl with mixing. Experiments done in duplicate.
Table 1. Effect of Albumin on Aggregation of Platelets From a Patient With Nephrotic Syndrome

<table>
<thead>
<tr>
<th>Stimulant (µM)</th>
<th>Albumin (g/dl)*</th>
<th>Aggregation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine</td>
<td>0.0†</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>82</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>0†</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>84</td>
</tr>
<tr>
<td>ADP</td>
<td>0.9</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>0</td>
</tr>
</tbody>
</table>

*Albumin concentration was the value of the original plasma albumin concentration (1.04 g/dl) plus added albumin.
†Patient's platelets without addition of albumin aggregated spontaneously without any addition of stimulant.
‡The extent of primary aggregation was 20%.

PRP (0.45 ml) was incubated with various concentration of albumin in 0.85% NaCl (0.05 ml) for 3 min, and 10 µl of stimulant was added.

was also remarkably inhibited by albumin, although primary aggregation was not much inhibited. Enhancement of platelet aggregation was gradually abolished as the hypoalbuminemia of the patient was corrected by the treatment of plasma transfusion and administration of prednisolone (Fig. 3).

Effects of albumin on MDA formation and release of arachidonic acid or its metabolites in response to collagen were investigated by adding albumin to washed platelet suspension obtained from a normal donor (Table 2). Albumin inhibited collagen-induced aggregation and MDA formation in proportion to the concentration of albumin added. The release of radioactivity from ¹⁴C-
Table 2. Effect of Albumin on MDA Formation and \textsuperscript{14}C-Arachidonic Acid Release

<table>
<thead>
<tr>
<th>Collagen (\textmu g/ml)</th>
<th>Albumin (g/dl)</th>
<th>\textsuperscript{14}C-Arachidonic Acid-Labeled Platelets</th>
<th>Washed Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aggregation (%)</td>
<td>MDA Formation (pmol/ml Platelets)</td>
<td>Aggregation (%)</td>
</tr>
<tr>
<td>50</td>
<td>0.00</td>
<td>94</td>
<td>604</td>
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<tr>
<td></td>
<td>0.69</td>
<td>76</td>
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<td>1.38</td>
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<tr>
<td></td>
<td>2.07</td>
<td>54</td>
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<td></td>
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<td>2.07</td>
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<td>3.45</td>
<td>16</td>
<td>207</td>
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Platelet suspension was incubated with 3 mM CaCl\textsubscript{2} and various concentrations of albumin for 3 min, and collagen was added. MDA formation and the release of radioactivity were measured as described in Materials and Methods. The results shown are means of duplicate determinations.

*Concentration in incubation mixture.
†The amount of radioactivity released from platelets without albumin in response to 50 \textmu g/ml collagen was defined as a unit, and the other data were referred to this unit.

arachidonic acid-labeled platelets was enhanced by low concentrations of albumin (Table 2). The enhancement, however, decreased when the amount of albumin was increased, and the release finally became less than that observed in the absence of albumin. In the presence of a given concentration of albumin, the amount of radioactivity released was dependent on and proportional to the amount of collagen added, as observed in the absence of albumin in Fig. 1.

DISCUSSION

When platelets are exposed to aggregating agents (thrombin, collagen, epinephrine, ADP), arachidonic acid is released from platelet membrane phospholipids probably via the activation of phospholipase A\textsubscript{2} and is metabolized by cyclooxygenase and lipoxygenase pathways. Some of the metabolites (endoperoxides and thromboxanes) cause the release reaction and aggregation.\textsuperscript{13,15} Arachidonic acid released from membranes and its metabolites are released outside of platelets.\textsuperscript{9,13,15,16} Accordingly, the total radioactivity released into the medium from \textsuperscript{14}C-arachidonic acid-labeled platelets by aggregating agents will correspond to the amount of arachidonic acid released from platelet membrane phospholipids apart from the transfer of arachidonic acid to plasmalogen phosphatidylethanolamine\textsuperscript{9} and may be used as an indicator of phospholipase A\textsubscript{2} activity. The measurement of MDA formation during platelet aggregation can be used as an indicator of synthesis of aggregating metabolites, endoperoxides, and thromboxanes. We used these simple methods for the investigation of platelet responses caused by various concentrations of collagen.

Low (nonaggregating) concentrations of collagen that induce neither aggregation nor serotonin release potentiate platelet aggregation.\textsuperscript{3} With these low concentrations of collagen small but appreciable amounts of radioactivity were released from \textsuperscript{14}C-arachidonic acid-labeled platelets (Fig. 1), but neither MDA formation (Fig. 2) nor serotonin release\textsuperscript{3} was detected. Furthermore, the analysis of released radioactive compounds showed that most (55\%-65\%)
of the radioactive compounds remained as arachidonic acid and only 5%-9% was converted to thromboxane B2 (a stable endproduct of the endoperoxide-thromboxane pathway). Indomethacin inhibited collagen-induced aggregation and MDA formation completely but did not abolish the release of arachidonic acid. The response to low (nonaggregating) concentrations of collagen was thus characterized by the release of arachidonic acid. The results support our hypothesis that the potentiation of platelet aggregability by a stimulant is at least partly due to some release of arachidonic acid from platelet membranes.3 There might be some threshold of arachidonic acid level beyond which arachidonic acid released can be utilized efficiently by the cyclooxygenase pathway to produce aggregating substances, endoperoxides, and thromboxanes. If a level of arachidonic acid released does not reach the threshold level, platelets may not aggregate and may be only potentiated. This potentiation is seen in the nephrotic syndrome.

Bang et al.4 suggested that enhanced aggregation in glomerular renal disease may arise as a consequence of loss in the urine of plasma proteins responsible for inhibition of aggregation. Silver et al.1 reported that albumin inhibits collagen-, ADP-, and arachidonic acid–induced aggregation and may be an important controlling factor in hemostasis. We found that enhanced platelet aggregation in the nephrotic syndrome was normalized in vitro by the addition of albumin to patient’s PRP (Table 1), and ex vivo by the correction of hypoalbuminemia by the treatment (Fig. 3). Furthermore, addition of albumin to the washed platelets from normal individuals inhibited collagen-induced aggregation and MDA formation (Table 2). The release of arachidonic acid or its metabolites into a surrounding medium decreased with the increase of the concentration of albumin when albumin concentration was more than 0.69 g/dl (Table 2). The complete absence of albumin, however, decreased the release instead of increasing the release. This may be explained by the fact that albumin is the main carrier protein for free fatty acids including arachidonic acid,17 and arachidonic acid cannot completely go into water phase (the surrounding medium) without the presence of albumin. In addition, Isakson et al.18 used a low concentration (0.1 g/dl) of fatty acid–free albumin to trap arachidonic acid released by hormonal stimulation from isolated perfused organs prelabeled with 14C-arachidonic acid and obtained a large release of radioactive materials. Thus the presence of albumin facilitates the release of free arachidonic acid into the surrounding medium, but at the same time albumin traps the free arachidonic acid and prevents it from being metabolized by cyclooxygenase, thus decreasing the levels of the metabolites, which are quite soluble and can diffuse out into the surrounding medium.9,13,15,16 In addition, under normal conditions ADP released by thromboxane A2 initiates further liberation of arachidonic acid,18 and it will be also metabolized to endoperoxides and thromboxanes. Therefore the decreased release of radioactivity observed at albumin concentrations greater than 0.69 g/dl (Table 2) probably reflected the decrease of the amounts of the metabolites formed and the decrease of the amount of arachidonic acid released secondarily by released ADP.

Enhanced platelet aggregation in the nephrotic syndrome may be partly attributed to a decrease of the amount of arachidonic acid trapped by albumin and an increase of arachidonic acid available for the synthesis of endoperoxides
and thromboxanes as a consequence of hypoalbuminemia. These explanations are compatible with the pathway of Lands et al.19 or isomerization of prostaglandin H2 into prostaglandin D2 in the presence of serum albumin20 and are in agreement with dose-dependent inhibition of aggregation as well as MDA formation by albumin (Tables 1 and 2). The potentiation of aggregation by low concentrations of collagen or epinephrine was short lived and decayed rapidly in PRP from healthy adults.3 The decay may partly be caused by the progressive binding of released arachidonic acid to albumin, since the bound arachidonic acid may no longer be available for the cyclooxygenase pathway to generate aggregating substances.

In fact, the potentiation by very low concentrations of stimulant was long lived in media of low albumin concentrations such as in PRP from patients with nephrotic syndrome (data not shown).

Our original study on the release of arachidonic acid as a platelet potentiator3 was corroborated by Kinlough-Rathbone et al., who advocated the role of arachidonic acid metabolites, endoperoxides and thromboxane A2, for platelet potentiation.21 The hypothesis that these metabolites are responsible for the potentiation of platelet aggregability is compatible with the finding that radioactive thromboxane B2 was detected in the milieu of the potentiated platelets that had been prelabeled with 14C-arachidonic acid. However, the major part of the radioactive compounds in the milieu remained as arachidonic acid itself. Furthermore, the potentiation could be prevented by an addition of albumin, which can trap arachidonic acid and thus prevent it from being metabolized by cyclooxygenase to endoperoxides and thromboxanes. Albumin cannot trap endoperoxides and thromboxanes and is not known to inhibit their activities. These findings suggest that the mechanism of release of arachidonic acid rather than its metabolites may be considered the key to the potentiation of platelet aggregability in normals as well as in those with pathologic conditions such as the nephrotic syndrome.

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ARACHIDONATE AND PLATELET POTENTIATION

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