The Role of Phospholipids in Platelet Function

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ABSTRACT

Platelet phospholipids undergo significant alterations during aggregation induced by thrombin or other agents. There is an early increase in phosphatidic acid, with a decrease in phosphatidyl inositol. De novo synthesis of most phospholipids from \(^{14}\)C-glycerol is decreased. Thrombin stimulates \(^{32}\)P-phosphate incorporation into di- and triphosphoinositides, suggesting increased phosphorylation of phosphatidyl inositol during aggregation.

Arachidonic acid for prostaglandin synthesis is released from platelet phospholipids. Thrombin induced aggregation results in release of arachidonic acid primarily from phosphatidyl choline and phosphatidyl inositol. The availability of free arachidonic acid may be regulated by platelet phospholipase A\(_2\) activity. The latter activity is stimulated by thrombin, requires calcium ions, and is inhibited by agents which elevate cyclic adenosine monophosphate.

Phospholipids are probably an essential component of the platelet surface lipoprotein procoagulant activity known as platelet factor 3. There is evidence that calcium ions may mediate binding between gamma carboxyglutamic acid residues on the amino terminal portion of prothrombin and negatively charged phosphate groups on phospholipid micelles. Binding of prothrombin to phospholipid on the platelet surface may orient the former such as to facilitate the prothrombinase activity of Factor Xa.

Platelet phospholipids and platelet factor 3 activity are decreased in some congenital and myeloproliferative disorders. Increases in these factors may be associated with thrombotic and arterial occlusive disorders.

Introduction

Platelet phospholipids may have a major role in several aspects of platelet function. Alterations in phospholipid composition and metabolism during aggregation may be vital to the membrane changes accompanying this process. Arachidonic acid released from platelet phospholipids by phospholipase A\(_2\) serves as the precursor for platelet prostaglandin synthesis. Platelet phospholipids may also accelerate some phases of the coagulation process by interacting with the plasma coagulation proteins.
Platelet Phospholipid Composition, Location and Metabolism

Phospholipids constitute approximately 13 percent of the dry weight of human platelets and 78 to 80 percent of the total lipids. The predominant species of phospholipids are phosphatidyl choline (38 to 42 percent), diacyl phosphatidyl ethanolamine (14 percent), plasmalogen phosphatidyl ethanolamine (14 to 18 percent), phosphatidyl serine (8 to 10 percent), phosphatidyl inositol (4 to 5 percent), sphingomyelin (15 to 17 percent), and lysophosphatidyl choline (1 percent). With the exception of sphingomyelin, which contains a high percentage of saturated fatty acids, particularly of 16 and 22 carbon chain length, most of the other phospholipids contain a predominance of unsaturated fatty acids.

The most functionally significant of these, arachidonic acid (20 carbons, four double bonds), appears to be in highest concentration in phosphatidyl inositol (33 to 42 percent) and phosphatidyl ethanolamine (32 to 37 percent). The contents of phospholipids and their fatty acids in the granule and membrane fractions of platelets appear to be similar to that of whole platelets, except for a lower concentration of phosphatidyl choline in the granule fraction. In another study, the alpha granules showed a higher ratio of plasmalogen phosphatidyl ethanolamine to diacyl phosphatidyl ethanolamine than did the whole platelet homogenates.

Selective hydrolysis during incubation of platelets with phospholipase C suggests that more phosphatidyl choline and sphingomyelin than other phospholipids are located in the outer membrane layer. Further studies regarding the localization of specific phospholipids within the platelet membrane were conducted by labelling them with 2,4,6 trinitrobenzenesulfonate (TNBS), which does not penetrate the cell surface. After 30 to 90 minutes of incubation with nonactivated platelets, only a small proportion of phosphatidyl ethanolamine reacted with TNBS; phosphatidyl serine did not react at all. After thrombin activation, there was increased labelling of phosphatidyl ethanolamine, but still no labelling of phosphatidyl serine. This pattern of TNBS labelling suggested that there is an asymmetric distribution of phospholipids in the platelet membrane, and that phosphatidyl ethanolamine may have a more significant in vivo role in hemostatic function than phosphatidyl serine.

Extension of these studies with phospholipid cross linking agents revealed clusters of phosphatidyl ethanolamine in the platelet membrane which could be critical for platelet function. The thrombin-induced increased reactivity of phosphatidyl ethanolamine with TNBS was not associated with any rearrangement of either phosphatidyl ethanolamine or phosphatidyl serine within the platelet membrane, suggesting that thrombin influences the reactivity of only those phospholipids on the platelet surface.

Human platelets can synthesize phospholipids de novo from phosphate, acetate, glycerol, and fatty acid precursors. Phosphatidyl ethanolamine synthesis in platelets occurs by reaction of cytidine diphasphoethanolamine with diglycerides, catalyzed by the enzyme ethanolamine phosphotransferase. Formation of phosphatidyl inositol in platelets occurs by formation of cytidine diphasphoglyceride from phosphatidic acid and cytidine triphosphate, followed by reaction of the CDP-diglyceride with myoinositol to form the inositides. The latter reaction is catalyzed by platelet CDP-diglyceride myoinositol transferase. Mechanisms for synthesis of the other phospholipids have not yet been studied in platelets.

When 14C-glycerol was incubated with platelets, incorporation occurred primar-
ily into phosphatidyl choline, phosphatidyl inositol, and phosphatidic acid; phosphatidyl serine and phosphatidyl ethanolamine were also labelled, but to a lesser extent. Thrombin decreased incorporation of glycerol into all phospholipids except for phosphatidyl serine, which showed a transient increase in labelling for 20 minutes. Other aggregating agents besides thrombin resulted in a decreased incorporation of glycerol into all classes of phospholipids, suggesting that this effect is not a specific thrombin mediated event, but rather is associated with the aggregation process. In other studies epinephrine induced aggregation was found to increase platelet phosphatidyl inositol synthesis. This occurred only following completion of the release reaction and was postulated to be a result of a reconstitution of membranes disrupted by secretion of the granules. Synthesis of platelet phosphoinositides was studied in greater detail in subsequent studies.

Phosphate labelled with \(^{32}\)P was incorporated most rapidly into diphosphoinositides and triphosphoinositides during the first hour of incubation. Labelling of phosphatidyl choline, phosphatidyl serine, and phosphatidyl ethanolamine was negligible during the first hour; however, at 12 hours these phospholipids contained 30 percent of the radioactivity. In a related study, it was found that human platelets incorporated \(^{32}\)P primarily into the polyphosphoinositides (di- and triphosphoinositide) where \(^{14}\)C glycerol did not appear in these phospholipids, suggesting that \textit{de novo} synthesis did not occur. Thrombin induced aggregation stimulated \(^{32}\)P incorporation into these phospholipids, but glycerol incorporation remained unchanged. These results suggested that only the phosphorylating reactions and not \textit{de novo} synthesis were stimulated by thrombin aggregation. The enzymes necessary for these reactions have all been shown to be localized in the platelet membrane.

When platelets, in which phosphatidyl inositol has been pre-labelled with \(^{32}\)P-phosphate, are activated by thrombin, there is an increased formation of labelled phosphatidic acid within two to eight seconds. This is accompanied by a simultaneous reduction in phosphatidyl inositol content, although within five to ten minutes there is an increase in labelling in phosphatidyl inositol. It was postulated that thrombin induces a very early stimulation of phospholipase C activity, resulting in degradation of phosphatidyl inositol, followed by phosphorylation of the diacylglycerol produced. These reactions may have an essential role in the membrane changes associated with the early phases of platelet activation, particularly the platelet shape transformation process. The time course of the changes in phosphatidic acid and phosphatidyl inositol show a close correspondence with platelet shape change. Agents which enhance shape change, such as fibrinogen and ADP, also increase phosphatidic acid formation.

Conversely, increasing cyclic adenosine monophosphate (cAMP) levels blocks the formation of phosphatidic acid and also inhibits shape change. Phosphatidic acid formation also seems to parallel thrombin-induced serotonin release. Previous evidence indicates that the platelet release reaction may be triggered by intracellularly bound calcium. The plasma membranes of thrombin treated platelets acquire an increased permeability to Ca\(^{2+}\) ions, and thrombin causes an increased turnover of platelet phosphatidic acid. Incubation of platelets with phosphatidic acid has also been shown to increase calcium permeability. It is, therefore, possible that the thrombin-induced release reaction is mediated by the ionophoretic properties of the resulting increased phosphatidic acid.
Phospholipid Arachidonic Acid as a Substrate for Prostaglandin Synthesis and Role of Phospholipase A<sub>2</sub>

Arachidonic acid is the major substrate for platelet prostaglandin synthesis. Greater than 95 percent of platelet arachidonic acid is esterified in phospholipids. The free carbonyl group of arachidonic acid is necessary for conversion to prostaglandins, and it has been observed that synthesis of the latter occurs most rapidly if phospholipids are first hydrolyzed. During aggregation up to 80 percent of platelet phospholipid arachidonic acid may be liberated and transformed. Thrombin treatment induces release of arachidonic acid primarily from phosphatidyl choline and phosphatidyl inositol, although other studies have reported some release from phosphatidyl serine and phosphatidyl ethanolamine also. After thrombin treatment, a marked increase in incorporation of labelled arachidonic acid into plasmalogens has been observed, and it was postulated that the latter may be an intermediate receptor of arachidonic acid transferred from phosphatidyl choline and phosphatidyl inositol.

Phospholipase A<sub>2</sub> activity has been detected in platelets, and there is indirect evidence that this activity may be localized on the inner membrane surfaces. Thrombin treatment stimulates phospholipase A<sub>2</sub> activity, and metabolic adenosine triphosphate (ATP) appears to be required for this stimulation. The release of arachidonic acid is independent of the platelet release reaction and therefore is not a function of activation of latent hydrolytic activity made available by the release reaction. Several lines of evidence suggest that platelet phospholipase A<sub>2</sub> has an absolute requirement for calcium ions. In the absence of external calcium, the ionophore, A23187, which mobilizes intracellular calcium, was five times more effective than thrombin in liberating arachidonic acid. Ionophore induced release of arachidonic acid does not require metabolic ATP and is independent of the release reaction. The requirement of calcium ions for phospholipase A<sub>2</sub> has been questioned by other investigators, who found that excess extracellular calcium actually inhibited both thrombin and ionophore A23187 stimulation of enzymatic activity. They suggest that the ability of the ionophore to stimulate phospholipase A<sub>2</sub> activity may actually be attributable to its ability to remove calcium ions from the site of enzyme activity, rather than the reverse.

There is evidence that platelet phospholipase A<sub>2</sub> may have a relative specificity for phosphatidyl choline, phosphatidyl inositol or phosphatidyl serine that contain arachidonic acid in the 2 position, thus selectively releasing this fatty acid rather than other long chain fatty acids which have inhibitory effects on prostaglandin synthesis.

Increased cyclic AMP can inhibit platelet aggregation induced by thrombin and other agents. It has been found that cAMP or agents which increase cAMP levels such as phosphodiesterase inhibitors or prostaglandins D<sub>2</sub>, E<sub>1</sub> or I<sub>2</sub> can inhibit platelet phospholipase A<sub>2</sub> activity and the resulting release of arachidonic acid. This inhibition of phospholipase A<sub>2</sub> by cAMP may be a mechanism for the regulation of the availability of precursors for synthesis of aggregatory agents by the platelet.

It has been theorized that phospholipase A<sub>2</sub> may have a key role in the processes of hemostasis and thrombosis. When vessel injury occurs, contact of collagen with platelet membranes may activate phospholipase A<sub>2</sub>, with subsequent increased availability of arachidonic acid to platelet cyclooxygenase and thromboxane synthetase, leading to platelet aggregation (figure 1). At the periphery of the vessel injury site, more of the arachidonic acid may be converted by in-

tact endothelial cells to prostaglandins H₂ and I₂, which then stimulate adenyl cyclase and increase cAMP levels. This in turn may inhibit phospholipase A₂ activity and limit further release of arachidonic acid.

Interaction of Platelet Phospholipids with Plasma Coagulation Factors

Removal of platelets from plasma is known to prolong clotting times, and the latter can then be shortened by addition of phospholipids. Platelet phosphatidyl serine is the most active in this regard, followed by phosphatidyl ethanolamine. The activity of phosphatidyl serine is less than that of whole disrupted platelets, however, and even addition of the total phospholipids extracted from platelets shortens the clotting time less than the platelets from which they were derived. In another study, it was found that 500 μg of extracted membrane phospholipids were required to produce an 11 second clotting time, whereas only 22.5 μg of platelet membrane vesicles were required for the same time when substituted for the extracted phospholipids.

It is clear that phospholipids are only one component of the in vitro procoagulant activity of the platelet lipoproteins surface usually referred to as platelet factor 3. Nevertheless, it has been found that negatively charged phospholipid surfaces in the form of liposomal vesicles can provide clot promoting activity approximating that of platelet membranes and are a valuable model for the study of the binding of plasma coagulation proteins. In these vesicles, the polar negatively charged phosphate groups are outwardly oriented towards the aqueous phase, whereas the non-polar long chain fatty acids are oriented inwardly (figure 2).
phospholipids may catalyze the coagulation reactions by binding the proteins and orienting them at the lipid-water interface.

The binding of coagulation factors and clot promoting activity of phospholipid micelles depends primarily on the net negative charge of these vesicles and not on the specific chemical structure of the phospholipids. The optimal charge per unit of surface area is also of importance, and again is independent of the type of phospholipid. The degree of unsaturation of the acyl residues of the phospholipids also is directly proportional to their clot promoting activity; this is probably a function of the relative ease with which large aggregates of unsaturated phospholipids can be dispersed into smaller particles as compared to saturated phospholipids, thus increasing the reactive surface.

Interaction of platelets with appropriate concentrations of thrombin, adenosine diphosphate (ADP), collagen or other agents results in a "release reaction" whereby ATP, ADP, serotonin, catecholamines, and other granule contents are secreted into the plasma. It has been suggested that during this process a phospholipoprotein unit membrane is exposed, which functions as a catalytic surface to accelerate coagulation. The phospholipids of these platelet lipoproteins have been implicated primarily in their interaction with activated factor X (Xa), factor V and Ca++ ions to form a prothrombinase which converts prothrombin to thrombin. Factor X and Xa have been shown to bind to negatively charged phospholipid micelles in the presence of Ca++. Factor Xa binds to platelets only after the release reaction has occurred, exposing the receptor sites (it has been estimated that there are 200 Xa receptor sites per platelet). The binding of Xa to platelets following the release reaction results in a 50,000 fold increase in the thrombin generating activity of Xa. This is in contrast to the only 50 fold increase in Xa activity that results when phospholipids are substituted for platelets. There is recent convincing evidence to indicate that the receptor site for Xa on the platelet surface is factor V rather than phospholipid. Factor V can bind to the surface of phospholipid bilayers, but it is more susceptible to inactivation when bound to phospholipid than when free. It is possible that this binding is a mechanism for regulating and decreasing activation of prothrombin rather than a procoagulant function.

Perhaps the best evidence for a role of phospholipid in thrombin generation is its capacity to bind prothrombin. This binding occurs only in the presence of Ca2+ or other divalent cations. The NH2 terminal region of prothrombin (known as Prothrombin Fragment 1) appears to be the region that mediates the binding to phospholipid. Isolated Prothrombin Fragment 1 can compete with the whole prothrombin molecule for phospholipid and thereby inhibit thrombin production. Prothrombin Fragment 1 contains the unique amino acid gamma carboxyglutamic acid; abnormal prothrombin lacking this amino acid has been demonstrated not to bind Ca++ or phospholipid. Structural investigations have shown that the first 10 glutamic acid residues from the NH2 terminal end of prothrombin or Prothrombin Fragment 1 are gamma carboxylated.

Recently, the physico-chemical characteristics of the binding of Prothrombin Fragment 1 to phospholipid have been investigated. The binding of Prothrombin Fragment 1 to vesicles of dioleoyl phosphatidyl choline and dioleoyl phosphatidyl glycerol was reversible and did not alter the vesicles, suggesting that binding probably occurred on the surface of the outer monolayer. Binding was proportional to Ca++ concentration in the range of 0.5 to 2.7 mM. The number of
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The binding sites on the phospholipid vesicles was dependent on the number of negatively charged phospholipid molecules on the external surface. When the ionic strength was increased, this resulted in a decrease in the Prothrombin Fragment 1—phospholipid association constant, suggesting an electrostatic mechanism for the binding.

A molecular model was proposed whereby 20 carboxylate groups of Prothrombin Fragment 1 are linked by Ca++ bridges to 20 phosphate groups of the phospholipid on the outer vesicular surface (figure 2). The transient local increase of Ca++ associated with the release reaction induced by platelet contact with collagen could promote the binding of prothrombin to exposed platelet or vascular tissue cell phospholipid in appropriate in vivo situations.

Relationships of Platelet Phospholipids to Disease, Diet and Drugs

Situations in which platelet factor 3 activity and/or platelet phospholipids are altered are listed in table I. Most patients with congenital hemostatic problems associated with impaired platelet factor 3 activity also have defective platelet release reactions, and the decreased platelet factor 3 may be secondary to the latter.27,77 Some of these patients have an abnormal platelet lipid pattern, with a relative decrease in phosphatidyl ethanolamine and an increase in phosphatidyl choline.68 Associated abnormalities in fatty acid composition were observed, with relative increases in the 18 and 20 carbon saturated and monounsaturated oleic, arachidic and eicosanoic acids, and decreases in the 22 carbon polyunsaturated docosatetraenoic acid and the 24 carbon monounsaturated nervonic acid.

Other studies have reported normal phospholipid composition in these patients and normal release of phospholipids after lysis in distilled water.35 Congenital impairment of platelet factor 3 activity has also been reported without any primary release or aggregation defect,25,36 and in association with glucose 6 phosphate dehydrogenase deficiency,71 and thrombasthenia.79 Some acquired situations where decreased platelet factor 3 availability can be hemostatically significant are in uremia,63,76 thrombocytopenia,74 poly-cythemia,1 myeloid metaplasia,21 dys-proteinemias,21,78 liver disease,48,78 and immune thrombocytopenias.19 No abnormalities of platelet lipids in uremia were observed in one study,2 but in another it was found that platelet phospholipids, particularly phosphatidyl serine and phosphatidyl choline, were decreased in uremia, polycythemia vera, myeloid metaplasia, and other disorders.35 A patient with an inhibitor against phospholipid procoagulant activity has been reported.60 The coagulation time in the

<table>
<thead>
<tr>
<th>TABLE I</th>
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<tr>
<td>Alterations in Platelet Factor 3 and/or Platelet Phospholipids</td>
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<tr>
<td>I. Decreased</td>
</tr>
<tr>
<td>A. Congenital</td>
</tr>
<tr>
<td>1. Associated with defective release reaction</td>
</tr>
<tr>
<td>2. Isolated deficiency</td>
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<tr>
<td>3. Glucose 6 phosphate dehydrogenase deficiency</td>
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<tr>
<td>4. Thrombasthenia</td>
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<tr>
<td>B. Acquired</td>
</tr>
<tr>
<td>1. Uremia</td>
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<tr>
<td>2. Thrombocytopenia</td>
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<tr>
<td>3. Polycythemia vera</td>
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<tr>
<td>4. Myeloid metaplasia</td>
</tr>
<tr>
<td>5. Dysproteinemias</td>
</tr>
<tr>
<td>6. Liver disease</td>
</tr>
<tr>
<td>7. Immune thrombocytopenias</td>
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<tr>
<td>8. Circulating inhibitor to phospholipid</td>
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<tr>
<td>II. Increased</td>
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<tr>
<td>A. Venous thrombosis</td>
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<tr>
<td>B. Atherosclerotic disorders</td>
</tr>
<tr>
<td>1. Type II hyperlipoproteinemia</td>
</tr>
<tr>
<td>2. Ischemia heart disease</td>
</tr>
<tr>
<td>3. Cerebrovascular accidents</td>
</tr>
<tr>
<td>III. Decreased Therapeutically</td>
</tr>
<tr>
<td>A. Diet-polyunsaturated fat</td>
</tr>
<tr>
<td>B. Drugs</td>
</tr>
<tr>
<td>1. Acetylsalicylic acid</td>
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<tr>
<td>2. Dipyridamole</td>
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<td>3. Clofibrate</td>
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<td>4. Halofenate</td>
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Platelet factor 3 assay was prolonged with the patient's platelets or with substitution of the phospholipids inosithin, phosphatidyl ethanolamine or phosphatidyl choline for the platelets.

Conversely, there is evidence for increased activity or alteration of platelet factor 3 phospholipids in disorders associated with thrombosis or atherosclerosis. In surgical patients developing venous thrombosis, total platelet phospholipids were higher both before and after surgery than in the non-thrombotic group. Patients with type II hyperlipoproteinemla were found to have increased platelet factor 3 present in their plasma and also released more platelet factor 3 after ADP stimulation than did normals. The platelets of the type II patients were also found to have an elevated total phospholipid content. Platelet factor 3 activity is also increased in patients with ischaemic heart disease.

Platelets from patients with cerebrovascular accidents also have increased clotting activity and an increased ratio of phospholipids to proteins. Their phospholipids show an increased palmitoleic acid content and a decrease in linoleic acid, a polyunsaturated fatty acid. Phosphatidyl ethanolamine is decreased in platelets of patients with chronic arterial occlusions, and the plasma concentration of this same phospholipid is increased, suggesting possible release from the platelets.

Animal experiments have indicated that platelet reactivity can be altered by dietary means. Rats fed a saturated fat or butter containing diet showed higher platelet factor 3 activities than those fed a corn oil containing diet. The saturated fat-fed rats had an increased platelet phospholipid oleic acid content. In vitro studies indicate that dioleoyl phosphatidyl choline has greater procoagulant activity than phosphatidyl choline composed of other fatty acid species. Human studies have revealed comparable findings.

Platelet factor 3 activity was increased in subjects on a saturated fat containing diet, whereas an unsaturated fat containing diet had the opposite effect.

Several drugs which inhibit platelet function also have been found to alter their phospholipid content. Acetylsalicylic acid (ASA) after either in vivo administration or in vitro incubation decreased platelet phospholipids by 22 percent; sphingomyelin was reduced by 17 to 27 percent and phosphatidyl choline by 10 to 12 percent. Dipyridamole, another inhibitor of platelet aggregation, was also found by these investigators to reduce platelet sphingomyelin by 38 percent and phosphatidyl choline by 21 percent. Clofibrate therapy of hyperlipidemia patients was found to decrease platelet total lipid content, to reduce the platelet factor 3 activity of platelet homogenates, and to inhibit the platelet release reaction. Incorporation of 14C-acetate into platelet phospholipids was reduced, but 14C-glucose incorporation was unchanged.

Hallofenate free acid, the metabolite of halofenate, another hypolipidemic agent, also decreased platelet factor 3 activity and decreased incorporation of both labeled acetate and glucose into phospholipids. Membrane receptor or ion transport changes resulting from these alterations conceivably could contribute to the inhibition of the platelet release reaction and aggregation by some of these drugs.

Acknowledgment

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