Phospholipid Transfer Between Plasma and Platelets In Vitro

By J. Heinz Joist, Georgina Dolezel, John V. Lloyd, and J. Fraser Mustard

Washed rabbit platelets were resuspended in plasma in which all of the major phospholipids had been isotopically labeled by injection of \(^{32}\text{PO}_4\) into rabbits. At certain time intervals during a 6-hr incubation at 37°C, aliquots were removed from the incubation mixture and the platelets were isolated and subjected to lipid extraction and phospholipid analysis. A continuous rise in platelet non-lipid-bound and lipid-bound radioactivity was observed throughout the incubation period. Two platelet phospholipids, lecithin and lyssolecithin, were significantly labeled, whereas little or no labeling of the other phospholipids was found. There was no detectable change in total or individual platelet phospholipid content. At 6 hr, 4% of total platelet phospholipid, 43% of platelet lyssolecithin, and 7% of platelet lecithin were labeled. Platelets incubated in plasma from rabbits with diet-induced hyperlipidemia took up and incorporated significantly more label into their phospholipids than did platelets in normal plasma. Labeling of both platelet lyssolecithin and lecithin could be due to uptake and metabolism of plasma lyssolecithin by platelets. However, labeling of platelet lecithin could at least in part be the result of direct exchange of this phospholipid with the plasma. Uptake and incorporation of endogenous plasma lyssolecithin by platelets and, possibly, direct exchange of platelet lecithin may be important mechanisms in the modification by plasma lipids of platelet membrane phospholipid fatty acid composition and platelet function.

There is evidence that an increase in plasma lipids in man may be associated with increased platelet factor 3 availability, increased response of platelets to aggregation- or release-inducing stimuli, and increased platelet turnover. Platelet adherence to the vessel wall and aggregation have been shown to be among the primary events in the formation of arterial thrombi, which are involved in causing some of the clinical complications of atherosclerosis. Thus, increased platelet sensitivity to aggregation- and release-inducing stimuli could be an important link between hyperlipidemia and atherosclerosis. The mechanism by which plasma lipids influence platelet function has not been determined.

In rats, diet-induced changes in the plasma fatty acid composition may lead to corresponding changes in the platelet fatty acid composition and such changes may be associated with increased thrombin-induced platelet aggregation and increased susceptibility to endotoxin-induced hepatic vein thrombosis. It has also been demonstrated in experimental animals and in man that such diet-induced changes in the platelet fatty acid composition occur pre-
dominantly in the platelet membrane phospholipids. This alteration could result from a direct exchange of fatty acids between phospholipids of plasma and platelets, a mechanism for which evidence has not been reported. We have examined a second possibility, i.e., whether preformed phospholipid molecules can exchange between plasma and platelets, a mechanism known to be operative in erythrocytes.14-16

MATERIALS AND METHODS

Chemicals

Carrier-free $^{32}$P0₄ was obtained from Atomic Energy of Canada, Ltd., Ottawa, Canada, as the phosphate ion in 0.1 N HCl. Organic solvents were reagent grade and were redistilled before use. Phospholipids used as reference materials for thin-layer chromatography (TLC) were stored at -20°C, either as the powder or as a solution in benzene. The powder was dissolved in CHCl₃:CH₃OH:HCl (200:100:0.1) prior to TLC. Phosphatidyl ethanolamine (PE) (bovine), phosphatidylserine (PS) (bovine), phosphatidylcholine (PC) (egg), lyso phosphatidylcholine (LPC) (egg), sphingomyelin (SPH) (bovine), and glycerophosphorylcholine (GPC) were purchased from Supelco, Bellefonte, Pa. TLC of 100 µg of each compound showed no contamination with other phospholipids. Inositol phosphate (MPI) was obtained from Koch-Light Laboratories, Buckinghamshire, England. TLC of 50 µg of this compound yielded a single spot with iodine vapor but was not stained with ninhydrin.

Adenosine diphosphate (ADP) was purchased as the sodium salt from Sigma Chemical Co., St. Louis, Mo., and dissolved in Tyrode solution, pH 7.3, modified to contain no calcium or magnesium ions.17

Labeling of Rabbit Plasma Phospholipids With $^{32}$PO₄ In Vivo

In preliminary experiments the time course of labeling of the plasma or platelet phospholipids was investigated. $^{32}$PO₄ (0.5 mCi/kg) was diluted with 2.0 ml 0.85% NaCl solution, the solution was neutralized by the dropwise addition of 0.1 N NaOH, and immediately injected into an ear vein of a New Zealand albino rabbit. In later experiments the dose of $^{32}$PO₄ was increased to 2.0 mCi/kg, and the material was injected intraperitoneally instead of intravenously since this gave a 50% increase in the extent of the labeling. Ten-milliliter blood samples were collected from an ear artery into one-sixth volume of acid citrate dextrose (ACD) solution after 18 hr and at 24-hr intervals thereafter. The platelets were isolated by differential centrifugation, washed twice with 1% EDTA in modified Tyrode solution (containing no Ca²⁺ or Mg²⁺), and lipid extraction of the final platelet sediment and of 0.8-mI aliquots of platelet-free plasma (prepared by centrifugation of platelet-poor plasma at 15,000 g for 1 min) was carried out. The aqueous phase was discarded and lipid-phase radioactivity and phosphorus content were determined.

Preparation of Platelet-free Plasma (PFP) Labeled In Vivo With $^{32}$PO₄

Blood was collected from anesthetized (sodium pentobarbital 30 mg/kg) rabbits (after a 12-hr fasting period) through a carotid artery cannula into ACD solution, using plastic syringes and tubes. The blood was centrifuged at 1500 g for 20 min to yield platelet-poor plasma (PPP), which was separated and again centrifuged at 100,000 g for 30 min in a Beckman ultracentrifuge to remove any remaining platelets or platelet-derived membranous material not sedimented by conventional high speed centrifugation.18 Chylomicron-rich plasma (0.5-1.0 ml) floating at the top was carefully removed from all plasma samples to avoid turbidity differences which would have affected tests of platelet aggregation to be carried out during the incubation experiments. PFP was then dialyzed at 5°C for 36 hr against 150 volumes of Tyrode solution containing 0.38% sodium citrate with three changes of the dialysate to remove free $^{32}$PO₄. Two-milliliter and 0.8-mI aliquots of dialyzed PFP were taken for automated cholesterol and triglyceride determination (Technicon N method)* and phospholipid analysis.

*We are indebted to Dr. Maurice Mishkel, McMaster University, Lipid Research Center, Hamilton General Hospital, for kindly performing these procedures.
PHOSPHOLIPID TRANSFER

Incubation Experiments

Platelets were isolated from blood of normal rabbits collected into ACD solution according to the method of Ardlie et al. After the second wash, they were resuspended into dialyzed 32P-labeled PFP in a siliconized glass vessel that had been prewarmed to 37°C for 10 min. Prior to the addition of the platelets, glucose (1 mg/ml final concentration), penicillin G (50 U/ml final concentration), and streptomycin (50 µg/ml final concentration) were added to the PFP, and the pH was adjusted to 7.35 by an automatic titrometer (Radiometer, Copenhagen, Denmark) that regulated the delivery of either room air or a mixture of room air and CO2 into the incubation vessel. This system provided a means of maintaining the pH between 7.32 and 7.38 throughout the incubation period, while the PRP was slowly stirred. Complete resuspension of the platelets was accomplished within 3-6 min. After 6 min, 3 hr, and 6 hr of incubation, two 3.0-ml aliquots of PRP were removed from the incubation mixture and centrifuged in an Eppendorf centrifuge at 15,000 g for 1 min. The supernatant plasma was removed, and aliquots were taken for lactic dehydrogenase (LDH) determination. The platelet sediments were washed twice with modified Tyrode solution containing 1% EDTA, and the platelet phospholipids were extracted. At the time intervals indicated, 1.0-ml samples of PRP were also taken for measurement of platelet aggregation.

Platelet Aggregation

Platelet aggregation upon the addition of 0.1 ml ADP (5 µM final concentration) was studied by a turbidimetric method described previously, using 1.0-ml aliquots of PRP. The output of the aggregometer (Payton Associates, Scarborough, Ont.) and sensitivity of the recorder were adjusted so that PRP had a light transmission value of 0 and PFP of 100%. The maximum height of the aggregation curve was measured in chart paper units and expressed as percentage change in optical density (OD).

Extraction of Phospholipid

Platelet or plasma phospholipids were extracted by the method of Bligh and Dyer as modified by Lloyd et al. Several additional modifications were introduced. PFP (0.8 ml) or platelet sediment from 3.0 ml of PRP resuspended in 0.8 ml of distilled H2O were transferred to a 12-ml graduated centrifuge tube. CHCl3 (1.0 ml) was added, followed after brief mixing by 2.0 ml of CH3OH. The contents were mixed and left for a minimum of 1 hr at room temperature with occasional remixing. The extract was then partitioned into two phases by adding 1.0 ml CHCl3 and 1.0 ml of 0.1 N HCl, mixing, and centrifuging at 1500 g for 10 min. The volume of the upper phase was recorded and 0.1 ml taken for measurement of radioactivity. The remainder of the upper phase was removed and discarded. (In one experiment the upper phases were stored at -20°C for concentration and paper chromatography.) The lower phase was removed with the aid of a Pasteur pipette (pierced through the interphase particles) and passed through a glass wool column to remove any contaminating interphase particles. The residual interphase protein was mixed with 2.0 ml CHCl3, left for 10 min at room temperature, and centrifuged at 1500 g for 10 min. As a result of this procedure, the protein formed a firm sediment on the bottom of the tube, so that the supernatant extract could be removed almost completely and combined with the previously separated lower phases after passing it through the same glass wool column. The column was rinsed with an additional 1.0 ml of CHCl3 which was added to the previous extraction mixture. The volume of the total lower phase extract was recorded, and 0.1 ml and 0.5 ml were taken for determination of radioactivity and phosphorus, respectively. The percentage recovery of phospholipid phosphorus during lipid extraction by this method was as follows: LPC 83, SPH 86, PC 93, PS 80, PE 95. The remaining lipid extract was incubated at 37°C and concentrated by evaporation of the solvent under nitrogen. The lipid was then dissolved in 0.2 ml CHCl3:CH3OH (2:1) for subsequent separation by TLC.

Thin-Layer Chromatography

Silica gel H (E. Merck, Canlab, Toronto, Ont.) as a slurry in borate buffer was spread to a thickness of 0.3 mm on glass plates. Before the samples were applied, the plates were activated by heating them in an oven at 95°C for 90 min. Cooling of the plates and subsequent application of the lipid extract were carried out under dry nitrogen in a perspex chamber. The lipids were separated by two-dimensional chromatography. The first solvent (CHCl3:CH3OH:CH3COOH:H2O
(30:15:8:1.6)) was allowed to proceed until the front of the solvent had reached the upper edge of the plate. The plate was then removed from the tank, dried in a stream of cold air, equilibrated under dry nitrogen for 30 min, and developed in the second solvent (CHCl₃:CH₃OH:7N NH₄OH:H₂O (17.5:30:0.35:0.9)) for the same distance.

**Detection and Identification of Phospholipids After Thin-Layer Chromatography**

Phospholipids were detected by exposing the plates to iodine vapor. The spots were identified by comparison of the mobility on TLC in the same system of known pure phospholipid reference compounds.

**Phosphate Assay**

The amount of phosphorus in phospholipids separated by TLC was determined according to the method of Rouser.

**Liquid Scintillation Counting**

Most samples were counted for 4 min, but samples with low radioactivity were counted again for 20 min, and the results were expressed as cpm (background subtracted). Samples (0.1 ml) of PRP or PFP were added to scintillation vials and mixed with 0.1 ml NCS solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.), followed by the addition of 2.0 ml absolute ethanol and 10 ml of scintillator solution containing 5 g of 2.5 diphenyloxazole and 0.3 g of 2, 2-1-phenylenebis (5-phenyloxazole) in 1 liter of toluene. Samples (0.1 ml) of upper phase or lower phase were directly mixed with 2.0 ml ethanol and 10.0 ml scintillator solution. Samples that had been subjected to phosphate assay were counted by Cerenkov radiation after decolorizing the samples by the method of Palmer. The counting efficiency in this system was 37%. Samples from each incubation experiment were counted on the same day.

**Paper Chromatography for Identification of Labeled Compounds in Aqueous Phase**

In one experiment the upper phases from duplicate platelet extracts derived from samples taken at 6 min, 3 hr, and 6 hr were combined and lyophilized. The material recovered was dissolved in 0.5 ml of distilled H₂O, and 0.1 ml of the solution was applied to Whatman No. 1 filter paper together with pure glycerophosphorylcholine (GPC) reference compound. Descending chromatography in N-propanol: ammonia: H₂O (6:3:1) was then carried out until the front of the solvent had reached a distance of 35 cm past the point of application of the sample. The paper was dried in a stream of cold air and stained for phosphorus according to the method of Vorbeck and Marinetti. The spots corresponding to the points of application of the samples and those which had newly appeared on the chromatogram were cut out first and the rest of the chromatogram was cut into pieces of a similar size. Each piece of the chromatogram was placed in a separate scintillation vial to which 5 ml of distilled H₂O were added. The vials were sealed and kept in a shaking device for 12 hr at room temperature. The pieces of paper were then removed from the vials and the eluates remaining in the vials were lyophilized. Two milliliters of 95% ethanol and 10.0 ml scintillator solution were then added to each vial and radioactivity was determined.

**Platelet Lysis**

This was studied by determining the amount of LDH lost from the platelets into the plasma during the incubation experiments. LDH was measured according to the method of Bergmeyer et al. The LDH value determined in dialyzed PFP was subtracted from the values measured in the supernatant PFP of the samples taken from the incubation mixtures after the platelets had been removed by centrifugation at 15,000 g for 1 min. The corrected values were expressed as percentage of that obtained using a sonicated sample of the incubation mixture after subtraction of the value of dialyzed PFP.
Fig. 1. Labeling of plasma (——) and platelet (---) phospholipids after intravenous injection of $^{32}$P-orthophosphate into a rabbit. $^{32}$PO$_4$ (0.5 mCi/kg) was injected into an ear vein of a New Zealand albino rabbit and serial 10-ml blood samples were collected into one-sixth volume of ACD solution through the ear artery at 18 hr and every 24 hr thereafter over a period of 6 days. The platelets were isolated by differential centrifugation and washed twice, and duplicate samples were subjected to lipid extraction. Duplicate samples of dialyzed PFP (obtained by centrifugation of PPP at 15,000 g for 1 min) were also subjected to lipid extraction. Radioactivity and phosphorus content of the lipid phases of the extracts were determined, and the specific activities (cpm/µg P) were determined for both platelet and plasma phospholipids. The data of one of two experiments with similar results are shown.

Dietary Induction of Hyperlipidemia in Rabbits

Six New Zealand albino rabbits were given a daily diet consisting of 170 g of regular chow (Ralston Purina Company of Canada) mixed with 30 g of frozen egg yolk (Canada Packers, Toronto, Ont.). Pellets were prepared in advance and stored at $-20^{\circ}$ C. The animals received water ad libitum. They were kept on this diet for a minimum of 16 wk. Lipid analysis of dialyzed PFP obtained from these rabbits revealed a mean cholesterol value of 364 ± 162 (SD) mg/100 ml (control rabbits 21 ± 4 mg/100 ml), a mean triglyceride value of 82 ± 16 mg/100 ml (control rabbits 61 ± 12 mg/100 ml), and phospholipid value of 214 ± 38 mg/100 ml (control rabbits 72 ± 2 mg/100 ml).

RESULTS

After intravenous administration of $^{32}$P-orthophosphate to rabbits maximum labeling of plasma phospholipids was found between 18 and 24 hr, whereas platelet phospholipid labeling reached its maximum around 72 hr (Fig. 1). Labeling of plasma phospholipids was subsequently shown to be considerably increased by increasing the dose of $^{32}$PO$_4$ from 0.5 to 2 mCi/kg and by intraperitoneal instead of intravenous injection.

Dialysis of $^{32}$P-labeled PFP against 50 volumes of citrated Tyrode solution (with three changes of the dialysate) over 36 hr resulted in the loss of more than 60% of the total plasma radioactivity (presumably representing either $^{32}$PO$_4$ or $^{32}$P-labeled small molecules (Table 1). Further dialysis was not associated with a significant further loss of radioactivity. When dialyzed $^{32}$P-PFP was subjected to lipid extraction, approximately 80% of the plasma radioactivity was recovered in the total extract. Most of the radioactivity was found in the lipid phase, but a small fraction (6.3%–14.4% of the total dialyzed plasma radioactivity) was in the aqueous phase (Table 1).
Table 1. Distribution of Radioactivity in Lipid Extracts of Dialyzed Rabbit Platelet-free Plasma Labeled In Vivo With $^{32}$P-Orthophosphate

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Platelet-free Plasma Before Dialysis</th>
<th>After Dialysis</th>
<th>Plasma Extract Aqueous Phase</th>
<th>Lipid Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>256,339</td>
<td>87,113</td>
<td>5,512 (6.3)</td>
<td>67,373 (77.4)</td>
</tr>
<tr>
<td>2</td>
<td>224,471</td>
<td>75,614</td>
<td>7,489 (9.9)</td>
<td>52,299 (69.2)</td>
</tr>
<tr>
<td>3</td>
<td>317,470</td>
<td>130,205</td>
<td>18,793 (14.4)</td>
<td>85,918 (66.0)</td>
</tr>
</tbody>
</table>

$^{32}$PO$_4$ (2.0 mCi/kg) was injected through an ear vein (experiments 1 and 2) or intraperitoneally (experiment 3) into rabbits. Blood was collected into ACD solution 21 hr later and platelet-free plasma (PFP) was prepared by differential centrifugation (see Methods). PFP was dialyzed against 150 volumes of Tyrode solution containing 0.38% sodium citrate for 36 hr at 5°C, with three changes of the dialysate. Dialyzed $^{32}$P-labeled PFP was subjected to lipid extraction and the extract partitioned; 0.1-ml aliquots of PFP (before and after dialysis) or lipid phase of the extracts were taken for determination of radioactivity. The figures are the means of duplicate measurements expressed as cpm/ml PFP or cpm/aqueous or lipid phase derived from 1 ml PFP. The numbers in parentheses represent recovery of radioactivity in aqueous or lipid phase as a percentage of that present in PFP.

When the individual phospholipid fractions were separated by TLC, all of the major identifiable phospholipids contained considerable amounts of radioactivity (Table 2). The highest specific activity (SA) was found in PE, followed by PS, PC, and LPC, while the labeling of SPH and MPI was less.

Table 3 summarizes the results of three experiments in which unlabeled washed rabbit platelets were incubated at 37°C in dialyzed PFP in which the phospholipids had been labeled with $^{32}$P in vivo. At 6 min there was a measurable amount of $^{32}$P in the lipid phase of the extracts (expressed as percentage of lipid-bound plasma $^{32}$P). This level was further increased at 3 and 6 hr. There was little $^{32}$P in the aqueous phase of the extracts at 6 min, but $^{32}$P in this fraction (expressed as percentage of total extracted plasma $^{32}$P) increased significantly during further incubation. When washed platelets incubated in dialyzed $^{32}$P-PFP for 3 hr were washed with either (1) EDTA-Tyrode solution, (2) EDTA-Tyrode solution containing 0.35% bovine serum albumin, or (3) unlabeled EDTA-PFP, similar amounts of platelet lipid-bound radioactivity were observed (1.24%, 1.31%, and 1.19%, respectively).

Table 3 also shows that the increase in $^{32}$P in the lipid phase was associated with an increase in phospholipid specific radioactivity. No significant change in

Table 2. Percentage Distribution of Phosphorus in, and Labeling of Individual Phospholipids in Dialyzed Plasma Obtained From Rabbits 21 hr After Injection of $^{32}$P-Orthophosphate

<table>
<thead>
<tr>
<th>Exp.</th>
<th>LPC SA %</th>
<th>PH SPH SA %</th>
<th>PC SA %</th>
<th>PS SA %</th>
<th>MPI SA %</th>
<th>PE SA %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>799</td>
<td>19.0</td>
<td>454</td>
<td>4.1</td>
<td>852</td>
<td>67.7</td>
</tr>
<tr>
<td>2</td>
<td>703</td>
<td>22.9</td>
<td>291</td>
<td>3.0</td>
<td>783</td>
<td>64.9</td>
</tr>
<tr>
<td>3</td>
<td>1176</td>
<td>20.5</td>
<td>722</td>
<td>3.9</td>
<td>1153</td>
<td>70.7</td>
</tr>
</tbody>
</table>

Aliquots (0.8 ml) of PFP were subjected to lipid extraction and two-dimensional TLC of the concentrated lipid phase material was carried out. The iodine-stained spots were scraped and phosphorus content and radioactivity were determined. Recovery of total radioactivity was greater than 90%. The specific radioactivities (SA) (cpm/µg P) were calculated from the actual cpm values. The figures for phosphorus distribution were derived from the means of duplicate sample analyses.

*In two experiments the spots for PS and/or MPI were accidentally not scraped. The percentages calculated for the other phospholipids are, therefore, slightly higher due to the approximately 5% loss of total phospholipid phosphorus.
PHOSPHOLIPID TRANSFER

Table 3. Transfer of Radioactivity From Dialyzed Rabbit Platelet-free Plasma Labeled In Vivo With 32P-Orthophosphate to Washed Rabbit Platelets

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1 1.35</td>
<td>0.1</td>
<td>0.07</td>
<td>0.57</td>
<td>2425</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>0.47</td>
<td>1.08</td>
<td>2425</td>
<td>35</td>
<td>301</td>
</tr>
<tr>
<td>6</td>
<td>1.02</td>
<td>1.97</td>
<td>103</td>
<td>103</td>
<td>4.25</td>
</tr>
<tr>
<td>2 1.04</td>
<td>0.1</td>
<td>0.10</td>
<td>0.67</td>
<td>2304</td>
<td>38</td>
</tr>
<tr>
<td>3</td>
<td>0.96</td>
<td>1.24</td>
<td>65</td>
<td>65</td>
<td>2.82</td>
</tr>
<tr>
<td>6</td>
<td>1.71</td>
<td>1.66</td>
<td>82</td>
<td>82</td>
<td>3.56</td>
</tr>
<tr>
<td>3 0.85</td>
<td>0.1</td>
<td>0.17</td>
<td>0.59</td>
<td>3048</td>
<td>64</td>
</tr>
<tr>
<td>3</td>
<td>0.82</td>
<td>0.89</td>
<td>101</td>
<td>101</td>
<td>3.31</td>
</tr>
<tr>
<td>6</td>
<td>0.97</td>
<td>1.17</td>
<td>128</td>
<td>128</td>
<td>4.20</td>
</tr>
</tbody>
</table>

Platelet non-lipid-bound 32P is that determined in the aqueous phase of the partitioned platelet extracts and is expressed as a percentage of the sum of the total aqueous and lipid phase 32P of the extract of 3.0 ml PFP. Platelet lipid-bound radioactivity is that detected in the lipid phase of the partitioned platelet extract and expressed as a percentage of that present in the lipid phase of the partitioned extract of 3.0 ml PFP. The relative specific radioactivity (Rel. SA) of platelet phospholipids was determined by dividing the specific radioactivity of the platelet phospholipids by the specific radioactivity of the plasma phospholipids (x 100). The results indicate that percentage of the platelet phospholipids which is derived from the plasma phospholipids.

Table 4. Labeling of Individual Platelet Phospholipids During Incubation of Unlabeled Rabbit Platelets in Rabbit Plasma Labeled In Vivo With 32P-Orthophosphate

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Incubation Time (hr)</th>
<th>LPC</th>
<th>SPH</th>
<th>PC</th>
<th>PS</th>
<th>MPI</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1</td>
<td>85</td>
<td>165</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>114</td>
<td>258</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>147</td>
<td>216</td>
<td>0</td>
<td>0</td>
<td>343</td>
<td>60</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>155</td>
<td>356</td>
<td>0</td>
<td>101</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>218</td>
<td>410</td>
<td>0</td>
<td>0</td>
<td>314</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>225</td>
<td>409</td>
<td>0</td>
<td>0</td>
<td>369</td>
<td>49</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>146</td>
<td>391</td>
<td>0</td>
<td>244</td>
<td>53</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>148</td>
<td>421</td>
<td>0</td>
<td>0</td>
<td>251</td>
<td>56</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>209</td>
<td>506</td>
<td>5</td>
<td>1</td>
<td>394</td>
<td>86</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Aliquots (3.0 ml) were removed from the incubation mixture in duplicate at the time intervals indicated and centrifuged. The platelet sediments were washed twice and subjected to lipid extraction. Phospholipid analysis by TLC and measurements of 32P and P content were carried out. The figures represent the mean values for total radioactivity (cpm) or specific radioactivity (SA in cpm/µg P) derived from the duplicate samples.
was a significant difference in the time course of labeling of PC and LPC of platelets (Fig. 2). A substantial fraction (34.9%) of platelet LPC was labeled rather rapidly with little further increase in the relative specific radioactivity of this platelet phospholipid (42.7% at 6 hr). In contrast, labeling of PC continued to rise throughout the total incubation period from 2.5% at 6 min to 7.0% at 6 hr.

To assess the effect of the washing procedure used in the initial separation of platelets from plasma on uptake of radioactivity by platelets, unwashed rabbit platelets were resuspended in dialyzed $^{32}$P-labeled PFP, and the results of platelet phospholipid analysis at 6 min were compared with those obtained using washed platelets (Table 5). Phospholipid relative specific radioactivity of washed platelets was significantly greater than that of unwashed platelets, but this difference was small.

To determine whether the rise in aqueous phase radioactivity in the platelet extracts observed during incubation with labeled plasma (Table 3) was due to hydrolysis by platelets of plasma LPC to GPC and fatty acids, concentrated aqueous phase material was subjected to paper chromatography. When the chromatogram was stained for phosphorus, only a single spot was visualized,
Table 5. Effect of Washing Procedure on Initial Uptake by Platelets of Radioactivity From Plasma Labeled In Vivo With $^{32}$P-Orthophosphate

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Plasma Phospholipid SA (cpm/µgP)</th>
<th>Platelet Phospholipid SA (cpm/µgP)</th>
<th>Rel. SA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>W U</td>
<td>U W</td>
</tr>
<tr>
<td>1</td>
<td>3048</td>
<td>64 48</td>
<td>2.10 1.58</td>
</tr>
<tr>
<td>2</td>
<td>2037</td>
<td>56 44</td>
<td>2.75 2.16</td>
</tr>
<tr>
<td>3</td>
<td>1416</td>
<td>32 26</td>
<td>2.26 1.84</td>
</tr>
<tr>
<td>Mean</td>
<td>51</td>
<td>39 2.37</td>
<td>1.86</td>
</tr>
<tr>
<td>SD</td>
<td>17</td>
<td>12 0.34</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Rabbit platelets separated from blood by differential centrifugation as described in Materials and Methods were either directly resuspended (without washing) in dialyzed $^{32}$P-labeled PFP, or washed twice and then resuspended in another sample of the same PFP. Aliquots (3.0 ml) were removed from both incubation mixtures in duplicate 6 min after resuspension of the platelets and centrifuged at 15,000 g for 1 min. The platelet sediments were washed twice with EDTA—Tyrode solution and subjected to lipid extraction together with aliquots of PFP. The total phosphorus content of the lipid phases was determined and phospholipid specific radioactivity (SA) and relative specific radioactivity (platelet phospholipid SA divided by plasma phospholipid SA x 100) were calculated for both washed (W) and unwashed (U) platelets. The figures are the means of duplicate sample analyses in each experiment. A paired t test was used for statistical analysis.

and this spot was located at a distance from the point of application identical to that found for a pure GPC-reference compound chromatographed on the same paper strip. The eluate from the paper containing this spot showed significant amounts of $^{32}$P, whereas $^{32}$P could not be demonstrated in any of the other eluates.

Platelets resuspended in the $^{32}$P-labeled, dialyzed PFP showed a normal aggregation response (≈ 50% change in OD) upon the addition of ADP (5 µM final concentration), which did not change significantly during the 6-hr incubation period (except for the first experiment in which the pH was not properly controlled). A small increase in LDH in the plasma was observed during the incubation period (0.44% ± 0.33% SD) of total LDH at 6 min to 2.17% ± 0.85% of total LDH at 6 hr).

Although it seemed unlikely that the small amount of $^{32}$P found in the aqueous phase of the plasma extracts after extensive dialysis of the plasma represented inorganic phosphate, the possibility was considered that labeling of platelet phospholipids was due to de novo phospholipid synthesis by platelets during the incubation period. Washed rabbit platelets were, therefore, resuspended in PFP to which $^{32}$PO$_4$ had been added in vitro to yield radioactivity in excess of that found in the aqueous phase of the extracts of PFP labeled in vivo. While increasing amounts of $^{32}$P were found in the aqueous phases of the platelet extracts during incubation, no $^{32}$P could be demonstrated in the lipid phase of the platelet extracts.

To assess the effect of increased plasma lipid levels on uptake and incorporation of plasma phospholipids by platelets, washed platelets from normal rabbits were incubated in dialyzed $^{32}$P-labeled PFP prepared from rabbits with diet-induced hyperlipidemia. Although this plasma contained approximately three times the amount of phospholipid found in normal rabbit plasma, there
was no significant change in the percentage distribution of the individual phospholipids and the specific activities of these phospholipids were similar to those observed with normal dialyzed $^{32}$P-labeled PFP. Hyperlipidemic plasma caused a significant increase in both non-lipid-bound and lipid-bound $^{32}$P in platelet extracts throughout the entire incubation period. Platelets in hyperlipidemic, dialyzed $^{32}$P-labeled PFP incorporated approximately $1\frac{1}{2}$–2 times as much label into both total phospholipids and PC as did platelets in normal dialyzed $^{32}$P-labeled PFP. In contrast, platelets exposed to hyperlipidemic plasma showed consistently less labeled LPC than did platelets exposed to normal plasma. As observed with normal dialyzed $^{32}$P-labeled PFP, there was little or no labeling of other platelet phospholipids upon incubation of platelets in hyperlipidemic, dialyzed $^{32}$P-labeled PFP.

DISCUSSION

Human blood platelets are capable of both de novo synthesis,29,30 and chain elongation30 of fatty acids and de novo synthesis of glycerophospholipids.31 Results of a study by Deykin and Desser30 could be interpreted as indicating that there is exchange of fatty acids and, perhaps, entire phospholipid molecules between platelets and plasma. However, in their study, only the transfer of these labeled lipids from platelets to plasma was studied, and the possibility was not excluded that some or, perhaps, all of the labeled platelet lipids found in the plasma were derived from a small proportion of lysed platelets. The data reported in this paper are derived from experiments in which unlabeled platelets were incubated in plasma in which all the major phospholipids had been labeled with $^{32}$PO$_4$ in vivo.

Rapid labeling of up to 40% of the platelet LPC was observed during the initial period of incubation with little further increase between 3 and 6 hr. In addition, there was a slower but continuous increase in labeling of PC, reaching a maximum of 7.0% of total platelet PC at the end of the 6-hr incubation period. In contrast, little or no labeling of the other major platelet phospholipids was observed.

In experiments of this kind, the possibility must be considered that phospholipids may become associated with the platelets by nonspecific adsorption onto the cell surface. However, several observations provide evidence against this possibility.

(1) Washed platelets incorporated only slightly (20%) more labeled phospholipid than did unwashed platelets, which indicated that removal of the original plasmatic atmosphere was clearly not the sole or predominant factor responsible for labeling of platelet phospholipids.

(2) Washed platelets incubated in labeled dialyzed plasma for 3 hr, and then washed in Tyrode solution containing protein or unlabeled plasma had essentially the same amount of associated lipid bound radioactivity as platelets washed in Tyrode solution that did not contain protein.

(3) Only two platelet phospholipids were labeled during the 6-hr incubation time with LPC being labeled at a significantly greater rate than PC.
In the experiments with hyperlipidemic plasma, the proportion of labeled LPC associated with the platelets was less than that observed during incubation with normal plasma, whereas the amount of labeled PC associated with the platelets was greater in hyperlipidemic plasma. Thus, it seems unlikely that our findings are attributable to nonspecific adsorption of labeled phospholipids to the platelet surface.

The possibility that labeling of platelet LPC and PC was due to utilization of residual plasma $^{32}$PO$_4$ (not eliminated during the dialysis procedure) in de novo platelet phospholipid synthesis was excluded. Although it is possible that this residual radioactivity in the aqueous phase of the dialyzed PFP represents a labeled, water soluble, high-energy phosphate donor for phospholipid synthesis such as $^{32}$P-ATP, it seems unlikely that this could account for the selective labeling of platelet LPC and PC.

There was also an increase in aqueous phase radioactivity, and we obtained some evidence that this radioactivity was associated with GPC. Elsbach et al.$^{32}$ have shown that human platelets take up and metabolize purified LPC added to plasma or to an artificial medium in which they are suspended. Metabolism of LPC by human platelets proceeded via two pathways: (1) acylation through the Lands pathway as previously shown by Cohen et al.$^{33}$ to yield PC, and (2) through deacylation to yield GPC and free fatty acid. Although we have no direct evidence from these experiments concerning the operation of the Lands pathway, our findings are consistent with a rapid metabolism of endogenous plasma LPC by platelets, resulting in the formation of both labeled GPC and PC. Thus, the two pathways described for human platelets could account for our findings with rabbit platelets. Whether labeling of PC was entirely due to conversion of LPC or at least in part due to direct uptake and incorporation of plasma PC by platelets remains to be elucidated.

Incubation of washed platelets in hyperlipidemic dialyzed $^{32}$P-labeled PFP resulted in a significant increase in uptake and incorporation by platelets of labeled plasma phospholipids over that seen in normal dialyzed $^{32}$P-labeled PFP. Among the various platelet phospholipids, only PC and LPC were significantly labeled. The question of why platelets exposed to hyperlipidemic plasma contained a smaller labeled fraction of LPC than platelets exposed to normal plasma cannot be readily answered. It could be that, in hyperlipidemic plasma, the platelets hydrolyze LPC more rapidly, convert LPC to PC more rapidly, or both. Another possibility is that, in hyperlipidemic plasma, direct exchange of PC predominates, accounting for the difference in the proportions of labeled PC and LPC. This mechanism, of course, would have to be accompanied by increased metabolism of LPC.

The results of these studies show that platelets, like other cells (RBC,$^{14,16}$ polymorphonuclear leukocytes,$^{24,35}$ liver cells,$^{26}$ brain,$^{37}$ aortic intima$^{38}$), can take up LPC (and possibly PC) from their suspending medium. This uptake may have implications in relation to the turnover of PC in the plasma membrane. Alteration of the fatty acid composition of platelet phospholipids by changes in the fatty acids of plasma LPC and PC might result in changes in platelet reactivity.
REFERENCES

28. Bergmeyer HV, Bernt E, Hess B: Lactic
PHOSPHOLIPID TRANSFER


Phospholipid transfer between plasma and platelets in vitro

JH Joist, G Dolezel, JV Lloyd and F Mustard

Updated information and services can be found at:
http://www.bloodjournal.org/content/48/2/199.full.html
Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml