Platelet lipid(s) bound to albumin increases endothelial electrical resistance: mimicked by LPA

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Minnear, Fred L., Sandeep Patil, Donald Bell, Jonathan P. Gainor, and Christine A. Morton. Platelet lipid(s) bound to albumin increases endothelial electrical resistance: mimicked by LPA. Am J Physiol Lung Cell Mol Physiol 281: L1337–L1344, 2001.—The objectives were to determine whether the permeability-decreasing activity of platelet-conditioned medium (PCM) is associated with a lipid bound to albumin and whether lysosphosphatidic acid (LPA) is present in the PCM. A decrease in permeability was assessed by an increase in electrical resistance across endothelial cell monolayers derived from bovine pulmonary arteries and microvessels. The Sephacryl S-200 fraction of PCM that contained albumin, the albumin immunoprecipitate from the PCM, and the methanol extract from the albumin immunoprecipitate all increased endothelial electrical resistance. Increased electrical resistance induced by PCM was not abolished by boiling and was mimicked by 1-oleoyl-LPA and 1-palmitoyl-LPA. Analysis of a methanol-chloroform extract of one sample of PCM by electrospray mass spectrometry revealed many fatty acids, ceramide, diacylglycerol, phosphatidic acid, and palmitoyl-LPA, but analysis of a second sample of PCM and the methanol extract of its albumin immunoprecipitate revealed no LPA, only lipids. These findings indicate that a bioactive lipid(s), possibly LPA, released from platelets and subsequently bound to albumin forms an active complex that decreases endothelial permeability.

MATERIALS AND METHODS

Materials. Platelet packs were purchased from the local American Red Cross blood bank. Bovine pulmonary artery endothelial cells were obtained from American Type Culture Collection (CCL 209) and Vec Technologies, and pulmonary microvascular endothelial cells were obtained from Vec Technologies. Minimum essential medium (MEM) and MCDB 131 medium were from GIBCO BRL, and fetal bovine serum was from Summit Biotechnology. Protein G Sepharose and Blue Sepharose were from Amersham Pharmacia Biotech. Electric cell-substrate impedance sensing (ECIS) wells were obtained from SELM, and an endothelial cell monolayer was obtained from Vec Technologies. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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from Applied Biophysics. LPA was from Avanti Polar Lipids. Avanti Polar Lipids analyzed the lipid profile of PCM with an Agilent Technologies model 5989A mass spectrometer with a 59987A electrospray interface. All other compounds and materials were purchased from Sigma.

Platelet isolation and preparation of PCM. Platelets were isolated from fresh nonirradiated platelet packs. Isolated platelets were concentrated to 2 × 10^9 platelets/ml in modified (Ca^{2+}/Mg^{2+}-free) Tyrode buffer (0.137 M NaCl, 0.003 M KCl, 0.012 M NaHCO_3, 0.006 M glucose, and 0.004 M Na_2HPO_4, pH 7.4) incubated in a plastic round-bottom tube for 2 h at room temperature, and centrifuged at 600 g for 20 min. The supernatant was designated PCM, divided into aliquots, and stored at -4°C for immediate use and at -80°C for later use.

Endothelial cell culture. Endothelial cells from bovine pulmonary arteries (CCL 209, starting at passage 16) and from bovine microvessels (starting at passage 3) were grown in complete culture medium consisting of MEM or MCDB 131 medium, 10 or 20% fetal bovine serum, and 50 μg/ml of gentamicin sulfate.

Measurement of endothelial resistance. Measurement of electrical resistance across endothelial cells grown as a monolayer is based on a technique developed to study the dynamic behavior of cells in culture. This novel methodology is known as ECIS (7, 18, 19, 26). Endothelial cells were cultured on small gold electrodes (5 × 10^-4 cm^2), and culture medium was used as the electrolyte. The small gold electrode, covered by confluent endothelial cells, and a larger gold counterelectrode (~2 cm^2) were connected to a phase-sensitive, lock-in amplifier. A 1-V, 4,000-Hz alternating current signal was supplied through a 1-MΩ resistor to approximate a constant current source of 1 μA. By treating the cell-electrode system as a simple series resistance-capacitance circuit, the measured change in electrical impedance can be partitioned into a measured in-phase voltage proportional to the resistance and an out-of-phase voltage proportional to capacitive reactance. Voltage and phase data were stored and processed with a personal computer. The same computer controlled the output of the amplifier and switched the measurements to different electrodes in each of two 8-well arrays during the course of an experiment. The small size of the cell-seeded electrode is the critical feature of the system. When electrodes of 10^-3 cm^2 or smaller are used, the impedance at the small electrode dominates the system, allowing the morphology of the cells located at this interface to be assessed.

ECIS wells were treated with 1% gelatin in 50 mM Tris (pH 8.0) for 10 min and washed three times with phosphate-buffered saline (Ca^{2+}/Mg^{2+}-free). Endothelial cells were seeded at 100,000 cells/ECIS well (8-mm diameter, 0.5 cm^2) in 400 μl of medium containing 20% (in MEM) or 10% (in MCDB 131 medium) serum. On the day of the experiment, the medium was removed from each well and replaced with serum-free medium. ECIS wells, consisting of one or two 8-well arrays, were placed in the ECIS incubator for 2 h to allow for equilibration at 37°C and 5% CO_2 and were then incubated with the desired mediators. The measurement of electrical impedance was obtained every minute for 30–60 min after treatment of the endothelial cells and is reported as the resistive portion of electrical impedance.

Gel filtration chromatography. PCM was concentrated two-fold and then applied to a calibrated Sephacryl S-200 column. Fractions were collected in 1-ml aliquots and analyzed by spectrophotometry at an absorbance of 280 nm. The column was calibrated with blue dextran, 2,000 kDa, catalase, 232 kDa, albumin, 67 kDa, and chymotrypsinogen, 25 kDa.

Albumin immunoprecipitation and methanol extraction. Albumin was precipitated from PCM with an antibody to human serum albumin. The antibody complexed to protein G Sepharose was incubated with PCM overnight at 4°C. The complex, consisting of antibody, albumin, and protein G Sepharose, was removed from the PCM by centrifugation at 100 g for 1 min and reconstituted in MCDB 131 medium before activity was tested for. For methanol extraction, 300 μl of 100% methanol were added to the ~300 μl of centrifuged albumin-bound beads and agitated at room temperature for 10 min. The methanol extract was centrifuged at 100 g, and the supernatant was collected. The addition of methanol, centrifugation, and collection of supernatant was repeated five times to yield 3 ml of pooled supernatant. The pooled methanol extract was air-dried at 37°C and reconstituted in distilled water to a volume of 300 μl.

Blue Sepharose chromatography. Platelet-conditioned plasma from the platelet packs was applied to a 30-ml Blue Sepharose column containing 100 ml of a solution of 0.1 M KCl and 0.05 M Tris (pH 7.0). The flow-through was retained, and then the lipids bound to albumin were extracted with 150 ml of 100% methanol and collected off the column. Methanol was evaporated under a stream of air at 37°C, and the residue was resuspended in 10 ml of distilled water. Aliquots of the reconstituted extract were incubated with 2% fatty acid-free human serum albumin. The albumin bound to the column was eluted overnight with 350 ml of buffer containing 1.5 M KCl and 0.05 M Tris (pH 7.0). The eluted albumin was concentrated by filtration (3,000 molecular weight cutoff membrane) in Tyrode buffer and brought to a volume of 10 ml. All samples were exchanged to Tyrode buffer and brought to the original 5-ml starting volume.

Gel electrophoresis. Aliquots of the various experimental fractions obtained from the PCM, Sephacryl S-200 column, albumin immunoprecipitate, and Blue Sepharose chromatography were loaded into a 3% stacker and separated by size by electrophoresis in a 4–15% sodium dodecyl sulfate-polyacrylamide gel. The SDS-PAGE gels were stained with Coomassie blue.

Electrospray mass spectrometry. Lipids in the PCM were extracted with the Bligh and Dyer (3) or modified Folch et al. (4) technique. The upper phase of the Bligh and Dyer (3) extract was extracted further with the modified Folch et al. (4) technique. These three extracts were analyzed with an Agilent Technologies model 5989A mass spectrometer with a 59987A electrospray interface. Detection was performed in the negative ionization mode at a quadrupole temperature of 110°C. Electronic potentials between the capillary exit voltage and the first skimmer lens were set low to prevent collision-induced dissociation of generated ions and thus increase the detection of singly charged molecular adducts. Confirmation of detection and mass accuracy was performed by injecting 1 μg of 18:1 or 16:0 LPA into a 20 μl/min stream of 65:35:8 (vol/vol/vol) chloroform-methanol-water with 0.1% NH_4OH (20% concentrated solution). Molecular ions of [M-H]^- were detected for both lysophospholipids, with no observation of collision-induced dissociation.

Statistics. Each electrical resistance study consisted of at least five different experiments with one or two cell monolayers per group per experiment. Data were analyzed with a two-way analysis of variance with repeated measures (29). Differences between treatments or groups from the control group were analyzed with the Newman-Keuls post hoc test. Significance was set at P < 0.05.

RESULTS

Patil et al. (19) recently reported that the active platelet factor is trypsin sensitive, is precipitable in ammonium sulfate, and resides in a fraction between 3
and 100 kDa. In preliminary experiments, PCM was applied to ion exchange columns to differentiate between positively and negatively charged proteins. Activity of the PCM as assessed by an increase in endothelial electrical resistance resided in the flow-through from a cationic (carboxymethyl) exchange column and in the bound, salt (1 M)-elutable fraction from an anionic (diethylaminoethyl) exchange column at pH 7.5 (data not shown). Binding to the anionic exchange column and the requirement of a high-salt elution are findings consistent with a very negatively charged protein such as albumin.

To approximate the size of the active protein fraction, PCM was applied to a calibrated Sephacryl S-200 gel filtration column. Fractionation yielded three peaks, with peaks 1 and 3 corresponding to the calibrated standards for catalase and albumin, respectively (Fig. 1A). SDS-PAGE revealed that peak 3 contained most of the albumin in the PCM (Fig. 1B). Fractions underlying the three peaks were pooled and re-concentrated back to the starting concentration of whole PCM. Only the pooled fractions underlying peak 3 increased endothelial electrical resistance (Fig. 1C).

Because albumin dominated the protein profile in the pooled fractions underlying peak 3 from the S-200 column, albumin was removed from the PCM by immunoprecipitation. An antibody to human serum albumin complexed to protein G Sepharose was incubated overnight at 4°C with PCM. SDS-PAGE shows that much of the albumin was removed from the PCM by immunoprecipitation (Fig. 2A); two successive immunoprecipitations removed most of the albumin (data not shown). Equal volumes of the albumin immunoprecipitate (including the protein G Sepharose beads) and whole PCM caused similar increases in endothelial electrical resistance (Fig. 2B). Activity was absent in the supernatant (Fig. 2B), which had much reduced levels of albumin (Fig. 2A).

Recently, Alexander et al. (1) reported that the lysophospholipid LPA is the active component in PCM. Because these authors found activity in the methanol extract of platelet-conditioned plasma, we extracted the albumin immunoprecipitate with methanol. Successive extractions with methanol yielded activity in the methanol extract and loss of activity in the extracted albumin immunoprecipitate (Fig. 3B). The methanol extract was devoid of albumin as demonstrated by SDS-PAGE (Fig. 3A). We also produced large volumes of methanol-extracted lipids from the platelet packs by applying platelet-conditioned plasma to a Blue Sepharose column. Methanol (100%) was applied to the albumin bound to the column (Fig. 4A). Because the methanol-extracted solution was a concentrated suspension, fatty acid-free human serum albumin was added to the methanol extract to form a complex of albumin-bound lipids before activity was tested for. Activity of the complex of fatty acid-free albumin and lipids was compared with that of the unbound extracted lipids; with the column-bound albumin, which was eluted from the column with 50 mM Tris and 1.5 M KCl; and with fatty acid-free albumin itself. The extracted lipids bound to fatty acid-free albumin increased endothelial electrical resistance (Fig. 4B). The original extracted lipids that were not bound to fatty acid-free albumin caused a slight increase in electrical resistance (data not shown). The methanol-extracted albumin subsequently eluted from
Because many lipids are resistant to heating, the PCM was heated to 100°C for 10 min. Heating the PCM produced a precipitate and a floccular supernatant, the latter containing permeability-decreasing activity (Fig. 4B). Centrifugation at 145,000 g for 30 min produced a clear supernatant that also contained activity. SDS-PAGE demonstrated that heating eliminated most of the protein bands of the whole PCM and produced a thickened, globlike band at the stacker-gel interface (Fig. 5A). On reduction with β-mercaptoethanol, bands became apparent at the molecular masses corresponding to reduced albumin and fibrinogen (Fig. 5A). The heat stability of PCM indicated that multimers of albumin retain the ability to bind biologically active lipids or that lipids are active in an unbound state after release from the heated multimerized albumin. In combination with other experiments, we have observed that the activity of heated PCM after dialysis at 3.5 kDa and after anionic or cationic exchange chromatography always resided with a protein fraction. Therefore, heated multimerized albumin must retain the ability to bind biologically active lipids.

To identify the lipids, with emphasis on phospholipids, PCM was extracted with the Bligh and Dyer (3) or modified Folch et al. (4) techniques, and in addition, the upper phase of the Bligh and Dyer (3) extract was
extracted further with the modified Folch et al. (4) technique. The molecular ions and possible identities are reported in Table 1. LPA was detected as palmitoyl (16:0)-LPA only by the modified Folch et al. extraction (Table 1). In addition, a number of lipids, fatty acids (many of which were polyunsaturated), ceramide, diacylglycerol, and phosphatidic acid were identified, and there were 10 unknown lipids. The same analysis was conducted with a second batch of PCM, which was as potent in activity as the first batch, and an active methanol extract of the albumin immunoprecipitated from that PCM. The lipid profiles were almost identical between the PCM and the methanol extract of the albumin immunoprecipitate; however, LPA was not detected in the second batch of PCM by the three extraction procedures (Table 2).

Direct application of 1-oleoyl-LPA or 1-palmitoyl-LPA caused an increase in electrical resistance of endothelial cell monolayers derived from pulmonary arteries (Fig. 6) and pulmonary microvessels (data not shown).

**DISCUSSION**

The objectives of the present study were to determine whether the active platelet factor that decreases endothelial permeability is associated with albumin, whether the activity is associated with a methanol-extracted lipid bound to albumin, and whether LPA is present in PCM. Our findings indicate that the permeability-decreasing activity of PCM is associated with albumin, is extractable from albumin with methanol, and is mimicked by 1-oleoyl-LPA and 1-palmitoyl-LPA. Palmitoyl (16:0)-LPA, a number of fatty acids, ceramide, diacylglycerol, phosphatidic acid, and 10 un-
known lipids were identified by electrospray mass spectrometry in one batch of PCM, but fewer lipids and no LPA were detected in an equally potent second batch of PCM and the methanol extract of the albumin immunoprecipitate from that PCM. We conclude that a bioactive lipid(s) bound to albumin mediates the PCM-induced decrease in permeability of the vascular endothelium in vitro. We speculate that platelets contribute to the maintenance of the vascular endothelial barrier through the release of biologically active lipids that bind to albumin, which serves as a protective carrier to the vascular endothelium.

In 1956, Wilbrandt et al. (30) reported that a protein fraction extracted from platelets reduces capillary permeability in the rat hind limb. Thirty-six years later, Haselton and Alexander (9) suggested that the active platelet factor is a heat-stable and trypsin-sensitive protein that exceeds 100 kDa. Patil et al. (19) confirmed these reported findings in a previous publication but determined via dialysis that the active protein

**Table 1. Electrospray mass spectrometry of Bligh and Dyer and modified Folch extracts of PCM**

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<tr>
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<td>Homogama linolenic (20:3)</td>
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<tr>
<td>846.7</td>
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**Table 2. Electrospray mass spectrometry of Bligh and Dyer extracts of PCM and methanol extract of albumin IP from same PCM**

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<tr>
<td>227.6</td>
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<td>Lignoceric acid (24:0)</td>
<td>368.64</td>
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</tr>
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<td>820.6</td>
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PCM, platelet-conditioned medium; m/z, mass-to-charge ratio; DAG, diacylglycerol; LPA, lysophosphatidic acid; PA, phosphatidic acid. Electrospray mass spectrometry was performed in negative ionization mode. Extraction was performed with the techniques of Bligh and Dyer (3) and Folch et al. (4).
by showing that heating PCM to 100°C for 10 min
demonstrated that the activity associates with albumin. Further-
more, activity was present in the methanol extract obtained from
both the albumin immunoprecipitate of the PCM and the albumin
bound to the Blue Sepharose column. These findings indicate that a
methanol-extracted lipid(s) bound to albumin is responsible for the
activity of PCM.

Haselton and Alexander (9) reported that the activity
of PCM is heat stable. We confirmed their findings by showing that heating PCM to 100°C for 10 min
produced a precipitate and a supernatant, the latter
containing activity. Heated PCM also contained multimers of albumin as viewed by SDS-PAGE. Because
many lipids are heat stable, the activity of the heated
PCM could reside in the unbound lipids that could be
released from the multimerized albumin. It is more
likely that the activity resided with the lipids bound to
the multimerized albumin because the activity of
heated PCM was retained in a protein fraction after
dialysis with a 3.5-kDa membrane and after anionic
and cationic exchange chromatography.

The recent publication by Alexander et al. (1) pro-
vided evidence that LPA may be the active factor. Activity
was present in the methanol extract but not in the
ether extract from platelet-rich plasma. Activity
was eliminated by enzymatic treatment with phospho-
lipase B that cleaves at the sn-1,2 positions and alkaline
phosphatase that cleaves at the phosphomonoester
bond but not by phospholipase A2 that cleaves at the
sn-2 position. 1-Oleoyl-LPA by itself decreased small-
solute permeability across endothelial cell monolayers
derived from bovine aorta. That a phospholipid such as
LPA or another biologically active lipid(s) may be the
active platelet component provides explanations for
some of the previously reported data. Shepard et al.
(23) have reported that a hydrophilic fraction and not a
hydrophobic fraction of an ether extraction of PCM
contains permeability-decreasing activity. A phospho-
lipid such as LPA as well as many lipids would be
retained in the hydrophilic fraction. Trypsin inhibits
the PCM-induced increase in endothelial electrical
resistance but does not noticeably alter the protein bands
identified on SDS-PAGE gels, although some bands are
diminished in concentration (19). Trypsin probably in-
hibits PCM activity by altering the confirmation of
albumin, exposing bound lipids to hydrolysis by lipases
that must be present in the PCM. Finally, the previ-
ously observed permeability-decreasing activity of bo-
vine serum albumin may be explained by LPA or a
biologically active lipid (13). Interestingly, albumin by
itself has been reported to decrease as well as increase
endothelial permeability. Albumin can decrease per-
meability by physically interacting with the glyocalyx
at the junctional surface (15). On the other hand,
albumin can also increase transcellular permeability
by binding to the albumin receptor, gp60, or albonin
and activating vesicular trafficking (28). The present
study, however, demonstrates that a biologically active
lipid(s) like LPA bound to albumin can profoundly
influence endothelial permeability and, in this case,
decrease permeability.

Characterization of the lipid profile in two separate
batches of methanol-chloroform-extracted PCM by
electrospray mass spectrometry revealed mixed results
with respect to detection of LPA. In the first batch of
PCM, palmitoyl (16:0)-LPA was identified. Palmitoyl-
LPA as well as stearoyl-LPA and arachidonoyl-LPA
have been shown to be the major molecular species of
LPA produced from thrombin-stimulated platelets (6).
Identifiable lipids in the first batch of PCM also in-
cluded fatty acids (many of which were polyunsaturat-
ed), ceramide, diacylglycerol, and phosphatidic acid. In addition, we were not able to identify 10 peaks in the mass spectra. In the second batch of PCM and the methanol extract of the albumin immunoprecipitate from that PCM, which was as potent in activity as the first batch, no LPA was identified. According to the lipid profile of this second batch of PCM, most of lipids were bound to albumin. Before this second analysis, we would have suggested that LPA was the active platelet factor. This most recent information, however, raises the possibility that other methanol-extracted lipids are bioactive.

Therefore, the identity of the lipid(s) with permeability-decreasing activity that is associated with platelets remains to be determined, although LPA and sphingosine 1-phosphate (Roberts JT, Vincent PA, Morton CA, and Minnear FL, unpublished observations) possess permeability-decreasing activity. The effects of LPA appear to be organ, tissue, or species specific. In contrast to the above findings in bovine endothelial cells from the lung and aorta, LPA increases permeability across porcine brain capillary endothelial cells (22) and human umbilical vein endothelial cells (27). Therefore, the present findings and those of Alexander et al. (1) that LPA can decrease endothelial permeability were initially a surprise, considering the previous reports in brain microvessels that LPA can function as a mitogen (10, 16). Many mitogens such as thrombin increase endothelial permeability. Nevertheless, the findings of the present study and those of Alexander et al. (1) indicate that a biologically active lipid(s) bound to albumin, possibly LPA, decreases the permeability of endothelial cell monolayers derived from bovine pulmonary arteries, bovine pulmonary microvessels, and bovine aorta.

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REFERENCES


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