Am J Physiol Lung Cell Mol Physiol
281: L1337–L1344, 2001.

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

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Received 8 December 2000; accepted in final form 20 July 2001

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 lysophosphatidic 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.

lysophosphatidic acid; gel electrophoresis; immunoprecipitation; methanol extraction; gel filtration chromatography; Blue Sepharose chromatography; electrospray mass spectrometry

MAINTENANCE OF THE VASCULAR ENDOTHELIUM as a semipermeable membrane for the passage of water and protein affords protection against the development of edema in various organ beds. Platelets contribute to this maintenance of the vascular endothelial barrier. A low platelet count in the blood causes purpuric hemorrhages in the skin (5, 11) and has been associated with an increase in the vascular endothelial permeability to protein in various organ beds (2, 8, 12, 14). Repletion with platelet-rich plasma reverses these changes (2, 8, 11, 12, 14). Recent findings support the hypothesis that activated platelets release a soluble factor that decreases endothelial permeability. For example, platelet-conditioned medium (PCM), which contains releasate from platelets, replicates the permeability-decreasing activity of whole platelets (1, 9, 19, 20, 23, 24). Proposed mediators that have been eliminated from contention as the active factor are serotonin, norepinephrine, cyclooxygenase metabolites, adenosine, and adenine nucleotides (9, 19, 20, 23). Both Haselton and Alexander (9) and Patil et al. (19) reported that the active platelet factor is associated with a protein because the activity is trypsin sensitive, is precipitable by saturated ammonium sulfate, and resides in a fraction between 3 and 100 kDa.

Alexander et al. (1) further proposed that lysophosphatidic acid (LPA; 1-acyl-2-hydroxyl-3-phosphoglyceride) is the active factor. Permeability-decreasing activity was present in the methanol extract but not in the ether extract from platelet-rich plasma. Synthetic 1-oleoyl-LPA by itself decreased the solute permeability across endothelial cell monolayers derived from bovine aorta. The biologically active phospholipid LPA is present in platelets, is in low concentration in whole blood, is released from activated platelets during coagulation, and is present in micromolar concentrations in serum. LPA as well as other lipids is usually bound to albumin on which it retains biological activity and is protected against hydrolysis by serum lipases (10, 16, 17, 25). Therefore, the initial objectives of the present study were to determine whether the active platelet factor is associated with albumin and whether it is extractable in methanol. Another objective was to identify the lipid profile of PCM and an albumin fraction of the PCM by mass spectrometry and to determine whether LPA is present.

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

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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 NaH_2PO_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 electrical 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 twofold 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 (26% concentrated solution). Molecular ions of $[M\text{-}H]^-$ 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. 1*A*). SDS-PAGE revealed that *peak 3* contained most of the albumin in the PCM (Fig. 1*B*). Fractions underlying the three peaks were pooled and reconcentrated back to the starting concentration of whole PCM. Only the pooled fractions underlying *peak 3* increased endothelial electrical resistance (Fig. 1*C*).

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. 2*A*); 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. 2*B*). Activity was absent in the supernatant (Fig. 2*B*), which had much reduced levels of albumin (Fig. 2*A*).

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. 3*B*). The methanol extract was devoid of albumin as demonstrated by SDS-PAGE (Fig. 3*A*). 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. 4*A*). 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. 4*B*). 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

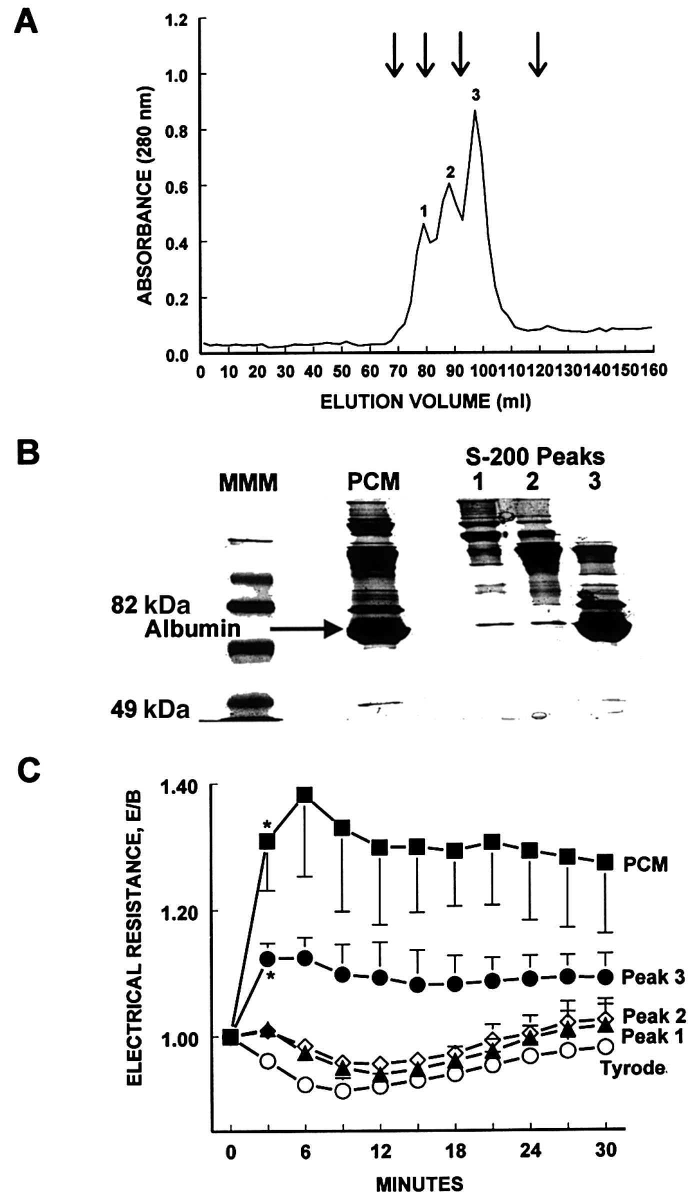

Fig. 1. Albumin fraction of platelet-conditioned medium (PCM) obtained by gel filtration chromatography retained permeability-decreasing activity. Concentrated PCM was applied to a Sephacryl S-200 gel filtration column. Fractionation yielded 3 peaks (*A*). Column was calibrated with (arrows, *left* to *right*) blue dextran (2,000 kDa), catalase (232 kDa), albumin (67 kDa), and chymotrypsinogen (25 kDa). Albumin resided primarily in the pooled fractions underlying *peak 3* from the Sephacryl S-200 column as demonstrated by SDS-PAGE (*B*). MMM, molecular mass markers. Pooled fractions underlying *peak 3* and PCM increased endothelial electrical resistance, whereas *peaks 1* and *2* had no activity (*C*). E/B, experimental-to-baseline ratio. Values are means ± SE; $n = 5$ cell monolayers per group. *$P < 0.05$ compared with Tyrode group (vehicle for PCM) from 3 to 30 min.

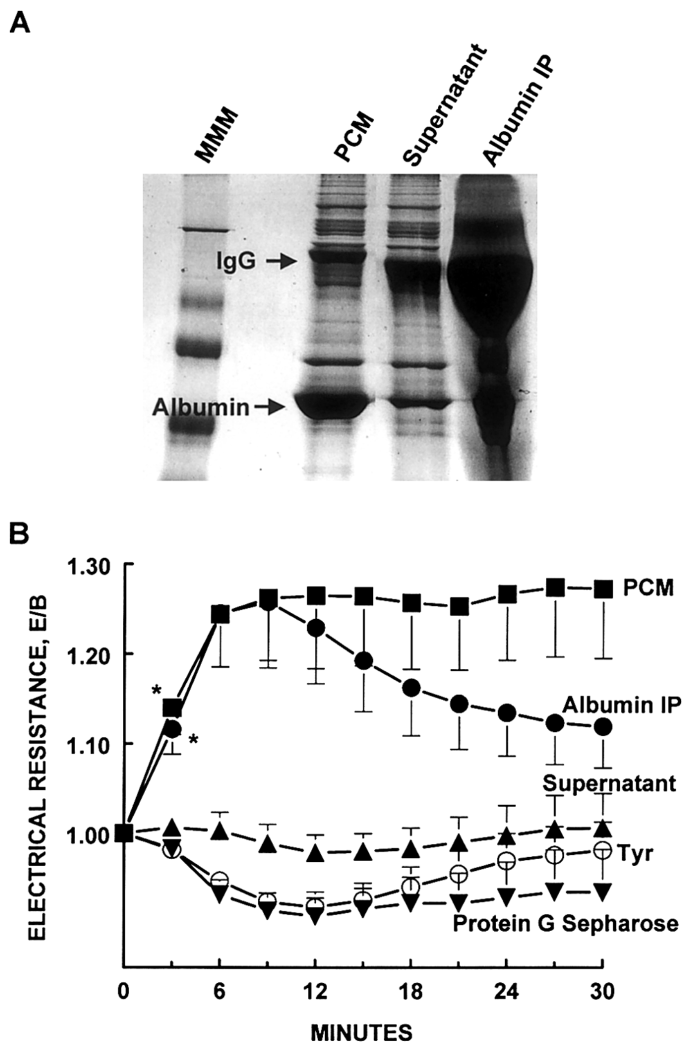

Fig. 2. Albumin immunoprecipitate (IP) from PCM retained activity. Immunoprecipitation of albumin with an antibody to human serum albumin removed most of the albumin from PCM as demonstrated by SDS-PAGE (*A*). Albumin immunoprecipitate, which included anti-human serum albumin antibody and protein G Sepharose, and PCM increased endothelial electrical resistance, whereas the remaining supernatant, protein G Sepharose alone, and Tyrode (Tyr) buffer had no effect on electrical resistance (*B*). Supernatant, PCM minus albumin immunoprecipitate. Values are means ± SE; n = 12 cell monolayers per group. $^*P < 0.05$ compared with Tyrode group from 3 to 30 min.

the column with Tris-KCl and fatty acid-free albumin itself had no activity (Fig. 4*B*).

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. 5*B*). 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. 5*A*). On reduction with β-mercaptoethanol, bands became apparent at the molecular masses corresponding to reduced albumin and fibrinogen (Fig. 5*A*). 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

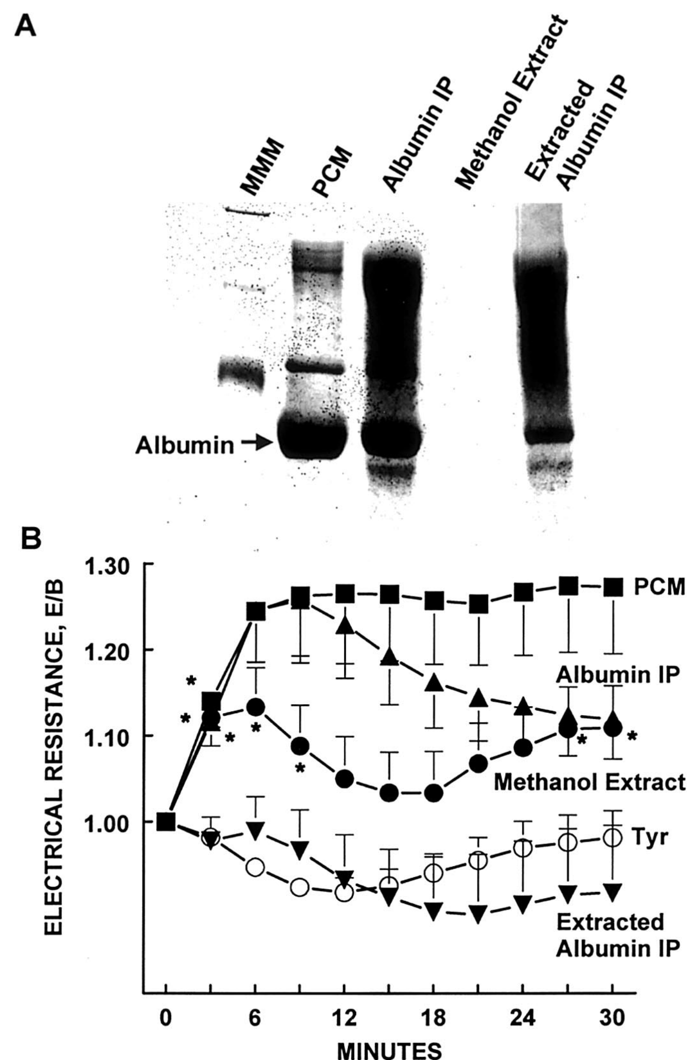

Fig. 3. Methanol extract of albumin immunoprecipitate from PCM contained activity. Much of the albumin was removed from PCM after immunoprecipitation with an anti-human serum albumin antibody (*A*). Methanol was added to the albumin immunoprecipitate to extract lipids from albumin. Methanol extract of albumin immunoprecipitate retained activity comparable with that of the albumin immunoprecipitate and whole PCM (*B*). Albumin immunoprecipitate after methanol extraction had no activity. Values are means ± SE; n = 12 cell monolayers/group. $^*P < 0.05$ compared with Tyrode group at designated time points for methanol extract group and from 3 to 30 min for PCM and albumin immunoprecipitate groups.

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).

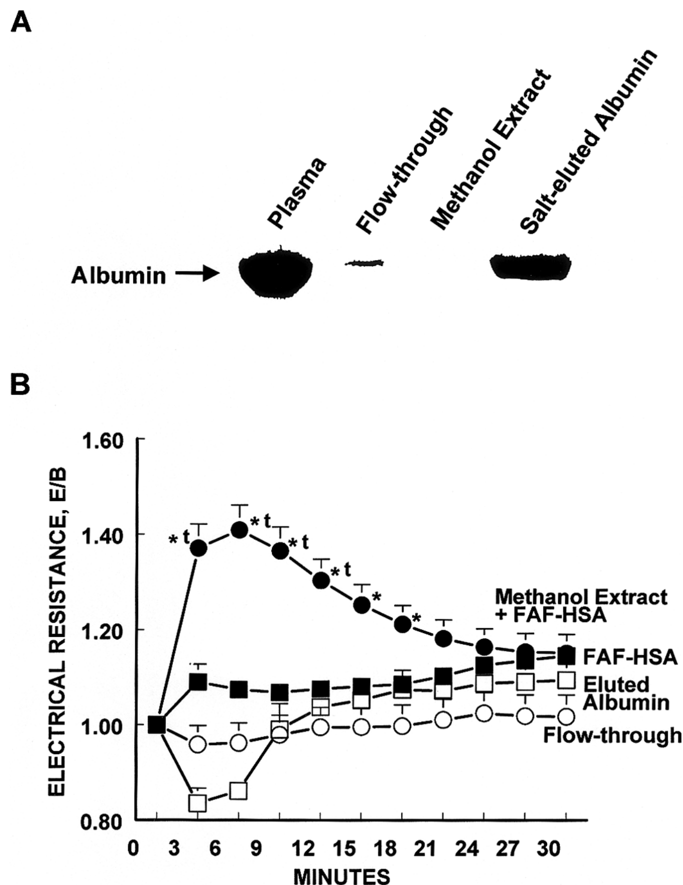

Fig. 4. Methanol extract of albumin bound to Blue Sepharose increased endothelial electrical resistance. Platelet-conditioned plasma was applied to a Blue Sepharose column. Albumin in plasma that bound to Blue Sepharose was extracted from lipids with 100% methanol (*A*). Albumin that remained bound was eluted with high concentration of salt. Fatty acid-free (FAF) albumin was added to the methanol extract. This complex increased endothelial electrical resistance (*B*). Methanol-extracted albumin that was subsequently eluted from the column lacked activity. HSA, human serum albumin. Values are means ± SE; $n = 5$ cell monolayers/group. $^*P < 0.05$ compared with flow-through group. $^tP < 0.05$ compared with eluted albumin group at designated times.

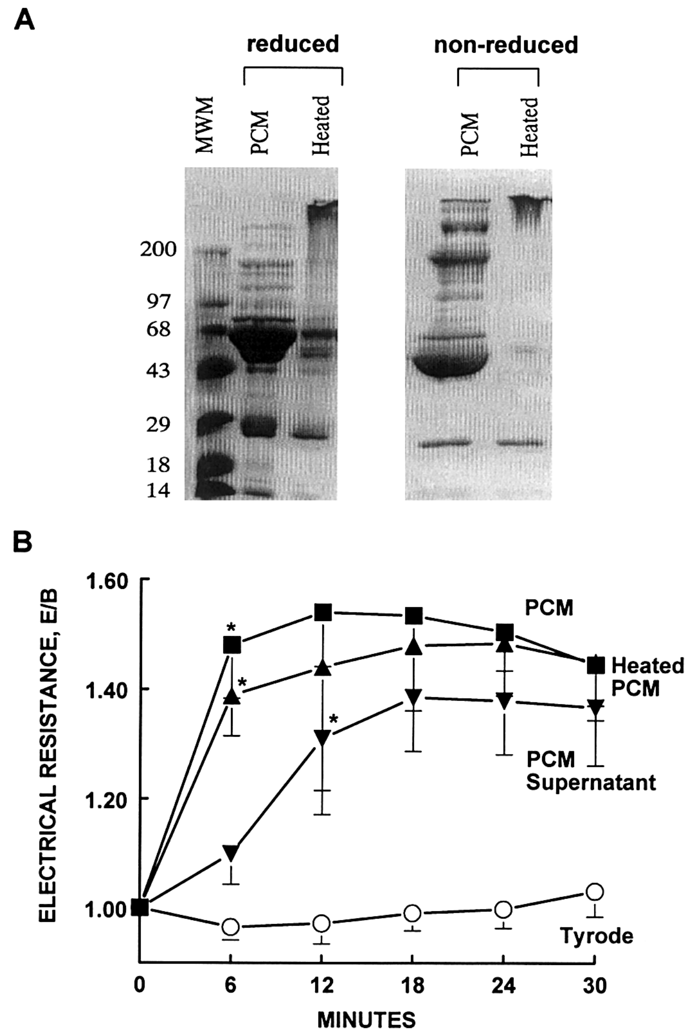

Fig. 5. PCM activity was resistant to heat. PCM was heated to 100°C for 10 min. Heating produced a floccular supernatant that was cleared after centrifugation. Heated PCM was devoid of most proteins, as viewed by SDS-PAGE (*A*, nonreduced). Nos. at *left*, molecular mass in kDa. PCM, the floccular supernatant (heated PCM), and the clear supernatant (PCM supernatant) were incubated with endothelial cell monolayers. All 3 solutions increased endothelial electrical resistance (*B*). Values are means ± SE; $n = 4$ cell monolayers/group. $^*P < 0.05$ compared with Tyrode group from 6 and 12 to 30 min.

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-

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

Bligh and Dyer Extract			Folch Extract		
m/z	Identity	Molecular Weight	*m/z*	Identity	Molecular Weight
171.1	Decanoic (10:0)	172.27	143.1	Octanoic (8:0)	144.21
227.6	Myristic (14:0)	228.38	166.1	Unknown	
241.4	Pentadecanoic (15:0)	242.41	219.3	Unknown	
255.3	Palmitic (16:0)	256.43	227.3	Myristic (14:0)	228.38
279.3	Linoleic (18:2)	280.48	253.4	Palmitoleic (16:1)	254.43
281.3	Oleic acid (18:1)	282.48	255.2	Palmitic (16:0)	256.43
282.9	Stearic (18:0)	284.48	277.3	Linolenic (18:3)	278.48
299.3	C_{18} sphingosine	299.50	279.3	Linoleic (18:2)	280.48
301.3	C_{18} sphingonine	301.51	281.4	Oleic (18:1)	282.48
303.3	Arachidonic (20:4)	304.52	283.3	Stearic (18:0)	284.48
305.3	Homogama linolenic (20:3)	306.53	297.3	C_{18} dihydrosphingosine	297.50
307.5	Eicosadienoic (20:2)	308.53	299.3	C_{18} sphingosine	299.50
397.4	D-*ribo*-Phytosphingosine	397.40	301.2	C_{18} sphingonine	301.51
530.7	Unknown		303.3	Arachidonic (20:4)	304.52
575.7	Unknown		305.3	Homogama linolenic (20:3)	306.53
593.7	C_{20} ceramide, 16:0–18:1 DAG	593.96	329.2	Unknown	
595.7	16:0–18:0 DAG	595.96	367.4	Lignoceric acid (24:0)	368.64
817.2	Unknown		409.3	16:0 LPA	410.10
818.9	Unknown		557.6	Unknown	
846.7	Unknown		573.7	Unknown	
			593.6	C_{20} ceramide, 16:0–18:1 DAG	593.96
			721.7	18:1–20:4 PA	722.90

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).

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 2. *Electrospray mass spectrometry of Bligh and Dyer extracts of PCM and methanol extract of albumin IP from same PCM*

Extract of PCM			Extract of Albumin IP		
m/z	Identity	Molecular Weight	*m/z*	Identity	Molecular Weight
227.6	Myristic (14:0)	228.38	149.1	Unknown	
241.4	Pentadecanoic (15:0)	242.41	165.0	Unknown	
255.1	Palmitic (16:0)	256.43	222.9	Unknown	
279.0	Linoleic (18:2)	280.48	255.1	Palmitic (16:0)	256.43
281.1	Oleic acid (18:1)	282.48	279.1	Linoleic (18:2)	280.48
283.2	Stearic (18:0)	284.48	281.0	Oleic acid (18:1)	282.48
299.1	C_{18} sphingosine	299.50	283.0	Stearic (18:0)	284.48
301.0	C_{18} sphingonine	301.51	296.8	Unknown	
303.0	Arachidonic (20:4)	304.52	298.9	C_{18} sphingosine	299.50
305.1	Homogama linolenic (20:3)	306.53	299.8	C_{18} sphingonine	301.51
367.1	Lignoceric acid (24:0)	368.64	303.2	Arachidonic (20:4)	304.52
480.0	Unknown		305.1	Homogama linolenic (20:3)	306.53
820.6	Unknown		312.9	Unknown	
			314.0	Unknown	
			367.1	Lignoceric acid (24:0)	368.64
			794.1	Unknown	

IP, immunoprecipitate. Electrospray mass spectrometry was performed in negative ionization mode. Extraction was performed with the technique of Bligh and Dyer (3).

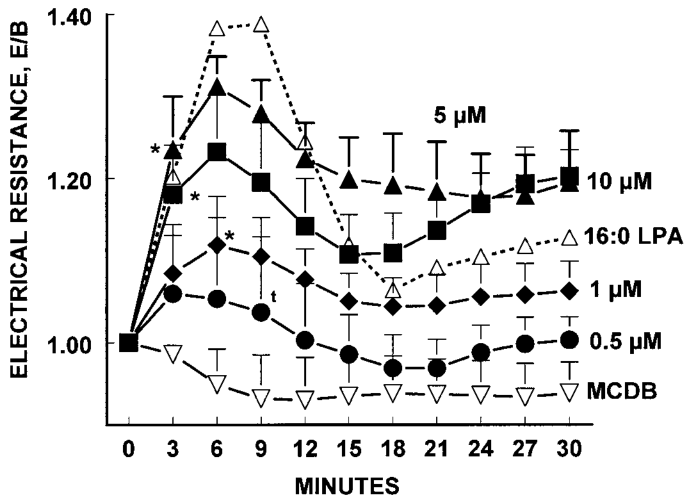

Fig. 6. Lysophosphatidic acid (LPA) mimicked activity of PCM. 1-Oleoyl (18:1)-LPA from 0.5 to 10 μM increased electrical resistance in a dose-dependent manner across endothelial cell monolayers derived from bovine pulmonary arteries. 1-Palmitoyl-LPA (16:0 LPA) at 5 μM increased endothelial electrical resistance to a similar extent as 5 μM 1-oleoyl-LPA. MCDB, MCDB 131 cell culture medium. Values are means ± SE; $n = 4$ cell monolayers/group. $^{*}P < 0.05$ compared with MCDB group from 3 and 6 to 30 min. $^{t}P < 0.05$ compared with MCDB group at 9-min time point.

fraction had a molecular mass between 3 and 100 kDa. An obvious criticism to the labeling of a protein as the active factor is how could platelets replenish the store of a protein continuously released into the plasma when it is known that platelets do not synthesize many proteins. This criticism prompted us to reevaluate the previous data and to consider viable alternatives to the composition of the active factor. There were also conflicting pieces of data. Patil et al. (19) and Haselton and Alexander (9) observed that the active factor was trypsin sensitive and precipitable in saturated ammonium sulfate, which would implicate a large charged protein, yet the activity was heat stable.

Assuming that the permeability-decreasing activity of PCM was associated with a protein fraction, we performed ion exchange chromatography to increase the specific activity of the protein fraction. Preliminary findings associated the activity to a negatively charged protein like albumin because the activity resided in the protein fraction bound to an anionic exchange column and required a high salt concentration to elute the activity from the column. Subsequent experiments performed in the present study that involved gel filtration chromatography, albumin immunoprecipitation of PCM, and Blue Sepharose chromatography demonstrated that the activity associates with albumin. Furthermore, 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) provided 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 phospholipase B that cleaves at the *sn*-1,2 positions and alkaline phosphatase that cleaves at the phosphomonoester bond but not by phospholipase A_2 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 phospholipid 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 inhibits PCM activity by altering the confirmation of albumin, exposing bound lipids to hydrolysis by lipases that must be present in the PCM. Finally, the previously observed permeability-decreasing activity of bovine 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 permeability by physically interacting with the glycocalyx at the junctional surface (15). On the other hand, albumin can also increase transcellular permeability by binding to the albumin receptor, gp60, or albondin 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 included 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.

We thank Jeff Moore of Avanti Polar Lipids for the electrospray mass spectrometric analysis of the lipid profile of platelet-conditioned medium and Wendy Hobb for editorial assistance.

This work was supported by American Heart Association Grant 97-0127A.

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