Supporting Information

Analytical Characterization of the Role of Phospholipids in Platelet Adhesion and Secretion

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Additional Detail Regarding Experimental Methods

Platelet-Rich Plasma Isolation from Whole Blood

Whole blood was centrifuged at 130xg for 10 min without brake, and the upper plasma layer was transferred to new tubes. Platelets were pelleted from the plasma by centrifugation at 500xg for 10 min and resuspended in fresh Tyrode’s buffer.

Device Fabrication for Adhesion Measurements

Briefly, microfluidic channels were fabricated in polydimethylsiloxane (PDMS) using standard photo-soft lithography techniques. The channel design was printed onto a transparent film (CAD/Art Services,), transferred onto a chrome mask plate (Nanofilm,), and patterned onto a layer of SU-8 photosresist (SU-8 100, Microchem; Newton, MA) on a silicon wafer. The cell culture channel dimensions were 400 µm (width) x 100 µm (height) x 2500 µm (length). Once a master (patterned SU-8 on a silicon wafer) was complete, 10:1 resin:curing agent mixture of Sylgard 184 (Ellsworth Adhesives) was degassed in a vacuum chamber and then cast onto the master, followed by overnight curing at 80ºC. Once cured, the PDMS layer was peeled off of the master, inlet and outlet holes were punched using a syringe needle, and the PDMS layer was washed to remove any remaining PDMS debris blocking the channels. Then, the PDMS layer was bonded to a clean glass substrate via oxygen plasma treatment to seal the channel. The device was brought into a bio-hood, and a 70 wt% ethanol solution was injected through the channel followed by a sterilized water rinse for sterilization purposes. This step was repeated three times, and after drying the channel, the device was exposed to UV light in the bio-hood for 5 minutes and then kept in the bio-hood until use.

Endothelial Cell Culture and Coating of the Microfluidic Device

The human endothelial cell line, Hy926, was purchased from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco’s Modified Eagle Medium (DMEM) with high glucose (formula: 4 mM L-glutamine, 4.5 g/L L-glucose, and 1.5 g/L sodium pyruvate (Gibco®, Carlsbad, CA)), supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (Sigma Aldrich, Milwaukee, WI) was used as the culture media, and cells were cultured in a T-flask with 5% CO₂ at 37ºC. Cells were fed every other day and split once a week. Endothelial cells were used only in passages three through twelve.

A day or two before the experiments, a microfluidic device was first incubated for 30 minutes with a 250 µg/mL human fibronectin solution in an incubator. Meanwhile, trypsinized endothelial cells from a T-flask were washed and re-suspended into the same culture media at a cell density of ~10⁷ cells/mL. The cell suspension was injected into the microfluidic device channel and kept in an incubator for 3 hours. The fresh cell culture media was then injected through the channel to remove any non-adherent endothelial cells remaining in the channel, and the device was maintained in an incubator, feeding the cells every 12 hours. A monolayer of endothelial cells was achieved within 48 hours (80% of the devices), and devices that did not yield a complete, uniform monolayer of endothelial cells within 48 hours were discarded (20% of the devices).

Quantitation of Secreted Platelet-Activating Factor

Platelet-secreted PAF was quantified using a previously published UPLC-MS/MS method. A Waters BEH C18 2.1 x 50 mm column was used for separation, and a Waters Acquity triple quadrupole mass spectrometer was used for multiple reaction monitoring of secreted PAF with an internal standard of PAF-d₄. Selected reaction monitoring (SRM) transitions for PAF and PAF-d₄ were 524.4 > 184.1 and 528.4 > 184.1, respectively. Calibration curves were created by normalizing peak areas of PAF standards to peak areas for 25 ng/mL PAF-d₄ to create response curves over the concentration range of 0.5 – 500 ng/mL PAF. 25 ng/mL PAF-d₄ was spiked into supernatants from washed phospholipid-incubated platelets immediately after supernatant collection, and PAF secreted from platelets was quantified using response-based calibration curves. Due to low recovery of PAF in Tyrode’s buffer, PAF secretion was measured from platelets incubated in phosphate buffered saline (free of Ca²⁺ and Mg²⁺, containing 1g/L glucose).

Effects of PC or SM Incubation on Platelet Function

Upon PC or SM incubation, enrichment was not significant in the phospholipid of interest. Exogenous PC reduced the number of secreted δ-granules from platelets as detected by CFMA (Fig. S1), whereas exogenous SM did not affect platelet δ-granule secretion (Fig. S2). Exposure to exogenous PC and SM suppressed platelet adhesion function (Fig. S3).
PC did not affect the quantity of the serotonin released from platelet granules or the kinetics of the release events (Figure S1). However, the number of δ-granules released per secretion event was significantly lower for PC-enriched platelets (N= 7.20±0.63 for control; N= 5.36±0.55 for PC condition p= 0.0337). Moreover the cumulative frequency of the release analysis showed that in early stages of secretion, the control and PC-treated platelets release granules with similar frequency, but that PC-enriched platelets slow granule release frequency before control platelets. This implies that the PC may influence the granular recruitment. In early moments of the exocytosis, the granules that are released immediately constitute the already docked granules. Release of the other granules requires recruitment of the granules to the site of fusion and docking. Both low N numbers and slower release frequency indicate the possible effect of PC on granule recruitment and docking. A possible effect of PC on vesicle recruitment in PC12 cells has been reported previously. Moreover, recent work has demonstrated that PC in axons of neurons shows a proximal to distal gradient which is regulated by actin dynamics. This type of inter-regulation may be possible between platelet PC and cytoskeleton where the disruption in PC localization interferes with actin function and recruitment to the membrane. Although this would reveal a unique regulatory pathway for platelet granular release, further work needs to be done to test this hypothesis. PC forms almost a flat layer, with minimal influence on membrane curvature. As expected, the % of fusion events with a foot (stable fusion pore) did not change with PC enrichment.

Incubation of the platelets with SM resulted no significant change in the platelet δ-granule secretion (Figure S2).

| Phospholipid Incubation Condition | Control | PC | SM |
|----------------------------------|---------|----|----|
| Transition used for relative quantitation | n/a | 782.9→86.1 | 703.8→184.1 |
| Instrumental Precision (RSD) | n/a | 15.94 | 7.635 |
| Biological Precision (RSD) | n/a | 17.58 | 23.59 |
| Total protein in pelleted platelets (µg/mL ± SD) | 117.3 ± 17.15 | 107.0 ± 16.09 | 109.7 ± 19.92 |
| Percent increase in platelets upon incubation (range in 4 replicates, 3 for PS) | n/a | 3.437 – 21.07 | -2.06 – 19.39 (not significantly enriched) |
| Average percent increase (Percent ± SD) | n/a | 12.13 ± 8.404 | 11.57 ± 9.378 (not significantly enriched) |
Figure S1. Effect of PC on δ-granule quantal release and release kinetics.

(A) PC did not influence the amount of serotonin released from single granules. Kinetics of the release (B) and (C) were also unchanged. (D) A lower number of granules exocytosed from single platelets with comparable fusion pore stability (E).

(F) Cumulative frequency analysis shows initially fast but later slowed granule trafficking in PC-enriched platelets.*p=0.05.
Figure S2. Effect of SM on δ-granule quantal release and release kinetics.

(A) SM did not change the amount of serotonin released from single granule, (B) and (C) kinetics of the release; (D) number of granules exocytosed from single platelet; (E) fusion pore stability or (F) cumulative frequency of the release. p>0.05
Figure S3. Platelet incubation with PC and SM suppressed platelet adhesion behavior.