SUPPLEMENT

Materials and Methods:

Murine phycoerythrin-labeled monoclonal anti-P-selectin and FITC-labeled PAC-1 antibodies were from BD Biosciences (San Jose, CA); calcein and FURA2 from Invitrogen (Carlsbad, CA); sterile filtered Hank's balanced salt solution and M199, BioWhittaker (Walkersville, MD); sterile tissue culture plates, Falcon Labware (Lincoln Park, NJ); human serum albumin, Baxter Healthcare (Glendale, CA); endotoxin-free PBS; 4-well Lab-Tek® II Chamber Slide System, Nalge Nunc International (Naperville, IL); collagen, Chrono-Log, Havertown, PA; PAF receptor antagonists, BioMol (Plymouth Meeting, PA). Other reagents were from Sigma.

Cell and organelle isolation

Blood was drawn into acid-citrate-dextrose and centrifuged (200xg, 20 min) without braking to obtain platelet-rich plasma that was filtered through two layers of 5 μ mesh (BioDesign, Carmel, NY) before 100 nM prostaglandin E_1 (PGE_1) was added and the cells recovered by centrifugation (500 x g, 20 min). The pellet was resuspended in 50 ml PSG (5 mM Pipes, 145 mM NaCl, 4 mM KCl, 50 μM Na_2HPO_4, 1 mM $MgCl_2$, and 5.5 mM glucose) containing 100 nM PGE_1. PGE_1 was removed by centrifugation just prior to use of the cells. Platelets were treated with varied concentrations of HAz-LPAF for 2h, washed, lysed by shear and mitochondria recovered by differential centrifugation [1].

Flow Cytometry and aggregation

P-selectin surface expression. Washed platelets (100 μl at 10^8/ml) were mixed with phycoerythrin-labeled anti-P-selectin antibody (1:100) and then treated with the stated reagents

for 10 min at room temperature. One volume of 2% formaldehyde was added, the cells incubated for 30 min, and then mixed with three volumes of PBS before FACScan analysis where platelets were gated by forward and side scatter. *PAC-1 surface expression*. Washed platelets were treated for 10 min with the stated reagents at room temperature before FITC-labeled anti-PAC-1 antibody was added. After 15 min, the fixed platelets were analyzed by flow cytometry.

Aggregation was monitored by changes in transparency of stirred suspensions of washed platelets containing 0.1 μg/ml μg collagen (Chrono-Log; Havertown, PA).

The truncated phospholipids, although soluble at the levels employed, were presented to platelets complexed to human serum albumin (0.025%) to reduce interaction with plasticware.

Microscopy

Washed platelets (1ml at 10^8/ml) were stimulated in six well glass coverslips for 10 min at 24 °C, washed thrice with PBS and fixed with 1.6% paraformaldehyde and 2.5% glutaraldehyde before post-fixation with 1% osmium tetroxide and staining with 0.5% and then 1.5% uranyl acetate. Samples were dehydrated with graded ethanol concentrations and hexamethydisilizane before gold coating for scanning electron microscopy. Cellular fluorescence after calcein-AM labeling was visualized with a 480 excitation/ 535 emission cube.

References:

1. Chen R, Yang L, McIntyre TM. Cytotoxic phospholipid oxidation products: Cell death from mitochondrial damage and the intrinsic caspase cascade. *J Biol Chem.* 2007;282:24842-24850.

Supplementary Figure 1. oxLDL induces platelet shape change. Platelets were treated with the stated agonists for 10 min before fixation and gold coating for visualization by scanning electron microscopy.

Supplementary Figure 2. A narrow concentration range of oxLDL particles stimulates platelet Ca^{++} flux. Platelets loaded with FURA2 were treated with the stated concentration of oxidized LDL particles and an increase in emission spectra recorded in a stirred fluorescent cuvette. The upper panel presents low oxLDL concentrations, the lower panel higher oxLDL concentrations.

Supplementary Figure 3. The PAF receptor antagonist CV3988 reduces oxLDL stimulation and thrombin synergy. Platelets were pre-incubated with CV3988, a PAF structural analog with some partial agonist activity, prior to stimulation with thrombin, oxLDL (A) or (B) HAz-LPAF, or their combination before intracellular free Ca^{++} was detected by FURA2 fluorimetry.

Supplementary Figure 4. A PAF receptor antagonist blocks platelet stimulation by HAz-LPAF. FURA2-labeled platelets were stimulated with the stated concentration of agonists with or without prior and continued exposure to 10 μM WEB2086 or the structurally unrelated ginkolide BN52021 PAF receptor antagonists.

Supplement Table I. Percent Change in Intracellular Calcium Values (n=3) for Figure 2.

Web2086	oxLDL	Thrombin	ADP	Collagen	oxLDL + Thrombin	oxLDL + ADP	oxLDL + Collagen
-	183.1±92.1	105.9±36.5	190.0±28.3	298.2±42.9	563.8±63.7	686.1±47.0	638.8±156.2
+	3.5±20.11	NA	NA	NA	156.7±41.4	329.9±102.8	334.1±183.3

Supplement Table II. Percent Change in Intracellular Calcium Values (n=3) for Figure 4.

HAz-LPAF	Thrombin	ADP	Collagen	HAz-LPAF + Thrombin	HAz-LPAF + ADP	HAz-LPAF + Collagen
439.6±62.8	297.5±77.2	291.8±45.6	531.1±20.4	982.4±3.9	935.3±120.5	775.8±43.1

Andrew P. Thomas and Fernando Delaville “The use of fluorescent indicators for measurements of cytosolic-free calcium concentration in cell populations and single cells” in Cellular Calcium, A Practical Approach Edited by J. G. McCormack and P. H. Cobbold, Oxford University Press, Oxford pp 1-54, 1991

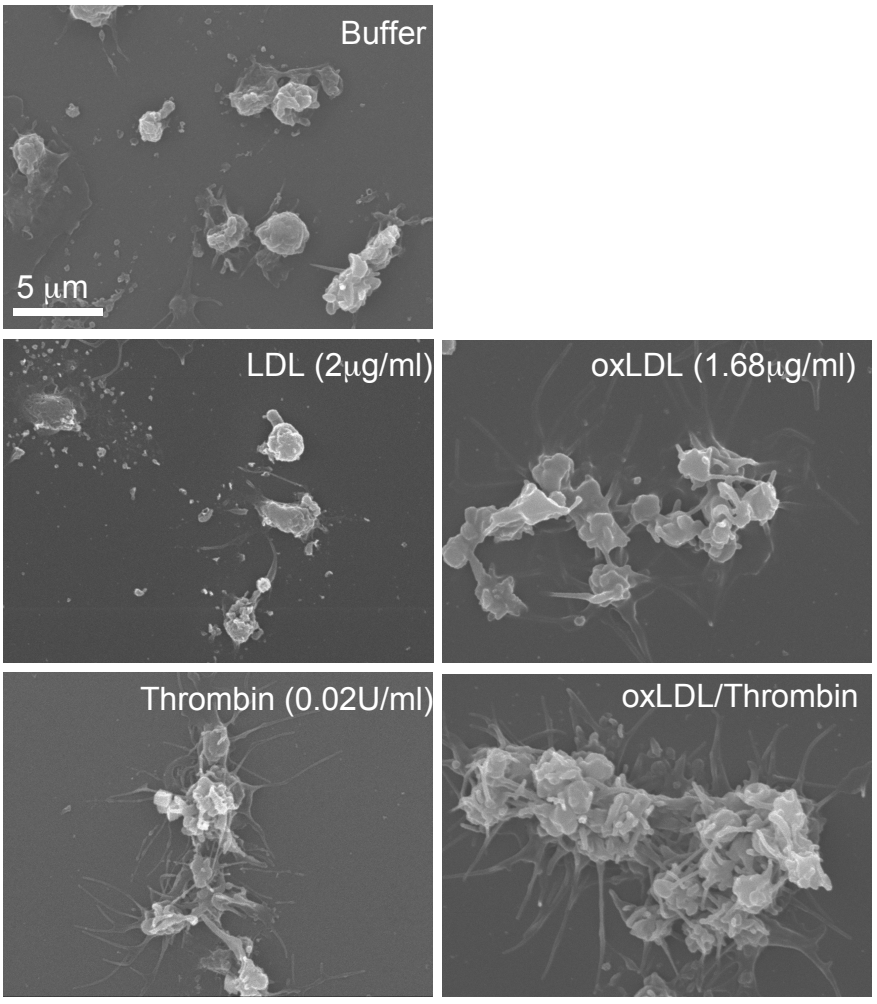
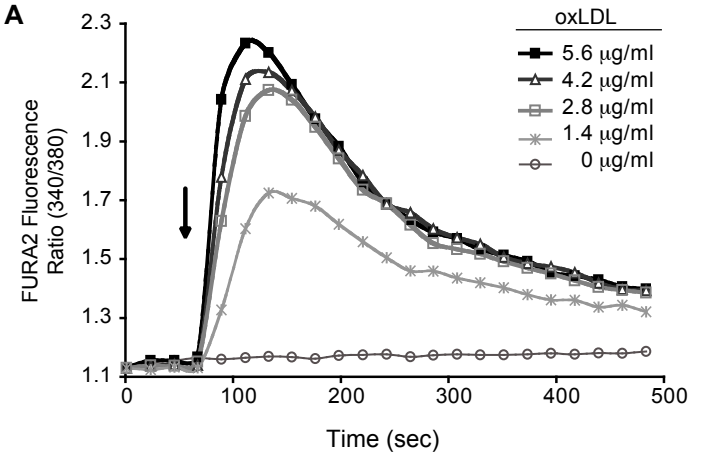


Figure 1
Supplement



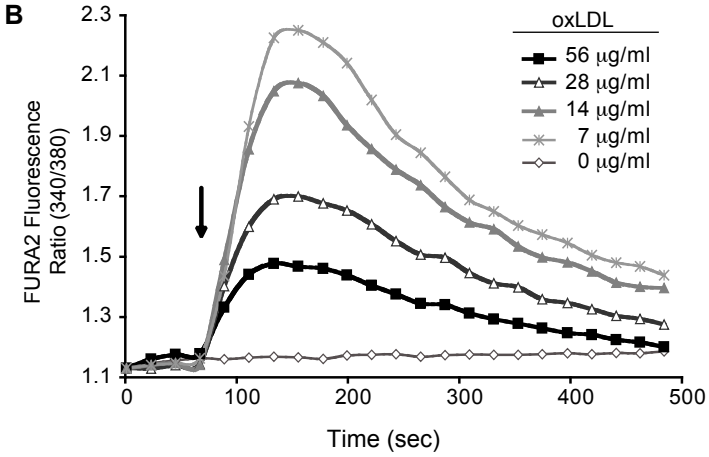


Figure 2
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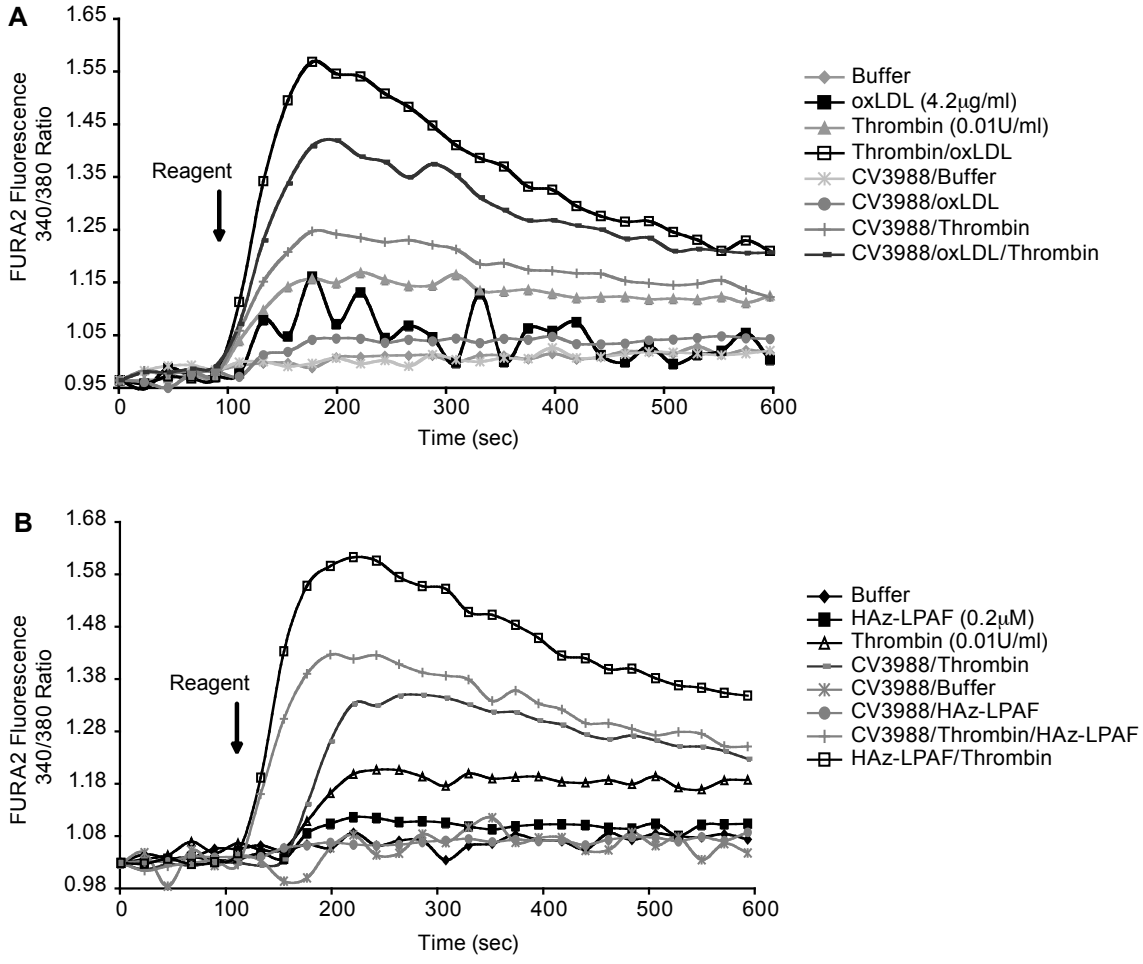

Figure 3
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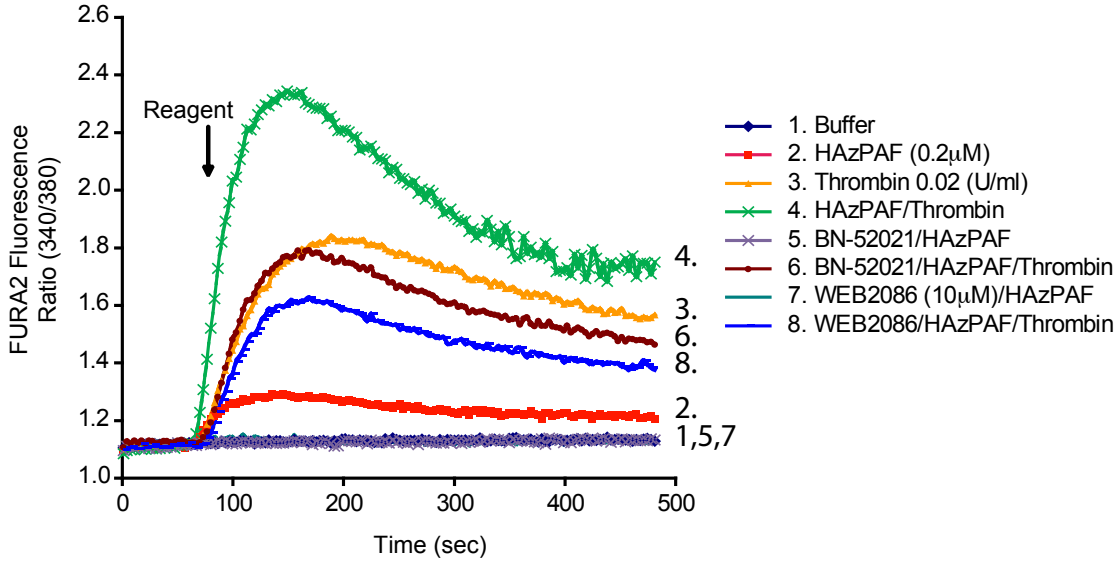

Figure 4
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