Platelet Activation by Low Concentrations of Intact Oxidized LDL Particles Involves the PAF Receptor

Rui Chen, Xi Chen, Robert G. Salomon, Thomas M. McIntyre

Objective—Mitochondrial depolarization aids platelet activation. Oxidized LDL (oxLDL) contains the medium length oxidatively truncated phospholipid hexadecyl azelaoyl-lysoPAF (H Az-LPAF) that disrupts mitochondrial function in nucleated cells, so oxLDL may augment platelet activation.

Methods and Results—Flow cytometry showed intact oxLDL particles synergized with subthreshold amounts of soluble agonists to increase intracellular Ca\(^{2+}\), and initiate platelet aggregation and surface expression of activated gpIIb/IIa and P-selectin. oxLDL also induced aggregation and increased intracellular Ca\(^{2+}\) in FURA2-labeled cells by itself at low, although not higher, concentrations. H Az-LPAF, alone and in combination with substimulatory amounts of thrombin, rapidly increased cytoplasmic Ca\(^{2+}\) and initiated aggregation. HAz-LPAF depolarized mitochondria in intact platelets, but this required concentrations beyond those that directly activated platelets. An unexpectedly large series of chemically pure truncated phospholipids generated by oxidative fragmentation of arachidonoyl-, docosahexaneoyl-, or linoleoyl alkyl phospholipids were platelet agonists. The PAF receptor, thought to effectively recognize only phospholipids with very short sn-2 residues, was essential for platelet activation because PAF receptor agonists blocked signaling by all these medium length phospholipids and oxLDL.

Conclusions—Intact oxLDL particles activate platelets through the PAF receptor, and the PAF receptor responds to a far wider range of oxidized phospholipids in oxLDL than anticipated. (Arterioscler Thromb Vasc Biol. 2009;29:363-371.)

Key Words: oxidized LDL • PAF • platelet • phospholipid • PAF acetylhydrolase

Platelet mitochondria maintain cellular energetics and viability, but these organelles also affect platelet activation. Thrombin produces a rapid decrease in mitochondrial transmembrane potential, thereby increasing reactive oxygen species formation and caspase 3 activation needed for maximal aggregation.\(^{2}\) Combining maximal amounts of thrombin with collagen\(^{3}\) or with activators of Bax, a proapoptotic protein that physically targets mitochondria, generates a subpopulation of highly activated platelets, and blockade of the mitochondrial permeability transition pore suppresses this form of platelet activation.\(^{7,6}\)

Oxidized low density lipoprotein (oxLDL) has a fundamental role in thrombotic disease and atherogenesis through activation of inflammatory cells that includes platelets,\(^{7,8}\) although the identity of the agonist(s) is unclear because oxLDL contains numerous bioactive compounds.\(^{9}\) Oxidation of lipoprotein particles, either chemically or enzymatically,\(^{10,11}\) fragments phospholipids that contain polyunsaturated fatty acyl residues. One such oxidatively-truncated phospholipid, hexadecyl azelaoyl lysoPAF (H Az-LPAF), derived from oxidation of common linoleoyl residues, rapidly enters nucleated cells, traffics to mitochondria, and initiates the mitochondrial-dependent pathway to apoptotic cell death.\(^{12}\)

We determined whether oxidized LDL contains agents that disrupt platelet mitochondrial function, thereby increasing cellular responsiveness. We prepared pure samples of a series of fragmented alkyl glycerophosphocholines—acylated lysoPAFs—found in oxidized LDL, and discovered that many of these stimulated platelets. However, these lipids, and intact oxLDL particles, were direct platelet agonists that did not require mitochondrial involvement. Instead, we find the range of PAF receptor ligands is larger than previously predicted and includes medium-length oxidatively truncated alkyl phospholipids found in oxLDL. We show low concentrations of intact oxLDL particles activate platelets only through their PAF receptor.

Materials and Methods

HAz-LPAF and PAF were from Avanti Polar Lipids; human LDL was oxidized with Cu\(\text{2SO}_4\) as described;\(^{13,14}\) fractions from oxidized arachidonoyl-, linoleoyl-, and docosahexaneoyl-glycerophosphocholine and those prepared synthetically were as described;\(^{14}\) other materials are described in the supplemental materials (available online at http://atvb.ahajournals.org).
Cell Isolation
Protocols using human blood were approved by the Cleveland Clinic IRB committee. Platelets were isolated as described in the supplemental materials.

Flow Cytometry
Surface proteins were quantified as described (supplemental materials). Platelet mitochondrial transmembrane potential was examined in washed platelets (10^7/mL) incubated (1 hour) with HAz-LPAF in serum-free RPMI, then with JC-1 (10 µg/mL, 15 minutes) before they were washed twice with PBS and analyzed by 2-color flow cytometry.

Microscopy
Electron microscopy was performed as described in the supplemental materials. For fluorescent microscopy, washed platelets (500 µL) were mixed with calcein-AM, treated with the specified reagents with mild shaking for 10 minutes at room temperature in a 4-well cover slip chamber before unattached cells were removed by washing.

Intracellular Ca^{2+} Measurement
Platelets (10^9/mL) were incubated for 30 minutes with 1 µmol/L FURA2-AM in PSG containing 1 µg/mL prostaglandin E1, collected by centrifugation, and suspended in Hank’s buffered salt solution. Fluorescence was continuously recorded at 25°C by alternating the excitation wavelength between 340 and 380 nm, and detecting the fluorescent emission at 510 nm with the bandwidth set at 2.5 nm for both emission and excitation.

Data Expression and Statistics
Experiments were performed at least 3 times with cells from different donors with representative experiments or mean±SE from all experiments shown. Supplemental Tables I and II provide quantitative Ca^{2+} data.

Results
Intact Oxidized LDL Particles Activate Platelets
Low amounts of intact oxLDL particles (5 µg/mL) stimulated mobilization of platelet alpha granules as shown by surface expression of P-selectin (Figure 1A). Platelets failed to respond to a subthreshold concentration of thrombin (0.02 U/mL), but did so when a low concentration of oxLDL was included in the incubation (Figure 1A and 1B). This combined response was greater than that induced by oxLDL alone, so oxLDL and thrombin signaling interact to promote platelet activation. Intact oxLDL particles alone also stimulated the adhesive response of platelets, and in combination with thrombin again evoked a response that was more robust than by either single agonist (Figure 1C). OxLDL particles, like thrombin, enhanced spreading and scanning electron microscopy showed the combination of agonists produced highly spread clusters or aggregates of platelets (supplemental Figure I).

oxLDL Priming and Activation Requires the PAF Receptor
Platelet activation by oxLDL was concentration dependent with concentrations from just under 1.5 µg/mL up to 5 µg/mL stimulating intracellular Ca^{2+} flux, but then activation was significantly reduced as the concentration of oxLDL increased above 7 µg/mL (supplemental Figure 2). Thrombin at 0.02 U/mL induced a modest increase in intracellular free Ca^{2+} (Figure 2A), oxLDL at 1.4 µg/mL was a better agonist than this very low level of thrombin, and the combination of oxLDL and thrombin greatly increased the rapidity and extent of platelet activation compared to either agonist alone. Similarly, oxidized LDL augmented the Ca^{2+} flux induced by ADP (Figure 2B) and collagen (Figure 2C). The inhibitory PAF receptor inverse agonist WEB2086 abolished signaling by oxLDL, without altering the response to thrombin, ADP, or collagen (Figure 2). WEB2086 also completely suppressed the cooperative effect of oxLDL on thrombin stimulated Ca^{2+}
flux (Figure 2), and largely suppressed the oxLDL enhancement of the response to ADP or collagen (Figure 2B and 2C).

Platelets Are Activated by the Truncated Phospholipid HAZ-LPAF

Agonist-induced mitochondrial depolarization enhances platelet reactivity. The synthetic oxidatively truncated alkyl phospholipid HAZ-LPAF depolarizes mitochondria of nucleated cells, and it depolarized mitochondria in intact washed platelets (Figure 3A). Platelets exposed to 2.5 μmol/L HAZ-LPAF were not different from control cells, but—as with nucleated cells—5 and 10 μmol/L HAZ-LPAF increased the population with depolarized mitochondria. Physical isolation showed that cytochrome c escaped from its mitochondrial compartment in platelets exposed to the higher concentrations of HAZ-LPAF (Figure 3B). Pure HAZ-LPAF increased platelet cytoplasmic Ca²⁺ levels in a concentration dependent way (Figure 3C), so this oxidatively truncated phospholipid is a direct platelet agonist. However, this Ca²⁺ response was unrelated to the mitochondrial dysfunction because HAZ-LPAF was ~25-times more potent at stimulating platelets than it was in depolarizing their mitochondria (compare Figure 3A with Figure 3C).

Submicromolar concentrations of HAZ-LPAF, like oxLDL, synergistically enhanced thrombin-induced increases in intracellular free Ca²⁺ (Figure 4A). HAZ-LPAF at 10⁻⁷ mol/L also enhanced platelet activation by suboptimal amounts of ADP (Figure 4B) and collagen (Figure 4C). HAZ-LPAF induced an immediate increase in intracellular Ca²⁺, in contrast to the delayed response to collagen, and it appears the response to the combination of HAZ-LPAF and collagen is a direct summation of independent events.

The response of platelets to HAZ-LPAF included changes in the conformation of surface gpIIb/IIIa to its active state recognized by PAC-1 antibody (Figure 5A). Again, the combination of HAZ-LPAF and trace amounts of thrombin was more effective than either agonist alone. HAZ-LPAF induced platelet aggregation, and this truncated phospholipid potentiated the effect of thrombin present at a concentration too low to induce aggregation by itself (Figure 5B).
synergistic effect of HAZ-LPAF on substimulatory amounts of thrombin comes from PAF receptor signaling because CV3988, a PAF receptor analog and receptor antagonist, suppressed this costimulus dependent aggregation. Suppression of the synergistic activation was not complete because CV3988 has partial agonistic activity16 (supplemental Figure III). Blockade of the PAF receptor by CV3988 also suppressed the ability of low concentrations of oxLDL to promote platelet aggregation by substimulatory amounts of thrombin (Figure 5B). Although CV3988 was only partially successful in reducing platelet activation and priming by HAZ-LPAF and oxLDL, this stimulatory effect derived from PAF receptor activation because the more effective PAF receptor antagonists BN52021 and WEB2086 abolished HAZ-GPC signaling and interaction with thrombin signaling (supplemental Figure IV).

Numerous Phospholipid Truncation Products Activate Platelets

We determined whether HAZ-LPAF was unique in its ability to stimulate platelets among the many oxidatively truncated phospholipids found in oxLDL, and so prepared pure samples of a series (Figure 6A) of phospholipids that are generated by the oxidative fragmentation of alkyl phospholipids with sn-2 docosahexenoyl, arachidonoyl, and linoleoyl residues.14,17 The backbone of this series of phospholipids is the lysoPAF (alkyl glycerophosphocholine) molecule that contains the sn-1 ether bond that is highly preferred by the PAF receptor. A large number of these structures at submicromolar concentrations induced a rapid increase in platelet cytoplasmic Ca\(^{2+}\) level (Figure 6B). The effectiveness of these oxidatively truncated alkyl phospholipids varied significantly, and did so in a largely nonobvious way. For instance, the most active group of truncated phospholipids included those with the shortest sn-2 residues tested—4 carbon oxobutyl and succinoyl residues—as expected, but also included 7 and 8 carbon long acidic \(\alpha,\beta\) unsaturated keto residues. While there was no distinct pattern to platelet activation by these phospholipids, HAZ-LPAF was a member of the most active group of the oxidatively truncated phospholipids. The PAF receptor antagonist WEB 2086 was completely effective in suppressing the response to HAZ-LPAF, and this blockade and inactivation of the PAF receptor also completely suppressed platelet activation by every other oxidatively truncated alkyl phospholipid we tested (Figure 6C). The structurally unrelated PAF receptor antagonists BN52021 and WEB2086 were equally effective in suppressing platelet activation by HAZ-LPAF (supplemental Figure IV), so the PAF receptor has a far wider range of agonist selectivity than previously determined using diacyl phospholipid homologs with unmodified chain fatty acyl residues.

Discussion

Thrombin is present at low concentrations during the initiation phase of thrombosis18 and low concentrations of oxLDL cooperated with substimulatory concentrations of thrombin—or collagen or ADP—to activate platelets. However, oxLDL particles by themselves also activated platelets because oxidative fragmentation of its pool of alkyl phospholipids generated PAF receptor agonists. Oxidation of polyunsaturated fatty acids in diacyl and alkyl phospholipids proceeds by identical mechanisms, but all the newly formed agonists arise from oxidation of the less abundant alkyl phospholipids.13 There are two unusual aspects of platelet stimulation by the lipid agonists of oxidized LDL. First is that intact oxidized LDL particles were potent platelet agonists. We13,19 and others20–23 have shown that oxidation of LDL generates a host of agonists for platelets, monocytes, and polymorphonuclear leukocytes. Among these agonists are

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** A pure oxidatively truncated phospholipid perturbs platelet homeostasis. **A,** Mitochondrial potential. Platelets were treated (n=3) with the stated concentration of HAZ-LPAF, and changes in mitochondrial potential were assessed using FURA2-loaded platelets (n=3).

**B,** Cytochrome C release. Platelets (n=366 Arterioscler Thromb Vasc Biol March 2009)

**C,** HAZ-LPAF induced a concentration-dependent transient increase in intracellular free Ca\(^{2+}\) levels in FURA2-loaded platelets (n=3).
very low amounts of platelet-activating Factor (PAF)\textsuperscript{24} and other very short-chained PAF analogs\textsuperscript{19,25} that are ligands for the PAF receptor that greatly prefers alkyl phosphatidylcholines. However, to assay and identify these agents the approach has been to extract, fractionate, and concentrate the biologically active species to remove the bulk of the inactive lipids that sequester the active lipids in inactive insoluble complexes. Here we find that either the relevant biologically active species are sufficiently soluble to allow them to partition between the lipoprotein particle and the platelet surface, or the \( \text{sn}-2 \) residue and the \( \text{sn}-1 \) ether bond of the newly formed oxidized alkyl phospholipids is available to PAF receptors on the platelet surface as found in the "lipid whisker" model for oxidized phospholipid interaction with CD36.\textsuperscript{26}

The second—and surprising—aspect of oxidized LDL activation of platelets is that the relevant receptor recognizing the lipids of oxLDL was the PAF receptor. Lysophosphatidic acid in oxLDL is described as the agonist in oxLDL that stimulates platelets via their LPA receptors,\textsuperscript{27} although this stimulation occurred at higher concentrations of oxLDL than required for PAF receptor-dependent signaling. Platelet activation by very low concentrations of oxLDL was abolished by PAF receptor antagonists, establishing the primacy of the PAF receptor in platelet responses to microgram quantities of intact oxidized LDL particles. Lysophosphatidic acid is some 10-fold less effective\textsuperscript{22} in stimulating platelets than oxidatively truncated phospholipids (supplemental Figure II) that act through the PAF receptor, and so stimulation of platelets at higher concentrations of oxLDL\textsuperscript{22} may have a larger contribution by this lysolipid when PAF receptor signaling becomes ineffective at higher oxLDL concentrations.

The PAF receptor, which has critical roles in inflammation, thrombosis, and vascular disease, is expressed by all cells of the innate immune system.\textsuperscript{28–30} This receptor displays marked ligand selectivity,\textsuperscript{28,31} allowing it to detect infinitesimal amounts of PAF in a sea of membrane phospholipids and lysophosphatidylcholines.\textsuperscript{32,33} A central feature of this selectivity is recognition of the unique short acetyl \( \text{sn}-2 \) residue of PAF, accompanied by a similarly strong recognition of the \( \text{sn}-1 \) ether bond and the choline headgroup. A model\textsuperscript{14} of PAF interaction with its receptor

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**Figure 4.** HAz-LPAF primes platelet reactivity. A low concentration of HAz-LPAF (0.1 µmol/L) increased intercellular Ca\textsuperscript{2+} levels in FURA2-loaded cells and aided the response to a low (A) 0.02 U/mL thrombin, (B) 1 µmol/L ADP, (C) 0.1 µg/mL collagen concentrations. Quantitative values are shown in supplemental Table II.
postulates a binding pocket that physically rejects longer sn-2 residues.

This modeling, however, is based on synthetic phospholipids where the sn-2 residue is a straight short-chained fatty acyl molecule, and this understanding was established before a molecular definition of the plethora of oxidatively truncated phospholipids found in oxidized lipoprotein particles,20–23,35 apoptotic cells,36 and platelet microparticles.37 Oxidized alkyl phospholipids break the paradigm established with these short straight-chained fatty phospholipids and introduce a host of new structures with a variety of oxy functions and carbon skeletons that can productively interact with the PAF receptor.

Examination of the PAF-like activity of a series of oxidatively truncated alkyl phospholipids shows that a short sn-2 residue is not an absolute criterion for an effective PAF receptor ligand. This means that oxidative fragmentation of any polyunsaturated fatty acyl residue, whether docosa-hexaenoyl or arachidonoyl residues that yield shorter 4 and 5 carbon fragments or linoleoyl residues forming 9 carbon long fragments, can generate effective PAF receptor agonists. Thus, PAF receptor agonists now also include longer sn-2 acidic residues such as the 9 carbon long HAz-LPAF, and 7- and an 8-carbon long keto acids (KDiA-GLPAF and KOdiA-LPAF, respectively). Only a few of the oxidatively truncated phospholipids bearing an ω-terminal aldehyde function were agonists, excluding a role for protein adduction by Schiff base formation in platelet activation by aldehydic phospholipids.

The pattern of platelet activation through the PAF receptor by ether-containing phospholipids is distinct from the pattern of effective CD36 ligands.38 This is epitomized by KODA-PC, the most efficient diacyl CD36 ligand,20,38 which failed to stimulate platelets (Figure 6). Although CD36 stimulation by oxLDL requires several hundred micrograms of oxLDL,39 CD36 could still bridge lower concentrations of oxidized LDL to platelets in a way that enhances platelet reactivity and promotes intravascular thrombosis.20 Platelets in different environments are exposed to vastly different levels of oxLDL, from the low number of modified particles in the circulation40 to the high levels sequestered in atherosclerotic lesions. OxLDL itself was a potent platelet agonist, but it also was synergistic with amounts of thrombin (0.02 U/mL) too low to be stimulatory by itself, so the PAF receptor ligands in oxLDL are positioned to promote events early in thrombosis. Additionally, OxLDL contains, at a minimum, various oxidatively modified phospholipids and cholesterol, isoprostanes and oxidized arachidonoyl residues,41 lyso-lipids generated by PAF acetylhydrolase hydrolysis of the newly minted oxidized phospholipids,42,43 and lysophosphatidic acid.9 As might be expected from this, the effect of oxLDL on inflammatory cells is complex, dependent on the particle concentration and extent and mode of oxidation.9

We observed that intact oxLDL particles functioned as full direct platelet agonists at a few µg/mL, and achieved a maximal response by ≈7 µg/mL. This response then faded as the oxLDL concentration increased, suggesting the additional

Figure 5. HAz-LPAF activates platelet traditional functional responses. A, HAz-LPAF alone and in combination with thrombin changed the conformation of IIb/IIIa to its activated form as measured by exposure of the PAC-1 epitope during flow cytometry. B, HAz-LPAF induced aggregation through the PAF receptor. Aggregometry of platelet rich plasma in responses to thrombin (0.005 u/mL) or oxLDL in the presence or absence of the PAF receptor antagonist CV3988 (1 µmol/L).
Figure 6. Truncated phospholipids created by oxidation of common phospholipids activate platelets through the PAF receptor. A, Reaction scheme depicting pathways forming specific phospholipid oxidation products starting from acyl lysoPAFs (alkyl glycerophosphocholines) containing the common arachidonoyl (AA), docosahexaneoyl (DHA), or linoleoyl (LA) polyunsaturated fatty acyl residues. B, Platelet stimulation by oxidized phospholipids. Intracellular Ca\(^{2+}\) as a function of time in platelets treated with 0.5 \(\mu\)mol/L of the stated phospholipid. C, Effect of PAF receptor inhibition. Platelets were pretreated with 10 \(\mu\)mol/L WEB2086, except for one sample of OB-LPAF that served as the positive control, before Ca\(^{2+}\) levels were determined.
presence of a less potent antagonist(s). One lipid of oxLDL that can interfere with platelet function is lysophosphatidylcholine,9,44 but its role is enigmatic. Its actions are concentration-dependent, either aiding or suppressing platelet function, and acts in receptor-dependent and independent ways. Other antiinflammatory agents present in oxLDL may also participate in forming the bell-shaped action profile, while the variety of agonists—particularly lysophosphatidic acid22—could produce the stimulation found at oxLDL higher concentrations.

Controversy exists regarding whether the circulating enzyme PAF acetylhydrolase/lipoprotein-associated phospholipase A2 that hydrolyzes PAF and oxidized phospholipids is antiinflammatory,29,47,48 or proinflammatory.49 These opposing views depend on whether one regards the substrate PAF or its lysoPAF product as the primary bioactive entity of oxidized LDL. Here we find several oxidatively truncated phospholipids that were less effective agonists than the lysolipid product, so their hydrolysis would enhance platelet activation. Conversely for the larger number of oxidatively truncated phospholipids that were more effective than the corresponding lysolipid hydrolytic product, hydrolysis by PAF acetylhydrolase would decrease PAF receptor activity. Resolution of these disparate views based on substrate selectivity is problematic.

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Disclosures
None.

References


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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 E₁ (PGE₁) 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 Na₂HPO₄, 1 mM MgCl₂, and 5.5 mM glucose) containing 100 nM PGE₁. PGE₁ 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 10⁸/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 10⁸/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:

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.

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

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Figure 1
Supplement
Figure 2
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Figure 3
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A

B

Reagent

FURA2 Fluorescence
340/380 Ratio

Time (sec)

Time (sec)
**Figure 4**

FURA2 Fluorescence Ratio (340/380) vs. Time (sec)

- **1. Buffer**
- **2. HAzPAF (0.2uM)**
- **3. Thrombin 0.02 (U/ml)**
- **4. HAzPAF/Thrombin**
- **5. BN-52021/HAzPAF**
- **6. BN-52021/HAzPAF/Thrombin**
- **7. WEB2086 (10uM)/HAzPAF**
- **8. WEB2086/HAzPAF/Thrombin**

Supplement