Integrative Physiology

Circulating Platelet-Activating Factor Is Primarily Cleared by Transport, Not Intravascular Hydrolysis by Lipoprotein-Associated Phospholipase A₂/PAF Acetylhylase

Jinbo Liu, Rui Chen, Gopal K. Marathe, Maria Febbraio, Weilin Zou, Thomas M. McIntyre

Rationale: The phospholipid platelet-activating factor (PAF) stimulates all cells of the innate immune system and numerous cardiovascular cells. A single enzyme (plasma PAF acetylhydrolase [PAF-AH] or lipoprotein-associated phospholipase [Lp-PL]A₂) in plasma hydrolyzes PAF, but significant controversy exists whether its action is pro- or antiinflammatory and accordingly whether its inhibition will slow cardiovascular disease.

Objective: We sought to define how PAF and related short-chain oxidized phospholipids turnover in vivo and the role of PAF acetylhydrolase/Lp-PLA₂ in this process.

Methods and Results: [3H-acetyl]PAF was hydrolyzed by murine or human plasma (t₁/₂, 3 and 7 minutes, respectively), but injected [3H-acetyl]PAF disappeared from murine circulation more quickly (t₁/₂, <30 seconds). [3H]PAF clearance was unchanged in PAF receptor⁻/⁻ animals, or over the first 2 half-lives in PAF-AH⁻/⁻ animals. [3H]PAF turnover was reduced by coinjecting excess unlabeled PAF or an oxidatively truncated phospholipid, and [3H]PAF clearance was slowed in hyperlipidemic apolipoprotein (apo)E⁻/⁻ mice with excess circulating oxidatively truncated phospholipids. [3H]PAF, fluorescent NBD-PAF, or fluorescent oxidatively truncated phospholipid were primarily accumulated by liver and lung, and were transported into endothelium as intact phospholipids through a common mechanism involving TMEM30a.

Conclusions: Circulating PAF and oxidized phospholipids are continually and rapidly cleared, and hence continually and rapidly produced. Saturable PAF receptor–independent transport, rather than just intravascular hydrolysis, controls circulating inflammatory and proapoptotic oxidized phospholipid mediators. Intravascular PAF has access to intracellular compartments. Inflammatory and proapoptotic phospholipids may accumulate in the circulation as transport is overwhelmed by substrates in hyperlipidemia. (Circ Res. 2011;108: 469-477.)

Key Words: PAF ■ oxidized phospholipids ■ phospholipid transport ■ lipoprotein-associated phospholipase A₂

The phospholipid platelet-activating factor (PAF) stimulates a single receptor (PAFR) expressed by platelets, but also by nearly every other cell of the innate immune system, and by numerous cells of the cardiovascular system. PAF and its receptor regulate inflammation, atherogenesis, cardiac rhythm, liver contractility, body temperature, and vascular tone. PAF is remarkably potent, activating cells at concentrations of 10⁻¹² mol/L, suggesting its presence is tightly controlled. However, its blood concentration can increase, for instance after ischemic stroke and inflammatory stress.

A single enzyme in blood metabolizes PAF⁵ and bioactive⁶ oxidatively modified phospholipids⁷ that accumulate in atherosclerosis and in response to other oxidative insults. Common names for this lipoprotein-associated enzyme are plasma PAF acetylhydrolase (PAF-AH) or lipoprotein-associated phospholipase (Lp-PL)A₂, but its gene name is group VII phospholipase A₂ (PLA2G7). PAF as a relatively soluble phospholipid is bound to lipoproteins, mainly albumin, with rapid exchange from lipoprotein particles allowing access both to plasma and the PAF receptor. There is significant controversy whether PLA2G7 is protective or promotes chronic inflammation, cardiovascular disease and atherogenesis, and direct manipulation of this activity in humans has not clarified the issue. A phase
illness and, that PAF catabolism primarily occurs in the intracellular compartment of tissues, reducing the role for circulating, although perhaps not intracellular, PLA2G7 in vivo PAF catabolism.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

In Vivo Phospholipid Metabolism
[3H-acetyl]PAF in 0.5% albumin in PBS, with or without a 1,000-molar excess of inactive enantiomeric PAF or a 2000-fold excess of synthetic AzPC, was injected into the retroorbital plexus.

Phospholipid Mass Spectrometry
Mass spectrometric analyses were performed on-line using electrospray ionization tandem mass spectrometry in the positive ion mode with multiple reaction monitoring using the molecular cation [MH]+ and the m/z 184 daughter phosphocholine ion.

Immunohistochemistry
Fluorescent NBD-PAF or NBD-AzLPAF were introduced by retroorbital injection. Organs were recovered 5 minutes later after extensively flushing the vasculature with PBS. Organs were excised, immediately frozen in liquid nitrogen, and embedded in OTC media for sectioning.

Statistics
The data represent the means±SD of the stated number of samples. The statistical analyses used a paired Student t test. For all of these hypotheses, the significance level was 0.05.

Results
PAF Disappeared From Circulation Faster Than Hydrolysis in Plasma
The half life of [3H-acetyl]PAF in human plasma was ≈7 minutes (Figure 1A), as we previously observed.23 We also observed that the turnover of [3H-acetyl]PAF was somewhat quicker in plasma than in whole blood where some 40% of plasma volume is displaced by blood cell volume. A similar pattern of efficient hydrolysis of [3H]PAF in plasma relative to blood held when murine material was examined (Figure 1A) where [3H]PAF was hydrolyzed in plasma with a t1/2 of ≈3 minutes.

We examined the rate of [3H-acetyl]PAF turnover in vivo with the expectation from the above observations that it would require approximately 3 minutes to hydrolyze half the [3H-acetyl]PAF we introduced into murine circulation by transport into tissues.20 Whether this uptake process is slower or faster than PLA2G7 action on PAF or oxidized phospholipids in plasma has not been examined, nor is it known whether transport of PAF and short-chain lipids occurs in species other than rat.

Here, we show that PAF is cleared from murine circulation in seconds as an intact molecule through saturable uptake by endothelium. This disappearance was faster than PAF hydrolysis in human or murine blood, and ablation of PLA2G7 had no immediate effect on PAF clearance. The half life of [3H-acetyl]PAF in human or murine blood, and ablation of PLA2G7 had no immediate effect on PAF clearance. The implications are that transport rather than intravascular hydrolysis primarily controls circulating PAF levels; that PAF and oxidized phospholipids are continually, and quickly in plasma than in whole blood where some 40% of plasma volume is displaced by blood cell volume. A similar pattern of efficient hydrolysis of [3H]PAF in plasma relative to blood held when murine material was examined (Figure 1A) where [3H]PAF was hydrolyzed in plasma with a t1/2 of ≈3 minutes.

We examined the rate of [3H-acetyl]PAF turnover in vivo with the expectation from the above observations that it would require approximately 3 minutes to hydrolyze half the [3H-acetyl]PAF we introduced into murine circulation by retroorbital injection. Instead we found that the rate of [3H-acetyl]PAF disappearance was significantly faster than this, with a t1/2 of less than 30 seconds (Figure 1B). We were technically unable to perform more blood collections over these short times to better define the precise t1/2, but it is apparent that the turnover in vivo is far faster than in ex vivo blood samples.

In Vivo PAF Clearance Was Independent of the PAF Receptor
PAF internalization by murine macrophages is a function of the PAF receptor,24 the only known entity to selectively recognize the structural features of PAF. The PAF receptor of circulating cells can account for little PAF clearance, as shown above, but microvascular endothelial cells express PAF receptors that are positioned to recognize and potentially clear circulating PAF. The rate of [3H]PAF clearance, however, was identical in parental BL6 mice and those with genetically ablated PAF receptors (Figure 1C).
In Vivo PAF Clearance Initially Was Independent of PAF-AH/Lp-PLA₂

Plasma PLA2G7 (PAF-AH/ Lp-PLA₂) is the sole enzyme in blood to effectively degrade PAF,²⁶ and a global PLA2G7 knockout has now been found to sensitize animals to necrotizing enterocolitis.²⁷ [³H]PAF clearance in PLA2G7⁻/⁻ mice was not different over the first minute when ≈75% of the label was cleared (Figure 1D). After this time, however, turnover was significantly delayed by loss of this enzyme.

Tissue Uptake and Intracellular PAF Catabolism

PAF that rapidly disappeared from the circulation might be internalized by tissues as the intact phospholipid and then rapidly metabolized as it enters, might be fully recovered from one or more of these organs as the intact phospholipid, or may be distributed between these 2 outcomes. We collected the major organs 5 minutes after [³H-acetyl]PAF injection, a time when vascular PAF had been fully cleared. We first extensively perfused the animals with blood to effectively degrade PAF,²⁶ and a global enzyme with 41% identity to PLA2G7 with a similar substrate preference) in liver PLA2G7⁻/⁻ mice, [³H-acetyl]PAF turnover in PAFR⁻/⁻ mice, [³H-acetyl]PAF turnover in PAFR⁻/⁻ mice and wild-type BL6 mice (n=5) performed on the same day with the same [³H]PAF preparation. Representative of 2 independent experiments. B, Half-life of [³H-acetyl]PAF in vivo. [³H-acetyl]PAF (10 μCi) in 100 μL of PBS containing 0.5% human serum albumin was injected into 10-week-old wild-type or PAF receptor–null male mice (n=5) through the retroorbital plexus. At the stated postinjection times, 100 μL of blood was collected by cardiac puncture, and intact [³H-acetyl]PAF was recovered by organic extraction for quantitation by liquid scintillation counting. Data represent 2 independent experiments. B, Half-life of [³H-acetyl]PAF in vivo. [³H-acetyl]PAF turnover in PL2G7⁻/⁻ mice. [³H-acetyl]PAF turnover in PL2G7⁻/⁻ mice (n=5) and wild-type BL6 mice (n=3) was assessed on the same day as above.

Vascular Endothelium Accumulated Vascular PAF

We sought to identify where intravascular PAF accumulated in the soft organs by introducing NBD-labeled PAF by the retroorbital route and collecting the organs 5 minutes later after exsanguination and buffer perfusion as before. We found sections of lung, liver and kidney fluoresced brightly in this experiment, heart less so, and brain not at all (Figure 3A). We also found clearly delineated patches of bright fluorescence in spleen. The accumulated fluorescence marks intact PAF, and its NBD-lysoPAF and NBD-phosphatidylcholine metabolites, because the NBD label in the lysoPAF backbone is in the nonhydrolyzable sn-1 alkyl residue. Thin layer chromatography confirmed (not shown) fluorescence was confined to these complex phospholipids and had not been converted to a neutral lipid.

Immunohistochemical detection of endothelial CD31 indicated that fluorescent PAF and its phospholipid metabolites primarily accumulated in endothelium (Figure 3B). The large vessel adjacent to an unstained bronchiale shows strong colocalization of fluorescent PAF and CD31 (an endothelial cell and platelet specific marker), but also that the phospholipid had been released into the subluminal compartment. Similarly, endothelium of a large renal vessel was strongly positive for the NBD label, as were numerous smaller vessels. The strongly punctate staining
of spleen was revealed to reflect the distribution of white pulp vessels with little staining away from the vessels (not shown).

A Short-Chain Phospholipid Competed for In Vivo PAF Clearance

We wished to determine whether clearance of trace quantities of [3H]PAF was saturable, and so would be slowed by high PAF concentrations. We cannot test this in vivo with PAF because of its strong vasoactivity, but the stereoisomer of PAF, although chemically identical, is not recognized by the PAF receptor and is not vasoactive. We observed that a 1000-fold molar excess of the enantiomeric stereoisomer of PAF reduced the rate of clearance of [3H]PAF as the t1/2 increased from less than 30 seconds to 110 minutes (Figure 4A, left).

An Oxidatively Truncated Phospholipid and PAF Shared a Clearance Mechanism

PAF is a short-chain phospholipid (the sn-2 residue is a 2-carbon acetyl residue) and oxidatively truncated phospholipids with short sn-2 residues accumulate in the circulation in response to hyperlipidemia or oxidative stress, which might slow PAF clearance through competition. We repeated the in vivo [3H]PAF clearance experiments in the presence of a large molar excess of chemically synthesized Az-lysoPAF, an abundant proapoptotic oxidatively truncated phospholipid. Excess Az-lysoPAF also significantly reduced the rate of clearance of intravascular [3H]PAF (Figure 4A, right). We next injected fluorescent Az-lysoPAF to determine whether this oxidatively truncated phospholipid was internalized, and whether this was by the same type of cells that acquired circulating PAF, to find that it also accumulated in endothelium and subendothelial structures (Figure 4B). We determined whether isolated liver tissue was able to accumulate extracellular PAF using precision-cut liver slices. In this approach, liver was sectioned into 1000-μm-thick slices with a Krumdieck Tissue Slicer that maintain organ ultrastructure while allowing cellular access to extracellular materials. Incubation of precision cut liver slices with NBD-
PAF for 1 minute showed this fluorescent phospholipid was rapidly accumulated by liver cells (Figure 4C), particularly in areas around the central vein. Inclusion of a 100-fold molar excess of PAF (here using biologically active PAF) greatly reduced fluorescent PAF uptake. The oxidatively truncated phospholipid Az-lysoPAF was similarly effective in reducing fluorescent PAF uptake. Both PAF and an oxidatively truncated phospholipid thus appear to compete for PAF uptake ex vivo in a structurally intact tissue.

Short-Chain Choline Phospholipid and PAF Import Share TMEM30a

Phospholipid import is undefined in mammals, but genetic approaches in *Saccharomyces cerevisiae* show choline phospholipid uptake requires Lem3/DRS1 or DRS2 heterodimers.30 Humans express TMEM30 mRNA whose sequence is similar to Lem3, but unknown protein function. We find phospholipid uptake is reconstituted by human TMEM30a or human TMEM30a/yeast Lem3 chimeras in Lem3 deletion mutants, and TMEM30a knockdown reduces PAF uptake by CHO and Jurkat cells.30a We found that human endothelial cells also express mRNA encoding TMEM30a, and that small interfering (si)RNA to this sequence reduced its mRNA compared to cognate scrambled RNA (Figure 5A). siRNA knockdown of TMEM30a also reduced uptake of fluorescent NBD-labeled phosphatidylcholine in a quantitatively significant way (Figure 5C). Endothelial cells internalize fluorescent BODIPY-labeled PAF (Figure 5D, top), which was suppressed in the presence of TMEM30a siRNA.
of excess unlabeled PAF (Figure 5D, middle) or the short-chain phospholipid Az-PC (Figure 5D, bottom). Short-chain choline phospholipids enter endothelial cells, in part, through a common carrier that includes TMEM30a.

Figure 6. Hyperlipidemia slows in vivo PAF clearance. A, Circulating PAF and Az-PC are increased in apoE−/− mice on a high-fat diet. Plasma was isolated from wild-type and apoE−/− mice (n=5) after 6 weeks on a high-fat diet, deuterated d4-PAF was added as an internal standard, and phospholipids were extracted and purified over an HyperSep NH2 cartridge before reverse-phase high-performance liquid chromatographic separation and analysis by electrospray ionization tandem mass spectrometry. C16:0-PAF was separated from isobaric lysophosphatidylcholine by normal-phase chromatography before reverse-phase chromatography. B, [3H-acetyl]PAF turnover. Clearance of [3H-acetyl]PAF was determined as in Figure 1 in wild-type or apoE−/− male mice after 6 weeks of a high-fat diet (n=5). C, Tissue distribution of intact [3H-acetyl]PAF. [3H]PAF turnover was determined 5 minutes postinjection as in Figure 2. All three panels present 1 of 2 independent experiments.

[3H]PAF Clearance Was Decreased in ApoE−/− Mice With Enhanced Intravascular Levels of Short-Chain Phospholipids

A bolus of short-chain phospholipids slowed [3H]PAF clearance in vivo, and a series of such short-chain phospholipids circulates in hyperlipidemic apolipoprotein (apo)E−/− animals.12 We found PAF and Az-PC concentrations also were higher in the circulation of apoE−/− animals fed a high-fat diet for 6 weeks compared to wild type animals on this diet (Figure 6A). We injected trace amounts of [3H]PAF into animals maintained on the high-fat diet for 6 weeks to determine whether endogenous short-chained phospholipids slowed PAF clearance. Indeed, [3H-acetyl]PAF disappeared significantly more slowly from the circulation of apoE−/− mice than BL6 control animals (Figure 6B). We examined the tissue distribution of [3H]PAF in apoE−/− and wild-type animals on a high-fat diet to determine whether uptake into all organs was uniformly altered. The data show all tissues of apoE−/− animals, except brain where significance was not attained, accumulated [3H]PAF more slowly than their wild-type counterparts (Figure 6C).

Discussion

PLA2G7 is the sole enzyme in plasma to appreciably catabolize PAF and short-chain phospholipid oxidation products. This is established by mutations in Japanese and other Asian populations where plasma from homozygous individuals who lack this enzyme cannot hydrolyze PAF, whereas plasma from heterozygous individuals hydrolyze PAF at half the rate of individuals with 2 wild-type alleles.31,32 Despite this, individuals with reduced levels of PLA2G7 activity do not display rampant inflammatory responses anticipated from uncontrolled PAF accumulation,33,34 nor does acute bronchoconstriction to inhaled PAF vary in these individuals.35 Additionally, a recent meta-analysis of 26,000 individuals revealed PLA2G7 variants, such as 379V, were associated with modest changes in enzymatic activity, but were not associated with cardiovascular risk markers, coronary atheroma, or coronary heart disease.36 Here, we find that circulating PLA2G7 is not the only way PAF is cleared from blood. [3H]PAF clearance initially occurred through tissue uptake by a system using TMEM30a that accepts choline phospholipids as transport substrates.
Accordingly, at early times [3H]PAF clearance was unaffected by ablation of PLA2G7. However, the knockout also shows this enzyme does significantly participate in [3H]PAF turnover at later times or lower concentrations. This observation elucidates a basis for incongruence between circulating PLA2G7 enzymatic activity and pathophysiologic measures.

We propose that circulating PAF and phospholipid oxidative products accumulate in the inflammatory apoe−/− hyperlipidemic model because saturable transport limits their clearance. In hyperlipidemia, a plethora of short-chain phospholipid oxidation products are available to compete and slow transport of PAF and the other biologically active phospholipids. In this way, hyperlipidemia can promote inflammation. Our studies were conducted in mice, where the PLA2G7 activity is 8.6 times that of humans,43 suggesting hydrolysis in human circulation would be even less effective, although human PAF turnover is yet to be investigated.

PAF was primarily transported as the intact molecule because a significant portion of PAF and Az-LPAF were recovered from tissue as intact molecules, particularly in lung. Additionally, intact PAF was recovered well after it would have been hydrolyzed had it remained in the circulation. By example, 3 minutes postinjection when ≈98% of intravascular PAF had been cleared from the circulation, only about half of this could have been hydrolyzed in plasma by this time.

In contrast, the majority of PAF was hydrolyzed in liver and kidney after internalization, and both hepatocytes and renal cells abundantly express type II intracellular PAF acetylhydrolase that also specifically hydrolyzes PAF.2,4 Liver Kupffer cells, as differentiated tissue macrophages, additionally retain a portion of the PLA2G7 they make,44 so intracellular PLA2G7 may contribute to intracellular PAF metabolism.

Uptake of intact PAF can have a biological consequence because the PAF receptor is present in intracellular compartments,43 and the PAF receptor of isolated nuclei stimulates a Ca2+ flux and initiates inflammatory gene transcription.46 These observations indicate intracellular PAF receptors in cells lacking robust hydrolytic activity have the potential to respond to extracellular PAF.

PAF is cleared from the circulation with great rapidity, so the presence of PAF in blood47 requires equally rapid secretion to counterbalance turnover. The concentration of circulating PAF increases with inflammatory or pathological insults,7,13,48 indicating either or both increased production and release in response to these insults. Pathways contributing to circulating PAF remain opaque, but likely include the combination of reduced PLA2G7 hydrolytic activity,49 increased intracellular PAF synthesis,50 and, at least in yeast, export facilitated by P-glycoprotein ABC transporters.51 Circulating PAF may be the product of mononuclear cells because of all the cells known to synthesize PAF, only monocytes release PAF.52,53

Reduced phospholipid uptake might also enhance circulating PAF concentrations, but molecular details of this internalization process are just now being defined. Uptake of PAF and a related structure edelfosine (PAF with an alkyl sn-2 residue) by genetically tractable yeast requires a heterodimeric complex of the P4-type ATPase Drs1 or Drs2,54 and lem3 (also discovered as ro3).55 Mutational analysis of the corresponding human ATPase homolog ATP8B1 shows it has no role in phospholipid import,56 but we find TMEM30a, a human lem3 homolog, reconstitutes phospholipid import in S cerevisiae and aids PAF uptake by cell lines.30a The finding here that TMEM30a is expressed by endothelial cells and facilitates phospholipid import suggest that the rapid clearance of circulating PAF reflects transport into endothelial cell rich organs including lung, liver, and kidney.

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Disclosures

None.

References

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**Novelty and Significance**

**What Is Known?**

- A single circulating enzyme, PAF acetylhydrolase/lipoprotein-associated phospholipase (Lp-PLA)₂, hydrolyzes PAF and oxidized phospholipids.
- Circulating PAF is thought to be controlled by intravascular PAF hydrolysis.
- Clinical trials of neither a Lp-PLA₂ inhibitor nor injection of recombinant PAF-AH achieved their primary end points.

**What New Information Does This Article Contribute?**

- Circulating PAF is primarily controlled by transport, not intravascular hydrolysis.
- PAF is cleared from murine circulation with a t₁/₂ of ∼30 seconds.
- PAF and oxidized phospholipids rapidly enter the circulation to maintain steady-state levels.
- PAF and oxidized phospholipids compete for uptake, and hyperlipidemia slows PAF clearance.

PAF activates vascular and innate immune cells at subpicomolar concentrations, so its presence in circulation must be tightly controlled. A single plasma enzyme encoded by *PLA2G7* hydrolyzes PAF and oxidatively truncated phospholipids in plaque and hyperlipidemic plasma, and its loss in Asian populations prevents PAF hydrolysis in blood. However, this population is not at risk for cardiovascular disease, nor was disease enhanced in subjects testing Darapladib that inhibits this enzyme. We found PAF is primarily cleared from murine circulation by transport, not intravascular hydrolysis. Uptake was rapid (t₁/₂, ∼30 seconds) and independent of the PAF receptor. Endothelial cell uptake of PAF and an antitumor phospholipid was aided by TMEM30a, a homolog of a yeast phosphatidylcholine transporter. PAF and a truncated phospholipid compete for uptake, so hyperlipidemia slowed clearance. PAF was transported as an intact phospholipid, and then rapidly hydrolyzed in liver, the primary recipient organ, and kidney, which express significant levels of PAF acetylhydrolase activity. Endothelial cells accumulate intact PAF and express intracellular PAF receptors, so intravascular PAF may affect intracellular signaling. Hydrolysis of PAF in protected intracellular compartments indicates inhibition of circulating Lp-PLA₂ by the inhibitor Darapladib should not slow PAF or oxidized phospholipid clearance, nor decrease PAF receptor signaling.
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Reagents
Platelet Activating Factor (1-O-hexadecyl-2-acetoyl-sn-glycero-3-phosphocholine, PAF), its enantiomer, 1-O-hexadecyl-sn-glycero-3-phosphocholine (C₁₆ lyso-PAF), LPC and 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (Az-PC), d₄-PAF were purchased from Cayman Chemical (Ann Arbor, MI). [¹H-acetyl]PAF was purchased from Perkin Elmer (Waltham, MA). Fluorescent NBD-PAF (1-[12-[7-nitro-2-1, 3-benzoazidazol-4-y] amino]dodecanoyl]-2-acetoyl-sn-glycero-3-phosphocholine) and fluorescent AzPAF (1-[7-nitro-2-1, 3-benzoazidazol-4-y] amino]dodecanoyl]-2-azalaoyl-sn-glycero-3-phosphocholine) were custom synthesized by Avanti Polar Lipids (Alabaster, AL). Anti-CD31 and Alexa647-conjugated anti-mouse were from BioLegend (San Diego, CA), while endotoxin free Human Serum Albumin was a product of Baxter Healthcare (Deerfield, IL). TMEM30a siRNA and its scrambled control were from Thermo Scientific Dharmacon (Lafayette, CO). HUVEC nucleofector kits were from Lonza (Allendale, NJ). MCDB105 medium and endothelial cell growth supplement are from Sigma (St. Louis, MO). Quantitect SYBR Green RT-PCR MASTER Mix is from Qiagen Inc (Valencia, CA). 3’TMEM30a primer catataacagtgtgcctc and 5’primer cgcggagatcagattga were from Invitrogen (Carlsbad, CA).

Animals
Wild-type and apoE⁻⁄⁻ mice in a C57BL6 background were purchased from Jackson Laboratory. PAFR⁻⁄⁻ mice were the very kind gift of Takao Shimizu (University of Tokyo) provided from a colony backcrossed for 10 generations and maintained by Jeffery Traver (Indiana University). PAF-AH⁻⁄⁻ backcrossed eight times into C57BL6 were generously provided by Diana Stafforini (Huntsman Cancer Center, University of Utah).

Cells
HUVEC were cultured in MDCB105 medium, with 15% FBS, 15% endothelial cell growth supplement and heparin (90 μg/ml) at 37°C in an atmosphere containing 5% CO₂. Trypsinized cells (5x10⁵) were mixed with siRNA (10 pM in 100 μl master mix) and nucleorporated by program A-34 before 48 h of culture. Lipid uptake in transfected cells (2 x 10⁶ cells/ml) was determined by physical suspension, washing in HBSS twice, and resuspending the cells in either 1 μM NDB-PC or NDB-PE at room temperature for 10 min. The labeled cells were washed twice with HBSS containing 1% (w/v) BSA before analysis by single color flow cytometry. TMEM30a was quantitated using the 3’TMEM30a primer catataacagtgtgcctc and the 5’primer cgcggagatcagattga.

In Vivo Phospholipid Metabolism
100 μCi of [¹H-acetyl]PAF was dried with nitrogen and dissolved in 1 ml PBS containing 0.5% human serum albumin by vortexing and ultrasonic dispersion for 2 min. 100 μl of the buffered [¹H-acetyl]PAF, with or without a 1,000-molar excess of enantiomeric PAF or a 2,000-fold excess of synthetic AzPC, was injected into mice via the retro-orbital plexus. Blood (~100 μl) was collected by cardiac puncture at 30 seconds, 1’, 2’ and 5 min post-injection, expressed into a microfuge tube, and a measured aliquot then immediately transferred to a glass tube containing methanol. Tissues were harvested 5 min post-injection by perfusing 10 ml PBS through the vascular system before lung, liver, spleen, kidney, heart and brain were recovered, in that sequence. Organs were immediately frozen with liquid nitrogen. Organs were then thawed, rapidly weighed and minced. [¹H]PAF was extracted from blood and organ homogenates by methanol/chloroform extraction before radiation was quantified by liquid scintillation counting.

Identification and Quantification of Phospholipids by Mass Spectrometry
After the addition of d₄-PAF to the monophasic extract as an internal standard, the lipids were extracted and the organic phase washed thrice. The chloroform extracts were dried under a stream of nitrogen, resuspended in 100 μl of methanol/water (85:15 v/v), absorbed to a HyperSep NH₂ column (Thermo Scientific) and the polar lipids isolated with a 100% methanol wash. The eluate was dried under nitrogen, resuspended in 200 μl methanol/water (85:15 v/v) and filtered. The phospholipid extract was injected onto a reverse phase C₁₈ HPLC column (2 x 150 mm, 5 μm, ODS; Phenomenex, Rancho Palos Verdes, CA) at a flow rate of 0.2 ml/min generated by a Waters Alliance 2690 HPLC (Waters, Wilmington, DE). Phospholipids were resolved using a ternary gradient system comprised of mobile phase A (water containing 0.2% formic acid) and mobile phase B (methanol containing 0.2% formic acid). The column was equilibrated with 85% mobile phase B/mobile phase A mixture and held at this composition for 29 min after the injection. HPLC column effluent was introduced into a Quattro Ultima triple quadrupole mass
spectrometer (Micromass, Manchester, UK). The mass spectrometer was configured with capillary voltage at 3.0 kV, cone voltage at 40 V, collision energy at 20 V, source temperature at 120 °C, and a desolvation temperature at 250 °C. The flow rate for the nitrogen in the cone gas and desolvation gas was 80 and 600 l/h, respectively. Collision-induced dissociation was obtained using argon gas. Mass spectrometric analyses were performed on-line using electrospray ionization tandem mass spectrometry (ESI/MS/MS) in the positive ion mode with multiple reaction monitoring mode. The multiple reaction monitoring transitions used to identify and quantify individual PC molecular species were the m/z for the molecular cation [MH]+ and the daughter ion m/z 184 (the phosphocholine group). Calibration curves were constructed with a fixed amount of d4-PAF internal standard and varying mol % of each authentic synthetic phospholipid prior to extraction and LC/MS/MS analysis.

**Immunohistochemistry**

10 µg of fluorescent NBD-PAF or NBD-AzPAF was dried under a stream of nitrogen, dissolved in 100 µl PBS with 0.5% human serum albumin and dispersed with ultrasonic irradiation for 5 min and introduced to the circulation through retro-orbital injection as before. Organs were recovered 1 minute or 5 min post retro-orbital injection after flushing the vasculature with 10 ml PBS. Lung, liver, spleen, kidney, heart and brain were excised as before, immediately frozen in liquid nitrogen, and embedded in OTC media for sectioning.