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Circulating Platelet-activating Factor is Primarily Cleared by Transport, not Intravascular Hydrolysis by Lipoprotein-associated Phospholipase A_2 / PAF Acetylhydrolase

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Abstract

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 A_2 (Lp-PLA_2)] in plasma hydrolyzes PAF, but significant controversy exists whether its action is pro- or anti-inflammatory 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-PLA_2 in this process.

Methods and Results—[^{3}H-acetyl]PAF was hydrolyzed by murine or human plasma ($t_{1/2}$ 3 and 7 min, respectively), but injected [^{3}H-acetyl]PAF disappeared from murine circulation more quickly ($t_{1/2}$ < 30 sec). [^{3}H]PAF clearance was unchanged in PAF receptor$^{-/-}$ animals, or over the 1st two half-lives in PAF-AH$^{-/-}$ animals. [^{3}H]PAF turnover was reduced by co-injecting excess unlabeled PAF or an oxidatively truncated phospholipid, and [^{3}H]PAF clearance was slowed in hyperlipidemic apoE$^{-/-}$ mice with excess circulating oxidatively truncated phospholipids. [^{3}H]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 pro-apoptotic oxidized phospholipid mediators. Intravascular PAF has access to intracellular compartments. Inflammatory and pro-apoptotic phospholipids may accumulate in the circulation as transport is overwhelmed by substrates in hyperlipidemia.

Keywords

PAF; oxidized phospholipids; phospholipid transport; lipoprotein-associated phospholipase A_2

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

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Disclosures
None

by numerous cells of the cardiovascular system. PAF and its receptor regulate inflammation, atherogenesis, cardiac rhythm, liver contractility, body temperature, and vascular tone.[1–4] PAF is remarkably potent—activating cells at concentrations of 10^{-12} M[5]—suggesting its presence is tightly controlled. However its blood concentration can increase, for instance after ischemic stroke[6] and inflammatory stress[7].

A single enzyme in blood metabolizes PAF[8, 9] and bioactive[10] oxidatively-modified phospholipids[27] that accumulate in atherosclerosis[11] and in response to other oxidative insults[12]. Common names for this lipoprotein-associated enzyme are plasma PAF acetylhydrolase (PAF-AH) or lipoprotein-associated phospholipase (Lp-PLA_2), but its gene name is group VII phospholipase A_2 (*PLA2G7*). PAF as a relatively soluble phospholipid is bound to lipoproteins, mainly albumin[13], with rapid exchange from lipoprotein particles[14] allowing access both to PLA2G7 and the PAF receptor.

There is significant controversy whether PLA2G7 is protective or promotes chronic inflammation, cardiovascular disease and atherogenesis,[15] and direct manipulation of this activity in humans has not clarified the issue. A phase III trial administering recombinant human plasma PAF acetylhydrolase to septic patients did not decrease mortality.[16] Conversely, chronic suppression of circulating enzymatic activity with the experimental drug Darapladib® did not reduce circulating oxidized phospholipids nor alter human plaque volume.[17] Still, plasma PAF acetylhydrolase is a strong and independent risk factor for cardiovascular disease,[18] and a large (~15,000 individuals) phase III trial is underway to determine if Lp-PLA_2 inhibition reduces incidence of first occurrence of major adverse cardiovascular events.

The focus on PAF-AH/Lp-PLA_2 in controlling the amounts of circulating PAF or oxidized phospholipids has yet to incorporate the results of a single early report indicating that PAF is rapidly cleared from rat circulation by transport into tissues.[19] 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 implications are that transport rather than intravascular hydrolysis primarily controls circulating PAF levels; that PAF and oxidized phospholipids are continually—and rapidly—released to the circulation to achieve steady state levels; that an excess of oxidized phospholipids promotes inflammation by competitively slowing PAF clearance; and, that PAF catabolism primarily occurs in the intracellular compartment of tissues, reducing the role for circulating—although perhaps not intracellular—PLA2G7 in *in vivo* PAF catabolism.

Methods

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

In vivo Phospholipid Metabolism

[^{3}H-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 retro-orbital 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 retro-orbital injection. Organs were recovered 5 min 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 ± S.D. of the stated number of samples. The statistical analyses used a paired Student's 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 [^{3}H-acetyl]PAF in human plasma was approximately 7 min (Fig. 1A), as we previously observed.[22] We also observed that the turnover of [^{3}H-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 [^{3}H]PAF in plasma relative to blood held when murine material was examined (Fig. 1A) where [^{3}H]PAF was hydrolyzed in plasma with a $t_{1/2}$ of ~ 3 min.

We examined the rate of [^{3}H-acetyl]PAF turnover *in vivo* with the expectation from the above observations that it would require approximately 3 min to hydrolyze half the [^{3}H-acetyl]PAF we introduced into murine circulation by retro-orbital injection. Instead we found that the rate of [^{3}H-acetyl]PAF disappearance was significantly faster than this, with a $t_{1/2}$ of less than 30 seconds (Fig. 1B). We were technically unable to perform more blood collections over these short times to better define the precise $t_{1/2}$, 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,[23] 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 [^{3}H]PAF clearance, however, was identical in parental BL6 mice and those with genetically ablated PAF receptors (Fig. 1C).

In Vivo PAF Clearance initially was Independent of PAF-AH/ Lp-PLA$_2$

Plasma PLA2G7 (PAF-AH/ Lp-PLA$_2$) is the sole enzyme in blood to effectively degrade PAF[25], and a global *PLA2G7* knockout has now been found to sensitize animals to necrotizing enterocolitis[26]. [^{3}H]PAF clearance in PLA2G7$^{-/-}$ mice was not different over the 1st minute when ~75% of the label was cleared (Fig. 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 two outcomes. We collected the major organs 5 min after [^{3}H-acetyl]PAF injection, a time when vascular PAF had been fully cleared. We first extensively perfused the animals with buffer to remove residual blood-borne label before harvesting the organs. Most soft tissues accumulated intact [^{3}H]PAF, with liver and lung accounting for the bulk of this accumulation (Fig. 2A). In part, preferential accumulation of [^{3}H]PAF by liver reflected organ size, and when accumulation was normalized by wet weight it is apparent that [^{3}H]PAF was preferentially accumulated by lung and spleen (Fig. 2B). These data show PAF was accumulated by least some organs as the intact phospholipid because the solvent extraction separates this lipid from its aqueous [^{3}H]acetate hydrolytic product. When tissue [^{3}H]acetate was quantified, it was apparent that the [^{3}H-acetyl]PAF had been extensively hydrolyzed in liver (Fig. 2C) where only about 3% of the label remained as the intact phospholipid 5 min post-injection (compare 2A with 2C). Similarly, only about 7% of the label remained as PAF in kidney, while lung retained nearly half its label as intact PAF. This pattern is congruent with the abundance of type II PAF acetylhydrolase (an intracellular enzyme with 41% identity to PLA2G7 with a similar substrate preference) in liver = kidney ≫ lung.[27]

Vascular Endothelium Accumulated Vascular PAF

We sought to identify where intravascular PAF accumulated in the soft organs by introducing NBD-labeled PAF by the retro-orbital route and collecting the organs 5 min 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 (Fig. 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, since the NBD label in the lyso-PAF backbone is in the non-hydrolyzable *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 (Fig. 3B). The large vessel adjacent to an unstained bronchiole shows strong co-localization 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 [^{3}H]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, while chemically identical, is not recognized by the PAF receptor[28] and is not vasoactive. We observed that a 1000-fold molar excess of the entiomeric stereoisomer of PAF reduced the rate of clearance of [^{3}H]PAF as the $t_{1/2}$ increased from less than 30 seconds to about 3 min (Fig. 4A*left*).

An Oxidatively Truncated Phospholipid and PAF Share a Clearance Mechanism

PAF is a short-chain phospholipid—the *sn*-2 residue is a two carbon acetyl residue—and oxidatively truncated phospholipids with short *sn*-2 residues accumulate in the circulation in response to hyperlipidemia[11] or oxidative stress[12], which might slow PAF clearance through competition. We repeated the *in vivo* [^{3}H]PAF clearance experiments in the presence of a

large molar excess of chemically synthesized Az-LysoPAF, an abundant pro-apoptotic oxidatively truncated phospholipid[29]. Excess Az-LysoPAF also significantly reduced the rate of clearance of intravascular [^{3}H-acetyl]PAF (Fig. 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 sub-endothelial structures (Fig. 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 1,000 micron 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 one minute showed this fluorescent phospholipid was rapidly accumulated by liver cells (Fig. 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 Import Shares TMEM30a

Phospholipid import is undefined in mammals, but genetic approaches in *S. 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 (Chen et al, submitted). We found that human endothelial cells also express mRNA encoding TMEM30a, and that siRNA to this sequence reduced its mRNA compared to cognate scrambled RNA (Fig. 5A). siRNA knockdown of TMEM30a also reduced uptake of fluorescent NBD-labeled phosphatidylcholine compared to cells transfected with scrambled RNA (Fig. 5B) in a quantitatively significant way (Fig. 5C). Endothelial cells internalize fluorescent BODIPY-labeled PAF (Fig. 5D*top*), which was suppressed in the presence of excess unlabeled PAF (Fig. 5D *middle*) or the short chain phospholipid Az-PC (Fig. 5D *bottom*). Short chain choline phospholipids enter endothelial cells, in part, through a common carrier that includes TMEM30a.

[^{3}H]PAF Clearance is Decreased in apoE$^{-/-}$ Mice with Enhanced Intravascular Levels of Short-chain Phospholipids

A bolus of short chain phospholipids slowed [^{3}H]PAF clearance *in vivo*, and a series of such short chain phospholipids circulate in hyperlipidemic apoE$^{-/-}$ animals.[11] 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 (Fig 6A). We injected trace amounts of [^{3}H]PAF into animals maintained on the high fat diet for 6 weeks to determine whether endogenous short chained phospholipids slowed PAF clearance. Indeed, [^{3}H-acetyl]PAF disappeared significantly more slowly from the circulation of apoE$^{-/-}$ mice than BL6 control animals (Fig. 6B). We examined the tissue distribution of [^{3}H]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 [^{3}H]PAF more slowly than their wild-type counterparts (Fig. 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, while plasma from heterozygous individuals hydrolyze PAF at half the rate of individuals with two 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. [^{3}H]PAF clearance initially occurred through tissue uptake by a system employing TMEM30a that accepts choline phospholipids as transport substrates. Accordingly, at early times [^{3}H]PAF clearance was unaffected by ablation of *PLA2G7*. However, the knockout also shows this enzyme does significantly participate to [^{3}H]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 oxidation 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[44], 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 since 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 min post-injection 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[45], so intracellular PLA2G7 may contribute to PAF metabolism.

Uptake of intact PAF can have a biologic consequence because the PAF receptor is present in intracellular compartments,[46] and the PAF receptor of isolated nuclei stimulates a Ca^{++} flux and initiates inflammatory gene transcription.[47] 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 blood[48] requires equally rapid secretion to counterbalance turnover. The concentration of circulating PAF increases with inflammatory or pathologic insults[7, 12, 49], 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[50], increased intracellular PAF synthesis[51], and, at least in yeast, export facilitated by P-glycoprotein ABC transporters[52]. Circulating PAF may be the product of mononuclear cells since of all the cells known to synthesize PAF, only monocytes release PAF[53, 54].

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[43], and lem3 (also discovered as ros3)[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 lem3p homolog—reconstitutes phospholipid import in *S. cerevisiae* and aids PAF uptake by cell lines (Chen, submitted). 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

Az	azelaoyl (nonadioyl)
Lp-PLA$_2$	lipoprotein-associated phospholipase A$_2$
LPAF	lyso-platelet-activating Factor, 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine
NBD	nitrobenzoxadiazole
PAF	Platelet-activating Factor, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine
PAF-AH	PAF acetylhydrolase
PAFR	Platelet-activating Factor Receptor

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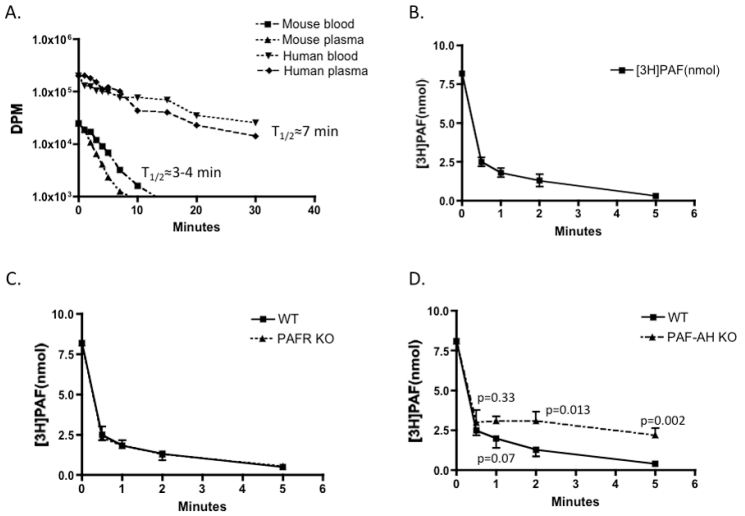

Figure 1. [^{3}H]PAF Clearance *in vitro* and *in vivo*

A. *In vitro* PAF hydrolysis by human or mouse (10 weeks old; n=5) plasma or blood. [^{3}H-acetyl]PAF (3.4 μCi) was added to 500 μl of plasma or whole blood to retain the ratio used in the following *in vivo* clearance experiments. Hydrolysis in aliquots taken at the stated times was stopped by mixing into an organic phase (methanol/chloroform; 2.5;1.25 vol/vol), splitting the resulting monophase with additional 0.1M acetic acid and chloroform (1 vol each) and recovering the hydrolyzed [^{3}H]acetate in the aqueous phase for quantitation by liquid scintillation counting. Data represent two independent experiments. **B**. Half-life of [^{3}H-acetyl]PAF *in vivo*. [^{3}H-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 retro-orbital plexus. At the stated post-injection times, 100 μl of blood was collected by cardiac puncture, and intact [^{3}H-acetyl]PAF was recovered by organic extraction for quantitation by liquid scintillation counting. **C**. [^{3}H]PAF turnover in PAFR$^{-/-}$ mice. [^{3}H-acetyl]PAF turnover in PAFR$^{-/-}$ mice and wild-type BL6 mice (n=5) performed on the same day with the same [^{3}H]PAF preparation. Representative of two independent experiments. **D**. [^{3}H]PAF turnover in PLA2G7$^{-/-}$ mice. [^{3}H-acetyl]PAF turnover in PLA2G7$^{-/-}$ mice (n=5) and wild-type BL6 mice (n=3) was assessed on the same day as above.

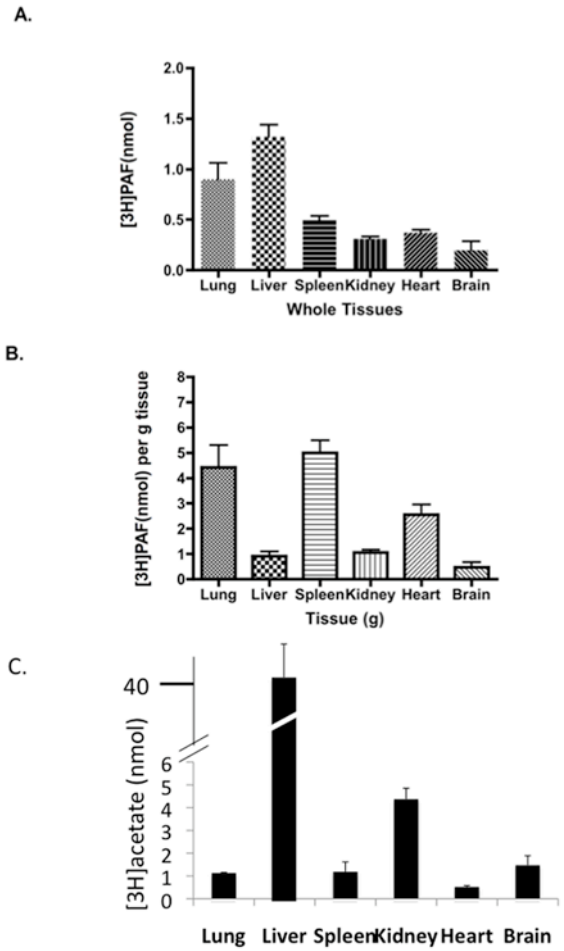

Figure 2. Tissue Accumulation of [^{3}H-acetyl]PAF and its [^{3}H]Acetate Hydrolytic Product

[^{3}H-acetyl]PAF (10 μCi) in 0.5% human serum albumin in PBS was injected (100 μl) into 10 week old anesthetized wild-type mice (n=5) through the retro-orbital plexus. After 5 minutes, the mice were perfused with 30 ml PBS, the organs were rapidly excised in the sequence presented from left to right in the figure, and immediately frozen in liquid nitrogen. The entire organ was weighed, PAF was extracted from weighed tissue specimens after mincing and separating [^{3}H]PAF from its [^{3}H]acetate hydrolytic product by methanol/chloroform extraction[20]. **A**. Intact [^{3}H-acetyl]PAF in whole organs. **B.** Intact [^{3}H-acetyl]PAF per gram of organ weight. **C.** Total [^{3}H]acetate present in each tissue. Data are from one of two independent experiments.

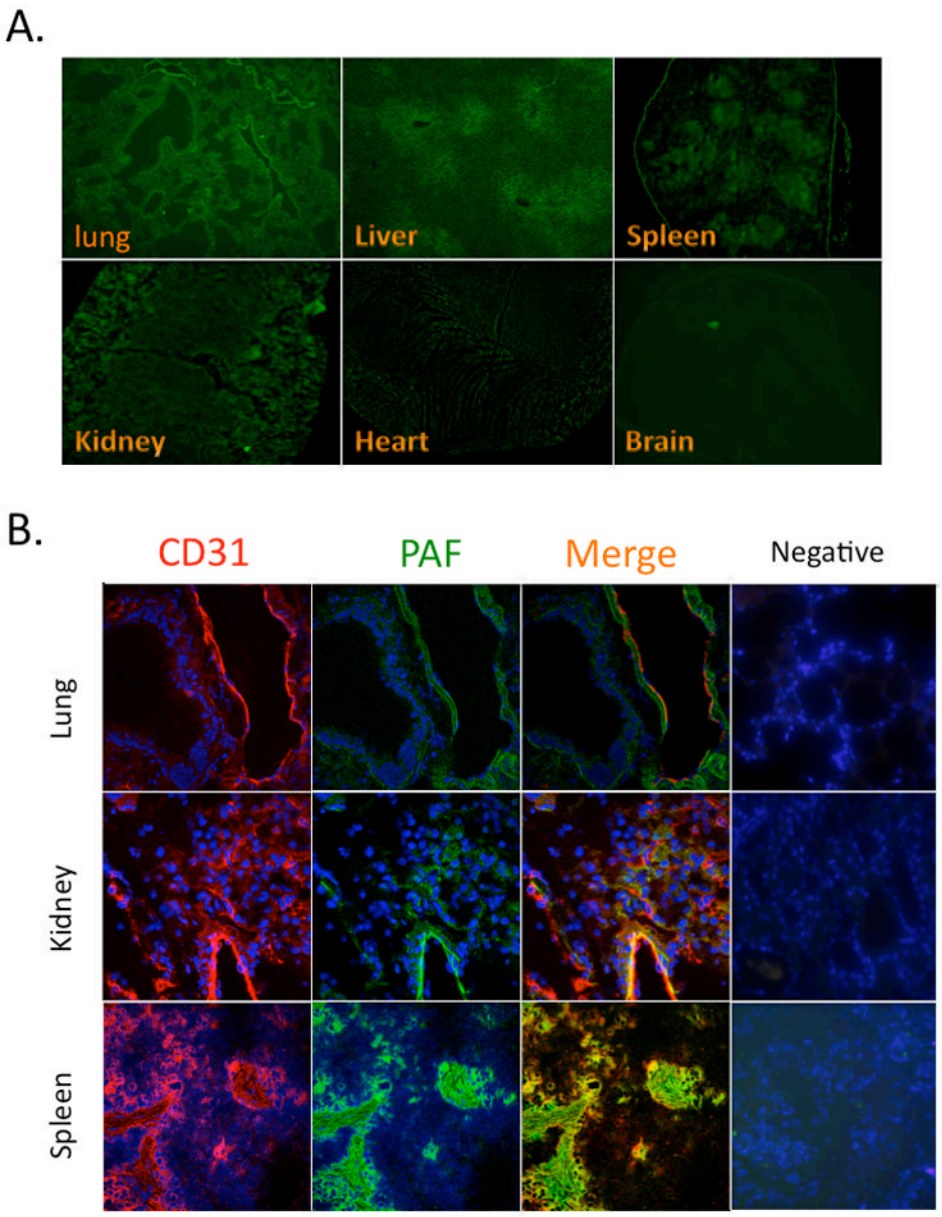

Figure 3. Tissue Distribution of Fluorescent NBD-PAF and Co-localization with Endothelium

A. Tissue distribution. Fluorescent NBD labeled-PAF (10 μg in 100 μl PBS containing 0.5% human serum albumin) was injected, or nothing, into 10 week old wild-type male mice through the retro-orbital plexus as before. The mice were extensively perfused with PBS 5 min post-injection, organs were extracted in sequence, and frozen immediately. The tissue was OTC embedded, sectioned, stained for endothelial cell CD31, and fluorescent micrographs were captured at 4X magnification. **B**. Endothelium and PAF co-staining. NBD-PAF labeled sections *(top*: lung; *middle*, kidney; *lower*, spleen) were stained, or not (*right*), with anti-CD31 and Alexa647-conjugated secondary antibody before fluorescent micrographs (63X magnification) were generated by confocal laser scanning microscopy.

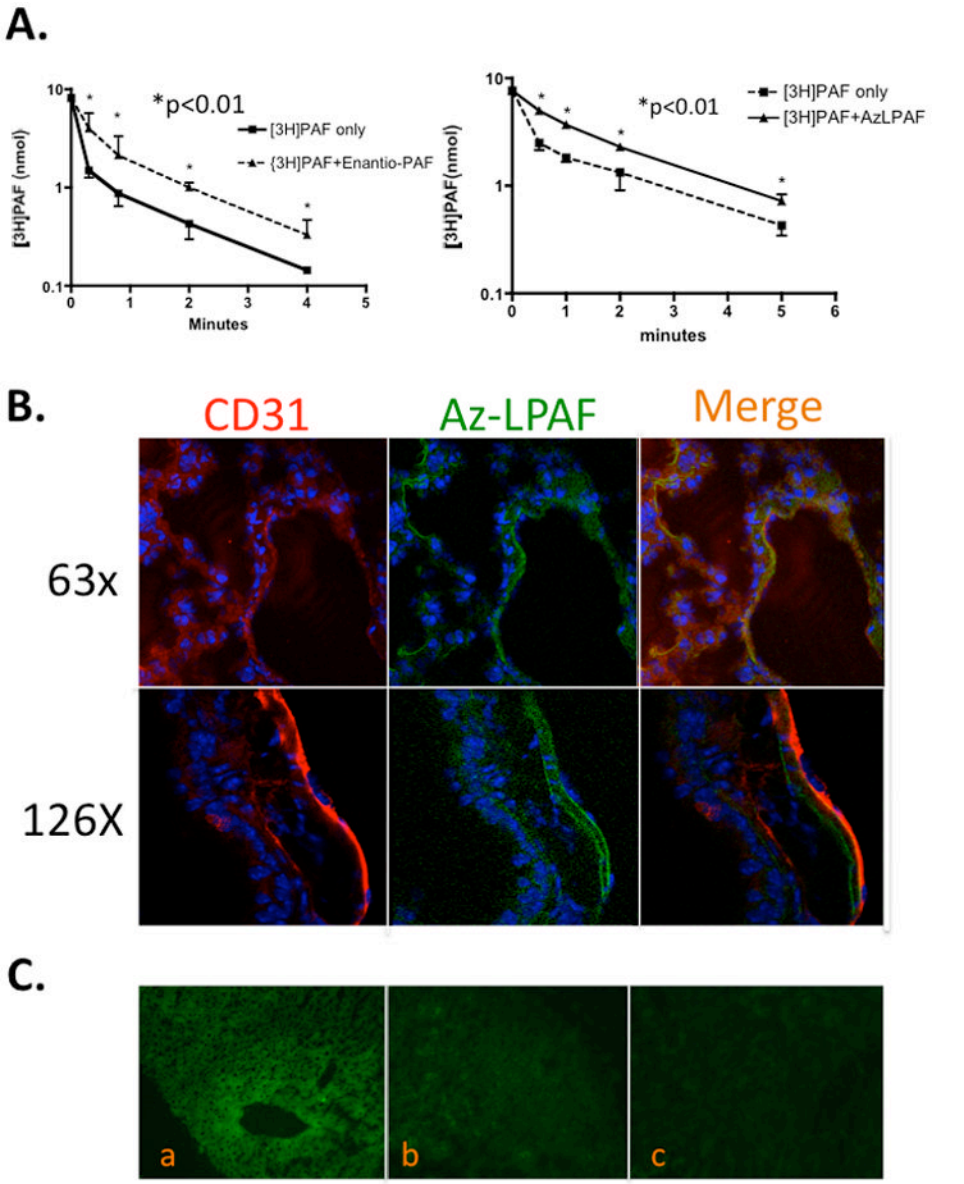

Figure 4. PAF and an Oxidatively Truncated Phospholipid Compete for Uptake in vivo

A. PAF or Az-lysoPAF competition for PAF [^{3}H]PAF clearance. *Left* A trace concentration of [^{3}H]PAF (10 μ Ci) was mixed with excess (10^{-6} M) enantiomeric PAF (3-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) in 0.5% human serum albumin in PBS. Enantiomeric PAF is chemically identical to PAF, but the PAF receptor is not activated by this stereoisomer and so blood pressure and the inflammatory response are not altered as they would be in response to micromolar PAF. Blood samples were collected at the stated times and processed to quantitate [^{3}H-acetyl]PAF as in Fig. 1. *Right*. A trace quantity of [^{3}H]PAF (10 μ Ci) was mixed with excess (2×10^{-6} M) synthetic Az-lysoPAF and [^{3}H]PAF clearance measured as before. **B**. Lung endothelium accumulates circulating Az-lysoPAF. NBD-labeled Az-lysoPAF was injected, animals were perfused with PBS 5 min later, and organs were harvested and processed and co-stained with anti-CD31 as in Fig. 3B. **C**. Short-chain phospholipids compete for labeled PAF uptake in precision-cut liver slices. Freshly isolated mouse liver was sectioned into 1,000 micron thick sections with a Krumdieck

Tissue Slicer. The liver slices were treated for 1 minute with 100 ng/ml of fluorescent NBD-PAF in the absence (*a*) or presence (10 μg/ml) of unlabeled PAF (*b*), or Az-lysoPAF (*c*). The treated slices were placed in 2 ml PBS containing 5% human serum albumin, washed 3 times, immediately frozen in liquid nitrogen, and then embedded in OTC. Sections were examined by fluorescence microscopy and micrographs captured at 10X magnification.

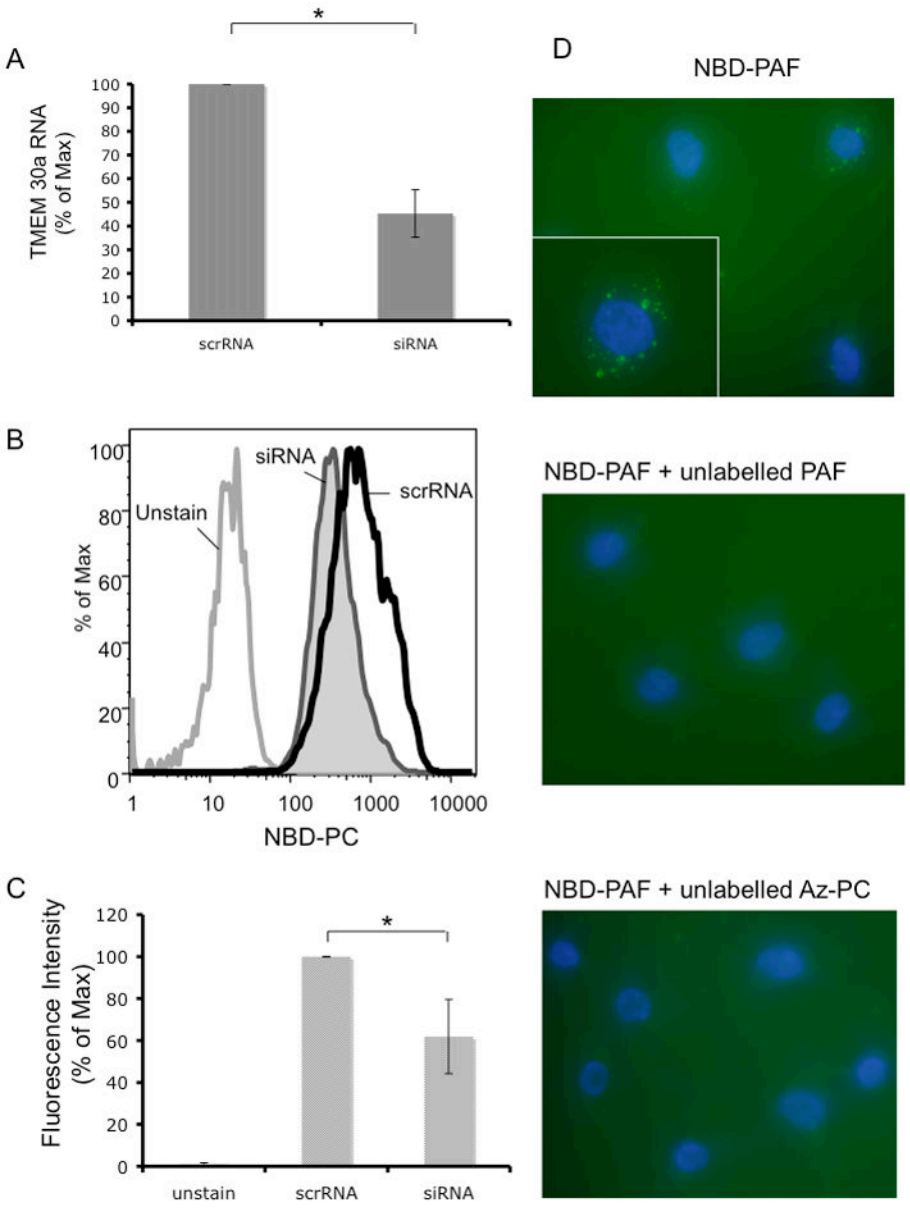

Figure 5. Endothelial Cell TMEM30a Aids Choline Phospholipid Uptake

(A) HUVEC were transfected by nuclear poration with human TMEM30a or scrambled siRNA, and 48 h later the amount of TMEM30a mRNA was determined by qPCR (n=3; $p< 0.01$). (B) Cells were suspended, washed with HBSS twice and resuspended (2×10^6 cells/ml) in 1 μM NBD-PC for 10 min. The labeled cells were washed twice with HBSS containing 1% (w/v) albumin before flow cytometry. (C) Summation of NBD-PC uptake in three experiments ($p<0.05$). (D) HUVEC on glass cover slips were treated with BODIPY-PAF (100 ng/ml) alone (*top*), or in the presence (10 μg/ml) of unlabeled PAF (*middle*) or Az-PC (*bottom*) for 1 min before the media was removed, the cells washed with 5% albumin in PBS and the cells imaged at 60× or 100× (inset) Shown is representative data from one of two independent experiments.

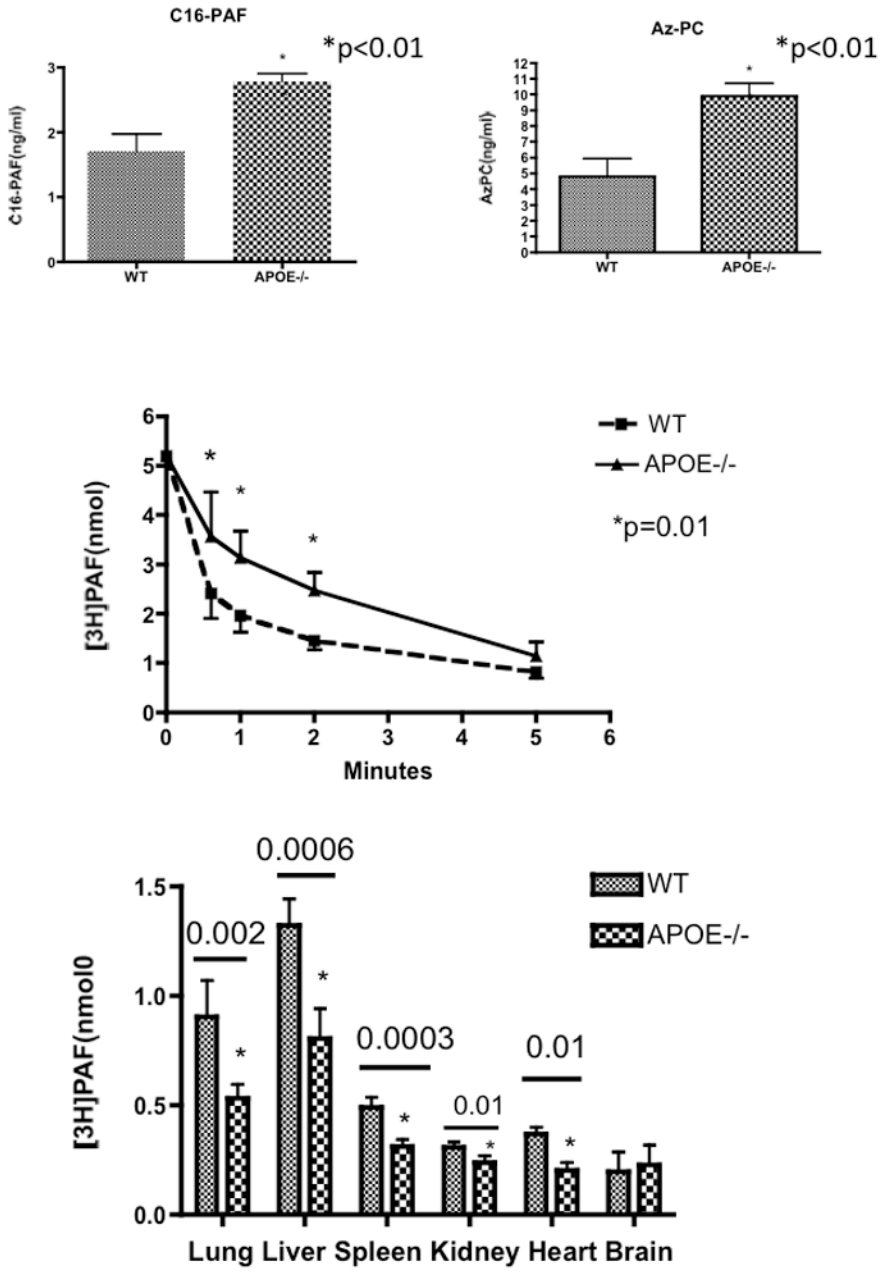

Figure 6. Hyperlipidemia Slows *in vivo* PAF Clearance

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 six weeks on a high fat diet, deuterated d_4-PAF was added as an internal standard, and phospholipids were extracted[20] and purified over an HyperSep NH_2 cartridge prior to reverse phase HPLC separation and analysis by electrospray ionization tandem mass spectrometry. $C_{16:0}$-PAF was separated from isobaric lysophosphatidylcholine by normal phase chromatography prior to reverse phase chromatography. **B**. [^{3}H-acetyl]PAF turnover. Clearance of [^{3}H-acetyl]PAF was determined as in Fig. 1 in wild-type or ApoE$^{-/-}$ male mice after six weeks of a high fat diet (n=5). **C**. Tissue distribution of intact [^{3}H-acetyl]PAF. [^{3}H]PAF turnover was determined 5 min post-injection as in Fig. 2. All three panels present one of two independent experiments.