To hydrolyze or not to hydrolyze: the dilemma of platelet-activating factor acetylhydrolase

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Abstract Mounting ambiguity persists around the functional role of the plasma form of platelet-activating factor acetylhydrolase (PAF-AH). Because PAF-AH hydrolyzes PAF and related oxidized phospholipids, it is widely accepted as an anti-inflammatory enzyme. On the other hand, its actions can also generate lysophosphatidylcholine (lysoPC), a component of bioactive atherogenic oxidized LDL, thus allowing the enzyme to have proinflammatory capabilities. Presence of a canonical lysoPC receptor has been seriously questioned for a multitude of reasons. Animal models of inflammation show that elevating PAF-AH levels is beneficial and not deleterious and overexpression of PAF receptor (PAF-R) also augments inflammatory responses. Further, many Asian populations have a catalytically inert PAF-AH that appears to be a severity factor in a range of inflammatory disorders. Correlation found with elevated levels of PAF-AH and CVDs has led to the design of a specific PAF-AH inhibitor, darapladib. However, in a recently concluded phase III STABILITY clinical trial, use of darapladib did not yield promising results. Presence of structurally related multiple ligands for PAF-R with varied potency, existence of multi-molecular forms of PAF-AH, broad substrate specificity of the enzyme and continuous PAF production by the so called bi-cycle of PAF makes PAF more enigmatic. This review seeks to address the above concerns.

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PLATELET-ACTIVATING FACTOR ACETYLHYDROLASE

Regulated inflammatory responses are defensive and homeostatic, while dysregulated responses are deleterious and impair homeostasis. A key molecule involved in both regulated and dysregulated inflammation is platelet-activating factor (PAF) (1, 2), structurally identified as 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (Fig. 1A), and its less pharmacologically active acyl analog, 1-acyl-2-acetyl-sn-glycero-3-phosphocholine (Fig. 1B). Several structural analogs of PAF, called PAF mimetics, are also documented and are implicated in inflammation. However, all of them are hydrolyzed to the biologically inactive lysoPAF/lysophosphatidylcholine (lysoPC) metabolites by a family of enzymes called PAF acetylhydrolase (PAF-AH) (1–4). The abundant and thoroughly characterized enzyme in this family is the plasma form of PAF-AH [also called lipoprotein-associated phospholipase A₂ (PLA₂) and group VII PLA₂]. The majority of the plasma enzyme (two-thirds) circulates in the plasma bound to LDLs, while the remaining plasma enzyme associates with HDLs and other lipoproteins (5). The plasma PAF-AH catalyzes the hydrolysis of acetate (in the case of PAF and acyl PAF) or other substituents at the sn-2 position that exist in oxidized phospholipids, including PAF mimetics. The plasma PAF-AH is a 440 amino acid long protein, with an apparent molecular mass of 45 kDa (5, 6) that varies with the extent of glycosylation. Three PAF-AH isoenzymes have been described to date. One of the enzymes belongs to the subclass PLA₂ classiﬁed under group VII that includes the plasma form, while the other two enzymes belonging to group VIII are intracellular (3, 7, 8). This localization of the enzymes is not strict, as plasma PAF-AH enzyme is also found intracellularly, and intracellular enzymes are also detected as circulating PAF-AH (9).

SPECTRUM OF SUBSTRATES FOR PAF-AH

A salient feature of PAF-AH, unlike other PLA₂s, is the absence of a calcium requirement for its full enzymatic

Abbreviations: APC, activated protein C; GPCR, G protein-coupled receptor; lysoPC, lysophosphatidylcholine; PAF, platelet-activating factor; PAF-AH, platelet-activating factor acetylhydrolase; PAF-R, platelet-activating factor receptor; PC, phosphatidylcholine; PLA₂, phospholipase A₂; PON-1, paraoxonase-1.

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Such modified phospholipids have been identified in hydroperoxides and isoprostane-containing PCs (complex phospholipids such as long chain phospholipid ray of related molecules. An extreme example includes specificity of PAF-AH is vastly relaxed beyond PAF to an ar-

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chain fatty acids at the sn-2 position of the phospholipids. This allows the plasma PAF-AH to circulate without causing any damage to the cellular components or to lipoproteins (2, 7). With a better understanding of PAF biology and the process of inflammation, it is now clear that the substrate specificity of PAF-AH is vastly relaxed beyond PAF to an array of related molecules. An extreme example includes complex phospholipids such as long chain phospholipid hydroperoxides and isoprostane-containing PCs (Fig. 2) (10). Such modified phospholipids have been identified in oxidized LDLs (11–13); in models of oxidative insult such as alcoholic blood (14), smokers blood (15), and electro-
negative LDLs (16); and in models of cutaneous inflamma-
tion (17). Notable among these lipids are the oxidatively truncated phospholipids butanoyl/butenoyl PAF/PC (11), azelaoyl PAF/PC (18, 19), palmitoyl glutaroyl PC, palmitoyl oxovaleryl PC (12), kodia PC (13), and many more (19) (Table 1). Although PAF is recognized by PAF receptor (PAF-R) at subnanomolar concentrations (11, 20), some of the above mentioned molecules also bind to PAF-R with varying affinities to activate PAF-R, hence popularly referred as PAF mimetics/PAF-like lipids (11, 19–21). However, these oxidized phospholipids accumulate during oxidative insult, as they are formed nonenzymatically, leading to exaggerated inflammatory responses that are also curtailed by the action of PAF-AH (6, 7, 11). Interestingly, noncanonical PAF-R ligands, such as lipoteichoic acid (a component of gram positive bacteria) and endotoxin lipopolysaccharide (a component of gram negative bacteria), may also bind to PAF- R, although with much lower affinity when compared with PAF (22–24).

**ATHEROSCLEROSIS AND INFLAMMATION**

The incidence of CVD in individuals without hypercho-

lesterolemia in the recent past provides a compelling reason to look beyond the traditional risk factors of atherosclerosis. Also, the failure of lipid lowering agents to effectively reduce the risk associated with CVD universally has prompted scientists across the globe to investigate atherosclerosis in a novel dimension, namely inflammation (25–27). A common feature of the various factors causing atherosclerosis is oxidative stress (28). Lipid molecules bearing PUFAs esterified to phospholipids are energetically favored targets for oxidation. Thus, LDL particles generally implied in CVD are oxidized and are no longer native to the body. It is the nature of the human body to effectively clear molecules of foreign nature. The innate immune system polices this task by mounting an inflam-
matory response (25, 26). In order to effectively eliminate oxidized LDL, monocytes/macrophages are recruited. It is during this phase that the trapped LDL undergoes further oxidation leading to endothelial dysfunction, formation of foam cells (26), and setting into motion a cascade of events leading to atherosclerotic streak formation. Macrophages, a prolific source of PAF-AH, may have a vital role in curtailing the PAF-R-mediated responses by hydrolyzing PAF-R ligands and other oxidized phospholipids during these events (29). In this regard, it is worth mentioning that paraoxonase-1 (PON-1), an esterase associ-
ated exclusively with HDL, was once claimed to hydrolyze oxidized phospholipids including PAF (30, 31). Later studies conclusively demonstrated that neither PAF nor oxidized phospholipids are hydrolyzed by PON-1, but trace amounts of the plasma form of PAF-AH copurifying with PON-1 during isolation was responsible for the observed hydrolysis (32). Moreover, recombinant PON-1, though containing significant esterase activities toward synthetic esters, is totally devoid of the claimed PAF or ox-
dized phospholipid hydrolyzing ability (33). What PON-1 does in HDL remains elusive.

**BETWEEN THE SUBSTRATE AND PRODUCT OF PAF-AH**

PAF and truncated oxidized phospholipids, including PAF mimetics, are among the commonly identified substrates of the plasma form of PAF-AH (2–4, 7, 9, 32, 33).
PAF is an early mediator of inflammation that is produced rapidly upon appropriate proinflammatory stimulus (1, 2). PAF activates a variety of cells of the innate immune system where it enhances the migratory and adhesive behavior of these cells, enabling them to transmigrate the endothelial barrier through juxtacrine signaling (34, 35). Being potent, PAF exerts its effects at subnanomolar concentrations through a single cell surface G protein-coupled receptor (GPCR) (11, 20). Thus, not only PAF synthesis but also its subsequent hydrolysis to the biologically inactive

### TABLE 1. Chemical structures of oxidatively truncated phospholipids

| LysoPAF | LysoPC |
|---------|--------|
| ![Chemical structure](image) | ![Chemical structure](image) |
| R¹ = C₁₆/₁₈ Fatty alcohol | R¹ = Any Fatty acid |
| 1-alkyl-2-butenoyl-sn-glycero-3-phosphocholine | 1-acetyl-butenoyl-sn-glycero-3-phosphocholine |
| 1-alkyl-2-butyryl-sn-glycero-3-phosphocholine | 1-acetyl-2-butyryl-sn-glycero-3-phosphocholine |
| 1-alkyl-2-oxovaleroyl-sn-glycero-3-phosphocholine | 1-acetyl-2-oxovaleroyl-sn-glycero-3-phosphocholine |
| 1-alkyl-2-glutaryl-sn-glycero-3-phosphocholine | 1-acetyl-2-glutaryl-sn-glycero-3-phosphocholine |
| 1-alkyl-2-(5-keto-6-octenediroyl) sn-glycero-3-phosphocholine | 1-acetyl-2-(5-keto-6-octenediroyl) sn-glycero-3-phosphocholine |
| 1-alkyl-2-azelaoyl sn-glycero-3-phosphocholine | 1-acetyl-2-azelaoyl sn-glycero-3-phosphocholine |

Free radical-mediated oxidative attack generates a host of truncated phospholipids. Oxidatively modified LDL, apoptotic cells are rich sources of these phospholipids. All of them are sensitive to PAF-AH and also recognize PAF-R with varying potency.
product, lysoPAF/lysoPC, by the action of PAF-AH is an important mechanism employed to prevent an exaggerated inflammatory response, and hence PAF-AH is aptly termed as “signal terminator” (36, 37).

Oxidative stress leads to an uncontrolled nonenzymatic chemical attack of the PUFA's in the phospholipids generating a pool of PAF mimetics and other oxidized phospholipids (11, 20, 38). Amid a sea of various other oxidation products produced during LDL oxidation, butanoyl and butenoyl species (Table 1) account for the bulk of the PAF activity, and these PAF mimetics were identified to be up to 1/10th as potent as PAF (11). In a case control study, mean plasma PAF levels of 23.8 pg/ml were reported in healthy subjects while CVD patients had elevated PAF levels of 49.7 pg/ml (39). Studies by Ninio and her coworkers detected the presence of PAF preferentially in intermediate LDL during LDL oxidation, where it reached up to 8.6 ± 5.7 pmol/mg in 3 h of oxidation (40). In another report, serum PAF levels were directly correlated with severity of anaphylaxis, where the PAF levels rose up to 805 ± 595 pg/ml in patients while control subjects had a basal levels of 127 ± 104 pg/ml (41).

The oxidized phospholipids could be a part of the cellular membrane or the lipoprotein particle. In either case, monitoring its effective removal by the action of PAF-AH is important due to the damage they cause by forming whiskers in membranes (42). Considering the proinflammatory properties of the substrates of PAF-AH, it appears important that the enzyme be maximally active to protect cells from uncontrolled oxidative/inflammatory damage. In this regard, it is not surprising that the enzyme is constitutively active. Moreover, overexpression of the enzyme confers cell survival, which otherwise would undergo cell death in response to an oxidative insult (43). Consistent with the concept that this enzyme serves a protective role, some forms of PAF-AH even change their subcellular location to potentially allow the removal of PAF/PAF mimetics at the very site of their origin. For example, measurable translocation of the type 2b enzyme from cytosol to plasma membrane in models of oxidative stress has been shown (44, 45).

LysoPC, the product of PAF-AH action when acyl PAF/diacyl phospholipid oxidation products are the substrates, has gained much attention in the past (46). Although acyl PAF is a moderate PAF-R ligand (it is just several fold less active than its alkyl counterpart), its hydrolytic product, lysoPC, is not an effective PAF-R agonist. However, lysoPC is believed to possess a variety of activities such as induction of cytokine synthesis (47), augmented migration of monocytes (48), chemoattraction in smooth muscle cells (49), etc.; all processes are potentially proatherogenic. Presently, a proinflammatory (hence proatherogenic) property is also ascribed to lysoPC/lysoPAF that not only questions the very anti-inflammatory nature of the PAF-AH, but also the proinflammatory nature of PAF/PAF mimetics (50, 51). However, a “proinflammatory” status cannot be assigned to lysoPC for two simple reasons. First, the normal serum concentration of lysoPC is between 140 and 150 µM (52); this value might increase by 40–50% owing to oxidation of phospholipids or may reach to millimolar levels in the case of hyperlipidemic subjects (53, 54). Most of this lysoPC is bound to albumin and to lipoprotein particles. However, it is important to note that the optimal concentration of lysoPC required to elicit reported biological responses is in the range of 10–50 µM. Thus, the concentration of lysoPC in the plasma is already higher than its action range and further addition of lysoPC should logically be ineffective. As mentioned, lysoPC binds to albumin, other plasma proteins, and even to cells; the real free lysoPC concentration in vivo will always be lower than the measured concentration. Second, the proatherogenic properties of lysoPC probably stem from the experiments that utilized commercial preparations of the molecule that are likely to be contaminated with PAF or PAF mimetics (55). Given the high potency of PAF and trace amount of PAF contaminants, lysoPC may manifest many of the PAF actions through PAF-R. In fact, lysoPC responses are blocked by PAF-R antagonists (55, 56). In a carefully performed study (55), it was proved that PAF present in trace amounts as contaminants in these commercial preparations was the reason for the inflammatory properties of lysoPC/lysoPAP. Moreover, the credibility of lysoPC receptor is seriously questioned (57, 58).

### RISK FACTOR OR A RISK MARKER?

An important observation that came from WOSCOPS (West of Scotland Coronary Prevention Study) caused increased ambiguity concerning the anti-inflammatory nature of the plasma PAF-AH. This study reported a correlation between elevated levels of PAF-AH and the severity of CVDs (59). On the contrary, additional trials failed to reproduce these findings [reviewed in (28)]. We believe that the increased level of the enzyme serves a protective function based on both in vitro and in vivo experiments and observation from PAF-AH-deficient subjects. For example, retroviral introduction of the plasma form of PAF-AH reduces atherosclerosis in a murine model (60). Endothelial cells exposed to electronegative LDL pre-treated with PAF-AH were protected from undergoing apoptosis, suggesting again the protective role of PAF-AH (16). Elevating the circulating levels of PAF-AH by exogenous administration was also found to be beneficial (61). Conversely, transgenic mice overexpressing the PAF-R exhibited increased bronchoconstriction (62) and susceptibility to develop spontaneous melanoma (63), while the PAF-R-null mice were less susceptible to systemic inflammation and acid inspiration-induced lung injury (64, 65). More importantly, in a recently concluded phase III STABILITY (Stabilisation of Atherosclerotic Plaque by Initiation of DarapladibTherapy) trial of 16,000 patients by GlaxoSmithKline involving a tightly controlled multi-center study with chronic coronary heart diseases, darapladib, a specific PAF-AH inhibitor, did not yield promising results. The drug failed to produce a statistically significant improvement in the risk of heart attack, stroke, or death, though it added greater reductions for some of the secondary
endocytosis. Currently, GlaxoSmithKline is thoroughly investigating this data for subgroup differences and an additional SOLID-TIMI 52 (Stabilization of Plaques using Darapladib-Thrombolysis in Myocardial Infarction 52) trial of 13,000 patients is ongoing. Better understanding of the complex inflammatory disorders in general, especially those that involve the PAF signaling system, is a prerequisite to developing novel drugs targeting PAF-AH (66).

The relevance of PAF and PAF-AH in health and disease is observed not just in CVDs, but in a host of diseases affecting the respiratory, dermal, gastrointestinal, pancreatic, and other systems. Both elevated and decreased activities of PAF-AH have been reported in a variety of diseased conditions. For example, dynamic variations in endogenous levels of PAF-AH occur over time in both experimental sepsis and in critically ill sepsis patients (67). In one study involving genetically deficient plasma PAF-AH mice, initially mice were protected from mortality when exposed to bacteria, but later developed significant necrotizing enterocolitis when compared with wild-type mice (68). On the other hand, decreased levels of the enzyme are associated with a number of diseases such as asthma, systemic Lupus erythematosus, and Crohn’s disease [reviewed in (4)]. More than the murine examples, humans from Asian populations, who are deficient in circulating plasma PAF-AH due to a common mutation at position 994 of the PLA2G7 gene resulting in valine to phenylalanine substitution (V279F), are at an increased risk suffering inactivation from modification of the residues that are oxidatively modified (68). On the other hand, decreased levels of the enzyme are associated with a number of diseases such as asthma, systemic Lupus erythematosus, and Crohn’s disease [reviewed in (4)]. More than the murine examples, humans from Asian populations, who are deficient in circulating plasma PAF-AH due to a common mutation at position 994 of the PLA2G7 gene resulting in valine to phenylalanine substitution (V279F), are at an increased risk to develop a range of inflammatory disorders (69). Unexpectedly, using recombinant PAF-AH in sepsis patients did not decrease the mortality rate, as reported by Opal et al. (70) in their phase III clinical trial. This study was carried out in comparison with activated protein C (APC), the only critical care drug that was available for sepsis. However, the population that Opal et al. (70) studied was at a low mortality risk when compared with the previous APC study. Unfortunately, the recombinant form of APC (commercialized as Xigris) that was in use until now, has recently been withdrawn, leaving the critical care physicians without a drug to treat sepsis (71). Outcomes from previous clinical sepsis trials using anti-PAF agents also were not of much promise. This has raised the question of whether the PAF signaling pathway should be targeted in sepsis (72). More recently other targets have also been identified, for example GPCRs coupled to endothelial Goq/Got1 signaling and spingosine-1-phosphate-dependent Gi signaling may play a critical role in mediating lethal responses to anaphylactic mediators such as PAF (73, 74).

The plasma PAF-AH is susceptible to oxidant attack and suffers inactivation from modification of the residues that contribute to enzymatic activity (75). Whether highly variable levels of circulating PAF-AH arise from oxidation in the general population is not currently known. In an elegant study (76), variation in PAF-AH levels is not due to variations in the efficiency of transcription, translation, and mRNA stability, but due to the presence or absence of the N-terminal domain. Additionally, expression of plasma PAF-AH is transcriptionally modulated by the mediators of inflammation (77), for example the promoter of the PAF-AH gene is positively regulated by PAF and negatively regulated by interferon γ and lipopolysaccharide [reviewed in (28)]. Because the final levels of PAF-AH activity are due to the result of both positive and negative modulators, it is difficult to assign precisely whether PAF-AH is a risk factor or a risk marker. The elevated levels of the enzyme probably help in curtailing the ill effects of the increased PAF and oxidized phospholipids during atherosclerosis by hydrolyzing it to a less harmful lysoPC/lysoPAF. Therefore, the increased serum concentration of the enzyme is most likely to be a potential risk marker.

MAKING AND BREAKING PAF AND PAF MIMETICS: BI-CYCLE OF PAF AND PAF MIMETICS

Despite the fact that lysoPC is not proatherogenic and PAF-AH is not a true risk factor, whether PAF-AH is a friend or a foe is still a debated issue (78, 79). It may be possible that overwhelming levels of acyl PAF and its mimetics may have a role in curtailing PAF-R activation. In fact, endothelial cells predominantly make acyl PAF when stimulated with proinflammatory agonists (80, 81). In any cell engaged in PAF biosynthesis, acyl PAF constitutes a major part of the PAF pool under normal conditions and alkyl PAF accounts for a very minor fraction. This is also true in cell free systems undergoing oxidation, such as oxidation of LDL (11). The acyl PAF can also bind and activate PAF-R at high concentration and is hydrolyzed by the same PAF-AH that also hydrolyzes alkyl PAF (1, 2, 4). The difference in the relative abundance of the two species of PAF is subjected to the availability of the precursor molecules. It is now believed that PAF (acyl and alkyl) is continuously produced at basal levels irrespective of a stimulus due to membrane remodeling (82). But the continuous PAF synthesis is counteracted by constitutive PAF-AH-mediated degradation (82). Also noteworthy is that acyl PAF is far less potent than alkyl PAF, and upon binding to PAF-R, presumably silences the cells. This is evident from the observation that PLA1 treatment of a lipid extract possessing PAF-like activity increases its activity several fold after the removal of diacyl PCs including acyl PAF (11). These two features are among a variety of remarkable mechanisms that the nature employs to ensure homeostasis. Thus, acyl PAF and acyl PAF mimetics may act as biological antagonists of PAF-R under normal conditions to keep PAF-sensitive cells quiescent.

Making and breaking alkyl and acyl PAF can be thought of as a bi-cycle of PAF (Fig. 3) involving two different cycles each producing the respective species of PAF. The rate of the reactions producing acyl PAF is higher than that of the alkyl PAF. Thus, it is apparent that under normal conditions, the two wheels of the PAF bi-cycle operate under different rates. Considering the potency of alkyl PAF acting at subnanomolar concentrations upon a suitable proinflammatory stimulus, it is obvious that the rate of production of alkyl PAF increases only by a small degree while the increased rate of acyl PAF production is
unlikely to make a significant difference as far as PAF-R activation is concerned (Fig. 3). Thus, the sheer abundance of acyl PAF enables it as the obvious target of PAF-AH action. Under normal conditions, though PAF-AH is engaged in hydrolyzing acyl PAF, low levels of alkyl PAF may transiently bind to the PAF-R. But, these low levels are unlikely to elicit an inflammatory response. Upon proinflammatory stimulation, the rate of acyl PAF synthesis goes up as does the synthesis of alkyl PAF. Now PAF-AH is busy in hydrolyzing overwhelming levels of acyl PAF, leaving alkyl PAF to effectively bind to PAF-R, resulting in a transient inflammatory response. Under oxidative insult conditions, free radical-mediated oxidative attack generates a plethora of oxidized phospholipids, all of which are PAF-AH substrates and some of which are also excellent PAF-R ligands (PAF mimetics). These oxidized phospholipids amplify the inflammatory responses. Failure of the PAF-AH to efficiently hydrolyze alkyl PAF and PAF mimetics due to their low abundance amid a pool of abundant acyl PAF and acyl PAF mimetics might lead to an exaggerated but unnecessary inflammatory response. This is evident in murine models of inflammation wherein massive amounts of recombinant PAF-AH (approximately 10-fold over the endogenous PAF-AH levels) had to be administered in order to generate a noninflammatory phenotype (36, 60). A dedicated PAF synthase is yet to be described (83). Thus we propose that the ambiguity is not concerning the nature of the products formed from PAF-AH hydrolysis or the elevated levels of PAF-AH during CVD, but rather the heterogeneous group of molecules that can act as agonists/antagonists for the PAF-R and as substrates for PAF-AH. We remain incompletely informed of the biology of this enigmatic molecule.

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