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Determination of phospholipase activity of PAF acetylhydrolase

Diana M. Stafforini [a,*], Thomas M. McIntyre [b]

[a] Huntsman Cancer Institute and Department of Internal Medicine, University of Utah, Salt Lake City, UT 84112, USA
[b] Department of Cell Biology, Lerner Research Institute, Cleveland Clinic Lerner College of Medicine, Cleveland, OH 44195, USA

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ABSTRACT

This article presents a radiometric assay to determine the enzymatic activity of platelet-activating factor (PAF) acetylhydrolase (PAF-AH), also known as lipoprotein-associated phospholipase A_2 and phospholipase A_2 group 7A. The method is based on the release of radioactively labeled acetate from sn-2-labeled PAF and separation of substrate and product using reversed-phase column chromatography on octadecyl silica gel cartridges. The assay is fast, convenient, reproducible, sensitive, and inexpensive. The instrumentation required includes standard laboratory equipment and a liquid scintillation counter. The assay is also useful to determine the activity of intracellular PAF-AH (PAF-AH II), provided that a few modifications are included. The enzymatic activity determined using PAF as the substrate is a direct indication of the ability of plasma samples, purified preparations, and cellular and tissue lysates to hydrolyze short- and medium-chain phospholipids that may or may not harbor oxidized functionalities. In addition, the assay can be used to test the suitability of other phospholipids, including species containing oxidized, long-chain sn-2 fatty acyl groups, as PAF-AH substrates. This versatile assay can be used to accurately determine PAF-AH activity in biological samples and preliminarily assess affinity and efficiency of the hydrolysis of potential substrates present in complex mixtures.

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Introduction

Platelet-activating factor (PAF) acetylhydrolase (PAF-AH) is a phospholipase A_2 (PLA_2) activity initially discovered by its ability to hydrolyze PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) in serum derived from IgE-sensitized rabbits (Fig. 1A) [1]. Subsequently, it was discovered that this enzyme is a member of a family of specialized PLA_2·s with high specificity for hydrolysis of PAF and structurally related phospholipids [2]. For hydrolysis to occur in a physiologically meaningful manner (i.e., with high affinity and/or high rate of product generation), the *sn*-2 acyl group present in the substrate must meet specific requirements. Its length has to be no longer than nine methylene groups and may or may not harbor acidic, aldehydic, or alcohol functionalities (Fig. 1B) [3]. In addition, this enzyme can hydrolyze glycerophospholipids with relatively long *sn*-2 chains *only if* they harbor oxidized functionalities, such as isoprostanes or fatty alcohols (Fig. 1C) [4–6]. Phospholipids containing long acyl groups at the *sn*-2 position, such as those found in plasma and organelle membranes, are not hydrolyzed. These combined features ensure that structural components of cellular membranes and lipoproteins remain intact, whereas products of oxidation and fragmentation are metabolized. This substrate specificity, combined with the fact that PAF-AH associates with lipoproteins [7], led to the alternate name "lipoprotein-associated PLA_2." Cloning and characterization of the gene encoding plasma PAF-AH [8], elucidation of its amino acid sequence and substrate specificity, and the ability of this enzyme to function in the absence of calcium resulted in formal classification of plasma PAF-AH as Group 7A phospholipase A_2 (PLA2G7A) [9,10].
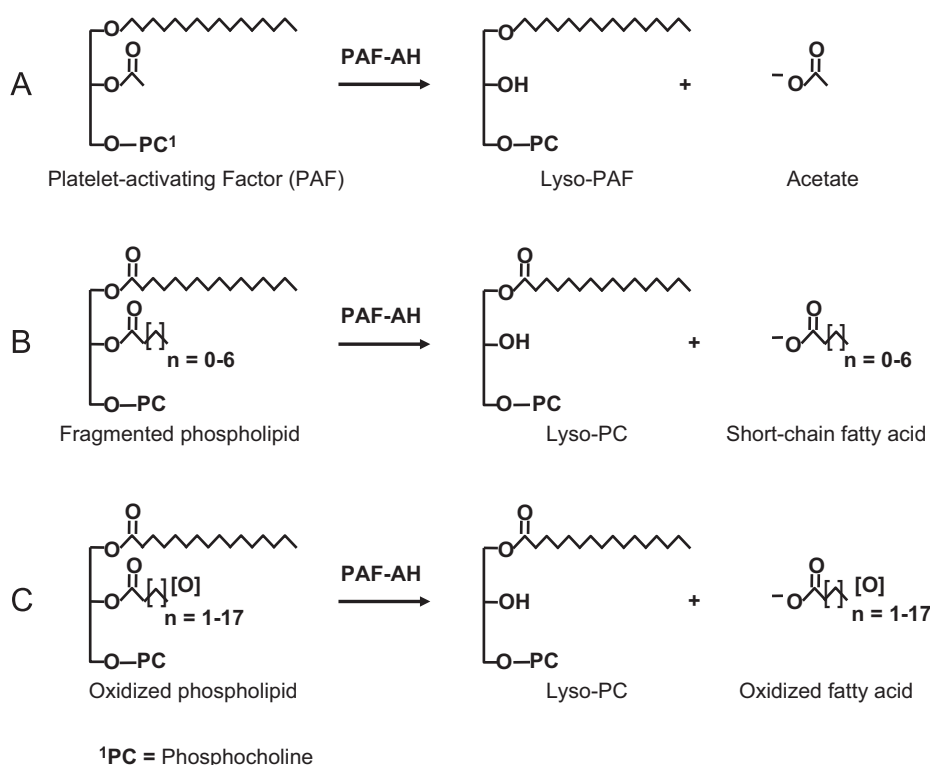
In addition to plasma, several tissues express an intracellular PAF-AH activity known as PAF-AH II that shares 43% homology with the plasma enzyme [11]. This protein is encoded by the *PAFAH2* gene and its substrate specificity is identical to that of plasma PAF-AH, that is, it hydrolyzes PAF and fragmented and oxidized phospholipids [5,12,13]. A second intracellular activity, PAF-AH Ib, is a heterotrimeric complex composed of two subunits (PAF-AH α1 and PAF-AH α2, or PLA2G8A and PLA2G8B) that can hydrolyze PAF and a third subunit that appears to play regulatory roles [14]. The heterotrimer is specific for PAF hydrolysis and does not recognize other phospholipids [15]. Further discussion of heterotrimeric PAF-AH is beyond the scope of this article.

A key feature that differentiates PAF-AHs from many PLA_2 activities is their calcium independence and unusually strong

Fig. 1. Reaction catalyzed by PAF-AH. (A) Hydrolysis of PAF to lyso-PAF and acetate. (B) Hydrolysis of short-chain phospholipids to lyso-PC and short-chain fatty acids. (C) Hydrolysis of oxidized phospholipids to lyso-PC and oxidized fatty acids. PC, phosphocholine.

selectivity for the sn-2 residue [2]. This indicates that these activities act continuously, for example to scavenge substrates, rather than acutely in response to activation signals. PAF-AH activity is inhibited by serine esterase inhibitors such as diisopropylfluorophosphate, Pefabloc, and phenylmethylsulfonyl fluoride [7,16]. This is due to the presence of a GXSXG motif characteristic of lipases and esterases, which confers sensitivity to nucleophilic agents such as those mentioned above [17]. Structural analogs of PAF have been shown to act as substrates and/or to competitively inhibit PAF-AH activity [18]. PAF receptor antagonists are also relatively good inhibitors of this activity, provided that their structure mimics that of PAF [4].

In this article, we describe a convenient method to assay the activity of the plasma form of PAF-AH and that of intracellular PAF-AH II [19,20]. Ideal enzyme assays should have high sensitivity, utilize commercially available and specific substrates, and be convenient and cost-effective [21]. The method described herein meets these requirements and has been optimized so that the rate of hydrolysis is dictated by the amount of enzyme added to the assay and the incubation time. This approach can be used to determine total PAF-AH activity in plasma and serum samples, in cell and tissue extracts, and in purified preparations.

Background of the methodology

The radiometric assay for determination of PAF-AH activity is based on utilization of 2-[acetyl-^{3}H]PAF as substrate; the [^{3}H]acetate generated by hydrolysis can be quickly and efficiently separated from labeled substrate because the product (acetate) is freely soluble in water, whereas the substrate (PAF) is amphipathic. Excess substrate is separated from released water-soluble products by reversed-phase column chromatography on octadecyl silica gel cartridges; the substrate binds to the column, and radioactive acetate passes through. Thus, the level of radioactivity released represents the amount of enzymatic activity in a given

fraction and can be conveniently quantified by liquid scintillation spectrometry [19,20]. The assay provides a measure of absolute enzymatic activity and thus can be utilized to make direct comparisons among laboratories.

Materials necessary for the determination of PAF-AH activity in plasma or serum

Reagents

1. PAF, 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Product No. 878110).
2. 1-O-Hexadecyl [acetyl-^{3}H]PAF (sp act 10–30 Ci/mmol) (Perkin Elmer, Product No. NET910250UC).
3. Bakerbond SPE octadecyl (C$_{18}$) disposable extraction columns. Each assay requires an individual column (J.T. Baker Product No. 7020-03).
4. Opti-Fluor liquid scintillation fluid (PerkinElmer Product No. 6013199).
5. Dilution buffer: Hepes buffer (0.1 M) or phosphate-buffered saline (PBS). Adjust the pH to 7.2 with 1 M sodium hydroxide. Store at 4 °C.
6. 0.5 M EDTA, pH 7.2. Store at room temperature.
7. Stop solution: acetic acid (10 M). Store at room temperature.
8. Wash solution: sodium acetate (0.1 M). Store at room temperature.

Instrumentation

1. Sonicator: a probe ultrasonic liquid processor with a power rating of 100 W or higher is necessary. Several models are available on the market (e.g., Labsonic homogenizers from Sartorius, Qsonica sonicators from Misonix, and Hielscher ultrasonic processors). Alternatively, one can use a less expensive ultrasonic jewelry and

eyeglass cleaner, although sonication time with this less energetic device must be increased.

2. Picofuge or microcentrifuge.
3. Water incubator set at 37 °C.
4. Vacuum manifold (optional): when it is necessary to perform many assays, the use of a multiplace vacuum manifold is a convenient and time-saving approach that allows simultaneous analysis of multiple samples. J.T. Baker offers 12- or 24-port vacuum manifolds (SPE-12G and SPE-24G) that can process 12 or 24 assays individually. If it is necessary to assay only a few samples, one can get satisfactory results by manually pushing the product of the reaction through a syringe attached to an octadecyl silica gel cartridge.
5. Beckman LS 6500 (or similar) liquid scintillation counter: it is convenient to use an instrument that can provide disintegrations per minute (dpm) to avoid the need to generate a quench curve.

Standard protocol

1. Mix 400 nmol of PAF with 4.5 μCi of 1-O-hexadecyl [acetyl-^{3}H]PAF. Both reagents are supplied as chloroform solutions by the manufacturers.
2. Evaporate the solvent using a stream of nitrogen, for approximately 2 min. If a source of nitrogen is not readily available, one can allow the solvent to evaporate at room temperature for several more minutes. **It is important to evaporate the solvent completely, regardless of the method used.** Add 4 ml of Hepes buffer or PBS, pH 7.2, and 10 μl of 0.5 M EDTA. The final concentration of PAF in this stock solution is 100 μM.
3. Sonicate for 5 min at 100 W, using a 4-mm (or similar) needle probe. **Keep the solution cold.** If an ultrasonic cleaner is used, we recommend longer (10-min) treatments. The resulting solution is sufficient for 100 assays and, if necessary, can be used repeatedly over a period of 1–2 weeks. The solution should be stored at −20 or −70 °C to avoid nonenzymatic hydrolysis. **The sonication step must be repeated each time the substrate is thawed.** Duplicate 10-μl aliquots should be counted to determine the specific radioactivity of the substrate; our working stock solutions contain approximately 20,000–22,000 dpm/nmol of PAF. The amount of nonenzymatic hydrolysis tends to increase with time. Stock solutions should be used before significant nonenzymatic hydrolysis of PAF takes place.
4. Thaw plasma/serum samples in a cold-water bath and prepare at least 100 μl of a 100-fold dilution of plasma/serum in Hepes buffer or PBS, pH 7.2. If possible, use the diluted sample to determine both enzymatic activity and protein content on the same day. **Discard any remaining diluted samples.** EDTA, citrate, and heparin can be used interchangeably as anticoagulants. Results obtained using plasma samples do not differ from those generated with serum. **Serum and plasma are blood products; use appropriate care when handling human samples.**
5. Mix two 10-μl aliquots of diluted samples to be assayed with 40 μl of 0.1 mM [acetyl-^{3}H]PAF in 1.5-ml polypropylene microcentrifuge tubes, at 4 °C. Avoid the use of glass tubes to minimize substrate binding to the glass surface. Cap the tubes, briefly mix, and centrifuge samples for 5 s in a picofuge or microcentrifuge.
6. Incubate the mixtures for 30 min at 37 °C **in a water bath.** No agitation is necessary.
7. Add 50 μl of 0.1 M acetic acid, **immediately followed by** 1.5 ml of 0.1 M sodium acetate solution. The mixtures can be left at room temperature for up to 1–2 h. **Failure to add sodium acetate rapidly after addition of acetic acid may result in high levels of nonenzymatic PAF hydrolysis.**

8. Precondition each octadecyl silica column by sequentially passing 3 ml of chloroform:methanol (1:2), followed by 3 ml of 95% ethanol and, finally, 3 ml of water through the cartridges. It is convenient (but not required) to precondition the columns during the 30-min incubation step (step 6). **If the cartridges are used repeatedly, the liquid waste generated during column preconditioning will contain radioactivity. Make sure you appropriately handle and discard this waste.**
9. If a vacuum manifold is used, place clean, 15-ml scintillation vials or other appropriate tubes to collect column effluents. If the assay is conducted manually, prepare an adequate number of clean scintillation vials for effluent collection.
10. Transfer the reaction mixtures from step 7 to individual octadecyl silica gel cartridges, pass the solutions through the columns, and collect the filtrates containing [^{3}H]acetate. **These filtrates should be saved.**
11. Wash the emptied 1.5-ml polypropylene microcentrifuge tubes used for the assays with 1.5 ml of sodium acetate solution. This step increases product recovery. Transfer the wash to the octadecyl silica gel cartridge corresponding to that assay and pass through the column, as in step 10. Collect this wash in the same vials used in step 10, so that the total volume of the filtrate is approximately 3 ml. Optional: repeat the wash step once more. **At this point, the samples are ready for radioactivity determination.**
12. Add 10 ml of Opti-Fluor and determine the amount of radioactivity in a liquid scintillation counter.

Characteristics of the assay

Initial studies conducted in the absence of a source of enzyme revealed that recovery of acetate from the cartridges is quantitative. When [^{3}H]acetate is substituted for [acetyl-^{3}H]PAF in the assay, more than 99% of the radioactivity is found in the aqueous phase that does not bind to the octadecyl silica cartridge. In addition, there is virtually complete removal of excess radiolabeled substrate because approximately 99% of the [acetyl-^{3}H]PAF present in the assay is retained by the reversed-phase cartridge.

The amount of protein permissible in the solution applied to the cartridges is limited, as large amounts of protein will prevent binding of PAF to the resin. This can result in what appears to be high enzymatic activity because the product becomes contaminated with substrate. In general, the use of 0.1 μl of human plasma (approximately 6 μg of protein) provides excellent signal-to-noise ratio and does not interfere with binding of PAF to the resin. However, it may be necessary to monitor this potential problem on special occasions. This can be achieved by performing mock assays terminated at zero time or using boiled or irreversibly inactivated sources of enzyme. Using this approach, we found that up to 1 μl of plasma (approximately 60 μg of protein) could be safely assayed without affecting PAF binding to the cartridges. However, in the presence of 10 μl of plasma (approximately 600 μg of protein), the columns retained only 96.4% of the radioactively labeled PAF. After any change in procedure, one should verify that the apparent product is not contaminated with substrate. The effluent should be extracted into CHCl$_3$ and examined by TLC [22] or HPLC [23].

The reversed-phase cartridge can be reused at least 10 times before the assay is adversely affected. In our experience, this number can be increased to 20 or more, provided that the amount of protein used is not excessive and that the cartridges are properly regenerated. This significantly decreases the cost per assay.

The reproducibility of the assay is illustrated in Fig. 2A, which depicts results obtained by assaying the same sample 10 times. We routinely test samples in duplicate and, typically, the results
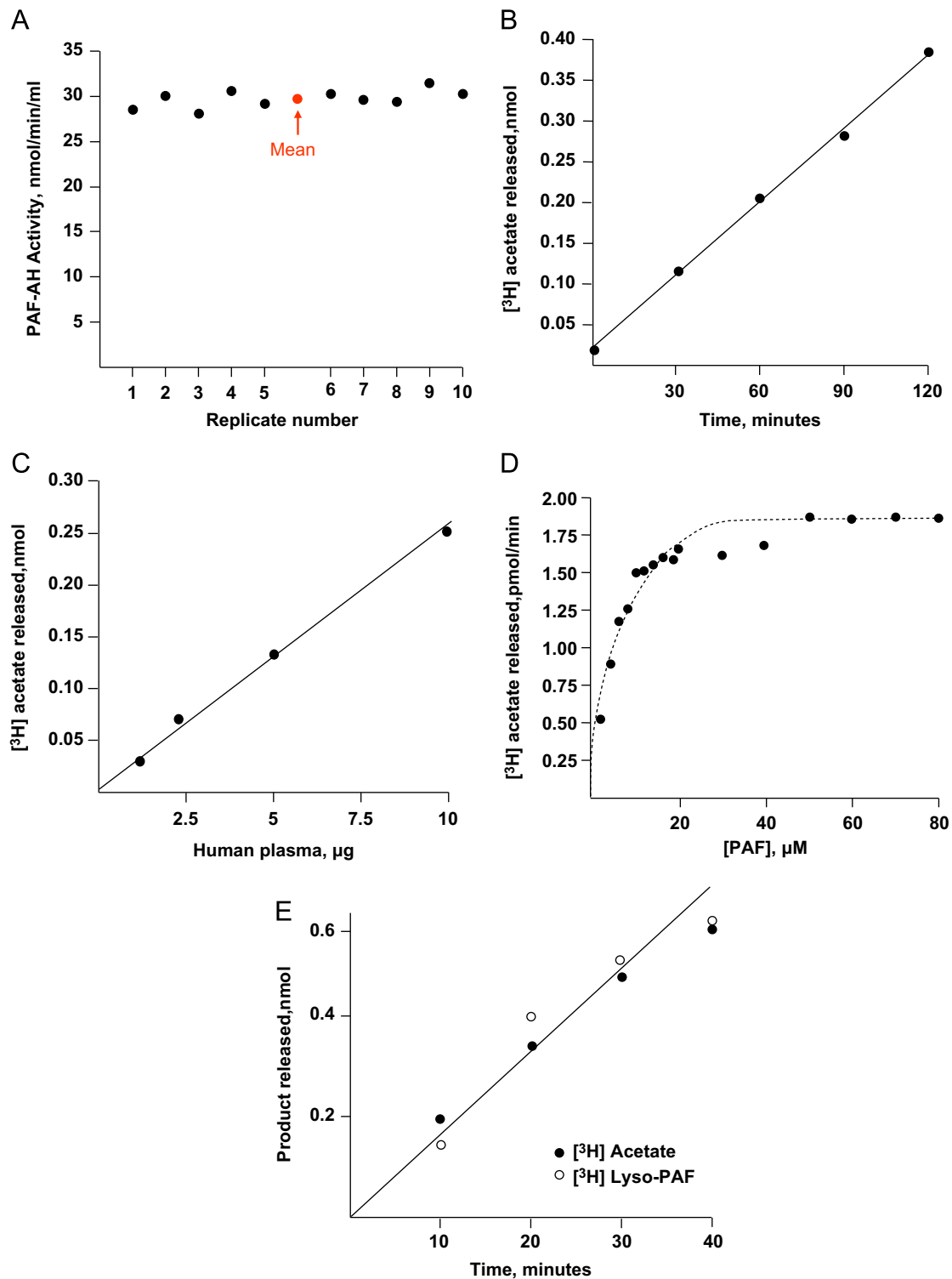
Fig. 2. Characterization of radiometric assay for the determination of PAF-AH activity. (A) The radiometric assay for PAF-AH is reproducible. We conducted 10 identical enzyme assays containing 0.1 μl (5 μg) of normal human plasma, using 80 μM [*acetyl*-³H]PAF as the substrate. We incubated the samples for 30 min at 37 °C. Mean enzymatic activity and standard deviation were 29.7 ± 1.0 nmol/min/ml. (B) Time course of PAF hydrolysis. We incubated 5 μg of human plasma with 80 μM [*acetyl*-³H]PAF for 0, 30, 60, 90, and 120 min at 37 °C. The products were separated from remaining substrate by reversed-phase chromatography, as described in the text. (C) Product generation is directly proportional to enzyme levels. The amount of plasma supplemented to each assay was varied between 0 and 10 μg of protein. We then supplemented 80 μM [*acetyl*-³H]PAF and incubated the samples for 30 min at 37 °C. (D) PAF hydrolysis is maximal when the concentration of PAF in the assay is 50 μM or higher. We incubated 5 μg of human plasma with various concentrations of [*acetyl*-³H]PAF (0–80 μM) for 30 min at 37 °C. The products were separated from the remaining substrate by reversed-phase chromatography, as described in the text. (E) Concordance between acetate and lyso-PAF production. We conducted parallel assays that contained purified PAF-AH supplemented with either 80 μM [*acetyl*-³ H]PAF or 80 μM [*alkyl*-³H]PAF and incubated the mixtures for 10, 20, 30, and 40 min, at 37 °C. Assays conducted with [*acetyl*-³H]PAF were processed as described in the text. The remaining assays that utilized [*alkyl*-³H]PAF as the substrate were subjected to total lipid extraction and separation by thin-layer chromatography using CHCl₃:CH₃OH:CH₃COOH:H₂O (50:25:8:4) as the solvent. The spot containing [*alkyl*-³H]lyso-PAF was scraped and quantitated by liquid scintillation counting.

agree within 5% of each other. The time course of acetate production is linear up to at least 120 min when the incubation is performed at either 25 (not shown) or 37 °C (Fig. 2B). However, the amount of product generated at room temperature is approximately half of that produced at 37 °C. The assay is linear as a function of the amount of plasma added, up to at least 10 μg (Fig. 2C). The effect of PAF levels on hydrolysis illustrates that the substrate concentration used in standard PAF-AH assays (80 μM) is saturating (Fig. 2D). As a rule, we adjust the amount of enzyme and incubation time so that no more than 7.5% of the substrate is hydrolyzed. Finally, there is complete concordance between the amount of [3H]acetate quantified using this assay compared with the levels of [alkyl-3H]lyso-PAF released from [alkyl-3H]PAF (Fig. 2E).

Expected results and calculations

Usually, PAF-AH activity in plasma/serum is expressed as nanomoles of substrate hydrolyzed per milliliter of plasma (or per mg of plasma protein), per minute at 37 °C. Data generated by scintillation counting must be corrected for quenching (unless the instrument is equipped with internal quench curves), incubation time, dilution factor, and volume of plasma used in the assay. Typically, we use the following conditions:

dilution factor of human plasma sample(s), $100 \times$;
volume of diluted plasma used in each assay, 0.01 ml;
incubation time at 37 °C, 30 min;
typical dpm in 10 μl of 0.1 mM [acetyl-3H] PAF, ~20,000;
typical dpm in negative (PBS) control, ~500;
typical average dpm in duplicate tests, ~4000;
activity in nmol/min/ml=(4000–500) dpm $\times$ 1/30 min $\times$ 1/0.01 ml $\times$ 1 nmol/20,000 dpm $\times$ 100.

Additional applications

Measurement of intracellular PAF-AH II activity

In addition to detecting plasma PAF-AH activity, this assay can be used for the determination of intracellular PAF-AH II activity. In this case, it is important to supplement the assays with Chaps (2 mM) and dithiothreitol (2 mM) [5]. A caveat is that the assay will also detect other calcium-independent PLA$_2$ activities such as G4B and iPLA$_2$·s (e.g., G6A) that are not specific with regard to the fatty acid being released [10] and that can, therefore, hydrolyze PAF. If necessary, this problem can be solved by assessing the extent to which long-chain phospholipids are hydrolyzed in a calcium-independent manner [24], using the experimental conditions of the PAF-AH assay. This value should be used to correct the activity obtained using the PAF-AH assay. An alternative and simpler approach is to saturate possible competing PLA$_2$ activities by supplementing PAF-AH assays with excess unlabeled, long-chain glycerophospholipids. However, care should be taken to ensure that the concentration of PAF on the surface of the micelles remains saturating. This can be accomplished by performing the assays at 60 and 80 μM [acetyl-H^3]PAF, for example, in the presence of a fixed amount of unlabeled long-chain glycerophospholipids. The activity values obtained under these two experimental conditions should not significantly differ from each other.

Assessment of other phospholipids as potential PAF-AH substrates

This assay can be utilized to directly assess hydrolysis of short-chain, synthetic oxidized phospholipids, such as 1-palmitoyl-2-(5′-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC). POVPC

is commercially available (Avanti Polar Lipids Product No. 870606 or Cayman Chemical Product No. 10031) and the sn-2-labeled molecule can be conveniently synthesized from 1-palmitoyl-2-[arachidonoyl-1-^{14}C]-sn-glycero-3-phosphocholine (PerkinElmer Product No. NEC765010UC), using previously described methods [5,25]. For phospholipids harboring longer sn-2 fatty acyl groups such as isoprostanes and fatty acid hydroperoxides, the simplest initial approach is to test the ability of these compounds to competitively inhibit hydrolysis of [acetyl-3H]PAF. The assays should be supplemented with Chaps (0.8 mM) for solubilization purposes [5]. A caveat is that the addition of phospholipids to the standard assay can decrease the effective concentration of PAF on the surface of the resulting (mixed) micelles. Thus, if inhibition is observed, it is essential to subsequently confirm that this response is indicative of substrate hydrolysis, using more direct methodologies [5,6]. Discussion of these approaches is beyond the scope of this article.

Assay automation and other modifications

Slight variations in this assay have been reported by a number of laboratories. The main modification is the methodology used to separate released product from unreacted substrate. For example, released acetate can be separated from PAF by solvent extraction and measurement of radioactivity in the aqueous phase [26–29]. This method is less desirable because extensive phase–phase extractions are likely to result in losses that may be difficult to account for. In addition, care must be taken to remove all CHCl$_3$ to avoid severe quenching during liquid scintillation counting. An alternative approach is to utilize a precipitation step to separate excess, labeled substrate (which forms a complex with albumin and trichloroacetic acid) from product (which remains soluble) [30]. This avoids the use of costly reagents and exposure to toxic solvents. The assay can be automated for use on a 96-well microtiter plate format, using activated charcoal to separate [acetyl-3H]PAF from [3H]acetate. The experimental conditions are similar to those described above. This assay requires special instrumentation, that is, a microplate scintillation counter [31–33].

Akiyama and co-workers proposed an interesting assay variation that can detect PAF-AH activity in a complex mixture of proteins using SDS–PAGE followed by transfer to polyvinylidene difluoride membranes [34]. The assay is based on measurement of decreased β-radioluminescence of [acetyl-3H]PAF bound to the membrane after incubation with radioactively labeled substrate. Whereas this assay is not practical for high-throughput screens, its advantage is that it can identify the size and occurrence of novel PAF-AH activities in complex mixtures. In addition, the assay can be used to test the suitability of almost any phospholipid substrate, provided that the sn-2 acyl residue is radioactively labeled. However, this approach is useful only when the enzymatic activity is insensitive to SDS treatment, a feature that may apply in a limited number of cases.

[alkyl-3H]PAF has also been used as a PAF-AH substrate. In this case, quantification of [alkyl-3H]lyso-PAF release is the measure of enzymatic activity [26,30]. Separation between substrate and product usually is accomplished by TLC or HPLC, followed by scintillation counting. This approach is time consuming and less accurate and does not appear to offer advantages compared with assays that utilize [acetyl-3H]PAF.

Other assays to determine PAF-AH activity

Fluorimetric assays

The first fluorescence-based assay for PAF-AH activity was described by Schindler and co-workers [35], who found that

a fluorophore-labeled ether lipid PAF analog (1-(N-dansyl-11-amino-1-undecyl)-2-acetyl-sn-glycerol-3-phosphorylcholine) was hydrolyzed by PAF-AH in a rat polymorphonuclear neutrophil extract. Subsequently, 1-palmitoyl-2-(7-nitrobenzoxadiazolyl) aminocaproyl phosphatidylcholine (C_6NBD PC) was used as a model fluorescent substrate [36,37]. This approach appears suitable to measure PAF-AH activity, as it has been reported to have properties generally comparable to those of the radiometric assay that uses [acetyl-^{3}H]PAF [36]. Whereas utilization of the fluorescent substrate can be convenient for investigators who prefer not to use radioactive isotopes, the assay has several drawbacks. First, a fluorimeter is required. Second, fluorescence, unlike radioactivity, is not an intrinsic property of a compound and fluorescent yield depends on environmental factors. Third, the fluorescence of the substrate (C_6NBD PC) is approximately 2% that of the product (NBD hexanoic acid) [38]. This can be highly significant as only a small fraction of the substrate is converted to product in an optimized enzyme assay. Fourth, the fluorescence of C_6NBD PC is affected by sonication and albumin content [38]. This problem can be solved by allowing the test sample to equilibrate with substrate for a time interval that is dependent on protein concentration and thus different for each sample [38]. Alternatively, the products of the reaction can be isolated using acidic lipid extraction and purification, before fluorescence determinations [38]. These steps can add to the time required and may result in lab-to-lab variations. Finally, the minimum quantity of NBD fatty acids that can be measured fluorimetrically has been reported to be 100 pmol [38]. In contrast, the standard radiometric assay can detect 20 pmol of [^{3}H]acetate; higher sensitivities can be attained if the specific radioactivity of the [acetyl-^{3}H]PAF substrate is increased.

Highest sensitivity without loss of specificity can be attained by a fluorimetric assay that utilizes authentic PAF as substrate [39]. After incubation of PAF with a source of PAF-AH, lyso-PAF is extracted from the reaction mixture using butanone. DEACZ (7-diethylaminocoumarin-3-carbonyl azide) is then utilized to derivatize the free hydroxyl group at the sn-2 position of lyso-PAF, to generate the 2-carbamoyl derivative. The products are subjected to HPLC and the derivatized lyso-PAF is quantified by a fluorescence detector. This method has high sensitivity as it can detect as little as 1 pmol of lyso-PAF and does not involve the use of radioactivity. However, it requires specialized instrumentation and is time consuming. For example, derivatization with DEACZ takes 3 h, and each sample needs to be individually subjected to HPLC [39].

Colorimetric assays

The first colorimetric assay used for determination of PAF-AH activity utilized 1-decanoyl-2-(4-nitrophenylglutaryl) phosphatidylcholine (DNGP) as the substrate [29]. Although similar results were obtained when PAF and DNGP were used to determine kinetic parameters of highly purified PAF-AH preparations, the reliability of this approach to determine PAF-AH activity in plasma samples or crude extracts that contain other PLA_2s remains to be determined. In addition, the response of this assay to variations in protein concentration and time have not been reported [29]. Many laboratories have utilized a commercially available colorimetric assay to determine PAF-AH activity. The initial assay was conceived, developed, and characterized by Kosaka and co-workers [40]. This approach is based on the use of 1-myristoyl-2-(4-nitrophenylsuccinyl) phosphatidylcholine, which, upon hydrolysis, releases 4-nitrophenyl succinate. This compound immediately degrades in aqueous solution and liberates 4-nitrophenol, which can be monitored spectrophotometrically [40]. Commercially available kits are marketed by Cosmo Bio

Co., Ltd. (Product No. ALF-274664847, Azwell Auto PAF-AH) and diaDexus (Lp-PLA_2 colorimetric activity (CAM) assay) and have been used in multiple clinical studies (see, for example, Refs. [41–46]). Limitations include that the substrate is hydrolyzed by nonspecific esterase activities such as that intrinsic to albumin, in addition to PAF-AH [40]. When this problem is corrected by supplementation with Chaps and sodium 1-nonane sulfonate, there is excellent correlation with the radiometric assay ($R^2 = 0.979$) [40,45]. Importantly, plasma from a subject who harbored two copies of the V279F inactivating mutation at the PLA2G7A locus [47] had essentially no enzymatic activity when the CAM assay was used [48]. This indicates that the CAM assay detects plasma PAF-AH activity exclusive of other plasma activities. The absolute activity determined using the spectrophotometric approach is higher compared with that obtained using radiometric assays [40,45,49–51]. A recent report compared results from studies that used colorimetric (diaDexus CAM and Azwell) assays and found that the mean plasma PAF-AH activities detected using these approaches were 151 ± 32 and 629 ± 141 nmol/min/ml, respectively [49]. In contrast, the mean activity found in similar studies that used radiometric assays was 42 ± 14 nmol/min/ml, a value that agrees favorably with studies in the authors' laboratories [5,52]. Mohler and co-workers [45] found good correlation between the colorimetric and radiometric assays when the data were log-transformed ($R^2 = 0.918$), but large differences in absolute hydrolytic rates using PAF versus 1-myristoyl-2-(4-nitrophenylsuccinyl) phosphatidylcholine as substrate. The basis for variations among these assays is unclear. Structural differences between PAF, which contains an sn-2 acetyl group, and substrates that contain 4-nitrophenyl [29,40] functionalities at the sn-2 position could affect accessibility to the active site and/or the rate of product generation. Whereas DNGP and PAF were recognized with equal affinities and were hydrolyzed at similar rates when purified PAF-AH was utilized [29], it is possible that specific kit components affect substrate availability and/or solubility when complex mixtures such as plasma and serum are assayed.

An additional colorimetric assay marketed by Cayman Chemical (Product No. 760901), Abnova (Product No. KA1354), and Interchim (Product No. Q91581) is based on the use of 2-thio-PAF as substrate. Upon hydrolysis by PAF-AH, free thiols are released and quantitated using 5,5'-dithiobis(2-nitrobenzoic acid) [53]. The dynamic range of the assay is limited only by the accuracy of the absorbance measurement. This assay was used in a study to assess the correlation between PAF-AH activity and the incidence of recurrent coronary events in postinfarction patients [54]. Advantages of this approach include accuracy and convenience, and the potential for use in high-throughput applications. However, there are several limitations. First, the price per assay using this commercially available kit is approximately three times higher compared with that of the radiometric assay. Second, the sensitivity of the Cayman assay ranges from 20 to 200 nmol/min/ml of PAF-AH activity. Although this is adequate for some studies, higher sensitivity may be required when samples are expected to express lower activity levels. PAF-AH activity in human plasma samples from pediatric healthy subjects can be as low as 25.0–27.7 nmol/min/ml [30,55]. Fatal anaphylaxis is associated with even lower activity levels (14.5 nmol/min/ml) [55]. Studies that involve populations that harbor inactivating mutations in the PLA2G7A locus are likely to include patients with low activity values [47,48,56]. Abnova, a company that commercializes this assay kit, states that it may be necessary to concentrate plasma samples to increase PAF-AH concentration. This can be cumbersome when multiple assays are required, it can lead to inactivation, and it adds to the cost. Thus, when activity is expected to be low, the radiometric assay is more appropriate and reliable. Third, different substrate affinities have been reported for PAF (19 μM) versus 2-thio-PAF (10 μM) using a preparation from bovine liver; it is not clear whether this feature also applies to plasma PAF-AH. Fourth,

the rate of [³H]acetate released from [acetyl-³H]PAF was reportedly higher than that of thiols generated from 2-thio-PAF [53]. To our knowledge, only two clinical studies utilized this assay and reported somewhat lower mean plasma PAF-AH activities (11.6 ± 4.1 [57], 15 ± 5.1 [57], and 26 ± 6 nmol/min/ml [54][1]) compared with results from similar studies that used radiometric assays. However, differences in the composition of the patient cohorts tested in each study could account for these variations.

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