methods

An improved assay for platelet-activating factor using HPLC-tandem mass spectrometry

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Abstract **We describe an improved assay for platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) using HPLC-tandem mass spectrometry (LC-MS/MS). The present method can readily detect as little as 1 pg (1.9 fmol) of PAF, a significant improvement over previously described LC-MS/MS methods, and gives a linear response up to 1,000 pg of PAF. Our method also overcomes the artifacts from isobaric lipids that have limited the usefulness of certain existing LC-MS/MS assays for PAF. In the course of these studies, we detected three novel lipid species in human neutrophils. One of the novel lipids appears to be a new molecular species of PAF, and the other two have chromatographic and mass spectrometric properties consistent with stearoyl-formyl-glycerophosphocholine and oleoyl-formyl-glycerophosphocholine. These observations identify previously unknown potential interferences in the measurement of PAF by LC-MS/MS. Moreover, our data suggest that the previously described palmitoyl-formyl-glycerophosphocholine is not unique but rather is a member of a new and poorly understood family of formylated lipids.**—Owen, J. S., R. L. Wykle, M. P. Samuel, and M. J. Thomas. **An improved assay for platelet-activating factor using HPLC-tandem mass spectrometry. *J. Lipid Res.* 2005.** 46: **373–382.**

Supplementary key words high-performance liquid chromatography tandem mass spectrometry • trace analysis • neutrophil

Platelet-activating factor (PAF) is a phospholipid autacoid with the chemical structure 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (1–3). The alkyl chain at *sn*-1 is most commonly hexadecyl, octadecyl, or octadec-*cis*-9-enyl, designated 16:0, 18:0, or 18:1, respectively. Other molecular species with different alkyl moieties occur in minor amounts (4–7). PAF has been implicated in the inflammatory response that follows injury (8–11) as well as in inflammatory conditions, including asthma and anaphylaxis (12, 13). More recently, PAF has been implicated in the growth and metastasis of epithelial cancers (14–17). Physiological roles have been proposed for PAF in fertility and embryonic development as well as in learning and memory (18).

Like most lipid mediators, PAF is not stored but is rapidly synthesized on demand in appropriately stimulated cells through the remodeling of membrane lipids (19, 20) and is rapidly broken down by acetylhydrolases in cells and in blood plasma (21). In consequence, PAF exists at very low concentrations in biological specimens, and studies on the biology of PAF have been limited by investigators' ability to quantify PAF with accuracy and precision. Early PAF measurements relied on bioassay. Nearly a decade passed between the discovery of PAF and the elucidation of its chemical structure, making physicochemical assay possible.

Currently, MS-based methods represent the state of the art in PAF assays in terms of sensitivity, precision, specificity, dynamic range, and versatility. The most sensitive approach described to date involves removal of the phosphocholine head group and its replacement with a nonpolar group. The resulting volatile derivative is then analyzed by GC-MS (22–28). As little as 50 fg of PAF can be detected using this approach (25). However, the requirement for derivatization makes these assays time-consuming, expensive, and susceptible to losses and artifacts. Such drawbacks have prompted the development of PAF assays using HPLC coupled to MS or tandem MS (LC-MS or LC-MS/MS, respectively) (29–34). This approach has the potential to deliver the advantages of GC-MS without the need for derivatization. However, LC-MS and LC-MS/MS techniques described to date have not approached the sensitivity obtainable with GC-MS, and most of them have shortcomings in specificity as well, as demonstrated below. Here, we describe an LC-MS/MS assay for PAF that shows significant improvements over previously described LC-MS/MS

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Abbreviations: d_3-16:0 PAF, 1-*O*-hexadecyl-2-[$^2H_3$]acetyl-glycerophosphocholine; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GPC, *sn*-3-glycerophosphocholine; LC-MS, high-performance liquid chromatography-mass spectrometry; LC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; lysoPC, lysophosphatidylcholine; MRM, multiple reaction monitoring; PAF, platelet-activating factor; PFPC, palmitoyl-formyl-glycerophosphocholine; PMN, polymorphonuclear neutrophil.

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methods in terms of both sensitivity and specificity. In the course of optimizing this improved PAF assay, we also found evidence for a novel PAF molecular species and two other novel choline phospholipid species. The latter two novel species appear to contain a formyl substituent in place of one of the fatty acyl substituents and thus, together with the palmitoyl-formyl-glycerophosphocholine (PFPC) species described by Harrison, Clay, and Murphy (34), may constitute a new and poorly understood family of lipids.

MATERIALS AND METHODS

Materials

1-*O*-Hexadecyl-2-[$^2H_3$]acetyl-glycerophosphocholine (d_3-16:0 PAF) was synthesized as described by Clay (35) from 1-*O*-hexadecyl-2-lyso-GPC (Bachem Bioscience, King of Prussia, PA) and [$^2H_6$]acetic anhydride (Aldrich Chemical, Milwaukee, WI). Unlabeled 16:0 PAF was purchased from Avanti Polar Lipids (Alabaster, AL). 18:0 PAF and 18:1 PAF were from Bachem. Concentrations of PAF standard solutions were determined by determination of phosphorus according to Rouser, Siakotos, and Fleischer (36). 1-Stearoyl-2-lyso-GPC was from Avanti. Calcium ionophore A23187 was from Calbiochem (La Jolla, CA). *N*-Formyl-methionyl-leucyl-phenylalanine (fMLP) was from Sigma. Ficoll was from Amersham Pharmacia Biotech (Uppsala, Sweden). Hypaque was from Nycomed (Princeton, NJ). Heparin was from Elkins-Sinn (Cherry Hill, NJ). Solvents were Optima grade (Fisher Scientific, Fair Lawn, NJ). Other chemicals were from Sigma Chemical (St. Louis, MO).

1-Lyso-2-stearoyl-GPC was prepared essentially as described by Polette et al. (37), with some modification. Briefly, 2 mg of 1,2-distearoyl-GPC (Avanti) was suspended by sonication in a two-phase system consisting of 1 ml each of ethyl ether and 0.1 M borate buffer, pH 6.4. *Rhizopus arrhizus* lipase (2×10^5 units; Sigma Chemical) was added, and the mixture was vortexed at room temperature for 1 h. The product was then immediately extracted from the reaction mixture (38) using solvents chilled to 4°C. Aliquots of the product, suitably diluted, were immediately analyzed by LC-MS/MS without any purification or storage to prevent acyl migration.

Neutrophil isolation and stimulation

Polymorphonuclear neutrophils (PMNs) were isolated from blood of healthy human volunteers. Briefly, blood was collected into 60 ml syringes containing 300 units of heparin and 2 ml of 100 mM EDTA, pH 7.4. PMNs were isolated by Ficoll-Hypaque density gradient centrifugation, followed by hypotonic lysis to remove residual red blood cells and washing to remove residual platelets (39). Isolated PMNs were counted using a Coulter counter and suspended in HBSS containing 24 mM Tris, pH 7.4, 1.4 mM $CaCl_2$, and no Mg^{2+}. Aliquots of the cell suspension (1×10^7 cells in 1 ml of HBSS in 1.5 ml polypropylene microfuge tubes) were warmed to 37°C in a shaking water bath for 20 min and then treated with A23187, fMLP, or no stimulus.

Stimulation was terminated by transferring the entire cell suspension to a disposable borosilicate glass test tube containing 2.2 ml of methanol acidified with 2.5% acetic acid. Control experiments showed that the acetic acid did not affect the amount of PAF or PFPC measured but did enhance recovery by preventing the formation of sodium adducts (data not shown). Internal standard (400 pg of d_3-16:0 PAF) was added, and lipids were extracted from the cell suspensions (cells and medium together) according to Bligh and Dyer (38).

LC-MS/MS

Data were collected on a Micromass Quattro II triple-quadrupole mass spectrometer equipped with a Z-spray™ source and operated in multiple reaction monitoring (MRM) mode with MassLynx NT™ software, version 3.5. Positive-ion and negative-ion electrospray ionization were used, with nitrogen as the nebulizing gas and the drying gas. The desolvation temperature was 150°C for normal-phase HPLC separations and 250°C for reversed-phase separations. The source temperature was 80°C for all experiments. Argon served as the collision gas at a pressure of 1 to 1.5×10^{-3} mbar and a collision energy of 23 V. Typical capillary voltages were 3.6 and −2.6 kV for positive and negative ion modes, respectively; typical cone voltages were 38 and −60 V. Resolution was set at 13.5 for both MS1 and MS2. PAF was detected in positive-ion mode as the transitions m/z 527 → 185 (d_3-16:0 PAF), m/z 524 → 184 (unlabeled 16:0 PAF), m/z 550 → 184 (18:1 PAF), and m/z 552 → 184 (18:0 PAF). In negative-ion mode, PAF was detected as m/z −511 → −62 (d_3-16:0 PAF), m/z −508 → −59 (unlabeled 16:0 PAF), m/z −534 → −59 (18:1 PAF), and m/z −536 → −59 (18:0 PAF).

HPLC separations were carried out on a Hewlett-Packard 1100 series instrument directly interfaced to the mass spectrometer and controlled by the MassLynx software. Normal-phase separations used a Thermo Hypersil-Keystone silica column, 2.1×150 mm, with 5 μm particle size. The normal-phase column was eluted isocratically with chloroform-methanol-water (60:55:5, v/v; solvent A) or with methylene chloride-methanol-water (120:60:9, v/v; solvent B) at a flow rate of 300–400 μl/min. A splitting tee directed 5% or 13% of the column effluent to the electrospray source. Reversed-phase separations used a Supelco Discovery Bio-Wide Pore C_{18} column, 1×50 mm, with 3 μm particle size. The reversed-phase column was eluted at 100 μl/min with a gradient of 100% solvent C to 100% solvent D over 5 min; hold at 100% D for 5 min; return to 100% C over 5 min; and hold at 100% C for at least 15 min to regenerate the column. Methanol-water-acetonitrile (57:23:20, v/v) containing 1 mM ammonium acetate was solvent C, and methanol containing 1 mM ammonium acetate was solvent D. A splitting tee directed 50% of the column effluent to the electrospray source for the first 15 min of each run; thereafter, the effluent was diverted to waste to avoid introducing large amounts of sphingomyelin into the source.

Catalytic hydrogenation

The d_3-16:0 PAF internal standard was omitted from these experiments to avoid possible artifacts caused by deuterium exchange during the hydrogenation reaction. Lipids were extracted (38) from A23187-stimulated PMNs and separated by normal-phase LC-MS/MS using solvent B. The fraction containing 16:0, 18:1, and 18:0 PAF (eluting between 6 and 9 min) was evaporated under a stream of nitrogen and dissolved in 200 μl of solvent C. Aliquots (10 μl) of this sample were analyzed by reversed-phase LC-MS/MS before and after catalytic hydrogenation. Peak areas of 18:1 PAF, the unknown peak, and 18:0 PAF were normalized to the area of the 16:0 PAF peak. For hydrogenation, a small amount of black platinum was added to the sample, and hydrogen gas was gently bubbled through the sample at ambient temperature and pressure for 1 h.

RESULTS AND DISCUSSION

Interference from PFPC

Our initial attempts to quantitate PAF by LC-MS/MS used the method of Savu et al. (33). This method takes ad-

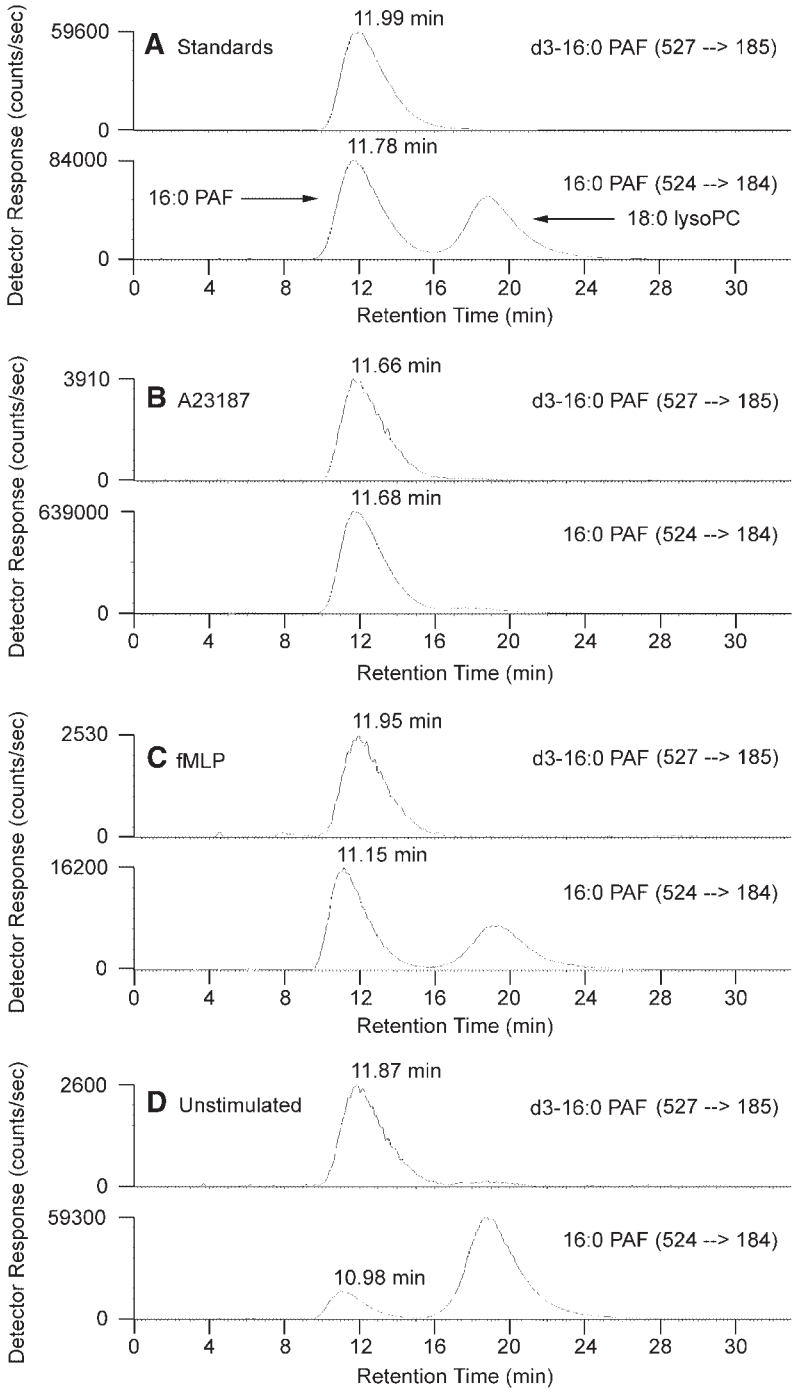

Fig. 1. Stimulus-dependent discrepancy in HPLC retention time of platelet-activating factor (PAF). A: 1-*O*-Hexadecyl-2-[$^2H_3$]acetyl-glycerophosphocholine (d_3-16:0 PAF) and unlabeled 16:0 PAF (10 ng each) were coinjected with total lipid extract from 1×10^7 unstimulated polymorphonuclear neutrophils (PMNs) on normal-phase HPLC-tandem mass spectrometry (LC-MS/MS). lysoPC, lysophosphatidylcholine. Other panels show LC-MS/MS chromatograms of total lipid extracts from 1×10^7 PMNs stimulated with 10 μM A23187 for 10 min (B), 1 μM *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) for 2 min (C), or no stimulus (D). PMN extracts contained 400 pg of d_3-16:0 PAF internal standard. In all panels, the column was eluted isocratically with chloroform-methanol-water (60:55:5, v/v) at a rate of 400 μl/min, and d_3-16:0 PAF and unlabeled 16:0 PAF were monitored as the indicated transitions in positive-ion mode. Chromatograms were modified by two rounds of Savitzky-Golay smoothing using a window of five scans, followed by first-order background subtraction to 40% below baseline. Peak retention times shown were calculated by the instrument software.

vantage of the fact that PAF, as a choline-containing phospholipid, yields abundant positive ions that efficiently fragment in collision-induced dissociation to give the characteristic product ion at m/z 184 (e.g., m/z 524 → 184 for 16:0 PAF). Trideuterated PAF internal standard, labeled on the acetate moiety (d_3-16:0 PAF), gives a singly deuterated product ion at m/z 185 (e.g., m/z 527 → 185 for d_3-16:0 PAF) (40) by a fragmentation mechanism recently elucidated (41). When d_3-16:0 PAF and unlabeled 16:0 PAF standards were coinjected on normal-phase LC-MS/MS with PMN lipid extract, the m/z 524 → 184 channel gave two peaks (**Fig. 1A**). The earlier peak was identified as the 16:0 PAF standard, based on its coelution with the d_3-16:0 PAF peak seen in the m/z 527 → 185 channel. The later eluting m/z 524 → 184 peak was identified as 18:0 lysophosphatidylcholine (lysoPC), which is present in the cell extracts, based on its coelution with authentic 18:0 lysoPC in separate experiments. Lipid extracts of PMN treated with the calcium ionophore A23187, a very robust stimulus for PAF biosynthesis in PMNs, contained only one major peak in the m/z 524 → 184 channel, which again coeluted with d_3-16:0 PAF (Fig. 1B). Surprisingly, however, when PMNs were unstimulated (Fig. 1D) or were stimulated with a more physiological, receptor-dependent agonist (chemotactic peptide fMLP; Fig. 1C), the earlier of the two m/z 524 → 184 peaks did not coelute exactly with d_3-16:0 PAF but instead eluted nearly 1 min earlier. These reproducible, stimulus-dependent discrepancies in retention time prompted us to investigate whether the earlier m/z 524 → 184 peak might contain compounds other than the expected 16:0 PAF.

Harrison, Clay, and Murphy (34) have noted the occurrence of PFPC in PMN extracts. This species is isobaric with 16:0 PAF in positive-ion MS/MS. However, 16:0 PAF and PFPC can be discriminated using negative-ion MS/MS, because they give product ions corresponding to their different acyl substituents (34). Specifically, both lipids give a $[M - CH_3]^-$ precursor ion at m/z −508, but 16:0 PAF gives the acetate product ion at m/z −59, whereas PFPC gives both the palmitate and formate product ions at m/z −255 and −45, respectively. When lipids from unstimulated PMN were analyzed by negative-ion LC-MS/MS, the material eluting just before d_3-16:0 PAF was confirmed to be PFPC, whereas the small amount of actual 16:0 PAF in the cells, barely detectable under these conditions, coeluted with d_3-16:0 PAF (**Fig. 2**). This system eliminated the problem of interference from PFPC but was found not suitable for routine use because of a lack of sensitivity. Indeed, the level of sensitivity shown in Fig. 2 could not be consistently attained in later experiments, and detection of PAF in negative-ion mode was typically 2 orders of magnitude less sensitive than in positive-ion mode.

No metabolic pathway leading to the biosynthesis of PFPC has been described; thus, it is possible that PFPC is not a natural product but an artifact of the extraction, storage, or processing of lipid samples. For example, formyl chloride is an intermediate in the breakdown of chloroform to phosgene. If our solvents contained traces

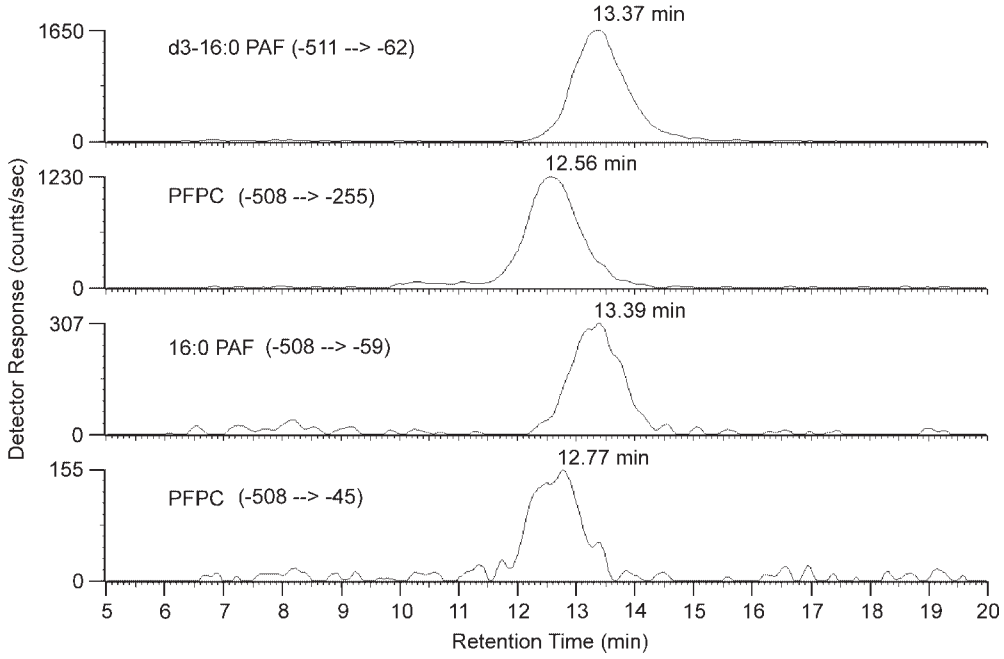

Fig. 2. Identification of palmitoyl-formyl-glycerophosphocholine (PFPC) by negative-ion multiple reaction monitoring (MRM). Total lipid extract from 3.5×10^7 unstimulated PMNs, containing 1.25 ng of d_3-16:0 PAF internal standard, was analyzed by normal-phase LC-MS/MS. The column was eluted isocratically with chloroform-methanol-water (60:55:5, v/v) at a rate of 300 μl/min, and d_3-16:0 PAF, unlabeled 16:0 PAF, and PFPC were monitored as the indicated transitions in negative-ion mode. The chromatogram was modified by two rounds of Savitzky-Golay smoothing using a window of seven scans, followed by first-order background subtraction to 40% below baseline. Peak retention times shown were calculated by the instrument software.

of formyl chloride or formic acid, some of the palmitoyl-lysoPC in samples could be formylated, yielding PFPC. Alternatively, if the PFPC we observe is the 1-formyl-2-palmitoyl-GPC isomer, it may be an oxidation product of PC plasmalogens containing palmitate at the *sn*-2 position, because the *sn*-1 vinyl ether bond can be oxidatively cleaved, yielding an *sn*-1 formyl group (42). We therefore investigated several approaches aimed at preventing the in vitro formation of PFPC in samples. Addition of formic acid (5%) to the water used in lipid extraction increased the amount of PFPC detected, but no treatment decreased PFPC levels below those seen using our usual lipid extraction and storage procedures (data not shown). Treatments tested included *1*) omission of acetic acid from the lipid extraction; *2*) use of newly purchased methylene chloride in place of chloroform for both lipid extraction and HPLC; *3*) addition of isopropyl alcohol to samples to titrate possible acylating species; *4*) analysis of samples immediately after extraction, with no storage time; *5*) flushing the headspace of sample vials with argon before storage, followed by use of argon instead of nitrogen to evaporate samples; and *6*) addition of EDTA or the antioxidants butylated hydroxytoluene or dimethyl sulfide, or combinations thereof, to the solvents used for lipid extraction. In these experiments, it was necessary to compare the effects of different treatments on the same PMN preparation, because PMNs from different blood donors appeared to contain vastly different concentrations of PFPC. Along these lines, Harrison, Clay, and Murphy (34) found that PMNs from smokers contain more PFPC than do PMNs from nonsmokers. Those authors also detected PFPC in PMN samples extracted and processed in the absence of any chlorinated solvents, again suggesting that possible reactive contaminants in chlorinated solvents do not explain the occurrence of PFPC. In studies not shown here, we have also detected PFPC in the cultured breast cancer cell line MCF-7. We tentatively conclude that PFPC is a natural product. In any event, its presence in lipid extracts was not prevented by straightforward chemical treatments and was therefore dealt with chromatographically.

No isocratic or gradient solvent system investigated was able to sufficiently resolve PFPC from PAF on normal-phase HPLC, and PFPC was often 10–20 times as abundant as PAF in unstimulated PMNs. However, C_{18} reversed-phase HPLC easily separated the two lipids. **Figure 3** shows a negative-ion MRM chromatogram of a total lipid extract from fMLP-stimulated PMNs. Detection of PAF and other choline-containing phospholipids by negative-ion MRM was more sensitive in reversed-phase HPLC than in normal-phase HPLC, likely because of improved peak shape and other factors, but for a given HPLC regime, positive-ion MRM remained by far the more sensitive detection method. The PFPC channel in this chromatogram (m/z $-508 \rightarrow -45$) detects two peaks, but in related experiments not shown here, we found that only the first peak, eluting at 4.05 min, is PFPC, because only this peak yields the palmitate product ion (m/z -255) on collision-induced dissociation. The second peak was consistently observed in the m/z $-508 \rightarrow -45$ channel but remains unidentified.

Interference from lysoPC

Figure 4A shows a reversed-phase LC-MS/MS chromatogram of PMN lipid extract in the positive-ion mode. The 18:0 and 18:1 PAF peaks in the upper two traces were identified by the retention times of authentic standards and confirmed by the presence of the acetate product ion (m/z -59) on the negative-ion MRM (data not shown). The double peaks for 16:0 PAF and d_3-16:0 PAF were commonly seen in this type of analysis and may reflect column overloading. To validate this method as a PAF assay, we measured PAF in aliquots of the same samples using both positive-ion and negative-ion detection (data not shown). Positive-ion MRM often detected significantly more "PAF" than did negative-ion MRM in the same sample, suggesting the presence of another positively interfering compound. In positive-ion MRM, 16:0 PAF is isobaric, not only with PFPC but also with a more abundant lipid, 18:0 lysoPC. Because one of the peaks in the 18:0 lysoPC channel (top trace) in Fig. 3 coeluted with 16:0 PAF, we investigated whether this peak may represent a form of 18:0 lysoPC. Lysophospholipids undergo acyl migration under conditions associated with lipid extraction and processing, such as exposure to polar solvents, acids, and silica, reaching an equilibrium that contains ~10% of the 1-lyso-2-acyl regio-

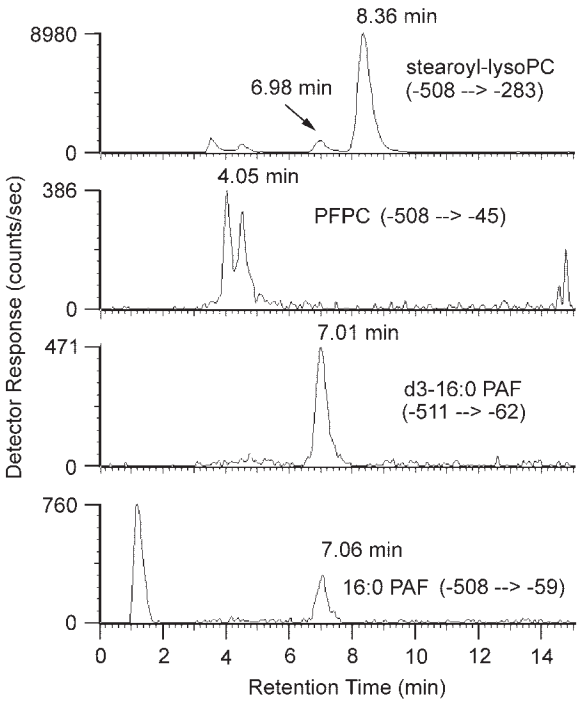

Fig. 3. Separation of PFPC from PAF by reversed-phase HPLC. Total lipid extract from 1×10^7 fMLP-stimulated PMNs, containing 400 pg of d_3-16:0 PAF internal standard, was analyzed by reversed-phase LC-MS/MS. The column was eluted using the gradient schedule described in Materials and Methods, and PFPC, d_3-16:0 PAF, unlabeled 16:0 PAF, and stearoyl-lysoPC were monitored as the indicated transitions in negative-ion mode. The chromatogram was modified by two rounds of Savitzky-Golay smoothing using a window of three scans, followed by first-order background subtraction to 40% below baseline. Peak retention times shown were calculated by the instrument software.

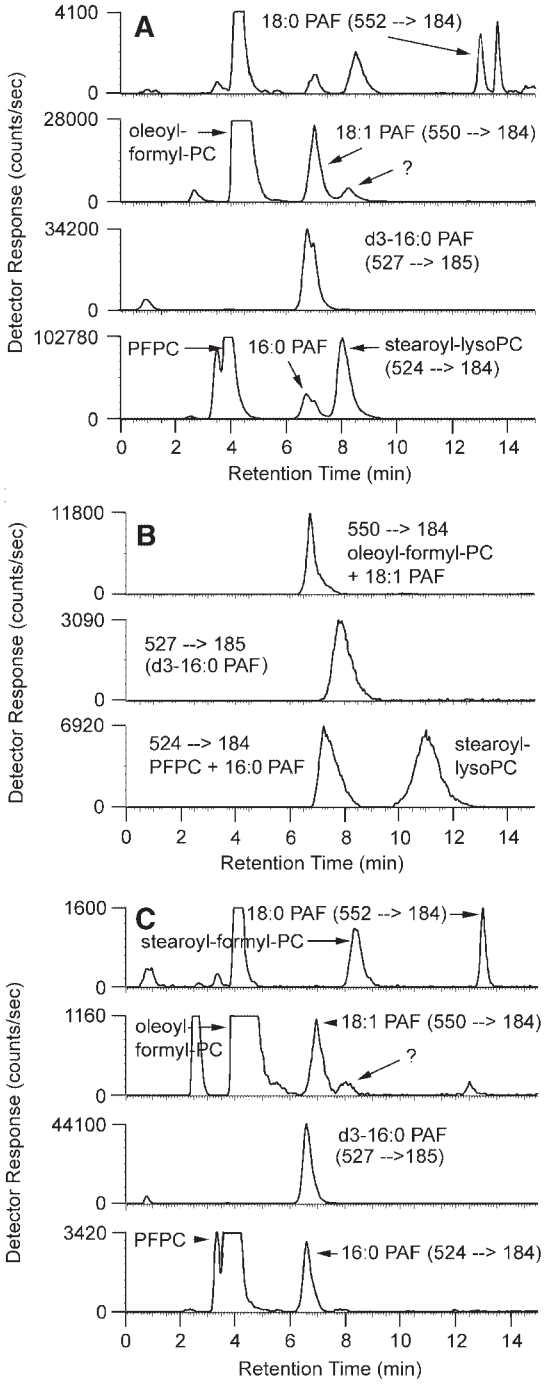

Fig. 4. Normal-phase HPLC sample cleanup removes interfering stearoyl-lysoPC. A: Total lipid extract from 1×10^7 unstimulated PMNs, containing 400 pg of d_3-16:0 PAF internal standard, was analyzed by reversed-phase LC-MS/MS. HPLC conditions were as described in Materials and Methods. B: The PAF-containing fraction from a lipid extract as in A was purified by normal-phase HPLC. The column was eluted isocratically with methylene chloride-methanol-water (120:60:9, v/v) at a flow rate of 300 μl/min. Approximately 5% of the effluent was diverted to the mass spectrometer for monitoring the elution of PAF and lysoPC in positive-ion mode. C: The PAF-containing fraction (6–9 min) was collected in a disposable borosilicate glass test tube, evaporated under a stream of nitrogen, and analyzed by reversed-phase LC-MS/MS in positive-ion mode. All chromatograms were modified by two rounds of Savitzky-Golay smoothing using a window of three scans, followed by first-order background subtraction to 40% below baseline.

isomer (37, 43, 44, 45). Reversed-phase HPLC, unlike normal-phase HPLC, separates the two regioisomers, with the 1-lyso-2-acyl isomer eluting first (37, 43, 44, 45). Preparation and analysis of a 1-lyso-2-stearoyl-GPC standard revealed that this lipid does indeed coelute with 16:0 PAF on reversed-phase HPLC under the conditions used here, causing significant positive interference (data not shown).

Final, optimized version of the assay

The use of normal-phase HPLC for sample cleanup enabled complete removal of interfering lysoPC. The mobile phase was optimized to allow baseline separation of the two lipids within a 15 min run time (Fig. 4B). Approximately 5% of the column effluent was diverted to the electrospray source to obtain this chromatogram. The remaining 95% of the effluent was collected between 6 and 9 min. This PAF fraction was then analyzed on reversed-phase LC-MS/MS (Fig. 4C). Note the absence of detectable lysoPC

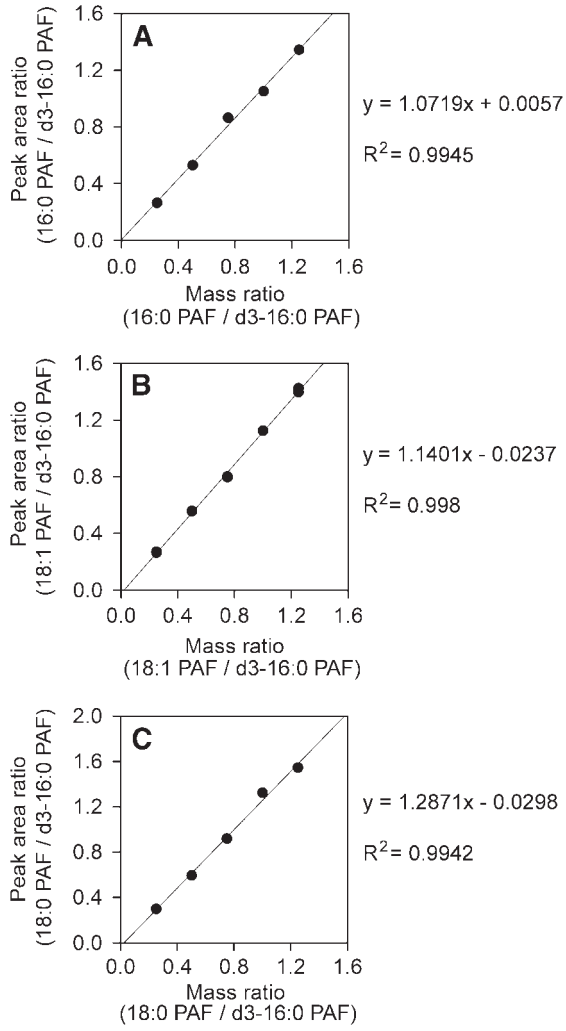

Fig. 5. Response curves for three common PAF molecular species. Deuterated PAF internal standard (400 pg) was coinjected with various amounts of unlabeled 16:0 PAF (A), 18:1 PAF (B), or 18:0 PAF (C) on reversed-phase LC-MS/MS. HPLC conditions were as described for Fig. 4. The ratio of PAF to d_3-16:0 PAF peak areas is plotted against the ratio of amounts injected. Results shown are from one experiment performed in duplicate.

from the purified sample (compare the *m/z* 524 → 184 channel in Fig. 4A, C). Lipid extracts purified as in Fig. 4B and analyzed as in Fig. 4C showed the same PAF levels whether positive-ion or negative-ion detection was used (data not shown). The sample cleanup procedure had the added advantage of improving the peak shape of 16:0 PAF and d_3-16:0 PAF, likely a benefit of reduced column loading. For cleanup of large batches of samples, only the first three to four samples are monitored by MRM to verify the stability of retention times. Thereafter, the mass spectrometer is placed in standby mode, and 100% of the column effluent is directed to an automatic fraction collector.

Fig. 4A, C are chromatograms from the same number of cell equivalents (1×10^7), yet much less PAF is detected in the purified sample shown in Fig. 4C. Specifically, when these PAF molecular species are quantified using the response factors from **Fig. 5**, the crude sample (Fig. 5A) appears to contain 614 pg of PAF, whereas only 41 pg of PAF is detected in the purified sample (Fig. 5C). This difference does not reflect the loss of PAF during the cleanup, because the d_3-16:0 PAF signal is actually slightly stronger in Fig. 5C, as a result of the improved sensitivity and peak shape obtained by injecting much less total lipid. Selective loss of unlabeled 16:0 PAF relative to d_3-16:0 PAF is also not likely, because the two compounds migrate identically on normal-phase HPLC (Fig. 1). Instead, the apparent loss of PAF upon sample purification reflects the observation, noted above, that most of the PAF signal seen in Fig. 5A is actually attributable to lysoPC. Recovery of PAF from the lipid extraction and normal-phase HPLC cleanup procedures, monitored by comparing the area of the d_3-16:0 PAF peak in sample chromatograms with injection of a known amount of d_3-16:0 PAF, was typically ~80% (data not shown).

Two putative novel formylated PC species

Under the reversed-phase HPLC conditions of Fig. 4A, C, PFPC elutes at 4.0 min and is detected in the 16:0 PAF channel. Note that the PFPC peak is much larger than the PAF peak and is shown truncated. When the full height of the PFPC peak is displayed, the PAF peak in Fig. 4C becomes invisible on the same vertical scale. Also shown in Fig. 4A, C, a large peak eluting at 4.3 min in the 18:1 PAF channel (positive ion *m/z* 550 → 184) was detected on negative-ion MRM as *m/z* −534 → −281 (oleate product ion) and as *m/z* −534 → −45 (formate product ion). These MS/MS characteristics, together with the peak's retention times on both normal-phase (Fig. 4B) and reversed-phase HPLC, strongly suggest that the peak is a novel formylated lipid, oleoyl-formyl-PC. Similarly, a major non-PAF peak in the 18:0 PAF channel in Fig. 4C, eluting at 8.4 min (and also visible in Fig. 4A), may represent another novel formylated lipid, stearoyl-formyl-PC, since this peak was also detected in negative-ion mode as *m/z* −536 → −283 (data not shown) and eluted several minutes before the isobaric PAF species on reversed-phase HPLC (Fig. 4A, C).

A putative novel isomer of 18:1 PAF

Isocratic elution of the reversed-phase HPLC column with various mixtures of methanol, water, and acetonitrile, supplemented with 1 mM ammonium acetate, were all able to resolve these formylated lipids from their respective isobaric PAF species, but gradient elution as described in Materials and Methods was used in all of the experiments shown here, for two reasons. Gradient elution was necessary to clean the column of the large amounts of sphingomyelins present in the PAF fractions purified by normal-phase HPLC. Gradient elution was also necessary for the resolution of 18:1 PAF from the unidentified peak eluting shortly afterward and marked in Fig. 4A, C with a question mark. This peak was not a signal "bleeding over" from a nearby *m/z* value, and negative-ion MRM experiments failed to find evidence for its being a formylated lipid or a lysoPC. The unidentified peak appeared to contain an acetate moiety, because it gave the characteristic *m/z* −59 product ion in negative-ion mode. Interestingly, PMNs stimulated with various agonists, including the calcium ionophore A23187, always produced this substance in the same proportion to 18:1 PAF, indicating that it is not merely a trace contaminant. The unidentified peak was not a plasmalogen, because it was not affected by exposure to HCl fumes under conditions in which an authentic standard PC plasmalogen was completely cleaved to the 1-lyso-2-acyl-GPC. These observations might suggest a 17:1 *sn*-1-acyl analog of PAF; however, negative-ion MRM showed no evidence of 17:1 (*m/z* −267) or 17:0 (*m/z* −269) fatty acid product ions in the peak. We tentatively conclude that the unknown peak is a novel isomer of 18:1 PAF containing a *trans* double bond and/or a double bond in a position other than the usual Δ^9 position. Consistent with this conclusion, when a PAF-containing HPLC fraction was subjected to catalytic hydrogenation, the amount of 18:0 PAF gained was equal to the sum of the amounts lost from 18:1 PAF and the unknown peak, under conditions in which loss of the latter two peaks was 95% complete (data not shown). This peak was also seen in lipid extracts of MCF-7 breast cancer cells.

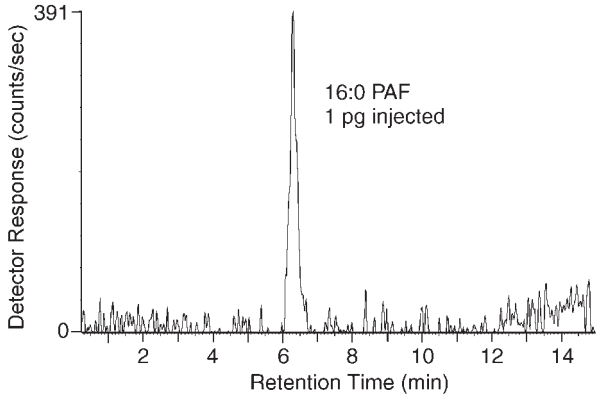

Fig. 6. MRM chromatogram of 1 pg (=1.91 fmol) of 16:0 PAF standard. One picogram of 16:0 PAF standard was injected on reversed-phase LC-MS/MS. HPLC conditions were as described for Fig. 4. The vertical axis shows the transition *m/z* 524 → 184. The chromatogram was modified by two rounds of Savitzky-Golay smoothing using a window of three scans, followed by first-order background subtraction to 40% below baseline. A signal-to-noise ratio of 6 was estimated from this chromatogram before background subtraction.

The alkyl moieties of ether lipids, including PAF, are derived from fatty alcohols, which are in turn derived from fatty acids. Besides oleic acid (18:1-*cis*-Δ^9), two 18:1 fatty acids found in dietary sources are vaccenic acid (*trans*-Δ^{11}) from beef and butterfat and petroselinic acid (*cis*-Δ^6) from certain vegetables and herbs (46). Margarine and shortening may also contribute various 18:1 fatty acids to the diet. Attempts to locate the double bond in the unknown peak using oxidative cleavage by $KMnO_4$ or reductive ozonolysis were unsuccessful because of a lack of sufficient pure material.

Response curves and limit of detection

Response curves (Fig. 5), generated by coinjecting 400 pg of d_3-16:0 PAF with various amounts of 16:0, 18:1, or 18:0 PAF using the LC-MS/MS conditions of Fig. 4C, were linear up to 1 ng of PAF injected. The relatively steep slope of the 18:0 PAF response curve likely reflects the fact that 18:0 PAF elutes at a later point in the gradient, and with a narrower peak shape, than the other PAF molecular species studied. Experiments with 16:0 PAF found the response was also linear down to 1 pg of PAF injected using the same amount of internal standard, for a dynamic range covering 3 orders of magnitude. One picogram (=1.91 fmol) of unlabeled 16:0 PAF standard injected on LC-MS/MS as in Fig. 4C was detected with a signal-to-noise ratio of ~6 (**Fig. 6**).

CONCLUSIONS

We describe an improved LC-MS/MS assay for PAF that shows superior sensitivity and specificity compared with previously published LC-MS and LC-MS/MS assays. Harrison, Clay, and Murphy (34) developed a PAF assay using negative-ion MRM; this assay is not susceptible to the interferences discussed here and was therefore extremely useful to us in the development and verification of the present method. Harrison, Clay, and Murphy (34) demonstrated detection of 500 pg of PAF with a good signal-to-noise ratio. It also should be noted that a more recent report by those authors achieves better sensitivity, on the order of a few picograms (47). However, we sought to develop a more sensitive method, because in our hands negative-ion detection of PAF was insufficiently sensitive for many biological applications, even in the presence of ammonium acetate, an additive reported to enhance the negative ionization of choline-containing phospholipids (48).

PAF, like all choline phospholipids, bears a permanent positive charge on the quaternary ammonium of its polar head group and therefore lends itself well to sensitive detection in positive-ion electrospray. Several positive-ion LC-MS and LC-MS/MS assays for PAF have been described, using either normal-phase or reversed-phase HPLC (30–33, 49). All of these positive-ion methods are susceptible to interference from isobaric compounds. Specifically, reversed-phase HPLC fails to resolve PAF from lysoPC, and normal-phase HPLC fails to resolve PAF from PFPC and related formylated lipids. Importantly, we find that these isobaric compounds may be present in amounts that dwarf the amount of actual PAF, particularly in unstimulated samples. Published reports using such methodologies must therefore be interpreted with caution. For example, Savu et al. (33), using normal-phase LC-MS/MS in positive-ion mode, found that human umbilical vein endothelial cells produce nanograms of PAF but undetectable amounts of the relatively inactive 1-acyl analog known as acyl-PAF. This result may be attributable to PFPC interference, because others, using an unrelated PAF assay, found these cells to produce large amounts of acyl-PAF but little PAF (50). On the other hand, Oda, Mano, and Asakawa (49) used reversed-phase LC-MS, which is susceptible to interference not from PFPC but from lysoPC. Such interference may explain the high levels of PAF reported by those authors in resting PMNs, more than 100-fold more PAF per cell than we detect here. The better methods using reversed-phase LC-MS or LC-MS/MS are those that use thin-layer chromatography or silica solid-phase extraction for sample cleanup, thus removing some of the interfering lysoPC (30–32). However, these methods may also be inadequate for samples containing low amounts of PAF, because lysoPC and PAF migrate close together on thin-layer chromatography. Thin-layer chromatography can also introduce significant losses when small samples are used. Our use of normal-phase HPLC for sample cleanup, followed by reversed-phase LC-MS/MS, capitalizes on the different strengths of both types of HPLC to eliminate interfering compounds and also gives better sensitivity than previous methods.

PFPC and the other formate-containing PC lipids described here appear to be natural products of unknown metabolic origin. These lipids are clearly of interest to investigators seeking to quantitate PAF and related compounds, because they can be a troublesome source of interference. However, formate-containing lipids may have biological significance as well. It is unknown whether these lipids, particularly at the relatively high concentrations observed here, might activate or antagonize the PAF receptor, lysophosphatidylcholine receptors, members of the Toll-like receptor family, or other lipid receptors. Harrison, Clay, and Murphy (34) found higher concentrations of PFPC in the blood of smokers than nonsmokers. Moreover, formate-containing lipids are among the products of plasmalogen oxidation (43). Together, these observations raise the possibility that formylated lipids are produced nonenzymatically in vivo under oxidative conditions and may thus serve as a marker or signal of oxidative stress. Alternatively, if there is an enzyme system that formylates lysophospholipids in vivo, this raises the question of whether formate-containing lipids may play some role in homeostasis. In either case (and the two scenarios are not mutually exclusive), there is much to be learned about this unusual family of lipids.

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