Quantitation of lyso-platelet activating factor molecular species from human neutrophils by mass spectrometry

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Abstract Two physicochemical methods have been developed for the quantitative analysis of lyso-platelet activating factor (lyso-PAF) based on gas-liquid chromatography-mass spectrometry (GLC/MS) and fast atom bombardment-mass spectrometry (FAB/MS) using stable isotope dilution. After addition of deuterated internal standards, lyso-PAF produced from neutrophils was purified by silicic acid chromatography and thin-layer chromatography (TLC). The GLC/MS assay employed phospholipase C or hydrofluoric acid for hydrolysis of the phosphocholine moiety to yield ether monoglycerides. Condensation of monoglycerides with acetone yielded the 1-O-alkyl-2,3-isopropylidene glycerol which could be analyzed by GLC/MS. The ions corresponding to M-15 fragments for both the labeled and unlabeled derivatives were monitored in a selected ion recording mode. Standard curves were found to be linear over the range tested (10–2000 ng) with a limit of detection found to be below 200 pg injected on column. For the FAB/MS assay, the unmodified lyso-PAF was well suited for direct analysis; however, the limit of detection (S/N > 3) using a glycerol matrix was found to be 5 ng placed on the probe tip. It was found that human neutrophils contain approximately 300 pg/10^6 cells which increased 2–3-fold during the 5-min period following challenge with 1.9 μM calcium ionophore, A23187. Two molecular species of lyso-PAF were identified as hexadecyl and octadecyl ethers at *sn*-1 with the octadecyl molecular species of lyso-PAF predominating in abundance after stimulation. — **Haroldsen, P. E., K. L. Clay, and R. C. Murphy.** Quantitation of lyso-platelet activating factor molecular species from human neutrophils by mass spectrometry. *J. Lipid Res.* 1987. **28:** 42–49.

Supplementary key words lyso-PAF • mass spectrometry • PAF • fast atom bombardment • GLC/MS • deuterium-labeled PAF

Lyso-platelet activating factor (1-O-alkyl-*sn*-glycero-3-phosphocholine) is a unique phospholipid in that it serves as both the direct precursor (1) as well as an initial metabolite (2) of the biologically active molecule PAF (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine). While lyso-PAF is essentially a biologically inactive molecule, except for the cytolytic properties it can display at high concentrations (3), interest in the molecule has followed recognition of the important biological properties that PAF plays as a lipid mediator of anaphylaxis and inflammation (4, 5). PAF has profound properties inducing rabbit platelet aggregation as well as release of stored mediators such as serotonin (6). Furthermore, PAF is considered to be a potent hypotensive agent (7) but in certain vascular beds, such as the pulmonary circulation, it can cause an increase in arterial pressure (8). Evidence suggests that this molecule is synthesized from lyso-PAF in cells such as the neutrophil, eosinophil, macrophage, and mast cells following activation of the acetyl transferase, possibly through phosphorylation of the inactive enzyme (9). Once formed, PAF is known to be rapidly deacetylated by plasma and tissue esterases leading to the formation of the inactive lyso-PAF (10). Hence, lyso-PAF can be considered to be both a metabolite and precursor of PAF.

Since lyso-PAF lacks innate biological activity as well as structural moieties that can be readily detected, for example, an ultraviolet light-absorbing chromophore, methods to detect this molecule have been based upon indirect techniques. Lyso-PAF can be acetylated to form PAF, which can then be detected by the release of labeled serotonin or by platelet aggregation (11). Alternatively, lyso-PAF can be acetylated with a radiolabeled acetyl group of known specific activity, and radioactivity content can then be determined following purification as the means of quantitation (12).

This report describes methods to measure the levels of lyso-PAF by gas-liquid chromatography/mass spectrom-

Abbreviations: The nomenclature used to designate the molecular species of lyso lecithins and lyso-PAF (e.g., 16:0e) indicates the total carbon chain at *sn*-1:the degrees of unsaturation in the radyl group at *sn*-1 and whether or not the radyl group at *sn*-1 is acyl (a) or ether (e) linkage. Phospholipid classes are abbreviated as follows: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine. GLC/MS, gas-liquid chromatography-mass spectrometry; PAF, platelet activating factor; FAB, fast atom bombardment; TLC, thin-layer chromatography.

etry (GLC/MS) and fast atom bombardment/mass spectrometry (FAB/MS). These methods were used to study the stimulation of the human neutrophil with the calcium ionophore A23187, which is thought to activate the calcium-dependent phospholipase A_2 and release lyso-PAF from phospholipid precursors as well as release lyso-PAF following deacetylation of PAF. Under conditions of this stimulation, human neutrophils have been shown to produce leukotriene B_4 (13), prostaglandin E_2 (14), and PAF (4); however, the concentrations of lyso-PAF, as well as its molecular species content during formation of these inflammatory lipid mediators, have not been measured.

MATERIALS AND METHODS

Materials

HPLC grade solvents obtained from commercial sources were used. TLC plates, silica gel GHL (250 μm), were obtained from Analtech. Calcium ionophore A23187 was obtained from Calbiochem and was dissolved in DMSO at a concentration of 1 mM. [^{14}C]Phosphatidylcholine (1-palmitoyl-2-[^{14}C]arachidonyl-PC, 55 mCi/mmol) was obtained from New England Nuclear. Silicic acid (CC4, 100–200 mesh) was obtained from Mallinckrodt. Lyso-PAF standards, hexadecyl (16:0e lyso-PAF) and octadecyl (18:0e lyso-PAF), were obtained as lyophilized powders from Sigma and Avanti Polar Lipids, respectively. 1-O-(9Z)-Octadecenyl-2-acetyl-*sn*-glycero-3-phosphocholine (Bachem) was hydrolyzed to 18:1e lyso-PAF using 0.4 N KOH at room temperature for 30 min. It was then purified by TLC as described below.

Cell preparation and incubation

Human neutrophils were isolated from freshly drawn adult venous blood by density gradient centrifugation on plasma-Percoll and then re-suspended in Krebs Ringer phosphate buffer at 10^7 cells/ml (15). Stimulation of cell suspensions at 37°C was initiated by addition of calcium ionophore A23187 to a final concentration 1.9 μM; in control cells an equal volume of DMSO (10 μl) was added. The stimulation period from 10–300 sec was terminated by the addition of two volumes of methanol which was rapidly cooled to −77°C. During this time, stable isotopically labeled lyso-PAF was added to the methanolic suspension as follows. For the FAB experiments, 50 ng of internal standard (label incorporated in the choline methyl position, see below) was added, while for the GLC/MS experiment, an alkyl-chain-labeled internal standard mixture was added which consisted of 5 ng of deuterated 18:0e lyso-PAF and 25 ng of deuterated 16:0e lyso-PAF in a fixed ratio (see below). In all experiments [^{14}C]phosphatidylcholine (1.6 nmol, 200,000 dpm) was added to ascertain the amount of hydrolysis of acyl phospholipids during the workup.

Isolation of lysophosphatidylcholine

Chloroform and additional methanol were added to the methanolic suspension in order to extract lipids by the method of Bligh and Dyer (16). The lipid extract was then fractionated on a short silicic acid column (1 g) and eluted sequentially with 3-column volumes of 25% methanol in chloroform (v/v) which removed neutral lipids and some PE; and 50% methanol in chloroform (v/v) which eluted PI and PS and the remaining PE; then with 3-column volumes of methanol and 4-column volumes of 30% water in methanol (v/v). These latter two fractions elute PAF, lyso-PAF, sphingomyelin, and PC. Initial fractions containing 25% and 50% methanol in chloroform were analyzed for radiolabeled free fatty acid content by TLC to assess acyl phospholipid hydrolysis. The 100% methanol and 30% water in methanol were combined, and concentrated in vacuo, and the residue was subjected to TLC on silica gel GHL plates with chloroform–methanol–acetic acid–water 50:30:8:5.0 which separated PAF from lyso-PAF by over 1 cm. The area of the TLC plate corresponding to the R_f of lyso-PAF was scraped and extracted three times with 1 ml of 10% water in methanol (v/v) followed by a Bligh-Dyer extraction to remove silicic acid. Samples were analyzed directly by FAB/MS or taken to dryness for hydrolysis prior to GLC/MS analysis.

Derivatization of lyso-PAF

For GLC/MS, the appropriate TLC fraction was treated with 1.0 ml of 0.2 M KOH in methanol at room temperature for 1 hr then treated with 0.3 ml of 50% hydrofluoric acid (29 M) for 4 hr at 25°C in 1.5-ml polypropylene centrifuge tubes to hydrolyze the phosphocholine group (17). The base hydrolysis was employed to degrade 1-acyl-lyso-phosphatidylcholine to glycerophosphocholine and fatty acid. Prior experiments indicated that no PAF or ether, acyl precursors were contained in this TLC fraction which would yield lyso-PAF by this hydrolysis step. However, this step did remove lysolecithin contamination. Following HF hydrolysis, 0.6 ml of $CHCl_3$–CH_3OH 90:10 was added and the solution was vortexed. The upper layer containing HF was removed by aspiration followed by washing with 0.3 ml of water. The organic phase was evaporated under N_2 and the isopropylidene derivatives were prepared essentially following the method of O'Donnell et al. (18). Briefly, the corresponding 1-O-alkyl-glycerols were treated at room temperature with 0.1 ml of 2,2-dimethoxypropane (Aldrich) and 0.1 ml of acetone containing 0.75% (v/v) concentrated HCl. Thirty minutes later the samples were dried by a stream of N_2 and suspended in 2,2-dimethoxypropane.

Determination of 1-O-octadecenyl-2-lyso-GPC stability to HF

Samples consisting of 9.5 ng of trideutero-1-O-octadecyl-2-lyso-GPC and varying amounts of 1-O-octadecenyl-2-

lyso-GPC were subjected to HF or phospholipase C hydrolysis. Briefly, 1 ml of pH 7.6 Tris-HCl buffer, 45 units of phospholipase C (*Bacillus cereus* Type XIII, Sigma), and 2 ml of diethyl ether were shaken with the sample at room temperature for 2 hr. The ether layer was removed and the sample was extracted with an additional 1 ml of diethyl ether. The ether extracts were dried under nitrogen and lyophilized to remove traces of water. The monoglycerides were resuspended in 0.25 ml of ether–hexane 1:9 and poured onto a 500-mg silicic acid column in a Pasteur pipette plugged with glass wool. The column was washed with 5 ml each of 10% and 20% ether in hexane. The monoglycerides were eluted with 3 ml of 100% ether followed by 2 ml of 10% methanol in $CHCl_3$. Extracts were derivatized as described above. In separate experiments, the monoglyceride fraction from the phospholipase C treatment was divided in half, with one half of the matched pair treated an additional 4 hr with HF (29 M) prior to derivatization. The ratios of ions for the 18:0e and 18:1e monoglyceride isopropylidenes were compared.

Mass spectrometry

FAB/MS. FAB/MS was performed with a VG micromass 7070E mass spectrometer with commercial saddle-field FAB gun operated at 5–6 KV (1 ma). Glycerol was used as the target matrix and xenon used as particle source. All analyses were carried out with the ion source at ambient temperature and 6 KV acceleration voltage. A Tracor Northern TN-1710 multichannel analyzer was used to acquire data over a range from m/z 400 to 550 amu during 50 scans (19). The detection limit of lyso-PAF by the FAB/MS method was estimated to be 5.0 ng added to the glycerol matrix which resulted in a signal-to-noise (s/n) ratio of 3. The detection limit was also estimated by the regression line of the 95% confidence limit of the standard curve and found to be 8 ng placed on the target surface.

GLC/MS. GLC/MS analysis was performed with either a Nermag 10-10 C quadrupole mass spectrometer or a VG Micromass 16 magnetic sector mass spectrometer, coupled to a capillary GLC and operated in the positive electron impact mode (70 eV). A portion of the isopropylidene derivatized samples was applied to the tip of a dry injector and injected onto a 30-meter DB-1 capillary column (0.25 mm, J & W Scientific) held at 250°C with an injector temperature of 275°C and helium as carrier gas. Using accelerating voltage scanning, the abundance of selected ions at m/z 341 and 344, 367, 369 and 372 was recorded to measure various molecular species of the isopropylidene ethers and their deuterated internal standards. The quadrupole mass spectrometer permitted the measurement of m/z 101 in addition to the above ions by selected ion recording.

Internal standard synthesis

The internal standards used for both GLC/MS and FAB/MS were derived from beef heart plasmalogen. Fresh heart was homogenized in cold saline and subjected to lipid extraction by the method of Bligh and Dyer (16). Approximately 25 mg of the resulting lipid extract was fractionated into separate phospholipid classes by silicic acid chromatography, and the phosphotidylethanolamine (PE) and phosphatidylcholine (PC) fractions were saved. Both PC and PE were separately subjected to mild (0.2 N KOH) base hydrolysis producing the respective lyso-ether phospholipids. The unsaturated fatty ether groups in lyso-PC were reduced with PtO_2 (10 mg) in CH_3OD using deuterium gas (D_2) at 1 atm. The Adam's catalyst was activated with D_2 for 5 min prior to use. The resulting 16:0e (approximately 75%) and 18:0e (approximately 25%) lyso-PAF molecular species were found to contain deuterium in the following atom %: 16:0e: D_0 (5.8%), D_1 (8.8%), D_2 (48.1%), D_3 (26.2%), D_4 (7.8%), D_5 (1.7%), D_6 (1.1%) and D_7 (0.4%); and 18:0e: D_1 (6.3%), D_2 (30.8%), D_3 (20.1%), D_4 (12.3%), D_5 (8.9%), D_6 (5.7%), D_7 (4.0%), D_8 (2.3%), D_9 (1.9%), D_{10} (1.2%) and D_{11} (0.7%). This lyso-PAF mixture was used for the GLC/MS assay.

The internal standard used for the FAB/MS assay was synthesized from the lyso-PE fraction. The lyso-PE (1.5 mg) was reduced with H_2 gas and PtO_2 (5–10 mg) and subjected to exhaustive methylation (20) on the amine moiety using CD_3I in the presence of catalytic amounts of KOH. The resulting 16:0e and 18:0e lyso-PAF molecular species were found to contain deuterium in the following atom % excess: 16:0e: D_0 (10%), D_9 (90%); 18:0e: D_0 (10%), D_9 (90%).

RESULTS

GLC/MS quantitation of lyso-PAF

Gas–liquid chromatography of native, intact lysophospholipids has not been possible due to the presence of the polar head group contained within the molecule. Removal of this polar head group by enzymatic or chemical means followed by derivatization to the 2,3-isopropylidene glycerol, renders these molecules amenable to GLC (18). The mass spectral behavior of these compounds has been previously described (21). Briefly, there are two major ions formed in the fragmentation of these molecules: a low mass oxonium ion, m/z = 101, formed by cleavage between the *sn*-1 and *sn*-2 carbons of the glycerol backbone characteristic to all molecular species (alpha cleavage); and the second major ion, at higher mass, arising from the loss of a methyl group from the molecular ion. These ions occur at m/z 341, 367, and 369 for the 16:0e, 18:1e, and 18:0e monoglyceride derivatives, respectively. The

abundance of these ions in a sample of isopropylidene derivatives derived from lyso-PAF isolated from human neutrophils stimulated by A23187 and analyzed by GLC/MS is shown in **Fig. 1.** As can be seen, m/z 101 indicates several components eluting from the gas chromatograph between 2 and 10 min. However, only those components at 7.0 and 8.3 min had the specific M-15 ions at m/z 341 and 369, indicating that the human neutrophil produced two major molecular species of lyso-PAF, 16:0e and 18:0e, respectively. The components at 4.2 and 5.5 min had retention times and full mass spectra consistent with methyl esters of palmitic acid and stearic acid, respectively. The fatty acid methyl esters undoubtedly arose during workup following methanolysis of acyl-lyso-PC which co-chromatographs with lyso-PAF in the TLC separation. Considering the fact that m/z 101 carries only a minor portion of the total ion current in the electron impact mass spectra of fatty acid methyl esters, one could estimate that these fatty acyl contaminants were present in a much higher concentration than the total molecular species of lyso-PAF. However, since a careful measurement of the ionization cross section of these two molecules and a relative formation of m/z 101 has not been made, a more quantitative assessment of the relative amounts of the fatty acid methyl esters and the isopropylidene derivatives of lyso-PAF cannot be made.

Using the abundance of the M-15 ions and deuterium-labeled internal standards, a quantitative assay for lyso-PAF was developed and standard curves were found to be linear over the range tested, 10–2000 ng of lyso-PAF, with correlation coefficients ≥ 0.990. However, for the purposes of the quantitative assay in studies of human neutrophils, the range of lyso-PAF concentrations used were between 10–60 ng (see Fig. 2). The limit of detection of lyso-PAF by the GLC/MS method was estimated by two different ways. The first method estimated a detection limit based on the observed signal-to-noise (S/N) for the M-15 ion; a signal-to-noise greater than 3 was observed when 200 pg of lyso-PAF derivative was injected on column. Another method to estimate detection limit of this assay involved the statistical analysis of the regression line shown in **Fig. 2.** Using the 95% confidence limits for the various values obtained at each point in the calibration curve, the lower limit of detection could be estimated as that point where the projected 95% confidence line intersected the value corresponding to the zero level of lyso-PAF. This limit was estimated to be 250 picograms. It should be noted that the limits of detection suggested above are primarily determined by the absolute amount of internal standard (and its unlabeled component) which determines the amplifier maximum gain in low level analysis. At no time was the sensitivity of the mass spectrometer limiting in measuring even the zero level of the calibration line in Fig. 2. Thus, one might be able to reduce the detection limits by employing less internal standard.

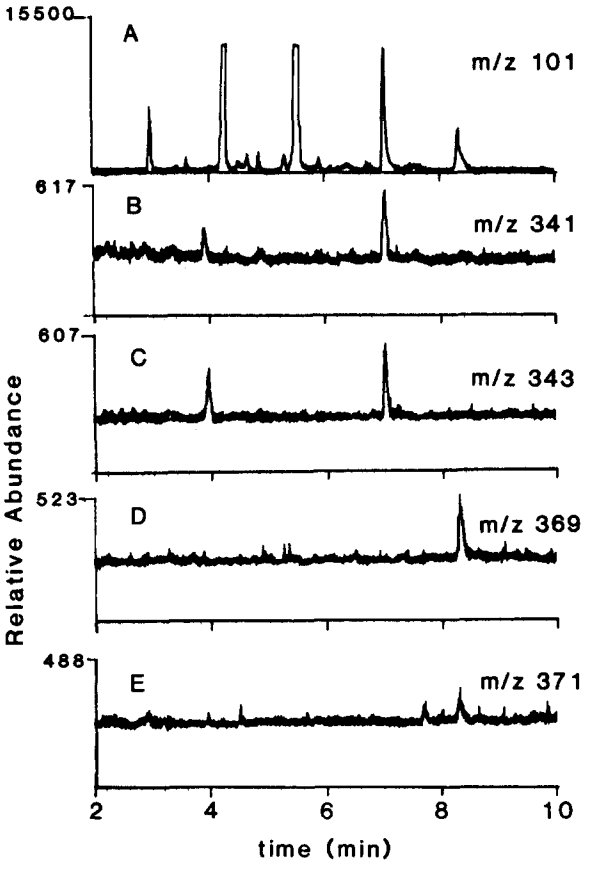

Fig. 1. Selected ion chromatograms for the isopropylidene derivatives of lyso-PAF molecular species derived from human neutrophils and those corresponding to the deuterium-labeled internal standard (16:0 and 18:0 D_3-lyso-PAF); A, m/z 101 corresponds to the alpha-cleavage ion of the isopropylidene derivatives of all lyso-PAF molecular species as well as an ion observed in the mass spectrum of fatty acid methyl esters; B, m/z 341 M-15 derived from 16:0e lyso-PAF; C, m/z 343 (M-15) derived from the deuterium-labeled internal standard for 16:0e lyso-PAF; D, m/z 369 (M-15) derived from 18:0e lyso-PAF; E, m/z 371 (M-15) derived from the deuterium-labeled internal standard for 18:0e lyso-PAF.

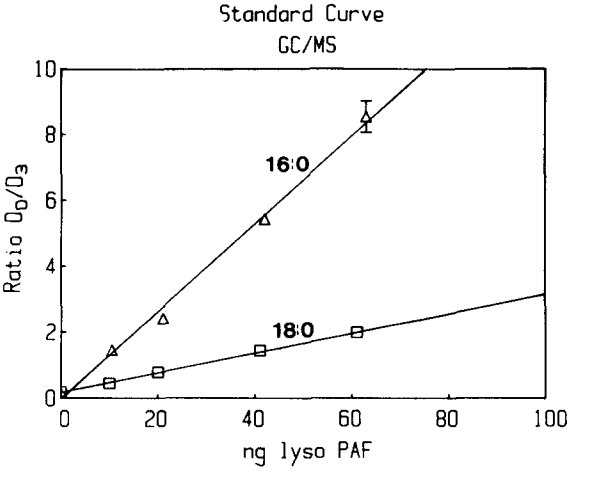

Fig. 2. Standard curves used for quantitation of 16:0e and 18:0e lyso-PAF molecular species by the GLC/MS procedure using 5 ng of D_3 (alkyl-labeled) 18:0e and 25 ng of D_3 (alkyl-labeled) 16:0e lyso-PAF. The ratio of the abundance of the M-15 ions for labeled (D_3) and unlabeled (D_0) isopropylidene derivative are indicated with different amounts (0–60 ng) of unlabeled lyso-PAF. Error bars are smaller than symbols if not shown (n = 3).

The suitability of the deuterium-labeled lyso-PAF as an internal standard for unsaturated lyso-PAF molecular species quantitated by the GLC/MS procedure is illustrated in **Fig. 3.** Using the HF technique to cleave the phosphocholine moiety from the 18:1e lyso-PAF yielded products proportional to the amount of D_3-18:0-lyso-PAF. However, the overall yield of 18:1e monoglyceride isopropylidene derivative was greater relative to the D_3-18:0e monoglyceride derivative when phospholipase C was employed to cleave an identical aliquot. In separate experiments, it was observed that the 18:1e-monoglyceride obtained by phospholipase C treatment was partially degraded by the 4-hr HF treatment. However, using controlled HF conditions described above, the deuterated saturated lyso-PAF compounds served as adequate, but not ideal, internal standards.

Quantitation of the lyso-PAF levels in resting and ionophore-stimulated neutrophils at various time points yielded the results seen in **Fig. 4** (levels reported as per million neutrophils). Levels in resting cells for both the hexadecyl and octadecyl molecular species were found to be approximately the same, with the combined levels on the order of 0.59 ng/10^6 cells. Upon stimulation, the levels of hexadecyl lyso-PAF increased 2.5-fold, while those of octadecyl lyso-PAF increased greater than 3.5-fold. The levels of both molecular species were significantly increased at 1 and 5 min post-stimulation. Evidence for unsaturated molecular species such as 18:1e lyso-PAF could not be found. These levels do not reflect artificial generation of lyso-PAF by chemical hydrolysis of ether phosphatidylcholines during the workup procedure, since in all experiments reported the amount of hydrolysis estimated by the production of radiolabeled free fatty acid from added ^{14}C-labeled PC was determined to be $\leq$ 0.1%. Since it was possible that PAF may be hydrolized faster than diacyl phosphatidylcholine, any experiment with greater than the 0.1% ^{14}C-labeled PC hydrolysis was discarded.

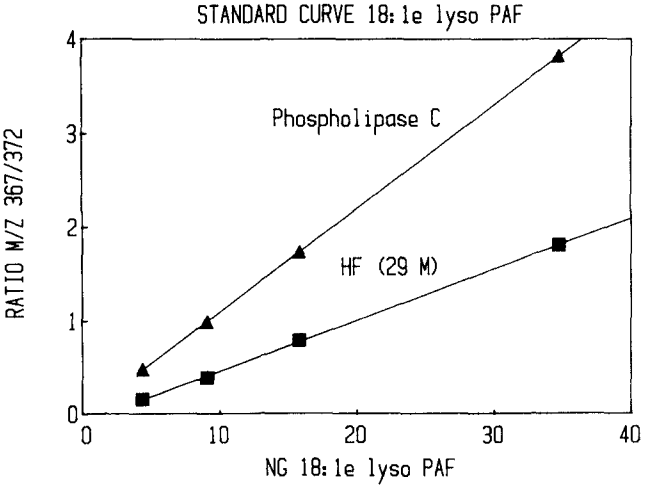

Fig. 3. Standard curves for phospholipase C-treated and HF-treated 1-O-octadecenyl-2-lyso-PAF (18:1e-lyso-PAF) using deuterium-labeled 18:0e-lyso-PAF as internal standard. Ions at m/z 367 and 372 refer, respectively, to M–15 ions of 18:1e isopropylidene and 18:0e isopropylidene monoglycerides. Slopes for the phospholipase C curve and HF curve were 0.109 and 0.054, respectively.

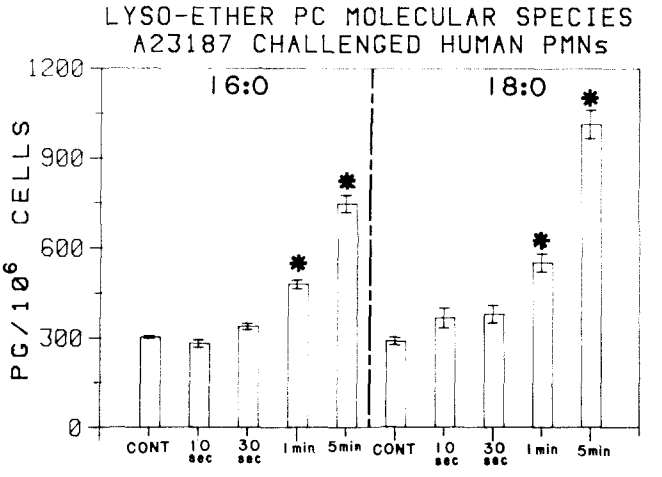

Fig. 4. Levels of lyso-PAF molecular species found by the GLC/MS procedure in resting or calcium ionophore-stimulated neutrophils (50×10^6 neutrophils per time point). Levels are presented as picograms per million cells produced at the times indicated following ionophore stimulation. *Statistically significant from control at the $P < 0.001$ level (n = 3).

In order to verify that the compounds being quantitated corresponded to their correct chemical structures, biologically derived samples were pooled and full mass spectra were obtained. **Fig. 5** shows the mass spectrum of the hexadecyl isopropylidene derivative of lyso-PAF isolated from human neutrophils which also contained a portion of the deuterated internal standard. The full mass spectra of this molecular species was identical to that of authentic hexadecyl lyso-PAF; however, since the deuterium-labeled internal standard eluted slightly ahead of the corresponding protium species, the ratio of the M–15 ions (m/z 341, 343, 344) was different from the ratios seen in Fig. 1.

FAB/MS quantitation of lyso-PAF

The GLC/MS procedure described above required degradation and derivatization of the molecules of interest, and thus the potential for artifacts did exist, particularly with concern to the unsaturated lyso-PAF species. Therefore, the use of a different but complimentary method for quantitation was used to verify the GLC/MS assay. FAB/MS has been shown to be especially well suited to the analysis of intact phospholipids (19) and this method was applied to the analysis of lysophosphatidylcholine derived from human neutrophils. The FAB mass spectra of human neutrophil-derived lysophosphatidylcholine molecular species as their pseudomolecular ions (MH^+) are shown in **Fig. 6.** The most

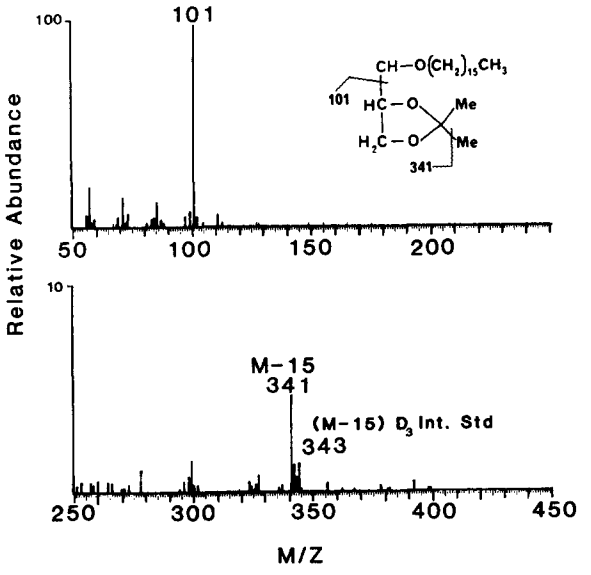

Fig. 5. Electron impact mass spectrum (70 eV) of the isopropylidene derivative derived from human neutrophil hexadecyl lyso-PAF generated by a 5-min challenge with 1.9 μM A23187. The ion at m/z 344 corresponds to M–15 from the added deuterated internal standard used for quantitative analysis.

abundant ion observed in this experiment was at m/z 184 as previously reported for phosphatidylcholines (19). These results indicate that both the ether and acyl lyso-PC molecular species were present in the TLC fraction as well as the deuterium-labeled internal standards. Quantitation of lyso-PAF species using the D_9 internal standard yielded results in close agreement to the GLC/MS method (**Table 1**). Again, there was no indication of the presence of unsaturated molecular species of lyso-PAF. It was found that the levels of both hexadecyl and octadecyl lyso-PAF species increased greater than 2-fold upon stimulation with ionophore. Standard curves were linear past the range used for quantitation (20–100 ng).

Direct comparison of the levels of lyso-PAF measured by both GLC/MS and FAB/MS by linear regression analysis yielded correlation coefficients for hexadecyl and octadecyl lyso-PAF species of $r^2 = 0.986$ and $r^2 = 0.985$, respectively, indicating good agreement between the two methods. However, the regression line did not intersect the origin but rather indicated that FAB/MS overestimated the lyso-PAF levels. This is also seen in Table 1 where the values measured by FAB are consistently higher than those by GLC/MS.

DISCUSSION

Recent investigations have suggested that lyso-PAF plays an important central role, perhaps as an intermediate between arachidonic acid and acetate, in the formation of several lipid mediators of the inflammatory response. It can be both a metabolite and a precursor of PAF. Therefore, it would be essential to have methods of high precision, accuracy, and sensitivity to quantitate the amounts of lyso-PAF in tissues or physiological fluids. The present methods used to quantitate lyso-PAF are somewhat limited. Polonsky and co-workers (11) have developed a method to acetylate lyso-PAF to PAF and measure its presence by biological activity. While this method offers a great deal of sensitivity, alternative molecular species of platelet activating factor do express different biological responses in the platelet aggregation assay or

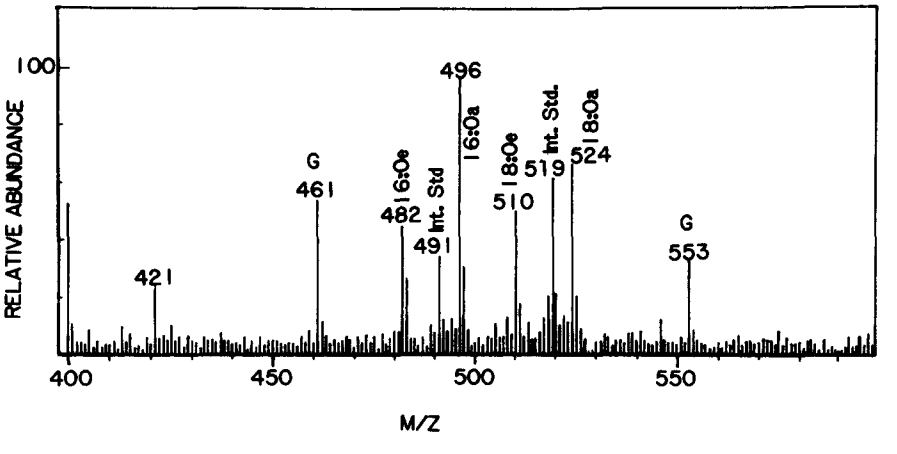

Fig. 6. Fast atom bombardment mass spectrum of lysophosphatidylcholine molecular species derived from human neutrophils after 5 min of calcium ionophore stimulation. Ions shown are pseudomolecular (MH^+) ions with the carbon chain length at *sn*-1 indicated as well as the acyl (a) or ether (e) linkage. The presence of both ether- and acyl-LPC molecular species are evident; G, glycerol cluster ions. Internal standard corresponds to nonadeutero internal standard for 16:0e (m/z 481) and 18:0e lyso-PAF (m/z 519).

TABLE 1. Comparison of lyso-PAF content in control and ionophore-stimulated human neutrophils as measured by GLC/MS and FAB/MS

Stimulus Time	GLC/MS	FAB/MS
	Hexadecyl (pg/10^6 cells)	
0 (control)	302 ± 9 (n = 6)[a]	354 ± 26 (n = 4)
1 min	479 ± 29 (n = 5)	537 ± 24 (n = 3)
5 min	746 ± 57 (n = 7)	718 ± 43 (n = 3)
	Octadecyl (pg/10^6 cells)	
0 (control)	289 ± 26 (n = 5)	440 ± 28 (n = 4)
1 min	552 ± 6 (n = 4)	597 ± 35 (n = 3)
5 min	1014 ± 95 (n = 5)	1065 ± 161 (n = 3)

[a]Standard error of the mean (number of replicates).

release of tritiated serotonin (22). Therefore, this method could have substantial error if presented with significant quantities of alternate molecular species of lyso-PAF; as shown in this study almost equivalent amounts hexadecyl and octadecyl lyso-PAF were formed by A23187-challenged human neutrophils. An alternative sensitive method involved the reacetylation to PAF with tritium-labeled acetate of known specific activity. Following separation of the newly synthesized PAF from unreacted acetate, the amount of PAF could be calculated from the radioactivity migrating into an appropriate TLC retention. While this method does not suffer from receptor recognition of alternative molecular species, other lysophosphatidylcholines such as acyl-lysophosphatidylcholine can lead to erroneous results when they are not separated. As seen in Fig. 6, such species can be present even in concentrations higher than lyso-PAF.

The quantitation of lyso-PAF by mass spectrometry has not been previously documented. The mass spectral methods employed here were based upon the use of stable isotope dilution to permit quantitation of lyso-PAF in the low picomolar range. The use of an internal standard chemically identical to the analyte of interest allows one to correct for losses incurred during the various purification steps, assuming that there is no significant isotope effect which would lead to separation of the internal standard from the native molecule. These methods, therefore, allow one to determine the exact levels of initially present lyso-PAF with a high degree of precision and accuracy. Furthermore, substantial selectivity of the method was realized due to the use of capillary GLC for prior separation and measurement of ions of reasonably high mass. The method based upon GLC/MS offers significant advantage in sensitivity, being two orders of magnitude more sensitive than the FAB/MS technique. However, in order to obtain this particular sensitivity, one must employ chemical degradation of the lyso-PAF to a molecule that could pass through a gas chromatograph. Therefore, substantial sample handling was necessary in order to carry out this quantitative analysis. The use of HF was employed for several reasons. First of all, it was found to be a substantially purer reagent as compared to phospholipase C. Phospholipase C from commercial vendors was found to contain a substantial amount of lipid impurities which added to the complexity of the gas chromatographic separation without an additional chromatographic purification step. Secondly, it was found that the hydrolysis at 4°C was highly reproducible in cleaving the phosphocholine moiety (23). Since there were no acyl groups in the lyso-PAF structure, acid-catalyzed acyl hydrolysis was not a concern. Furthermore, as shown above, the authentic molecular species of PAF were found to be suitably stable to the HF hydrolysis step. The FAB technique required a minimal amount of sample isolation and preparation; however, the detection limit for lyso-PAF was substantially higher (5–10 ng). Although the results obtained by both methods are complimentary, the levels measured by the FAB/MS method appeared to be consistently higher than those measured by the GLC/MS method. This discrepancy was thought to be due to the presence of ions at every mass unit arising from the glycerol matrix in the FAB experiment, as has been observed previously (23). However, the speed of analysis by this technique could offset the need for high accuracy in some experiments.

The production of lyso-PAF following stimulation of human neutrophils has been reported by other investigators. However, the levels of the lyso-PAF were not reported but rather estimated indirectly by the PAF bioassay following reacetylation (24). We report here that two molecular species of lyso-PAF increased significantly following stimulation of human neutrophils with calcium ionophore, in a time-dependent manner. It is interesting to note that the levels of the 18:0e lyso-PAF which increased 3.5-fold above control levels were greater than that found for the 16:0e lyso-PAF in spite of the fact that the 16:0e phosphatidylcholine molecular species were somewhat more abundant than the saturated 18:0e phosphatidylcholine molecular species (25). This observation was in keeping with the finding that the molecular species of PAF found to be produced by human neutrophils was predominantly the 16:0e PAF (17), suggesting a substrate specificity for this lyso-PAF molecular species. Furthermore, there was no indication that 18:1e lyso PAF was formed even though the precursor phospholipid is as abundant.

The detection and quantitation of lyso-PAF should not be interpreted to represent solely those levels of PAF that have been synthesized by these cells following stimulation and subsequently hydrolyzed by acetyl hydrolase. Lyso-PAF could arise from multiple biochemical events such as activation of a phospholipase A_2 and its operation on a 1-O-alkyl-2-acyl-glycerophosphocholine, action of transacylase on a similar substrate, or by de novo synthesis.

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