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Quantification of distinct molecular species of the 2-lyso metabolite of platelet-activating factor by gas chromatography–negative-ion chemical ionization mass spectrometry

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ABSTRACT

The biological activity of platelet-activating factor (PAF) is comprised by a few molecular species of phosphatidylcholine which contain a fatty alcohol connected by an ether linkage to the *sn*-1 position of the glycerol backbone and an acetate ester at the *sn*-2 position. The various molecular species of PAF differ in chain length and degree of unsaturation in the fatty alcohol residue side-chain. PAF is rapidly hydrolyzed to lyso-PAF by an acetylhydrolase enzyme which is quite active in a number of cells that synthesize PAF. We describe a method for quantitation of lyso-PAF which involves conversion to its propionate derivative in the presence of an internal standard (deuterium-labelled PAF), digestion to the diglyceride with *Bacillus cereus* phospholipase C, conversion to the pentafluorobenzoate derivative and capillary column gas chromatographic–negative-ion methane chemical ionization mass spectrometric analysis. Distinct molecular species of lyso-PAF can be individually quantitated at levels of 1 ng or less. These methods are applied to the demonstration of lyso-PAF accumulation in renal tissue from transplanted allografts undergoing acute rejection, in renal tissue from kidneys subjected to cold storage and autotransplantation, and in intestinal mucosa subjected to warm ischemia and reperfusion.

INTRODUCTION

Platelet-activating factor (PAF) is a biologically active phospholipid which exerts its effect on responsive cells via membrane-bound receptors [1,2]. Among the diverse effects of PAF is activation of a number of cells in addition to platelets including polymorphonuclear leukocytes, monocytes, and macrophages. PAF also increases microvascular permeability, induces hypotension, decreases cardiac output, stimulates hepatic glycogenolysis, and induces uterine contraction [1,2].

Structurally, PAF is a phosphatidylcholine molecule with a long-chain fatty alcohol connected by an ether linkage to the *sn*-1 position of the glycerol backbone and by an acetate esterified to the *sn*-2 position [3–5]. PAF analogues with long-chain fatty acyl groups esterified to the *sn*-1 position [6,7] have little biological activity [2], and analogues with fatty acyl groups longer than three carbons in the *sn*-2 position also have greatly reduced biological activity [2,3]. Several distinct biologically active molecular species of PAF are recognized that differ in chain length and degree of unsaturation of the fatty alcohol residue in the *sn*-1 position [2,3]. The initial structural elucidation of PAF from rabbit leukocytes re-

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vealed predominantly octadecyl (C18:0) fatty alcohol residues in the *sn*-1 position with smaller amounts (10%) of hexadecyl residues [4]. PAF obtained from human amniotic fluid during labor contains exclusively C18:0 fatty alcohol residues [8]. In contrast, PAF from ionophore A23187-stimulated human neutrophils was found to contain only hexadecyl (C16:0) fatty alcohol residues [5]. FMLP-stimulated human neutrophils have been reported to contain several fatty alcohol residues in the *sn*-1 position, with C16:0 being the most abundant (40%) followed by C18:1 (18%) and C18:0 (16%) [9,10].

A critical tool in the determination of the role PAF plays in the many physiologic and pathophysiologic processes in which it is suspected to participate is the ability to measure the amount of PAF in biologic samples. PAF is often quantitated by bioassay on platelets after chromatographic isolation [2]. It is difficult to be certain of the specificity of such assays because many substances will induce serotonin release from platelets, and assay precision is limited by the difficulty in accounting for variable losses during chromatographic isolation. Bioassay also fails to distinguish among various molecular species of PAF. Gas chromatographic–mass spectrometric (GC–MS) measurements employing heavy isotope-labelled analogues of PAF circumvent these difficulties. Several groups have successfully measured PAF by stable-isotope dilution GC–MS using deuterium-labelled PAF as an internal standard [11–13].

PAF is rapidly inactivated by hydrolysis to its 2-lyso derivative [14–20]. The acetylhydrolase enzyme which catalyzes this hydrolysis is quite active in many cells that synthesize PAF and in plasma [16], and the level of activity of the enzyme is thought to participate in the regulation of net PAF production in some cells [19–21]. Quantitation of lyso-PAF is difficult because the substance lacks biological activity [2]. Derivatization of lyso-PAF with UV and fluorescent chromophores followed by high-performance liquid chromatographic analysis has been employed to measure lyso-PAF levels in plasma [22]. We describe here an MS method for measurement of lyso-PAF which involves conversion to the propionate derivative with propionyl chloride, enzymatic hydrolysis to the diglyceride, formation of the pentafluorobenzoate derivative, and GC–MS analysis under conditions that permit simultaneous visualization of various molecular species of PAF itself and of a deuterium-labelled PAF as the internal standard. This method is applied to the quantitation of lyso-PAF from several biological samples.

EXPERIMENTAL

Materials

The authentic reference compounds 1-O-hexadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (C16:0-PAF), 1-O-octadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (C18:1-PAF), 1-O-oleoyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (C18:1-PAF), 1-O-hexadecyl-2-lyso-*sn*-glyceryl-3-phosphorylcholine (C16:0-lyso-PAF) and 1-O-octadecyl-2-lyso-*sn*-glyceryl-3-phosphorylcholine (C18:0-lyso-PAF) were obtained from Biomol Research Labs. (Plymouth Meeting, PA, USA). Tritium-labelled PAF (1-O-octadecyl-[9,10-^{3}H(N)]-2-acetyl-*sn*-glyceryl-3-phosphorylcholine) and tritium-labelled lyso-PAF (1-O-alkyl[1′,2′-^{3}H]-2-lyso-*sn*-glyceryl-3-phosphorylcholine) were obtained from NEN Research Products (Boston, MA, USA). Propionic acid and propionyl chloride were obtained from Aldrich (Milwaukee, WI USA). The compounds [$^2H_{10}$]propionic anhydride, [$^2H_4$]acetic acid, and [$^2H_3$]acetyl chloride were obtained from MSD Isotopes (Pointe-Claire, Dorval, Canada). All organic solvents were obtained from Burdick and Jackson (Muskegon, MI, USA). Conical, screw-cap 5- and 10-ml tubes were obtained from VWR (Chicago, IL, USA).

Preparation of [$^2H_3$]-C16:0-PAF and [$^2H_3$]-C18:0-PAF standards

Either 1.0 mg of C16:0-lyso-PAF or 1.0 mg of C18:0-lyso-PAF was added to a 1-ml Reacti Vial (Pierce, Rockford, IL, USA) as a 10 mg/ml solution in chloroform–methanol (10:1) and concentrated to dryness under nitrogen. Then 0.2 ml of

perdeuteroacetic acid was added, and the vial was vortex-mixed. Then 0.05 mg of [$^2H_3$]acetyl chloride was added, and the tube was vortex-mixed and allowed to stand at room temperature for 30 min. Analysis of aliquots of the reaction mixture on Analtech (Newark, DE, USA) silica gel G plates in chloroform–methanol–water–ammonium hydroxide (67.5:52.5:11:4) using visualization with iodine vapor at various times that indicated conversion of lyso-PAF (R_F 0.66) to PAF (R_F 0.75) was roughly 70% complete at 5 min, 90% complete at 15 min, and apparently totally complete at 30 min. At the end of the 30-min incubation, the reaction mixture was concentrated to dryness under nitrogen. Then the reaction was performed a second time (to reduce the blank value of the deuterium-labelled standards) by adding 0.2 ml of perdeuteroacetic acid, vortex-mixing, adding 0.05 ml of [$^2H_3$]acetyl chloride, and incubating at room temperature for 30 min. The reaction mixture was then again concentrated to dryness under nitrogen and reconstituted in chloroform–methanol (10:1). The blank value (apparent contamination with non-deuterated PAF) of the resultant deuterium-labelled PAF standards was determined to be as low as 1 part per thousand in some preparations by digestion to the diglyceride, conversion to the pentafluorobenzoate derivative, thin-layer chromatographic (TLC) purification, and GC–MS analysis as described below. The amount of residual (unreacted) lyso-PAF in the [$^2H_3$]-C16:0-PAF standard was determined to be as low as 5 parts per thousand by subjecting the standard to propionylation, as described below, followed by digestion to the diglyceride, conversion to the pentafluorobenzoate, TLC purification, and GC–MS analysis. The [$^2H_3$]-C16:0-PAF was used as an internal standard for both PAF and lyso-PAF measurements during the processing steps described below and was added in amounts of 20 ng to each sample. The [$^2H_3$]-C18:0-PAF was used to prepare the corresponding diglyceride pentafluorobenzoate derivative, and this material was used as an external standard (*ca.* 20 μg per lane) on the outer lanes of TLC plates on which purification was performed before GC–MS analysis of PAF and lyso-PAF derivatives from biological samples.

Preparation of standards of the 2-propionate derivatives of C16:0-lyso-PAF and C18:0-lyso-PAF

Either 1.0 mg of C16:0-lyso-PAF or 1.0 mg of C18:0-lyso-PAF was added as a chloroform–methanol solution (10:1) to a 5-ml Reacti Vial and concentrated to dryness under nitrogen. Then 0.1 ml of propionic acid was added, and the tube was vortex-mixed. Then 0.05 ml of propionyl chloride was added, and the tube was vortex-mixed again and incubated for 45 min at 60°C. The reaction mixture was then concentrated to dryness under nitrogen, and 0.2 ml of chloroform–methanol (10:1) was added. The contents of the tube were again concentrated to dryness under nitrogen. This process was repeated a second time to remove the last trace of acid from the reaction mixture (which otherwise interferes with subsequent steps in sample processing). TLC analysis on channelled Analtech silica gel G plates with a pre-adsorbent strip in chloroform–methanol–water–ammonium hydroxide (55:52.5:11:4) indicated that conversion of lyso-PAF species (R_F 0.45) to its 2-propionate derivative (R_F 0.56) was essentially complete as estimated by iodine staining under these conditions. The 2-propionyl-lyso-PAF derivative migrated slightly ahead of PAF (R_F 0.52) in this system.

Preparation of standards of the 2-[$^2H_5$]propionate derivatives of C16:0-lyso-PAF and C18:0-lyso-PAF

Either 0.1 mg of C16:0-lyso-PAF or 0.1 mg of C18:0-lyso-PAF was added as a chloroform–methanol solution (10:1) to a 5-ml Reacti Vial and concentrated to dryness under nitrogen. Then 0.05 ml of [$^2H_{10}$]propionic anydride was added, and the tube was vortex-mixed. Then 0.01 ml of pyridine was added, and the tube was vortex-mixed again and incubated for 90 min at 90°C. The reaction mixture was then repeatedly concentrated to dryness under nitrogen, as described above, and finally reconstituted in 0.2 ml of chloroform–methanol (10:1). The contents of the tube were again concentrated to dryness un-

der nitrogen, and the resultant [$^2H_5$]propionate derivatives were purified by TLC analysis as described above. Conversion of lyso-PAF to the [$^2H_5$]propionate derivative was about 90% complete as estimated by iodine staining under these conditions.

Preparation of standard 1-O-hexadecanoyl-2-acetylglycerophosphorylcholine

A 2-mg amount of 1-O-hexadecanoyl-2-lysophosphorylcholine was added to a 5-ml silanized, conical Reacti Vial in 1.0 ml of chloroform–methanol (10:1) and concentrated to dryness under nitrogen. Acetic acid (0.4 ml) and acetyl chloride (0.1 ml) were then added and the tube was vortex-mixed and allowed to stand at room temperature for 40 min. Extent of reaction was assessed by TLC, and the product was concentrated and reconstituted in chloroform–methanol (10:1) as described above.

Preparation of standard diglyceride pentafluorobenzoate derivatives of C16:0-PAF, [$^2H_3$]-C16:0-PAF, C18:0-PAF, C18:1-PAF, 2-propionyl-C16:0-lyso-PAF, 2-propionyl-C18:0-lyso-PAF, 2-[$^2H_5$]propionyl-C16:0-lyso-PAF, 2-[$^2H_5$]propionyl-C18:0-lyso-PAF, and 1-O-hexadecanoyl-2-acetylphosphorylcholine

Each standard phosphorylcholine molecular species was added (*ca.* 0.5 mg) as a solution in chloroform–methanol (10:1) to a silanized, conical 10-ml screw-cap tube and concentrated to dryness under nitrogen. A 1-ml volume of diethyl ether was then added, and digestion to the diglyceride was performed with *Bacillus cereus* phospholipase C, as described below. The diglyceride was then converted to the pentafluorobenzoate with pentaflurobenzoyl chloride as described below, concentrated to dryness, reconstituted in 0.1 ml of hexane, and applied to an Analtech silica gel G plate (250 μm) which was channelled, prescored, and contained a pre-adsorbent lane for sample application. Plates were developed in hexane–ethyl acetate (9:1). Plates were then broken, and the migration position of the standards in the outer lanes was determined with iodine staining. The standards were then harvested from the inner (non-iodine-exposed lanes) and eluted with hexane–ethyl acetate. The diglyceride pentafluorobenzoate derivatives from various molecular species of PAF exhibited an R_F of about 0.37 in this system, and the analogous derivatives from the 2-propionyl-lyso-PAF compounds exhibited an R_F of about 0.46. The extracts were concentrated to dryness under nitrogen in 1-ml Reacti Vials and reconstituted in heptane. Aliquots of the heptane solutions of these derivatives were used as external standards in GC–MS analyses of biological samples to determine the retention times of the standard analytes at the beginning of each run.

Extraction, processing, and derivatization of biological samples for measurement of PAF and lyso-PAF

PAF and lyso-PAF were first extracted from samples by the method of Bligh and Dyer [23]. Aliquots (1 ml) of fluid samples (from supernatants of tissue incubations) were placed in well silanized, conical, screw-cap 10-ml vials. Alternatively, tissue samples (up to 100 mg) were placed in such vials and covered with 1 ml of Krebs–Ringer–bicarbonate buffer. An internal standard consisting of 20 ng of [$^2H_3$]-C16:0-PAF and/or of 30 nCi of [$^3H_2$]-lyso-PAF was added in 0.01 ml each of chloroform–methanol (10:1). (In addition to the biological samples, standard curves for both PAF and lyso-PAF were also prepared which consisted of five points with amounts of 0, 1, 2, 5 and 10 ng of each of the PAF or lyso-PAF standards.) Chloroform (1.25 ml) was then added to each sample, and the samples were vortex-mixed for 30 s. Methanol (2.5 ml) was then added, and the samples were vortex-mixed for 1 min. Fluid samples were then allowed to stand for 30 min at room temperature, and tissue samples were subjected to bath sonication for 30 min. Chloroform (1.25 ml) and water (1.25 ml) were then added, and the samples were vortex-mixed for 1 min, with careful adjustment of vortexer speed to avoid solvent contact with the screw cap. The samples were then centrifuged for 5 min on a table-top centrifuge at 800 *g*. The upper (aqueous) layer and any tissue debris were then

removed and discarded. The lower (chloroform) layer was concentrated to dryness under nitrogen, and the sample was then reconstituted in chloroform–methanol (10:1; 1 ml).

Samples in which the content of lyso-PAF was to be measured were then subjected to propionylation to form the 2-propionyl derivative of lyso-PAF. (This step was usually omitted in samples in which amounts of PAF less than 5 ng were to be measured because the propionylation reaction resulted in extraneous peaks on GC–MS analysis of PAF derivatives that complicated the measurement of lower levels of PAF. In samples containing higher levels of PAF, samples need not be split, and both lyso-PAF and PAF can be measured simultaneously.) For the propionylation reaction, the sample was placed in a silanized conical 5-ml tube with a screw cap and concentrated to dryness under nitrogen. Propionic acid (0.1 ml) was added, and the tube was vortex-mixed for 1 min. Propionyl chloride (0.05 ml) was then added, and the tube was vortex-mixed for 15 s and then incubated for 45 min at 60°C. The sample was then concentrated to dryness under nitrogen, and 0.2 ml of chloroform was added. This step was repeated twice to remove residual derivatizing reagent and acid. The sample was then reconstituted in chloroform–methanol (10:1; 1 ml), and an aliquot (10%) of the sample was subjected to liquid scintillation spectrometry to determine the recovery of $[^3H_2]$-lyso-PAF. In a typical experiment recovery averaged 70 ± 3%. In cases where PAF and lyso-PAF measurements were performed separately, 20 ng of the internal standard $[^2H_3]$-C16:0-PAF were added at this point, and lyso-PAF GC–MS data were subsequently corrected for $[^3H_2]$-lyso-PAF recovery.

Samples were then applied to disposable 3-ml silicic acid columns (J. T. Baker, Phillipsburg, NJ, USA) in a vacuum manifold (Supelco, Bellefonte, PA, USA). Columns had been pre-conditioned by elution with ethyl acetate (2 ml) and then methanol (2 ml). After elution of the sample application solvent, successive elutions (to waste) were performed with chloroform–acetic acid (100:1; 2 ml) and chloroform–methanol (1:1; 2 ml). The sample was then eluted from the column with 4 ml of chloroform–methanol–water (1.25:2.50:1.0), which was collected in a silanized, conical 10-ml screw-cap tube. To this tube, chloroform (1.05 ml) and water (1.05 ml) were added, and the tube was vortex–mixed for 1 min and centrifuged for 5 min on a table-top centrifuge at 800 *g*. The upper (aqueous) layer was aspirated to waste, and the lower (chloroform) layer was concentrated to dryness under nitrogen.

Digestion of the 2-propionyl-lyso-PAF and PAF samples with *B. cereus* phospholipase C (Sigma, St. Louis, MO, USA) was then performed to form the diglycerides from these molecules. To the dry sample in a silanized, conical 10-ml screw-cap tube was added 1 ml of diethyl ether, and the tube was then vortex-mixed for 1 min. Sodium borate buffer (0.25 ml) was then added. (The composition of this buffer was 300 ml of water, 9.15 g of sodium tetraborate, 0.97 g of sodium chloride, and 35 mg of $CaCl_2 \cdot H_2O$, pH 8.0). Then 25 U of *B. cereus* phospholipase C (Sigma P-4014) were added, and the sample tube was capped and incubated for 90 min at 37°C in a shaking water bath. The tube was then centrifuged for 5 min on a table-top centrifuge at 800 *g*, and the upper (ether) layer containing the diglyceride product was aspirated into a silanized, conical, screw-cap 5-ml tube.

The diglycerides derived from 2-propionyl-lyso-PAF or PAF were then converted to the pentafluorobenzoate derivatives. To the dry sample in a silanized, conical 5-ml screw-cap tube was added 0.05 ml of 4% pyridine in toluene, and the tube was vortex-mixed for 1 min. Then 0.01 ml of pentafluorobenzoyl chloride (Sigma) was added, and the tube vortex-mixed for 30 s and incubated for 90 min at 80°C. The sample was then concentrated to dryness under nitrogen in a 50°C water bath, and 0.5 ml of water was added. The tube was then vortex-mixed for 1 min, and 0.8 ml of hexane was added. The tube was again vortex-mixed for 1 min and then centrifuged for 1 min at 800 g on a table-top centrifuge. The lower (aqueous) layer was aspirated to waste, and the upper (hexane) layer was washed twice more with 0.5-ml aliquots of water to remove all residual salts from the derivatization reaction. The hexane lay-

er was then concentrated to dryness under nitrogen, and the sample was reconstituted in fresh hexane (0.01 ml).

The diglyceride pentafluorobenzoate derivatives formed from 2-propionyl-lyso-PAF or from PAF were then purified by TLC on Analtech silicic acid G plates (250 μm) that were pre-scored, channelled, and contained a pre-adsorbent band for sample application. Before sample application, plates were developed in hexane–ethyl acetate (9:1), oven-dried at 100°C for 30 min, and allowed to cool to room temperature. To the outer channelled lanes at both sides of the plate, 20 μg of external standard were applied. The external standard was the diglyceride pentafluorobenzoate derivative prepared from [$^2H_3$]-C18:0-PAF. (This material was used as external standard because the molecular ion of its diglyceride pentafluorobenzoate derivative is distinct from that of any of the derivatives of PAF or 2-propionyl-lyso-PAF from biological samples. Because the external standard is used in large amounts (micrograms) compared to the amounts of biological products to be measured (nanograms), even trace contamination of sample lanes with external standard would prevent accurate quantitation if the external standard were identical to the analytes to be quantitated.) Samples were applied in 0.1 ml of hexane to the pre-adsorbent portion of each lane in the two central quartiles of the plate. The plate was then developed first in hexane, removed from the developing chamber, and allowed to dry at room temperature for 15 min. The plate was then developed in hexane–ethyl acetate (9:1). The plate was then allowed to dry at room temperature, and the two ends of the plate containing the external standards were broken away from the two central quartiles. The ends of the plate were then stained with iodine vapor to visualize the external standard, and its position was marked on the plate. A 2.5-cm band was then marked on the central quartiles of the plate which extended 0.5 cm below the lower margin of the external standard spot and 1.5 cm above the upper margin of the external standard spot. The width of the external standard spot itself was about 0.5 cm. From each individual sample lane, silicic acid within the marked band was scraped from the plate onto glassine weighing paper with a razor blade. This silicic acid was placed into silanized, conical 5-ml screw-cap tubes to which 1 ml of hexane–ethyl acetate (9:1) was added. The tubes were vortex-mixed for 1 min, and water (0.25 ml) was then added. The tubes were vortex-mixed again for 1 min and then centrifuged for 1 min at 800 *g* on a table-top centrifuge. The upper layer was aspirated into a silanized 1-ml Reacti Vial, concentrated to dryness under nitrogen, reconstituted in 0.10 ml of hexane, again concentrated to dryness under nitrogen, and reconstituted in 0.020 ml of heptane.

Gas chromatography–mass spectrometry

The diglyceride pentafluorobenzoate derivatives prepared from 2-propionyl-lyso-PAF or PAF were analyzed on a Hewlett-Packard 5890 Series II gas chromatograph interfaced with a Hewlett-Packard 5988 mass spectrometer operated by a Hewlett-Packard RTE-A computer system as previously described [24]. Samples were introduced into the chromatograph via a Grob-type injector operated in the splitless mode with helium as the carrier gas (head pressure 14 kPa; total flow-rate 10 ml/min; injector temperature 290°C) and analyzed on an 8 m × 0.32 mm I.D. cross-linked methylsilicone capillary GC column with 0.17 μm film thickness (Ultraperformance, Hewlett-Packard). The GC oven temperature was programmed from 85 to 285°C at 40°C/min starting 0.8 min after injection and then held at 285°C. The interface temperature was maintained at 290°C. The mass spectrometer was operated in the negative-ion chemical ionization (NICI) mode (ionization voltage 230 eV; source temperature 100°C) with methane (source pressure 0.2 kPa) as reagent gas. The major ion in the NICI mass spectrum of the pentafluorobenzoate derivatives of the diglycerides derived from PAF or from 2-propionyl-lyso-PAF was the molecular ion. Peak areas for each analyte were integrated with the RTE-A data system and were expressed as a ratio to the area observed for the internal standard peak (derivative of [$^2H_3$]-C16:0-PAF) in the same injection. The raw peak-area ratios

were corrected by subtracting the corresponding ratio in the blank sample, to which no analyte except the internal standard had been added. In cases where lyso-PAF and PAF quantitations were being performed on separately processed samples, the corrected peak areas for lyso-PAF derivatives were then multiplied by the reciprocal of the fractional recovery of the $[^3H_2]$-lyso-PAF spiked into each sample to monitor the efficiency of extraction and propionylation.

Animal studies

All animal studies were performed according to protocols approved by the Washington University Committee for the Humane Care of Laboratory Animals.

Renal allograft studies

Outbred mongrel dogs were anesthetized with sodium pentobarbital (30 mg/kg), and simultaneous unilateral nephrectomy was performed in paired animals, as previously described [25]. Removed kidneys were flushed with lactated Ringers solution and chilled to 4°C. Kidneys were then cross-transplanted between animals using the carotid artery and jugular vein as arterial and venous connections. The ureter was cannulated with polyethylene tubing and exteriorized. Kidneys were positioned into a subplatysmal pocket, and the wounds were closed. After five days without immunosuppressant medications, the allograft and native contralateral kidney were removed, flushed with chilled Ringers solution and processed for PAF and lyso-PAF measurements. Tissue slices of the cortex and medulla were prepared with a Stadie–Rigs microtome with a uniform thickness of less than 0.5 mm. Tissue slices (100 mg) were incubated in 2.5 ml of Krebs–Ringer–bicarbonate buffer at 37°C under 95% O_2–5% CO_2 for 30 min. The buffer was then removed, and aliquots of methanol (5 ml) containing 20 ng of $[^2H_3]$-C16:0-PAF as an internal standard were added to the buffer and to the tissue.

Renal autograft studies

Single kidneys were removed from anesthetized dogs and immediately flushed with 200 ml of Collins solution. The incisions were closed, and the removed kidneys were stored at 4°C for 48 h. The kidneys were then autotransplanted into the donor and reperfused for 60 min. At the end of the reperfusion period, autotransplanted kidneys and the contralateral control kidney were removed and flushed, and slices from the inner and outer cortex and from the medulla were prepared and incubated as described above.

Intestinal ischemia and reperfusion studies

Adult mongrel dogs were fasted for 18 h and anesthetized with sodium pentobarbital (30 mg/kg). The abdomen was incised at the midline. A segment of distal ileum (30 cm) 15 cm proximal to the ileocecal junction was isolated, and the remaining bowel was removed, as previously described [26]. After heparinization (800 μg/kg), an arterial circuit was established between the femoral artery and the artery perfusing the ileal segment. A blood flow probe and a pressure transducer were introduced into the circuit. After aortic pressure and intestinal blood flow stabilized, a section of the intestinal segment (*ca.* 5 g) was removed (pre-ischemic sample) and placed in chilled saline for subsequent incubation. Intestinal perfusion pressure was then reduced by 30 mmHg via a variable clamp. After 3 h of reduced perfusion pressure, another section of the intestinal segment was removed (ischemic sample) for subsequent incubation. The intestinal perfusion was then raised toward the pre-ischemic level by opening the variable clamp, and perfusion was continued for 1 h. A final intestinal segment (reperfusion sample) was then removed for subsequent incubation. Immediately after removal of the intestinal segments, the mucosa was removed with a glass microscope slide. The tissue was cut into pieces of about 15 mg and gently blotted. About 200 mg of tissue per condition were then placed in a 25-ml Erlenmeyer flask containing 2.5 ml of Krebs–bicarbonate buffer. Incubation was performed in a shaking 37°C water bath for 30 min. At the end of this period, the medium was removed and PAF and lyso-PAF production was assessed as described above.

RESULTS

The derivatization scheme is summarized in Fig. 1. Lyso-PAF and PAF were co-extracted by the method of Bligh and Dyer [23] from biological samples and treated with priopionyl chloride. The lyso-PAF was converted to its 2-propionyl derivative under these conditions and PAF underwent no reaction. Phospholipase C from *B. cereus* was then used to catalyze hydrolytic removal of the phosphorylcholine head group to yield the diglycerides of PAF or 2-propionyl-lyso-PAF. The diglycerides were then converted to the pentafluorobenzoate derivatives with pentafluorobenzoyl chloride. This halogenated aromatic derivative had excellent electron-capture properties.

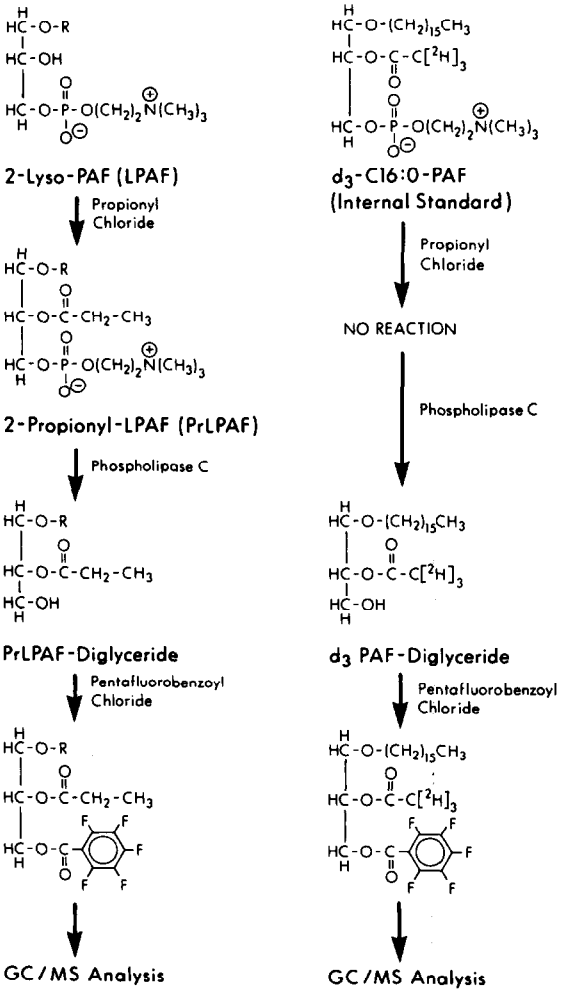

Fig. 1. Derivatization scheme for lyso-PAF and an internal standard. For C16:0-lyso-PAF, R = $(CH_2)_{15}CH_3$, and for C18:0-lyso-PAF, R = $(CH_2)_{17}CH_3$.

Fig. 2 illustrates the behaviour of these derivatives on capillary column GC–NICI-MS analysis. Derivatives of various molecular species of both PAF and lyso-PAF could be visualized on the same chromatographic run. The NICI mass spectrum of the derivatives of all of these compounds was dominated by the molecular anion. The molecular ions for these derivatives prepared from various molecular species of PAF and lyso-PAF standards were as follows: *m/z* 552 for C16:0-PAF; *m/z* 555 for [$^2H_3$]-C16:0-PAF; *m/z* 566 for C16:0-lyso-PAF; *m/z* 578 for C18:1-PAF; *m/z* 580 for C18:0-PAF; and *m/z* 594 for C18:0-lyso-PAF. Each of these derivatives also exhibited a distinct GC retention time. Absolute retention times varied from run to run with the age and condition of the GC column, but representative values from a typical run are illustrated in Fig. 2, where retention times for derivatives prepared from various parent standards were 4.82 min for the [$^2H_3$]-C16:0-PAF internal standard, 4.96 min for C16:0-lyso-PAF, 5.27 min for C18:0-lyso-PAF, 4.80 min for C16:0-PAF, 5.08 min for C18:1-PAF, and 5.12 min for C18:0-PAF.

Quantitation of each analyte involved integration of the peak area, division by the peak area for the internal standard in the same injection, subtraction of the corresponding ratio from the blank sample which contained only internal standard, and interpolation from a standard curve. A typical standard curve for C16:0-lyso-PAF is described by the equation $y = 0.328 + 0.2469x$ ($r = 0.99$), where y is the ion current ratio (*m/z* 566/*m/z* 555 and x is the amount of C16:0-lyso-PAF (ng) in amounts between 1 and 10 ng. A typical standard curve for C18:0-lyso-PAF is described by the equation $y = 0.7605 + 0.6192x$ ($r = 0.99$), where y is the ion current ratio (m/z 594/m/z 555) and x is the amount of C18:0-lyso-PAF (ng). Although as little as a few picograms of these derivatives of lyso-PAF or PAF can be visualized on GC–MS when injecting progressive

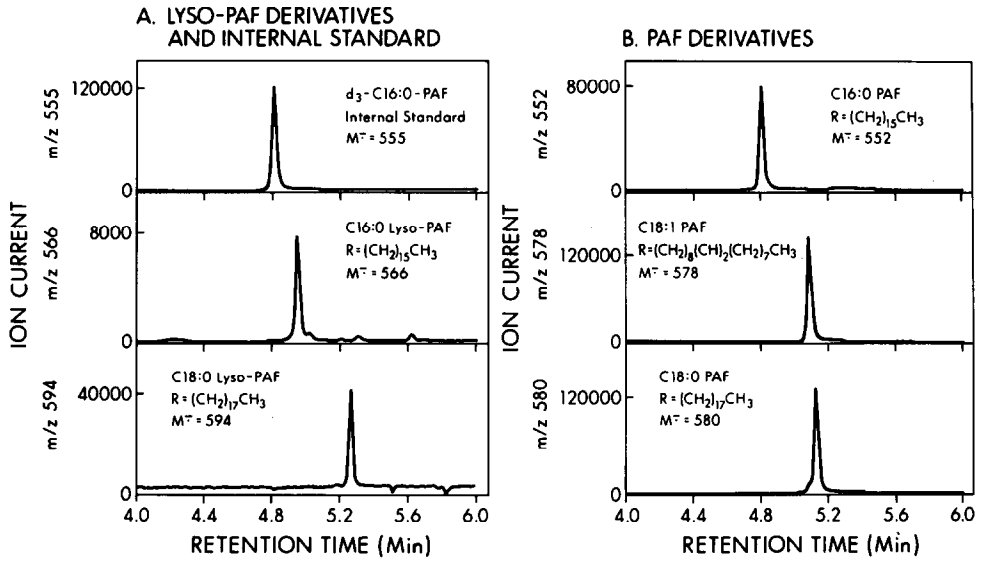

Fig. 2. Gas chromatographic–mass spectrometric analysis of derivatives of various molecular species of lyso-PAF and PAF. Standard PAF and lyso-PAF molecular species were obtained from commercial sources specified in the Experimental section and were converted to the diglyceride pentafluorobenzoate derivatives as outlined in Fig. 1. These derivatives were then analyzed by capillary GC–NICI-MS under conditions described in the Experimental section.

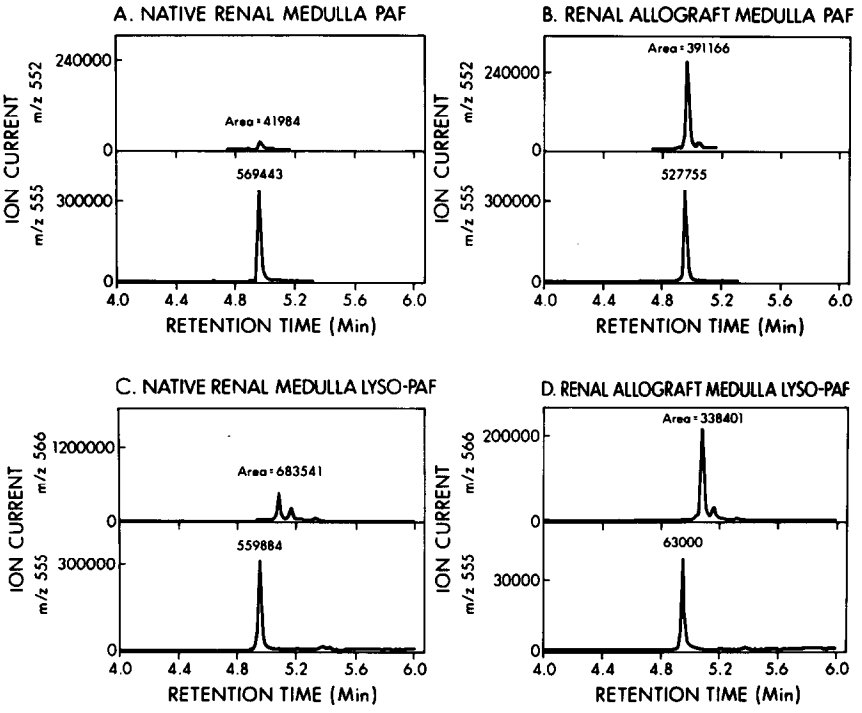

Fig. 3. Production of PAF and lyso-PAF by renal allografts and native kidneys. Dogs were subjected to unilateral nephrectomy and renal allotransplantation and maintained for five days without immunosuppressant medication. The renal allograft and contralateral native kidney were then removed, and slices from the renal medulla were prepared and incubated as described in the Experimental section. Samples for measurement of lyso-PAF and PAF were processed as outlined in Fig. 1. GC–MS tracings for C16:0-PAF from (A) native renal medulla and (B) allograft renal medulla and GC–MS tracings for C16:0-lyso-PAF from (C) native renal medulla and (D) allograft renal medulla are shown. No significant signal for C18:0-PAF was obtained from these samples.

dilutions of standards, quantitation of lyso-PAF from biological samples in practice was found to require at least 0.5–1.0 ng of material in the original sample to yield a final signal that is clearly above extraneous chemical noise from biological materials. For standard curve samples carried through the complete work-up, 1 ng of standard C16:0-lyso-PAF analyte yielded a signal that is about nine- to thirty-fold above blank values.

That these methods are applicable to biological samples is illustrated in Figs. 3–6. As illustrated in Fig. 3A and B, renal allograft rejection is associated with a rise in renal medullary C16:0-PAF accumulation from 0.74 to 8.68 ng per 0.1 g of tissue. This difference of 7.95 ng per 0.1 g of tissue presumably reflects the PAF synthetic capacity of leukocytes infiltrating the allograft that are not present in native renal tissue. Both native renal tissue and renal allograft tissue exhibit robust PAF acetylhydrolase activity, however [21]. This is reflected by the fact that there is a 62.24 ng per 0.1 g of tissue difference in the accumulation of C16:0-lyso-PAF by allograft renal medulla (78.40 ng per 0.1 g of tissue) compared to native renal medulla (16.61 ng per 0.1 g of tissue). The absolute difference in lyso-PAF accumulation is therefore roughly eight times greater than the difference in PAF accumulation.

As illustrated in Fig. 4, the apparent signal for C16:0-lyso-PAF from renal tissue in fact arises from the propionylated lyso-PAF derivative rather than from pre-formed 1-palmitoyl-2-acetyl-*sn*-3-glycerylphosphorylcholine (the 1-acyl analogue of C16:0-PAF), although the 3-pentafluorobenzoate derivative of the diglyceride of either compound yields a molecular ion at *m/z* 566. Fig. 4A illustrates the ion current at *m/z* 566 for a renal tissue sample which had not been subjected to propionylation, and Fig. 4B illustrates the result from the same sample after propionylation. Propionylation clearly produced a strong, distinct signal for a material with an ion at *m/z* 566 which eluted at exactly the same retention time (4.96 min) observed for the external standard of the pentafluorobenzoate of the diglyceride from the 2-propionyl derivative of C16:0-lyso-PAF, which had been injected separately.

Fig. 5 illustrates that there are substantial regional differences in lyso-PAF content within the renal cortex. Fig. 5A illustrates the GC–MS signal for the derivative of C16:0-lyso-PAF from the outer renal cortex from a kidney that had been removed, stored at 4°C for 48 h, and autotransplanted back into the original donor. Fig. 5B illustrates the much greater lyso-PAF signal from the inner cortex from the same kidney. Much

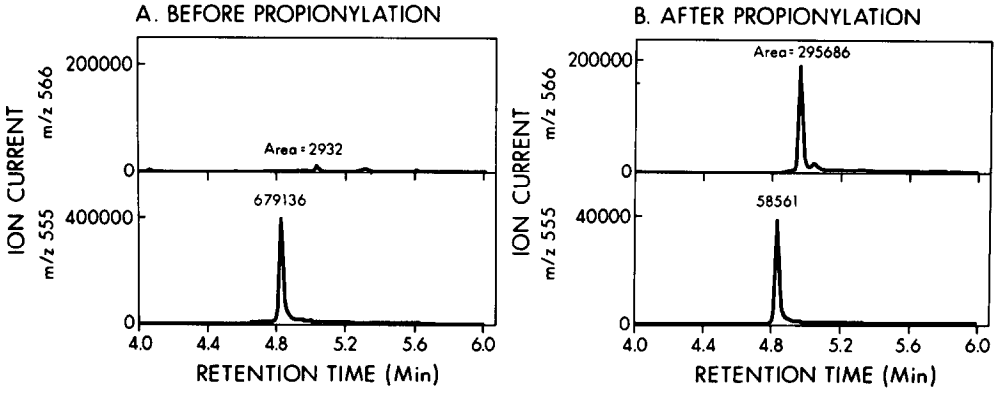

Fig. 4. Formation of the putative 2-propionyl derivative of lyso-PAF requires propionylation. Renal allograft tissue samples were prepared, incubated, and processed as in Fig. 3, except that samples were split into two aliquots after addition of deuterium-labelled internal standard. One aliquot (A) was not subjected to propionylation with propionyl chloride in propionic acid and the other aliquot (B) was subjected to propionylation. Both samples were then subjected to silicic acid chromatography, phospholipase C digestion, esterification with pentafluorobenzoyl chloride, and TLC purification, as described in the Experimental section, and were then analyzed by GC–MS, as illustrated in the figure.

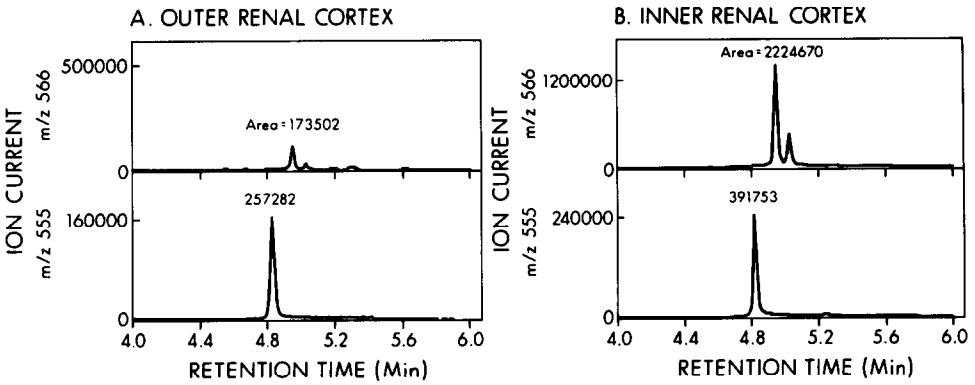

Fig. 5. Lyso-PAF production by various regions of renal cortex after cold storage and autotransplantation. Dogs were subjected to unilateral nephrectomy, the removed kidney was stored at 4°C for 48 h and then autotransplanted back into the original donor, reperfused for 60 min, and removed, as described in the Experimental section. Slices from the outer (A) and inner (B) cortex of the renal autograft were prepared and incubated as described in the Experimental section. Samples for measurement of lyso-PAF were processed as outlined in Fig. 1 and were analyzed by GC–MS, as shown in the figure.

greater amounts of lyso-PAF are therefore derived from the region of renal cortex most proximate to the medullary junction. In this context, it is striking that transplanted kidneys with prolonged cold ischemia times exhibit loss of function after reperfusion that is associated with morphologic damage to the nephron at the S-3 segment [27,28]. This segment lies in the inner cortex at the corticomedullary junction.

Fig. 6 illustrates temporal differences in lyso-PAF accumulation in a segment of intestinal mucosa subjected to warm ischemia and then reper-

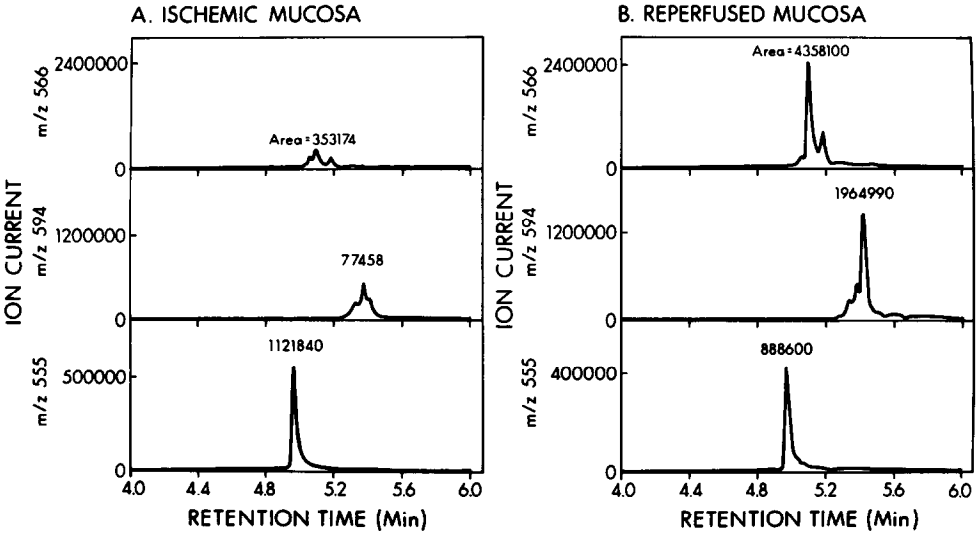

Fig. 6. Lyso-PAF production by intestinal mucosa during ischemia and reperfusion. Loops of canine intestine were vasculary isolated *in vivo*, subjected to periods of reduced perfusion (ischemia) for 3 h, and then reperfused at full arterial pressure for 1 h, as described in the Experimental section. Sections of the intestinal segment were removed during the period of ischemia (A) and during the period of reperfusion (B), and mucosa from them was removed and incubated as described in the Experimental section. Samples were processed for measurement of lyso-PAF as outlined in Fig. 1 and analyzed by GC–MS, as illustrated in the figure above.

fusion. Fig. 6A illustrates the relatively low levels of C16:0-lyso-PAF and C18:0-lyso-PAF from the segment of intestinal mucosa during the period of ischemia, and Fig. 6B illustrates the much higher levels of both molecular species of PAF that were produced during reperfusion. It has previously been observed that lipid mediators derived from the metabolism of arachidonic acid are also produced in much greater quantities by intestinal mucosa during the period of reperfusion after ischemia than during the ischemic period itself [26], and both arachidonate metabolites and lyso-PAF are often coordinately generated by hydrolysis of a common phospholipid precursor, 1-O-alkyl-2-arachidonoyl-*sn*-3-glycerylphosphorylcholine [1,2].

As discussed above, a potentially confounding factor in these analyses is the contribution of preexisting 1-O-acyl-2-acetyl-glycerophosphorylcholine molecules within tissues to the signal for the corresponding 1-O-alkyl-2-propionylglycerophosphorylcholine species formed by the propionylation of lyso-PAF. Although, as demonstrated in Fig. 4, this contribution is minor for renal tissues, this situation may vary among tissues. The molecular ion for both 1-O-hexadecanoyl-2-acetyl-3-pentafluorobenzoyl-*sn*-glycerol and 1-O-hexadecyl-2-propionyl-3-pentafluorobenzoyl-*sn*-glycerol is *m/z* 566. In addition, these two compounds exhibit identical retention times on GC under the conditions described here (not shown). As in Fig. 4, the degree of contribution of the 1-O-acyl-2-acetyl species to the signal for the lyso-PAF derivative can be assessed by comparing the apparent lyso-PAF signal before and after propionylation. In situations where substantial contribution is found to occur, the contribution can be eliminated by derivatizing lyso-PAF with [$^2H_{10}$]propionic anhydride rather than with propionyl chloride. As illustrated in Fig. 7, the resultant 1-O-hexadecyl-2-[$^2H_5$]propionyl-3-pentafluorobenzoyl-*sn*-glycerol exhibits a molecular ion at *m/z* 571 (rather than *m/z* 566), and the corresponding 1-O-octadecyl species exhibits a molecular ion at *m/z* 599 (rather than *m/z* 594). These ions are distinct from those produced by the 1-O-hexadecanoyl-2-acetyl and 1-O-octadecanoyl-2-acetyl species, respectively.

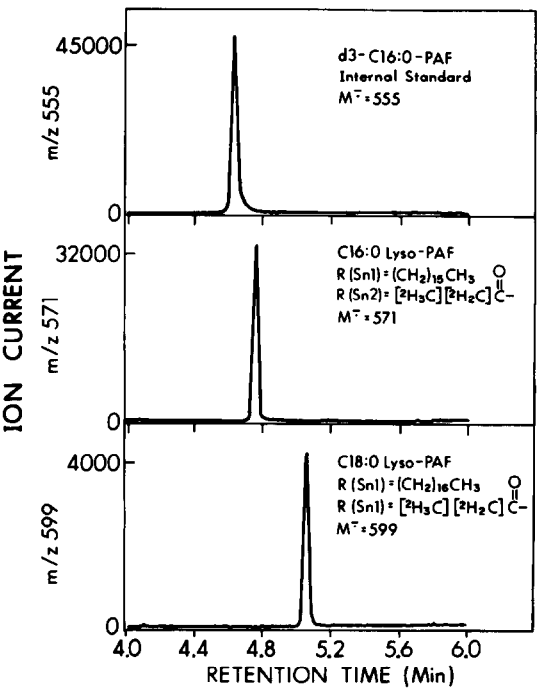

Fig. 7. Gas chromatographic–mass spectrometric analysis of 2-[$^2H_5$]propionyl derivatives of molecular species of lyso-PAF relative to the derivatized [$^2H_3$] internal standard. Standard lyso-PAF molecular species were obtained from the commercial sources specified and were converted to the 2-[$^2H_5$]propionyl-3-pentafluorobenzoyl derivatives; standard [$^2H_3$]-PAF was converted to the corresponding 3-pentafluorobenzoyl derivative, as described in the Experimental section. These derivatives were then analyzed by capillary GC–NICI-MS under the conditions described in Fig. 2.

DISCUSSION

The method described here permits simultaneous quantitation of distinct molecular species of lyso-PAF and PAF under the same GC–MS conditions and has been successfully applied to the quantitation of lyso-PAF from tissue sources. Modifications to the assay that might improve its performance would be to employ an internal standard, lyso-PAF, with a heavy isotope label in the 1-O-alkyl chain. Possible interference from 1-acyllysophospholipids with odd-numbered fatty acyl carbon chains could be eliminated by a saponification step to hydrolyze acyl linkages before the propionylation step. The 1-O-alkyl ether is stable to conditions of saponification [6]. Both

odd-numbered alkyl ether chains in PAF [29] and odd-numbered acyl side-chains in choline phospholipids [6] occur with very low abundance, however, and our preliminary experience with saponification before propionylation indicates that 1-acyl-lysophospholipids with odd-numbered carbon chains are not a major source of interference with the assay in the tissues studied here.

One possibile simplification of the GC–MS method for measurement of lyso-PAF is to derivatize the 2-propionyl-lyso-PAF with pentafluorobenzoyl chloride direclty without forming the diglyceride intermediate [14,15]. This approach involves high concentrations of derivatizing reagent (ten-fold that of the present method), long incubation times (4 h rather than 90 min), and higher temperatures (120°C rather than 80°C) and has been successively employed in qualitative structural studies [14,15]. The applicability of this approach to quantitative measurements, in particular the impact of these conditions on the blank value and stability of the [$^2H_3$]-C16:0-PAF internal standard, has not been examined. In studies with standard compounds, we found that the direct derivatization approach yields standard curves similar to the present method and has no adverse influence on the blank value or stability of the deuterium-labelled internal standard (not shown). Attempts were also made to apply this method to tissue samples that had been subjected to the Bligh and Dyer [23] extraction, spiked with internal standard, passed over disposable silicic acid columns, and then split into two aliquots. One aliquot was processed by the method described here involving phospholipase C digestion to the diglyceride, and the other aliquot was subjected to the direct derivatization procedure. In several attempts, only the tissue sample aliquots subjected to phospholipase C digestion and not those subjected to direct derivatization yielded a signal for the internal standard (not shown). This likely reflects consumption of the derivatizing reagent by large amounts of competing reactant in the tissue sample aliquots not subjected to enzymatic digestion. Although it may be possible to circumvent this problem with higher concentrations of derivatizing reagent and more extensive sample pre-purification, it is not clear that such measures would result in a procedure with substantial time and cost savings over enzymatic digestion.

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