

Analysis of Lipids by Mass Spectrometry and Other Methods

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June 2, 2014

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Liquid chromatography/mass spectrometry analyses of macrophage glycerophospholipids

A cell extract corresponding to 10^7 cells was used for these analyses. The following internal standards were added: 600 pmol each of 1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine, 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoinositol, before lipid extraction according to the method of Bligh and Dyer [1]. After evaporation of organic solvent under vacuum, the lipids were redissolved in 100 μ l methanol/water (9:1, v/v) and injected into a high-performance liquid chromatograph equipped with a binary pump Hitachi LaChrom Elite L-2130 and a Hitachi Autosampler L-2200 (Merck). The column was a Supelcosil LC-18 (5-mm particle size, 250 x 2.1 mm) (Sigma-Aldrich) protected with a Supelguard LC-18 (20 x 2.1 mm) guard cartridge (Sigma-Aldrich). Mobile phase was a gradient of solvent A (methanol/water/*n*-hexane/32% ammonium hydroxide, 87.5:10.5:1.5:0.5, by vol.) and solvent B (methanol/*n*-hexane/32% ammonium hydroxide, 87.5:12:0.5, by vol.). The gradient was started at 100% solvent A; it was decreased linearly to 65% solvent A, 35% solvent B in 20 min, to 10% solvent A, 90% solvent B in 5 min, and to 0% solvent A, 100% solvent B in an additional 5 min. Flow rate was 0.5 ml/min, and 80 μ l of the lipid extract was injected. The liquid chromatography system was coupled online to a Bruker esquire6000 ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The total flow rate into the column was split and 0.2 ml/min entered into the electrospray interface of the mass spectrometer. Nebulizer gas was set to 30 pounds per square inch, dry gas to 8 l/min, and dry temperature to 365°C. Ethanolamine phospholipids (PE) and phosphatidylinositol (PI) were detected in negative ion mode with the capillary current set at +3500 V over the initial 25 min as $[M-H]^-$ ions. Choline phospholipid (PC) species were detected over the elution interval from 25 to 35 min in positive ion mode, as $[M+H]^+$ ions, with the capillary current set at -3500 V.

PE and PI molecular species were identified by multiple reaction monitoring MS/MS experiments on chromatographic effluent by comparison with previously published data [2-8]. Cutoff parameter was set at m/z 150 and fragmentation amplitude at 1 arbitrary unit. Because of the lability of vinyl ether linkages in acid media, plasmalyl (1-alkyl) and plasmenyl (1-alk'1'-enyl) glycerophospholipids were distinguished by acidifying the samples before lipid extraction. For the identification of acyl chains of PC species, ionization was carried out in negative mode with postcolumn addition of acetic acid at a flow rate of 100 ml/h as $[M+CH_3CO_2]^-$ adducts, and acyl chains were identified by MS³ experiments. Stereospecific assignment of fatty acyl chains was carried out by comparing the relative intensities of the 1-lysophospholipid and 2-lysophospholipid compounds arising in the fragmentation experiments (the signal of the latter predominates over that of the former in ion-trap MS) [2-8].

Liquid chromatography/mass spectrometry analyses of lysophospholipids

A cell extract corresponding to 10^7 cells was used for these analyses. The following internal standards were added: 200 pmol each of 1-tridecanoyl-*sn*-glycero-3-phosphocholine and 1-miristoyl-*sn*-glycero-3-phosphoethanolamine, before lysophospholipid extraction with *n*-butanol. After evaporation of the organic

solvent under vacuum, the lipids were redissolved in 100 μ l methanol/water (9:1, v/v) and injected into a high-performance liquid chromatograph equipped with a binary pump Hitachi LaChrom Elite L-2130 and a Hitachi Autosampler L-2200 (Merck). The column was a Supelcosil LC-Si protected with a Supelguard LC-Si guard cartridge (Sigma-Aldrich). Mobile phase was a gradient of solvent A (chloroform/methanol/water/32% ammonium hydroxide, 75:24:5:0.5, by vol.) and solvent B (chloroform/methanol/water/32% ammonium hydroxide, 55:39:5.5:0.5, by vol.). The gradient was started at 100% solvent A, it was decreased linearly to 50% solvent A in 2 min, it was maintained for 8 min, and finally it was decreased to 0% solvent A in 2 min. Flow rate was 0.5 ml/min, and 80 μ l of the lipid extract was injected. The liquid chromatography system was coupled online to a Bruker esquire6000 ion-trap MS (Bruker Daltonics, Bremen, Germany). The total flow rate into the column was split and 0.2 ml/min entered into the electrospray interface of the mass spectrometer. Nebulizer gas was set to 30 pounds per square inch, dry gas to 8 l/min, and dry temperature to 365°C. Ethanolamine and inositol lysophospholipids were detected in negative ion mode with the capillary current set at +3500 V as $[M-H]^-$ ions. Choline lysophospholipids were detected in positive ion mode as $[M+H]^+$ ions, with the capillary current set at -4000 V. Quantification of inositol lysophospholipid species was carried out by comparison with an external calibration curve made with 1-palmitoyl-*sn*-glycero-3-phosphoinositol [8].

Gas chromatography/mass spectrometry analyses of fatty acid methyl esters

After incubations, the cells were washed twice with phosphate-buffered saline, and a cell extract corresponding to 107 cells was scraped in ice-cold water and sonicated in a tip homogenizer twice for 15 s. Before extraction and separation of lipid classes, internal standards were added. For total phospholipids, 10 nmol of 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine was added; for TAG, 10 nmol of 1,2,3-triheptadecanoylglycerol was added, and for CE, 30 nmol of cholesteryl tridecanoate was added. Total lipids were extracted according to Bligh and Dyer [1], and the resulting lipid extract was separated by thin-layer chromatography using n-hexane/diethyl ether/acetic acid (70:30:1, by vol.) as the mobile phase. Spots corresponding to the various lipid classes were scraped and phospholipids were extracted from the silica with 800 μ l methanol followed by 800 μ l chloroform/methanol (1:2, v/v), and 500 μ l chloroform/methanol (2:1, v/v). TAG and CE were extracted with 1 ml chloroform/methanol (1:1, v/v) followed by 1 ml of chloroform/methanol (2:1, v/v). Glycerolipids were transmethylated with 500 μ l of 0.5 M KOH in methanol for 30 min at 37°C. 500 μ l of 0.5 M HCl was added to neutralize. Cholesteryl esters were transmethylated as follows. Each fraction was resuspended in 400 μ l methyl propionate and 600 μ l of 0.84 M KOH in methanol was added for 1 h at 37°C. Afterward, 50 μ l and 1 ml of acetic acid and water, respectively, were added to neutralize. Extraction of fatty acid methyl esters was carried out with 1 ml n-hexane twice.

Analysis of fatty acid methyl esters was carried out in a Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass-selective detector operated in electron impact mode (EI, 70 eV) equipped with an Agilent 7693 autosampler and an Agilent DB23 column (60 m length x 250 μ m internal diameter x 0.15 μ m film thickness). Oven temperature was held at 50°C for 1 min, then increased to 175°C at a rate of 25°C/min, then increased to 215°C at a rate of 1.5°C/min, and the final ramp being reached at 235°C at a rate of 10°C/min. The final temperature was maintained for 5 min, and run time was 39.67 min. Data analysis was carried out with the Agilent G1701EA MSD Productivity Chemstation software, revision E.02.00 [9,10].

Liquid chromatography/mass spectrometry analyses of eicosanoids

A small amount of butylated hydroxytoluene in methanol (0.01%, w/v) was added to the supernatants to prevent eicosanoids degradation. Deuterated PGE₂ and LTB₄ (400 pmol each) were added as internal standards before extraction. Eicosanoids were extracted using Bond Elut Plexa solid phase extraction columns (Agilent),

following the manufacturer's instructions. Columns were pretreated with 3 ml methanol and 3 ml water. Supernatants were acidified with 0.5% acetic acid and 10% methanol was also added before sample loading. Samples were washed with 3 ml 10% methanol and lipid products were eluted with 1.5 ml 100% methanol twice. Lipid products were concentrated under vacuum and redissolved in 100 μ l of solvent A (see below).

90 μ l of the extract was injected into an Agilent 1260 Infinity high-performance liquid chromatograph equipped with an Agilent G1311C quaternary pump and an Agilent G1329B autosampler. The column was a Supelcosil LC-18 (250 x 2.1 mm, 5 μ m particle size) protected with a Supelguard LC-18 (20 x 2.1 mm) guard cartridge (Sigma-Aldrich). The mobile phase consisted of a gradient of solvent A (water/acetonitrile/acetic acid, 70:30:0.02, by vol) and solvent B (acetonitrile/isopropanol, 50:50, by vol). The gradient was started at 100% solvent A, which was decreased linearly to 75% at 3 min, 55% at 11 min, 40% at 13 min, 25% at 18 min, and 10% at 18.5 min. The last solvent mixture was held for an additional 1.5-min period, and finally the column was reequilibrated with 100% solvent A for 10 minutes before the next sample injection.

The flow rate through the column was fixed at 0.6 ml/min and this flow entered into the electrospray interface of an API2000 triple quadrupole mass spectrometer (Applied Biosystems). The parameters of the source were set as follows: ion spray voltage, -4500 V; curtain gas, 25 pounds per square inch; nebulizer gas, 40 pounds per square inch; desolvation gas, 80 pounds per square inch; temperature, 550°C. The analyzer mode was set to scheduled multiple-reaction monitoring with negative ionization, defining for each analyte the m/z of the parent ion as Q1 mass, the m/z of its daughter ion fragment (transition) as Q3 mass, and associating it to the chromatographic retention time to improve the number of analytes collected in a single chromatographic run. The retention time window was set to 120 s. The declustering potential and collision energy for each analyte was optimized by the use of analytical standards. Other parameters were fixed for all analytes: entrance potential, -10 V; focusing potential, -350 V; collision cell exit potential, -10 V. Quantification was carried out by integrating the chromatographic peaks of each species and comparing with an external calibration curve made with analytical standards.

- Other Methods -

Cells – Human monocytes were isolated from buffy coats of healthy volunteer donors obtained from the Centro de Hemoterapia y Hemodonación de Castilla y León (Valladolid, Spain) as described previously [3,11]. Briefly, blood cells were diluted 1:1 with phosphate-buffered saline, layered over a cushion of Ficoll-Paque, and centrifuged at 750 x g for 30 min. The mononuclear cellular layer was recovered and washed three times with phosphate-buffered saline, resuspended in RPMI 1640 medium supplemented with 40 μ g/ml gentamicin, and allowed to adhere in sterile dishes for 2 h at 37°C in a humidified atmosphere of CO₂/air (1:19). Nonadherent cells were removed by washing extensively with phosphate-buffered saline, and the remaining attached monocytes were used the following day.

Resident peritoneal macrophages from Swiss male mice (University of Valladolid Animal House, 10–12 wk old) were obtained by peritoneal lavage using 5 ml cold PBS, as described elsewhere [12, 13]. The cells were plated at 2 x 10⁶ per well (6-well plates) in 2 ml RPMI 1640 medium with 10% heat-inactivated serum, 100 U/ml penicillin, and 100 mg/ml streptomycin, and allowed to adhere for 20 h in a humidified atmosphere of 5% CO₂ at 37°C. Wells were then extensively washed with PBS to remove nonadherent cells. Adherent macrophages were then used for experimentation. When inhibitors were used, they were added to the incubation media 30 min before stimulating the cells with zymosan. All procedures involving animals were undertaken in accordance with the Spanish National Committee on Biosafety and Animal Care, under the guidelines established by the Spanish Ministry of Agriculture, Food, and Environment, and the European Union.

U937 monocyte-like cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). For experiments, the cells were incubated at 37°C in a humidified atmosphere of CO₂/air (1:19) at a cell density of 0.5-1 x 10⁶ cells/ml in 12-well plastic culture dishes (Costar). Cell differentiation was induced by adding PMA to a final concentration of 35 ng/ml for 24 h [14,15]. U937 cell differentiation was confirmed morphologically by light microscopy and by conversion of cells to an adherent cell population.

RAW 264.7 macrophage-like cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine at 37°C in a humidified atmosphere of CO₂/air (1:19). Cells were plated at 10⁶/well, allowed to adhere overnight, and used for experiments the following day. [6, 16].

PLA₂ activity assays – Ca²⁺-dependent PLA₂ activity was measured using a modification of the mammalian membrane assay described by Diez et al. [17]. Briefly, monocyte homogenates were incubated for 1–2 h at 37°C in 100 mM HEPES (pH 7.5) containing 1.3 mM CaCl₂ and 100,000 dpm [³H]AA-labeled membrane, used as a substrate, in a final volume of 0.15 ml. Prior to assay, the cell membrane substrate was heated at 57°C for 5 min to inactivate CoA-independent transacylase activity. The assay contained 25 µM bromoenol lactone to completely inhibit endogenous Ca²⁺-independent PLA₂ activity. After lipid extraction, free [³H]AA was separated by thin-layer chromatography, using *n*-hexane/ethyl ether/acetic acid (70:30:1) as a mobile phase.

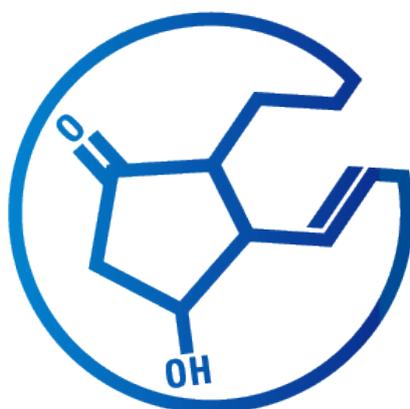
For Ca²⁺-independent PLA₂ activity, the cell homogenates were incubated for 2 h at 37°C in 100 mM HEPES (pH 7.5) containing 5 mM EDTA and 100 mM labeled phospholipid substrate (1-palmitoyl-2-[³H]palmitoyl-glycero-3-phosphocholine, sp. act. 60 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO), in a final volume of 150 µl. The phospholipid substrate was used in the form of sonicated vesicles in buffer. The reactions were quenched by adding 3.75 volumes of chloroform/methanol (1:2). After lipid extraction, free [³H]palmitic acid was separated by thin-layer chromatography, using *n*-hexane/ethyl ether/acetic acid (70:30:1) as a mobile phase. In some experiments, Ca²⁺-independent PLA₂ activity was also measured using a mixed-micelle substrate or the natural membrane assay. For the mixed micelle assay, Triton X-100 was added to the dried lipid substrate at a molar ratio of 4:1. Buffer was added, and the mixed micelles were made by a combination of heating above 40°C, vortexing, and water bath sonication until the solution clarified. The natural membrane assay was carried out exactly as described above, except that CaCl₂ was omitted, and 5 mM EDTA was added instead. All of these assay conditions have been validated previously with regard to time, homogenate protein, and substrate concentration [18-26].

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