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Putative biomarker for phospholipid accumulation in cultured cells treated with phospholipidosis-inducing drugs: Alteration of the phosphatidylinositol composition detected using high-performance liquid chromatography-tandem mass spectrometry

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ABSTRACT

We developed a high-performance liquid chromatography–tandem mass spectrometric method for phospholipid biomarker discovery and applied it to a cell-based assay system for the screening of phospholipidosis-inducing drugs. We studied the compositions of phospholipid molecules exceeding 100 species in cultured cells and found a characteristic alteration in the composition by treatment with cationic amphiphilic drugs possessing phospholipidosis-inducing potency. The compositions of phosphatidylinositol in RAW264 cells were significantly affected by the drug treatment. Similar alterations were also found in THP-1 cells. These phenomena were not observed when cells were treated with warfarin, which does not have phospholipidosis-inducing potency. Structural analysis of the altered phosphatidylinositols by a product ion scan revealed the presence of certain fatty acyl chains. Based on our findings, we proposed a prediction parameter (PP) for phospholipid accumulation calculated from the relative compositions of phosphatidylinositol species. As the dosage of imipramine (a cationic amphiphilic drug) increased, both the PP and cellular phospholipid content increased. Our results suggest that PP has potency as a biomarker for phospholipid accumulation in cells treated with drugs.

1. Introduction

Drug-induced phospholipidosis (DIPL), which is caused by long-term treatment with cationic amphiphilic drugs (CADs), is a pathological condition in which phospholipids are hyperaccumulated in cells. A hallmark of DIPL is the formation of lamellar bodies, which are concentric structures assembled with phospholipid, in subcellular organelles such as lysosomes. The formation of lamellar bodies is found in various organs and tissues including lung and liver. For example, amiodarone (AM) increases the risk of pneumonitis and hepatitis [1]. Therefore, for both new drug development and clinical use of drugs, it is important to estimate how much phospholipid is accumulated by the drug administration. One of the major methods for *in vitro* estimation is a fluorescent assay using biomembrane stains [2–5]. On the other hand, the *in vivo* gold standard for diagnosis of DIPL is the detection of lamellar bodies with electron microscopy in biopsy samples, which is also applicable for cellular assay systems. This method is superior to other methods in the direct detection of accumulated phospholipid, but the method is invasive, laborious, and requires a long time. Therefore, biomarkers to monitor the progression of DIPL have been developed. These include phenylacetylglycine [6], sets of genomic biomarkers [7–9], and bis(monoacylglycero)phosphate (BMP), which is a type of phospholipid and a structural isomer of phosphatidylglycerol (PG) [10]. Mortuza et al. [10] reported that the content of BMP was increased in lymphocytes, serum, lung, and kidney of rats by AM treatment. Baronas et al. [11] also reported that di-docosahexaenoyl BMP in rat urine was increased by treatment with AM. BMP is believed to be a good biomarker because it is specific for phospholipid accumulation and enables

Abbreviations: AM, amiodarone; BMP, bis(monoacylglycero)phosphate; CAD, cationic amphiphilic drug; CPZ, chlorpromazine; DiI–C18, Vybrant DiI cell-labelling solution; DDPC, didecanoyl phosphatidylcholine; DMPC, dimyristoyl phosphatidylcholine; DHPC, 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine; DIPL, drug-induced phospholipidosis; EDTA, ethylenediaminetetraacetic acid; HPLC-MS/MS, high-performance liquid chromatography–tandem mass spectrometry; IMI, imipramine; MRM, multiple reaction monitoring; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; TAM, tamoxifen; WF, warfarin.

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non-invasive sampling. BMP is measured using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) in negative or positive ion mode [10–12]. Unfortunately, the detection sensitivity is not always high because the specific product ion for BMP (monoacylglycerol) is unstable; its ester bonds between the fatty acid and glycerol are easily cleaved in the collision cell of a mass spectrometer. Therefore, BMP measurement requires more skilful operation in the optimisation of the collision setting compared to other phospholipid classes. From this viewpoint, a more easily detectable phospholipid-biomarker is desired.

In the present study, we investigated the phospholipid composition in cultured cells using HPLC-MS/MS and found a characteristic alteration of the phospholipid composition by treatment with CADs. The fatty acyl chain structures of the altered phospholipids were also investigated with HPLC-MS/MS. Our results suggest that the composition of phosphatidylinositol (PI) can be a putative biomarker for phospholipidosis.

2. Materials and methods

2.1. Chemicals

Tamoxifen citrate salt (TAM; >99%), warfarin sodium salt (WF; >97%), imipramine hydrochloride (IMI; >98%), methanol (LC-MS grade), 25% ammonia solution (reagent grade), and RPMI-1640 supplemented with L-glutamine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Chlorpromazine hydrochloride (CPZ; USP grade), AM hydrochloride (>98%), and ammonium formate (97%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). EDTA-2Na (>99.5%) was a product of Dojindo Laboratories (Mashiki, Kumamoto, Japan). 1,2-Diheptadecanoyl-*sn*-glycero-3-phosphocholine (DHPC; >99%) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Dimyristoyl phosphatidylcholine (DMPC; >99%) and didecanoyl phosphatidylcholine (DDPC; >99%) were obtained from NOF Corporation (Tokyo, Japan). Vybrant DiI cell-labelling solution (DiI-C18) was from Invitrogen (Carlsbad, CA, USA). Other chemicals of analytical grade were from local suppliers.

2.2. Cell culture

RAW264 cells, U-937 cells and THP-1 cells were provided from RIKEN BRC (Tsukuba, Japan) and grown in RPMI-1640 with L-glutamine containing 10% foetal bovine serum and penicillin streptomycin (GIBCO; Invitrogen, 100 Units/mL for penicillin and 100 μg/mL for streptomycin) at 37 °C in a humidified atmosphere with 5% CO_2. The cells were treated with CADs (CPZ, 10 μM; IMI, 25–100 μM; AM, 10 μM, TAM, 10 μM) or non-CAD (WF, 100 μM) for 24 h and then collected to extract the lipid components.

2.3. Sample pre-treatment

The total lipids in the cells were extracted according to a reported method [13] with minor modifications. Chloroform–methanol (2:1, 5 mL) was added to 500 μL of the cellular suspension in a centrifuge tube. The mixture was vigorously shaken and centrifuged at 1200 × *g* for 5 min. The upper layer was then transferred to another centrifuge tube and mixed with chloroform–methanol (1:2, 2 mL) and saline (1 mL). The mixture was re-extracted in the same manner. The lower layers of the first and the second extractions were mixed together with saline (1.5 mL). The mixture was again vigorously shaken and centrifuged. The lower layer was collected and dried under a nitrogen stream. The dried residuals were reconstituted with 2-propanol–methanol (2:1) and then subjected to HPLC-MS/MS measurement.

2.4. Measurement of phospholipid by HPLC-MS/MS

A Quattro Premier triple quadrupole mass spectrometer hyphenated with a 2795 separation module (Waters, Milford, MA, USA) was used. The experimental conditions were the following: column, Xterra MS C8 (3 cm × 2.1 mm ID, 3.5 μm, Waters); column temperature, 40 °C; injection volume, 5 μL; mobile phase, biphasic gradient with ammonium formate (10 mM) and methanol (initial, 70% methanol; 1.5 min, 90% methanol; 15 min, 90% methanol; 16.5 min, 70% methanol; 18 min, 70% methanol); flow rate, 0.3 mL min^{-1}; electrospray voltage, +3 kV; cone voltage, +40 V; and collision energy, 40 eV. The multiple reaction monitoring (MRM) methods for each phospholipid class were as follows: phosphatidylcholine (PC), product ion at *m*/*z* 184.1; phosphatidylethanolamine (PE), neutral loss of 141.0; phosphatidylserine (PS), neutral loss of 185.0; PG, neutral loss of 189.0; and PI, neutral loss of 277.0. The structural information for the fragmentations is shown in Supplementary Fig. S1. The mass numbers of the precursor ions were obtained from the theoretical molecular masses. A list of MRM transitions is provided in Supplementary Table S1. In preliminary studies, we screened approximately 20 *m*/*z* values detecting peak(s) from approx. 50 *m*/*z* values (corresponding to 32:0–44:12) for each phospholipid class. Then, the screened lipids of the cells were measured before and after drug treatments. Phospholipids with the same polar head group were monitored simultaneously in each run. Therefore, each sample required five injections to cover all five phospholipid classes. The peak area values obtained were summed up for all peaks in each class, and then the ratios of the respective peak areas to the sum were calculated. Principal component analysis was conducted with Microsoft Excel 2007 after normalisation and standardisation.

Relative quantification of the target PI molecules was performed using DHPC as an internal standard, of which the MRM conditions were as follows: electrospray voltage, −2.5 kV; cone voltage, −40 V; collision energy, 40 eV; precursor ion mass, 806.0; and product ion mass, 269.0 (for heptadecanoic acid).

Fatty acyl chain analysis was also conducted using HPLC-MS/MS in the product ion scan mode. The mobile phase was ammonia solution (0.05%), which was diluted from 25% ammonia solution and methanol. The injection volume was 10 μL. The other HPLC conditions, including the gradient program, were as mentioned above. The product ion scan settings were as follows: electrospray voltage, −2.8 kV; cone voltage, −30 kV; and collision energy, 30 eV.

2.5. Electron microscopy

The RAW264 cells treated with CPZ for 24 h were fixed in phosphate-buffered 2% glutaraldehyde and 2% paraformaldehyde for (1 h), washed with phosphate buffer, post-fixed in phosphate-buffered 1% osmium tetroxide for 30 min at 4 °C, dehydrated using a series of ethanol concentrations, and embedded in Epon resin (TAAB Laboratories Equipment Ltd., Berkshire, UK). Ultra-thin sections (60-nm thick) were cut using a diamond knife (Diatome Ltd, Bienne, Switzerland) on a Leica EM UC6 (Vienna, Austria), placed on copper grids, and stained with 4% uranyl acetate and 0.3% lead citrate. The samples were observed with an H-7650 electron microscope (Hitachi, Tokyo, Japan) operating at an accelerating voltage of 80 kV.

2.6. Flow cytometry for the measurement of accumulated phospholipid

DiI-C18 was added to the foetal bovine serum and maintained overnight at 37 °C. RAW264 cells (passage numbers less than 20) were seeded on normal plastic well plates at 5×10^4 cells mL^{-1}. After 24 h incubation, the culture medium was exchanged to one