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Advanced Lipid Extraction Method for the Determination of the Phospholipase D Activity

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Phospholipase D is a ubiquitous enzyme that plays an important role in various lipid mediated cellular signaling pathways and produces rare phospholipids, phosphatidylethanol or phosphatidylbutanol, instead of phosphatidic acid with unique catalytic activity transphosphatidylation in the presence of primary alcohols. The reaction products, phosphatidylethanol or phosphatidylbutanol are used as markers of *in vitro* phospholipase D activity in many studies. For the sensitive detection of the phospholipase D products, we developed an advanced lipid extraction method that facilitates recovery of the compounds. With the new method, the activity change of phospholipase D by agonists could be detected more easily and the recovery rate was also increased. The increase of detected enzyme activity change was about double fold compared to the conventional lipid extraction method. This method provides selective force for the phospholipase D products in the extraction procedure.

Key words: Phospholipase D, Phosphatidylethanol, Phosphatidylbutanol, Enzyme activity, Lipid extraction, HPLC

INTRODUCTION

Phospholipase D (PLD) catalyses hydrolysis of the terminal phosphodiester bond of membrane glycerophospholipids, resulting in the formation of phosphatidic acid (PA) and a related head group (Bligh and Dyer, 1959; Exton, 1998; Gomez and Jeire, 1998). For the *in vitro* activity study of this enzyme, short-chain aliphatic alcohols (ethyl or butyl alcohols) are added in the reaction mixture (Gunnarsson *et al.*, 1998). PLD produces rare phospholipids, phosphatidylethanol (PEt) or phosphatidylbutanol (PBt), instead of PA with unique catalytic activity transphosphatidylation in the presence of primary alcohols. The reaction products, PEt or PBt are used as markers of *in vitro* PLD activity in many studies. The main product of the PLD reaction, PA, is subsequently converted into lyso-PA (LPA) by phospholipase A_2, (PLA_2) or DAG by PA-phosphohydrolase.

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For the detection of PEt or PBt to determine PLD activity, the liquid scintillation counting (LSC) method after thin layer chromatography (TLC) development is widely (Exton, 1998; Kemken *et al.*, 2000) used. Instrumental analysis for the rare phospho-lipids were not usually adopted because of the characteristics of the rare phospholipids. Recently, analysis of PEt or PBt with high performance liquid chromatography (HPLC) using various detectors like the evaporative light scattering detector (ELSD), fluorescence detector or mass spectrometry (MS) were reported by several research groups (Kim *et al.*, 1999; Kobayashi and Kanfer, 1987; Oh *et al.*, 2000; Park *et al.*, 2000). Analysis of the PLD product with gas chromatography (GC) and GC-MS was reported also (Yon and Han, 2000).

Sample preparation is very important to insure reproducibility and accuracy of the experimental results. The lipid extraction method with chloroform and methanol for the sample preparation, first developed by Bligh and Dyer (Varga *et al.*, 2000), is generally used for PLD activity tests. The method is simple, fast and gives good results for most experimental purposes. However, for research regarding lipid mediated cellular signaling events such as the PLD activity test, the method needs to be improved in

several aspects including improvement of recovery rate for the PLD products and enrichment of the polar lipids such as PBt or PEt.

With the new lipid extraction method, the change of PLD activity could be detected about double fold in sensitivity compared to the conventional method and the contamination of other cellular lipids such as phosphatidylcholine could be reduced. This method will provide a more sensitive method for the detection of minute changes of PLD and cellular signaling events.

MATERIALS AND METHODS

Materials

MCF-7 human breast cancer cells were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). 1,2-Dipalmitoyl-sn-Glycero-3-Phospho-butanol (PBt) was purchased from Avanti Polar-Lipids, Inc. (Alabaster, AL, USA). Dulbeccos Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin solution were from Gibco BRL (Gaithersburg, MD, USA). Tamoxifen citrate was from Sigma (St. Louis, MO, USA). [^{3}H]-palmitate was purchased from Amersham Bioscience (Uppsala, Sweden). All other chemical agents used were of analytical grade.

Cell culture

MCF-7 cells were grown in DMEM medium supplemented with 10% FBS. The cells were maintained at 37°C in a water-saturated atmosphere with 95% air and 5% CO_2. When confluence was reached, the cells were trypsinized and subcultured to a new tissue culture plate.

Lipid extraction

MCF-7 cells grown on 6 well plates were washed twice with 2 mL of ice-cold phosphate buffered saline (PBS) and transferred to 2 mL Eppendorff tubes. The cells were harvested with centrifugation for 10 min at 3,000 rpm (865×g), 4°C. Cell pellets were resuspended with 700 μL of water and 70 μL of 2N HCl was added. To the mixtures, 800 μL of ethylacetate were added and the mixtures were shaken vigorously for 10 min. After standing for 10 min. at room temperature, the phases were separated with 30 min. centrifugation at 10,000 rpm (9,615×g), 25°C. Upper organic layers were transferred to new Eppendorff tubes and to the lower aqueous layer, 700 μL of ethylacetate was added. The mixtures were mixed again and the upper organic layers were retrieved and pooled after centrifugation. The organic phases pooled were kept in -20°C for 2 to 8 hr. and centrifuged for 5 min. at 10,000 rpm (9,615×g), 25°C. The lower aqueous phases were removed carefully with micropipette. The organic phases were dried with vacuum drier and resuspended with a small volume of methanol and chloroform (1:1, v/v) for TLC separation. For the comparison of efficiency of the new method, the conventional lipid extraction method was adopted (Yon and Han, 2000). The cells were quickly washed with ice-cold PBS and suspended in ice-cold methanol. Cellular lipids were extracted with a mixture of chloroform and methanol (Varga *et al.*, 2000).

Determination of PLD activities

PLD activities were determined by the formation of phosphatidylbutanol (PBt) as described elsewhere (Yon and Han, 2000). Briefly, MCF-7 cells cultured on 6 well plates were metabolically labeled with 1 μCi/mL of [^{3}H]-palmitate in a serum-free medium for 24 h. The cells were pretreated with 0.3% (v/v) butanol for 15 min. before stimulation with 50 M of tamoxifen for 30 min. After stimulation, the cells were quickly washed twice with ice-cold PBS. Lipids were extracted according to the method of Bligh and Dyer (1959) and the newly developed method. PBt was separated by a thin-layer chromatography (TLC) using a solvent system of ethyl acetate/iso-octane /acetic acid/water (110:50:20:100, vol/vol). The regions corresponding to the PBt standard bands were identified with 0.002% (w/v) primulin in 80% (v/v) acetone, scraped and counted using LSC.

HPLC analysis and recovery test

HPLC analysis was performed with a Waters Nova-Pak silica column (3.9×300 mm, Waters Corp., Milford, MA, USA), using Waters 510 solvent delivery system (Waters Corp., Milford, MA, USA) and an Alltech 2000 evaporative light scattering detector (ELSD, Alltech Associations, Inc., Deerfield, IL, USA). Samples were analyzed with solvent gradient system from chloroform-methanol-ammonium hydroxide (90:10:0.5 v/v) to chloroform-methanol-water-ammonium hydroxide (75:22:2.7:0.5 v/v) at a flow rate of 1 mL/min (Table I). The ELSD drift tube temperature was set to 45°C under a nebulizing N_2 flow of 1.7 L/min. For the recovery test of PBt, a total 1 μg of PBt was added in each cell suspension and lipid extraction was performed by the two different lipid extraction methods. The dried samples were resuspended with 100 μL of methanol and chloroform (2:1, v/v) and injected in the HPLC-ELSD system for analysis.

Table 1. Solvent gradient condition for HPLC analysis

Time	0	12	17	30	45	50	60
% B	0	18	40	50	100	100	0

A: chloroform-methanol-ammonium hydroxide (90:10:0.5 v/v).
B: chloroform-methanol-water-ammonium hydroxide (75:22:2.7:0.5 v/v)
Flow rate : 1 mL/min.

Statistical analysis

All experiments were performed at least three to six times. Data were analyzed using one-way ANOVA, and considered to be significantly different at $p<0.05$.

RESULTS AND DISSCUSSION

PLD is a key enzyme in the lipid mediated cellular signaling pathways and involved in many signaling events (Exton, 1998; Gomez and Jeire, 1998). The enzymatic activity of PLD is subject to change by various stimulants and the changes of the enzymatic activity of PLD induce many other signaling events in the cells (Kim *et al.*, 1999; Oh *et al.*, 2000; Park *et al.*, 2000; Shin *et al.*, 1998). To know the roles of PLD in the signaling pathways, detection of the changes of PLD activities is very important.

HPLC analysis of PBt

Fig. 1 shows the HPLC profiles of total lipid extracts prepared from the conventional and the new lipid extraction methods. The chromatograms indicated that the total amounts of the cellular lipids extracted by the new method was less than the amounts by the conventional method.

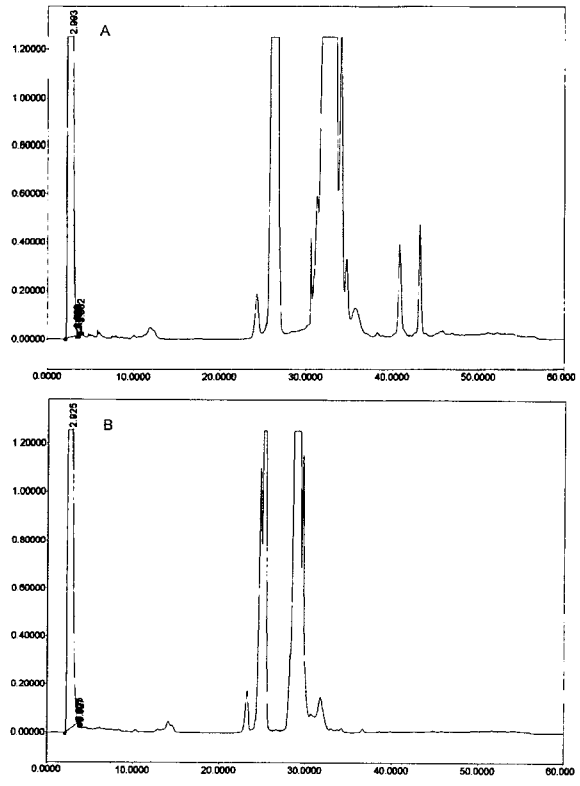

Fig. 1. HPLC-ELSD chromatograms of the total lipid extracts of MCF-7 cells. A: Total lipid extract of MCF-7 cells prepared from conventional method. B: Total lipid extract of MCF-7 cells prepared from new method. HPLC conditions are described in the Materials and Methods.

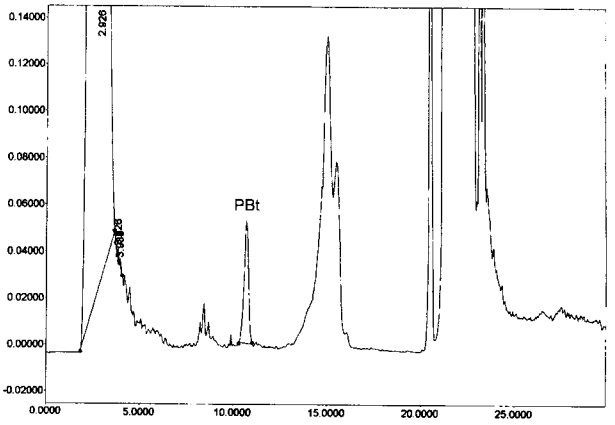

Fig. 2. HPLC-ELSD chromatogram of PBt in the lipid extract of MCF-7 cells. Ten microgram of PBt was spiked in the suspension of MCF-7 cells and lipid extraction was performed with new lipid extraction method. HPLC conditions are described in the Materials and Methods.

PBt can be detected as a defined peak around retention time of 10 min (Fig. 2).

PBt recovery rate test

The HPLC-ELSD system showed a linear response against PBt standards with in a given concentration range of 1 to 0.25 μg (Fig. 3). PBt was added in the cell suspensions before the extraction solvents were mixed. After lipid extraction by conventional and new methods, the samples were dried and redissolved in 100 μL of dissolving solvent (chloroform:methanol=1:2, v/v) and injected in to the HPLC-ELSD system. The average recovery rate of PBt using new lipid extraction method was 20% and relative standard deviation was 15%. When we use conventional lipid extraction method developed by Bligh and Dyer (Varga *et al.*, 2000), the average recovery rate was about 10%, representing that the recovery rate in this new lipid extraction method is about 2 times higher than that in the

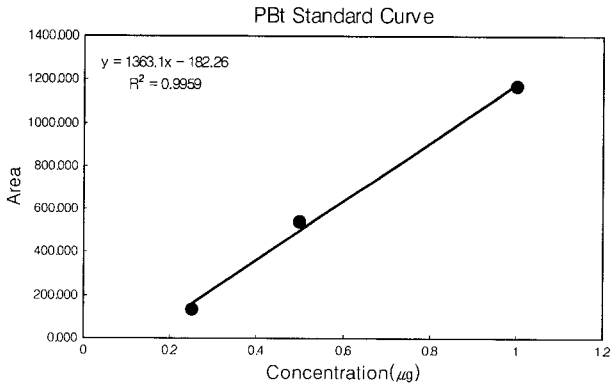

Fig. 3. Standard curve for PBt standard molecule using HPLC-ELSD. ELSD showed linear responses againt PBt within the concentration range of 0.2 to 1. HPLC conditions are described in the Materials and Methods.

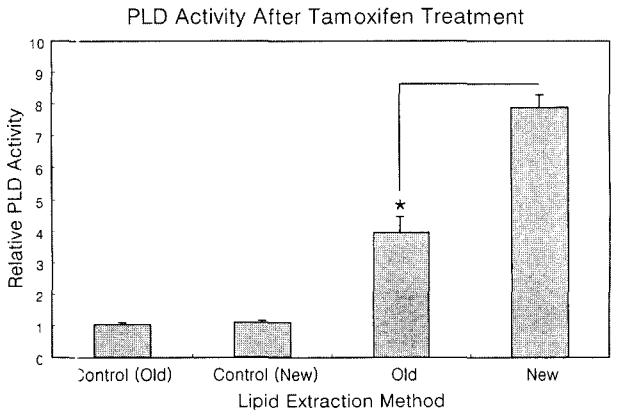

Fig. 4. PLD activity changes induced by tamoxifen. New method showed higher sensitivity against same agonist compared to conventional method. Procedures for PLD activity test was described in the Materials and Methods. Data are mean±S.D. from three separate experiments. $p<0.05$.

conventional method.

PLD activity test

PLD activities of the samples prepared from conventional and new lipid extraction methods were measured using LSC. The PLD activities increased by tamoxifen were 2 to 3 fold maximum in MCF-7 cells with conventional lipid extraction methods. With the new method, the PLD activity change was 7 to 8 fold increase compared to the control sample (Fig. 4). The total counts of the samples prepared from the new method were lower than that from the conventional method, and this result was consistent with the result from HPLC analysis of the total lipid extracts.

The new lipid extraction method showed quite low contamination of other cellular lipids such as phosphatidylcholine, the major phospholipid of cell membrane in the HPLC-ELSD analysis. This result is consistent with the low total count of the lipid extract with LSC analysis. The recovery rates for PBt or PEt with new method are about 2 fold higher than the conventional method. These results suggest that the new method provides a more acidic environment in the extraction procedure and enriches polar lipids like PBt and PEt.

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