Alteration of Phospholipids during the Mitophagic Process in Lung Cancer Cells

Jae Won Lee†, Kyung Mi Cho†#, Jae Hun Jung‡, Quangdon Tran¶, Woong Jung§, Jongsun Park¶, and Kwang Pyo Kim*†

1Department of Applied Chemistry, The Institute of Natural Science, College of Applied Science, Kyung Hee University, Yongin 17104, Republic of Korea
2Department of Pharmacology, Metabolic Diseases and Cell Signaling Laboratory, Research Institute for Medical Sciences, College of Medicine, Chungnam National University, Daejeon 35245, Republic of Korea
3Department of Emergency Medicine, School of Medicine, Kyung Hee University, Seoul 05278, Republic of Korea

Introduction

Mitochondria have critical roles in various cellular processes, including energy production, metabolism, proliferation, differentiation, and cell death [5, 19, 42]. In mitochondria, ATP generation via oxidative phosphorylation occurs through the electron transport chain, fatty acid oxidation, and TCA cycle [12]. The maintenance of healthy mitochondria is critical for cellular function and survival, and mitophagy controls the healthy mitochondria by balancing biogenesis and the turnover of mitochondria [13, 20]. Mitophagy is a lysosomal degradation process that selectively recognizes and eliminates damaged mitochondria to regulate their number and quality [2, 40]. Mitochondrial division is part of this degradation mechanism by creating smaller organelles, which helps autophagosomes engulf the mitochondria. The autophagosomes then fuse with lysosomes to deliver the mitochondria for degradation [44]. The targeted removal of mitochondria via mitophagy is essential to maintain mitochondrial quality, cell viability, and homeostasis.

Previous studies have reported that mitochondrial dysfunction is related to several diseases [26, 33]. In particular, two Parkinson’s disease-associated genes, PINK1 and Parkin,
are involved in the maintenance of healthy mitochondria through mitophagy [9]. Treatment with carbonyl cyanide m-chlorophenylhydrazone (CCCP) induces the recruitment of Parkin to degrade mitochondria and promotes mitophagy. Thus, CCCP treatment has been widely applied to study the mechanism of cellular mitophagy [7, 11, 41].

In cells, the dynamic behaviors of mitochondria, including their movement, fusion, and division, are controlled by their structure [16]. In particular, recent studies have reported that phospholipids in mitochondria have roles in mitochondrial dynamics by interacting with dynamin GTPases and altering the biophysical properties of the mitochondrial membranes [8, 46]. Altered composition of phospholipids also promotes mitophagy [16, 29]. Therefore, it would be interesting to profile the mitophagic cellular phospholipids that comprise the cell membranes, which are highly complex and include many hundreds of distinct lipid species [35].

Recent advances in mass spectrometry (MS) allow detailed profiling of phospholipids to study their roles in cellular signaling and disease pathogenesis [14, 21]. In our laboratory, a MALDI-TOF/MS-based analysis method has been established for phospholipid profiling of various biological samples [18, 37]. Furthermore, this method has been used to classify subsets of several cancer types [17, 23, 24]. In this study, we applied this MALDI-TOF/MS technique to analyze phospholipids in mitophagy-induced lung cancer cells. The altered phospholipids in the mitophagic cells were investigated to find correlations between mitophagy and cellular phospholipids.

Materials and Methods

Antibodies and Reagents

Anti-LC3B, anti-p62, and anti-actin antibodies were purchased from Sigma-Aldrich (USA). Anti-Beclin1 antibody was obtained from Aviva Systems Biology (USA) and anti-Fundc1 antibody was obtained from Novus Biologicals (Littleton, USA). Horseradish-peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG secondary antibodies were purchased from Komabiotech (Korea). CCCP was purchased from Sigma-Aldrich. JC-1 dye was purchased from Molecular Probes (USA). HPLC-grade methanol, chloroform, and water were purchased from Burdick and Jackson (USA). 2,5-Dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (CHCA), 9-aminoacridine, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich. P-40, 120 mM NaCl, 25 mM sodium fluoride, 40 mM β-glycerol phosphate, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzanidene, and 2 μM microcystin-LR. Lysates were centrifuged for 15 min at 12,000 × g. The cell extracts were resolved by 15%, 12%, and 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, USA). The membranes were blocked for 1 h in 1× Tris-buffered saline buffer (TBS; 140 mM NaCl, 2.7 mM KCl, 250 mM Tris-HCl, pH 7.4), containing 5% skimmed milk and 0.2% Tween-20, followed by overnight incubation with primary antibodies (diluted 1,000-fold) at 40°C. The secondary antibody was horseradish-peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Komabiotech), diluted 5,000-fold in the blocking buffer. Protein expression was visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Thermo Fisher Scientific, USA).

Confocal Imaging Analysis

H460 cells were grown on glass coverslips until they were 50–70% confluent and then infected and/or transfected with the indicated plasmids. The cells were fixed in 4% paraformaldehyde at room temperature for 10 min and permeabilized in 0.2% Triton X100 at room temperature for 5 min. Coverslips were mounted with Vectashield (Vector Laboratories, USA) and cells were visualized by using a Zeiss confocal microscope.

Mitochondrial Membrane Potential Measurement

Cells were collected by trypsinization and stained with JC-1 dye (Molecular Probes, USA) for 30 min. Then, the cells were treated with 30 μM CCCP for 10 min and analyzed by a Becton Dickinson FACS Canto system.

Extraction of Phospholipids

Phospholipids were extracted from control and mitophagy-induced H460 cells using the Bligh and Dyer method [6]. H460 cells with or without CCCP treatment were collected from each culture dish and then the number of cells was counted by using a hemocytometer. The total number of cells was 8 × 10⁷. They were detached using trypsin for 3 min. After centrifugation, cells were rinsed with PBS twice to remove the debris of culture media and trypsin, and transferred into 3 ml of CHCl₃:MeOH (1:2 (v/v)) in 15 ml conical glass tubes. Each sample was vortexed intensely for 1 min, sonicated for 10 min, and cooled on ice for 10 min. After adding 2.3 ml of CHCl₃:H₂O (1:1.3 (v/v)), the samples were vortexed and centrifuged at 2,500 × g for 10 min. The lower organic phase containing phospholipids was collected and dried by using...
a speed vacuum. All experiments were performed in triplicates.

**MALDI-TOF/MS Analysis**

To make a binary matrix solution for lipid MALDI-TOF/MS analysis, 3.5 mg each of DHB and CHCA was dissolved in 1 ml of 70% methanol containing 0.1% TFA for positive-ion mode or 9-
aminoacridine (5 mg/ml; dissolved in 6:4 isopropanol:acetonitrile (v/v)) for negative-ion mode. The matrix and phospholipids were mixed and pipetted onto stainless steel 384-well target plates (Bruker Daltonics, USA), and then dried in a vacuum desiccator for homogenous sample distribution and crystallization of the sample and matrix. After sample spotting, each spot was analyzed by MS. MALDI-TOF/MS analysis was performed by using an Ultraflex III TOF/TOF mass spectrometer (Bruker Daltonics) equipped with a 200 Hz smart beam laser as the ionization source. The parameters to acquire spectra were as follows: delay, 180 ns; ion source 1, 25 kV; ion source 2, 21.65 kV; and lens voltage, 9.2 kV. The mass range of MALDI-TOF was 550–1,100 Da molecules and an average of 1000 shots/spot was applied. Before each data acquisition, an external calibration was conducted by using calibration standards with mass-to-charge (m/z) ranges of 510–810 Da (positive-ion mode) and 564–906 Da (negative-ion mode). MALDI LIFT analysis was performed directly on the sample spot after the MALDI-TOF/MS analysis. LIFT data were annotated using the lipid database Lipidomics Gateway (http://www.lipidmaps.org).

**Preprocessing of MALDI-TOF/MS Data**

The spectra of each group were obtained 30 and 15 times in the positive and negative ion mode, respectively. All preprocessing steps were carried out using a MALDIquant R package. The peak intensity of single spectra was transformed to a square root scale for variance stabilization and smoothed using a moving average algorithm. The spectrum background was evaluated using the statistics-sensitive non-linear iterative peak-clipping algorithm and used for baseline correction. The intensities of multiple spectra were normalized using the probabilistic quotient normalization method. Features with signal-to-noise ratios greater than 5 were selected as peaks. Peaks affiliated with the same mass were aligned by the statistical regression-based approach using the identification of landmark peaks and estimation of a non-linear warping function.

**Statistical Analysis of MALDI-TOF/MS Data**

In the statistical analyses, MetaboAnalyst 2.0 was used to identify differentially regulated lipids (DRLs) [43]. Missing values in the data obtained from MALDIquant were replaced by half of the minimum positive value in the data. The intensity values of each peak across multiple spectra were mean-centered and divided by the standard deviation. Principal component analysis (PCA) was performed to differentiate the control and mitophagy-induced H460 cells. In the volcano plots, the differentially regulated phospholipids were identified in the comparison of control and mitophagy-induced H460 cells using the following criteria: (i) p values from the t-tests less than 0.01 and (ii) absolute fold changes greater than 1.3. To show the correlation between samples and features, hierarchical clustering of the differentially regulated phospholipids was performed using Euclidean distances and Ward linkage. The Pearson’s correlation coefficients of the features were calculated and clustered to identify correlating peaks.

**Results**

**Induction of Mitochondria-Specific Autophagy by CCCP in Human H460 Lung Cancer Cells**

Mitochondria-specific autophagy, called mitophagy, is a cellular metabolic process that mediates the selective elimination of dysfunctional mitochondria [38]. Mitophagy is triggered by many stresses that may cause depolarization of the outer mitochondrial membrane (OMM). CCCP is a chemical uncoupler of mitochondria that can induce depolarization of the OMM [15]. In the current study, H460 cells were treated with 30 µM CCCP for 8 h to induce mitophagy. To confirm that mitophagy occurred in these cells, the expression of autophagy marker proteins (LC3 and Beclin1) and mitophagy marker proteins (p62 and Fundc1) was analyzed by immunoblot analysis with the corresponding antibodies. As shown in Fig. 1A, the level of LC3B-II was markedly increased in H460 cells treated with CCCP, indicating the initiation of autophagic flux. Another autophagic mediator, Beclin-1, was also found to be upregulated (Fig. 1A, upper panel). p62 and Fundc1 are well established as cargo proteins that recognize mitochondria to be cleared by the autophagic process and deliver them to the autophagosomes [15, 27, 30]. The decreased levels of p62 and Fundc1 in CCCP-treated samples indicated that mitophagy had occurred in these cells (Fig. 1A, lower panel). In agreement with these findings, mitochondrial membrane potential decreased by CCCP treatment (Fig. 1B). To further confirm these observations, confocal microscopic analysis was performed. As expected, the accumulation of GFP-LC3B punctae was enhanced in CCCP-treated H460 cells (Fig. 1C). Moreover, the mitochondrial localization of GFP-Parkin, which is associated with depolarization and mitophagy [38], was also stronger in CCCP-treated cells (Fig. 1D). Taken together, these data show that treatment of H460 cells with CCCP depolarized the mitochondria, leading to mitophagy.

**MALDI-TOF/MS Analysis of Phospholipids in Mitophagic Lung Cancer Cells**

MALDI-TOF/MS was applied to analyze alterations in phospholipids in the mitophagy-induced H460 cells. Fig. 2A
summarizes the experimental workflow. Using the Bligh and Dyer method, phospholipids were extracted from control and mitophagy-induced H460 cells. The lipid extracts were subjected to MALDI-TOF/MS analysis in positive and negative ion modes. Lipid identification was performed based on LIFT mode and LIPID MAPS. MALDI-LIFT (MS/MS) analysis was directly performed on the sample spot after MALDI-MS to determine the lipid structure. Lipidomics Gateway (http://www.lipidmaps.org) was used to identify lipids based on major fragment ions of the MS/MS spectrum after manual monoisotope selection. For example, the fragmentation spectrum of m/z 798.6; [PC 34:1] [M+K]+ showed peaks corresponding to specific choline head groups containing phosphate (m/z 184), potassium adducted phosphate (m/z 163), and other distinct peaks corresponding to phospholipids (Fig.S1). Other phospholipids were also identified by using LIFT mode and their fragmentation spectra. Data processing was performed using MALDIquant and R package. Finally, MetaboAnalyst was applied for the statistical analysis of datasets.

MS-based lipidomic analysis was performed (biological triplicates and 10 technical replicates), generating a total of 45 lipid profiles for H460 cells (whole cell lysates of H460, n = 30 in positive mode, n = 15 in negative node) and H460 cells treated with CCCP (whole cell lysates of H460/CCCP, n = 30 in positive mode, n = 15 in negative mode).

We compared the representative MALDI-TOF/MS chromatograms of phospholipids from control and mitophagy-induced H460 cells in positive-ion mode (Fig. 2B).
Altered peaks were evident in the chromatograms of mitophagy-induced H460 cells compared with the control. Next, we applied principal component analysis to estimate whether phospholipid profiling based on MALDI-TOF/MS is applicable for the comparison of control and mitophagic lung cancer cells. In the PCA score plot of positive-ion mode data, the two groups of control and mitophagic lung cancer cells were well
separated (Fig. 3A). The MALDI spectra of phospholipids separated two groups with 69.8% total variances, including principal component (PC) 1 (55.3%) and PC 2 (14.5%). Furthermore, these two groups were also differentiated in the PCA score plot of negative-ion mode data with the variances of PC 1 (64.8%) and PC 2 (12.6%) (Fig. 3B). This indicated that the composition of cellular phospholipids in the lung cancer cells was altered by mitophagy.

Identification and Quantification of Phospholipids That Were Altered in the Mitophagic Lung Cancer Cells

MALDI-TOF/MS was used to analyze several phospholipid species in the control and mitophagic lung cancer cells. In the positive-ion mode, 23 phosphatidylcholines (PCs), 4 sphingomyelins (SMs), and one lysophosphatidylcholine (LPC) were analyzed with three adducted ions, a hydrogen ion (H\(^+\)), a sodium ion (Na\(^+\)), and a potassium ion (K\(^+\)). Fig. 4A shows the hierarchical clustering and heat map indicating upregulated (red) or downregulated (green) differentially regulated phospholipids (DRLs) in the mitophagic lung cancer cells compared with control cells. Among the 28 lipid species, 11 were selected as DRLs with fold change (FC) > 1.3 and p value < 0.01 in the volcano plot (Fig. 4B). These DRLs are listed in Fig. 4C. Compared with the control, nine lipids (PC {28:0} [M+H\(^+\)], PC {30:1} [M+H\(^+\)], PC {32:2} [M+H\(^+\)], PC {30:0} [M+H\(^+\)], SM {38:1} [M+H\(^+\)], SM {d40:2} [M+H\(^+\)], PC {P-38:3} [M+H\(^+\)], PC {34:4} [M+H\(^+\)], and PC {34:3} [M+H\(^+\)]) were downregulated and two lipids (PC {34:5} [M+Na\(^+\)] and PC {34:1} [M+K\(^+\)]) were upregulated in the mitophagic lung cancer cells.

Next, 11 phosphatidylinositols (PI) and one lysophosphatidylinositol were analyzed as [M-H\(^-\)] ions in the negative-ion mode. The hierarchical clustering and heat map showed the upregulated and downregulated lipid species in the mitophagic lung cancer cells (Fig. 5A), and three lipids (PI {20:4/18:2} [M-H\(^-\)], PI {18:0/22:6} [M-H\(^-\)], and PI {18:1/22:6} [M-H\(^-\)]) were selected as DRLs (fold change (FC) > 1.3, p value < 0.01) in the volcano plot (Fig. 5B). These three PIs were downregulated in the mitophagic lung cancer cells (Fig. 5C).

The above statistical analyses revealed that phospholipids such as PC, SM, and PI were downregulated in mitophagic lung cancer cells compared with control cells. To quantitatively characterize the upregulated or downregulated phospholipids, we presented a bar graph of individual species in the control and mitophagic lung cancer cells.

**Fig. 4.** Quantification of phospholipids (determined in positive-ion mode) altered in mitophagic lung cancer cells. (A) Hierarchical clustering of each sample data set showing differentially regulated phospholipids (DRLs) analyzed in the positive-ion mode. (B) Volcano plot including DRLs in mitophagic cells compared with control, determined in positive-ion mode (fold change > 1.3, p value < 0.01). (C) The list of DRLs.
In the quantification of individual PC species, almost all compounds were downregulated in the mitophagic cells. In our experiments, various species of SM and PI were also downregulated in the mitophagic cells.

**Discussion**

PC, which is one of major components of the membrane bilayer, is associated with various cellular progresses [10, 34]. In particular, a PC synthetic defect is related to mitophagy in muscle disease [29]. In normal muscle fibers, the mitochondria are distributed uniformly. Choline kinase is the primary enzyme in de novo biosynthesis of PC. Loss of choline kinase activity decreases the PC levels of the mitochondrial membrane, leading to the dysfunction of mitochondria. These mitochondria are eliminated by mitophagy, which can induce the sparse mitochondria in the muscle fiber [28]. Similar to this previous report, our results also indicated that mitophagic cells exhibit quantitative downregulation of PCs. Thus, PC appears to be essential for the maintenance of healthy mitochondria, and a lack of PC can induce mitophagy.

SM is one of the sphingolipids with essential roles in various cellular signaling pathways [3, 22, 31]. Furthermore, sphingolipids are highly associated with autophagy [1, 39, 45]. It was previously reported that ceramide and sphingosine-1-phosphate promote autophagy in cancer cell lines [25, 32]. In sphingolipid metabolism, ceramide can be regulated via the hydrolysis of SM by sphingomyelinase. Thus, downregulated SM in the mitophagy-induced H460 cells might be correlated with ceramide that can induce mitophagy [36]. Phosphatidylinositol 3-kinase, which phosphorylates PI at position 3 of the inositol ring, is known as a lipid kinase that regulates autophagy [4]. Thus, the downregulation of PI seems to be consistent with altered lipid metabolism in the mitophagic cells.

In conclusion, MALDI-TOF/MS was used to analyze alterations in phospholipids in control and mitophagy-induced H460 cells. Several phospholipids, including PC, SM, and PI, were successfully analyzed in the positive and negative ion modes. The MALDI-TOF/MS data were subjected to PCA for the differentiation of control and mitophagic cells, and the two were discriminated well in the PCA score plot. Hierarchical clustering and the heat map also presented the pattern of up/downregulated phospholipid species in the mitophagic cells. The volcano

---

**Fig. 5.** Quantification of phospholipids (determined in negative-ion mode) altered in mitophagic lung cancer cells. (A) Hierarchical clustering of each sample data set showing differentially regulated phospholipids (DRLs) analyzed in the negative-ion mode. (B) Volcano plot including DRLs in mitophagic cells compared with control, determined in negative-ion mode (fold change > 1.3, p value < 0.01). (C) The list of DRLs.
Phospholipids Altered in the Mitophagic Process 1797

November 2016
Vol. 26
No. 10

plot indicated that almost all the molecular species of PC, SM, and PI were downregulated in the mitophagic cells. Furthermore, the quantitative downregulation of these lipid species was represented in a bar graph. Finally, our data indicate that mitophagy can induce altered metabolism of cellular phospholipids. These results were consistent with the previous reports of lipid metabolism correlated with mitophagy. MALDI-TOF/MS was an effective method to analyze altered phospholipids in mitophagic cells. In the future, more detailed profiling of various phospholipids is required to study the lipid metabolism of mitophagic cells in more depth.

Acknowledgments

This work was supported by the Bio and Medical Technology Development Program (Project No. 2012M3A9B6055305) through the National Research Foundation of Korea funded by the Korean Ministry of Education, Science and Technology, Korea.

References


Fig. 6. Bar graph showing individual species of PC (A), SM (B), and PI (C) in control and mitophagy-induced H460 cells. *, fold change > 1.3, p value < 0.01.


