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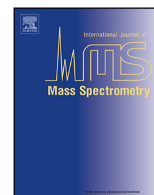
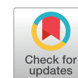


Full Length Article

Metabolomic study of mouse embryonic fibroblast cells in response to autophagy based on high resolution gas chromatography–mass spectrometry



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ABSTRACT

Autophagy is a lysosome-based degradation process, which is vital for cellular homeostasis. In recent years, its dysfunction has been reported to be closely related with various human diseases. Even though studies about autophagy from genomics and proteomics have been extensively conducted, the downstream metabolites and metabolic pathways associated with autophagy are rarely elucidated. In this study, we developed a high resolution gas chromatography-quadrupole orbitrap mass spectrometry (GC-Q Orbitrap MS) method to conduct the metabolomic analysis of mouse embryonic fibroblast cells (MEFs) in response to autophagy by comparing wide type MEFs (WT MEFs) with Atg7-knockout MEFs (Atg7$^{-/-}$ MEFs) under starvation. After data acquisition and multivariate statistical analysis, 32 metabolites with significant difference ($p < 0.05$, VIP > 1) were successfully identified by the library search and retention index (RI) calculation. Further metabolic pathway analysis proved that 34 metabolic pathways were significantly altered, which were mainly involved in carbohydrate, lipid, amino acid and other metabolism. Our results not only proved that carbohydrate metabolisms play an important role during autophagy along with amino acid metabolism and lipid metabolism, but also provided more autophagy-related target molecules, which contributes to the mechanism study of autophagy.

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1. Introduction

Autophagy is a lysosome-based degradation process of cellular components including dysfunctional proteins and damaged organelles under various stress, which is an evolutionarily conserved process and is vital for maintaining cellular homeostasis [1–3]. Since the mechanisms of autophagy were firstly reported, its key roles in a series of physiological processes have been revealed. Especially in recent years, autophagy has been reported to be closely related with various human diseases such as cancers, neurodegenerative disorders, aging, innate and adaptive immunity and so on [4,5]. However, more and more evidence proved that autophagy can prevent diseases with a cytoprotective mechanism, while the dysfunctional autophagy may lead to pathology [6,7]. Thus better understanding of autophagy from the molecular level is significant to ultimately realize the regulation of autophagy and find news to prevent or treat diseases.

Current researches on autophagy mainly focuses on upstream autophagy-related genes (Atg) or proteins [8–10], while the downstream metabolites associated with autophagy are rarely studied. Metabolomics is an emerging '-omics' approach of system biology, which targets on the comprehensive or targeted profiling of small molecule metabolites in cells, tissues, or whole organisms [11,12]. Since metabolites can reveal physiological processes that are happening or have happened and most of the biological effects will be magnified at the downstream metabolic level [13], study of autophagy from the perspective of metabolomics will contribute to revealing autophagy-related target molecules and deducing autophagy related metabolic pathways, which will be helpful to fully understand the molecular mechanisms of autophagy. Atg7, one of the core Atg genes, is essential in the formation of the autophagophore. It has been proven that autophagy will exhibit lower levels or even be blocked without Atg7 [14,15]. In our previous work, we have conducted metabolomic analysis and lipidomic analysis of mouse embryonic fibroblast cells (MEFs) with and without Atg7 under acute starvation based on liquid chromatography-mass spectrometry (LC–MS) [16,17], which have

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preliminarily revealed the metabolites and metabolic pathways related with autophagy, especially the lipid metabolism.

However, LC–MS is not good at detection non-polar metabolites, which limits the comprehensive analysis of the related metabolic pathways. In addition, due to the limited databases and standard spectra, identification of unknown metabolites based on LC–MS is usually time-consuming and exhausted [18,19]. Compared with LC–MS, gas chromatography-mass spectrometry (GC–MS) facilitates the identification of metabolites with commercial libraries and offers a broad coverage of compound classes including polar and nonpolar compounds organic and amino acids, sugars, sugar alcohols, lipophilic compounds [20]. Hydrophilic compounds can even be separated efficiently and detected successfully after derivatization [21,22]. Particularly in recent years, the combination of GC with high resolution MS offers notably fast scan and high mass accuracy, which further promotes the applications of GC–MS in metabolomics researches. Currently, GC–MS based metabolomics has been widely applied in a variety of biological samples such as serum [23], plasma [24,25], urine [21,26], cerebrospinal fluid (CSF) [27] to explore the underlying mechanisms or potential biomarkers of various physiological or pathological processes [28].

Therefore, in the current study, a high resolution GC-quadrupole orbitrap mass spectrometry (GC-Q Orbitrap MS) method was developed to explore the metabolic difference between wild-type (WT) MEFs and $Atg7^{-/-}$ MEFs under 2 h' starvation to reveal the metabolites and metabolic pathways associated with autophagy. We successfully identified 32 metabolites with significant difference during autophagy and further analyzed the related metabolic pathways, which were mainly associated with amino acid, carbohydrate and lipid metabolism. Our results provided more autophagy-related target molecules and gave new insights into the molecular mechanism of autophagy from the perspective of metabolomics.

2. Materials and methods

2.1. Chemical reagents

Methanol (HPLC grade), *n*-hexane (HPLC grade), heptadecanoic acid (≥98%), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA, for GC derivatization), methoxyamine hydrochloride, pyridine (anhydrous), *n*-decane (C10), *n*-eicosane (C20) and Earle's Balanced Salt Solution (EBSS) were obtained from Sigma-Aldrich China Inc. (Shanghai, China). Ultra-pure water was from HangzhouWahaha Group Co., Ltd. (Zhejiang, China). Dulbeco's modified Eagle's medium (DMEM), Phosphate buffered solution (PBS) and Trypsin-EDTA (0.25%) were purchased from Hyclone (Beijing, China). Fetal Bovin serum and GluMAX were from Life Technologies Corporation (Grand Island, NY, USA). *n*-Alkane Solution (C10-C39) was from ANPEL Laboratory Technologies (Shanghai) Inc.

2.2. Cell culture experiments and sample preparation

Wild-type and $Atg7^{-/-}$ MEF cell lines were cultured in DMEM supplemented with 10% FBS and 1% (v/v) penicillin/streptomycin at 37 °C in an atmosphere of 5% CO_2. The medium was changed every 24 h.

Wild-type and $Atg7^{-/-}$ MEFs were seeded in 10 cm culture dishes respectively for about 20 h till the cell density was about 70%–80%. EBSS was used to afford the starvation condition to induce autophagy. After exposed to EBSS for 2 h, cells (four dishes as replicates, n = 4) were washed by cold PBS and cold water subsequently. Then 1.8 mL cold methanol (containing 10 μg/mL heptadecanoic acid as the internal standard) were added to extract metabolites. Briefly, the cells were scraped, homogenized and transferred into 2 mL tubes. After 5 min sonication, samples were centrifuged at 14,000 rpm at 4 °C for 15 min. The supernatants were then aspirated and filtered through a 0.22 μm PTFE membrane. After that, 400 μL of the filtered supernatant was transferred to a new Eppendorf tube for following vacuum-dried (without heating) in Savant™ SPD111 SpeedVac™ centrifugal vacuum concentrators (Thermo Fisher Scientific).

The dried sample was dissolved in 60 μL of methoxyamine solution (20 mg/mL in pyridine), and then under ultrasound treatment for 5 min at room temperature to dissolve as many metabolites as possible. Subsequently, the sample was placed in the Thermo-Shaker (Thermo Fisher Scientific) at 37 °C for 2 h oximation reaction followed by silylation reaction with 60 μL of MSTFA also in the Thermo-Shaker at 37 °C for 1 h. Ultimately, the derivatized samples were centrifuged again at 14,000 rpm for 15 min at 4 °C to discard the compounds insoluble in the derivatization solution, avoiding producing unrepeatable results and making the syringe for GC injection blocked. Then the supernatant was transferred to a conical insert in 2-mL glass vial for subsequent GC-Q Orbitrap MS analysis.

2.3. GC-Q Orbitrap MS

1 μL of the derivatization sample was analyzed by the high resolution GC-Q Orbitrap mass spectrometry (Thermo Scientific™ Q Exactive™ GC hybrid quadrupole-Orbitrap GC–MS/MS, Thermo scientific, USA). Helium was used as the carrier gas in the constant flow rate of 1.2 ml/min and the split ratio was 25:1. The separation was achieved by a TG-5HT capillary column (30 m × 250 μm × 0.25 μm) and the oven temperature program was set as follows: initially kept at 70 °C for 2 min, increased at the rate of 10 °C/min to 180 °C and kept for 5 min, then ramped to 320 °C at 20 °C/min, held for 5 min. The temperature of inlet, transfer interface and ion source were set at 280 °C, 280 °C and 250 °C respectively. The ionization mode of metabolites was electron impaction (EI, −70 eV) and the voltage of detector was set at 1600 V. Mass signals were acquired in full scan mode with the range of *m/z* 50–600 after 3 min filament on delay.

The quality control (QC) samples were prepared by mixing equal aliquots (50 μL) of all analytical samples, then treated and analyzed in the same way as other samples to investigate the repeatability and reproducibility of the methods. The instrument was tuned and calibrated before analysis to ensure the mass accuracy within 2 ppm. Blank samples (hexane, n = 2) and QC samples (n = 3) were injected prior to the sample analysis to ensure system equilibrium. The samples were randomly processed alternately according to the sample types. A blank sample and a QC sample were injected every 4 samples to monitor the reproducibility and stability of the method. All the derivatized samples were analyzed within 24 h. The *n*-Alkane mixture solution was injected to obtain the retention times of *n*-alkanes for calculating the Kovat's retention index (RI) of metabolites instantly after running all the samples.

2.4. Data processing

The acquired MS data were firstly transformed into Trace Finder Software (Thermo Fisher Scientific, USA) to do the data pretreatment process including ion peaks filtration, deconvolution, peaks matching, retention time alignment and so on. A data set containing ion peaks (represented as peak @RT *m/z*) was generated and were further applied for data processing by SMICA. Peaks with RSD (relative standard deviation) of the area in QC samples larger than 30% were removed from the data set. Besides, all the peak areas were corrected by internal standard in the same sample to obtain the corrected peak areas. After that the corrected data set were imported into SMICA to do the principal component analysis (PCA)

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