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研究課題名（和文）Development of a novel technique for direct analysis of single lipid droplets in mammalian cells by nanoESI-MS

研究課題名（英文）Development of a novel technique for direct analysis of single lipid droplets in mammalian cells by nanoESI-MS

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研究成果の概要（和文）：単一のLDレベルでの質量分析による脂肪滴の分析は、依然として分析上の課題です。 この作業では、単一のLD内のホスファチジルコリンとトリグリセリドの分離とプロファイリングのためのチップ内溶媒マイクロ抽出質量分析と呼ばれる新しい手法を開発しました。 この方法は、哺乳類細胞のLDを分析し、さまざまな条件で誘発されたLDのトリグリセリドとホスファチジルコリンのプロファイルを比較するために使用され、成功しています。 私たちの方法は、脂質生産のメカニズム、脂質パッケージング、およびそれらの病態生理学的役割についての理解を深めるために.

研究成果の学術的意義や社会的意義
新しい脂質評価法を開発し、人類の健康増進に貢献する。単一のLDレベルでの質量分析による脂肪滴の分析は、脂質生産のメカニズム、脂質パッケージング、およびそれらの病態生理学的役割についての理解を深めるために、基礎的な脂質生物学などの分野に適用される可能性があります。本研究成果は、このような生命システムを脂質分子種から網羅的に捉えることを可能とし、代謝異常が病態の背後に潜む疾患の分子メカニズムの解明に貢献できると考えられます。

研究成果の概要（英文）：The analysis of lipid droplets (LDs) by mass spectrometry (MS) at the single LD level is still an analytical challenge. In this work, we developed a novel technique termed in-tip solvent microextraction mass spectrometry for the separation and pro-filing of phosphatidylcholines and triglycerides within a single LD. This method has been successfully used to analyze LDs in mammalian cells and to compare the profiles of triglycerides and phosphatidylcholines in LDs induced at different condi-tions. Our method has the potential to be applied to such fields as fundamental lipid biology, to further our understanding on the mechanisms of lipid production, lipid packaging, and their pathophysiological roles.

研究分野：Analytical Chemistry

キーワード：Lipid droplet　Mass spectrometry　Microextraction

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１．研究開始当初の背景

Lipid droplets (LDs), known as an energy reservoir organelle in almost all types of eukaryotic cells, are crucial for lipid metabolism[1]. LDs have crucial roles in lipid metabolism and excessive intracellular accumulation of LDs is related to various prevalent human metabolic diseases, including obesity, diabetes, steatosis, arteriosclerosis and non-alcoholic fatty liver disease (NAFLD)[2]. All lipid droplets share the same structure-a hydrophobic oil core of the storage lipids, which mainly comprise triacylglycerols (TAGs) and cholesterol esters (CEs), is shielded by a phospholipid monolayer that contains specific proteins[3]. LDs were originally thought to be only a passive cellular storage site for excess lipids. However, in recent years, proteomic, ultra-structural and functional studies have shown that LDs are multi-functional organelles with important roles in multiple biological processes. Emerging evidence also links aberrant storage of neutral lipids in LDs to human metabolic syndrome. Many aspects of LDs have been studied, including composition analysis[4]. The recent research reveals that the composition of single LDs might vary and influence the associated proteins[5]. However, no direct evidence on the molecular composition of individual LDs is available.

Mass spectrometry (MS) is widely used in metabonomic, proteomics, clinical medicine, and food safety as a useful analytical tool because of its high sensitivity and rapidity of respond. Nanoelectrospray ionization is a development of electrospray ionization (ESI) for performing limited sample analysis[6]. Nanoelectrospray only requires very low amounts (μL) of very low concentration samples (nmol/mL). The technique also has an increased tolerance to high aqueous solvents and salt contamination. Therefore, nanoelectrospray mass spectrometry (nanoESI-MS) is suitable for single cell analysis[7]. Moreover, even the analysis of single organelles became possible. Direct organelle mass spectrometry (DOMS) developed by Horn et al allows lipid compositions analysis in single LDs isolated from plant tissues[8]. However, only triglycerides molecules were detected since the vast majority of lipids contained in LDs are TAGs (e.g. ~97% TGs, 1% phospholipids, 2% proteins in a 1-μm diameter LD)[8-9]. Therefore, without separation, it is difficult to simultaneously detect TGs and phospholipids in single LDs due to ion suppression effects.

２．研究の目的

Here, we purpose to develop a novel technique for direct analysis of single lipid droplet in mammalian cell by using nanospray mass spectrometry. In the meantime, we aim to separate PCs and TGs in a single cellular lipid droplet and apply the method to different kinds of LDs to figure out differences between them. This approach will help facilitate new studies about LD heterogeneity and the molecular nature of subcellular compartments in cellular systems.

３．研究の方法

Herein, in this study, we developed a novel technique of in-tip solvent microextraction mass spectrometry (ITSME-MS) to separate PCs and TGs in a single cellular LD (Figure 1). A single lipid droplet with a small amount of buffer solution was sucked into a nanotip using a three-dimensional micromanipulator. The nanotip was subsequently backfilled with the organic solvent suitable for lipid extraction and subjected to nanoESI-MS analysis. A gradient solvent system at the tip zone was formed, from aqueous buffer solution to increasingly hydrophobic organic solvent. As a result, PCs were detected earlier than TGs as it dissolved better in water. This method has been successfully applied for the analysis of LDs in HepG2 cells by mass spectrometry and to compare the lipid profiles in three different kinds of LDs at single LD level. The developed method should find wide applications, such as lipid biology and disease diagnosis, and allows a better understanding of the mechanisms of lipid production, packaging into cytosolic LDs, and pathophysiology.

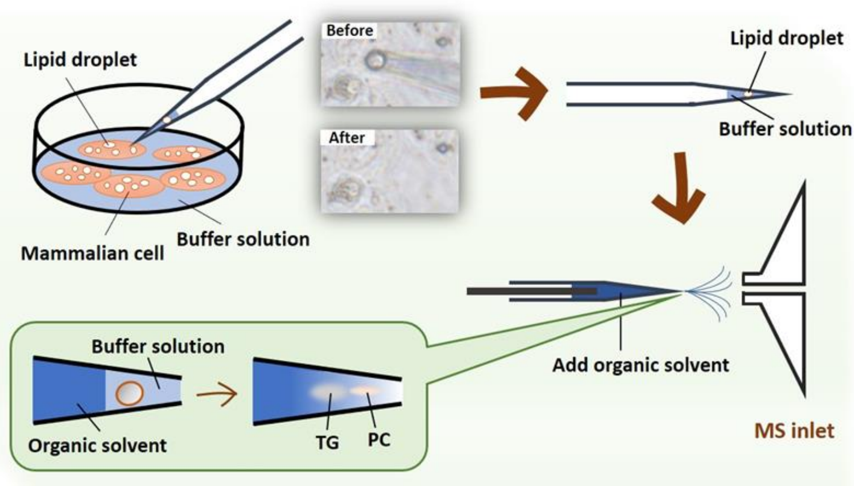

Figure 1. Schematic of single LD analysis with in-tip solvent microextraction mass spectrometry

４．研究成果

1) Single lipid droplet analysis with ITSME-MS

In human and other mammalians, excess fuels are changed into fat for storage. Vigorous fat storage can be seen in the liver as well as the adipose tissue. When human hepatic cells are incubated with fatty acids (FAs), numerous LDs appear in the cell, being a hepatic steatosis model. Firstly, HepG2 cells were incubated with 500 µM oleic acid (OA) for 48 h to produce LDs. The glass nanospray emitter was used to get a single LD from cells by using multifaceted nanomanipulator. The images before and after LD sampling are shown in Figure 2a and 2b. The nanospray emitter was backfilled with organic solvents (methanol:isopropanol=1:1, with 0.1% TFA). The use of this solvent enabled extracting the lipid contents out of LDs and succeeding direct analysis of lipid contents by nanoESI-MS. LD contains TGs as the major core lipid and PCs as the major surface lipid. A series of TGs and PCs could be detected from a single LD in positive-ion mode by our method. Moreover, PCs and TGs were separated in time order. As shown in Figure 2c, PC 34:1 (*m/z* 760.5848) appeared at 0.054 min and reached the maximum at 0.07 min, but TG (54:3) started at 0.07 min and maximized at 0.10 min. The mass spectra at 0.07 min and 0.10 min were exhibited in Figure 2d and 2e. We detected 15 TGs and 8 PCs successfully based on accurate mass.

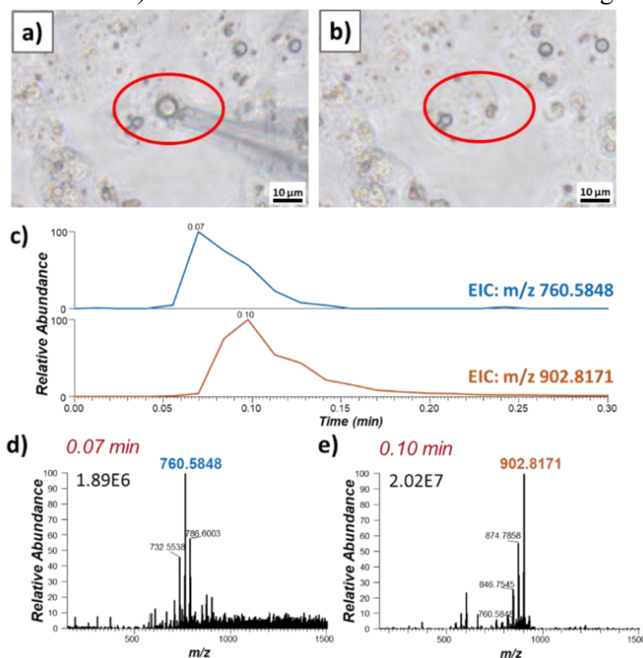

Figure 2. Separation of PCs and TGs in single lipid droplet with in-tip solvent microextraction mass spectrometry. Bright field images of before (a) and after (b) lipid droplets were captured. (c) The extracted ion chronogram of m/z 760.58 (PC 34: 1) and m/z 902.82 (TG 54: 3) during MS analysis in positive mode. (d, e) Representative MS spectra at 0.07 min and 0.10 min, respectively.

It is noteworthy that the intensity of TGs is about 10 times higher than PCs. The size of LDs tested by our method is about 7 µm in diameter. Based on a simple calculation reported by Anke Penno et al[10], the ratio between phospholipid and TG would be 0.1-0.2%. Therefore, if no separation was utilized, it could be hard to detect TGs and PCs simultaneously on one mass spectrum. Thus, this approach can directly show molecular distribution at organelle level in more comprehensive manner than previously achieved.

2) Mechanism study of ITSME-MS

The standard solution of PC 34:1 (16:0/18:1) and TG 45:0 (15:0/15:0/15:0) were prepared to test the methodology developed in this work. About 0.5 µL NH_4COOH solution was put in the capillary from the opposite side to the tip, and was kept to the tip area. Subsequently, the mixture of PC 34:1 and TG 45:0 (2 µL) was added into the capillary as well (Figure 3a), and immediately tested by nano-ESI MS. As shown in Figure 3b, PC 34:1 appeared earlier than TG 45:0 and peaked at 0.05 min, whereas TG 45:0 reached the maximum more slowly until 0.14 min. The mass spectra at 0.05 min and 0.14 min were shown in Figure 3c and 3d. In comparison, the mixture of PC 34:1 and TG 45:0 was also tested directly by nano-ESI. PC 34:1 and TG 45:0 appeared almost at the same time and TG 45:0 even reached its maximum (0.05 min) earlier than PC 34:1 (0.27 min). Besides, the buffer aqueous solution was

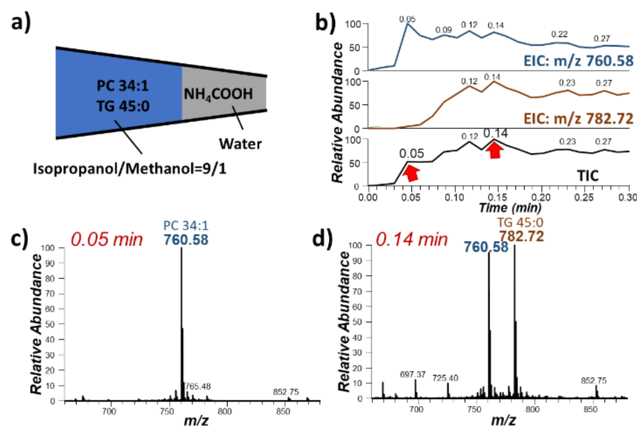

Figure 3. Separation of PC 34:1 and TG 45:0 by in-tip solvent microextraction mass spectrometry. (a) Schematic diagram of the nanospray capillary tip area. (b) The extracted ion chronograms (EIC) of PC 34:1 (m/z 760.58) and TG 45:0 (m/z 782.72) and total ion chronogram (TIC) during MS analysis in positive mode. (c), (d) Representative MS spectra at 0.05 min and 0.14 min.

replaced by pure water to investigate the role of NH_4COOH. The time difference was still observed between PC 34:1 and TG 45:0 appeared as before. However, the signal-to-noise ratio was inferior to before. Therefore, addition of NH_4COOH into the system both maintains cell morphology and improves the ionization of PCs and TGs during the MS detection.

One possible explanation is the formation of gradient mixture in the tip area during the MS analysis. In the area nearer to the tip, water concentration will be higher. PC 34:1 has both hydrophilic and hydrophobic parts, although TG 45:0 has only the hydrophobic part. PC 34:1 is more soluble in water and moves faster than TG 45:0 in the gradient mixture. This explains how PCs appear earlier than TGs.

3) Comparison of lipid profiles in lipid droplets induced at different conditions

Two major dietary fatty acids (FAs), OA and palmitic acid (PA), are preferentially stored as fat in the liver if found in excess[11]. Thus, we used OA and PA to induce LDs in HepG2 cells. Besides, mammalian cells cultured in the presence of fetal bovine serum (FBS) also generate a number of small LDs[12]. It is hard to isolate and purify the targeted LDs by using conventional high-speed centrifugation. One advantage of our approach is to directly analyze only one single LD. Here we examined three different kinds of LDs induced by FBS, OA and PA, respectively. LDs induced by FBS are treated as control group. Oil-red-O staining is commonly used to visualize intracellular lipid accumulation. As shown in Figure 4a, 4d and 4g, treatment with OA and PA both caused fat accumulation in the cytosol and very small amount of LD were produced in the control group.

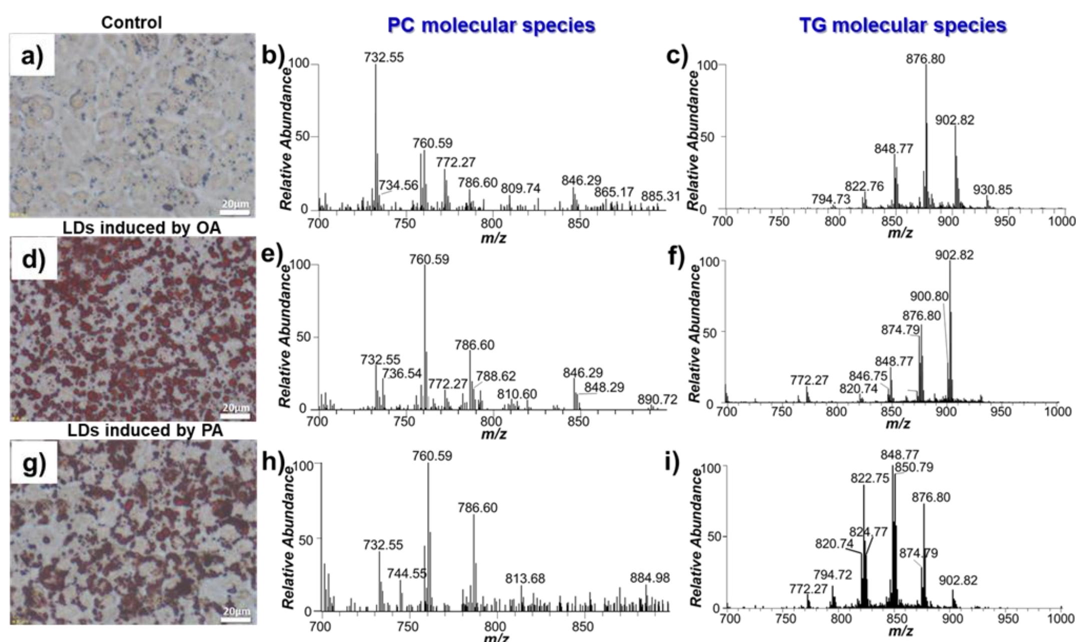

Figure 4. Lipid profiling of three different kinds of LDs. Representative morphological images of Oil-Red-O lipid staining of LDs in control group (a), LDs induced by OA (d) and LDs induced by PA (g). PC molecular species profiles obtained from LDs in control group (b), LDs induced by OA (e) and LDs induced by PA (h) using in-tip solvent microextraction mass spectrometry. (c, d, f) Display the TG profiles obtained from LDs in control group (c), LDs induced by OA (f) and LDs induced by PA (i) using in-tip solvent microextraction mass spectrometry.

The PC and TG molecular species of these three different kinds of LDs were analyzed with in-tip solvent microextraction mass spectrometry. The spectra of PC molecular species and TG molecular species acquired from these three kinds of LDs were shown in Figure 4b, 4c, 4e, 4f, 4h and 4i. There were crucial differences in the relative content of PC and TG molecular species detected between these three different kinds of LDs. For example, the most abundant TG molecular was *m/z* 876.80 in the control LDs, whereas *m/z* 902.82 and *m/z* 848.77 were the most in LDs induced by OA and PA (*m/z* 876.80, *m/z* 902.82 and *m/z* 848.77 corresponded to TG 52:2, TG 54:3 and TG 50:2, respectively).

A similar phenomenon also appeared in LDs induced by PA. TG 48:0, TG 48:1, TG 48:2 and TG 50:1 which contained 16:0 increased obviously. However, the most abundant TG molecule was not TG 48:0 which was made up by three palmitic acid (16:0) acyl chain. It indicates that OA is more inclined to participate in the synthesis of TG compared with PA. In addition, while OA and PA incubation were associated with an increase of FA18:2 and FA 16:1, respectively. FA 18:2 was converted from OA (FA 18:1) and FA 16:1 was converted from PA (FA 16:1) by the desaturase which existed in the human cells. These results indicate that our approach could be used to distinguish LDs with similar morphology by lipidomic profiling.

Similar results discussed above were also observed for PCs species analysis. Compared with control LDs, PC 36:2 and PC 36:3 increased in the LDs induced by OA, and PC 32:1 increased in the LDs induced by PA. The detailed compositional information of PCs was identified through MS/MS

data. The acyl chain of PC 36:2 and PC 36:3 contained oleic acid (FA 18:1). As well, PC 32:1 and PC 32:2 both had palmitic acid (FA 16:0). Our results proved that excess fatty acids not only related to the synthesis of storage lipid (TGs), also participated in the synthesis of membrane functional lipid (PCs).

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5．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　1件／うち国際共著　1件／うちオープンアクセス　0件）

1．著者名 Zhao Yaoyao、Chen Zhen、Wu Yue、Tsukui Takayuki、Ma Xiaoxiao、Zhang Xinrong、Chiba Hitoshi、Hui Shu-Ping	4．巻 91
2．論文標題 Separating and Profiling Phosphatidylcholines and Triglycerides from Single Cellular Lipid Droplet by In-Tip Solvent Microextraction Mass Spectrometry	5．発行年 2019年
3．雑誌名 Analytical Chemistry	6．最初と最後の頁 4466～4471
掲載論文のDOI（デジタルオブジェクト識別子） 10.1021/acs.analchem.8b05122	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

〔学会発表〕　計4件（うち招待講演　0件／うち国際学会　2件）

1．発表者名
Yaoyao Zhao, Yue Wu, Zhen Chen, Zijun Gao, Xinrong Zhang, Hitoshi Chiba, Shu-Ping Hui
2．発表標題
HepG2細胞内の単一脂肪滴に関するnanoESI-MS直接脂質分析
3．学会等名
第43回日本医用マススペクトル学会年会
4．発表年
2018年

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2．発表標題
単一脂質滴のトリグリセリド構成に及ぼす中鎖脂肪酸の影響
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Yaoyao Zhao, Zhen Chen, Yue Wu, Xinrong Zhang, Hitoshi Chiba, Shu-Ping Hui
2．発表標題
Lipid profiling of single lipid droplets in HepG2 cells by using nanospray mass spectrometry (nano-ESI MS)
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Separating and profiling phosphatidylcholines and triglycerides from single lipid droplet in HepG2 cells by in-tip solvent microextraction mass spectrometry
３．学会等名
68th American Society for Mass Spectrometry Conference（国際学会）
４．発表年
2019年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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