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Comprehensive and quantitative analysis of lysophospholipid molecular species present in obese mouse liver by shotgun lipidomics

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Abstract

Shotgun lipidomics exploits the unique chemical and physical properties of lipid classes and individual molecular species to facilitate the high-throughput analysis of a cellular lipidome on a large scale directly from the extracts of biological samples. A platform for comprehensive analysis of lysophospholipid (LPL) species based on shotgun lipidomics has not been established. Herein, after extensive characterization of the fragmentation pattern of individual LPL class and optimization of all experimental conditions including developing new methods for optimization of collision energy, and recovery and enrichment of LPL classes from the aqueous phase after solvent extraction, a new method for comprehensive and quantitative analysis of LPL species was developed. This newly developed method was applied for comprehensive analysis of LPL species present in mouse liver samples. Remarkably, the study revealed significant accumulation of LPL species in the liver of *ob/ob* mice. Taken together, by exploiting the principles of shotgun lipidomics in combination with a novel strategy of sample preparation, LPL species present in biological samples can be determined by the established method. We believe that this development is significant and useful for understanding the pathways of phospholipid metabolism and for elucidating the role of LPL species in signal transduction and other biological functions.

Keywords

Aqueous phase lipidomics; lipotoxicity; lysophospholipid; mass spectrometry; *ob/ob* mice; shotgun lipidomics

Lysophospholipid (LPL) species belong to the category of glycerophospholipids[1]. Individual species of LPL contains a phosphate and a characteristic polar head group linked to the glycerol backbone, and has only a single acyl chain connected to glycerol backbone at either *sn*-1 or 2 position. Similar to their parent glycerophospholipids, LPL species can be classified into different classes based on their polar head groups. The majority of LPL species are membrane-derived signaling molecules produced by phospholipases and widely

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Notes

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distributed in mammalian systems[2, 3]. These species possess diversified cellular functions, including serving as extracellular mediators, induction of cellular proliferation, involvement in development of nervous and vascular systems, transduction of intracellular signal, and association with apoptosis[4–8]. For example, lysophosphatidic acid (LPA) species regulate critical biological functions and disease processes through interaction with multiple LPA receptors[9, 10].

Therefore, detection and quantification of LPL species in biological samples are very important in understanding the role of LPL species in cellular and molecular biology. Determination of the changes of these LPL species under pathological conditions could allow for delineation of biosignatures for diseases, thereby providing not only deep insight into the biochemical mechanisms underpinning the diseases, but also biomarkers for diagnosis and prognosis of the diseases at their earliest stages.

Most phospholipid (PL) species exist in the organic phase after organic solvent extraction utilizing the methods such as Bligh-Dyer, Folch, or others[11–14]. Many analytical methods have been applied for analysis of PL species of a cellular lipidome on a large scale directly from the organic extracts of biological samples[15–19]. However, there are many lipid classes such as LPL species which disperse to the aqueous layer to a great degree after organic solvent extraction[20, 21]. Therefore, methods have been separately developed for analysis of a certain class or a category of LPL species[22–24].

Analysis of LPL species by combining high performance liquid chromatography (HPLC) with electrospray ionization-tandem mass spectrometry (ESI-MS/MS) generally is a powerful tool to detect and quantify a large number of LPL species. The applications of this strategy have been reported in many recent publications. For example, LC-ESI-MS/MS was utilized to measure the content of LPL species in mouse serum and mammalian cells[23], soy protein isolate[25], and human plasma[24]. However, in most of these studies, the investigators analyzed the organic phase of the samples, from which separation of the LPL and PL species by HPLC is time-consuming. Shotgun lipidomics have exploited the unique chemical and physical properties of individual PL classes to facilitate high-throughput analysis of a cellular lipidome on a large scale[15, 26–30]. However, analysis of lipid species is conducted directly from the organic extracts of biological samples. Apparently, a systematic strategy for analysis of the majority of those lipid classes in the aqueous phase is still lacking due to the difficulties in recovery and enrichment of those lipids classes, and in removing the high concentrations of salts in the aqueous phase.

In the classic procedure of Bligh and Dyer extraction, the aqueous phase usually is discarded and cannot be directly used for analysis of the lipids present in the phase by mass spectrometry because of the presence of high salt content. These problems were best solved by the developed method in this study. A simple isolation and enrichment strategy through solid phase extraction (SPE) had been exploited for the LPL species present in the aqueous phase and those LPL species present in both aqueous and organic phases were analyzed by multi-dimensional MS-based shotgun lipidomics (MDMS-SL)[15, 26].

The developed procedure after partial separation and enrichment with an SPE column enabled us to identify and quantify lysophosphatidylserine (LPS), LPA, lysophosphatidylglycerol (LPG), and lysophosphatidylinositol (LPI) species which are largely present in the aqueous phase without the concerns of high salt interference. Thus, lipidomics analysis of both organic and aqueous phase extracts become true with only one-time extraction, which achieves much broader analysis of lipid species without increases in the sample sizes. By applying the developed method, we determined the changes of individual species of six LPL classes including lyso choline glycerophospholipid with acyl or ether linked species (LPC), lysophosphatidylethanolamine (LPE), LPS, LPA, LPG, and LPI in the liver of *ob/ob* mice in comparison to their wild-type littermates.

MATERIALS AND METHODS

Internal Standards and Chemicals

Internal standard (IS) species including 17:0 LPC, 17:1 LPS, 17:1 LPE, 17:1 LPI, 17:1 LPG, and 13:0 LPA were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). These IS species represent the physical properties of the six lipid classes of interest as closely as possible and absent or present in very minimal amounts in lipid extracts. They were dissolved in methanol/chloroform (1:1, *v/v*) or methanol as a premixed solution and stored at −20 °C in glass tubes.

Methanol and ammonium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA). Formic acid was obtained from Electron Microscopy Sciences (Hatfield, PA). All other solvents used for lipid extraction and sample preparation were obtained from Burdick and Jackson (Muskegon, MI). HybridSPE Cartridges and all other chemicals reagents at least at analytical grade were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Animal Model of Obesity

Leptin-deficient *ob/ob* (Lep^{-}/Lep^{-}) mice and wild-type (Lep^{+}/Lep^{+}) littermates (male, 10, 12, and 16 weeks of age, 4 animals per group, C57BL/6J) were used in the study. Genotyping of *ob/ob* mice was performed by PCR. Moreover, wild-type rats were also used in this study for method development. They were housed in a temperature-controlled room (20 – 22 °C) with 12-hour light/dark cycles and were fed with standard rodent chows. Female Sprague Dawley rats (400 – 500 g body weight) were also used in the study. Animals were euthanized by asphyxiation with CO_2 followed by cervical dislocation. Rat plasma was harvested immediately after sacrifice. Murine liver was excised quickly and perfused with ice-cold phosphate-buffered saline (PBS) to remove blood, blotted with Kimwipes (Kimberly-Clark, Roswell, GA) to remove excess buffer, and then immediately freeze-clamped at the temperature of liquid N_2. All of tissue samples were stored at −80 °C until lipid extraction and analysis. All animal protocols used in this study were approved by the Institutional Animal Care and Use Committee at the Sanford-Burnham Medical Research Institute.

Lipid Extraction

A powder sample (approximately 25 mg) from individual liver sample of mouse or rat was weighed and further homogenized in 0.5 ml of ice-cold diluted (0.1x) PBS with a Branson Sonifier S-450 Digital Ultrasonic Cell Disruptor/Homogenizer. Protein assay on each individual homogenate was performed with a Pierce BCA Protein Assay kit (Pierce, Rockford, IL). The IS species including 17:0 LPC, 17:1 LPS, 17:1 LPE, 17:1 LPI, 17:1 LPG, and 13:0 LPA (1,055, 80.5, 184, 88.8, 4.13, and 45 pmol/mg protein, respectively) were added before extraction of liver samples. A similar set of IS species including 17:0 LPC, 17:1 LPS, 14:0 LPE, 17:1 LPI, 17:1 LPG, and 13:0 LPA (90.0, 0.02, 5.0, 1.5, 0.06, and 0.3 nmol/ml plasma, respectively) was added before extraction of plasma samples. Other necessary IS species for global analysis of cellular lipidomes could also be added to each sample based on the protein concentration or sample volume prior to extraction of lipids. Thus, the lipid content could be normalized to the protein content or sample volume and quantified directly. In the experiments for determination of extraction recovery, the identical amounts of internal standards were added after extraction. Then, the determined content of individual LPL species was compared with that as described above to determine the extraction recovery.

Lipid extraction was performed by the modified method of Bligh and Dyer[11] as described previously[31]. The organic phase and aqueous phase were collected into a new glass tube separately. An equal volume of 4% formic acids in methanol was added to the aqueous phase and mixed for 30 seconds using a vortex mixer. Then, the aqueous phase solution was loaded onto a HybridSPE cartridge, which was used to recover and enrich LPL species from biological matrices. The SPE column was washed twice with 2 ml of methanol solution and the lipids trapped in the SPE column from the aqueous phase were eluted with 8 ml of 10% ammonia methanol solution for LPI, LPS, and LPG. Then the column was further eluted with 8 ml of 20% ammonia methanol solution for LPA. The organic phase from Bligh and Dyer extraction and the methanol eluent from SPE (hereafter called aqueous phase) were dried under nitrogen stream separately. Finally, the dried film of organic phase was reconstituted with a volume of 500 μl/mg of tissue protein in chloroform/methanol (1:1, *v*/*v*) and the dried film from aqueous phase was reconstituted with a volume of 200 μl/mg of tissue protein or a volume of 2 μl/μl of plasma in methanol. The resuspensions were stored at −20 °C before analysis by MS.

Mass Spectrometry

Extracted murine liver or rat plasma lipids were prepared for nano-electrospray analysis by diluting individual lipid extract for 20 to 100 folds using methanol for aqueous phase and using a mixture of chloroform/methanol/isopropanol (1:2:4, *v*/*v*/*v*) for organic phase. The diluted samples were then used for MS analysis separately.

Data were acquired on a triple-quadrupole mass spectrometer (Thermo Scientific TSQ Vantage mass spectrometer, San Jose, CA) equipped with an Advion Nanomate nanospray ion source (Ithaca, NY). The Nanomate was controlled using the accompanying ChipSoft software (version 8.3.1). The source voltage and the nitrogen gas pressure were adjusted as needed to maintain a stable spray and good signal, generally the source voltage was 1.15 kV

and the nitrogen gas pressure was 0.55 psi. Experimental sequences for the TSQ mass spectrometer were programmed and controlled using the Xcalibur system software as previously described[16, 32]. The collision gas pressure was set at 1.0 mT and the collision energy (CE) varied with the classes of lipids as well as the scan modes. Typically, a 3 to 5-min period of signal averaging in the profile mode was employed for each tandem MS mass spectrum. Quantification of lipid species by MS was conducted following the principles as previously described[33].

Determination of Optimal Collision Energy for Quantification of LPL Species

Rat liver extracts from both aqueous and organic phases prepared as described above without addition of IS species were equally divided into at least 8 fractions. Different amounts (e.g., varied from 10 to 500 pmol/mg protein for 17:1 LPS in series) of IS species in a mixture were added to each of the fractions. Then a trial and error approach in combination with ramping of varied CE values was used to determine the optimal CE for individual LPL class.

Specifically, a particular precursor-ion scan (PIS) or neutral-loss scan (NLS) used for quantification of an LPL class (Table 1) was acquired from each of the solutions prepared above at the fixed collision pressure of 1 mT with varied CE from 10 to 40 eV at 5 eV per step for first ramping of determination. The tandem MS mass spectrum, which represented the maximal total ion intensity during the CE ramping, was used to estimate the mass content of individual species of the lipid class. Then, the acquired data from all the solutions at different CE in the CE ramping were plotted as I_S/I_X *vs.* C_S/C_X, where, I_S and I_X were the peak intensities of the IS and the species of X of a particular LPL class present in a solution, which both were determined at a particular CE, and C_S and C_X were the concentrations of the corresponding IS and the species as estimated above, respectively. In theory, the only variables here were the I_S and C_S, and the I_x and C_x should be constant under the experimental conditions. The slope of the obtained linear plot represented the effects of CE on instrument response factor of the LPL species of interest (i.e., X). The CE corresponding to the plot with a slope of one represented the optimal CE with the estimated mass content of individual species described above. This step yielded a set of optimal CE values with small differences from the lines with the slope of one from individual species of a class. These determined CE values were averaged and a certain range (± 10 eV) of this averaged CE was ramped again with a fine step of 2 eV. The mass spectrum with the most abundance of ion intensity was used to estimate the content of individual species again. The procedure described above was repeated with this newly obtained content of individual species until the determined content of individual species was within ± 5% of variation in comparison to that of the previously determined one. The averaged CE determined from this last trial was used as an optimal CE for the specific LPL class.

Data Analysis

All tandem MS mass spectra were automatically acquired by a customized sequence subroutine operated under Xcalibur software. Data processing including ion peak selection, baseline correction, data transfer, peak intensity comparison, ^{13}C de-isotoping, and quantitation were conducted using a custom programmed Microsoft Excel macros as

described previously[16]. All data are presented as the means ± SD of at least four separate animals. Statistical significance was determined by a two-tailed student t-test in comparison to control, where *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

RESULTS AND DISCUSSION

Optimization of the Scan Modes of MS/MS for Identification of LPL Species

Any lipid class, possessing sufficient ionization efficiency and informative fragments after collision-induced dissociation (CID) in MS analysis, can be identified and quantified by MDMS-SL as extensively described previously[15, 16, 26, 33, 34]. Therefore, primary experiments were performed to elucidate the informative fragments of individual LPL class and optimize the MS/MS conditions for their identification and quantification. Through product-ion ESI-MS analysis of standards after CID, we characterized the fragmentation pattern of each LPL class of interest. From these fragmentation patterns, specific MS/MS scan modes corresponding to the polar group or backbone of each class of LPL were elucidated.

For example, product-ion analysis of LPI standards (e.g., 16:0 and 17:1 LPI) displayed an informative fragmentation pattern (Figure 1A and B). This fragmentation pattern was extensively validated through product-ion analysis of other LPI species from preparations of biological samples. The fragmentation pattern showed the presence of a common abundant fragment at *m/z* 241 and a unique fragment ion corresponding to the neutral loss of 316 Da. This fragmentation pattern indicated that all LPI species could be detected by both PIS of 241 (PIS241) and NLS of 316 (NLS316). Once the identity of the head group was identified, the acyl chain of LPI species could be derived from the *m/z* of the ion.

It should be specifically pointed out that although all the LPI species could be sensitively detected through either PIS241 or NLS316, the reversed statement that an ion detected with either PIS241 or NLS316 corresponds to an LPI species was not true since possible artificial ions could be detected by these scans. As discussed previously[26], an extra scan should be employed to definitively identify a species by using PIS and NLS techniques. Therefore, both PIS241 and NLS316 were used for identification of LPI species present in lipid extracts of biological samples.

Product ion ESI-MS analysis of LPS standards (i.e., 16:0 and 17:1 LPS) displayed the fragmentation pattern of LPS species (Figure 1C and D), in which a common abundant fragment at *m/z* 153 and a unique fragment ion corresponding to the neutral loss of 87 Da were present. These features indicate that PIS153 and NLS87 could be used to identify and quantify individual LPS species as discussed above. Similarly, through characterization of the fragmentation patterns of other LPL classes, at least two scan modes of MS/MS for each class of LPL species were identified (Figure S1), which were summarized in Table 1. The fragmentation patterns of LPC and LPE species have been elucidated previously[35]. When these characterized scan modes were used to detect the existence of LPL species after solvent extraction, it was found that LPC and LPE species could be detected from the organic extracts, whereas the species of LPI, LPG, and LPS classes were essentially undetectable from the extracts. Moreover, the low abundance of LPA makes it hard to be

identified and quantified together with LPC and LPE in organic extracts since a residual fragment ion at *m/z* 153 could be generated in the ion source from LPC species, which interferences with the analysis of LPA species as previously described[36]. On the other hand, it is well known that LPS species can readily yield corresponding LPA species after loss of serine in the ion source so that the ion source-generated LPA interferences the measurement of LPA if LPA and LPS coexist[23, 37]. Taken together, these considerations led us to develop a new strategy to comprehensively analyze LPL species (see next subsection).

It should be noted that due to the lack of commercial availability for *sn*-2 acyl LPL species, determination of regioisomeric species of LPA, LPE, LPG, LPI, and LPS was not conducted in the current study. Discrimination of regioisomers of LPC species from their sodium adducts has previously been demonstrated[35]. That approach could be easily integrated into the current method.

Optimization of the Procedure Using HybridSPE to Recover and Enrich LPL Species from the Aqueous Phase

Many lipid classes are extractable with organic solvents utilizing classic extraction methods such as Bligh-Dyer, Folch, etc. Because LPL species are comprised of only a single fatty acyl chain and a polar head group, their structures render these lipids more hydrophilic than their corresponding PL species containing two aliphatic chains. Therefore, many LPL classes (e.g., LPS, LPI, and LPG) are largely present in the aqueous phase after organic solvent extraction.

Hybrid SPE has previously been successfully used for preparation of biological samples to remove PL interferences prior to LC-MS analysis[38–41]. The principle of HybridSPE for removal of PL species was based on the Lewis acid-base interaction between Hybrid SPE Zirconia ions and PL species[41]. In this study, this type of column was exploited to enrich and recover LPL species in the aqueous phase following the same principle. Specifically, the phosphate moiety of LPL species is a strong Lewis base (electron donor) that interacts with Zr atoms coated on the silica surface in the SPE column under acidic conditions. The bound LPL species then could be eluted with a basic solution. The varied pH conditions were achieved by variation of the concentration of formic acid or ammonium hydroxide solution in methanol in the study. In the procedure of method development, after loading of aqueous phase onto the column and extensive desalting under acidic conditions, elution was initially conducted stepwise with 1, 5, 10, 15, and 20% ammonium hydroxide solution in methanol and the eluents were subject to ESI-MS analysis for LPL species. It was found that the majority species of LPG, LPI, and LPS were eluted with 10% ammonium hydroxide solution and LPA was largely eluted after washing with 20% ammonium hydroxide in methanol. Although LPC, and LPE could also be detected from the eluent with 10% ammonium hydroxide, their contents in the aqueous phase were relatively much lower than those in the organic phase. Therefore, these two LPL classes were directly analyzed from the organic extracts. The recovery of the sample preparation was greater than 85% in the fractionation of either 10 or 20% ammonium hydroxide in methanol. We found a similar recovery for each LPL class under both types of elution conditions. Therefore, 10% ammonium hydroxide in methanol was chosen in the method. The detail recovery

information of each LPL class was provided in Table S1. The elution of LPA with 20% ammonium hydroxide solved the conflict of in-source fragmentation generated from LPC and LPS.

Optimization of Collision Energy for Quantification of LPL Species

Tandem MS analysis of individual species of a lipid class is a process depending on the structure of individual lipid species[26]. This has led scientists to use at least two IS species for general quantification of individual species of a class when a tandem MS approach was exploited[42, 43]. Ramping CE in a large range at a fixed collision pressure might be used to minimize the differential effects of the collision process on different molecular species of a class, which usually is time consuming. Alternatively, selection of the representative CE to balance the differential effects was frequently employed when numerous species of a class are commercially available. This latter method was frequently employed in our previously studies[44–46]. Unfortunately, there are not many species of each LPL class commercially available for this purpose. Therefore, in the current study, we developed a new method through a trial and error approach in combination with ramping CE energy by using biological samples to achieve this goal as described under “MATERIALS AND METHODS” and demonstrated below with LPS as a representative by using NLS87 for quantification of its species.

It was found that a linear plot of I_S/I_X *vs.* C_S/C_X was essentially obtained for all the species of LPL classes. Figure 2 showed a few linear plots of the selected LPS species varying with different number of carbon atoms and number of double bonds obtained from a CE ramping at individual step of 5 eV. The results demonstrated that, as anticipated, the plots were linear, but the slope of these plots varied. The slope of nearly 1 for each species of LPS represented the optimal CE with minimal effects on different molecular species of LPS. As shown in Figure 2, the optimal CE of 20 eV for 18:0 LPS, of 20 eV for 18:1 LPS, of 22 – 25 eV for 20:4 LPS, and of 20 – 25 eV for 22:6 LPS was obtained. Taken all the species together, an average of the optimal CE for the species of LPS was determined at the CE of 22 eV for the first ramping. Fortunately, the tandem MS mass spectrum by using this CE to determine the content of individual LPS species again yielded a very small difference (< 5%) from those obtained from the previous trial. Therefore, the CE of 22 eV was determined as an optimal value for quantification of LPS species by using one IS and NLS87. The detail information of optimal CE for each scan mode along with other types of information for all determined LPL classes was summarized in Table 1.

To examine the optimized experimental conditions for quantification of LPL species, tandem MS analysis was first performed to determine the peak intensities from an equimolar mixture of two species of each class. The spectra showed that essentially identical ion peak intensities were obtained from the equimolar mixture after ^{13}C de-isotoping of the species (Figure S2).

Determination of Dynamics Range for Quantification of LPL Species

Next, we determined the linearity of quantification of LPL species by using one IS for each LPL class with the optimized CE in the method as described above. For example, different

amounts of 17:1 LPS (from 10 to 500 pmol/mg protein) along with other LPL IS species in a premixed solution were added to the fixed amount of rat liver homogenates during lipid extraction. The mixtures were analyzed by using the settings listed in Table 1 (Figure 3). The linearity of peak intensity ratios of individual LPS species and standards after ^{13}C de-isotoping *vs.* their corresponding molar ratios in the mixtures was analyzed by linear log plots (i.e., $\log[I_X/I_{IS} - b] = \log[C_X/C_{IS}] + c$, which is derived from $(I_X/I_{IS}) = a(C_X/C_{IS}) + b$) as discussed previously[47] (Figure 3E). An essentially identical linear correlation was well obtained for all the LPS species present in rat liver, indicating that the ionization response factors obtained from this method were molecular species independent. The results also demonstrated a broad linear dynamic range of over 4 orders and the low limit of quantification at less than 1.5 fmol/μl. Similar experiments to other LPL classes were also performed to determine the linear dynamic ranges, low limits of detection and quantification, etc. (Table 1). The linear dynamic range of quantification of the method covers the extraction efficiency since as demonstrated with a variety of mass spectra and the determined dynamic range of quantification, the extraction efficiency obviously exceeded the range of this linear dynamic range. The relative standard deviations of sample analysis were around ± 5% for modest to abundant LPL species and ± 10 to 15% for low abundance LPL species, leading to within ± 5% for the total content of individual class. LPL species present in the rat liver were showed in the Figure 4.

Application of the Developed Method for Biological Samples

The developed method was applied for analysis of LPL species present in the liver of *ob/ob* mice and their littermates. Six LPE, three LPA, seven LPG, seven LPS, six LPI, and eleven LPC species present in mouse liver extracts were identified and quantified (Figure 5). The contents of LPL species in mouse liver were in great agreement with those published recently[48], thereby providing a validation of the developed method to a certain degree.

The amounts of individual species of each LPL class in the lipid extracts of the liver from wild-type and *ob/ob* mice were compared (Figure 5). It was revealed that significant changes of these LPL species were present in *ob/ob* mouse liver in comparison to their wild-type controls. Specifically, the content of the majority of LPI, LPE, LPC and LPG species was significantly increased whereas the content of LPA and LPS species was less increased in *ob/ob* mouse liver compared with that of the controls (Figure 5, Figure S3). For example, the LPI content changed from 404.6 ± 33.2 in wild-type mouse liver to 624.2 ± 21.5 pmol/mg protein in *ob/ob* mouse liver. The content of the majority of LPS species in *ob/ob* mouse liver was not significantly different from that of the controls (Figure 5F and Figure S3A).

It is well known that LPA species function as signal molecules that can induce cell growth through interaction with LPA subtype receptors. The content of LPA was only increased slightly, which was very different from the majority of other LPL classes that were increased substantially in *ob/ob* mouse liver. This is likely due to that LPA is consumed for biosynthesis of TAG to a great extent. The significant accumulation of all LPL classes indicates lipotoxicity under obesity conditions. These results are consistent with the fact that lysophospholipids inhibit fatty acid oxidation in the liver and reduce energy expenditure, thereby leading to diet-induced obesity with a high fat diet as previously demonstrated[49].

Additionally, the developed methodology was also applied for analysis of LPL species present in biofluid samples. Table S2 tabulated the determined LPL content of rat plasma. We found that the determined contents of LPL were well comparable to those available in the literature[50] (Table S2).

In summary, a highly accurate, efficient, sensitive, and reproducible method for comprehensive analysis of LPL molecular species by MDMS-SL was developed and showed to be very powerful for identification and quantification of LPL classes and individual molecular species in biological samples. We believe that the developed method should greatly contribute to identifying the role of LPL molecular species in biological systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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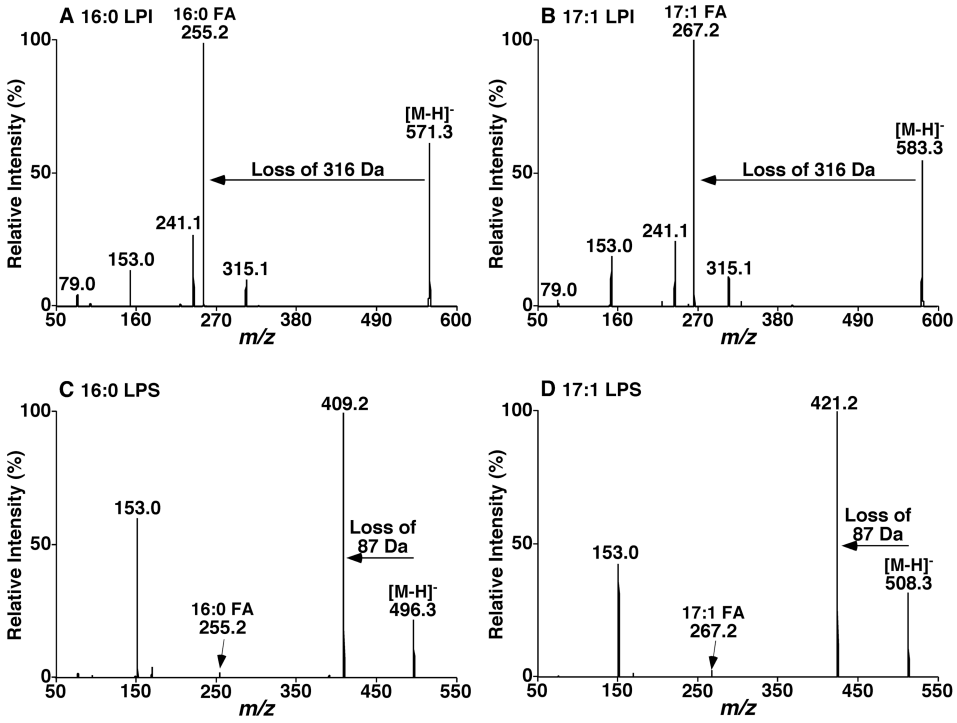

Figure 1.

Elucidation of fragmentation patterns of lysophospholipid classes with representative synthetic species. Product ion ESI-MS analyses of synthetic 16:0 lysophosphatidylinositol (LPI) (A), 17:1 LPI (B), 16:0 lysophosphatidylserine (LPS) (C), and 17:1 LPS (D) at collision pressure of 1 mT and collision energy of 35 and 22 eV for LPI and LPS species, respectively, were performed as described under “MATERIALS AND METHODS”. Tandem MS analysis was performed to identify the common fragmentation patterns of LPI (A and B) and LPS (C and D), which were used for identification and quantification of these LPL species based on the principles of multi-dimensional mass spectrometry-based shotgun lipidomics. FA denotes fatty acyl (or fatty acid).

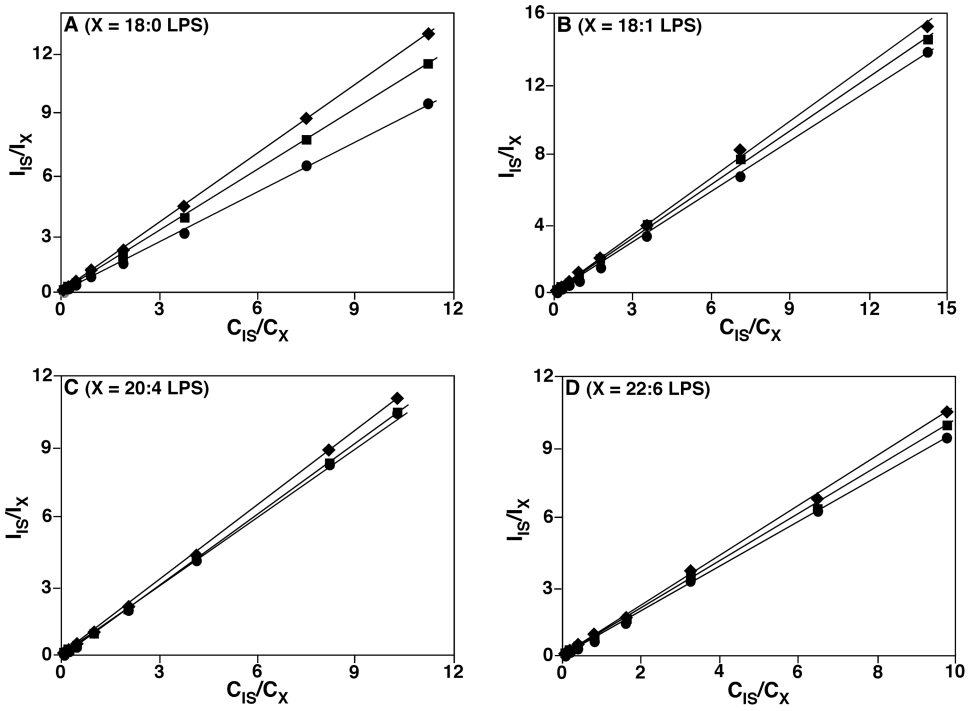

Figure 2.

Demonstration of optimizing collision energy for quantification of lysophospholipid (LPL) species. A series of solutions containing an identical amount of lipid extract from rat liver, but varied amounts of 17:1 lysophosphatidylserine (LPS) as an internal standard (IS) for quantification of LPS species were prepared. These solutions were subject to tandem MS analyses of neutral loss of 87 Da (NLS87) at different collision energy as described under "MATERIALS AND METHODS". Plots of I_S/I_X acquired from NLS87 *vs.* C_S/C_X at CE of 15 (diamonds), 20 (squares), and 25 (circles) eV were illustrated for LPS species (X) of 18:0 (A), 18:1 (B), 20:4 (C), and 22:6 (D), where I_S was the peak intensity of IS (i.e., 17:1 LPS) at the concentration of C_S; whereas I_X was the peak intensity of species X which was present in rat liver extract and estimated as described under "MATERIALS AND METHODS". Since I_X was virtually constant under the experimental conditions, the slopes of the plots only depended on the values of CE. The values of these slopes were used to determine the optimized CE value for individual MS/MS scan mode as described under "MATERIALS AND METHODS". The optimized CE value for individual MS/MS scan mode was summarized in Table 1.

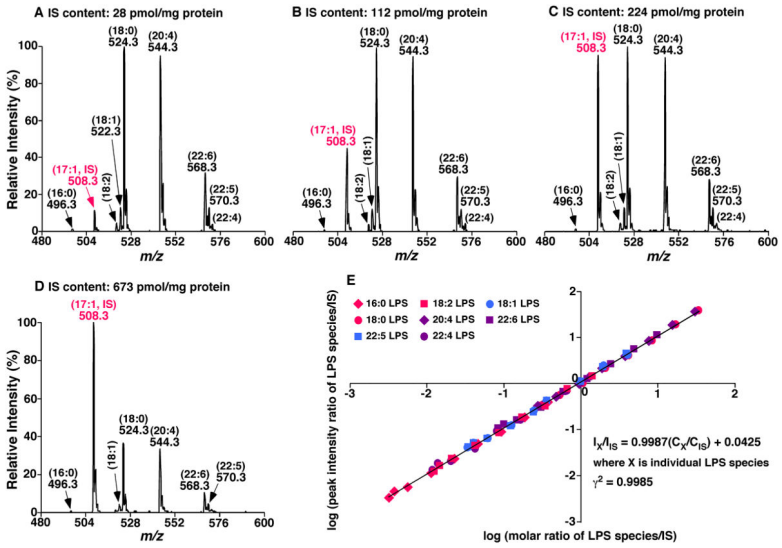

Figure 3.

Representative determination of linear dynamic range of the method with optimized collision energy for quantification of lysophosphatidylserine (LPS) species. A fixed amount of lipid extract from rat liver was added with different amount of 17:1 LPS as an internal standard (IS) (28 pmol/mg protein, panel A; 112 pmol/mg protein, panel B; 224 pmol/mg protein, panel C; and 672 pmol/mg protein, panel D) for quantification of LPS species present in rat liver extracts. Linear regression (panel E) of peak intensity ratios (I_X/I_{IS}) of liver LPS species (X) and IS vs. their molar ratio (C_X/C_{IS}) was performed and the linear result was plotted as its logarithm format to demonstrate the true linearity of the data as previously described[47].

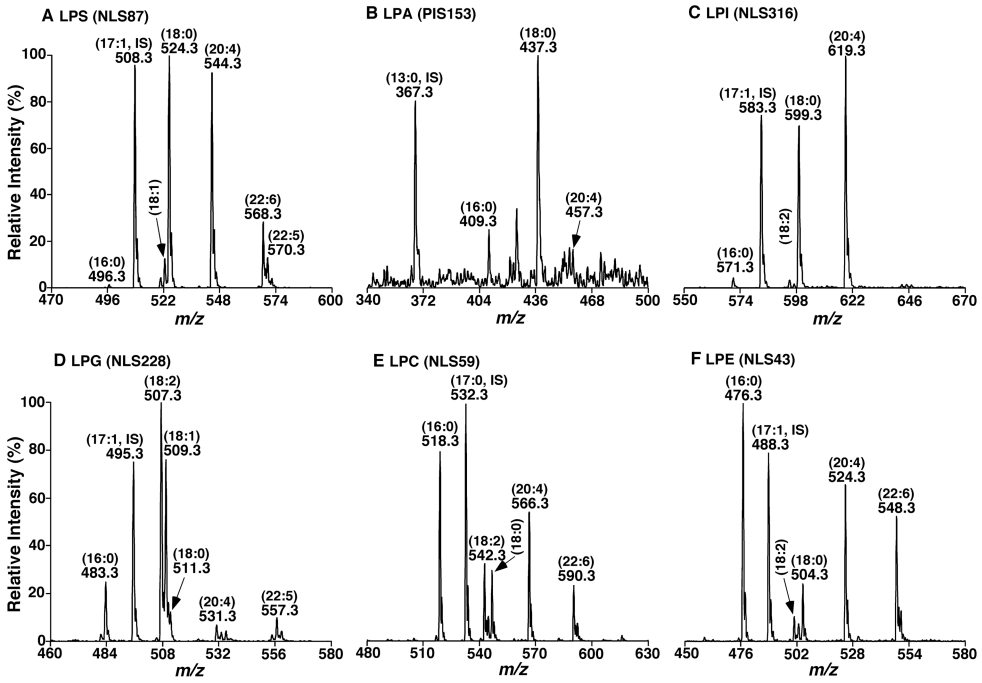

Figure 4.

Representative MS/MS measurement of the content of lysophospholipid (LPL) species in lipid extracts from rat liver. Representative MS/MS spectra of NLS87 (lysophosphatidylserine, LPS, panel A), PIS153 (lysophosphatidic acid, LPA, panel B), NLS316 (lysophosphatidylinositol, LPI, panel C), NLS228 (lysophosphatidylglycerol, LPG, panel D), NLS59 (lysophosphatidylcholine, LPC, panel E), and NLS43 (lysophosphatidylethanolamine, LPE, panel F) were acquired from lipid extracts of rat liver samples as described under “MATERIALS AND METHODS” with the experimental settings summarized in Table 1. NLS, PIS, and IS stand for neutral loss scan, precursor-ion scan, and internal standard, respectively.

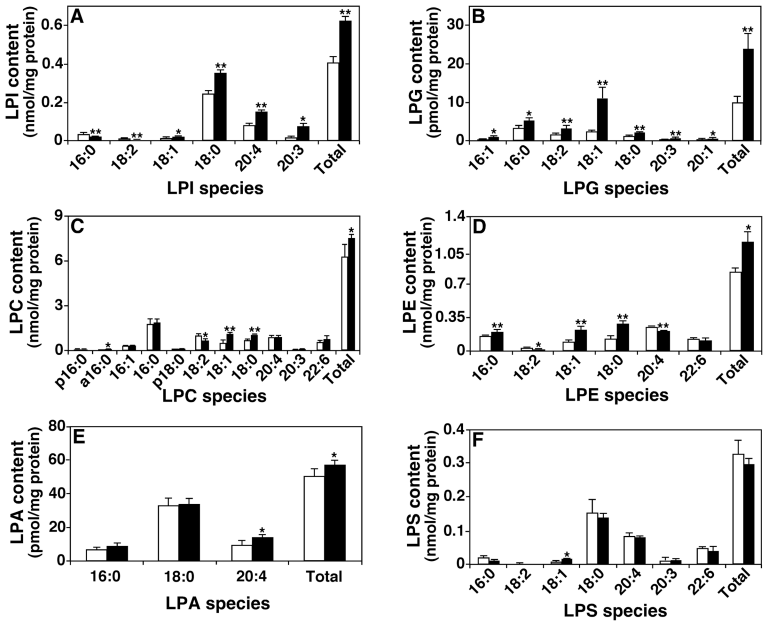

Figure 5.

Comparison of the content of individual lysophospholipid (LPL) species in lipid extracts from wild-type *vs. ob/ob* mouse liver samples. The content of individual LPL species in lipid extracts of wild-type (open bars) *vs. ob/ob* (solid bars) mouse liver samples was determined by tandem MS analysis as illustrated in Figure 4, each of which was obtained by ratiometric comparison of individual ion peak intensity with that of internal standard (IS) of the class. LPI, LPG, LPC, LPE, LPA, and LPS denote lysophosphatidylinositol, lysophosphatidylglycerol, lyso choline glycerophospholipid, lysophosphatidylethanolamine, lysophosphatidic acid, and lysophosphatidylserine, respectively. The prefix “a” and “p” stand for plasmanyl- and plasmenyl-containing species, respectively. All data are presented as the means ± SD of four different animals. Statistical significance was determined by a two-tailed student t-test in comparison to control, where $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Table 1

The MS/MS scan modes and method information for analysis of individual lysophospholipid classes

Ion Mode	Class	Quantitation	Qualification	CE	LOQ	LOD	DR
Negative	LPS	NLS87	PIS153	22/35	1.5	0.5	1.5–15000
	LPA	PIS153	PIS79	30/35	0.5	0.17	0.5–500
	LPI	NLS316	PIS241	35/35	1.2	0.4	1.2–5000
	LPG	NLS228	PIS153	32/35	0.15	0.05	0.15–300
Positive	LPC	NLS59	NLS205 (PIS184)	22/30(35)	15	5	15–50000
	LPE	NLS43	NLS163	20/25	3.9	1.3	3.9–50000

CE: Collision Energy (eV); LOQ: limit of quantitation (fmol/μL); LOD: limit of detection (fmol/μL); DR: dynamic range (fmol/μL).