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UltraPerformance Convergence Chromatography-High Resolution Tandem Mass Spectrometry for Lipid Biomarker Profiling and Identification

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Abstract

Lipids represent biologically ubiquitous and highly dynamic molecules in terms of abundance and structural diversity. Whereas the potential for lipids to inform on disease/injury is promising, their unique characteristics make detection and identification of lipids from biological samples analytically demanding. We report the use of ultraperformance convergence chromatography (UPC²), a variant of supercritical fluid chromatography, coupled to high resolution, data-independent tandem mass spectrometry for characterization of total lipid extracts from mouse lung tissue. The UPC² platform resulted in lipid class separation and when combined with orthogonal column chemistries yielded chromatographic separation of intra-class species based on acyl chain hydrophobicity. Moreover, the combined approach of using UPC² with orthogonal column chemistries, accurate mass measurements, time-aligned low- and high-collision energy total ion chromatograms, and positive and negative ion mode product ion spectra correlation allowed for confident lipid identification. Of great interest was the identification of differentially expressed ceramides that were elevated 24-hours post whole thorax lung irradiation. The identification of lipids that were elevated 24-hours post-irradiation signifies a unique opportunity to investigate early mechanisms of action prior to the onset of clinical symptoms in the whole thorax lung irradiation mouse model.

Keywords

Supercritical fluid chromatography; convergence chromatography; lipidomics; lung irradiation; ceramides

Introduction

Lipids have significant potential to inform on physiology due to the pivotal role lipids play in many biological processes including cellular integrity and permeability, energy storage

and metabolism, and signaling pathways (Shevchenko & Simons, 2010). Heightened interest in the mechanism by which disruption of lipid metabolism and homeostasis contributes to a variety of human diseases and injuries (e.g., cancer, diabetes, neurodegenerative disorders, infectious diseases, and pulmonary conditions) has led to a substantial increase in lipidomic research (van Meer et al., 2008). The field of lipidomics, broadly described as the comprehensive biochemical characterization of “all” lipids (referred to as the lipidome) within a cell, tissue, or organism presents a variety of analytical challenges resulting primarily from the dynamic range, vast structural diversity, and spatial localization of biological lipids (Wenk, 2010).

A variety of analytical techniques have been used in lipidomic studies including direct infusion mass spectrometry (i.e., shotgun lipidomics) (Yang et al., 2009) and coupling of chromatographic techniques, in particular gas chromatography (GC) (Kind et al., 2009) and liquid chromatography (LC) (Nygren et al., 2011), to mass spectrometry (MS). LC separations including both normal phase (Hutchins et al., 2008) and reverse phase (Damen et al., 2014) platforms are the most prevalent separation techniques coupled to MS for lipidomic investigations. The expansion of the application of hydrophilic interaction chromatography (HILIC) separations has added additional selectivity with solvents amenable to ionization (Cífková et al., 2012; Zhu et al., 2012). But, even with the variety in column chemistry, particle size, and solvent systems, reverse phase, normal phase, and HILIC-based LC-MS platforms often require multiple chromatographic separations to cover the diversity in polarity, hydrophobicity, and amphiphilicity observed in biological lipids.

The desire for LC-MS biomarker profiling efforts to analyze lipids of substantial diversity in a minimal number of chromatographic runs demands separations that can efficiently cover lipid diversity. An alternative to GC and LC separation techniques is the use of supercritical fluid chromatography (SFC) (Grand-Guillaume Perrenoud et al., 2014; Nováková et al., 2014). SFC involves the use of a supercritical fluid as the mobile phase. The advantages of using a supercritical fluid for chromatographic separation is it provides lower viscosity and higher diffusivity over LC solvents which translates to greater separation efficiency and unlike GC is not limited to volatile, low molecular weight molecules (Klesper et al., 1962; Poe & Schrodén, 2009; Taylor, 2009). The tunable nature of the supercritical fluid which includes solvent character optimization via adjustment of pressure, temperature, and addition of a co-solvent can be exploited to tailor chromatographic separation to deliver GC-like separations and LC-like solvation.

SFC coupled to mass spectrometry for analysis of lipids has been shown to have utility (Lísa & Hol apek, 2015; Matsubara et al., 2009; Uchikata et al., 2012; Wada et al., 2011; Yamada et al., 2013; Zhou et al., 2014). The reports by Zhou (Zhou et al., 2014) and Lisa (Lísa & Hol apek, 2015) highlighted the advantages of a SFC variant referred to as ultraperformance convergence chromatography (UPC²) coupled to a quadrupole time-of-flight (QTOF) mass spectrometer for chemical profiling of glycerolipids from bovine milk and broad lipid class separation of porcine brain extract, respectively. To date, the use of SFC for chromatographic separation let alone its combination with high resolution tandem mass spectrometry has not been demonstrated for lung lipidomic biomarker identification. Disruption of lipid metabolic enzymes and pathways has been associated with various lung

diseases/injuries (e.g., pulmonary emphysema, cystic fibrosis, acute lung injury, allergic asthma, and pulmonary bacterial and viral infections) (Uhlir & Gulbins, 2008; Wenk, 2005). Particular lipids of interest include glycerophospholipids which comprise ~90% of lung surfactant (Haworth & Levy, 2007) and sphingolipids, most notably ceramides (Cer), which are associated with key physiological processes such as cell signaling (e.g., apoptosis) (Uhlir & Gulbins, 2008).

Two mouse models associated with lung injury include the stress-induced whole thorax lung irradiation (WTLI) model and the genetic knockout model for cellular retinol-binding protein, type 1 (*Rbp1*($-/-$)). Lung injury from high-dose irradiation is considered a delayed effect of acute radiation exposure and characterized depending on the severity of the insult by diffuse interstitial pneumonitis followed by progressive fibrosis (Jackson et al., 2012). Correspondingly, *Rbp1*($-/-$) mouse lungs are characterized by varying degrees of hyperplasia and when exposed to irradiation exhibit exacerbated lung dysfunction (unpublished results). We report on the use of UPC² high resolution data-independent tandem mass spectrometry to characterize total lipid extracts from mouse lung samples from WTLI and *Rbp1*($-/-$) mouse models.

Material and Methods

Reagents and Materials

Carbon dioxide (CO₂) (99.9% grade) was purchased from Air Gas (Linthicum Heights, MD). LC-MS grade methanol, acetonitrile, water, formic acid and ammonium formate were purchased from Fisher Scientific (Pittsburg, PA). HPLC grade tert-Butyl methyl ether (MTBE) was purchased from Sigma Aldrich (St. Louis, MO).

All lipid standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphate (PA(18:1/18:1)), 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine (PC(16:0/16:0)), 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (PC(18:1/18:1)), 1,2-di-(eicosatetraenoyl)-sn-glycero-3-phosphocholine (PC(20:4/20:4)), 1-hexadecanoyl-2-(docosahexaenoyl)-sn-glycero-3-phosphocholine (PC(16:0/22:6)), 1-octadecanoyl-2-(docosahexaenoyl)-sn-glycero-3-phosphocholine (PC(18:0/22:6)), 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (PE(18:1/18:1)), 1-hexadecanoyl-2-(docosahexaenoyl)-sn-glycero-3-phosphoethanolamine (PE(16:0/22:6)), 1-octadecanoyl-2-(docosahexaenoyl)-sn-glycero-3-phosphoethanolamine (PE(18:0/22:6)), 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1'-rac-glycerol) (PG(16:0/18:1)), 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho-L-serine (PS(18:1/18:1)), 1-hexadecanoyl-sn-glycero-3-phosphocholine (LPC(16:0)), 1-octadecanoyl-sn-glycero-3-phosphocholine (LPC(18:0)), N-(hexadecanoyl)-sphing-4-enine-1-phosphocholine (SM(d18:1/16:0)), N-(hexadecanoyl)-sphing-4-enine (Cer(d18:1/16:0)), N-(octadecanoyl)-sphing-4-enine (Cer(d18:1/18:0)), N-(octadecenoyl)-sphing-4-enine (Cer(d18:1/18:1)), N-(eicosanoyl)-sphing-4-enine (Cer(d18:1/20:0)), N-(docosanoyl)-sphing-4-enine (Cer(d18:1/22:0)), N-(tetracosanoyl)-sphing-4-enine (Cer(d18:1/24:0)), and N-(tetracosenoyl)-sphing-4-enine (Cer(d18:1/24:1)) were used as qualitative standards. 1,2-diheptadecanoyl-sn-glycero-3-phosphate (PA(17:0/17:0)), 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine (PC(17:0/17:0)), 1-heptadecanoyl-sn-

glycero-3-phosphocholine (LPC(17:0)), 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine (PE(17:0/17:0)), 1,2-diheptadecanoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (PG(17:0/17:0)), 1,2-diheptadecanoyl-sn-glycero-3-phospho-L-serine (PS(17:0/17:0)), N-heptadecanoyl-D-erythro-sphingosine (Cer (d18:1/17:0)), N-heptadecanoyl-D-erythrosphingosylphosphorylcholine (SM (d18:1/17:0)) were used as internal standards.

Animal Models

The WTLI mouse model has been published previously (Jackson et al., 2012). Briefly, female C57BL/6J mice were allowed to acclimate to the facility for 1-2 weeks prior to radiation exposure. Animals were anesthetized (100 mg kg⁻¹ ketamine; 10 mg kg⁻¹ xylazine) and randomized to groups to receive a single, uniform dose of 15 Gy delivered to the whole lung at a dose rate of 69 cGy min⁻¹ (320-kVp, HVL = 1.00 mm Cu; Precision X-ray Inc., North Branford, CT). Sham-irradiated mice were included as controls. Lung tissue was collected from the mouse at the time of necropsy 1 day post irradiation, perfused, and immediately stored at -80 °C. All experiments were performed with prior approval from Duke University. The *Rpb1*(-/-) mouse model has been published previously (Pierzchalski et al., 2013). Briefly, female, virgin, C57BL/6 wild-type or *Rbp1*(-/-) mice aged 1.5 to 4 months were used according to institutional guidelines of the University of Maryland, Baltimore. WT mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA), and *Rbp1*(-/-) mice were bred in-house from breeders obtained from Pierre Chambon and Norbert Ghyselinck (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Institut National de la Santé et de la Recherche Medicale, Illkirch, France). Lung tissue was collected and immediately stored at -80 °C upon necropsy. *Rbp1*(-/-) lung samples were not perfused.

Gene expression

Total RNA was isolated with a Purelink miniRNA plus kit (Invitrogen; Life Technologies, Grand Island, NY, USA). RNA was quantified by a NanoDrop 2000 spectrophotometer (Thermo, Wilmington, DE, USA). RNA was reverse-transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems; Life Technologies). Quantitative polymerase chain reaction (qPCR) was done with predesigned and optimized primers (Applied Biosystems). mRNA gene expression was measured with a Step One Plus real-time PCR (Applied Biosystems). mRNA gene expression was calculated by the comparative Ct method with 18s as the reference gene.

Sample preparation

Total lipid extracts from mouse lung samples were prepared using the MTBE lipid extraction protocol (Matyash et al., 2008) with slight modifications. Briefly, 400 µL of methanol:water (3:1, v/v) was added to 30-40 mg of lung tissue and homogenized with ceramic beads (Precellys®24-Dual, PeqLab, Wilmington, DE). Internal standards and 1 mL of MTBE were added followed by room temperature shaking for 1 hour. Then, 250 µL of water was added and left to sit at room temperature for 15 minutes. Phase separation was completed by centrifugation at 10 minutes for 10000 RPM. The upper phase was removed and dried at room temperature under nitrogen. The recovered lipids were reconstituted in 500 µL of

chloroform:methanol (2:1, v/v). Prior to analysis samples were further diluted with acetonitrile:isopropanol:water (65:30:5, v/v/v). The internal standard and lipid standard mixture concentrations were set at 500 ng/ml final concentration.

Chromatography

SFC was performed on a Waters ACQUITY UPC² system (Milford, MA). The UPC² was equipped with a binary solvent delivery pump, auto-sampler, column oven, and back pressure regulator. Mobile phase A was CO₂ and mobile phase B was methanol:acetonitrile (1:1, v/v) with 15 mM ammonium formate. The gradient was 5% B for 1 minute, ramp to 50% B over 11.5 minutes, held for 4 minutes, ramped to 5% B in 1 minute and equilibrated for 2.5 minutes for a total run time of 20 minutes. The flow rate was 1.85 mL/min and the back pressure was held at 1500 psi. The column oven was maintained at 50 °C and held two columns: ACQUITY UPC² BEH (2.1 × 50 mm; 1.7 µm) and HSS C₁₈ SB (2.1 × 50 mm; 1.8 µm). The auto-sampler was kept at 5 °C and a 1 µL injection was used for all samples.

Ultra Performance Liquid Chromatography (UPLC) was performed on a Waters ACQUITY UPLC system (Milford, MA). The reverse phase C18 separation was achieved using a C18 CSH (1.7 µm; 2.1 × 100 mm) column (Waters, Milford, MA). Mobile phase A was 10 mM ammonium formate with 0.1% formic acid in water/acetonitrile (40:60, v/v) and mobile phase B was 10 mM ammonium formate with 0.1% formic acid in acetonitrile/isopropanol (10:90, v/v). The gradient was ramped from 40 % to 43 % B in 2 minutes, ramped to 50 % in 0.1 minutes, ramped to 54 % B in 9.9 minutes, ramped to 70 % in 0.1 minutes, and ramped to 99 % B in 5.9 minutes. The gradient was returned to initial conditions in 0.5 minutes and held for 1.9 minutes for column equilibration. The flow rate was 0.4 mL/min. The column was maintained at 55 °C and the auto-sampler was kept at 5 °C. A 5 µL injection was used for all samples. The HILIC separation was achieved using a BEH HILIC (1.7 µm; 2.1 × 100 mm) column (Waters, Milford, MA). Mobile phase A was 10 mM ammonium acetate in water/acetonitrile (5:95, v/v) and mobile phase B was 10 mM ammonium acetate in water/acetonitrile (50:50, v/v). The gradient was ramped from 0.1 % to 20 % B in 10 minutes, and then ramped to 80 % B in 3.0 minutes. The gradient was returned to initial conditions in 0.1 minutes and held for 2.99 minutes for column equilibration. The flow rate was 0.5 mL/min. The column was maintained at 30 °C and the auto-sampler was kept at 5 °C. A 5 µL injection was used for all samples.

Mass Spectrometry

All mass spectrometry experiments were performed on a Waters SYNAPT G2 QTOF mass spectrometer (Milford, MA). The instrument was operated in both positive (+) and negative (–) ion mode electrospray. The capillary voltage was 3.0 kV (+) and 2.5 kV (–) and sampling cone voltage was 30 V (+/–). Nitrogen at a flow of 650 L/h was used as the desolvation gas with a constant desolvation temperature of 400 °C. The source temperature was set at 125 °C. Data were acquired over the *m/z* range of 100–1200. The mass spectrometer was operated in MS^E mode with alternating low- and high-collision energies. The first scan was set at low-collision energy (4 eV) and used to collect precursor ion spectra. The second scan was set at high-collision energy and ramped from 25–40 eV which was used for generation of product ion spectra. Argon gas was used for collision-induced

dissociation. An integral LockSpray unit which collected a reference scan every 10 s lasting 0.3 s was used to ensure high mass accuracy data acquisition. Leucine Enkephalin was used as the lock-mass at a concentration of 0.1 mg/L in water-acetonitrile (1:1, v/v) with 0.1 % formic acid. The reference calibrant was sprayed at a flow rate of 10 μ L/min using the fluidics system of the instrument. A single point lock-mass at m/z 556.2771 (+) and 554.2615 (–) were used for all analyses. Data were acquired and analyzed with Waters MassLynx v4.1 software.

Bio-informatics

UPC² tandem mass spectrometry data was processed using Progenesis QI version 2.0 (Nonlinear Dynamics, Newcastle, United Kingdom). Raw data files were directly imported into Progenesis QI where retention time alignment, peak picking, deconvolution of adducts, relative abundance, and preliminary identification were performed. The Progenesis QI vector alignment quality was manually processed and files were aligned (sensitivity, chromatographic peak width, and retention time limits) by vendor default values. Relative abundance was based on normalization to internal standards. Preliminary identification was based on precursor ion accurate mass (< 10 ppm) database searching of LIPIDMAPS. The processed and normalized data generated from Progenesis QI which included peak area, m/z value, and retention time was exported into SIMCA-P +13.0 (Umetrics, Umea, Sweden) for multivariate analysis. The multivariate analysis included unsupervised principal component analysis (PCA) and supervised orthogonal projections to latent structures discriminate analysis (OPLS-DA). The features selected from the multivariate analysis were assigned putative structures based on *de novo* analysis of retention times from two different column chemistries, MS^E low- and high-collision energy scan correlation, positive and negative ion tandem mass spectra interpretation, and for a number of selected lipids authentic standard verification. The statistical analysis involved the use of three biological replicates (n=3) per group and established statistical significance as a proof-of-principal of the UPC² tandem mass spectrometry platform. Increased numbers of biological replicates will be necessary in order to provide more rigorous statistical analysis. On-going efforts are currently underway to increase the number of biological replicates in order to further validate the present data.

Results and Discussion

UPC²

Supercritical or near-critical CO₂ was used as the mobile phase with varying co-solvent to resolve the lipid species in total lipid extracts from mouse lung over a 20 minute gradient (Figures 1 and 2). Two different column chemistries, C18 and Silica (referred to as BEH), were utilized in order to chromatographically separate extracted lipids based on acyl chain hydrophobicity and head group polarity. Lung total lipid extracts yielded highly abundant, early eluting glycerolipids in the positive ion mode (Figure 1), and fatty acids in the negative ion mode (Figure 2), whereas glycerophospholipids were present in both ionization modes.

Figure 1 shows the total ion chromatograms (TIC) and extracted ion chromatograms (XIC) for the C18 and BEH columns acquired in the positive ion mode. The XIC from several high abundant lysophosphocholines (LPC), glycerophosphocholines (PC),

lysophosphoethanolamines (LPE), glycerophosphoethanolamines (PE), and sphingomyelins (SM) were used to assess the chromatographic separation of lipid classes as affected by the C18 and BEH columns under the UPC² conditions. Supporting Information (SI) Table S1 displays the identified LPC, PC, LPE, PE, and SM based on accurate mass, lipid class, equivalent carbon number (ECN), and elution order per column. The elution profile for the C18 separation resembled the model used to describe reverse phase separations of triglycerides (TG) and intra-class glycerophospholipids (Lísa et al., 2011; Lísa & Holcapek, 2008). The ECN reverse phase model predicts for lipids within a specific class a proportional relationship between lower ECN and less chromatographic retention. The ECN is dependent not only on the cumulative number of acyl carbons but also on the degree of saturation within the acyl chains. The elution profile for the BEH separation displayed the opposite elution order for intra-class glycerophospholipids. Whereas the C18 separation was explained using the ECN reverse phase model, the BEH separation (Figure 1B) had an inverse relationship between the ECN value and chromatographic retention analogous to HILIC separations (Cífková et al., 2012). The glycerophospholipid UPC² C18 and BEH elution profile observed for the lung total lipid extracts was confirmed for a series of PC and PE lipid standards (Figure S1). The standard mixture displayed coherent and predictable elution profiles based on ECN as described above.

The total lipid extracts were also analyzed in the negative ion mode on both column chemistries. Representative negative ion mode C18 TIC and corresponding XIC are shown in Figure 2. Highlighted in Figure 2 and displayed in Table S2 was the glycerophospholipid C18 separation in the negative ion mode. The elution profile in the negative ion mode C18 separation did not differ from the positive ion mode C18 separation although different adducts and abundances were observed. Of note, the C18 UPC² platform provided efficient separation of Cer as evident by near baseline resolution for a number of individual Cer. The Cer elution order on the C18 column was described by the ECN reverse phase model where the lower the ECN value corresponded to earlier elution time (Table 1).

In order to demonstrate the complementary and contrasting chromatographic separation of UPC² for Cer, a comparison between two commonly used lipidomic UPLC chromatographies, reverse phase C18 (Damen et al., 2014) and HILIC (Cífková et al., 2012), was juxtaposed to UPC² C18 and BEH separations (Figure 3). The standard Cer mixture contained seven ceramides with a common d18:1 sphingosine and a varying amide linked fatty acid (16:0, 18:0, 18:1, 20:0, 22:0, 24:0, and 24:1). The UPLC HILIC and UPLC reverse phase C18 separations (Figure 3C and 3D) yielded distinct elution profiles: the UPLC HILIC separation displayed complete coelution of the Cer mixture in under 1 minute, and the UPLC reverse phase C18 separation demonstrated thorough separation of the mixture between 11 and 16 minutes adhering to the ECN reverse phase model. The UPC² C18 separation (Figure 3A) of the Cer mixture had an identical elution profile as the reverse phase C18 separation yet was accomplished in under 3.5 minutes marking a distinct difference in elution time. The UPC² C18 separation had baseline resolution of five of the ceramide standards and full-width half height resolution for the other two ceramides (Cer22:0 and Cer24:1). The UPC² BEH separation of the Cer mixture (Figure 3B) displayed a co-elution profile where the ceramide standards were partially resolved over 15 seconds and fully eluted in less than 2.7 minutes.

Data-Independent Tandem Mass Spectrometry

The mass spectrometry acquisition platform involved the use of accurate mass data-independent tandem mass spectrometry referred to as MS^E. MS^E alternates rapidly between low- and high-collision energy scans resulting in an efficient means of generating precursor and product ion spectra over the entire chromatographic gradient.

An example of MS^E data was shown in Figure 4. Displayed were the corresponding TIC of the low- and high-collision energy scans bracketing the glycerophospholipid retention times on the BEH column in the positive ion mode. The low-energy scan highlighted the most abundant glycerophospholipid precursor ions (Figure 4B). The high-collision energy scan (Figure 4A) highlighted the most abundant product ions resulting from the gas-phase dissociation of the low-energy scan precursor ions. The time aligned low- and high-collision energy TIC visualized valuable structure information such as the identity of the glycerophospholipid head group. For example, the presence of an abundant product ion at m/z 184.1 observed in the high-collision energy scan indicated the glycerophospholipids at these particular retention times contained a phosphocholine head group. As such, searching for the presence of the product ion at m/z 184.1 allowed for localization of glycerophospholipids containing a phosphocholine to specific retention times. This particular strategy was akin to performing a precursor ion scan on a tandem quadrupole mass spectrometer and likewise was advantageous for identifying precursor ions with a common functional group. Additionally, the MS^E strategy was instructive for identifying diagnostic neutral losses that were specific to precursor ions having conserved labile structural features (analogous to neutral loss scan). The neutral loss of 141 Da was observed in the high-collision energy scan (Figure 4A) which classified these glycerophospholipids as having a phosphoethanolamine head group and consequently facilitated identification of the glycerophospholipids at these specific retention times as PE.

Confirmation of the structural identity of the glycerophospholipids of interest required more detailed interrogation of the high-collision energy spectra. Putative identifications were achieved for a number of lipid species via *de novo* interpretation of the high-collision energy spectra generated from the MS^E experiments. One such example was the putative structure assignment for the precursor ion at m/z 792.5 ($[M+H]^+$; t_R of 9.17 min on the BEH column) (Figure 4A inset, Figure S2).

In addition to our interest in glycerophospholipids, we also investigated Cer. Cer produce a characteristic product ion unique to its sphingoid base upon positive ion mode gas-phase dissociation (Haynes et al., 2009; Sullards et al., 2007). The product ion's m/z value is dependent on the acyl chain length and degree of saturation of the sphingoid base. For example, Cer containing the following sphingoid bases, sphingadienine (d18:2) or sphingosine (d18:1), yield characteristic product ions at m/z values 262.3 and 264.3, respectively. Figure 5A shows likely cleavage sites for the formation of the Cer product ions referenced above. The positive ion mode high-energy collision scan (on the C18 column) for the retention time bracketing the elution of Cer (t_R =2.5-4.0 min) generated chromatographic peaks corresponding to product ions at m/z values 262.3 and 264.3 (Figure 5B). This indicated that most of the Cer (at least the most abundant species) contained either a sphingadienine (d18:2; m/z 262.3) or sphingosine (d18:1; m/z 264.3) base. The XIC for

precursor ions associated with product ions at m/z values 262.3 and 264.3 (positive ion mode; C18 column) resulted in a number of abundant and chromatographically separated Cer that contained either a d18:2 or d18:1 (Figure 5C and D). Table S3 details the mass accuracy, ECN, t_R , characteristic product ion, and putative identification for Cer in Figure 5.

Structure assignment for Cer(d18:2/20:0) and Cer(d18:1/20:0) is detailed in Table S4 and Figure 6. There are several possible isomeric structures when accurate mass alone is used for confirmation of Cer(d18:2/20:0) and Cer(d18:1/20:0). As seen in Table S4, Cer structural isomers produce a series of diagnostic product ions. Thus, where accurate mass alone falls short in narrowing the search for structure identification, tandem mass spectrometry provides further insight for structure assignment. The structural isomers identified via the accurate mass precursor search varied in the length of the acyl chain and degree of saturation of the sphingoid base. *De novo* interpretation of the positive and negative ion high-collision energy spectra provided complementary and additional structure insight. The identification of diagnostic product ions from the precursor ion at m/z 592.6 (+ ion mode) and 590.6 (– ion mode) permitted confident assignment of the sphingoid base and secondary acyl chain which corresponded to Cer(d18:2/20:0) (Table S4A, Figure 6A and B). The aforementioned structure determination pipeline was applied to the ion pair at m/z 594.6(+)/592.6(–) with structure assignment being Cer(d18:1/20:0) (Table S4B, Figure 6C and D). Of note, Cer(d18:2/20:0) was chromatographically separated from Cer(d18:1/20:0) which allowed for less complex high-collision energy spectra and more straightforward *de novo* interpretation. The same experimental analysis was performed for all identified Cer.

Bio-informatics

Differential expression analysis was performed using Waters Progenesis QI. The raw data files were separated into 4 groups per model based on column chemistry and ionization polarity: BEH positive, BEH negative, C18 positive, and C18 negative. Statistical analysis was achieved by importing grouped data files processed by Progenesis QI into Simca P for multivariate analysis. The multivariate analysis of the total lipid extracts demonstrated that the principal component analysis (PCA) plots discriminated lipid profiles from lungs that were exposed to 15 Gy WTLI (1 day post-exposure) from lungs that were sham irradiated (Figure 7A and 7B). Table S5 lists m/z values with associated lipid classification and putative identification, where possible, that were differentially expressed between the lungs exposed to WTLI and sham irradiated. Of interest were a number of Cer that were up-regulated at 24-hours post WTLI. In total, ten Cer were identified that were differentially expressed all of which were elevated in mouse lung exposed to WTLI (Figure 7C and 7D).

Our studies showed cellular retinol-binding protein, type 1 (*Rbp1*), a chaperone protein for the essential nutrient vitamin A and a tumor suppressor gene (Esteller et al., 2002; Kuppumbatti et al., 2001; Napoli, 2012; Pierzchalski et al., 2013), was acutely reduced in lung after radiation (Figure S3). As such, we interrogated cellular retinol-binding protein, type 1-null (*Rbp1*(–/–)) mouse lung to additionally inform on the mechanism of radiation-induced lung damage. The multivariate analysis of the total lipid extracts from the *Rbp1*(–/–) model demonstrated that the PCA plots discriminated the lipid profiles from wild type lungs from the *Rbp1*(–/–) lungs (Figure S4A and B). Table S6 lists m/z values with

associated lipid classification that were differentially expressed between the wild type and *Rbp1*($-/-$) mouse lungs.

Biomarker Analysis and Interpretation

The UPC² platform demonstrated chromatographic separation of lipid classes and when combined with high resolution tandem mass spectrometry and bioinformatics permitted identification of potential lung lipid biomarkers. The analytical pipeline described herein resulted in effective and well-characterized detection of a variety of lipid classes. The profiling data identified lipids associated with radiation-induced lung damage. The identification of potential lipid biomarkers at early time-points in the WTLI model is encouraging for understanding early mechanistic pathways for injury progression and also for early prognosis of lung injury. Moreover, the identification of early time-point lipid markers 1 day post 15 Gy WTLI highlighted the role lung lipid homeostasis and metabolism may have in response to the radiation insult. These lipid markers may have diagnostic and/or prognostic potential and will be investigated further for their biological utility. Additionally, these profiling data have the potential to inform on the mechanism of damage as there are a number of differentially expressed lipids that are common to the WTLI and *Rbp1*($-/-$) models, including fatty acids, Cer, and PC (Table S5, S6). The acute reduction in cellular retinol-binding protein, type 1 (*Rbp1*) expression following irradiation (Figure S3) is significant due to the importance of cellular retinol-binding protein, type 1 (*Rbp1*) in regulating retinoic acid. Retinoic acid is an active metabolite of vitamin A that regulates lung homeostasis and morphogenesis (Maden & Hind, 2004; Mollard et al., 2000; Napoli, 2012). Previous reports have identified retinoic acid as important to lung fibrosis, inflammation, membrane damage, and cellular metabolism (Druilhe et al., 2008; Pierzchalski et al., 2013; Tabata et al., 2006; Wu et al., 2013). These profiling data from the WTLI and *Rbp1*($-/-$) models have potentially generated insight towards dissecting the initial molecular events of radiation-induced lung injury and indicate these events include disruption of lipid metabolism and homeostasis of retinoic acid.

Cer are of great interest owing to their important role as signaling molecules and, as such, we have focused an in-depth discussion on their significance. Elevated levels of Cer occur under many situations of cellular stress stimuli such as radiation, ischemia, oxidative stress, and chemotherapeutic agents (Arana et al., 2010). Increased levels of Cer in lung tissue 24-hours post 15 Gy WTLI indicates cellular stress stimuli (e.g., oxidative stress) impacts the lungs immediately following the radiation insult. The mechanistic role for increased levels of Cer have been linked to alteration of the physical membrane properties and/or specific interaction with intracellular enzymatic targets (e.g., phosphatases and phospholipases) or ion channels (Uhlir & Gulbins, 2008). Lung function due to increased levels of Cer have been reported to be impacted via increased inflammation (compromised endothelial barrier, increased pulmonary emphysema), susceptibility to infection (pathogen uptake), and cell survival (increased apoptosis) (Maceyka & Spiegel, 2014; Uhlir & Gulbins, 2008). Consequently, detection and characterization of Cer from lung tissue was particularly relevant.

The analysis of Cer from biological samples has been achieved via a variety of analytical techniques which have mostly been centered on the use of an analytical separation (thin-layer chromatography, GC, and LC) coupled to MS (Sullards et al., 2011). Targeted LC-MS assays have focused on the detection of various molecular species of dihydroceramide (Cer(d18:0); sphinganine), ceramide (Cer(d18:1); sphingosine), and phytoceramide (Cer(t18:0); 4-hydrophosphinganine) which have been reported to be the most abundant and biologically germane in mammals (Haynes et al., 2009). In addition, untargeted lipidomic assays have taken the approach of characterizing a broad scope of lipids of which Cer were included in the suite of lipids that were detected and accessed for differential expression (Telenga et al., 2014).

In regards to lung fluid or tissue, targeted and untargeted mass spectrometry-based assays have been applied to the detection of Cer(d18:0), Cer(d18:1), and Cer(t18:0) in sputum (Telenga et al., 2014), bronchoalveolar lavage fluid (Tibboel et al., 2013), plasma (Alberg et al., 2013), lung epithelial cells (Zehethofer et al., 2014), and lung tissue (Petrache et al., 2005). Elevated Cer in lung-associated samples have been linked to increased risk of lung cancer (Alberg et al., 2013), increased risk factor for chronic obstructive pulmonary disease (Telenga et al., 2014), and suggested to be a pathogenic mediator and therapeutic target for pulmonary emphysema (Petrache et al., 2005; Tibboel et al., 2013). Based on the results presented herein numerous Cer were differentially expressed (all elevated) in mouse lungs exposed to 15 Gy WTLI when compared to non-irradiated controls. The lungs were collected 24-hours post WTLI and represented an early time point at which histopathology or physiological function parameters were not yet indicative of injury onset (Jackson et al., 2012).

A unique aspect of the UPC² tandem mass spectrometry assay as described was the detection and structural identification of Cer with a sphingadienine base (Cer(d18:2)). Cer(d18:2) was present in lung tissue and similar to Cer(d18:1) was differentially expressed (elevated) between lungs that were irradiated and non-irradiated controls. The detection of Cer(d18:2) at similar relative abundance as that of Cer(d18:1) indicated Cer(d18:2) in the lungs was abundant and its elevated expression correlated with the radiation insult. Sphingadienine bases are commonly found in yeast and plants but have been reported in mouse brain, human aorta, plasma, and erythrocyte membranes (Colsch et al., 2004; Pruett et al., 2008).

To date the biological significance of Cer(18:2) especially in regards to mammalian systems has received little attention. Its presence in yeast and plants have been associated with membrane structure and a key intermediary of the synthesis of complex sphingolipids (Markham et al., 2013). It's been suggested its uptake from dietary ingestion via glucosylceramides containing Cer(d18:2) in wheat and soy play a role in colon cancer (Sullards et al., 2000). Also of particular relevance is the role of increased unsaturation on the biophysical properties of Cer especially given the role ceramide-rich platforms play in cell signaling and disease (Stancevic & Kolesnick, 2010). The effects of Cer unsaturation were determined to have increased miscibility in a fluid model and decreased ability to form gel domains (Pinto et al., 2011). It was concluded that the different biophysical properties of each Cer as dictated by unsaturation and acyl chain length markedly influenced membrane

fluidity. Moreover, it was suggested that the biophysical properties conferred by varying Cer structures may be important for understanding cell signaling in specific tissues and cells. Of note, Pinto (Pinto et al., 2011) did not consider the use of Cer(d18:2) rather varying structures of Cer(d18:1) where the unsaturation was introduced in the non-sphingoid acyl chain yet the relationship between Cer structure and biophysical properties would likely extend to all Cer.

A greater understanding of what Cer are present in terms of structure and abundance are of vital importance for characterizing the role Cer play in biological systems. Consequently, the use of UPC² high resolution tandem mass spectrometry enabled characterization of lung lipids and laid the foundation for on-going efforts to validate the biomarker status of Cer (and other lipid species) in lung injury and disease.

Conclusion

UPC² high resolution tandem mass spectrometry coupled to bioinformatics was used to characterize total lipid extracts from mouse lung samples from two different animal models. The UPC² platform yielded lipid class chromatographic separation of the total lipid extracts. In particular, the UPC² C18 separation was uniquely suited for chromatographically resolving Cer differing in acyl chain length and degree of unsaturation. Determination of lipid structures was achieved via the use of data-independent tandem mass spectrometry. MS^E yielded accurate *m/z* values for precursor and diagnostic product ions that enabled confident assignment of structures. Potential lipid markers were statistically generated via the use of Waters Progenesis QI coupled to multivariate statistical analysis. Of note, various Cer were determined to be elevated in the WTLI mouse model. Determination of Cer as being differentially expressed in lung tissue 24-hours following 15 Gy WTLI provides a unique opportunity for gaining an understanding of the initial molecular events triggered by the radiation insult that potentially impacts the latency and severity of lung injury from radiation exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Cer	ceramides
DG	Diacylglycerols
ECN	equivalent carbon number

GC	gas chromatography
HILIC	hydrophilic interaction chromatography
HPLC	high performance liquid chromatography
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
LPC	lysophosphoscholines
LPE	lysophosphoethanolamines
MS	mass spectrometry
MS^E	data-independent tandem mass spectrometry
MTBE	tert-Butyl methyl ether
PA	glycerophosphates
PC	glycerophosphocholines
PCA	principal component analysis
PE	glycerophosphoethanolamines
PG	glycerophosphoglycerols
PI	glycerophosphoinositols
PS	glycerophosphoserines
QTOF	quadrupole time-of-flight
Rpb1 (-/-)	retinol-binding protein mouse genetic knockout
SFC	supercritical fluid chromatography
TG	Triacylglycerols
TIC	total ion chromatograms
UPC²	ultraperformance convergence chromatography
WTLI	whole thorax lung irradiation
XIC	extracted ion chromatograms

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Figure 1.

UPC² separation of mouse lung lipid extracts from the WTLI model. TIC and XIC are shown for MS detection in positive ion mode for the C18 (A.) and the BEH (B.) columns. The XIC for representative glycerophospholipids for each column was shown above the TIC with the m/z value and lipid class designated. Refer to Table S1 for lipid identification.

Figure 2. UPC² separation of mouse lung lipid extracts from the WTLI model with TIC and XIC shown for the negative ion mode C18 column. The bottom panel was the TIC for the full gradient. The middle panel (t_R 2-13 min) highlighted the regions where Cer and glycerophospholipids were observed. The top two panels were the XIC for the most abundant Cer (left panel; t_R 2.60-3.75 min) and glycerophospholipids (right panel; t_R 5.75-12.75 min) observed upon negative ion mode. The precursor ion and lipid class was designated above each peak in the XIC. Refer to Table 1 and Table S2 for lipid identification.

Figure 3.

UPC² and UPLC separation of a Cer standard mixture. Positive ion mode chromatograms as shown for UPC² C18 (A.), UPC² BEH (B.), UPLC HILIC (C.), and UPLC reverse phase C18 (D.). The Cer standard is labeled above each chromatographic peak. The Cer standard mixture included Cer(d18:1/16:0), Cer(d18:1/18:0), Cer(d18:1/18:1), Cer(d18:1/20:0), Cer(d18:1/22:0), Cer(d18:1/24:0), and Cer(d18:1/24:1).

Figure 4. MS^E high- (A.) and low- (B.) collision energy TIC from the WTLI model. The most abundant precursor ion and its corresponding lipid class is designated for each peak in the low-collision energy scan. The high-collision energy scan is labeled with the most abundant product ion. The product ion at m/z 184.1 was diagnostic for phosphocholine (PCho) and the neutral loss of 141 Da was diagnostic for phosphoethanolamine (PEtN). The inset mass spectrum was the high-collision energy spectrum for the precursor ion at m/z 792.5.

Figure 5.

MS^E XIC for Cer. A.) Likely cleavage sites for the formation of diagnostic Cer product ions containing the following sphingoid bases: sphingadienine (d18:2): m/z 262.3 and sphingosine (d18:1): m/z 264.3. B.) The high-collision energy scan for Cer product ions in the positive ion mode on the C18 column (t_R 2.5-4.0 minutes). C.) XIC for the precursor ions associated with the product ion at m/z 262.3 (d18:2). D.) XIC for the precursor ions associated with the product ion at m/z 264.3 (d18:1).

Figure 6.

High-collision energy spectra for the precursor ions corresponding to Cer(d18:2/20:0) (A and B) and Cer(d18:1/20:0) (C and D). A.) Positive ion mode high-collision energy spectrum of the ion at m/z 592.6 ($[M+H]^+$ for Cer(d18:2/20:0)). B.) Negative ion mode high-collision energy spectrum of the ion at m/z 590.6 ($[M-H]^-$ for Cer(d18:2/20:0)). C.) Positive ion mode high-collision energy spectrum of the ion at m/z 594.6 ($[M+H]^+$ for Cer(d18:1/20:0)). D.) Negative ion mode high-collision energy spectrum of the ion at m/z 592.6 ($[M-H]^-$ for Cer(d18:1/20:0)). Inset structures in each mass spectrum correspond to the likely cleavage site upon gas-phase dissociation. Nomenclature adopted from literature (Haynes et al., 2009; Shaner et al., 2009; Sullards et al., 2007).

Figure 7.

Bioinformatics and multivariate analysis of lung lipids from the WTLI mouse model comparing lipids detected in mouse lungs exposed to 15 Gy WTLI (1 day post exposure) and non-irradiated controls. PCA plot for positive (A.) and negative (B.) ions detected on the UPC² tandem mass spectrometry platform. Normalized abundance profiles (C.) and putative structure assignment (D.) for Cer identified to be differentially expressed in the WTLI mouse model.

Ceramides identified in the negative ion mode C18 chromatogram from Figure 2. Number of IDs corresponded to the number of possible Cer structures based on searching LIPID MAPS (10 ppm search criteria). Asterisk (*) next to the putative ID denotes authentic standard was used to confirm identification.

m/z [M-H] ⁻	m/z Theoretical	Mass Accuracy (ppm)	Lipid Class	CN:DB	ECN	t_R	Elution Order	Number of IDs	Putative ID	[M+H] ⁺ Confirmation
534.4885	534.4892	1.3	Cer	34:2	30	2.65	1	4	Cer(d18:2/16:0)	536.5194
536.5054	536.5048	1.1	Cer	34:1	32	2.75	2	3	Cer(d18:1/16:0)*	538.5204
562.5239	562.5205	6.0	Cer	36:2	32	2.84	3	5	Cer(d18:1/18:1)*	564.5294
564.5375	564.5361	2.5	Cer	36:1	34	2.94	4	4	Cer(d18:1/18:0)*	566.5480
590.5519	590.5518	0.2	Cer	38:2	34	3.02	5	5	Cer(d18:2/20:0)	592.5664
592.5670	592.5674	0.7	Cer	38:1	36	3.13	6	3	Cer(d18:1/20:0)*	594.5811
618.5827	618.5831	0.6	Cer	40:2	36	3.21	7	2	Cer(d18:2/22:0)	620.5935
644.6003	644.5987	2.5	Cer	42:3	36	3.27	8	none	Cer(d18:2/24:1)	646.6117
620.5979	620.5987	1.3	Cer	40:1	38	3.33	9	3	Cer(d18:1/22:0)*	622.6100
646.6134	646.6144	1.5	Cer	42:2	38	3.37	10	1	Cer(d18:1/24:1)*	648.6268
648.6281	648.6300	2.9	Cer	42:1	40	3.52	11	2	Cer(d18:1/24:0)*	650.6464