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Multidimensional Mass Spectrometry-based Shotgun Lipidomics Analysis of Vinyl Ether Diglycerides

Kui Yang¹, Christopher M. Jenkins¹, Beverly Dilthey¹, and Richard W. Gross^{*,1,2,3}

¹Department of Medicine, Division of Bioorganic Chemistry and Molecular Pharmacology, Washington University School of Medicine, St. Louis, MO 63110, USA

²Department of Developmental Biology, Division of Bioorganic Chemistry and Molecular Pharmacology, Washington University School of Medicine, St. Louis, MO 63110, USA

³Department of Chemistry, Washington University, St. Louis, MO 63130, USA

Abstract

Diglycerides play a central role in lipid metabolism and signaling in mammalian cells. Although diacylglycerol molecular species comprise the majority of cellular diglycerides that are commonly measured using a variety of approaches, identification of extremely low abundance vinyl ether diglycerides has remained challenging. In this work, representative molecular species from the three diglyceride subclasses (diacyl, vinyl ether and alkyl ether diglycerides) (hereafter referred to as diradylglycerols) were interrogated by mass spectrometric analysis. Product ion mass spectra of the synthesized diradylglycerols with varied chain lengths and degrees of unsaturation demonstrated diagnostic fragmentation patterns indicative of each subclass. Multidimensional mass spectrometry-based shotgun lipidomics (MDMS-SL) analysis of mouse brain and heart lipid extracts were performed using the identified informative signature product ions. Through an array of tandem mass spectrometric analyses utilizing the orthogonal characteristics of neutral loss scanning and precursor ion scanning, the differential fragmentation of each subclass was exploited for high yield structural analyses. Although molecular ion mass spectra readily identified diacylglycerol molecular species directly from the hexane fractions of tissue extracts enriched in non-polar lipids, molecular ion peaks corresponding to ether-linked diglycerides were not observable. The power of MDMS-SL utilizing the tandem mass spectrometric array analysis was demonstrated by identification and profiling of individual molecular species of vinyl ether diglycerides in mouse brain and heart from their undetectable molecular ion peaks during MS¹ analysis. Collectively, this technology enabled the identification and profiling of previously inaccessible vinyl ether diglyceride molecular species in mammalian tissues directly from extracts of biologic tissues.

Keywords

plasmalogen; vinyl ether; vinyl ether diglycerides; shotgun lipidomics

*Corresponding author, rgross@wustl.edu.

Introduction

Lipid 2nd messengers regulate multiple responses to cellular stimulation through a diverse variety of chemical mechanisms. These include their prominent roles as specific ligands for cellular receptors, regulation of protein kinases (*e.g.*, protein kinases C) and binding to ion channels regulating their electrophysiological characteristics [1–4]. Critical to understanding the complex roles of lipids in biologic systems is the identification and quantitation of signaling lipids that orchestrate signal transduction. Through innovative mass spectrometric analytical strategies in conjunction with enabling technological advances in instrumentation and bioinformatics, our understanding of the roles of specific lipid 2nd messengers and their biologic sequelae have grown exponentially during the last decade [5–10]. In large part, this has resulted from identification and quantitation of the diverse array of extremely low abundance lipid 2nd messengers that mediate cellular responses during agonist stimulation.

Years ago, we demonstrated that individual protein kinase C isoforms are differentially regulated by distinct subclasses of diglycerides which are comprised of diacylglycerol, vinyl ether diglycerides and alkyl ether diglycerides (hereafter collectively referred to as diradylglycerols, Scheme 1) [11,12]. Specific diradylglycerol molecular species are critical metabolites that regulate lipid metabolism and signaling. For example, ethanolamine phosphotransferase exhibits a remarkable substrate specificity for the vinyl ether linkage in alkenyl-acylglycerols (*i.e.*, vinyl ether diglycerides) resulting in the synthesis of plasmenylethanolamines which are abundant in both brain and heart [13]. Although each diglyceride lipid subclass has markedly different physical properties, stereo-electronic features and functions in cellular metabolism and signaling, the importance of individual subclasses of diglycerides and their downstream sequelae remain poorly understood. In large part, progress has been hampered by the difficulty in analysis of extremely low abundance molecular species of vinyl ether diglycerides although diacylglycerol molecular species comprising the majority of cellular diglycerides have been commonly measured using a variety of mass spectrometric approaches [14–19]. To traverse this difficulty, we synthesized representative molecular species of the two ether-linked subclasses of diradylglycerols and examined their fragmentation patterns along with those of commercially available diacylglycerol molecular species using different adduct ions. We identified signature fragmentation patterns indicative of each of the three diglyceride subclasses from the analysis of their ammonium adducts which proved suitable for the tandem mass spectrometric array analysis utilizing neutral loss scanning (NLS) and precursor ion scanning (PIS) techniques by Multidimensional Mass Spectrometry-based Shotgun Lipidomics (MDMS-SL) approaches developed in our laboratory [20–26]. Although vinyl ether diglyceride molecular species were not observed in molecular ion MS¹ spectra of lipid extracts of biological tissues (*e.g.*, mouse brain and heart), the use of diagnostic product ions in the MDMS-SL approach facilitated high analytical specificity and increased S/N by over 100-fold thereby enabling the identification and profiling of individual vinyl ether diglyceride molecular species through an array of MS² spectra. Utilization of this approach offers new opportunities for identifying the roles of individual molecular species of vinyl ether diglycerides in lipid metabolism, their regulation of specific isoforms of protein kinases C (and other C1 domain containing proteins), and alterations in

membrane molecular dynamics that modulate the function of critical transmembrane proteins.

Materials and methods

Reagents

1-O-1'-(Z)-hexadecenyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 1-O-1'-(Z)-octadecenyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-*sn*-glycerol and 1-stearoyl-2-arachidonoyl-*sn*-glycerol were purchased from Avanti Polar Lipids. 1-O-hexadecyl-*sn*-glycero-3-phosphocholine and 1-O-octadecyl-*sn*-glycero-3-phosphocholine were obtained from Cayman Chemical. Arachidonoyl chloride was purchased from Santa Cruz Biotechnology. Oleoyl chloride, N,N-dimethylglycine (DMG) hydrochloride, 4-(dimethylamino)pyridine, and *Bacillus cereus* phospholipase C (PLC) were obtained from Sigma Aldrich. Triethylamine was purchased from Fisher Scientific. Solvents for mass spectrometric analysis were purchased from Burdick and Jackson (Muskegon, MI).

Synthesis of Plasmalyncholine Molecular Species

Both 1-O-hexadecyl-2-oleoyl-*sn*-glycerol and 1-O-octadecyl-2-arachidonoyl-*sn*-glycerol were synthesized by acylation of lyso-platelet activating factor using the appropriate fatty acyl chlorides. Briefly, 1-O-hexadecyl-*sn*-glycero-3-phosphocholine (20 μ mol) or 1-O-octadecyl-*sn*-glycero-3-phosphocholine (20 μ mol) were dissolved in 2 ml of freshly distilled chloroform over P₂O₅. Next, either oleoyl chloride (100 μ mol) or arachidonoyl chloride (100 μ mol) and triethylamine (60 μ mol) were added in the presence of 4-dimethylaminopyridine (5 μ mol). The reaction mixture was incubated under nitrogen atmosphere for 5 hr at 30°C, reaction products extracted by a modified Bligh and Dyer procedure and the resultant chloroform layer was evaporated under a nitrogen stream. The dried lipid mixture was initially dissolved in chloroform prior to dilution (1:5, v/v) in acetonitrile/methanol/water (300:200:40). The resuspended reaction mixture was injected onto a Whatman SCX HPLC column (4.6 $\times$ 250 mm) equilibrated with acetonitrile/methanol/water (300:200:40) and reaction products were purified using isocratic elution. Both 1-O-hexadecyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 1-O-octadecyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine eluted at ~11 min. Column eluents containing the desired molecular species were extracted into chloroform, and analyzed by mass spectrometry for structural confirmation.

Synthesis of Vinyl Ether and Alkyl Ether Diglycerides

To the dried commercially available plasmalyncholines or synthesized plasmalyncholines, 200 μ l of 20 mM HEPES, pH 7.4 was added and the mixture was sonicated for 5 min at 40% power using a 1s duty cycle. Diradylglycerol molecular species were generated by incubating small unilamellar vesicles of 1-O-1'-(Z)-hexadecenyl-2-oleoyl-*sn*-glycero-3-phosphocholine (50 nmol in 0.2 ml), 1-O-1'-(Z)-octadecenyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (50 nmol in 0.2 ml), 1-O-hexadecyl-2-oleoyl-*sn*-glycero-3-phosphocholine (~10 nmol in 0.2 ml), or 1-O-octadecyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (~10 nmol in 0.2 ml) in 20 mM HEPES, pH 7.4 containing 2.5 units of PLC for 3 min at 37°C. Reactions were terminated by addition of 2 ml of chloroform/methanol (1:1) and phase

separation was induced by addition of 0.8 ml of 25 mM LiCl. The samples were vortexed, centrifuged at 2500×g for 2 min and the resultant chloroform layer was removed and dried under a stream of nitrogen. The resultant diglycerides were analyzed by mass spectrometry as described below.

Extraction of Diradylglycerols from Mouse Brain and Heart

Four-month old wild type (WT, C57BL/6J) male mice were purchased from Jackson Laboratories (Bar Harbor, ME). The animals were sacrificed by cervical dislocation in a protocol approved by the Washington University Animal Studies committee. Extraction of diradylglycerols and other lipids from brain and heart was performed by addition of 2 mL of cold isopropanol/hexane (3:2, v/v) to the weighed frozen tissue and incubation at -20°C for half an hour [27]. The cold extract was brought to room temperature and incubated for an additional 15 min. The solvent phase was removed and saved. The tissue residue was re-extracted with isopropanol/hexane (3:2, v/v) at room temperature for 15 min twice. The three extracts were combined and centrifuged to remove insoluble material. The extract was dried under a N_2 stream prior to reconstitution in anhydrous chloroform.

Hexane-methanol Liquid-Liquid Partitioning for Enrichment of Diradylglycerols

Methanol-saturated hexane (solvent H) and hexane-saturated methanol (solvent M) were prepared by mixing hexane, methanol and water at 1:1:0.1 (v/v/v). After centrifugation, the upper phase was removed as solvent H, the lower phase was removed as solvent M, and the interface was discarded. A portion of the above lipid extract was dried under a N_2 stream to near dryness. To the dried lipid residue, 2 mL of solvent M were added followed by vortexing for 1 min. Next 2 mL of solvent H were added and the mixture was vortexed for 1 min. After centrifugation, the upper hexane layer was carefully removed avoiding any contamination from the interface. To the remaining methanol layer, 2 mL of solvent H were added and vortexed for 1 min. After centrifugation, the upper hexane layer was removed and combined with the previous one. To the combined hexane layers, 3 mL of solvent M were added to back extract the carried-over polar lipids. After centrifugation, the upper hexane layer was carefully removed and dried under a N_2 stream. The residue was reconstituted in anhydrous chloroform (hereafter called hexane extract) and stored in -20°C before mass spectrometric analysis.

Acid Treatment of Lipid Samples

A portion of the above hexane extract was evaporated under a N_2 stream to near dryness. To the dried residue, 900 μl of 95% methanol and 100 μl of 1 N aqueous sulfuric acid were added and incubated at 70°C for 5 min [28]. Then, 1 mL of hexane was added followed by centrifugation and removal of the upper hexane layer. To the remaining methanol layer, an additional 1 mL of hexane was added and the mixture vortexed. After centrifugation, the upper hexane layer was removed and combined with the previous one. The combined hexane layers were dried under a N_2 stream and reconstituted in anhydrous chloroform.

Preparation of Infusion Solution and Generation of Diradylglycerol Adduct Ions for Mass Spectrometric Analysis

Synthetic diradylglycerol species or hexane extracts of tissue samples were diluted in solvent (chloroform/methanol/isopropanol, 1:2:4 (v/v/v)) for analysis through direct infusion into a TSQ mass spectrometer using a Nanomate device. For high mass accuracy analysis, analytes were dissolved in chloroform/methanol (1:1 (v/v)) for syringe injection into the Orbi-trap mass spectrometer. For analysis of diradylglycerol Na⁺ adducts, diluted lipid solutions prepared in glass tubes were directly injected into the mass spectrometer. Diradylglycerol Li⁺ adducts were prepared by diluting the lipid solution in the presence of 8% (v/v) LiOH/methanol (prepared by 200-fold dilution of a saturated LiOH methanol solution). For the analysis of diradylglycerol NH₄⁺ adducts, diluted lipid solutions in the presence of 10 mM CH₃CO₂NH₄ were utilized during direct infusion. Additionally, synthetic diradylglycerol species or hexane extracts of tissue samples were derivatized using DMG as previously described [17] followed by hexane-methanol partitioning to obtain DMG-derivatized diradylglycerols in hexane while excess reagents used during the derivatization reaction were removed by extraction into methanol. For the analysis of DMG-derivatized diradylglycerols, the diluted solutions in the presence of 0.1% (v/v) formic acid were used for the protonated DMG-derivatized species analyses, or in the presence of 8% (v/v) of the above LiOH/methanol solution for the lithiated DMG-derivatized species analysis.

Mass Spectrometric Analysis

Lipidomic analyses of synthetic diradylglycerols or hexane extracts of tissue samples were performed as described previously [9,21] after sample preparation as described above. Mass spectrometric analysis was performed using a TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an automated nanospray apparatus (i.e., Nanomate HD, Advion BioSciences, Ithaca, NY) for direct infusion as previously described [29]. The capillary temperature of the mass spectrometer was set at 150 °C. The spray voltage and gas pressure of the Nanomate were set at 1.3 kV and 1.1 psi, respectively. Mass spectrometric analyses including full mass MS¹ spectra and tandem mass spectrometric analysis were all performed in the positive-ion mode. Tandem mass spectrometric analyses including product ion full mass MS² spectra, neutral loss scanning and precursor ion scanning of diradylglycerol NH₄⁺ adducts were performed at a collision energy of 20 eV and a collision gas pressure of 0.6 mTorr. This combination of collision energy and gas pressure was demonstrated to be optimal for the greatest analytical sensitivity by analysis of NH₄⁺ adducts of synthetic diradylglycerol standards. The gas pressure utilized for other adduct analyses was 1 mTorr, and the collision energy was 40, 25, and 35 eV for analyses of diradylglycerol Li⁺ and Na⁺ adducts, protonated DMG-derivatized species, and lithiated DMG-derivatized species, respectively.

The NH₄⁺ adducts of synthetic diradylglycerols and their protonated DMG-derivatives were also analyzed using a hybrid mass spectrometer (LTQ-Orbitrap, Thermo Scientific) in the positive-ion mode with sheath, auxiliary, and sweep gas flows of 4, 1, and 0 arbitrary units, respectively. The capillary temperature was set at 275°C, the electrospray voltage was 4.0 kV, and tube lens were set to 100 V. The instrument was calibrated as instructed by the

manufacturer prior to the analysis of diradylglycerol standards. The isolation width was set to 2 (m/z), normalized collision energy was set at 20%, and resolution was at 30–60K during CID for high mass accuracy analysis of product ions by MS² and MS³ full mass spectra. Source fragmentation was on with energy set at 20 V for high mass accuracy analysis of product ions of NH₄⁺ adducts of vinyl ether diglyceride species by full MS¹ analysis due to their low ion intensities of precursor ions transferred during CID. Instrument control and data acquisition were performed using the Thermo Xcalibur version 2.1 software.

Results

Mass Spectrometric Analysis of Diacyl, Vinyl Ether and Alkyl Ether Diglycerides

Since diradylglycerols do not contain a charged moiety, their efficient ionization requires the addition of a positively charged adduct ion to promote ionization and generation of diagnostic fragment ions during electrospray ionization and fragmentation. First, we examined the utility of NH₄⁺, Li⁺, Na⁺ counter-ions as effective adduct ions for ionization. Ionization efficiency was greatest using NH₄⁺ while Li⁺ and Na⁺ had lower ionization efficiencies. Formation of informative fragment ions of each of diacyl, vinyl ether or alkyl ether diglyceride subclasses was greatest with NH₄⁺ (Figure 1), and was also observed with Li⁺ adducts but at lower sensitivity (Figure 2 and Figure S1 in the Electronic Supplementary Material, ESM). In sharp contrast, Na⁺ adducts of vinyl ether and alkyl ether diglycerides did not fragment under the conditions employed (Figure 2 and ESM Fig. S1). We also examined DMG-derivatized protonated and lithiated adducts of each of the three subclasses of diradylglycerols. Only protonated DMG –derivatized vinyl ether diglycerides generated intense informative fragment ions (Figure 3 and ESM Fig. S2).

Direct infusion of each of the six synthetic standards in the presence of 10 mM CH₃CO₂NH₄ into a triple quadrupole mass spectrometer demonstrated the presence of the anticipated NH₄⁺ adduct molecular ions. Next, fragmentation of the molecular ion was performed at different collision energies and gas pressures to identify optimal conditions for the generation of diagnostic product ions that could be utilized to discriminate each of the three subclasses. Product ion spectra of the NH₄⁺ adduct ions of each of the three subclasses of diradylglycerol standards showed informative fragmentation patterns. Prominent product ion peaks resulting from fragmentation of 1-palmitoyl-2-oleoyl-*sn*-glycerol (16:0–18:1 diacylglycerol, Scheme 1 A) demonstrated the neutral loss of NH₃ and fatty acid either from the *sn*-1 or *sn*-2 position generating the cationic dehydrated 18:1- or 16:0-monoacylglycerol product ions at m/z 339.3 or m/z 313.3, respectively, paired in nearly equal intensities (Figure 1). Similarly, fragmentation of 1-stearoyl-2-arachidonoyl-*sn*-glycerol (18:0–20:4 diacylglycerol) revealed the cationic dehydrated monoacylglycerol product ions at m/z 341.3 and m/z 361.2 but with preferential loss of NH₃-arachidonic acid (m/z 341.3) vs. stearic acid (m/z 361.2).

Analysis of product ion peaks resulting from fragmentation of 1-O-1'-(Z)-hexadecenyl-2-oleoyl-*sn*-glycerol (16:0–18:1 vinyl ether diglyceride, Scheme 1 B) also revealed a predominant peak at m/z 339.3 corresponding to the cationic dehydrated 18:1-monoacylglycerol product ion. In contrast to the fragmentation patterns of diacylglycerol NH₄⁺ adducts (*i.e.*, generating paired monoacylglycerol product ions), the neutral loss of

NH_3 and *sn*-2 fatty acid was completely absent for vinyl ether diglyceride NH_4^+ adducts which discriminates these two subclasses. Furthermore, product ion spectra of vinyl ether diglycerides demonstrated a unique fragment ion at m/z 240.3 corresponding to the 16 carbon alkyne- NH_4^+ adduct which further facilitates subclass discrimination. Similarly, fragmentation of the synthetic 1-O-1'-(Z)-octadecenyl-2-arachidonoyl-*sn*-glycerol (18:0–20:4 vinyl ether diglyceride) demonstrated a prominent product ion at m/z 361.2 (corresponding to cationic dehydrated 20:4-monoacylglycerol) and a moderate intensity unique fragment ion at m/z 268.3 (corresponding to the 18 carbon alkyne- NH_4^+ adduct). The highly similar fragmentation patterns of these two synthesized vinyl ether diglyceride molecular species, independent of their chain lengths and degrees of unsaturation, permits the accurate profiling of vinyl ether diglyceride molecular species of varied *sn*-2 fatty acids in biological samples.

Similar to the NH_4^+ adducts of vinyl ether diglycerides, product ion analysis of the NH_4^+ adducts of the alkyl ether diglycerides, 1-O-hexadecyl-2-oleoyl-*sn*-glycerol (16:0–18:1 alkyl ether diglyceride, Scheme 1 C), and 1-O-octadecyl-2-arachidonoyl-*sn*-glycerol (18:0–20:4 alkyl ether diglyceride), also demonstrated the complete absence of the fragmentation pathway via neutral loss of NH_3 and *sn*-2 fatty acid, revealing the presence of only the cationic dehydrated 18:1- and 20:4-monoacylglycerol fragment ions at m/z 339.3 and m/z 361.2, respectively, which discriminates them from the diacylglycerol subclass. Moreover, no peaks corresponding to alkene- or alkyne- NH_4^+ adducts were present in the product ion spectra of alkyl ether diglycerides (Figure 1) which therefore discriminates them from the vinyl ether diglycerides.

Product ion analysis of the lithiated DMG-derivatized molecular species of each of the three diglyceride subclasses demonstrated structurally informative product ions but in relatively low intensities (ESM Fig. S2). Product ion analysis of the protonated DMG-derivatized molecular species of both diacylglycerol [17] and alkyl ether diglyceride subclasses displayed one single predominate peak resulting from the neutral loss of DMG (Figure 3). In contrast, the fragmentation of protonated DMG-derivatives of vinyl ether diglycerides demonstrated informative fragment ions including the combined neutral losses of DMG and *sn*-2 fatty acids resulting in the product ions at m/z 279.2 and m/z 307.2 for DMG-derivatized 16:0–18:1 and 18:0–20:4 vinyl ether diglycerides, respectively (Figure 3). The product ion peaks at m/z 424.3 and m/z 446.1 corresponded to the loss of 16:0 and 18:0 vinyl alcohols from DMG-derivatized 16:0–18:1 and 18:0–20:4 vinyl ether diglycerides, respectively. However, when these informative product ions identified from the vinyl ether diglyceride standards were utilized in NLS and PIS of protonated DMG-derivatized diglycerides to identify vinyl ether diglyceride molecular species from lipid extracts of biological samples, a much lower sensitivity compared to analysis utilizing NH_4^+ adducts was observed (data not shown) likely due to the competitive multiple fragmentation pathways in protonated DMG-derivatives *vs* the two major pathways in NH_4^+ adducts of vinyl ether diglycerides (compare Figure 3 to Figure 1).

Collectively, these results substantiate the chemical identity of each of the ether-linked diradylglycerol subclasses as well as their diacyl counterparts, identify the utility of their NH_4^+ adduct ions for highly sensitive ionization and informative product ion generation,

and demonstrate the unique MS² fragmentation patterns of each diglyceride subclass enabling the use of MDMS-SL for analysis of vinyl ether diglyceride molecular species in biologic samples (*vide infra*).

High Mass Accuracy Mass Spectrometry of Diradylglycerol Molecular Species

To substantiate the aforementioned assignments of product ions in fragmentation of diradylglycerols, we performed high mass accuracy mass spectrometry of molecular ions and product ions using an LTQ-Orbitrap mass spectrometer. High mass accuracy analysis with the NH₄⁺-diradylglycerols and protonated DMG-derivatized vinyl ether diglycerides substantiated the integrity of vinyl ether and alkyl ether diglyceride species we synthesized for the study and the assignments of the product ions determined with the TSQ mass spectrometer (Table 1). The results demonstrated that the masses of each of the molecular ions and the assignments of product ions were accurate within 4 ppm substantiating the fragmentation patterns discussed above. High mass accuracy mass spectrometric analysis of product ions provided compelling evidence for the proposed differential fragmentation patterns of the three diradylglycerol subclasses and the signature fragment ions that discriminated each diradylglycerol subclass. Accordingly, NH₄⁺ adduct ions of each diradylglycerol subclass and their corresponding diagnostic fragment ions were used for subsequent MDMS-SL experiments of lipid extracts of mouse brain and heart.

Multidimensional Mass Spectrometry-based Shotgun Lipidomics Analysis of Mouse Brain and Heart Extracts

Although brain and heart tissues have high contents of ethanolamine lipids containing 1-O-1'-Z alkenyl-2-acyl linkages (*e.g.*, plasmalogen lipids) [30–32], detection and identification of individual vinyl ether diglyceride molecular species has proven difficult. To demonstrate the utility of the observed diagnostic fragmentation patterns of the synthetic diradylglycerols in biologic samples, we performed MDMS-SL on mouse brain and heart extracts. First, diradylglycerols were enriched by liquid/liquid partitioning into hexane as described in Materials and methods. This resulted in the removal of polar lipids and other metabolites into the methanol phase while the hexane layer was markedly enriched in non-polar lipids. This enrichment procedure greatly facilitated the analysis of diradylglycerols by MDMS-based SL.

Brain hexane extracts were directly infused into the TSQ mass spectrometer revealing multiple diacylglycerol molecular ions in the full mass MS¹ scan (Figure 4). However, the full mass MS¹ scan did not reveal any ion peaks corresponding to either alkyl ether or vinyl ether diglyceride molecular species due to their extremely low abundance. Next, we used the diagnostic fragment ions identified from the vinyl ether diglycerides synthesized for this study (Figure 1). NLS of 257, 283 and 285 amu gave robust signals likely corresponding to 16:0, 18:1 and 18:0 vinyl ether diglycerides, respectively, resulting from the neutral loss of NH₃ and the corresponding vinyl alcohol (note that the vinyl alcohol is a resonance form of the fatty aldehyde). To substantiate these assignments, additional experiments were performed to exclude the possibility that isobaric odd chain length fatty acids in diacylglycerols could be responsible for the signals observed during NLS. To this end, we compared PIS of cationic dehydrated 18:1-, 20:4-, 20:1-, 22:6- and 22:4-monacylglycerol

product ions with the corresponding NLS of NH₃-fatty acids (*i.e.*, 18:1, 20:4, 20:1, 22:6 and 22:4 fatty acids) (ESM Fig. S3). As demonstrated in Figure 1 by the product ion spectra of three subclasses of synthetic diradylglycerol standards containing 18:1 or 20:4 fatty acyl at *sn*-2 position, cationic dehydrated 18:1- or 20:4- monoacylglycerol product ions (at *m/z* 339 or *m/z* 361, respectively) are present for all three subclasses of diradylglycerols resulting from the neutral loss of NH₃ and *sn*-1 moieties (*i.e.*, fatty acid, vinyl alcohol, or fatty alcohol for diacyl, vinyl ether, or alkyl ether diglyceride subclass, respectively). In contrast, the neutral loss of NH₃ and *sn*-2 fatty acid is solely present in diacylglycerols while this fragmentation pathway via loss of NH₃ and *sn*-2 fatty acid is completely absent for both vinyl ether and alkyl ether diglycerides. Therefore, the presence of the ion peaks in PIS and their absence during NLS in ESM Fig. S3 indicate that the observed peaks are ether-linked diglyceride and not odd chain length fatty acid containing diacylglycerol molecular species.

Next, to confirm the presence of vinyl ether diglycerides, we utilized the diagnostic fragment ions identified from the vinyl ether diglyceride synthetic standards (Figure 1). Specifically, PIS of the diagnostic fragment ions at *m/z* 240 and *m/z* 268 (for 16:0 and 18:0 vinyl ether diglycerides, respectively) and by inference *m/z* 266 (for 18:1 vinyl ether diglycerides) were employed to discriminate vinyl ether diglycerides from alkyl ether diglycerides which do not produce these diagnostic ions during fragmentation (Figure 1). The strong similarities in the NLS of 257, 283 and 285 amu and PIS of *m/z* 240, *m/z* 266 and *m/z* 268 based on the informative/diagnostic ions corresponding to 16:0, 18:1 and 18:0 vinyl ether diglycerides provide compelling evidence for the presence of multiple vinyl ether diglyceride molecular species in brain (compare Figure 4 to ESM Fig. S4). The presence of vinyl ether diglycerides in brain was also substantiated by the acid lability of the vinyl ether linkage. The peaks identified as vinyl ether diglycerides were dramatically reduced by brief acid treatment (data not shown). Collectively, these results demonstrated that brain contains multiple molecular species of vinyl ether diglycerides and performed profiling of these species through specific NLS and PIS for vinyl ether linkages (Figure 4 and ESM Fig. S4).

Identification of individual vinyl ether diglyceride molecular species is achieved utilizing the specific NLS or PIS of the vinyl alcohol determining the vinyl ether linkage (*e.g.*, 16:0 vinyl ether containing species present in NLS 257 and PIS 240), and the precursor ion mass determining the total carbon number and the degree of unsaturation (*e.g.*, 34:1 vinyl ether diglyceride species at *m/z* 596), from which the chemical composition of fatty acyl chain is readily derived (*e.g.*, 18:1 fatty acyl chain derived from the 16:0 vinyl ether containing 34:1 diglyceride species, which can be further confirmed using PIS of the cationic dehydrated monoacylglycerol product ion, *i.e.*, PIS 339 for 18:1 acyl containing species). The identified vinyl ether diglyceride species in brain include p16:0–18:1 at *m/z* 596, p16:0–20:4 at *m/z* 618, p18:1–18:1 at *m/z* 622, p16:0–20:1/p18:0–18:1 at *m/z* 624, p16:0–22:6 at *m/z* 642, p18:1–20:4 at *m/z* 644, p16:0–22:4/p18:0–20:4 at *m/z* 646, p18:1–20:1 at *m/z* 650, p18:0–20:1 at *m/z* 652, p18:1–22:6 at *m/z* 668, p18:0–22:6 at *m/z* 670, p18:1–22:4 at *m/z* 672 and p18:0–22:4 at *m/z* 674 (Figure 4 and ESM Figures S3 and S4).

Previously, vinyl ether diglycerides have been shown to be preferred substrates for CDP-DAG ethanolamine phosphotransferase activity in rabbit myocardium and to have distinct effects on activation of myocardial protein kinase C (PKC) [11,13]. Accordingly, we used

the developed methodology to identify the presence of vinyl ether diglycerides in mouse myocardium. Mouse myocardial hexane extracts were directly infused into the TSQ mass spectrometer revealing multiple diacylglycerol molecular ions in the MS¹ full mass scan. Similar to the results with brain extracts, the MS¹ scan did not reveal any peaks corresponding to either vinyl ether or alkyl ether diglyceride molecular species due to their low abundance. Next, we used the diagnostic fragment ions identified from the vinyl ether diglycerides synthesized for this study (Figure 1) to perform MDMS-SL analysis. Neutral loss scanning of 257 amu, precursor ion scanning for m/z 240, precursor ion scanning for m/z 385, and neutral loss scanning of 345 amu unambiguously identified the presence of 16:0–22:6 vinyl ether diglyceride (Figure 5). The predominance of this molecular species likely reflects the abundance of 22:6 fatty acids in murine myocardium in both the plasmalogen and plasmalogen ethanolamine pools.

Discussion

In the present work, we have analyzed representative molecular species of three diradylglycerol (1-O-1'(Z) alkenyl, 1-O-alkyl, and diacyl) subclasses containing either oleic acid or arachidonic acid at the *sn*-2 position to determine informative product ions to enable the utilization of MDMS-SL in the identification and profiling of individual vinyl ether diglyceride molecular species in biological samples. We compared the utility of several adduct ions as well as derivatized moieties to determine the optimal counter ion(s) to generate informative fragment ions. The results demonstrated the utility of NH₄⁺ adduct ions to yield diagnostic fragmentation patterns for each of the three diradylglycerol subclasses. These results were utilized for MDMS-SL analysis of vinyl ether diglycerides directly from extracts of mouse brain and heart.

Due to the diminutive amounts of vinyl ether and alkyl ether diglycerides in biologic samples, it was necessary to enrich the tissue extract by liquid/liquid partitioning. Utilization of hexane/methanol partitioning allows the vast majority of polar lipids and other polar metabolites to enter the methanol phase resulting in their removal while simultaneously enriching the targeted non-polar lipids in the hexane phase. This strategy allows for the analysis of non-polar lipids directly from extracts of biologic tissues and fluids without prior chromatographic purification.

The utility of MDMS-SL is easily appreciated through its comparison with the molecular ion MS¹ scans of mouse brain and heart extracts which did not contain any observable signal for vinyl ether or alkyl ether diglyceride molecular species. The power of MDMS-SL was harvested from informative transitions identified through the analysis of synthetic diradylglycerols prepared for this study. Once diagnostic fragment ions were identified by product ion analysis, the utilization of NLS and PIS techniques using a triple quadrupole mass spectrometer results in over 100-fold increases in S/N as well as remarkably enhanced analytical specificity in NLS and PIS. The utilization of synthetic diradylglycerols identified multiple differences in fragmentation patterns both in the mass of fragment ions and the relative intensities of fragment ion peaks. Moreover, a suite of diagnostic product ions for 16:0 and 18:0 vinyl ether diglycerides at m/z 240 and 268, respectively (and by inference at m/z 266 for 18:1 vinyl ether diglycerides), were identified enabling their use for PIS. In

addition, the highly sensitive loss of vinyl alcohol-NH₃ (e.g., loss of 257 and 285 amu for 16:0 and 18:0 vinyl ether diglycerides, respectively, and by inference loss of 283 amu for 18:1 vinyl ether diglycerides) were identified enabling their use for NLS. The combination of NLS and PIS analyses identifies vinyl ether diglyceride molecular species and discriminates them from their isomeric alkyl ether diglyceride species and isobaric odd chain length diacylglycerol species. Through this strategy, numerous vinyl ether diglyceride molecular species were observed and profiled directly from the hexane extracts of brain and heart tissues.

The combination of NLS and PIS also allow the detection of unknown molecular species in a class or subclass of compounds once diagnostic fragment ions have been identified. Examples include determination of the presence or absence of odd chain length aliphatic chains, oxidized fatty acids, or fatty acids containing additional constituents in the aliphatic chain (e.g., nitro fatty acids). It should be recognized that MRM also results in marked increases in S/N. However, utilization of MRM requires *a priori* knowledge of the specific individual molecular species that are present in the sample as well as their diagnostic transitions.

We specifically point out that for non-polar lipid analysis both the ionization efficiency of molecular ions as well as their fragmentation patterns are influenced by the chain length and the degree of unsaturation of the aliphatic chain as well as the covalent linkage at the *sn*-1 carbon of the glycerol backbone. The developed method can be used to identify ions undetectable in MS¹ scans and facilitates identification and profiling of individual molecular species of vinyl ether diglycerides that may contain unknown aliphatic constituents and/or covalent modifications. Thus, the developed approach enables identification and profiling of vinyl ether diglyceride molecular species as well as direct comparisons of alterations in vinyl ether diglyceride profiles, although determination of the absolute amounts requires the utilization of internal standards and knowledge of the relative ionization efficiencies of each analyte. Absolute quantitation can be performed using stable isotope labeled standards of the targeted compounds, SILAC based methods, or synthesized vinyl ether diglyceride internal standards that are absent in biological samples. The identical fragmentation patterns in the product ion analysis of our synthesized 16:0–18:1 and 18:0–20:4 vinyl ether diglycerides indicate the independence of the fragmentation kinetics of individual molecular species having differential acyl chain lengths and degrees of unsaturation, which permits the utilization of one internal standard for accurate quantitation of each vinyl ether linkage diglyceride molecular species in combination with the ionization efficiency correction.

In summary, we have identified diagnostic fragmentation patterns in each of the three subclasses of diradylglycerols through tandem mass spectrometric analysis of their NH₄⁺ adducts. This strategy was employed to identify and profile vinyl ether diglycerides molecular species directly from extracts of brain and heart. We anticipate that utilization of this approach will facilitate the identification of the roles of vinyl ether linked diglycerides in lipid metabolism and signaling and their maladaptive alterations in disease states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Tandem mass spectra of product ions of NH₄⁺ adducts of synthetic diacyl, vinyl ether, alkyl ether diglycerides. The tandem mass spectra are presented for diacyl (D), vinyl ether or plasmeryl (p) and alkyl ether (a) 16:0–18:1 diglycerides (left panel) and for 18:0–20:4 diglycerides (right panel). The fragment ions at *m/z* 339 (left panel) and at *m/z* 361 (right panel) correspond to the cationic dehydrated 18:1 and 20:4-monoacylglycerol product ions, respectively, which are generated from the neutral loss of NH₃ and corresponding *sn*-1 moieties (16:0 or 18:0 fatty acid for diacylglycerols, vinyl alcohol for vinyl ether diglycerides, and alcohol for alkyl ether diglycerides). The fragment ions at *m/z* 240 (left panel) and at *m/z* 268 (right panel) are signature product ions for vinyl ether diglycerides that are absent in the spectra of diacylglycerols and alkyl ether diglycerides, which are NH₄⁺ adducts of the 16 carbon and 18 carbon terminal alkynes derived from 16:0 and 18:0 vinyl alcohols, respectively.

Figure 2.

Tandem mass spectra of product ions of Li^+ or Na^+ adducts of synthetic diacyl, vinyl ether, and alkyl ether diglycerides. The tandem mass spectra are presented for the Li^+ adducts of diacyl (D), vinyl ether or plasmenyl (p) and alkyl ether (a) 18:0–20:4 diglycerides (left panel) and their corresponding Na^+ adducts (right panel). Structurally informative fragment ions are present in tandem mass spectra of the diglyceride Li^+ adduct (*e.g.*, the product ion at m/z 311 representing the Li^+ adduct of 20:4 fatty acid; the product ions at m/z 347, m/z 331 and m/z 333 representing the fragment ions after neutral loss of 20:4 fatty acid from diacyl, vinyl ether, or alkyl ether diglyceride molecular ions, respectively; and the product ion at m/z 341 resulting from the neutral loss of 20:4 fatty acid lithiated salt from the diacylglycerol molecular ion). In contrast, Na^+ adducts of vinyl and alkyl ether diglycerides do not fragment under the experimental conditions employed, while the Na^+ adducts of diacylglycerol species do fragment but generate only low abundance structural product ions.

Figure 3.

Tandem mass spectra of product ions of protonated DMG-derivatized synthetic diacyl, vinyl ether, alkyl ether diglycerides. The tandem mass spectra are presented for diacyl (D), vinyl ether or plasmeryl (p) and alkyl ether (a) 16:0–18:1 diglyceride-DMG derivatives (left panel) and for 18:0–20:4 diglyceride-DMG derivatives (right panel). Product ion scanning of both diacyl and alkyl ether diglyceride-DMG derivatives demonstrates the neutral loss of DMG as the predominant fragment ion (*i.e.*, m/z 577 or m/z 563 for diacyl or alkyl ether 16:0–18:1 diglyceride, respectively; and at m/z 627 or m/z 613 for diacyl or alkyl ether 18:0–20:4 diglyceride, respectively). In contrast, in addition to the fragment ion at m/z 561 or m/z 611 from the neutral loss of DMG, product ion scanning of protonated vinyl ether diglyceride-DMG derivatives presents structurally informative fragment ions that include the fragment ion at m/z 279 or m/z 307 resulting from the neutral loss of DMG and 18:1 or 20:4 fatty acid, and the fragment ion at m/z 424 or m/z 446 resulting from the neutral loss of 16:0 or 18:0 vinyl alcohol, for 16:0–18:1 or 18:0–20:4 vinyl ether diglycerides, respectively.

Figure 4.

Identification and profiling of vinyl ether diglyceride molecular species in mouse brain tissues. Hexane extracts of mouse brain were prepared as described in Materials and methods. Brain hexane extracts were diluted in the presence of 10 mM $\text{CH}_3\text{CO}_2\text{NH}_4$, and the NH_4^+ adducts of diradylglycerols were analyzed in the positive-ion mode utilizing a full MS^1 scan and the NLS as described in Materials and methods. The top mass spectrum represents the MS^1 full scan that reveals predominantly the diacylglycerol molecular ions that are also present in the tandem mass spectrum of neutral loss (NL) of both NH_3 and H_2O . The three bottom spectra are the tandem mass spectra from neutral loss of NH_3 and a vinyl alcohol (or alternatively an isobaric odd chain length fatty acid, or an isomeric fatty alcohol which were excluded (see text)). Note that the ion peaks detected in NLS of 257, 283 or 285 amu are either undetectable or barely detectable in the full MS analysis and NLS of NH_3 and H_2O , as shown by dashed lines.

Figure 5.

Identification of vinyl ether diglyceride molecular species present in mouse heart. Hexane extracts of mouse heart were prepared as described in Materials and methods. Heart hexane extracts were diluted in the presence of 10 mM $\text{CH}_3\text{CO}_2\text{NH}_4$, and the NH_4^+ adducts of the diradylglycerols were analyzed in the positive-ion mode utilizing full MS^1 scan, NLS and PIS as described in Figure 4 and ESM Figures S3 and S4. The top full MS^1 scan reveals predominantly diacylglycerol molecular ions that are also present in the tandem mass spectrum of neutral loss (NL) of NH_3 and H_2O . NLS of 257 amu represents the loss of NH_3 and 16:0 vinyl alcohol. PIS of m/z 240 corresponds to the tandem mass spectrometric analysis of the NH_4^+ adduct of the 16 carbon saturated terminal alkyne derived from 16:0 vinyl alcohol. PIS of m/z 385 corresponds to the tandem mass spectrometric analysis of 22:6-monoacylglycerol product ion derivatives. NLS of 345 amu represents the loss of NH_3 and 22:6 fatty acid which is absent for vinyl ether diglyceride species with 22:6 fatty acid at *sn*-2. Collectively, these tandem mass spectrometric analyses prove the presence of 16:0–22:6 vinyl ether diglyceride species in the studied mouse heart.

A. 1-palmitoyl-2-oleoyl-*sn*-glycerol (16:0-18:1 diacylglycerol) B. 1-O-1'-(Z)-hexadecenyl-2-oleoyl-*sn*-glycerol (16:0-18:1 vinyl ether diglyceride) C. 1-O-hexadecyl-2-oleoyl-*sn*-glycerol (16:0-18:1 alkyl ether diglyceride) **Scheme 1.**

Structure of representative molecular species of three subclasses of diglycerides

Table 1

Theoretical masses of assigned product ions and their measured masses by high mass accuracy mass spectrometry of synthetic diradylglycerol species

diradylglycerol species	molecular ion or product ion	theoretical mass	measured mass	ppm
D16:0–18:1/ NH_4^+	molecular ion	612.5567	612.5567	0.0
	loss of NH_3	595.5302	595.5298	−0.6
	loss of $(\text{NH}_3+\text{H}_2\text{O})$	577.5196	577.5194	−0.3
	loss of $(\text{NH}_3+sn-1 \text{ moiety})$	339.2899	339.2895	−1.2
	loss of $(\text{NH}_3+sn-2 \text{ fatty acid})$	313.2743	313.2738	−1.5
D18:0–20:4/ NH_4^+	molecular ion	662.5723	662.5721	−0.4
	loss of NH_3	645.5458	645.5459	0.2
	loss of $(\text{NH}_3+\text{H}_2\text{O})$	627.5352	627.5351	−0.2
	loss of $(\text{NH}_3+sn-1 \text{ moiety})$	361.2743	361.2739	−1.0
	loss of $(\text{NH}_3+sn-2 \text{ fatty acid})$	341.3056	341.3051	−1.4
	20:4 ketene/ H^+	287.2375	287.237	−1.7
	20:4 ketene- $\text{H}_2\text{O}/\text{H}^+$	269.2269	269.2264	−2.0
p16:0–18:1/ NH_4^+	molecular ion	596.5618	596.562	0.4
	loss of NH_3	579.5352	579.5353	0.1
	loss of $(\text{NH}_3+\text{H}_2\text{O})$	561.5247	561.5243	−0.7
	loss of $(\text{NH}_3+sn-1 \text{ moiety})$	339.2899	339.2892	−2.1
	Alkyne (16C)/ NH_4^+	240.2691	240.2683	−3.4
p18:0–20:4/ NH_4^+	molecular ion	646.5774	646.5776	0.3
	loss of NH_3	629.5509	629.551	0.2
	loss of $(\text{NH}_3+\text{H}_2\text{O})$	611.5403	611.5401	−0.4
	loss of $(\text{NH}_3+sn-1 \text{ moiety})$	361.2743	361.2734	−2.4
	Alkyne (18C)/ NH_4^+	268.3004	268.2995	−3.4
a16:0–18:1/ NH_4^+	molecular ion	598.5774	598.577	−0.7
	loss of NH_3	581.5509	581.551	0.2
	loss of $(\text{NH}_3+\text{H}_2\text{O})$	563.5403	563.5404	0.1
	loss of $(\text{NH}_3+sn-1 \text{ moiety})$	339.2899	339.2896	−0.9
a18:0–20:4/ NH_4^+	molecular ion	648.5931	648.5930	−0.1
	loss of NH_3	631.5665	631.5665	−0.1
	loss of $(\text{NH}_3+\text{H}_2\text{O})$	613.5560	613.5562	0.4
	loss of $(\text{NH}_3+sn-1 \text{ moiety})$	361.2743	361.2740	−0.7
	20:4 ketene/ H^+	287.2375	287.2371	−1.4
	20:4 ketene- $\text{H}_2\text{O}/\text{H}^+$	269.2269	269.2266	−1.2

diradylglycerol species	molecular ion or product ion	theoretical mass	measured mass	ppm
p16:0–18:1-DMG/H ⁺	molecular ion	664.5880	664.5876	−0.6
	loss of DMG	561.5247	561.525	0.6
	loss of (DMG+ <i>sn</i> -2 fatty acid)	279.2688	279.2683	−1.8
	loss of <i>sn</i> -1 vinyl alcohol	424.3427	424.3425	−0.4
p18:0–20:4-DMG/H ⁺	molecular ion	714.6036	714.6035	−0.2
	loss of DMG	611.5403	611.5403	0.0
	loss of (DMG+ <i>sn</i> -2 fatty acid)	307.3001	307.2996	−1.6
	loss of <i>sn</i> -1 vinyl alcohol	446.3270	446.3268	−0.5