In Situ Structural Characterization of Phosphatidylcholines in Brain Tissue Using MALDI-MS/MS

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Phosphatidylcholine (PC) is one of the most abundant classes of phospholipids and is a major component of membranes in biological systems. Recently, PCs have been detected by direct tissue analysis using MALDI-TOFMS. However, these studies did not allow for the structural characterization of PCs in tissue. In the current study, an in situ method for detection and structural analysis of PC species in brain tissue was developed using a MALDI-TOF/TOF mass spectrometer. Initial profiling of lipids in tissue is performed by MALDI-TOFMS, which allows for the assignment of PC species. However, to confirm the structure of the PC species detected in tissue, MALDI-MS/MS analysis was employed. In this work, protonated, sodiated, and potassiated PC species were detected in brain tissue using DHA matrix. MALDI-MS/MS analysis of these species yielded fragments that verified a phosphocholine head group, but did not supply any fragments that would permit the identification of acyl substituents. To obtain more structural information, lithium adducts of PC species were produced using DHA matrix dissolved in 100 mM lithium chloride. MALDI-MS/MS analysis of lithiated PC species produced fragments that allowed for the identification and positional assignment of acyl groups in PC species. (J Am Soc Mass Spectrom 2005, 16, 2052–2056) © 2005 American Society for Mass Spectrometry

Phosphatidylcholines (PC), a class of glycerophospholipids, are main building blocks of biological membranes accounting for ~50% of phospholipid mass in cell membranes of eukaryotic cells [1, 2]. PCs also play an important role in cell signaling [3], and the disruption of PC homeostasis in mammalian cell leads to cell death [4]. The basic structure of phosphatidylcholine (1,2-diacyl-sn-glycero-3-phosphocholine) is in Figure 1. PC consists of a phosphocholine head group and a glycerol backbone with two acyl (fatty acid) tails. The two acyl groups are attached to the glycerol backbone at the sn-1 and sn-2 position. The polar phosphocholine head group gives PC a strong zwitterionic character.

Several studies have employed matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) for the detection and structural analysis of PCs [5–8]. Due to the presence of a positively charged quaternary amine, PC species are easily ionized in positive ion mode. Fragments observed in MALDI-MS spectra, corresponding to the loss of trimethylamine, phosphocholine head group, and acyl groups, have been used for structural characterization of PCs [5, 6]. MS² of PCs using MALDI-FTICR/MS have produced abundant

ions at $m/z = 184$ attributed to the head group of PC [6]. Additionally, post-source decay (PSD) MALDI-MS has also been employed for the structural analysis of PCs [7]. In this study, sodiated PC species were analyzed with PSD coupled with MALDI-MS and produced fragments, corresponding to the loss of trimethylamine, phosphocholine head group, and acyl groups.

MALDI-MS methods have been developed for the in situ analysis of biomolecules, typically peptides and proteins, from tissue [9–11]. Due to its high sensitivity, tolerance for salts and other contaminants, and a wide mass range with little fragmentation, MALDI is the ionization method of choice for the direct analysis of complex samples such as tissue. In these methods, matrix is deposited directly onto a tissue section and then mass analyzed with no additional sample preparation. Direct tissue analysis using MALDI produces mass spectral profiles, which can be employed to map the location of molecules in tissue. Recently, MALDI-MS has been used for direct analysis of phospholipids in lens tissue [12] and in rat brain tissue [13, 14]. In these studies, positive ion MALDI mass spectra were dominated by PC species, which showed different regional distribution in the relative abundance of PC species in tissue.

In this work, in situ analysis of PCs in rat brain tissue was conducted using a MALDI-TOF/TOF mass spectrometer. Tandem MS analysis of protonated, sodiated, and potassiated PC species yielded fragments that

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Figure 1. Phosphatidylcholine.

correspond to the loss of trimethylamine and the head group from PC and the phosphocholine molecular ion. To generate fragments with more pertinent structural information, lithiated PC species were analyzed. Lithium adducts were produced by using 2,6-dihydroxyacetophenone matrix dissolved in 100 mM lithium chloride. Tandem MS analysis of lithiated PC species yielded fragment mass peaks that correspond to the loss of trimethylamine, the phosphocholine head group, and acyl groups.

Experimental

Mass Spectrometer

A MALDI-TOF/TOF (4700 Proteomics Analyzer, Applied Biosystems, Framingham, MA) was used in this study for both MS and MS/MS analyses and has been described in detail previously [15]. A Nd:YAG laser (355 nm) at a repetition rate of 200 Hz was employed for ionization. For MS analysis, mass spectra were the sum of 400 laser shots and acquired in reflectron mode. For MS/MS analysis, mass spectra were the sum of 1000 laser shots, a collision energy of 1 keV with air as the collision gas was used to induce fragmentation. The following lipid standards were used to calibrate the mass spectrometer: brain phosphatidylcholines (99%, porcine, Avanti Polar Lipids, Alabaster, AL) and sphingomyelins (99%, porcine, Avanti Polar Lipids).

Tissue Sectioning

All animal use and handling in this work abide by the Guide for the Care and Use of Laboratory Animals (NIH). Male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN) between 300 and 420 g were euthanized by an intraperitoneal injection of sodium pentobarbital (>65 mg/kg). The brains were quickly removed from the skull and frozen in dry ice-chilled isopentane for 15 s, before storage at -80°C . Frozen brain tissue was cut into thin sections (14 μm thickness) in a cryostat (CM 3050 S; Leica Microsystems Nussloch GmbH, Nussloch, Germany). Serial brain sections were alternately placed onto a MALDI sample target and poly-L-lysine coated microscopic slide. The tissue sections on the microscopic slides were stained with cresyl violet and a rat brain atlas [16] was used for the assignment of the brain region analyzed by mass spectrometry.

Sample Preparation

The MALDI matrix used in this study was 2,6-dihydroxyacetophenone, DHA, (Fluka, Buchs, Switzerland). Lithium chloride (Sigma-Aldrich, St. Louis, MO) was dissolved in 50% ethanol at a concentration of 100 mM. Matrix solutions were prepared in 50% ethanol or 100 mM LiCl in 50% ethanol at a concentration of 30 mg/mL for DHA. For tissue analysis, 0.1 μL of matrix solution was deposited directly on the section, resulting in a matrix spot size between 800 and 1500 μm , and allowed to air-dry before insertion into the mass spectrometer.

Lipid Assignment

In MALDI-TOF mass spectra, the PC species number equals the total length and number of double bonds of both acyl chains, while the SM species number corresponds to the length and number of double bonds of the acyl chain attached to the sphingosine base. Assignment of PC species based upon product-ion spectra is as follows: PC sn-1 acyl group/sn-2 acyl group.

Results and Discussion

Experiments were conducted analyzing PCs in situ from rat brain tissue with DHA matrix by MALDI-TOFMS and MALDI-TOF/TOFMS. Figure 2 illustrates a typical mass spectrum of rat brain tissue with DHA matrix. This mass spectrum was obtained from the cerebral cortex region of the brain in positive ion reflectron mode. The most dominant mass peaks in Figure 2 assigned to PCs were the following two species: PC 32:0 and PC 34:1. The molecular ions, $[M + H]^+$, $[M + Na]^+$, and $[M + K]^+$, were detected for both species. Mass peaks associated with a sphingomyelin (SM) species, SM18:0, was also observed. These results are in good agreement with a previous study [13] using MALDI-TOFMS for direct tissue analysis of the cerebral cortex region of rat brain.

To confirm the assignment of PC species, MALDI-MS/MS analysis was conducted on the mass peaks

Figure 2. (a) MALDI mass spectrum of cerebrum cortex with DHA matrix.

observed in Figure 2. A product-ion spectrum of $[\text{PC } 32:0 + \text{H}]^+$ ($m/z = 734.585$) in positive ion mode with DHA matrix contained only one major fragment peak observed at $m/z = 184.072$. This mass peak corresponds to phosphocholine ($\text{C}_5\text{H}_{15}\text{O}_4\text{N}$), which is formed by the loss of the polar head group of PC. This result is in agreement with previous MS/MS studies using MALDI-FTICR/MS⁶, electrospray-MS⁷(ESI-MS)^{17–19}, and PSD²⁰ MALDI-MS⁷. MALDI-MS/MS analysis of all other protonated PC species in Figure 2 yielded a similar fragmentation pattern, in which the only major peak was phosphocholine.

Additional experiments were performed in which sodiated and potassiated PC species were analyzed by MALDI-TOF/TOF. A product-ion spectrum of $[\text{PC } 32:0 + \text{K}]^+$ ($m/z = 772.524$) consisted of fragment peaks at m/z 713.491, 162.958, and 551.602. The mass peak at 713.491 Da is produced by the neutral loss of trimethylamine $[\text{N}(\text{CH}_3)_3]$ from the parent ion, while the mass peak at 162.958 Da, corresponds to a potassiated five-membered cyclophosphane ring ($\text{C}_2\text{H}_5\text{O}_4\text{P} + \text{K}$). The fragment peak at $m/z = 551.602$ is assigned as $[\text{PC } 32:0 - \text{N}(\text{CH}_3)_3 - \text{C}_2\text{H}_5\text{O}_4\text{P} - \text{K} + \text{H}]^+$ and attributed to the net loss of the phosphocholine head group and potassium from the parent ion. Further analysis of potassiated and sodiated PC species in Figure 2 produced product-ion spectra with similar fragment peaks with the major difference being the substitution of sodium for potassium in sodiated PC species. Previous MS/MS studies of sodiated species using ESI/MS²⁰, fast atom bombardment mass spectrometry (FAB-MS)²¹, and PSD²² MALDI-MS⁷ have yielded similar fragment peaks. However, in some of these previous studies^{7, 21} additional fragment peaks corresponding to the loss acyl groups were also observed. In our work, MALDI-MS/MS analysis of sodiated and potassiated PC species did not produce any significant fragment peaks corresponding to the loss of acyl groups.

To gain more structural information on PC species by ESI-MS/MS analysis, previous studies^{17, 18} have generated lithium adducts of PC species. In these previous works it was demonstrated that lithiated adducts of PC species were more extensively fragmented than protonated PC species; thus yielding more pertinent structural information, allowing for the assignment of acyl groups. In the current work, the matrix, DHA, was dissolved in 100 mM LiCl and deposited directly onto the tissue sections to produce lithium adducts. Figure 3a illustrates a typical MALDI-TOF mass spectrum of rat brain tissue with DHA/LiCl matrix. This mass spectrum was obtained from the cerebral cortex region of the brain in positive ion reflectron mode. The two major peaks in this mass spectrum are the lithium adducts of PC32:0 and PC34:1. Figure 3a is in agreement with Figure 2a, in which the same major PC species are observed except that they are lithium adducts.

Figure 3b shows a product-ion spectrum of $[\text{PC } 32:0 + \text{Li}]^+$ ($m/z = 740.514$) in positive ion mode with

DHA/LiCl matrix. Several fragment peaks providing structural information are recorded. The mass peak at $m/z = 681.536$ corresponds to the neutral loss of trimethylamine while the mass peaks at 557.560 $[\text{M} + \text{Li} - \text{N}(\text{CH}_3)_3 - \text{C}_2\text{H}_5\text{O}_4\text{P}]^+$ and 551.516 Da $[\text{M} + \text{H} - \text{N}(\text{CH}_3)_3 - \text{C}_2\text{H}_5\text{O}_4\text{P}]^+$, correspond to the net loss of the phosphocholine head group with and without lithium. Structural information enabling the identification of the acyl groups of the PC specie is provided by the mass peaks at 484.349, 478.320, and 425.311 Da. These mass peaks are attributed to losses of palmitic acid, the lithium salt of palmitate, and trimethylamine with palmitic acid as neutral molecules and are assigned as follows: 484.349 $[\text{M} + \text{Li} - \text{C}_{16}\text{H}_{32}\text{O}_2]^+$, 478.320 $[\text{M} + \text{H}$

Figure 3. (a) MALDI mass spectrum of cerebrum cortex with DHA matrix in 100 mM LiCl. MALDI/TOF-TOF mass spectra of (b) PC32:0+Li mass peak and (c) PC34:1+Li mass peak.

– $C_{16}H_{32}O_2$] $^+$, 425.311 [M + Li – N(CH₃)₃ – $C_{16}H_{32}O_2$] $^+$. Based upon the fragment peaks observed in Figure 3b, the major PC species contributing to the mass peak assigned as [PC 32:0 + Li] $^+$ in Figure 3a is PC 16:0/16:0. The fragment peaks observed for lithiated PC species in tissue by MALDI-TOF/TOF analysis are in similar agreement with ESI-MS/MS studies [17,18] of lithiated PC species.

One additional note for Figure 3b is the presence of interfering peaks corresponding to a sphingomyelin specie (SM 18:0 + Li). These peaks are marked with asterisks in Figure 3b and were recorded at 737.565 and 678.539 Da. These mass peaks are assigned as follows: 737.565 [SM 18:0 + Li] $^+$, 678.539 [SM 18:0 + Li – N(CH₃)₃] $^+$. This emphasizes the care that has to be taken in assigning mass peaks with direct tissue analysis in which several lipid species are present and can overlap in the same mass selection range.

Figure 3c is a product-ion spectrum of [PC 34:1 + Li] $^+$ (m/z = 766.578) in positive ion mode with DHA/LiCl matrix. The major fragment peaks in this mass spectrum correspond to the neutral loss of trimethylamine (707.534 Da) and the net loss of the phosphocholine head group with and without lithium (583.540 [M + Li – N(CH₃)₃ – $C_2H_5O_4P$] $^+$ and 577.525 Da [M + H – N(CH₃)₃ – $C_2H_5O_4P$] $^+$). Several additional fragment peaks were observed corresponding to the neutral losses of acyl groups and trimethylamine. The mass peaks were assigned as follows: 510.33 [M + Li – $C_{16}H_{32}O_2$] $^+$ loss of palmitic acid, 504.342 [M + H – $C_{16}H_{32}O_2$] $^+$ loss of the lithium salt of palmitate, 451.279 [M + Li – N(CH₃)₃ – $C_{16}H_{32}O_2$] $^+$ loss of trimethylamine and palmitic acid, 484.358 [M + Li – $C_{18}H_{34}O_2$] $^+$ loss of oleic acid, 478.357 [M + H – $C_{16}H_{32}O_2$] $^+$ loss of the lithium salt of oleate, 425.278 [M + Li – N(CH₃)₃ – $C_{18}H_{34}O_2$] $^+$ loss of trimethylamine and oleic acid. Based upon the fragment peaks observed in Figure 3c, the major PC specie contributing to the mass peak assigned as [PC 34:1 + Li] $^+$ in Figure 3a is PC 16:0/18:1. The positional assignment of acyl groups accordingly as sn-1 or sn-2 substituents is based upon previous fragmentation studies [7,17] of PC species in which the abundance of ions corresponding to the loss of the sn-1 substituent is greater than the abundance of ions reflecting the loss of the sn-2 substituent. Previous studies of the distribution of PC species in rat brain have identified PC 16:0/18:1 as one of the most abundant PC species, which further supports assignment based upon direct tissue analysis by MALDI-TOF/TOF [22,23].

Conclusions

In this work, a method for in situ detection and structural analysis of PC species in brain tissue was developed using a MALDI-TOF/TOF mass spectrometer. Initial profiling of lipids in tissue is conducted by MALDI-TOFMS and allows for the assignment of PC species. To confirm the structure, lithium adducts of PC species were analyzed by MALDI-MS/MS and yielded fragments that allowed for the identification and posi-

tional assignment of acyl groups in PC species. Future studies will include analyzing additional anatomical regions in rat brain tissue and other organs for PC distribution. Additional studies using MALDI-MS/MS will be conducted for the identification of other classes of phospholipids and gangliosides by direct tissue analysis.

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