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Isolation and mass spectrometry characterization of molecular species of lactosylceramides using liquid chromatography-electrospray ion trap mass spectrometry

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Abstract

Reverse-phase liquid chromatography/electrospray ion trap mass spectrometry (LC-ESI-MS^n) was established for identification of the molecular species of lactosylceramides. Lactosylceramides derived from porcine blood cells were separated on a CapcellPak C_8 column using a mixture of methanol and 1 mM ammonium formate from the C_{16} to C_{26} fatty acyl chains based on the length of total carbon chains and the nature of sphingoid bases (w″) and fatty acyl chains ($Y'_0 - w''$) was identified by MS^3 as their $[M + H]^+$ ions. The same number of fatty acyl moieties appeared in the order of unsaturated, (2-)hydroxylated, and saturated components. The molecular species of lactosylceramides derived from porcine blood cells totaled more than 33 and included mainly $C_{24:0}$-$d_{18:1}$, $Ch_{24:0}$-$d_{18:1}$, $Ch_{24:1}$-$d_{18:1}$, $C_{24:1}$-$d_{18:1}$, and $C_{22:0}$-$d_{18:1}$ in addition to 28 minor species from $C_{16:0}$ to $C_{26:0}$ fatty acyl moieties. The molecular species of lactosylceramides in the membrane microdomain fraction of HL-60 cells (70% were differentiated into macrophage-lineage cells) were identified as $C_{24:0}$-$d_{18:1}$, $C_{24:1}$-$d_{18:1}$, $C_{22:0}$-$d_{18:1}$, $C_{16:0}$-$d_{18:1}$, and more than 21 other minor species. Our results suggest that reverse-phase LC-ESI-MS^n is a useful and simple method for identification of lactosylceramide molecular species.

Keywords: Lactosylceramide; Molecular species; Fatty acyl chain moiety; Long-chain base moiety; LC-ESI-MS^n

Studies on molecular membrane biology have focused on the dynamics of cellular proteins and the lipid microenvironment at the cell surface [1]. It is known that glycosphingolipids play important roles in cell–cell interaction, recognition, transmembrane signaling, and cellular growth and differentiation in animal cells [2]. These compounds self-aggregate easily based on their nature and cluster with sphingomyelin with or without cholesterol and various signal transducer molecules to form various types of microdomains called "lipid-raft" at plasma membrane [3–6]. Although the lipid composition of the rafts in different cells is not clear, lactosylceramides are known to localize with "sphingolipid-sterol rafts" and trigger raft-related signal transduction processes. In neutrophils, lactosylceramide-enriched microdomains on the cell surface coupled with tyrosine protein kinase LYN (Lyn)[1] induce the activation of Lyn via lactosylceramides and lead to superoxide generation [3].

To understand the biological activity and physical behavior of lactosylceramides, it is important to deter-

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[1] *Abbreviations used:* Lyn, tyrosine protein kinase LYN; FAB, fast atom bombardment; CAD, collisional-activated dissociation; CID, collision-induced dissociation; ESI, electrospray ionization; APCI, atmospheric pressure chemical ionization; DFP, diisopropylphosphorofluoridate; SIM, single ion monitoring; 3D, three-dimensional.

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mine their structural heterogeneity. Lactosylceramides contain lactose and a ceramide moiety. Various classes of the sphingoid base have been identified, such as the dihydro type (sphinganine), the additional hydroxyl group type (phytosphingosine), and the 2-amino-4-ene-1,3-diol type with 14–22 carbon chains (sphingenine). The commonest type in animal tissues is C_{18} sphingenine ($-d_{18:1}$). In addition, significant variation can also occur in fatty acyl moiety with regard to chain length, unsaturation, and hydroxylation. Lactosylceramides containing saturated fatty acyl chains display relatively high thermotropic and pressure-induced transition, but the introduction of a single *cis* double bond into the fatty acyl chains markedly reduce these properties, especially when the double bonds are located near the middle of the fatty acyl chain [6].

Mass spectrometry (MS) is a powerful tool for lipid structure analysis. Several methods incorporating tandem MS for identification of intact ceramides and neutral sphingolipids such as glucosyl- and lactosylceramides have been published. In early 1990s, Costello and Vath [7] used fast atom bombardment tandem mass spectrometry (FAB-MS/MS) and reported valuable data about the structure of glycosphingolipids, such as the molecular weight of fatty acyl chain and sphingoid base, by analyzing the MS/MS spectra of $[M + H]^+$ and $[M - H]^-$ ions. Ann and Adams [8,9] and Duarte et al. [10] also reported that, in the presence of alkaline metal, $[M + metal]^+$ ions were formed with ceramides and glucosylceramides and these showed a much higher sensitivity than the $[M + H]^+$ ions. In addition, they provided more informative MS/MS spectra for the location of double bonds at high-energy CAD, by using charge remote cleavage. At present, electrospray ionization tandem mass spectrometry (ESI-MS/MS) and atmospheric pressure chemical ionization tandem mass spectrometry (APCI-MS/MS) are used for determination of the structure of intact ceramides and lactosylceramides with low-energy CID [11–13] or high-energy CID [14]. Lee et al. [15] and Pettus et al. [16] reported analysis of the molecular species of ceramides by reverse-phase LC-ESI/MS/MS and normal-phase LC-APCI/MS/MS, respectively.

We report here the design of a simple and useful method for separation and MS^n characterization of molecular species of lactosylceramides using reverse-phase HPLC-ESI ion trap mass spectrometry.

Materials and methods

Materials

Lactosylceramide derived from porcine blood cells was purchased from Matreya (Pleasant Gap, PA). The synthetic lactosylceramide for $C_{24:1}$-$d_{18:1}$ was provided by Professor Sandro Sonnino (Center of Excellence on Neurodegenerative Diseases, Department of Medical Chemistry, Biochemistry and Biotechnology, University of Milan, Italy). All reagents used in this study were of the highest grade, especially those used for HPLC, spectrometry, and amino acid analysis. Chloroform, acetone, sodium chloride, methanol, ethylenediaminetetraacetic acid (EDTA), Triton X-100, Tris–HCl, vitamin D_3, and diisopropylphosphorofluoridate (DFP) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Lithium chloride anhydrous and ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were obtained from Nacalai Tesque (Kyoto, Japan). Ammonium acetate, ammonium formate, and primulin were from Sigma–Aldrich (St. Louis, MO). Complete is a product of Roche (Switzerland). Ultrapure water was prepared using the Millipore Milli-Q purification system (Bedford, MA).

Mass spectrometric analysis

All analyses were performed in positive mode using a LCQ DECA XP ion trap mass spectrometer (Thermo Electron, San Jose, CA) with a conventional electrospray ionization source.

Infusion-ESI-MS^n analysis

Lactosylceramide at a concentration of 5–10 ng/μl was dissolved in methanol with or without alkaline and ammonium salts (1 mM LiCl, 1 mM NaCl, and 10 mM ammonium acetate). Sample solution was directly infused at a rate of 3–5 μl/min into the ESI source without separation of the molecular species of lactosylceramides. The temperature of the ion transfer tube was set at 150–400 °C. The spray voltage was set at 5 kV and the nitrogen sheath gas pressure used was 10 U. In MS^n ($n = 2$–4) analysis, the collision energy was fixed at 100%.

HPLC-ESI-MS^n analysis. Reverse-phase HPLC was carried out with the Magic2002 system (Michrom BioResources, Auburn, CA). The column was Capcellpak C_8 UG120, 1.0 mm ID × 150 mm (Shiseido Fine Chemicals, Tokyo). The mobile phases were as follows: solvent A was 1 mM ammonium formate in methanol:water (76:24 v/v) and solvent B was 1 mM ammonium formate in methanol:water (96:4 v/v). The elution program was isocratic elution with 80% B for 5 min, increase to 95% B, and then hold for 30 min. The flow rate was 50 μl/min. The HPLC was joined to the mass spectrometer, LCQ DECA XP. The MS conditions were as follows: ion transfer tube temperature 250 °C, spray voltage 5 kV, sheath gas pressure 20 U. MS^n ($n = 4$) analysis was performed on data-dependent experiments (DDE) with collision energy 80%.