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Atypical surface behavior of ceramides with nonhydroxy and 2-hydroxy very long-chain (C28–C32) PUFAs

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ABSTRACT

Unique species of ceramide (Cer) with very-long-chain polyunsaturated fatty acid (VLCPUFA), mainly 28–32 carbon atoms, 4–5 double bonds, in nonhydroxy and 2-hydroxy forms (n-V Cer and h-V Cer, respectively), are generated in rat spermatozoa from the corresponding sphingomyelins during the acrosomal reaction. The aim of this study was to determine the properties of these sperm-distinctive ceramides in Langmuir monolayers. Individual Cer species were isolated by HPLC and subjected to analysis of surface pressure, surface potential, and Brewster angle microscopy (BAM) as a function of molecular packing. In comparison with known species of Cer, n-V Cer and h-V Cer species showed much larger mean molecular areas and increased molecular dipole moments in liquid expanded phases, which suggest bending and partial hydration of the double bonded portion of the VLCPUFA. The presence of the 2-hydoxyl group induced a closer molecular packing in h-V Cer than in their chain-matched n-V Cer. In addition, all these Cer species showed liquid-expanded to liquid-condensed transitions at room temperature. Existence of domain segregation was confirmed by BAM. Additionally, thermodynamic analysis suggests a phase transition close to the physiological temperature for VLCPUFA-Cers if organized as bulk dispersions.

1. Introduction

Intensive research over the past decades has implicated ceramide (Cer) in the regulation of a great variety of key cellular functions. However, the paradigm that a single Cer species is responsible for many different cell functions has been challenged by the recognition that "ceramide" constitutes a family of related molecules, subject to metabolism by nearly 30 enzymes and with dozens of structurally distinct molecular species [15]. Saturated and monounsaturated fatty acids from C_{14} to C_{24} are typical acyl chains of sphingolipids in most mammalian tissues, although shorter chain ceramides originated from various transacylation reactions also occur naturally [6]. Notable exceptions are skin and testis sphingolipids, which contains glucosyl-Cer species with fatty acids up to C_{34} long [39] and mammalian testis and spermatozoa, which have sphingomyelin (SM) and Cer with high proportions of very long chain (C_{24} to C_{36}) polyunsaturated fatty acids (VLCPUFA) of the n−6 or the n−3 series [12,35], i.e., elongated versions of well-known C20 or C22 PUFA of the n−6 or the n−3 series, such as arachidonic, docosapentaenoic or docosahexaenoic acids.

In many tissues, of which myelin and skin sphingolipids are known examples, an important part of the sphingolipid species contains a 2-hydroxyl group at the second carbon atom of their fatty acyl chain [14]. This biochemical modification also occurs in the VLCPUFA of rodent testis sphingolipids including SM [37], gangliosides [36,40], and Cer [43]. In rat testis, the ratio between 2-hydroxy and nonhydroxy VLCPUFA (h-V and n-V, respectively) in SM and Cer increases from the onset of spermatogenesis to adulthood [43]. This is due to the fact that n-V Cer and SM species are exclusive components of precursor spermatocytes, whereas h-V Cer and SM species appear as they differentiate to spermatids, as well as in spermatozoa [43]. In mature gametes, n-V SM and h-V SM, but no Cer, occur endogenously on the sperm head and, intriguingly, considerable amounts of solely h-V Cer species are located on the tail [30]. Almost complete hydrolysis of the head-located SM occurs after inducing the acrosomal reaction [44], this leading to gametes that are considerably enriched in n-V, and especially in h-V, ceramides. Thus, whereas in testicular germ cells VLCPUFA-containing ceramides play a role as biosynthetic precursors

Abbreviations: Cer, ceramide; VLCPUFA, very long chain polyunsaturated fatty acid; h-V, 2-hydroxy VLCPUFA; n-V, nonhydroxy VLCPUFA; SM, sphingomyelin; $\mu_{\perp}$, dipole moment perpendicular to the interface; π, surface pressure; π_t, transition pressure; ΔV, surface potential; BAM, Brewster angle microscopy; Cs^{-1}, compressibility modulus; LC, liquid-condensed; LE, liquid-expanded; MMA, mean molecular area; S, solid phase; *L*, semi-empirical average molecular length; *V*, semi-empirical molecular volume; *Th*, semi-empirical film thickness

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of complex sphingolipids including SM, in acrosome-reacted spermatozoa they are final products. Based on previous studies on different species of SM being converted to Cer by the action of sphingomyelinase [8,19,38], such a massive increase in the Cer/SM mole ratio may be envisaged to have an important impact on the sperm surface properties.

Although there are studies extensively describing Cer properties in Langmuir monolayer [4,6,7,9,18,23], the effects on lipid bilayers of diverse Cer species in pure and mixed form [34], and the impact of actively generating Cer from SM by sphingomyelinase on different features of the membrane physical state [8,19,38], the properties and behavior of the relatively novel n-V and h-V Cer species remain to be established. The aim of this study was to define specific molecular parameters of individual molecular species of these sperm-associated Cers, as well as their thermodynamic properties. In Langmuir monolayers, surface pressure, surface potential, and imaging by Brewster angle microscopy (BAM) were measured to determine their average molecular organization at the membrane interface. Our results showed that n-V Cer species differ significantly from h-V Cer species in most of their surface properties and that both behave atypically if compared with the more ubiquitous molecular species of Cer previously described in the literature [6].

2. Materials and methods

2.1. Chemicals

16:0 Cer, 18:1 Cer, 24:1 Cer and 2-hydroxy 24:0 Cer were from Avanti Polar Lipids Co (Alabaster, AL). The n-V Cer and h-V Cer were obtained from adult Wistar rat testes using a combination of TLC and HPLC procedures. The silica gel was from Merck, the HPLC column was from Rainin LC. The gas chromatography and HPLC equipments were both from Varian Inc., USA. All solvents were HPLC grade (JT Baker; UVE, Dorwill, Argentina).

2.2. Separation of ceramides

Lipid extracts were prepared from rat testes [2] and spotted on 500 μm-silica gel TLC plates under N_2 along with commercial standards. Chloroform:methanol:ammonia (90:10:2, by vol.) resolved rat testis Cer into two bands. The lower one was almost exclusively made up by h-V Cer species, whereas the upper one contained all Cer species with nonhydroxy fatty acids, this including common Cer species with C_{16} to C_{26} fatty acids and those with nonhydroxy VLCPUFA [43]. The separated h-V and n-V fractions were recovered and treated with N_2-saturated 0.5 N NaOH in anhydrous methanol at 50 °C for 10 min in order to remove any potential lipid contaminant with ester-bounded fatty acids from the Cer samples, neutralized, partitioned into chloroform, and subjected again to TLC. This time chloroform:methanol:ammonia:water (90:10:05:0.5, by vol.) was used, as it allows the fraction containing Cer species with C_{16} to C_{26} fatty acids to lag behind the Cer species containing nonhydroxy VLCPUFA [11]; this propensity facilitates partial purification of the latter.

Silica gel plates impregnated with 10% $AgNO_3$ and chloroform:methanol (80:20, by vol.) were then used to separate the Cer species with C_{16} to C_{26} fatty acids from those containing VLCPUFA. This resulted in partial resolution of the latter ceramides into two bands: the upper one contained the main very long chain tetraenoic fatty acid (n − 28:4) and the lower one mainly the pentaenoic fatty acids (n − 30:5 and n − 32:5). Although with the expectedly lower Rf values due to the hydroxyl group, an essentially similar separation resulted when h-V Cers were subjected to this procedure. After this pre-separation, each of the Cer subfractions was subjected to HPLC to obtain the six major VLCPUFA-containing Cer species from rat testis (Fig. 1).

Lipids were located on TLC plates under UV light after spraying with 2,7-dichlorofluorescein, and rapidly transferred to glass tubes for elution. This was done by successively extracting the silica with nitrogen-saturated chloroform:methanol:water (5:5:1), centrifuging, collecting the supernatants, and partitioning them with 4.5 volumes of water [2]. The eluates were finally washed with methanol:1 M NaCl (1:1 by vol.) to remove Ag^+ ions. The organic phases were reduced in volume under N_2, filtered to remove traces of particulate matter, dried, and dissolved in methanol for HPLC injection.

HPLC was performed at 35 °C using a stainless steel column (250 mm × 4.6 mm ID) packed with 5 μm particles covered with octylsilane (C8). The solvent, at a flow rate of 1 mL/min was methanol:1 mM potassium phosphate buffer, pH 7.4, in a 96:4 (v/v) ratio. Cer peaks were detected at 205 nm using a Prostar 335 photodiode array detector and collected as they emerged from the column. Chloroform and water were then added to the eluates and Cer species were recovered in the chloroform phase resulting after phase partition.

Individual species were identified and quantified by means of gas chromatography after adding appropriate amounts of n − 16:0 Cer and h − 24:0 (2S–OH) as internal standards to the samples, subjecting them to methanolysis, and recovering by TLC the nonhydroxy and 2-hydroxy fatty acid methyl esters to be subjected to GC. The former were analyzed directly and the latter after conversion into trimethylsilyl derivatives [29,43].

2.3. Monolayer compression isotherms

Langmuir isotherms were obtained using a 90 cm^2 compartment of a specially designed circular Monofilmmeter Teflon trough (Mayer Feintechnik, Germany) filled with 80 mL of 145 mM NaCl, pH ~5.6, equipped with a platinized Pt plate for measuring the surface pressure. The surface potential of the film was simultaneously measured by a surface ionizing electrode formed by a ^{241}Am plate positioned 5 mm above the monolayer surface, and a reference calomel electrode connected to the aqueous subphase.

The whole system was enclosed in an acrylic box, saturated with N_2 gas to prevent lipid peroxidation, and surrounded by a metallic grid connected to ground to reduce external interference in surface potential measurements. Experiments were performed using a subphase of 145 mM NaCl kept at 21 °C and 8 °C (±0.5 °C) by means of an external circulating water bath (Haake F3C). Absence of surface active impurities in the subphase solution or the spreading solvent was ascertained as described elsewhere [32].

Lipid monolayers were obtained by spreading adequate aliquots of Cer solutions onto the aqueous surface. After allowing solvent evaporation for 5 min, the surface pressure–area isotherms were

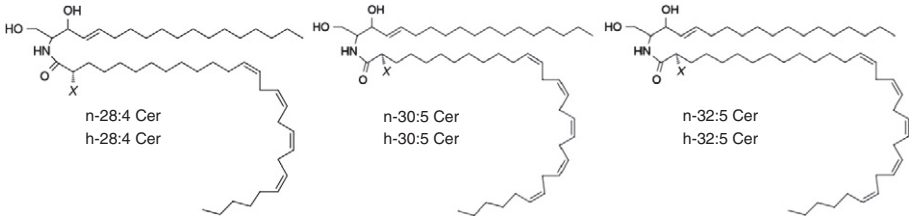

Fig. 1. Schematic structures of the ceramides (Cer) containing nonhydroxy (n) and 2-hydroxy (h) very-long-chain polyunsaturated fatty acids (VLCPUFA) studied in this work. *X* represents the presence of – H or – OH, respectively, in the second carbon atom of the amide-bound fatty acid. The depictions are shown for illustrative purposes and are not meant to represent the molecular organization adopted by the chains at the interface.