Ceramides and Sphingomyelins with High Proportions of Very Long-chain Polyunsaturated Fatty acids in Mammalian Germ Cells*

Natalia E. Furland, Samanta R. Zanetti, Gerardo M. Oresti, Eduardo N. Maldonado, and Marta I. Aveldano

From the Instituto de Investigaciones Bioquímicas de Bahía Blanca, Consejo Nacional de Investigaciones Científicas y Técnicas and Universidad Nacional del Sur, 8000-Bahía Blanca, Argentina

Very long-chain polyunsaturated fatty acids (VLCPUFA) have previously been shown to be components of sphingomyelin (SM) of mammalian testis and spermatozoaa. Here we examined the fatty acids of testicular ceramide (Cer) in comparison with those of SM in some mammals with a special focus on the rat testis. In bull, cat, dog, rabbit, mouse, and rat, VLCPUFA were found in both testicular lipids, Cer having a higher percentage of VLCPUFA than SM. Rat testis had the highest percentage of VLCPUFA in both lipids, the major ones being 28:4n-6 and 30:5n-6. VLCPUFA-containing SM and Cer occurred in cells located in the seminiferous tubules, where germ cells had a higher percentage of these species than Sertoli cells. Seminiferous tubule fractionation showed that SM and Cer of mitochondria and lysosomes had mostly saturates and negligible VLCPUFA, the latter being important in the SM and Cer of microsomes and other membrane fractions. VLCPUFA were absent from the SM and Cer of rat prepuberal testis, increased with the onset of spermatogenesis to account for nearly 15 and 40% of the total fatty acids of testicular SM and Cer, respectively, remained at those levels throughout the adult life of fertile rats and tended to decrease at advanced ages. Four conditions that lead to selective death of germ cells in vivo, namely experimental cryorchidism, post-ischemic reperfusion, focalized x-ray irradiation and treatments with the antineoplastic drug doxorubicin, caused the VLCPUFA to disappear from the testicular SM and Cer of adult fertile rats, showing that these lipids are specific traits of spermatogenic cells.

The main sphingolipids of eukaryotic cells, sphingoglycolipids and the sphingophospholipid sphingomyelin (SM), play influential roles in membrane structure and function. They have been a focus of great interest in the past decade because of some of their metabolites, ceramides, sphingoid bases, and phosphorylated sphingoid bases, have emerged as important messengers in cell signaling (1, 2). Ceramide (Cer), or N-acylsphingosine, constitutes the basic structure of sphingolipids. It has been the subject of thousands of studies, involved as it is, among other processes, in cell differentiation, stress, growth suppression, senescence, and apoptosis (3, 4). Sphingomyelin and Cer serve many different functions at distinct locations in the cell, since changes in their molar ratio alter the membrane physical properties, thereby governing microdomain formation, membrane vesiculation and fusion, vesicle efflux, vesicular trafficking, and other aspects of membrane dynamics (5, 6). Sphingomyelin and Cer are thus multifunctional lipids, the Cer involved in these functions mostly being generated from specific SM by specific sphingomyelinases or by de novo synthesis.

Normal sphingolipid de novo biosynthesis, as well as catabolism, produce several intermediates that parallel the “mediators” produced from complex sphingolipid turnover, including Cer (7). Thus, the steady-state levels of Cer in cells may be modified by a set of enzymes in different locations that form, remove, or modify the lipid. This complexity, and the fact that, with only a few exceptions like the skin (8), Cer is naturally a minor lipid class in most animal tissues and cells, may explain why general information concerning the fatty acids of this lipid is in general scarce. Our interest in the fatty acids of the ceramides of testis and spermatooza began with the expectation that they should differ from those of other tissues. We searched for very long chain polyunsaturated fatty acids (VLCPUFA) in this lipid on the basis that Poulos and co-workers (9, 10) had identified this type of fatty acid, i.e. tetraenoic, pentaenoic, and hexaenoic fatty acids with up to 34 carbon atoms, as normal fatty acids of the testis and spermatooza in a number of mammals including man, showing they were specific constituents of SM. In the testis from adult fertile mammals, continuous rounds of spermatogenesis occur in the seminiferous tubules. Germ cells differentiate from diploid spermatogonia to haploid spermatocytes and finally to spermatooza with the structural and metabolic support of Sertoli cells. During normal spermatogenesis, the number of successfully maturing germ cells is determined by the supportive capacity of somatic Sertoli cells, supernumerary germ cells normally undergoing apoptosis, an important process in the regulation of the germ cell population (11). Given the relationship between Cer and apoptosis, this physiological “background” of apoptosis was a second reason...
for our interest in investigating testicular Cer and its fatty acids in relation to those of SM. Tilly and Kolesnick (12) predicted that SM-derived Cer and sphingosine 1-phosphate could have a potential impact on gonadal physiology by mediating apoptosis and its control in normal and pathological conditions, a concept that received confirmation in experiments measuring these metabolites in sections of human seminiferous tubules (13). The balance between cell growth stimulation and arrest in the mature testis involves a delicate equilibrium between bio-synthesis, remodeling, catabolism and recycling of lipids, including sphingolipids and their fatty acids. The biochemical characteristics of the native SM and Cer present in germ cells from fertile adult animals have not yet been studied in detail.

In this work we have examined, using standard chromatographic procedures, the fatty acids of the SM and Cer isolated from the testes of some mammals, with a special focus on the rat testis. Taking advantage of the knowledge that the population of germ cells in the testis is specifically sensitive to certain cell stressors, we studied their effects on the testicular SM and Cer fatty acids. We provide qualitative and quantitative data showing that both testicular lipids have far from negligible proportions of VLCPUFA and present evidence that these lipids are associated to healthy cells of the spermatogenic lineage in fertile adult animals.

**EXPERIMENTAL PROCEDURES**

**Testis**—Four-month-old Wistar rats were used in all studies involving adult animals. The testes were obtained from anesthetized animals (5 mg/kg acepromazine and 50 mg/kg ketamine, intraperitoneal) which were sacrificed immediately thereafter. Protocols for animal experimentation were performed in accordance with the legal requirements of a local institutional animal research committee. The testes from animals other than rats and mice were obtained from local slaughterhouses immediately after the animal death (bulls, rabbits) or from a local veterinary office (dogs, cats) after standard surgical procedures. Testes were decapsulated and after removing visible blood vessels were rinsed in saline. Lipid extracts were prepared using mixtures of chloroform–methanol (14). One testis from an adult rat (600 – 800 μg of total lipid phosphorus) was used to study Cer and its fatty acids, and 8 – 10 testes per sample were pooled to analyze the Cer of 14-day-old rats or mice. Seminiferous tubules from 20 adult rat testes per sample were used to analyze small subcellular fractions like mitochondria.

**Rat Seminiferous Tubules, Cellular and Subcellular Fractions**—Seminiferous tubules were isolated from adult rat testes after successive dissociation and washings with Dulbecco’s modified Eagle’s medium containing collagenase type I, glucose, and bicarbonate to separate tubules from interstitial cells (15). Two major cellular fractions were separated in bulk from the tubules, one enriched in germ cells and the other mostly containing Sertoli cells, after digestion with collagenase, trypsin, and hyaluronidase, followed by osmotic shock and filtration (16). The purity of the fractions obtained was satisfactory as evaluated by light microscopy, although the yield of Sertoli cells was low. Subcellular fractions were also prepared from seminiferous tubules, after removing interstitial cells. Crude nuclear and mitochondrial fractions were obtained by centrifuging sucrose homogenates from 20 testes at 1000 × g and 10,000 × g respectively. A clear supernatant and a buoyant membrane fraction floating on top of the tubes resulted from the latter centrifugation. The clear supernatant was centrifuged at 100,000 × g at 4 °C for 1 h to obtain a crude microsomal fraction. The buoyant membrane fraction was analyzed for comparison, with no further fractionation. Protocols that had been developed specifically to isolate pure nuclei (17) and mitochondria (18), employing gradients of Nycodenz® and Percoll®, respectively, were applied to the crude nuclear and mitochondrial fractions. A small lysosomal fraction was obtained as a pellet during the purification of the mitochondrial fraction.

**In Vivo Studies**—Four different conditions that have been documented to result in the specific death of cells of the germinal lineage in Wistar rats were chosen to study their consequences on testicular SM and Cer: unilateral cryptorchidism, post-ischemic reperfusion, x-ray irradiation, and treatment with the antineoplastic drug doxorubicin. All procedures were performed in anesthetized animals. Unilateral cryptorchidism (19, 20) was induced in mature rats by displacing one of the testes, together with its ipsilateral epididymis, to the abdominal cavity through the inguinal channel, and practicing a small suture in the latter to prevent the testis from descending. The contralateral scrotal testes were used as controls. Ischemia was induced by a clockwise 720° torsion applied to the spermatic cord by open surgery (21) for 1 h, followed by a return to the natural position to allow for reperfusion of the tissue. Exposure of testes to ionizing radiation was performed according to published procedures and doses (22, 23). Briefly, anesthetized animals were irradiated locally at a distance of 100 cm with a single total dose of 6.5 gray of X-rays produced by a linear accelerator. For the study of doxorubicin effects, a series of four doses (3 mg/kg, once a week during 4 weeks) (24) were administered.

The animals were sacrificed at different intervals after these interventions. The testes were evaluated histologically using standard hematoxilin-eosin staining procedures. The intervals chosen to study the long-term consequences of the described treatments on testicular SM and Cer were those showing a significant reduction in the population of germ cells in seminiferous tubules (e.g. as in Fig. 6). In all procedures described, the weight of the experimental testes had decreased significantly compared with that of controls (e.g. as in Fig. 7). The animals subjected to X-rays and doxorubicin proved to be infertile.

**Lipid Separation and Analysis**—Most of the solvents used were HPLC-grade (JT Baker, Phillipsburg, NJ) or UVE, Dorwill, Argentina). After preparation of lipid extracts, centrifugation, and partition (14), the organic phases containing the lipids were recovered and the solvents were evaporated under N₂. Aliquots were taken for total lipid phosphorus measurement (25). The extracts were spotted (as bands) on TLC plates (500 μm, silica gel G) kept under N₂ along with commercial standards (Sigma). Ceramides were resolved using chloroform/methanol/acetic acid, 95:4.5:0.5, by vol. (8) or chloroform/methanol/ammonia/water (90:10:05:0.5, by vol.) as solvents. The latter solvent was used to advantage to resolve the Cer into three groups of species.
exposing them to NH3 vapors in a closed tank and then spraying SM. The basis of 500 sphingoid base in both or the phosphorylcholine weight in weight of their fatty acids alone, with no correction for the amounts of SM and Cer are expressed on the basis of the sive hexane extractions. To facilitate and simplify comparisons, methanol/water/hexane (1:1:1, by volume), doing three succes-

The VLCPUFA of bovine retina rod outer segments (28, 29) and rat seminiferous tubule total lipids (30), including GC mass spectrometry. The spectra of the main VLCPUFA of rat testicular SM and Cer (included in Fig. 2) were obtained using a Hewlett-Packard 6890 GC connected to a 5972 mass spectrometer (hyperbolic quadrupole), equipped with a capillary column (HP5-MS, 30 m × 0.25 mm × 0.25 μm). Helium (1 ml/min) was the carrier gas, and the fragments were analyzed using an NBS 75 K Hewlett-Packard Mass Spectrometer Chem Station library. For fatty acid composition analysis, a Varian 3700 gas chromatograph and a Varian Star Chromatography Worksta-

RESULTS

SM and Cer Fatty Acids—In the total lipid from mammalian testis, the most abundant fatty acids have long been known to be saturated, monounsaturated, and polyunsaturated fatty acids (PUFA) with 18 to 22 carbon atoms belonging to the n-6 (18:2n-6, 20:4n-6 and 22:5n-6) or the n-3 (18:3n-6, 20:5n-3, 22:6n-3) series, depending on species. The sum of PUFA with 24 – 34 carbon atoms, referred to as “VLCPUFA” in this work, amounted to an average of 5% of the fatty acids of the total lipid of testis in the mammals analyzed. Various polar and non-polar lipid classes, including glycerophospholipids, neutral glyc-

Very Long-chain PUFA in Germ Cell Sphingolipids
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TABLE 1
Fatty acid composition of sphingomyelin and ceramide from rat testis and spermatozoa

| Fatty acid | SM   | Cer |
|-----------|------|-----|
| 14:0      | 0.25 ± 0.07 | 0.29 ± 0.10 |
| 14:1      | 0.06 ± 0.03 | 0.11 ± 0.04 |
| 15:0      | 0.09 ± 0.03 | 0.17 ± 0.11 |
| 15:1      | 0.03 ± 0.01 | 0.12 ± 0.04 |
| 16:0      | 36.54 ± 2.68 | 13.94 ± 1.72 |
| 16:1      | 0.37 ± 0.28 | 1.33 ± 0.74 |
| 17:0      | 0.80 ± 0.20 | 0.46 ± 0.15 |
| 17:1      | 0.09 ± 0.06 | 0.33 ± 0.11 |
| 18:0      | 26.02 ± 0.75 | 14.06 ± 1.76 |
| 18:1      | 1.17 ± 0.32 | 2.23 ± 0.84 |
| 19:0      | 0.14 ± 0.05 | 0.14 ± 0.11 |
| 19:2      | 0.28 ± 0.11 | 0.61 ± 0.51 |
| 20:0      | 1.60 ± 0.19 | 0.92 ± 0.08 |
| 20:1      | 0.05 ± 0.03 | 0.04 ± 0.04 |
| 21:0      | 0.08 | 0.07 |
| 22:0      | 3.76 ± 0.41 | 4.08 ± 0.49 |
| 22:1      | 0.30 ± 0.04 | 0.30 ± 0.08 |
| 23:0      | 0.79 ± 0.16 | 0.96 ± 0.15 |
| 23:1      | 0.25 ± 0.05 | 0.35 ± 0.12 |
| 24:0      | 4.55 ± 0.52 | 10.58 ± 0.70 |
| 24:1      | 5.22 ± 0.55 | 7.26 ± 0.34 |
| 24:2n-6   | 1.48 ± 0.12 | 0.71 ± 0.07 |
| 26:0      | 0.00 | 0.08 |
| 24:4n-6   | 0.16 ± 0.03 | 0.12 ± 0.03 |
| 24:5n-6   | 0.14 ± 0.04 | 0.09 ± 0.05 |
| 25:4n-6   | 0.01 ± 0.04 | 0.20 ± 0.20 |
| 25:5n-6   | 0.02 | 0.08 ± 0.03 |
| 26:4n-6   | 0.18 ± 0.04 | 0.29 ± 0.15 |
| 26:5n-6   | 0.08 ± 0.03 | 0.09 ± 0.01 |
| 27:4n-6   | 0.20 ± 0.04 | 0.17 ± 0.18 |
| 27:5n-6   | 0.08 | 0.05 |
| 28:4n-6   | 8.24 ± 0.90 | 19.00 ± 1.76 |
| 28:5n-6   | 0.56 ± 0.10 | 2.26 ± 0.63 |
| 29:4n-6   | 0.20 ± 0.02 | 0.40 ± 0.09 |
| 29:5n-6   | 0.02 ± 0.02 | 0.15 ± 0.03 |
| 30:4n-6   | 0.24 ± 0.03 | 0.43 ± 0.09 |
| 30:5n-6   | 4.66 ± 0.47 | 13.39 ± 1.30 |
| 31:5n-6   | 0.36 ± 0.05 | 0.31 ± 0.09 |
| 32:5n-6   | 0.93 ± 0.10 | 3.90 ± 0.32 |
| VLCPUFA   | 16.06 ± 1.63 | 40.84 ± 3.09 |

FIGURE 1
Percentage of total VLCPUFA in sphingomyelins and ceramides in the testis from various mammals. The bars represent the sum of PUFA with 24 to 32 or 34 carbon atoms, according to the species. At least three animals per group were analyzed.

VLCPUFA was consistently higher in Cer than in SM, and that the rat was the species with the highest amount of these fatty acids in both lipids.

All animals studied contained different amounts of individual VLCPUFA, although a component common to all was 28:4n-6. Mice contained small amounts of n-3 VLCPUFA, which were virtually absent from rat, whereas cat, dog, and rabbit SM and Cer had relatively higher amounts of n-6 trienes such as 28:3n-6 and were richer in 30:4n-6 than the corresponding lipids from the rat testis shown in Table 1. The bull testicular SM and Cer had VLCPUFA of the n-6 and the n-3 series, 30:6n-3, 32:6n-3, and 34:6n-3 being the major ones. It is interesting to point out that the VLCPUFA of bull testicular SM and Cer were the same as those previously identified as important acyl groups of the phosphatidylcholine present in bovine retina rod outer segments (28, 29).

In rat, most of the VLCPUFA present in SM and Cer belonged to the n-6 series and had an even number of carbon atoms in their chains, although odd-chain VLCPUFA also occurred (Table 1), as was also observed in other testicular lipids (31). The longest VLCPUFA of rat testicular SM and Cer was 32:5n-6, and the most abundant in both lipids were 28:4n-6 and 30:5n-6 (Table 1 and Fig. 2).

The mass spectra of these two fatty acids, as methyl esters, are included in Fig. 2. Most of the fragments produced from 28:4 and 30:5 were similar to those formed from 20:4n-6 and 22:5n-6, respectively (not shown), the spectra showing coincidence of over 98% between the two major VLCPUFA of SM and Cer and these two well known, abundant, and ubiquitous fatty acids from rat tissues. In addition, peaks with an m/z ratio of 430 and 456 were observed in the spectra, which coincide with the expected weight of the molecular ion for the methyl esters of 28:4n-6 and 30:5n-6, respectively.

One of the solvent systems used for the separation of rat ceramides on TLC plates resolved them into three bands, each showing a different, almost clear-cut, fatty acid composition. The band with the lower Rf contained the major long (C16–C18) chain saturated fatty acids; the middle band had very long (C24) saturated and monounsaturated acyl groups; and the band migrating in front contained most of the VLCPUFA present in testicular Cer (Fig. 2). Analysis of the sphingoid base contained in each band showed that all three had sphingosine as the predominant long-chain base, followed by minor amounts (5%) of sphinganine, indicating that the fatty acids and not the base determined their tendency to separate, suggesting that the fatty acids overrule or hinder the interaction between the relatively polar base and the silanol groups of the support. This TLC behavior is shared by other lipids containing VLCPUFA, including phospholipids, like retina phosphatidylcholine (32) and testicular SM. In all cases the band containing VLCPUFA migrates more than the bands containing other fatty acids.

Cellular and Subcellular Distribution of Testicular VLCPUFA-containing SM and Cer—In rat, SM and Cer with VLCPUFA were found to be absent from tissues or cells other than testis and spermatozoa. In the testis, most of the SM and Cer rich in VLCPUFA were contributed by cells located within the seminiferous tubules (Fig. 3), the extratubular cells having SM and Cer with much lower amounts of these fatty acids. A separation of rat seminiferous tubule cells into two fractions containing Sertoli and germ cells showed that the latter were significantly richer in VLCPUFA-containing species of SM and Cer than the former (Fig. 3). This was a good direct indication that cells of the germinal lineage were the main contributors of these peculiar species to the total SM and Cer of testis.

The preparation of subcellular fractions from seminiferous tubules showed that the fatty acid composition of SM
and Cer differed markedly among fractions, the percent of VLCPUFA in both lipids increasing in the order: lysosomal, mitochondrial, nuclear, and microsomal fractions (Fig. 4). The mitochondrial fraction contained only 2 and 4% VLCPUFA in its SM and Cer, respectively. The same lipids of microsomes were much richer in these fatty acids than those of mitochondria but in turn had less VLCPUFA than the starting homogenate of seminiferous tubules. A serendipitous finding was that, of all fractions, one that was going to be discarded, floating on top of the clear supernatant overlying the first crude mitochondrial fraction, was the one containing the SM and Cer richest in VLCPUFA (25 and 58% of the fatty acids, respectively). This fraction, containing membranes and vesicles, could have contained plasma membranes and cisternal elements of the Golgi apparatus. The enrichment in VLCPUFA of the SM and Cer of this fraction in comparison with those of microsomes would agree with the generally recognized fact that the endoplasmic reticulum is the main cellular site of the de novo biosynthesis of Cer in cells, whereas the major site of SM synthesis from Cer (SM synthase) is the lumen of Golgi apparatus vesicles (33). Coincidentally, in cultured Sertoli cells, the trans-Golgi cisternae and the trans-Golgi network were shown to be the main sites of synthesis of SM (34).

VLCPUFA-containing SM and Cer and Age—Before the age of sexual maturity, VLCPUFA were absent altogether from the SM and Cer of rat testis, both these lipids containing only saturated, monounsaturated, and dienoic fatty acids (Fig. 5). The first VLCPUFA in SM and Cer were detected between
P25 and P27, i.e. concomitantly with the onset of spermatogenesis and the appearance of the first round of spermocytes, that in the rat starts between P20 and P26 (35). In young and adult fertile rats, in which rounds of spermatogenesis occur continuously, VLCPUFA remained at virtually constant levels in SM and Cer throughout adult life. Only in really senile (28-month-old) rats did the proportion of VLCPUFA in both testicular lipids tend to decline. A similar decrease was previously shown to affect the VLCPUFA of phosphatidylcholine in the retina of rats of similarly advanced age (36). In both cases, the aging-related decreases may be associated with a partial loss of the highly specialized cells in which these lipids occur.

**In Vivo Germinal Cell Line Deletion and Testicular VLCPUFA-containing SM and Cer**—After determining that SM and Cer with VLCPUFA were not found in testis before differentiated germinal cells appeared, we set up experimental conditions to induce these cells in fertile animals to die off. We used the following models of spermatogenic cell death: experimental cryptorchidism, post-ischemic reperfusion, x-ray irradiation of the testis, and administration of the chemotherapeutic drug doxorubicin. Independently of the initial mechanism of germ cell derangement, or the type of germ cell initially affected, all four conditions had in common a conspicuous outcome: the drastic involution of the testes due to the selective loss of cells from the germinal lineage. An example of this is given in Fig. 6 for the long-term consequences of the irradiation with a single dose of x-rays.

The fastest involution was observed for cryptorchidism. In agreement with previous data (37, 38), in few days this resulted in histological evidence of selective loss of the most differentiated cells of the germinal line, sparing spermatogonia and Sertoli cells. A few days after surgery, the cryptorchid testes showed as the most marked change a dramatic decrease in the weight and in the content of total lipid P (Fig. 7). The data in this figure show that the total amount per testis of SM and Cer decreased due to cryptorchidism at virtually the same rate as the total phospholipid. The fatty acid composition of both these sphingolipids (right panels of Fig. 7) changed dramatically, the most conspicuous change being that both lost their initially important amounts of VLCPUFA.

The results in Fig. 8 compare the eventual long term effects of the four germ cell-depleting conditions studied on the amounts per testis of SM and Cer and on their fatty acid composition. All of them resulted in due time in a reduced amount of both sphingolipids and in a marked fall in the percentage of VLCPUFA these lipids originally had.

Concomitantly with the changes shown in Fig. 8, cryptorchidism, ischemia-reperfusion, x-ray irradiation, and doxorubicin administration resulted in a marked reduction in testicular weight and in lipid phosphorus. In all four cases, this

![FIGURE 4. Major groups of fatty acids of the SM and Cer of subcellular fractions isolated from rat seminiferous tubules.](image)

![FIGURE 5. Changes in the major groups of fatty acids from rat testicular SM and Cer (percent) during postnatal development, adulthood, and aging.](image)

![FIGURE 6. Light micrographs of normal rat testis in comparison with rat testes obtained 6 weeks after irradiation with a single dose of 6 gray of x-rays per cm² at a distance of 1 m.](image)
depletion was accompanied by a massive reduction of the 22:5n-6 originally bound to glycerophospholipids, and by the disappearance of the 3'-sulfated, 1-alkyl, 2-acyl-sn-glycero3-galactolipid known as “seminolipid” from the testis.

DISCUSSION

The presented results show that VLCPUFA-containing SM and Cer are qualitatively distinctive and quantitatively important constituents of cells produced in the mammalian testis. To identify the cellular origin of these lipids we reasoned that, if they were specific components of cells of the germinal lineage, they should: 1) be absent from the testis at developmental

stages previous to the onset of spermatogenesis; 2) be present in mature testis, where they would be associated with cells located within seminiferous tubules and, within these tubules, be less concentrated in Sertoli than in spermatogenic cells; 3) decline slowly with natural aging; and 4) tend to disappear from the testis of healthy, fertile males in conditions that would result in the selective death of spermatogenic cells and their loss from the testis. Our results show that testicular SM and Cer carrying VLCPUFA meet all these criteria; these species thus behaving as a kind of lipid “markers” of mature cells of the germ cell line.

In the adult rat testis, the disappearance of preexisting VLCPUFA from the SM and Cer in the four experimental conditions studied in this work, cryptorchidism, ischemia-reperfusion, x-ray irradiation, and administration of doxorubicin, chosen because they had been documented to cause selective death of germ cells by the mechanism of apoptosis, was a strong piece of evidence obtained in vivo of the consequences of such death. Although the end results were similar, an important difference between the four germ cell-killing conditions studied was the rate at which the reduction of the mentioned lipid species occurred. This fact may be explained considering the type of spermatogenic cell mostly affected in each case. Complete loss of the mentioned lipids took just a few days in the case of cryptorchidism (Fig. 7, 8), because the dying elements in this case were mainly preexisting spermatocytes and spermatids (37, 38),

FIGURE 7. Effects of unilateral cryptorchidism. On the left panels, the effects of this condition on rat testicular weight and total lipid phosphorus (P), as well as on the amounts per testis of SM and Cer fatty acids are shown. On the right panels, the changes in the proportions of the main groups of fatty acids of SM and Cer, calculated from the fatty acid composition, are given. The letters S, M, D, and V refer to the sums of saturates, monoenes, dienes, and very long-chain polyenes, respectively.

FIGURE 8. Comparison of the effects of cryptorchidism, post-ischemic reperfusion, x-ray irradiation, and doxorubicin administration on the amount and composition of SM and Cer fatty acids. The results correspond to samples obtained at different intervals after the experimental injury (10 days, 6 weeks, 6 weeks, and 9 weeks, respectively). On the right panels the percentages of the main groups of fatty acids of SM and Cer, calculated from the fatty acid composition, are given. The letters S, M, D, and V, refer to the sums of saturates, monoenes, dienes, and very long-chain polyenes, respectively.
the most temperature-sensitive cells of the germ lineage (sparing spermatogonia, Sertoli cells, and interstitial cells). It took much longer for lipid and fatty acid to be similarly depleted after x-ray irradiation or doxorubicin administration (Fig. 8), because the predominant elements initially dying in these cases were spermatogonia (39, 40). Consistent with the fact that a complete spermatogenic cycle takes about 60 days in the rat, several weeks had to elapse to give the preexisting differentiated germ cells an opportunity to exit from the testis before the lipid and VLCPUFA depletion could be observed. Thus after these periods, not only were spermatogenic cells absent from the testis, but spermatooza had also disappeared from the epididymis and the rats were sterile.

Even though the reduction of testicular VLCPUFA-Cer was certainly confirmed in the present work as a long term consequence of germ cell death, we were initially also interested in detecting possible apoptosis-related Cer increases, an event that is often associated with transient increases in Cer levels. SM-derived Cer has been proposed to participate in the apoptosis-mediated control of the number of germ cells in the seminiferous tubule (11, 12), and exogenously added Cer has been shown to act as a pro-apoptotic second messenger in seminiferous tubules (13). Using the present approach, we observed a small (20–40%), transient Cer increase in whole testis at time points close to those specified by the respective authors as accumulating the largest number of apoptotic cells per testis (e.g. 1 day in cryptorchidism, 2 days in doxorubicin-treated rats). The Cer-associated fatty acid most responsible for this “peak” of Cer increase was C-16:0, the VLCPUFA not increasing significantly at any point in time. This observation sounded reasonable, since an apoptosis-related Cer rise should involve molecular species of this lipid more ubiquitous than the VLCPUFA-containing Cer.

Evidence exists in other non-neuronal tissues indicating that C-16:0 Cer represents the Cer species elevated during apoptosis under a variety of stimuli (e.g. Refs. 41–43). Although Cer can be generated from SM in practically all cellular compartments via the activation of sphingomyelinases (SMases), it is the mitochondrial Cer that is associated with apoptosis, the Cer generated in other cellular compartments having no effect on this death mechanism (44). Our observation that mitochondria from adult rat seminiferous tubules have SM and Cer with virtually no VLCPUFA, although indirectly, adds support to the idea that VLCPUFA-containing species of these testicular sphingolipids probably are not involved as mediators in pro-apoptotic mechanisms.

At present it is difficult to draw definitive conclusions concerning the biological role of ubiquitous, simple ceramides with specific fatty acids such as C-16 or C-18 or C-24 Cer, in defined physiological and pathological situations in vivo, owing in part to the complexity of the metabolic, cell biological, and signaling pathways acting upon their levels in cells (2, 45). The SM and Cer with VLCPUFA of testis not only are tissue-specific but specific of spermatogenic cells. Although the total amounts of Cer in testis, as in any tissue, represent a balance between Cer-generating and Cer-removing processes, we would like to propose that the small, VLCPUFA-rich, ceramides present in healthy, non-stressed adult testes in basal, “steady state” conditions, are mostly those generated de novo in the biosynthetic route leading to VLCPUFA-containing SM. Since the biological function of spermatogenesis is to ensure a continuous production of fertile spermatooza, and since these gametes do contain VLCPUFA-rich SM (9, 10), it is apparent that an important purpose of making these sphingolipid species in testis is that they eventually end up as components of these gametes.

Ceramide synthase (CerS), the enzyme that acylates sphinganine to form dihydroceramide, and also sphingosine to form ceramide, in contrast to the rest of the enzymes of sphingolipid metabolism, has multiple genes, six in humans and mice, five of them located in different chromosomes (46). Although the reason for such CerS multiplicity is not clear at present, these enzymes are thought to be involved in regulating the synthesis of ceramides containing specific fatty acids in specific cells. Interestingly, the expression (mRNA) of one of these CerS (the number 3) is limited almost solely to testis (47). It would be worth studying the substrate specificity of this enzyme toward VLCPUFA, since it could be responsible for the unusual fatty acid composition of germ cell ceramides.

Albeit constituents of germ cells, the precise cellular site in adult seminiferous tubules where SM and Cer with VLCPUFA are normally synthesized remains to be investigated. Since Sertoli cells normally provide germ cells with the necessary physical support and proper milieu (48), which may include nutrients, intercellular signaling molecules, and perhaps also lipids, the former cannot be excluded as a possible source of these sphingolipid species. Sertoli cells in culture isolated from 19-day-old rats have been shown to display an active synthesis of SM, characterized using specific inhibitors and radioactive substrates as tools (34). However, since at that early age of the rat SM and Cer with VLCPUFA are still not present in the testis (Fig. 5), the question of where and how these species are synthesized in the adult seminiferous tubules remains open. There are technical difficulties in trying to answer these questions using testicular cells in culture. On the one hand, given the close interdependence that exists in adult life between Sertoli and germ cells, cultures of any of these cell types from the mature testis is difficult to establish. On the other, although easy to establish and deal with in culture, Sertoli cells isolated from immature testis will probably not synthesize all fatty acids and lipids typically formed in sexually mature animals.

The question on how spermatogenic cells acquire their VLCPUFA-containing SM and Cer is a dilemma of similar magnitude to that of how these cells acquire their abundant PUFA-rich glycerophospholipids, not yet resolved. Thus, although germ cell glycerophospholipids are severalfold richer in 22 carbon PUFA (e.g. 22:5n-6) than Sertoli cells (49), the latter are much more active than the former at expressing the desaturases and elongases that convert the essential fatty acids 18:2n-6 and 18:3n-6 to 22:5n-6 and 22:6n-3, respectively (50). This led these latter authors to postulate the occurrence of some form of transport of
PUFA-rich lipids from Sertoli to germ cells, thus linking the active fatty acid desaturation/elongation systems and lipid synthesizing ability of Sertoli cells to one of their metabolic functions in supporting germ cells. This possibility could also apply to the VLCPUFA-containing SM of germ cells.

There are a few questions concerning the synthesis and turnover of VLCPUFA-containing lipids that could be investigated in whole seminiferous tubules. Specific desaturases must be expressed to synthesize 20:4n-6 and 22:5n-6, and specific elongases must modify these polyenes to produce 28:4n-6 and 30:5n-6. How and where this synthesis takes place, at which precise point in the biosynthetic sequence and in what form the ultra-long fatty acids are N-acyl bound to sphingoid base(s) by Cer synthase to assemble ceramides that become substrates for SM synthase, await investigation.

Aside from the biochemical issues, questions on the biophysical properties of the germ cell sphingolipids arise, such as how the SM and Cer with such a mismatch in their unsaturated fatty acids that could be easily isolated by TLC, are kept from mixing among cell compartments, and others.

The biological aim of normal spermatogenic cells in accruing VLCPUFA-rich SM and Cer may be to ensure that these sphingolipids end up in the final cellular product of their differentiation, spermatозoa. Since SM and Cer do contain significant amounts of VLCPUFA in these gametes (52), it is probably in these highly differentiated cells where VLCPUFA-containing sphingolipids fulfill their ultimate physiological function. This possibility opens new exciting questions about the properties and purpose of both lipids in sperm membranes. Awareness of the natural occurrence of far from negligible amounts of sphingolipids with “atypical” fatty acids in the extensively studied seminiferous tubules and spermatозoa may facilitate investigation of their involvement in male reproductive physiology, including a possible role as protagonists in fertilization-related events.

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