Sequential depletion of rat testicular lipids with long-chain and very long-chain polyenoic fatty acids after X-ray-induced interruption of spermatogenesis[S]

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Abstract When a single dose of X-rays is applied to the adult rat testis, stem spermatogonia are damaged, and spermatogenesis is interrupted. Supported by Sertoli cells, spermatogenic cells that endure irradiation complete their differentiation and gradually leave the testis as spermatozoa. In this study, the in vivo changes taking place a number of weeks after irradiation revealed cell-specific features of testicular lipid classes. A linear drop, taking about six weeks, in testis weight, nonlipid materials, free cholesterol, and 22:5n-6-rich glycerophospholipids took place with germ cell depletion. Sphingomyelins and ceramides with nonhydroxy very long-chain polyenoic fatty acids (n-VLCPUFA) disappeared in four weeks, together with the last spermatocytes, whereas species with 2-hydroxy VLCPUFA lasted for six weeks, disappearing with the last spermatids and spermatozoa. The amount per testis of 22:5n-6-rich triacylglycerols, unchanged for four weeks, fell between weeks 4 and 6, associating these lipids with spermatids and their residual bodies, detected as small, bright lipid droplets. In contrast, 22:5n-6-rich species of cholesterol esters and large lipid droplets increased in seminiferous tubules up to week 6, revealing they are Sertoli cell products. At week 30, the lipid and fatty acid profiles reflected the resulting permanent testicular involution. Our data highlight the importance of Sertoli cells in maintaining lipid homeostasis during normal spermatogenesis.—Oresti, G. M., P. L. Ayuza Aresti, G. Gigola, L. E. Reyes, and M. I. Aveldaño. **Sequential depletion of rat testicular lipids with long-chain and very long-chain polyenoic fatty acids after X-ray-induced interruption of spermatogenesis. *J. Lipid Res.* 2010.** 51: **2600–2610.**

Supplementary key words ceramide • cholesterol ester • fatty acid • lipid droplet • phagocytosis • polyunsaturated fatty acid • spermatogenic cell • Sertoli cell • sphingomyelin • triacylglycerol • very long chain PUFA • X-rays

The mammalian testis is known to be highly susceptible to a variety of anticancer agents, including a number of chemotherapeutic drugs and X-ray irradiation. Precisely because of its efficacy to kill rapidly dividing cells, exposure of the testis to these agents, whether incidental or therapeutic, selectively targets the population of spermatogenic cells and is a potential cause of male infertility. While somatic cells, including Sertoli cells, are apparently unaffected, spermatogonia that are mitotically dividing and spermatocytes during the early phases of meiosis are the testicular cells most vulnerable to X-rays (1). Relatively less susceptible are nondividing spermatogonial stem cells on the one hand and postmeiotic cells on the other. Spermatids and spermatozoa, with their much more compact nuclei, are notably resistant. Germ cell death induced by X-ray irradiation mostly occurs via apoptosis (2, 3), which results from radiation-induced free radical generation damaging DNA. The protein P53, the known intracellular sensor of DNA damage, is required for this response (1, 4). P53 upregulates in germ cells the production of Fas, the surface receptor that, on binding the Fas ligand produced by Sertoli cells, activates apoptosis via a caspase-mediated cascade (5, 6).

A few weeks after having locally irradiated the testis with X-rays, spermatogenesis recommences from type A spermatogonial stem cells that had not been affected at irradiation time in some mammalian species like mice (1) but not in others like LBNF1 rats, which are in this regard a good model of the human testicular sensitivity to X-rays (7). In these rats, despite the presence and normal proliferation of apparently undamaged type A spermatogonia, it is their further differentiation that is after some time impeded,

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Abbreviations: ADG, ether-linked triglyceride; CE, cholesterol ester; Cer, ceramide; CGP, choline glycerophospholipid (diradyl); DPG, diphosphatidylglycerol; EGP, ethanolamine glycerophospholipid; GC, gas chromatography; GPL; glycerophospholipid; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; TAG, triacylglycerol; VLCPUFA, very long-chain polyunsaturated fatty acids (n- and 2-OH are used as prefixes to denote nonhydroxy and 2-hydroxy VLCPUFA, respectively).

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of three figures.

and consequently, they die by apoptosis (7). This vicious circle phenomenon, described as "the proliferation-apoptosis block" (8) lasts for several weeks and results in permanent loss of fertility if time elapses and the animals are untreated. Thus, spermatogenesis is interrupted at its origin. Yet, the large preexisting population of nondividing germ cells that is unaffected by or able to overcome the effects of irradiation continues its programmed proliferation and differentiation at the normal pace of spermatogenesis, orderly leaving the seminiferous tubules in the form of spermatozoa. The seminiferous epithelium is gradually depopulated of spermatogenic cells until it contains only a few spermatogonia and Sertoli cells, and eventually, only Sertoli cells.

In a previous study (9), irradiation with X-rays was used among other agents that are established to be pro-apoptotic to germ cells but not to Sertoli cells as experimental tools to show that the unique species of sphingomyelin (SM) and ceramide (Cer) that contain very long chain polyunsaturated fatty acids (VLCPUFA) in rat seminiferous tubules (e.g., 28:4n-6, 30:5n-6, and 32:5n-6) belong to cells of the spermatogenic lineage. This was clearly an "all-or-none" phenomenon, the disappearance of germ cells 6 weeks after a single dose of X-rays correlating with the disappearance of these species, but when and why had these species disappeared was not known. In addition to "normal", nonhydroxylated (n-) VLCPUFA, the SM (10) and, more recently, also the Cer (S. R. Zanetti et al., unpublished observations) of rat testis and spermatozoa were found to contain important proportions of species with 2-hydroxylated versions of these unique fatty acids (2-OH VLCPUFA). The present study had as its main purpose to survey how germ cell-associated SM and Cer species with each of these types of VLCPUFA are affected as germ cells gradually decrease in number but progress in their differentiation until they are all gone from the testis and only Sertoli cells remain in seminiferous tubules.

The major glycerophospholipids (GPL) that contain 22:5n-6 were included in this study because they are known to be major membrane lipids of germ cells (11). The proportion of 20:4n-6 decreases while that of 22:5n-6 increases with differentiation from spermatocytes to spermatids (12), and both germ cell types have lipids considerably richer in 22:5n-6 and poorer in 20:4n-6 than Sertoli cells (13). For this reason, germ cell losses occurring after irradiation, with spermatocytes disappearing first, then spermatids, then spermatozoa, may be expected to evolve with opposite changes in the proportions of these testicular PUFA. The inclusion in this study of triacylglycerols (TAG) and cholesterol esters (CE) showed that these lipids, also rich in 22:5n-6, are probably catabolic products of different cell types within seminiferous tubules, the CE being mainly generated by Sertoli cells.

MATERIALS AND METHODS

Animals and irradiation

Adult four-month-old male Wistar rats weighing between 300 and 350 g, under general anesthesia induced by intraperitoneal injection of ketamine and acepromazine (0.72 and 0.22 mg/kg, respectively) were used. The animals were immobilized at their extremities on a tray and irradiated locally with X-rays (total dose 6.5 Gy) using a Saturne 41 linear accelerator (GE-Medical Systems) projecting a 5 × 5 cm field that included both testes. There was a distance of 100 cm between source and scrotal skin, and the dose rate was of 2.0 Gy/min. Both irradiated and nonirradiated animals were euthanized at intervals from 1 to 30 weeks after irradiation to obtain the testes. All procedures with animals were carried out in accordance with guidelines issued by an institutional Animal Research Committee, whose guidelines are in accordance with the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (ILAR), National Academy of Sciences (Bethesda, MD, 1996). After weighing, some of the organs were fixed in 10% formaldehyde, embedded in paraffin, cut into 3 μm sections, and stained with hematoxylin-eosin for histological examination.

Lipid separation and analysis

After weighing, capsules and visible vessels were removed from testes, which were then homogenized using mixtures of chloroform-methanol (14). The solid nonlipid materials that remained after thorough lipid extraction were recovered and kept in a desiccator until a constant weight was obtained. This "lipid-free dry weight" was recorded as an estimate of the global weight of proteins, nucleic acids, and other nonsoluble materials present in the tissue. After preparation of lipid extracts, aliquots were taken for total lipid fatty acid composition, total lipid phosphorus, and phospholipid composition analyses. The latter was performed after HPTLC (15). The lipid spots were located with the aid of iodine vapors, scraped from the plates, and quantified by phosphorus analysis (16).

For preparative isolation of lipid classes, most of the lipid extracts were spotted on TLC plates (500 μm, silica gel G) under N_2, along with commercial standards (Sigma Chemical Co., MO). The polar lipids remained at the origin of the plates, and the neutral lipids were resolved in two steps. Chloroform/methanol/aqueous ammonia (90:10:2 by vol) was run up to the middle of the plates to separate the ceramides. Then, these solvents were evaporated and the plates were developed again by running n-hexane/diethyl ether (80:20 by vol) up to the top of the plates to resolve CE, TAG, and triglycerides with an ether bond, mostly alkyl-diacylglycerols (ADG). After TLC, zones with the lipids of interest were located under UV light after spraying with 2′,7′-dichlorofluorescein in methanol and scraped into tubes for elution. This was done by three successive extractions of the silica support with chloroform/methanol/water (5:5:1 by vol), centrifuging, collecting the solvents, and partitioning with four volumes of water to recover the lipids.

The total phospholipid fraction was subjected to further separation and analysis. Aliquots were set aside to study the total GPL fatty acid composition. Most of the rest was used to preparatively isolate SM and major GPLs using chloroform/methanol/acetic acid/0.15 mol/l NaCl (50:25:8:2.5 by vol). The GPLs were resolved into classes by two-dimensional TLC (16), with choline and ethanolamine glycerophospholipids (CGP, EGP) then separated into their two major (phosphatidyl and plasmenyl) subclasses. The eluted, dried CGP or EGP were briefly (1 min) agitated with a solution of 0.5 N HCl in acetonitrile, rapidly evaporating this solvent and resolving the resulting lipids by means of chloroform/methanol/water (65:25:4 by vol). This separated the major 1,2-diacyl-GPL (containing a minor amount of 1-alkyl-2-acyl-GPL) from the 2-acyl-GPL formed from the corresponding plasmalogens (1-alk-1′-enyl, 2-acyl-CGP or EGP).

A mild alkali treatment was performed on the SM and Cer samples to remove any potential lipid contaminant containing

ester-bound fatty acids. Both lipids were taken to dryness and treated (under N_2) with 0.5 N NaOH in anhydrous methanol at 50°C for 10 min. After this alkaline treatment, SM and Cer were recovered again by TLC. This procedure, involving alkaline methanolysis, was also used to obtain, as methyl esters, the fatty acids ester-bound to total GPL (thus excluding the fatty acids amide-bound to SM from this group). All solvents used in this study were HPLC-grade (JT Baker, NJ; UVE, Dorwill, Argentina), and most procedures were carried out under N_2.

Fatty acid analysis

The fatty acids of all lipid classes were quantified by gas chromatography (GC) after adding an internal standard (methyl heneicosanoate) and conversion of the eluted, dried lipids into methyl ester derivatives. With the mentioned exception of total GPL, the fatty acid methyl esters (FAME) were prepared by transesterification with 0.5N H_2SO_4 in anhydrous methanol under N_2 (17) and kept overnight at 45°C in Teflon®-lined, screw-capped tubes. Before GC, FAMEs were routinely purified by TLC using hexane/ether (95:5 by vol) on silica gel G plates that had been prewashed with methanol/ethyl ether (75:25 by vol) and dried. After adding methyl heneicosanoate and methyl 2-OH lignocerate as internal standards, the FAMEs derived from SM and Cer were separated by running hexane/ether (80:20 by vol) up to the middle of the plates, followed by hexane/ether (95:5 by vol) up to near the top, to recover the 2-hydroxy and nonhydroxy FAME (S. R. Zanetti et al., unpublished observations). For elution of all FAME from the silica support, this was transferred to tubes and agitated with water/methanol/hexane (1:1:1 by vol), centrifuging to recover the upper hexane layers and repeating twice the hexane extractions.

Before GC, the 2-OH FAME from SM and Cer were converted into O-trimethylsilyl (O-TMS) derivatives by adding a mixture of N,O-bis (trimethylsilyl)trifluoroacetamide (BSTFA) and 5% trimethylchlorosilane (TMCS) (Fluka reagent, Sigma-Aldrich). The samples were kept overnight in small conical tubes with Teflon®-lined caps at 45°C under N_2 with the reactants. The latter were evaporated, and the TMS ethers, taken up into hexane, were subjected to GC.

The n-VLCPUFA of SM and Cer were identified by procedures and criteria described in detail in previous work (9). The 2-OH VLCPUFA were thoroughly characterized previously by Robinson et al. (10) as components of rat testicular SM and by Sandhoff et al. (18) as components of a novel series of glycosphingolipids in mouse testis. Here this identity was confirmed by subjecting the O-TMS derivatives of intact and per-hydrogenated 2-OH FAME to mass spectrometry (supplementary Fig. III). A Hewlett-Packard 6890 GC connected to a 5972 mass spectrometer equipped with a capillary column (HP5-Ms, 30 m × 0.25 mm id) was used. 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 quantification, a Varian 3700 Gas Chromatograph equipped with two (2 m × 2 m) glass columns packed with 10% SP 2330 on Chromosorb WAW 100/120 (Supelco, Inc.) was used. The column oven temperature was programmed from 150°C to 230°C at a rate of 5°C/min (190°C to 230°C in the case of 2-OH fatty acids) (S. R. Zanetti et al., unpublished observations) and then kept at the upper temperature for at least 30 min for elution of VLCPUFA. Injector and detector temperatures were set at 220 and 230°C, respectively, and N_2 (30 ml/min) was the carrier gas. The fatty acids were detected with two flame ionization detectors operated in the dual-differential mode and connected to a Varian Star Chromatography Workstation (version 4.51). Statistical analyses of the results were performed using the two-tailed Student's *t*-test. All data are expressed as mean ± SD from a number of at least four different animals.

Staining of lipid droplets

Fixed pieces of testis were rinsed in PBS containing 30% sucrose overnight at 4°C for cryoprotection, placed in a small amount of OCT compound (Crioplast®, Biopack), and immersed in liquid nitrogen. Frozen sections 5–7 μm thick were prepared with a cryostat, picked up on silanized glass coverslips, allowed to dry at room temperature for 20 min, and stored in a refrigerator until stained. The tissue sections were fixed in 4% paraformaldehyde in PBS for 5 min, washed, and covered with a solution of a 1.5 μg/ml in PBS of Nile Red (Molecular Probes, Eugene, OR) for 15 min at room temperature. Better selectivity for cytoplasmic lipid droplets was obtained when the tissue sections were viewed for yellow-gold fluorescence (450–500 nm band pass excitation filter, a 510 nm center wavelength chromatic beam splitter, and a 528 nm bong-pass barrier filter). Cell nuclei were stained with a 5 μM solution of Hoescht 33342 in PBS for 30 min, then briefly washed with PBS and mounted with fluoromount (Dako). A Nikon Eclipse E-600 fluorescence microscope equipped with 40× NA 1.0 objective was used. Photography and digitalization were obtained using a model ST-7 SBIG CCD camera driven by CCDOPS software, version 5.02 (SBIG Astronomical Instruments).

RESULTS

Post-irradiation changes in testicular histology, weight, and main lipids

After exposure to a single dose of 6.5 Gy of X-rays localized on the testis, spermatogenic cells gradually disappeared from the seminiferous tubules as weeks postirradiation passed (**Fig. 1**). Up to week 2 postirradiation, cells in their meiotic phase (pachytene spermatocytes), round and elongated spermatids, spermiogenesis-related residual bodies, and spermatozoa were all present. At week 4, spermatocytes were no longer observable, while their successor spermatids, normal proportion of residual bodies, and spermatozoa had become the main representatives of the germ cell line within the tubules. At week 6 and thereafter, only Sertoli cells were present in the seminiferous tubules, and no germ cells other than a few occasional spermatogonia remained. Sertoli cells were apparently viable, as their typically shaped nuclei were clearly intact and visible at all stages, in agreement with the fact that they persistently outlived the germ cells. From week 6 to 30, the interstitium was expanded at the expense of the reduced seminiferous tubules, in agreement with previous results showing that this reflected a hormonally dependent extratubular accumulation of fluid (19). Spermatogenesis was not reestablished in the period studied. The animals became infertile; no offspring resulted after verified mating from weeks 26 to 30 with different females of proven fertility. This coincided with the histological observation that spermatozoa were detectable in part of the epididymal lumena only up to week 6 postirradiation (data not shown) and was rare or absent thereafter.

Concomitantly with the described changes, an almost linear decrease in testicular fresh weight took place, lasting about 6 weeks, followed by an additional decrease with a lower slope in the next 6–30 weeks (Fig. 1A). In this regard, our Wistar rats exposed to 6.5 Gy of X-rays responded

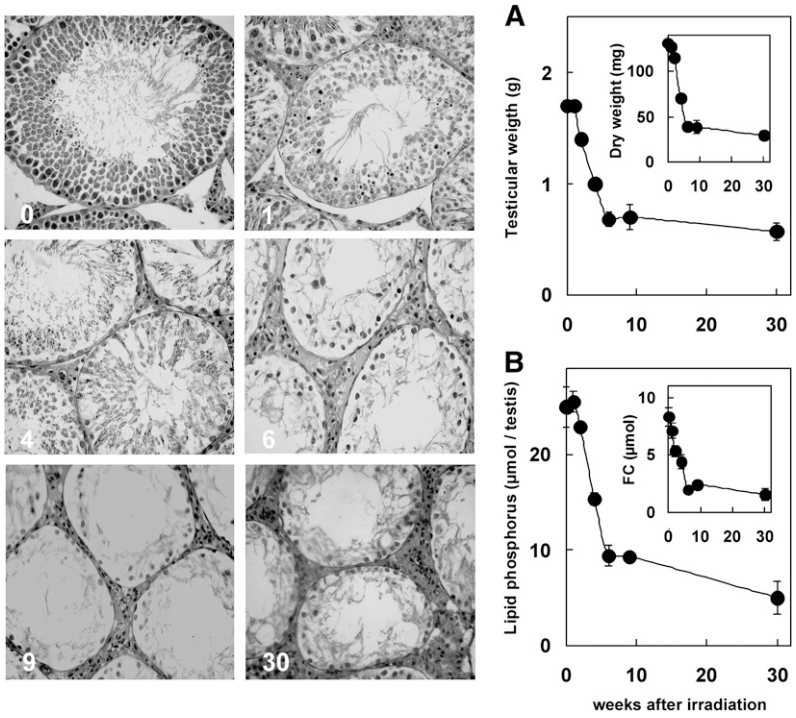

Fig. 1. Long-term consequences of a single dose of X-rays (6.5 Gy) locally applied to the adult rat testis. On the left, photomicrographs of tissue sections stained with hematoxylin-eosin (200×) in a nonirradiated control (0) and at different time points (1, 4, 6, 9, and 30 weeks) after irradiation. A: Postirradiation reduction in testicular fresh weight and, in inset, lipid-free dry weight. B: Concomitant decrease in the amount per testis of total phospholipids and, in inset, free cholesterol (FC). Data are expressed as mean ± SD, n = 4.

comparably to the X-ray-sensitive rat strain LBNF1 exposed to 6 Gy (7, 20).

The "lipid-free dry weight," an estimate of the global weight of proteins, nucleic acids, and other molecular materials not soluble in organic solvents, decreased 70% and 80% after 6 and 30 weeks, respectively, compared with the preirradiation values (Fig. 1A). At the same two time points, the fresh weight had decreased less, 60 and 70% of the initial values, respectively. This 10% difference was in part ascribable to the increased volume of fluid collected extratubularly with time (19), which affects the wet (but not the dry) weight.

In parallel with the testicular weight changes, the total amount of major cell membrane lipids (i.e., phospholipids and free cholesterol) decreased markedly (Fig. 1B). Both dropped to values 60–64% lower and 78–82% lower, respectively, than the initial ones after 6 and 30 weeks. The eventual loss of nearly 80% of these two lipid classes was comparable to the 80% decrease of nonlipid constituents, a reasonable fall considering that all these materials originally constituted the matter of the spermatogenic cells that left the testis as spermatozoa after completing their differentiation.

Changes in phospholipid classes and subclasses

Although all testicular phospholipids decreased with time after irradiation, the major classes did so at different rates (**Fig. 2**). The phospholipid composition did not show significant alterations during weeks 1–4, but several significant differences emerged after week 6. The classes decreasing the most in the whole period examined were CGP and EGP, with the plasmenyl subclasses relatively less affected than the corresponding phosphatidyl subclasses. This accounted for increased percentages of the phospholipid classes that decreased relatively less, such as diphosphatidylglycerol, phosphatidylserine, and phosphatidylinositol. An interesting extreme of this trend was SM, whose relative contribution to the total testicular phospholipid changed from 6% to 15% between nonirradiated controls and 30 weeks postirradiation (Fig. 2). The phospholipid profile at week 30 was that of an aspermatogenic adult rat testis, its seminiferous tubules populated only by Sertoli cells (Fig. 1), which agrees with the fact that the latter contain more PS, PI, and particularly, much more SM than germ cells (13).

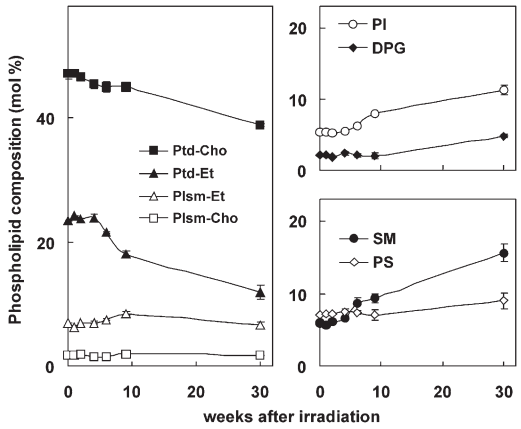

Fig. 2. Changes in testicular PL composition (%) after interruption of spermatogenesis at its origin. After separation of PL into classes, lipid phosphorus was measured. Although all PL decreased (Fig. 1B), it did so at different rates, as indicated by the increased percentage of plasmalogens and SM. Data are expressed as mean ± SD, n = 4. DPG, diphosphatidylglycerol; Pdt-Cho, phosphatidylcholine; Pdt-Et, phosphatidyl- ethanolamine; Plsm-Cho, plasmenylcholine; Plsm-Et, plasmenyl ethanolamine; PI, phosphatidylinositol; PL, phospholipid; PS, phophatidylserine; SM, sphingomyelin.

Sphingomyelins, ceramides, and their fatty acids

The total amount per testis of SM decreased gradually (**Fig. 3A**), paralleled by a reduction in the metabolically related Cer, in the period covering weeks 1–6 after irradiating the testis. The fall in both lipid classes was marked by a key change in their fatty acid composition: the species that contain (total) VLCPUFA gradually disappeared from the testis in this interval, in agreement with the fact that these lipids are associated with germ cells (9, 21), which compose the majority of the cell population in the testis. As species with these fatty acids decreased, SM and Cer with saturated (mostly 16:0), monoenoic (18:1, 24:1), and traces of dienoic (18:2n-6) fatty acids (Fig. 3B) became progressively more apparent, thus associating these species with the permanent somatic cells that persisted after germ cells were depleted, mostly Sertoli cells in the tubules and extratubular cells.

The germ cell-associated, VLCPUFA-containing SM and Cer showed a further remarkable change in the proportion among species that contain nonhydroxylated (n) VLCPUFA and 2-hydroxylated (2-OH) VLCPUFA (Fig. 3C). The former disappeared from the testis two weeks earlier than the latter. Thus, both lipids had virtually no species with n-VLCPUFA at postirradiation week 4, a point at which species with 2-OH VLCPUFA still abounded, revealing the occurrence in rat testis of species of SM and particularly of Cer with a high 2-OH VLCPUFA/VLCPUFA ratio.

The order of lipid decrease in Fig. 3 paralleled the order of cell decrease: species with n-VLCPUFA were depleted together with the last spermatocytes, whereas species 2-OH VLCPUFA predominated in the interval in which differentiating spermatids prevailed as the last germ cells (Figs. 1). Both kinds of cells and both kinds of VLCPUFA-rich species were undetectable in the testis at postirradiation week 6 and thereafter.

Decreased glycerophospholipids versus unchanged or increased neutral lipids

To compare the amounts of main GPL with amounts of SM and neutral lipids on the same basis, all were studied by measuring their fatty acids. The content of total GPL fatty acids per testis (**Fig. 4A**) summarizes the changes undergone by all individual phospholipid classes analyzed, as illustrated for the main contributors, CGP and EGP. All of the GPL, as well as SM, decreased with time.

In contrast to the massively decreasing GPL, the amount per testis of TAG remained virtually unchanged for four weeks, suggesting that the glycerides continued to be produced in cells that were unaffected by irradiation. Interestingly, most of TAG disappeared from the testis between postirradiation weeks 4 and 6 (Fig. 4A). In its timing, the sharp decrease of TAG coincided with the phase in which 2-OH VLCPUFA-containing SM and Cer predominated but also dropped; the main cellular elements present during those two weeks were the last spermatids, related residual bodies, and spermatozoa.

Although the ADGs made a relatively small contribution to the total changes, they differed from TAG in their slower pace of decrease after four weeks (Fig. 4A).

The CEs differed from the rest of the lipids in that their amount per testis increased from the very first week after irradiation and steadily collected throughout the whole

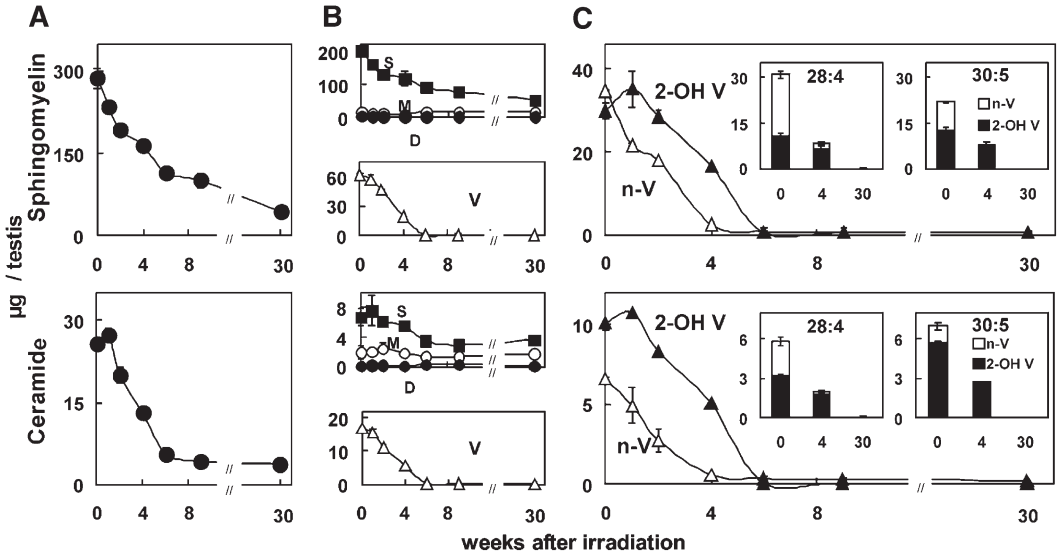

Fig. 3. Time-course of the in vivo changes affecting testicular sphingomyelin and ceramide fatty acids starting one week after a single exposure of the testis to X-rays. A: Depicts total fatty acids. B: Depicts all SM or Cer fatty acids, grouped into saturated (S), monoenoic (M), dienoic (D), and very long chain polyenes (V), where "V" represents nonhydroxy VLCPUFA + 2-hydroxy VLCPUFA. C: Depicts nonhydroxy VLCPUFA (n-V) and 2-hydroxy VLCPUFA (2-OH V) separately. Insets: Depict the amounts of two major n-V (gray bars) and two major 2-OH V (black bars) of SM and Cer at weeks 0, 4, and 30. Note that species of SM and Cer with n-V disappeared from the testis 2 weeks earlier than the corresponding species with 2-OH V fatty acids. Data are expressed as mean ± SD, n = 4. Cer, ceramide; SM, sphingomyelin; VLCPUFA, very long-chain polyunsaturated fatty acids.

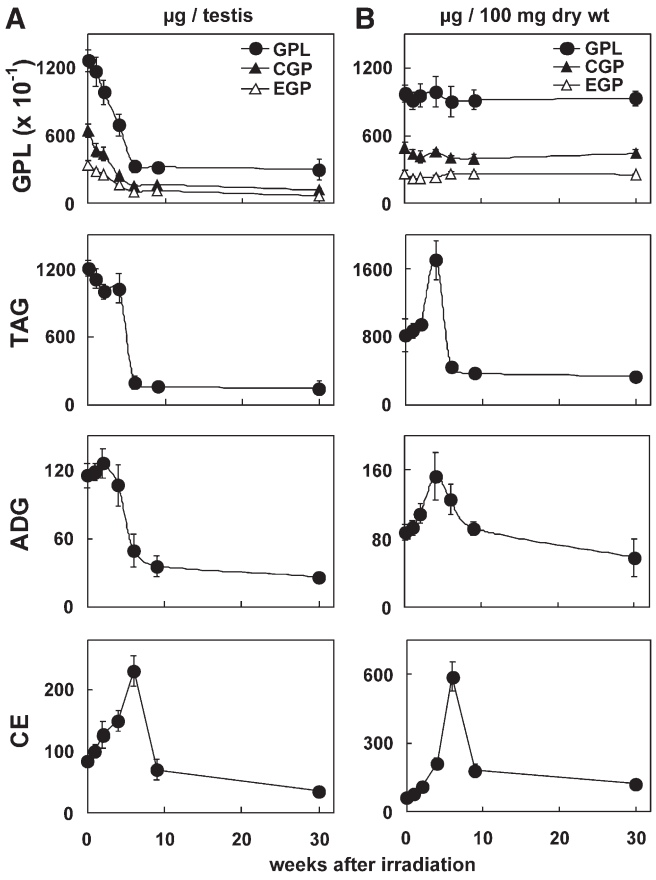

Fig. 4. Time-course of the in vivo changes affecting testicular GPL, TAG, CE, and ADG, starting one week after a single exposure to X-rays. Comparison of amounts per testis (A) and amounts per milligram of lipid-free dry weight (B). While the amount of GPL per testis dropped drastically, the amount of neutral glycerides did not change much for four weeks, and the amount of CE increased continuously for six weeks after irradiation. Data are expressed as mean ± SD, n = 4. ADG, ether-linked triglyceride; CE, cholesterol ester; GPL; glycerophospholipid; TAG, triacylglycerol.

period of six weeks (Fig. 4A) during which germ cells were depleted. It was only after postirradiation week 6 that CEs slowly started to decrease and only at week 9 that the amount of CE per testis was close to the initial amount.

We decided to express our lipid data on a "per testis" rather than on a "concentration" basis for the reasons shown in Fig. 4. Because cell cohorts were gradually disappearing from the testis over time, the tissue weight (wet and dry) also decreased. Proteins and DNA (constituents of the lipid-free dry weight usually put in the denominator to express lipid concentration) decreased at the same pace as main cell membrane lipid components, phospholipids, and cholesterol (Fig. 1B). As a result, the concentration of GPL did not change significantly throughout the 30-week period examined (Fig. 4B), while that of the three neutral lipids increased markedly during the first weeks, due to the combination [(unchanged or increased amount)/(decreased tissue weight)]. When expressed per testis, however, it was clearly apparent that despite the decreasing number of germ cells and phospholipids, TAG production was virtually unchanged for four weeks, ADG production increased slightly, and CE production increased continuously during the six weeks.

Major PUFA ester-bound to testicular lipids

Long-chain PUFA. Although the amount of all fatty acids ester-bound to testicular lipids eventually decreased after irradiation, they did so at significantly different rates among lipid classes. GPL species with the major PUFA, 22:5n-6 and 20:4n-6, decreased at similar rates between weeks 0 and 4, whereas those with 22:5n-6 were dramatically reduced between weeks 4 and 6. This led to a marked reversion of the 22:5n-6/20:4n-6 ratio in testicular GPL (**Fig. 5**). From week 6 onwards, the GPL of the cells residing in the testis had 20:4n-6 as their major PUFA.

As with total TAG (Fig. 4), the amount per testis of 22:5n-6-containing species of TAG (Fig. 5) was virtually unchanged between weeks 0 and 4, but it fell drastically between weeks 4 and 6.

In contrast, the amounts of 22:5n-6-rich species of CE tended to build up right from the very first week after irradiation, making the main contribution to the increased mass of total testicular CE. These CE species reached their maximum amount at week 6, when the 22:5n-6-rich TAG had already fallen to its minimum, strongly suggesting that each neutral lipid class belonged to a different cell type. Because the zenith of CE occurred at the point when only Sertoli cells but no spermatids or spermatozoa remained in the testis (Fig. 1), one may conclude that most of the produced CE accumulated in the latter.

Very long chain PUFA. In GPL and TAG, 24:5n-6 and 24:4n-6 paralleled the changes already described for the major 22:5n-6 and 20:4n-6 during weeks 0–4 postirradiation: a gradual decline of both VLCPUFA occurred as the cell numbers decreased, and the predominant cell types changed. This was followed by a sharper loss of 24:5n-6 and longer pentaenes over that of the corresponding tetraenes during weeks 4–6 (Fig. 5). Pentaenes and tetraenes with 26 to 32 carbon atoms were no longer detectable in testicular GPL or TAG after week 6 postirradiation.

In ADG, the amounts of their major VLCPUFA, 24:5n-6 and 24:4n-6, tended to increase up to postirradiation week 2 and decrease subsequently, just like the corresponding pentaenes and tetraenes with longer chains. In the testis of healthy fertile adult rats, these lipids were previously shown to belong to cells located within seminiferous tubules and to be composed mostly of 1-O-alkyl, 2-3-diacyl, *sn*-glycerols (22). Because of their seminiferous tubule localization, the fact that they are negligible in isolated germ cells suggested that they may be mostly produced by Sertoli cells (23). Considering their unusual fatty acid and glycerol backbone, it had been suggested that these lipids could directly or indirectly be related to the biosynthesis of plasmalogens or other ether-linked lipids, such as seminolipid (22). However, the fact that the amount of species with 22:5n-6 increased steadily in ADG for 4 weeks (Fig. 5) suggests that a part of the total ADG could also have a catabolic origin: 22:5n-6-rich plasmalogens, after hydrolysis of their phosphoryl base, could give rise to 1-alk-1´-enyl,2-

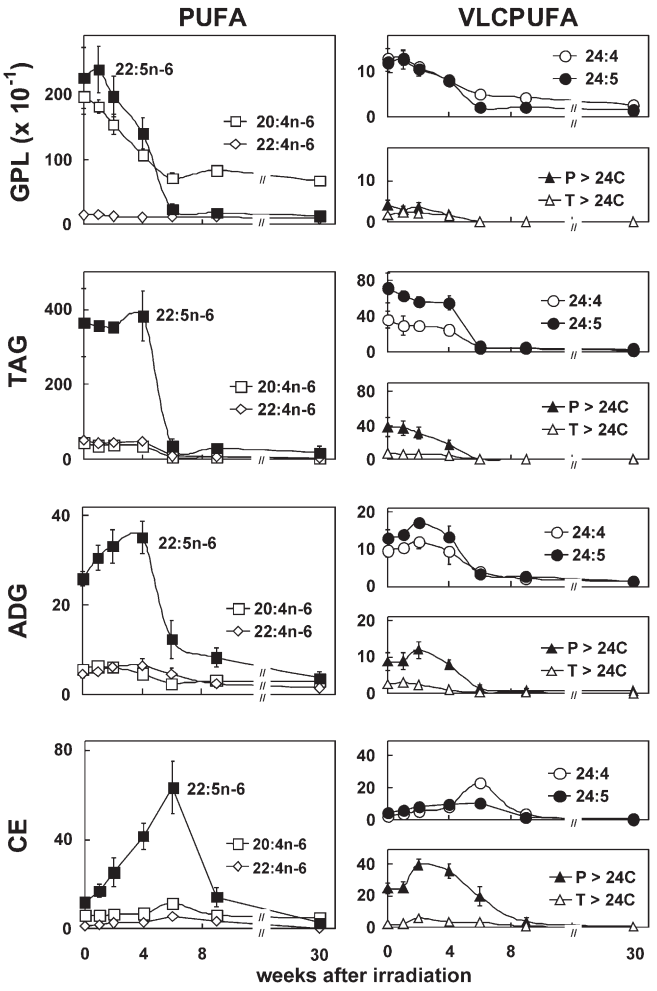

Fig. 5. Long-term postirradiation changes affecting the amounts of major PUFA and VLCPUFA of rat testicular lipids. The amounts are expressed as μg per testis. Tetraenes and pentaenes longer than 24:4n-6 and 24:5n-6, added up, are indicated as T > C24 and *P* > C24, respectively. The changes in 22:5n-6 per testis paralleled, and mostly determined, the lipid changes shown in Fig. 4. Data are expressed as mean ± SD, n = 4. ADG, ether-linked triglyceride; CE, cholesterol ester; GPL; glycerophospholipid; TAG, triacylglycerol; VLCPUFA, very long-chain polyunsaturated fatty acid.

acyl, *sn*-glycerols, which could be acylated at *sn-3* to provide an ADG, thus contributing to the total ADG fraction, as isolated here.

In CE, the amounts of 24:4n-6 and 24:5n-6 were maintained at their usually low levels after irradiation, except for an as yet unexplainable increase of 24:4n-6 detected at week 6, but both fatty acids decreased to virtual disappearance later on. By contrast, longer pentaenes and tetraenes (those with 26 to 32 carbon atoms) remained unchanged during the first week, collected in CE for a period of four weeks, and then decreased slowly thereafter (Fig. 5). This time course clearly differed from the considerable, long-lasting and almost linear buildup of CE species with 22:5n-6.

Neutral lipids and lipid droplets

The use of Nile Red as a maker allowed the detection of two different kinds of lipid inclusions in rat seminiferous tubules (**Fig. 6**). The most abundant was a population of small lipid droplets associated with the luminally facing aspect in the majority (~70%) of the tubules. These were associated with elongated spermatids in differentiation to spermatozoa and coincided with the particles known as "residual bodies." The second type of inclusion (~30% of the tubules) was a basally located population of larger lipid droplets. The latter collected in Sertoli cells in cytoplasmic areas close to their nuclei and proximal to the basal membrane surrounding the seminiferous tubules (Fig. 6). Neither type of inclusion was detectable in the tissue at postirradiation weeks 9 and 30.

In control tubules and up to week 4 postirradiation, when spermatids, residual bodies of spermiogenesis, and spermatozoa in formation interacting with Sertoli cells persisted, both types of lipid droplets coexisted. At week 6, when only Sertoli cells remained, just the basally located large lipid droplets abounded. Taken together, this time course of lipid droplet "disappearance" and the profiles of TAG and CE changes (Figs. 4 and 5) suggested that the smaller, spermiogenesis-related lipid droplets located close to the apex of Sertoli cells mark the residual bodies and that these structures may contain most of the abundant 22:5n-6-rich TAG that are unchanged for four weeks. By contrast, the larger oil droplets, concentrated near the basal area of seminiferous tubules in Sertoli cells up to week 6, must contain the progressively increased 22:5n-6-rich CE.

Long-term effects of X-rays on testicular lipid fatty acids

After postirradiation week 9 and by week 30, a new steady-state condition was established in which the aspermatogenic testis had a new and stable lipid and fatty acid composition. **Fig. 7** summarizes the situation of eight selected fatty acids and the sum of C24–C32 PUFA at postirradiation week 30 compared with untreated controls for the main lipid classes (other than SM and Cer) that were surveyed in this study. Changes in fatty acid profiles as a function of time are shown in supplementary Figs. I and II. The new major fatty acid after testicular involution was 18:1n-9; several GPL classes (PE, PS, PI, and notably, DPG) and the neutral lipids showed a significant increase in the percentage of 18:1n-9. The new major PUFA in most lipids was 20:4n-6, and a relative increase in its precursor 18:2n-6 was noticeable in some cases. All lipid classes eventually contained minor amounts of 22:5n-6 and 24:5n-6 and lacked C26-C32 VLCPUFA altogether.

DISCUSSION

The presented results focus on spermatogenic cells of the adult rat testis in vivo and their interactions with Sertoli cells from the perspective of lipids. Because a long period starting one week after irradiation is covered, this study does not include cells that were directly damaged by irradiation. The cells that were killed by apoptosis because of X-rays, a process that takes 12–16 h (24), disappeared from the testis in a day or two, depriving the testis of their descendants. This study mostly deals with spermatogenic cells that, not having been affected by irradiation or hav-

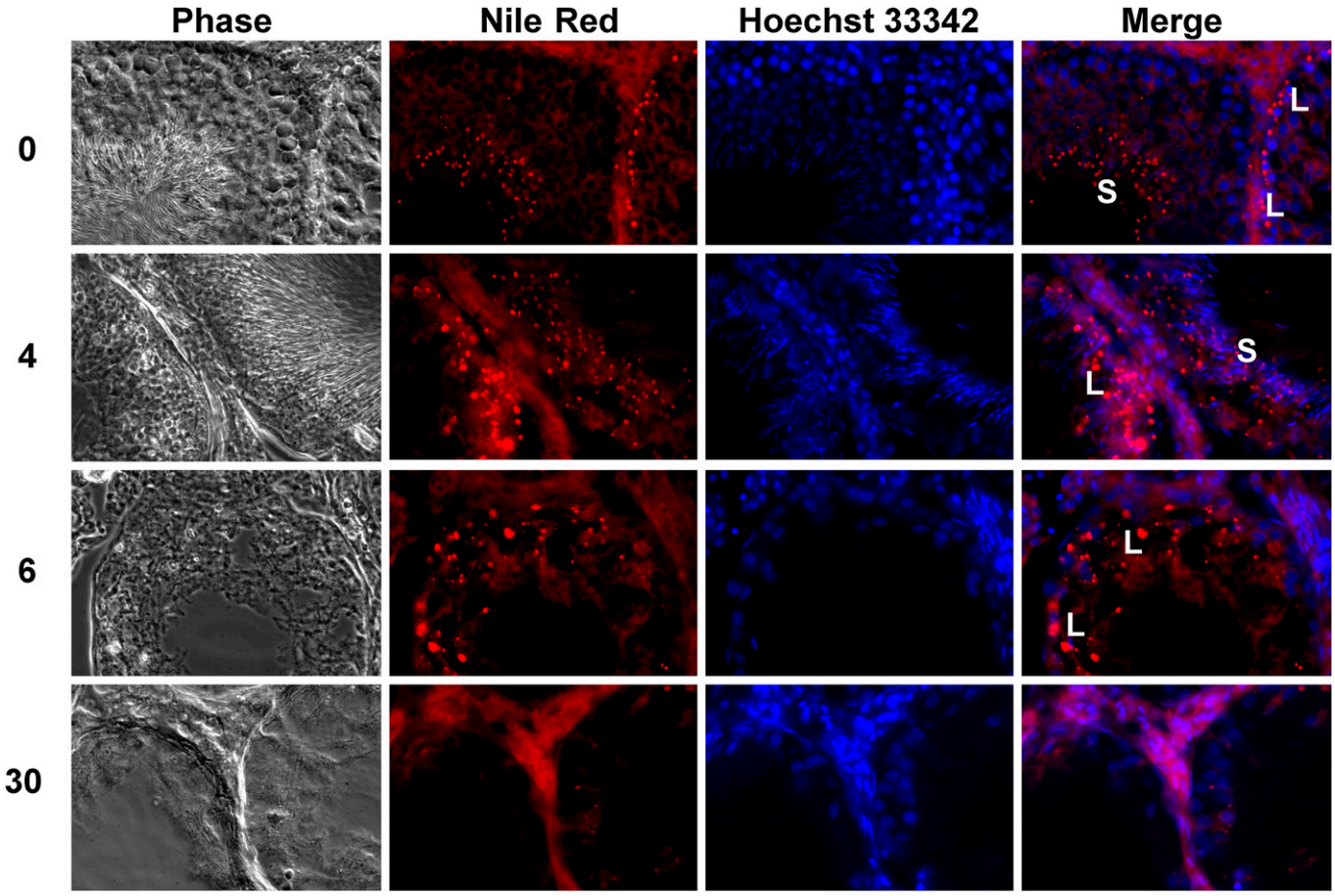

Fig. 6. Testicular lipid droplets detected with Nile Red staining. In control tubules (0), two populations of lipid droplets coexisted [labeled L (large) and S (small)], the former located near the basal membrane (associated with Sertoli cells), and the latter facing the luminal aspect of the tubules (associated with residual bodies formed from elongated spermatids in spermiogenesis). At week 4 postirradiation, the S droplets persisted, and the L droplets had increased in size. At week 6 (just Sertoli cells present), only L droplets remained. At week 30, no lipid inclusions were detectable in Sertoli cells. Hoechst 33342 was used to reveal cell nuclei.

ing succeeded to overcome its effects, continued their differentiation at the normal pace of spermatogenesis and in due time left the testis, as spermatozoa, toward the epididymis. As this cell population could not be replaced with new ones from the surviving spermatogonia that were able to proliferate but failed to differentiate (8, 25), the number of germ cells gradually waned.

Sphingomyelins and ceramides

The fact that sphingolipids with VLCPUFA belong to spermatogenic cells is now established (S. R. Zanetti et al., unpublished observations) (9, 10, 18, 21). A remarkable finding of the present study was that, after interruption of spermatogenesis, species of SM and Cer with n-VLCPUFA vanished from the testis two weeks before the species with 2-OH VLCPUFA. The fact that the former only lasted as long as spermatocytes were present, whereas the latter were the main species during weeks 4–6 postirradiation when the last spermatids and spermatozoa predominated as cell components, strongly suggested an association between each type of species and normal germ cell differentiation. This conclusion was supported by subsequent work showing that isolated pachytene spermatocytes contain mostly n-VLCPUFA in their SM and Cer, whereas the same lipids of round spermatids and spermatozoa are rich in species of SM and Cer with 2-OH VLCPUFA (23).

The information regarding the biosynthesis of VLCPUFA-containing species of SM and Cer in the testis of adult animals is still limited. The present results show that adult Sertoli cells in vivo, in the absence of germ cells, do not synthesize these lipids. In rat (9) and mouse (21) prepubertal testis, with seminiferous tubules mostly populated by Sertoli cells, species with these fatty acids are also absent. These lipids are probably biosynthesized in developing spermatogenic cells, concomitantly with their growth and proliferation, which entails an intense membrane biogenesis and obviously requires a copious amount of membrane lipids. In our view, most of these lipids are biosynthesized de novo in the less differentiated spermatocytes, then modified (e.g., the 2-hydroxylation of VLCPUFA) in the daughter cells as their differentiation proceeds.

Glycerophospholipids

The modifications of main GPL-associated PUFA support this interpretation, because among germ cells, the larger but less differentiated spermatocytes contain GPL

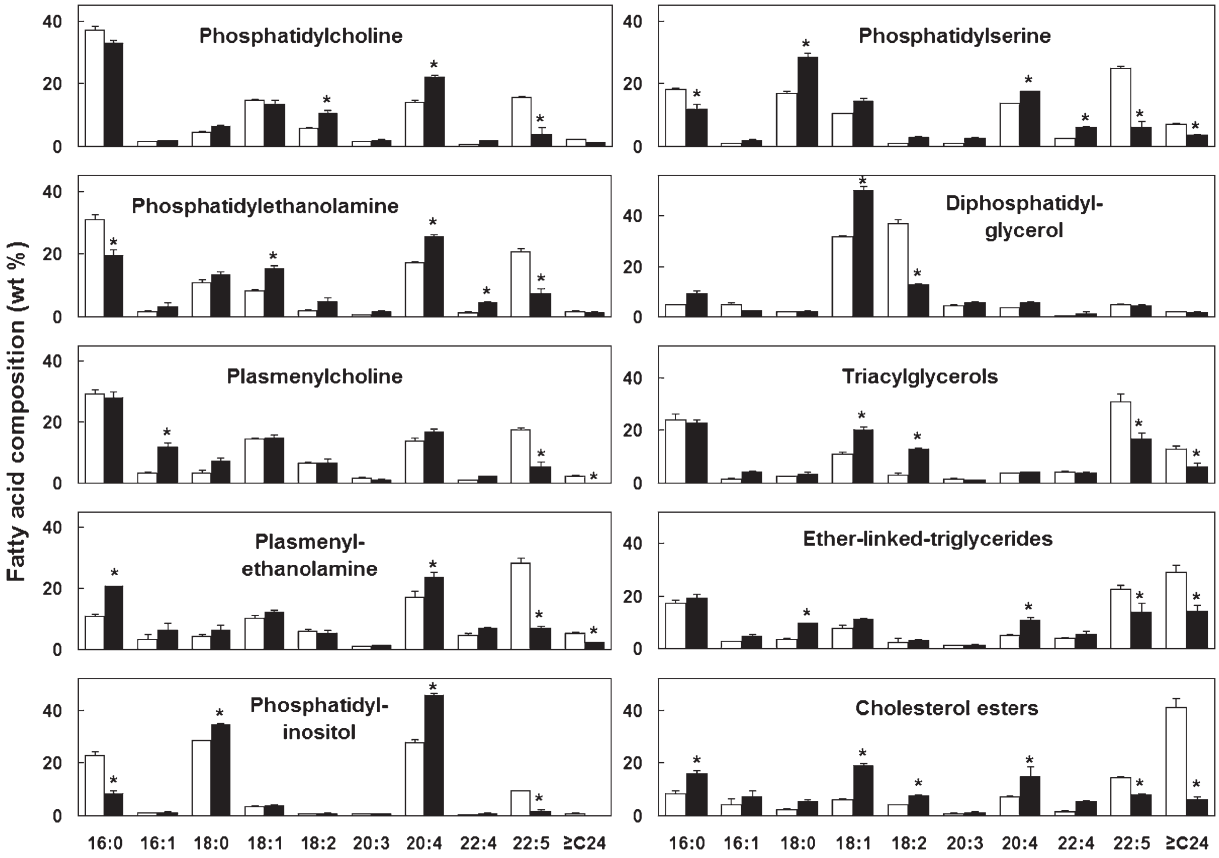

Fig. 7. Summary of the long-term changes after irradiation on the abundance (%) of main fatty acids of the rat testicular lipid classes of this study (other than sphingomyelin and ceramide shown in Fig. 3). White bars: nonirradiated testes; black bars: 30 weeks after irradiation. All lipids, polar and neutral, were richer in 20:4n-6 and poorer in 22:5n-6 and VLCPUFA in the latter condition. Data are expressed as mean ± SD, n = 4. The asterisks indicate statistically significant differences compared with nonirradiated testes ($P < 0.05$, Student's *t*-test). VLCPUFA, very long-chain polyunsaturated fatty acid.

with similar proportions of 20:4n-6 and 22:5n-6, whereas the more differentiated spermatids and spermatozoa have a much larger 22:5n-6/20:4n-6 ratio (12, 13) In the present study, GPL-associated 22:5n-6 and 20:4n-6 declined at similar rates during weeks 0–4, in agreement with the interpretation that spermatocytes formed during that period were gradually exhausted, mostly because they differentiated into spermatids. Interestingly, as it was the case with SM species with a high 2-OH/n-VLCPUFA ratio, GPL species with a high 22:5n-6/20:4n-6 ratio predominated, but fell intensely between weeks 4 and 6 postirradiation on a background of virtually unchanged 20:4n-6. This abrupt fall correlates with the fact that during such interval, 22:5n-6-rich spermatids differentiated into spermatozoa, which carried with them the last 22:5n-6-rich GPL as they normally left the testis.

Triacylglycerols

An initially intriguing observation of this study was that, in the presence of the massive decreases affecting the amounts per testis of 22:5-rich GPL, those of the 22:5-rich TAG were unchanged for four weeks. This implied that these TAG continued to be produced throughout the first four weeks in cells that had not been affected by irradiation. Again, previous work by Coniglio et al. (12) gave us a clue, as spermatids contain more 22:5-rich TAG than spermatocytes do. The rat testis still had spermatids being formed at week 4 postirradiation, virtually as the only germ cell type lasting in the tissue. Thus, the unchanged TAG were formed concomitantly with spermiogenesis, which functioned at its normal pace for four weeks at the expense of the germ cell precursors that had completed their differentiation.

Recent findings not only strengthened this idea but added a new element to consider. The large population of small, bright lipid droplets, which in this study were detected toward the lumena of seminiferous tubules, mostly composed the residual bodies that are normally released in the last stages of spermiogenesis at the same pace as condensing spermatids become spermatozoa. These remnant particles contained 22:5n-6-rich TAG and GPL (23). The unaffected presence of these particles observed here up to week 4 after irradiation explains the virtually unchanged amounts per testis of 22:5n-6-rich TAG during that period and the fact that both TAG and small lipid droplets disappeared concomitantly from the testis during the next two weeks.

Cholesterol esters

Despite the dramatic involution the testis undergoes during the first six weeks after irradiation, the mass per

testis of CE in that period increased three times, which represents an almost 10-fold increase in CE concentration compared with total phospholipids. The amount per testis of CE species with 22:5n-6 increased continuously and almost linearly during that period. Because the 22:5n-6-rich TAG had already been totally exhausted at postirradiation week 6 and because large lipid droplets containing neutral lipids located near the basal area of seminiferous tubules were present when virtually no germ cells (or TAG) remained in the testis, it may be concluded that these lipid inclusions contained the accumulated 22:5n-6-rich CE and that these lipids are mostly Sertoli cell products.

During normal spermatogenesis, Sertoli cells are known to endocytose (i) apoptotic bodies that are generated regularly from supernumerary or defective proliferating germ cells and (ii) residual bodies that are shed from elongating spermatids as they differentiate into spermatozoa. Both kinds of remnant particles consist of germ cell-derived materials: membranes, including intracellular organelles, macromolecules (nucleic acids, proteins), and lipids, densely packed together. This implies that Sertoli cells regularly incorporate, with apoptotic and residual bodies, a large amount of germ cell membrane-derived free cholesterol and (PUFA-rich) GPL and SM, in addition to nonmembrane neutral lipids, such as TAG. In our view, Sertoli cells, as any phagocyte would do, hydrolyze the overabundant lipids to free fatty acids, convert the fatty acids into CoA esters in the endoplasmic reticulum, and combine such acyl groups with the incorporated free cholesterol to produce CEs, which are collected temporarily in the form of cytoplasmic lipid droplets.

A recent study showed that isolated germ cells (pachytene spermatocytes and round spermatids), as well as differentiating spermatid-derived residual bodies, contribute little CE to the total CEs present in seminiferous tubules (23), supporting the proposed association of CE with Sertoli cells. In addition to nonesterified cholesterol, spermatocytes and spermatids contained 22:5n-6-rich GPL, while residual bodies concentrated 22:5-rich GPL and TAG. Phagocytosis and processing by Sertoli cells of germ cell-derived apoptotic bodies and residual bodies could account for the 22:5-rich CE shown here to be formed for six weeks (i.e., as long as spermatogenesis was sustained). By the same token, because spermatocytes contain sphingomyelins with high proportions of n-VLCPUFA (23), the fraction of the total CE that contains n-VLCPUFA, lasting less than four weeks in the postirradiated testis, could arise from phagocytosis and processing of spermatocyte-derived apoptotic bodies.

Acyl-CoA:cholesterol acyl transferase (ACAT) is the endoplasmic reticulum-bound enzyme responsible for the esterification of cholesterol when it is present at concentrations exceeding metabolic demands in cells. A variety of cells and tissues including testis (26) express the ACAT gene, of which two main isoforms, ACAT-1 and ACAT-2, have been identified (27). With the aid of a selective ACAT-1 inhibitor, both isoforms were shown to be active in murine seminiferous tubules (28). One of these enzymes is likely to be responsible for the formation of the highly unsaturated CE we associate in this study with Sertoli cells. Rather than the enzyme itself, which has already been proposed to play a role in the maintenance of lipid homeostasis in the testis (28), the present study calls attention to the possible origin of its fatty acid substrates and shows the fatty acids of the unique CE species it produces.

CONCLUDING REMARKS

Although this study focuses on lipids, we must consider that in addition to cholesterol and fatty acids, Sertoli cells must maintain the homeostasis of a great variety of molecules after phagocytosis of apoptotic and residual bodies. Sertoli cells must also dismantle macromolecules (proteins and nucleic acids), transport them to and process them in appropriate subcellular sites, convert them into secondary metabolites (aminoacids, small peptides, and nucleosides), and then possibly reutilize or send them out through ATP-driven transporters. This requires a great deal of metabolic energy. Coincidentally, after phagocytosis of apoptotic germ cells, Sertoli cells produce the highest levels of ATP compared with other testicular cells (29) and are able to efficiently β-oxidize germ cell-derived lipids. Thus, lipids originally synthesized in germ cells can be utilized by Sertoli cells to supply their own stringent energetic demands as supporters of spermatogenesis.

The importance of long-chain polyenoic fatty acids (22:5n-6 and/or 22:6n-3, according to the species) ester-bound to GPL has long been recognized for a healthy and successful development of germ cells. Accordingly, animals deficient in FADS2, the desaturase that initiates the desaturation-chain elongation cascade by which essential fatty acids are transformed into these PUFA, are subject to hypogonadism and sterility (30). The VLCPUFA amide bound to SM and Cer are lipid components of germ cells, opening important new avenues for research regarding their biochemistry and molecular biology, biophysical properties, and functional roles in male mature gametes.

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