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Changes in Lipids Containing Long- and Very Long-Chain Polyunsaturated Fatty Acids in Cryptorchid Rat Testes[1]

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ABSTRACT

The aim of the present study was to examine the effects of experimental cryptorchidism on rat testicular phospholipids and neutral lipids that contain long-chain (C_{18}-C_{22}) and very long-chain (VLC) (C_{24}-C_{32}) polyunsaturated fatty acids (PUFA). The weight of the cryptorchid testis was nearly half that of the contralateral control at postsurgical Days 7–10 owing to the depletion of germ cells. Concomitantly, the amounts of major glycerophospholipids (GPL) and sphingomyelin (SM) per testis decreased. Both these lipids lost their characteristic long-chain and very long-chain PUFA, notably 22:5n-6 and 28:4n-6, respectively, which suggests that these species are linked to the membranes of germ cells. In contrast, the amounts and concentrations of triglycerides (TG; triacylglycerols and 1-*O*-alkyl-2,3-diacylglycerols) and cholesterol esters (CE) increased several fold in the surviving cells (mainly Sertoli cells) in the cryptorchid testis. All these neutral lipids, but especially CE, accumulated large amounts of the major PUFA of the testis, 22:5n-6, as well as pentaenes with longer carbon chains (i.e., 24:5n-6 in TG and 28:5n-6 in CE). This accretion suggests that neutral lipids may store preformed PUFA coming from dying germ cell GPL and also VLCPUFA no longer needed as a source of PUFA destined to assemble new germ cell GPL. The lipid adjustments observed in cryptorchidism suggest a possible role for Sertoli cell CE in the turnover and conservation of PUFA within seminiferous tubules.

Sertoli cells, spermatid, spermatogenesis, stress, testis

INTRODUCTION

In most mammalian species, the temperature of the testes is tightly regulated, to assure optimal reproductive function [1, 2]. One important cause of infertility in the cryptorchid testis is prolonged exposure to a relatively high abdominal temperature, which leads to the disruption of spermatogenesis [3–6]. This condition has been reported extensively as a stress factor that rapidly induces apoptotic death of developing germ cells [7–9], with virtually no damage to the Sertoli cell population [10, 11].

The adult mammalian testis and spermatozoa have long been known to be extremely rich in lipids containing polyunsaturated fatty acids (PUFA) with 18 to 22 carbons atoms, the latter predominating in the adult testis (22:6n-3 and 22:5n-6, depending on the species) [12–16]. These long-chain PUFA increase several-fold in the lipids of testis with the onset of spermatogenesis [17, 18]. In the rat, 20:4n-6 and 22:5n-6 are typically the most abundant PUFA that are esterified to the major testicular glycerophospholipids (GPL). Germ cell ethanolamine and choline glycerophospholipids (EGP and CGP, respectively) are richer in 22:5n-6 than their counterparts in Sertoli cells [19]. Dietary deficiencies that lead to a decrease in these PUFA in GPL result in impaired spermatogenesis [20, 21]. Taken together, these factors point to 22:5n-6 as an important constituent of the GPL of rat germ cells.

Tetraenoic and pentaenoic PUFA of the n-6 series with very long chains (VLCPUFA)—elongated versions of 20- and 22-carbon (n-6) tetraenes and pentaenes—have also been characterized as specific components of mouse and rat testicular lipids [22–24]. These fatty acids, which have four or five double bonds and 24 to 32 carbon atoms, occur in different proportions in various classes of lipids in the rodent testis. In rat seminiferous tubules, the major PUFA are 20:4n-6 and 22:5n-6, followed by 24:4n-6 and 24:5n-6. These fatty acids are present in the major GPL and in triglycerides (TG), which are glycerolipids that in rodents comprise triacylglycerols (TAG) and 1-*O*-alkyl-2,3-diacylglycerols (alkyl-DAG) [25]. The cholesterol esters (CE) from rat seminiferous tubules are unusual in that they contain pentaenoic PUFA (e.g., 22:5n-6) and VLCPUFA (e.g., 28:5n-6 and 30:5n-6) as the major acyl chains [25]. Recently, we have observed that the sphingomyelin (SM) and ceramide (Cer) of rat seminiferous tubules contain PUFA with 28–32 carbon atoms, with 28:4n-6 and 30:5n-6 being the major VLCPUFA [26].

In the present study, we examined the effects of cryptorchidism on the lipids and fatty acids of the testis. Experimental cryptorchidism is considered to be a good model in which to study the consequences of spermatogenic arrest because it selectively affects the germinal cell lineage sparing the Sertoli cells [27, 28]. We predicted that this model could help us to discriminate the location and possible functions of the PUFA and VLCPUFA- rich lipids of the rat testis. We demonstrate that the amount of germ cell phospholipids (PL) decreases and their fatty acid composition changes as the germ cells disappear. Another finding of this study is that neutral lipids, especially CE, accumulate in the remaining tissue. These results highlight a novel aspect of cellular interactions in the seminiferous tubules, regarding lipid and fatty acid metabolism.

MATERIALS AND METHODS

Animals and Procedures

Male Wistar rats, 4 mo of age and weighing 300–350 g, were used in the present study. The animals were anesthetized by i.p. injection of a combination of ketamine (50 mg/kg) and acepromazine (5 mg/kg). Experimental cryptorchidia was performed following the surgical procedures of Farooqui et al. [27] and Barqawi et al. [28]. Briefly, after a midline incision, one testis was

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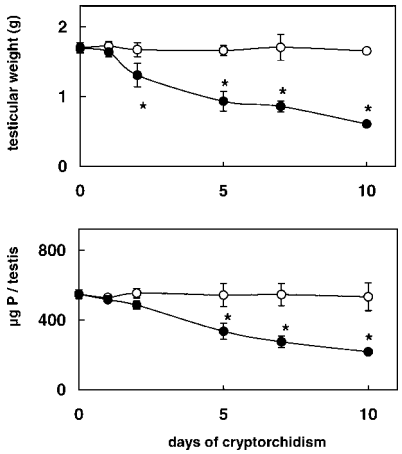

FIG. 1. Time-course of the changes in the weight and in the total amount of lipid phosphorus in the cryptorchid rat testis (closed circles), in comparison with the corresponding contralateral testis (open circles). Each point represents the average (mean values ± SD) of four animals. The asterisks indicate statistically significant differences from the corresponding contralateral testis ($P < 0.05$, Student *t*-test).

pulled through the internal inguinal canal and placed in the abdominal cavity. To prevent relocation of the testis in the scrotum, the inguinal canal was sutured using a monofilament nylon. The contralateral testis remained in the scrotum as a control. In a second group of rats (sham-operated group), the same surgical manipulations were carried out, except that the testis was returned to the scrotum after being pulled through the inguinal canal. The animals were killed 1–10 days after surgery and the testes were removed for histological examination and lipid extraction. After weighing, some of the organs were fixed in 10% formaldehyde, embedded in paraffin, and cut into 3-µm sections. The sections were stained with hematoxylin-eosin. All procedures were carried out in accordance with guidelines issued by the Animal Research Committee of the University of the South in accordance with the *Guide of the Care and Use of Laboratory Animals* of the Institute for Laboratory Animal Research (ILAR) of the National Academy of Science (Bethesda, MD).

Lipid Separation and Analysis

The testes were decapsulated and lipid extracts were prepared according to the procedure of Bligh and Dyer [29]. The solvents used in the preparative and analytical steps were HPLC-grade (JT Baker, NJ and UVE, Dorwill SA, Argentina). After the preparation of lipid extracts, the organic phases that contained the lipids were recovered, and the solvents were evaporated under N_2. Lipid phosphorus was determined in aliquots of these extracts using the chemicals and reactions described by Rouser et al. [30]. To isolate the neutral lipids, most of the lipid extracts were spotted (as bands) onto TLC plates (500 µm, silica gel G) under N_2 along with commercial standards (Sigma Chemical Co., St. Louis, MO). Neutral lipids were separated into classes using chloroform:methanol:acetic acid (95:4.5:0.5 by vol.) [31] up to the middle of the plates (to separate Cer), followed by n-hexane:diethyl ether:acetic acid (80:20:2 by vol.) up to the top of the plates (to separate the TG and CE). The total PL, which remained as the major lipid band at the origins of these plates, was collected by scraping the silica into tubes. Elution was carried out by thoroughly mixing this silica three times with chloroform:methanol:water (5:5:1 by vol.) and centrifuging the samples. The three eluates were pooled, mixed with four volumes of water, and separated into phases, to recover the lipids in the organic phase. After drying, the polar lipid samples, mostly containing PL, were dissolved in chloroform:methanol and aliquots were taken for the fatty acid analysis of total PL. The remainders of these PL samples were used for the preparative isolation of the major PL classes, cholineglycerophospholipids (CGP) and ethanolamine glycerophospholipids (EGP), which were separated by two-dimensional TLC [30], and SM, which was separated by mono-dimension TLC using chloroform:methanol:acetic acid:0.15 M NaCl (50:25:8:2.5) [32] as the solvent. After TLC, the zones that contained the neutral or polar lipid classes were located under UV light after spraying the plates with an N_2-driven solution of dichlorofluorescein in methanol, and the samples were scraped into tubes for elution, as described above.

The sphingolipids SM and Cer were dried and treated (under N_2) with one volume of 0.5 N NaOH in anhydrous methanol at 50°C for 10 min, to remove any potential lipid contaminant with ester-bound fatty acids. After mild alkaline treatment, one volume of chloroform and one volume of 0.5 N HCl were added and the tubes were centrifuged to recover the organic phases. These samples were rapidly dried and the lipids were separated once again by TLC.

After the isolation of the glycerophospholipids GCP and EGP, they were separated into their two major subclasses (1,2-diacyl- and 1-alkenyl,2-acyl-) by brief exposure of the lipids to HCl to release the aldehydes present in the latter. The dried lipid was vortexed for 1 min with one volume of 0.5 N HCl in acetonitrile:chloroform (1:1), followed by neutralization with one volume of 1.2 N sodium bicarbonate and rapid centrifugation, partitioning, drying of the organic phase, and spotting onto TLC plates. The resulting diacyl-glycerophospholipids and 2-acyl-lysoglycerophospholipids were separated by TLC using chloroform:methanol:water (65:25:4 by vol.). After elution, an aliquot was used for phosphorus quantitation and the remainder for fatty acid analysis of the subclasses.

Fatty Acid Analysis

The fatty acid compositions of the lipids were determined by gas-chromatography (GC) of their fatty acid methyl ester derivatives. The methyl esters from the fatty acids esterified to glycerol in total GPL were obtained by subjecting aliquots from the total polar lipid fraction to mild alkaline methanolysis, as described above (fatty acids that were amide-bound to sphingolipids were excluded from this preparation). In the other cases, the lipids were converted to methyl esters by placing the dry lipid samples overnight at 45°C with one volume of 0.5 N H_2SO_4 in anhydrous methanol under N_2 in Teflon-lined, screw-capped tubes [33]. Methyl heneicosanoate was added as an internal standard for quantitative analysis. Before GC, all of the methyl ester samples were purified by TLC using hexane:ether (95:5 by vol.) on silica gel G plates that had been previously washed with methanol:ethyl ether (75:25 by vol.). The methyl ester spots were located under UV light after spraying with dichlorofluorescein, scraped into tubes, and recovered into hexane after thoroughly mixing the silica support with methanol:water:hexane (1:1:1 by vol.; three successive hexane extractions). The combined hexane extractions were dried, the methyl esters were dissolved, and aliquots of the methyl ester solutions were taken for analysis. Nitrogen gas was used to protect the samples throughout the procedures used.

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., Bellefonte, PA) was used. The column oven temperature was programmed from 155°C to 230°C at a rate of 5°C/min, and then kept at the upper temperature for about 30 min to allow VLCPUFA to elute from the column. The injector and detector temperatures were set at 220°C and 230°C, respectively, and N_2 (30 ml/min) was the carrier gas. The fatty acid peaks were detected with flame ionization detectors, operated in the dual-differential mode, and quantified by electronic integration (using a Varian workstation). Statistical analyses of the results were performed using the two-tailed Student *t*-test, with $P < 0.05$ regarded as statistically significant. All the data shown in the figures are expressed as means ± SD obtained using at least four different animals per condition.

RESULTS

Changes in Weight and Histology

In comparison with their contralateral counterparts, the weights of the cryptorchid testes decreased progressively starting as early as Day 2 after surgery (Fig. 1). By Day 10, the mean weight of the cryptorchid testes was nearly half the initial value.

The histological analysis of the cryptorchid testes provided an explanation for the testicular weight loss (Fig. 2). The earliest microscopic changes were detected by Day 2, at which time most of the seminiferous tubules remained structurally intact and only a few were found to have a slightly diminished germ cell population. At Days 5 and 7, most of the tubules had lost their normal structures, and many of the germ cells in the seminiferous epithelium were missing. On Day 10, all the seminiferous tubules were devoid of germ cells, the remaining cells being mostly Sertoli cells and spermatogonia. The contralateral testis in the scrotum showed no histologic changes. As expected with tissue involution, no lymphocytic or other inflammatory cell infiltration was observed in any of the cryptochid testes studied.

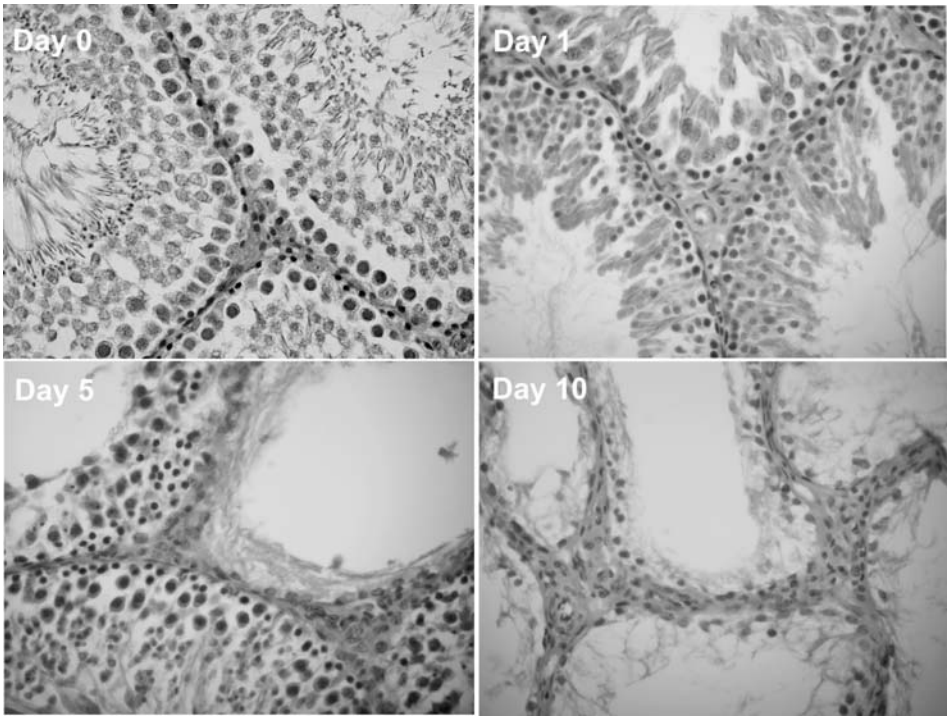

FIG. 2. Light micrographs stained with hematoxylin-eosin (original magnification ×400) of preparations of rat testes subjected to experimental cryptorchidism for the periods indicated. Note that the diameters of the tubules decrease and the numbers of tubules per field increase, concomitantly with the loss of germ cells. A significant number of germ cells had disappeared by Day 5, and by Day 10, the seminiferous epithelium consisted mainly of Sertoli cells and spermatogonia.

Major Lipid Classes

Experimental cryptorchidism resulted in a rapid and significant decrease in the amount of lipid phosphorus per testis (~44% by Day 10; Fig. 1). Since this decrease in total PL almost paralleled the testicular weight loss (~47% by Day 10), the concentration of lipid phosphorus per gram of tissue did not show significant changes, despite the marked decrease observed in the total amount of testicular PL (Fig. 3). Concomitantly, cryptorchidism resulted in a remarkable accumulation of the neutral lipids TG (TAG and alkyl-DAG) and CE. This increase was obvious when expressed as mg of lipid per testis, and was much more apparent when expressed as mg of lipid per gram of tissue (Fig. 3).

Major Phospholipid Fatty Acids

The amounts per testis of the three major PL, CGP, EGP, and SM, decreased to similar extents, which was consistent with both the reduction in total lipid phosphorus and the depletion of germ cells induced by cryptorchidism. However, this decrease affected differentially the major fatty acids of these PL. All the fatty acids showed tendencies to decrease (Fig. 4), albeit at different rates. The major 16:0, 20:4n-6, and 22:5n-6 ester-bound to GPL decreased more than the other fatty acids. The latter of these PUFA decreased the most and the most rapidly, with the result that the GPL at Day 10 had a significantly higher 20:4/22:5 ratio than the control GPL.

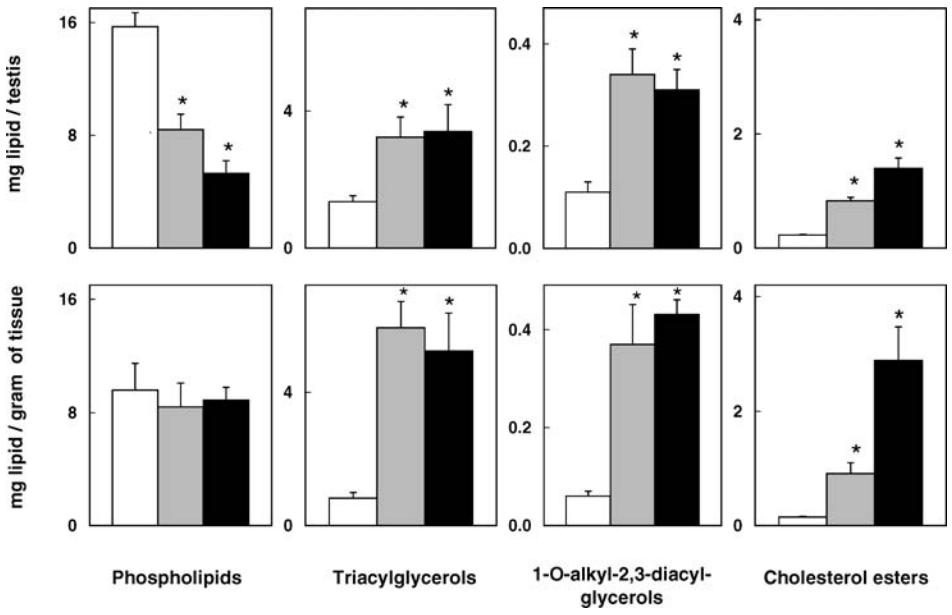

FIG. 3. Effects of unilateral cryptorchidism on the amounts and concentrations of the major lipid classes in the adult rat testes examined in the present study. Note the significant accumulation of neutral lipids in the cryptorchid testis, especially when expressed per gram of testis. White bars, control testes (Day 0); gray bars, 5 days of cryptorchidism; black bars, 10 days of cryptorchidism. The asterisks indicate statistically significant differences from the corresponding contralateral testes, which were used as controls ($P < 0.05$). The contralateral testes did not show significant differences from the Day 0, untreated controls.

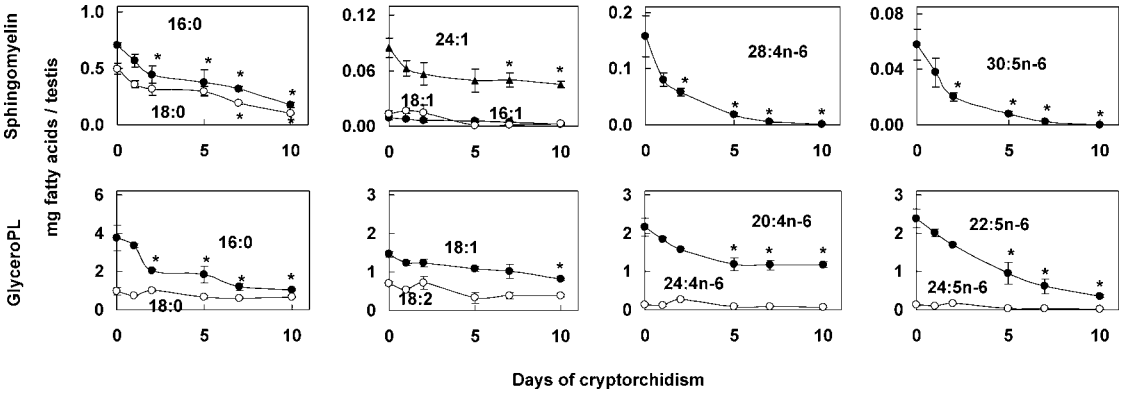

FIG. 4. Effects of unilateral cryptorchidism on the amounts of the major fatty acids of sphingomyelin (SM) and glycerophospholipids (GlyceroPL) in adult rat testes. * $P < 0.05$ compared to Day 0, untreated animal testes.

The amount of total SM per testis also decreased in the cryptorchid testis at a rate similar to those of the total lipid phosphorus and major GPL, concomitant with the depletion of germ cells. On Days 0 and 10, SM comprised 11% and 9%, respectively, of the total PL. Even though the amount of all SM fatty acids decreased in the cryptorchid testis, the species with VLCPUFA (especially 28:4n-6 and 30:5n-6) diminished the most and the most rapidly (Fig. 4). Ten days after surgery, these fatty acids had selectively disappeared from the SM of the rat testis. Testicular Cer was present as a very minor lipid in the neutral lipid fraction, probably representing a precursor in the biosynthesis of SM. As did SM, Cer contained saturated fatty acids and VLCPUFA, of which the major one was 28:4n-6, followed by 30:5n-6. In both SM and Cer, VLCPUFA decreased at higher rates than the other fatty acids due to cryptorchidism, to the point that by Day 10, both sphingolipids contained virtually undetectable amounts of VLCPUFA. Thus, saturated and monoenoic fatty acids became the major fatty acids of the SM (Fig. 4) and Cer (not shown) that remained in the testis as a consequence of cryptorchidism. These results suggest that the molecular species of SM and Cer that contain VLCPUFA 28:4n-6 and 30:5 n-6 are specific components of the germ cells.

Separation of the two major GPL classes, CGP and EGP, into their two major subclasses (plasmenyl- and phosphatidyl-ethanolamine and plasmenyl- and phosphatidyl-choline; Fig. 5) revealed that the subclasses that contributed the most to the observed PL reduction were phosphatidylcholine (PC) and phosphatidylethanolamine (PE). In all four subclasses, but especially in PC and PE, 22:5n-6 was the fatty acid that decreased the most. In contrast, the percentage of 20:4n-6 showed little change in all the GPL (Fig. 5). Thus, this fatty acid was the major PUFA of these lipids on Day 10 postsurgery (Figs. 4 and 5).

Farooqui et al. [27] and Barqawi et al. [28] have demonstrated that apoptotic rather than necrotic events occur in the germ cells in the model used in the present study. The reductions in GPL and sphingolipids in the absence of accumulations of lipid metabolites, such as free fatty acids, lysophospholipids, and diacylglycerols, although indirect, are consistent with an apoptotic mechanism of germinal cell disappearance. Since these reductions paralleled the decrease in the number of germ cells, the PL that remained in the testis represent those present in the surviving cells, the bulk of which were Sertoli cells.

Neutral Lipids and Their Fatty Acids

In contrast to the decreases in GPL, cryptorchidism resulted in increased amounts and concentrations of the neutral lipids (Fig. 3). The two subclasses of TG accumulated progressively

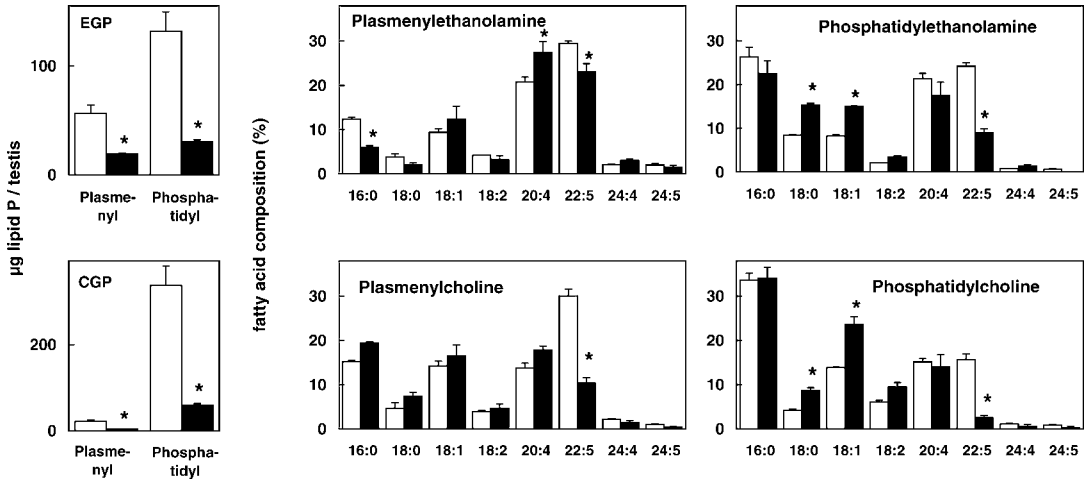

FIG. 5. Changes in the amounts (left panels) and percentages of the major fatty acids (right panels) of the two major subclasses of rat testicular ethanolamine and choline glycerophospholipids (EGP and CGP, respectively) as a consequence of cryptorchidism. White bars, control, untreated testes; black bars, 10 days of cryptorchidism. * $P < 0.05$ compared to control testes.

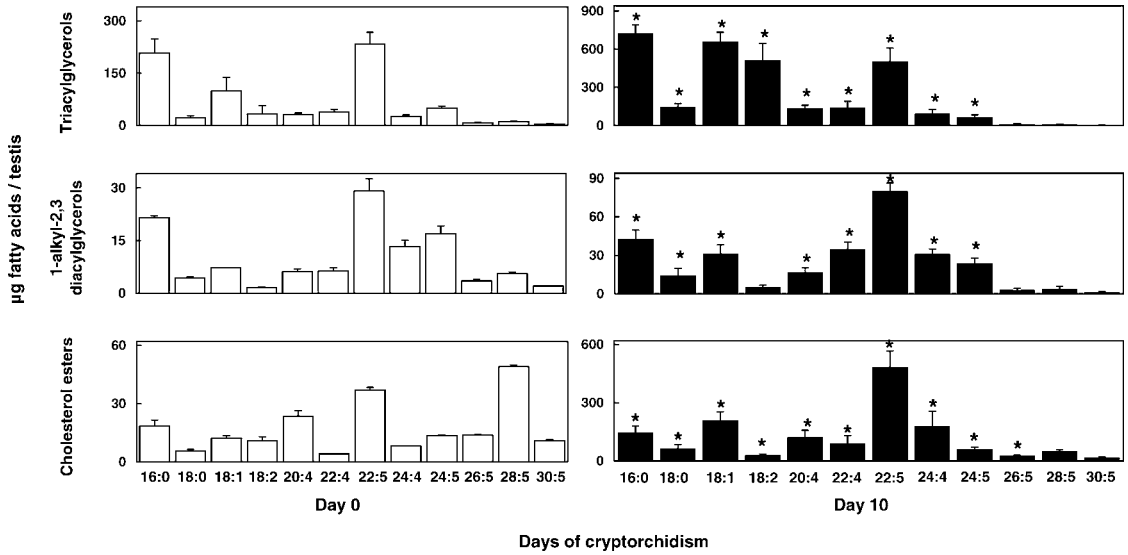

FIG. 6. Effects of cryptorchidism on the amounts of the major fatty acid constituents of the two major subclasses of testicular TG and cholesterol esters. White bars, control testes (Day 0, untreated animals); black bars, 10 days of cryptorchidism. * $P < 0.05$ compared to control testes.

for 5 days, thereafter reaching plateau levels. The cholesterol esters differed from TG in that they started to accumulate from Day 1, being one of the first and most sensitive lipid changes observed, and continued to increase throughout the period of the study. By Day 10, the amount of CE per testis had increased 6-fold, and its concentration per gram of tissue had increased as much as 20-fold compared to the controls.

Although all the TAG fatty acids increased, 16:0, 18:1, and 18:2n-6 accumulated to a greater extent than did the PUFA 20:4n-6, 22:4n-6, and 22:5n-6 and the very long-chain PUFA, 24:4n-6 and 24:5n-6 (Fig. 6). This was different from the case of alkyl-DAG, for which the fatty acids that accumulated the most were 22:5n-6 and the 24-C VLCPUFA.

The CE accumulated nearly all the fatty acid types, although the VLCPUFA and PUFA were the most abundant (Fig. 6). The former (e.g., 28:5n-6) increased over the first few days, then reached a plateau and later on tended to decrease (data not shown), whereas PUFA, in particular 22:5n-6, increased continuously, with dramatic build-up in the 5–10-day postsurgical period.

Since any tissue accumulation of CE requires the presence in cells of free fatty acids and free cholesterol, we speculate that both components are abundant in the cryptorchid testes, possibly as a result of the breakup of germ cell membranes. The fact that the most actively esterified fatty acid was 22:5n-6 supports this interpretation, which also requires that both the accumulating CE and the necessary esterases are present in the surviving cells.

DISCUSSION

Our findings show that experimentally induced unilateral cryptorchidism in adult rats results in remarkable qualitative and quantitative modifications to the testicular lipids and their fatty acids. These changes are apparently related to the rapid and selective depletion of germinal cells. The rapid testicular involution observed in the present study is consistent with previously published results using the same model. These latter studies have shown that cryptorchidism results in the rapid suppression of mainly spermatocytes and spermatids, which are the most susceptible elements of the germinal cell line to temperature stress [8, 34, 35], while sparing spermatogonia and all somatic cells, which are mostly Sertoli cells [8, 36]. The changes observed in the major lipid classes in the present study parallel these events. The most dramatic change, the rapid loss of large amounts of 22:5n-6-rich GPL (including the diacyl- and alkenyl-acyl-subclasses of EGP and CGP) and virtually all of the VLCPUFA-rich SM and Cer, may be ascribed to germ cell depletion. In the surviving cells, massive increases in TG and CE clearly occur, increasing the concentration of not only the VLCPUFA that they normally contain, e.g., 28:5n-6 [25], but also the PUFA that are typical of GPL, in particular 22:5n-6.

The fatty acid changes observed in the GPL of the cryptorchid rat testis are consistent with the fact that testicular GPL rich in PUFA, especially 22:5n-6 [37, 17], are much more abundant in germ cells than in Sertoli cells [19]. Furthermore, vigorous accretion of 22:5n-6 occurs in the testes of mice [17] and rats [38] in the period between birth and the onset of spermatogenesis. In the present study, we confirm that the total and individual GPL rich in 22:5n-6 are important constituents of the normal germ cell population by showing that selective elimination of these cells leads to significant reductions in both GPL and 22:5n-6.

The presence of molecular species of SM with VLCPUFA in mammalian testes and spermatozoa has been demonstrated previously [39, 40]. In the present study, we show that 28:4n-6, followed by 30:5n-6, are the major VLCPUFA in the SM of adult rat testes, in agreement with previous data [40]. Although with different percentages, the minor testicular Cer showed the same characteristic fatty acids as SM, and after 10 days of cryptorchidism, the same almost complete loss of their VLCPUFA occurred. The reductions in these typical fatty acids concomitantly with germ cell death in the cryptorchid testes of fertile rats indicate that they are normal constituents of the most mature cells of the germinal cell line. This is consistent with the findings that: 1) SM and Cer of sexually immature rat testes lack these fatty acids altogether; and 2) VLCPUFA are important constituents of the SM and Cer of mature, highly differentiated spermatozoa [41].

Interestingly, seminolipid, a lipid that is so specific for testicular germ cells and spermatozoa that it may be considered a lipid marker of these cells, has also been observed to disappear from the testis by Day 10 of

cryptorchidism (data not shown). This lipid (3-sulfogalactosyl-1-alkyl-2-acyl-sn-glycerol), which is known to contain almost exclusively 16:0 as the fatty acid at *sn-2*, is synthesized in the early phase of spermatocyte development and is maintained in all subsequent germ cell stages, including mature spermatozoa, of which it is a quantitatively important and functionally relevant lipid [42–44]. Genetic deletion of the enzymes involved in the synthesis of seminolipid or of its immediate precursor result in impaired spermatogenesis [45, 46]. Cerebroside sulfotransferase (CST)-null mice, generated by gene targeting, have been used to show that the same enzyme is involved in the synthesis of both brain sulfogalactosylceramide and testicular seminolipid [46]. Cst(−/−) mice lack the former lipid in the brain and the latter lipid in the testis. Notably, whereas Cst(−/−) mice of both sexes show neurological abnormalities, only the males are infertile due to a block in spermatogenesis before the first meiotic division [46], which underlines the essential nature of seminolipid in normal spermatogenesis. In the present study, the fact that cryptorchidism induced the total loss of this 16:0-rich lipid from adult rats within 10 days correlates with the drastic loss of PUFA-rich GPL and VLCPUFA-rich SM and supports our interpretation that the latter, as seminolipids, were originally components of germ cells.

Along with saturated, monoenoic and dienoic fatty acids, the TG (TAG and alkyl-DAG) of rat testis initially contained PUFA of the n-6 series, particularly tetraenes with 20–28 carbon atoms and pentaenes with 22–32 carbon atoms. The main PUFA in both lipids were 22:5n-6, 24:4n-6, and 24:5n-6, all three being particularly high in alkyl-DAG, as shown previously [25]. Cryptorchidia caused a marked increase in the content and a change in the fatty acid composition of both neutral lipids, mainly evidenced by the accumulation of 22:5n-6. Previous work with rat seminiferous tubules has shown that [^{14}C]24:4n-6 is actively desaturated to [^{14}C]24:5n-6, and that both forms are actively esterified to TG, suggesting that these lipids rich in 24-C VLCPUFA may function as a dynamic chemical repository of PUFA [47]. Through chain shortening or 'retro-conversion,' 24:4n-6 and 24:5n-6 could be convenient and readily available sources of the major PUFA of the germ cell GPL 20:4n-6 and 22:5n-6. This hypothesis is supported by the fact that dietary modifications in the n-3:n-6 PUFA ratio arising from fish oil-rich diets result in relative deficiencies in n-6 fatty acids in mouse tissues and plasma [25]. This type of dietary pressure induced drastic depletion of both neutral glycerolipids and their n-6 PUFA from the testes, whereas the GPL retained their n-6 PUFA-rich pattern. Taken together, these observations support the idea that under normal conditions, TG may act as metabolically active donors of polyunsaturated acyl groups for the biosynthesis of GPL, a markedly active process that is sustained throughout the adult life of the rat. When this synthesis is interrupted, as in the present model, the PUFA of TG are no longer able to play this role and thus they start to accumulate in these lipids. This would explain in part the increased amounts of PUFA, such as 22:5n-6, 24:4n-6, and 24:5n-6, in the TG. The selective accumulation of 22:5n-6 in the TG suggests that these lipids are also able to act as acceptors of fatty acids that originate in the breakdown of pre-existing GPL.

The CE of rat testis, which are shown in the present study to be rich in 22:5n-6 and VLCPUFA, particularly 28:5n-6 and 30:5n-6, belong to cells located within the seminiferous tubules [25], most probably Sertoli cells. These cells have a higher ratio of esterified to unesterified cholesterol than do germinal cells [19]. The fact that the VLCPUFA of adult CE are not observed in the testicular CE of sexually immature rats [25] strongly suggests they are involved in functions that appear after the onset of spermatogenesis. Under normal circumstances, one of these functions could be, as suggested for TG, to act as a temporary store of PUFA and VLCPUFA, thus functioning as donors of PUFA that will eventually become the acyl groups of GPL [25]. Although the behavior of CE during cryptorchidism resembled that of the neutral glycerides, the massive accumulation of the former suggests even more directly a relationship with the acyl groups of GPL. It is possible that under stress circumstances similar to that in the present study, CE are formed as a way to protect the surviving cells from the harmful accumulations of GPL-derived free fatty acids and free cholesterol formed in the membranes of dying cells.

As the amount of GPL formerly associated with the disappearing germ cells of cryptorchid testis decreased, TG and CE rich in PUFA tended to accumulate in the surviving cells, mostly Sertoli cells. Supporting evidence for the accumulation of neutral lipids in the latter may be found in the histologic observations presented by other authors, in which they show lipid droplets in the Sertoli cell cytoplasm under several pathological conditions that lead to spermatogenesis arrest [48, 49]. This is consistent with the fact that damaged or dead germ cells are phagocytosed by Sertoli cells, which hydrolyze and are able to reutilize some of the resulting elements [50–52].

Well-regulated cholesterol ester metabolism is essential for normal spermatogenesis, as revealed by the phenotype of *h*ormone *s*ensitive *l*ipase (HSL)-deficient mice [53, 54]. In these mice, oligospermia is associated with CE accumulation owing to a complete lack of cholesterol ester hydrolase (CEH), an activity that is mediated by the testis-specific isoform of HSL. Durham and Grogan [55] characterized two isoforms of CEH in rat testes: a temperature-stable form and a temperature-labile form. The latter is present only in Sertoli cells and its activity is inhibited by a testicular temperature above 37°C [55, 56]. Interestingly, this form of the enzyme displays considerable substrate specificity, being less active towards CE with shorter (C16–18) fatty acids than towards CE with longer fatty acids (especially one with a very long chain, 24:1) [55]. Exposure of the testes to the abdominal temperature leads to a rapid inactivation of this thermolabile isoform of the CEH [56, 57]. Moreover, 4 days of cryptorchidism in rats leads to reductions in HSL activity, as well as in the amounts of HSL mRNA and protein [58]. The effect of cryptorchidism on this enzyme may explain the rapid increases in the amounts and concentrations of testicular CE observed in the present study.

An increased level of CE results either from the inhibition of CE hydrolase or from the stimulation of CE esterase activities. In cryptorchidism, the marked increase of testicular CE first involved a moderate increase in its VLCPUFA and later a dramatic and persistent increment in CE 22:5n-6 and 20:4n-6. We interpret this behavior as reflecting a dual biochemical role of CE. In the first phase, since VLCPUFA are potential precursors of PUFA after CE hydrolysis and chain shortening, they may have accumulated in CE because they were no longer required as a source of acyl chains destined to be membrane GPL. In the second phase, 22:5n-6 and 20:4n-6–formerly linked to testicular GPL and now concentrating in CE–could reflect a stimulated esterification of fatty acids to cholesterol. This enzyme reaction could be a convenient way not only of protecting the surviving cells but also of retaining the polyenoic acyl chains in the testis. Thus, both enzyme activities, which are not mutually exclusive, are apparently operative in the testis, the esterase becoming evident as an outcome of germ cell death.

The cryptorchid model, which represents an experimental situation that induces selective death of germ cells and survival of somatic cells (accumulating the neutral lipids TG and EC), allows discrimination of the main lipids of the major groups of cells in the rat seminiferous epithelium. Whereas 22:5n-6-rich glycerophospholipids and VLCPUFA-rich SM and Cer, as the 16:0-rich seminolipid, are tied to the fate of germ cells, the PUFA and VLCPUFA-rich TG and CE are closely associated with an important function of Sertoli cells.

While this paper was in progress, other models of specific germ cell death used in our laboratory, involving irradiation with x-rays (unpublished data) and the administration of the antineoplastic agent doxorubicin (unpublished data), have shown similar trends (albeit at different rates and to different extents) for the effects of cryptorchidism on the lipids of rat testes, i.e., PL depletion and CE accumulation, which lend support to the present conclusions. Cryptorchidism resulted in the fastest cellular and lipid biochemical changes (days as opposed to weeks). If cryptorchidism is not corrected, the three situations have in common that they eventually lead to testicular involution and atrophy. However, one important difference is that spermatogonia are preserved for some time in cryptorchidism, while they are damaged irreversibly and rapidly die in the other two models. If cryptorchidism is treated with adequate and rapid surgical correction, the surviving spermatogonia are potentially able to reinitiate spermatogenesis, and the testis may be eventually repopulated by spermatogenic cells. Although it may be predicted that the changes observed in the present study for testicular lipids are potentially reversible if the cryptorchid testis is replaced into its normal position, this is difficult to assess experimentally, as it would require further surgical manipulation of the animals. Since the cellular and biochemical effects conferred by this condition may be ascribed mainly to the higher temperature that the testis encounters in the abdominal cavity, a good model that allows some insight into cryptorchidism and the reversibility of its effects is mild testicular hyperthermia. Preliminary work from our laboratory has shown that mild hyperthermia promotes similar effects on cells and lipids to those described in the present study, and that these changes tend to revert to normal after an appropriate number of weeks.

Although it seems obvious that the selective death of specific germ cells during cryptorchidism would parallel the disappearance from the testis of lipids and fatty acids that are typical of these cells, we have shown that the germ cell population that is most rapidly eliminated in this condition is the one that contains polar membrane phospholipids rich in polyunsaturated fatty acids with long and very long chains. We also show that after cryptorchidism-induced death of germ cells, some of the fatty acids that originally composed the germ cell membrane lipids end up in neighboring cells that survive this condition. These fatty acids, once esterified in triglycerides and cholesterol esters, are at the same time innocuous to the surviving cells and potentially useful as a source of acyl groups to replace phospholipids in the case of timely treatment of cryptorchidism. In our view, the formation of these neutral lipids is one of the many biochemical mechanisms that are rapidly triggered in the surviving cells of the testis, in order to tolerate and eventually overcome the stress imposed by this adverse condition.

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