Very Long Chain PUFA in Murine Testicular Triglycerides and Cholesterol Esters

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ABSTRACT: Very long chain (VLC) PUFA of the n-6 and n-3 series are known to occur in mammalian testis. The aim of this work was to characterize further two testicular lipid classes with VLCPUFA, cholesterol esters (CE) and total triglycerides (TG) in rat and mouse testis. The VLCPUFA predominating in these lipids were a series of n-6 pentaenes and tetraenes with 24 to 32 carbons, including small amounts of odd-chain PUFA, 28:5n-6 and 24:5n-6 prevailing in CE and TG, respectively. Most of the VLCPUFA of TG were concentrated in a small fraction of TG, made up by 1-*O*-alkyl-2,3-DAG. This TG subclass was absent altogether from the TG of sexually immature testis. The TG and the CE with VLCPUFA only occurred in testis of adult fertile animals. The proportion of VLCPUFA in total TG and CE was higher in rodents than in other mammals. In the n-6 PUFA-rich adult mouse testis, the amounts of testicular triacylglycerols decreased significantly after consumption of fish oil for 2 wk. Whereas 18:2n-6 was significantly reduced, the amounts of 22:5n-6 and longer n-6 PUFA were less affected in all major testicular lipids including PC and PE, where they were unchanged. The 1-*O*-alkyl-2,3-DAG and their n-6 VLCPUFA were virtually unaffected by the diet. The VLCPUFA-containing molecular species of CE and TG may represent a form of storage of cholesterol and polyenoic FA required to sustain spermatogenesis. *Via* chain-shortening, VLCPUFA stored in the neutral lipids may serve as precursors of the major C_{22} PUFA typical of cell membrane glycerophospholipids, protecting testicular cells against shifts in FA composition induced by dietary changes.

Paper no. L9052 in *Lipids 38*, 73–80 (January 2003).

A group of very long chain (VLC) PUFA of the n-6 and n-3 series was characterized several years ago in the mammalian genital tract. In rat testes (1) and in mouse spermatocytes and spermatids in culture (2,3), 24:4n-6 and 24:5n-6 were shown to be actively formed from labeled 20:4n-6. In rat testis, Grogan (4) showed that 20:4n-6 is the precursor of these and of even longer PUFA, including tetraenes and pentaenes with up to 30 carbon atoms. In testis and spermatozoa of a number of mammalian species, Poulos *et al.* (5) reported the occurrence of n-6 and n-3 VLC tetraenoic, pentaenoic, and hexaenoic FA. Complete homologous series of n-6 tetraenes and pentaenes up to C_{34}, including VLCPUFA with even and odd numbers of carbons, were characterized in the total lipid of rat seminiferous tubules (6). In the same article, such VLCPUFA were shown to be synthesized *in situ* by successive elongation of 24:4n-6 and 24:5n-6, two FA connected by an active 6-desaturation.

VLCPUFA were characterized in the retina of several vertebrates (7) and in the brain of rats (8) and humans (9,10). The VLCPUFA of retina are important components of the dipolyunsaturated molecular species of PC of retinal rod outer segments (11). In the PC of both retina (12) and brain (10), VLCPUFA are specifically esterified to the *sn*-1 position of the glycerol backbone. The proportion of VLCPUFA-containing molecular species of PC in human brain increases with development (9) and in the rat retina decreases with aging (13). As with the n-6 VLCPUFA of testis (4,6), the n-3 VLCPUFA of retina (14,15) are actively synthesized *in situ* by successive elongation of ordinary PUFA such as 20:5n-3 and 22:6n-3, with an important role being played by the 6-desaturation of 24:5n-3 to 24:6n-3.

The VLCPUFA of testis and spermatozoa occur in specific lipids. They were first reported to be located in unique molecular species of sphingomyelin (SM) (16). Robinson *et al.* (17) confirmed this finding in a variety of species and extended it by showing the occurrence, in rat and boar SM, of 2-hydroxylated derivatives of VLCPUFA. Further work in rat seminiferous tubules (6) detected the presence of VLCPUFA not only in SM but also in two neutral lipids, cholesterol esters (CE) and triglycerides (TG). The aim of this work was to study further the FA of these two lipid classes. It is shown that the testicular tissue of rodents contains important proportions of VLCPUFA of the n-6 series in these two lipids. Two subclasses of TG are described, the major one being the triacylglycerols (TAG), and the minor one containing TG with *O*-alkyl bonds. The latter is shown to concentrate most of the VLCPUFA present in testicular TG.

Since the levels of n-6 PUFA in tissue lipids may be affected by the consumption of diets enriched in n-3 FA, and since recently (18) we observed that the amount and proportion of 18:2n-6 and other n-6 PUFA in mouse plasma decreased after 2 wk on a fish oil-rich diet concomitantly with a reduction of TG, CE, and PC, we were interested in seeing how this condition would affect the FA of the same lipid classes from testis. In contrast to the liver, the testis did not incorporate much n-3 PUFA. Instead, it preserved the n-6 PUFA, especially the major 22:5n-6 of phospholipids (PL). The n-3-rich diet induced changes in the proportions of the TG and of their n-6 PUFA (18:2, 22:5, and VLCPUFA),

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Abbreviations: CE, cholesterol esters; LH, luteinizing hormone; PL, phospholipid; SM, sphingomyelin; TAG, triacylglycerols; TG, total triglycerides; VLC, very long chain. Fatty acids are named by the convention of: number of carbon atoms:number of double bonds.

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which suggest that the levels of 22:5n-6 were sustained at the expense of the acyl groups stored in these lipids.

MATERIALS AND METHODS

The rat and mouse testicular tissues used in this study were obtained rapidly after the animals' death, whereas those from domestic animals were collected after standard surgical procedures. In the case of mice, testes from two groups of animals were analyzed: controls, having eaten the standard rodent lab chow diet, and fish oil-fed mice, receiving an n-3 FA-enriched diet for 2 wk. This diet consisted of 23% protein, 63% carbohydrates, 5% of a vitamin mixture, and 9% fish oil (w/w). The FA compositions of control and fish oil diets were as follows: saturates, 19.3 and 25.8%; monounsaturates, 22.4 and 29.6%; n-6 PUFA, 57.9 and 13.7%; and n-3 PUFA, 0.4 and 30.8%, respectively (18). The protocol for the study conformed to accepted norms of animal care and experimental procedures. The testes were decapsulated, and lipid extracts were prepared from the entire tissue or from portions of it according to the procedure of Bligh and Dyer (19). The extracts were dried under a stream of N_2, and the neutral lipids were separated on silica gel G TLC plates by using hexane/diethyl ether/acetic acid (80:20:1, by vol) up to the middle of the plates, to separate the TG from the CE, followed by *n*-hexane/diethyl ether (95:5, vol/vol) up to the top, in order to ensure separation of CE from the solvent front. All lipid bands were located under UV light after spraying the plates with 2′,7′-dichlorofluorescein in methanol.

The VLCPUFA of TG and CE were identified using the criteria described in previous work (6,7,11). The FA composition of lipids was determined by GC of their FAME derivatives. These were prepared by placing the lipid samples with 14% BF_3 in methanol overnight at 45°C under N_2 in Teflon® sealed tubes. Before GC, the methyl esters were purified by TLC using hexane/ether (95:5, vol/vol) on silica gel G plates that had been prewashed with methanol/ether (75:25, vol/vol). The methyl esters were recovered from the silica after thorough mixing and partitioning between water/methanol/hexane (1:1:1, by vol; three hexane extractions). Under the present GC conditions, the minor n-6 pentaenes with odd-numbered chains tended to coelute with (not resolve from) the corresponding hexaenes of the n-3 series with even-numbered chains having one carbon less (minor in mice and negligible in rats) (e.g., 27:5n-6 with 26:6n-3, 29:5n-6 with 28:6n-3, and so on). Something similar occurred between the n-6 tetraenes with odd chains and the n-3 pentaenes with even chains having one carbon less (e.g., 21:4n-6 with 20:5n-3, and so on) (Tables 1 and 2). To estimate the sums of n-6 PUFA and n-3 PUFA given in Figure 3, the methyl esters were resolved according to unsaturation by argentation TLC. Silica gel G plates, precleaned with methanol/ether (75:5, vol/vol) and dried, were impregnated by spraying with aqueous $AgNO_3$ (2 g per 10 g of silica), dried, and activated. The previously purified PUFA fractions were spotted and resolved into fractions by means of chloroform/methanol (90:10, vol/vol). HPLC-grade solvents were used throughout. A Varian 3700 gas chromatograph equipped with two (2 m × 2 mm) 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 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. Injector and detector temperatures were set at 220 and 230°C, respectively, and N_2 (30 mL/min) was the carrier gas. The FA peaks were detected with FID, operated in the dual-differential mode, and quantified by electronic integration (Varian Workstation). Statistical analyses of the results were performed using the two-tailed Student's *t*-test.

RESULTS

FA of TG and CE of murine testis. The FA compositions of CE and TG from (adult, fertile) rat and mouse testis are compared in Table 1. Both species contained VLCPUFA in TG and CE, most of them belonging to the n-6 series. These were virtually the only VLCPUFA type in the rat, the mouse containing somewhat more components of the n-3 series than the rat. In both animals, the single lipid class most highly enriched in molecular species with VLCPUFA was CE. The sum of VLCPUFA accounted for as much as nearly 40 and 20% of the FA of rat and mouse CE, respectively. The mouse had a significantly larger proportion of VLCPUFA of n-3 series (22:6n-3 and longer) than the rat in this lipid. In both animals, the most abundant VLCPUFA of testicular CE was 28:5n-6 (Table 1, Fig. 1).

In total testicular TG (Table 1), docosapentaenoic (22:5n-6) and linoleic (18:2n-6) acids were the major PUFA, the former predominating in the rat and the latter in the mouse. The sum of VLCPUFA (24 to 32 carbons) represented between 5 and 20% of the FA of total TG. Most of this sum was accounted for by 24:4n-6 and 24:5n-6, with a series of longer tetraenes and pentaenes up to 32 carbons long being responsible for the rest.

The solvents described in Materials and Methods for the separation of the neutral lipids resolved the TG into two subfractions, the minor upper one accounting in rodents for about 10–20% of the FA of total testicular TG. This subfraction of TG did not release glycerol after treatment with lipoprotein lipase, as did the major band of TG, containing the TAG. On TLC, the minor band of TG had an R_f corresponding to a standard of 1-*O*-palmityl-2,3-distearoyl-glycerol. After mild alkaline treatment, it produced FAME and a product having the same TLC migration, in two different solvents, as standards of two well-known 1-alkyl-diols, batyl and chimyl alcohol. It did not release fatty aldehydes after treatment with HCl fumes. It was concluded that the small subfraction of TG was a TG with an *O*-alkyl bond, 1-*O*-alkyl-2,3-DAG. This subclass concentrated most of the VLCPUFA that is present in the testicular TG as a class. Figure 1 (upper panel) shows the FA profiles of this lipid from rat and mouse testis. Pentaenoic FA of the n-6 series, from 22:5 to 30:5, were its major constituents, followed by tetraenoic FA from 20:4n-6 to 28:4n-6. The major acyl

TABLE 1
FA Composition of Major Neutral Lipid Classes from Murine Testis[a]

	Total triglycerides (TG)		Cholesterol esters		
	Rat	Mouse (C)	Rat	Mouse (C)	Mouse (FO)
14:0	0.8 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	1.2 ± 0.1	2.0 ± 0.2
15:0	0.1 ± 0.03	0.2 ± 0.04	0.4 ± 0.1	—	—
16:0	23.9 ± 0.4	13.4 ± 0.9	8.2 ± 1.2	7.0 ± 1.0	5.8 ± 0.8
17:0	0.2 ± 0.03	0.2 ± 0.04	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.4
18:0	2.6 ± 0.5	2.9 ± 0.3	2.0 ± 0.6	3.4 ± 0.6	2.6 ± 0.1
14:1	0.5 ± 0.1	0.04 ± 0.02	—	0.3 ± 0.1	0.4 ± 0.4
15:1	0.02 ± 0.01	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.3 ± 0.3
16:1	1.6 ± 0.1	4.2 ± 0.1	1.8 ± 0.4	2.9 ± 0.3	2.9 ± 0.1
17:1	0.1 ± 0.01	0.2 ± 0.02	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.4
18:1	11.3 ± 0.8	25.3 ± 0.5	5.4 ± 0.3	16.8 ± 3.3	14.3 ± 2.4
20:1	0.6 ± 0.1	0.1 ± 0.02	0.5 ± 0.1	0.3 ± 0.02	0.3 ± 0.1
18:2n-6	3.9 ± 0.6	28.1 ± 1.9	4.4 ± 0.2	2.9 ± 0.5	1.6 ± 0.3*
18:3n-6	0.1 ± 0.1	1.6 ± 0.5	0.2 ± 0.1	0.3 ± 0.2	0.5 ± 0.4
18:3n-3	0.5 ± 0.04	0.7 ± 0.1	0.2 ± 0.04	3.1 ± 0.5	2.8 ± 0.2
18:4n-3	—	0.3 ± 0.1	—	1.0 ± 0.2	0.7 ± 0.3
20:2n-6	—	0.3 ± 0.1	—	0.6 ± 0.1	0.9 ± 0.1
20:3n-9	—	0.1 ± 0.01	0.2 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
20:3n-6	1.6 ± 0.2	0.3 ± 0.04	0.7 ± 0.1	2.0 ± 0.2	0.5 ± 0.2
20:4n-6	3.8 ± 0.2	0.9 ± 0.2	11.8 ± 0.4	4.2 ± 0.5	1.2 ± 0.1*
20:4n-3	—	0.2 ± 0.03	—	1.1 ± 0.2	1.1 ± 0.1
20:5n-3, 21:4n-6, 22:3n-6	0.5 ± 0.03	0.5 ± 0.04	1.1 ± 0.3	1.1 ± 0.1	2.1 ± 0.2
22:4n-9, 22:3n-6	0.2 ± 0.02	0.2 ± 0.03	0.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.3
22:4n-6	4.8 ± 0.3	2.1 ± 0.4	2.2 ± 0.1	7.3 ± 1.1	1.8 ± 0.1*
22:5n-6, 23:3n-6	28.4 ± 0.7	5.9 ± 0.7	15.8 ± 0.2	7.5 ± 0.2	5.1 ± 0.7
22:5n-3, 23:4n-6	0.4 ± 0.01	0.4 ± 0.1	0.4 ± 0.05	2.7 ± 0.6	6.0 ± 0.9
22:6n-3, 23:5n-6, 24:3n-6	0.7 ± 0.25	2.0 ± 0.6	2.1 ± 0.2	10.6 ± 1.5	15.1 ± 2.7*
24:4n-6	3.5 ± 0.4	1.4 ± 0.1	3.4 ± 0.1	1.1 ± 0.3	0.9 ± 0.3
24:5n-6	6.3 ± 0.4	4.4 ± 0.4	5.7 ± 0.2	3.4 ± 0.9	1.8 ± 0.2*
25:4n-6, 24:5n-3	—	0.1 ± 0.03	—	0.3 ± 0.1	0.4 ± 0.4
25:5n-6, 24:6n-3	0.1 ± 0.08	0.4 ± 0.03	0.1 ± 0.1	0.7 ± 0.2	1.1 ± 0.4
26:4n-6	0.5 ± 0.1	0.1 ± 0.02	0.7 ± 0.1	0.7 ± 0.05	0.3 ± 0.3
26:5n-6	0.9 ± 0.1	1.1 ± 0.1	5.4 ± 0.2	3.2 ± 1.0	2.1 ± 0.4
27:4n-6, 26:5n-3	0.2 ± 0.03	0.03 ± 0.01	0.4 ± 0.04	0.4 ± 0.1	0.9 ± 0.1
27:5n-6, 26:6n-3	0.1 ± 0.04	0.3 ± 0.1	1.7 ± 0.1	0.5 ± 0.1	1.4 ± 0.3
28:4n-6	0.2 ± 0.1	0.05 ± 0.02	0.2 ± 0.1	0.7 ± 0.3	0.5 ± 0.4
28:5n-6	1.2 ± 0.2	0.7 ± 0.03	17.9 ± 2.5	8.9 ± 2.4	7.2 ± 0.3
29:5n-6, 28:6n-3	0.1 ± 0.04	0.1 ± 0.02	0.7 ± 0.2	1.9 ± 0.7	2.4 ± 0.6
30:4n-6	—	—	0.04 ± 0.03	0.7 ± 0.2	0.3 ± 0.4
30:5n-6	0.4 ± 0.1	0.2 ± 0.04	3.5 ± 0.6	0.8 ± 0.5	0.9 ± 0.6
31:5n-6	0.02 ± 0.01	0.01 ± 0.002	0.6 ± 0.1	0.1 ± 0.02	0.4 ± 0.4
32:5n-6	—	—	0.2 ± 0.1	0.1 ± 0.1	—
33:5n-6	—	—	0.1 ± 0.04	—	—
LC-PUFA	44.7 ± 0.8	42.4 ± 1.5	39.0 ± 1.3	42.8 ± 3.2	37.7 ± 3.7
VLCPUFA	13.6 ± 1.3	9.5 ± 0.6	40.9 ± 2.5	24.6 ± 6.6	22.4 ± 4.6

[a]Results are given as percentages of the total FA in each lipid (mean values ± SD from at least four animals). In the case of mice, (C) refers to controls and (FO) to animals consuming fish oil. Abbreviations: LC-PUFA, sum of PUFA with 18–22 carbons; VLCPUFA, sum of PUFA with >22 carbons. The asterisks point to significant differences due to FO consumption. Except for small amounts of 20:5, 22:5, and 22:6n-3, PUFA of the n-3 series were undetectable in rats and detectable though minor components in mice.

chains had an even number of carbon atoms, but odd-chain PUFA were found in both series. Separation of the pentaenoic and tetraenoic FA from both CE and TG by argentation TLC, followed by catalytic hydrogenation, gave rise in both cases to a complete homologous series of saturated FA (20:0 to 28:0 and 22:0 to 32:0), including the odd-chain saturates.

Most of the CE and TG with VLCPUFA observed in this work were contributed by cells located within the seminiferous tubules, as ascertained in rats by comparing the FA profile of lipids from whole testis, from seminiferous tubules, and from the cells that are discarded after the preparation of seminiferous tubules.

In rats, the FA composition of CE and TG, with the typical abundance of 22:5n-6 as well as VLCPUFA, was maintained

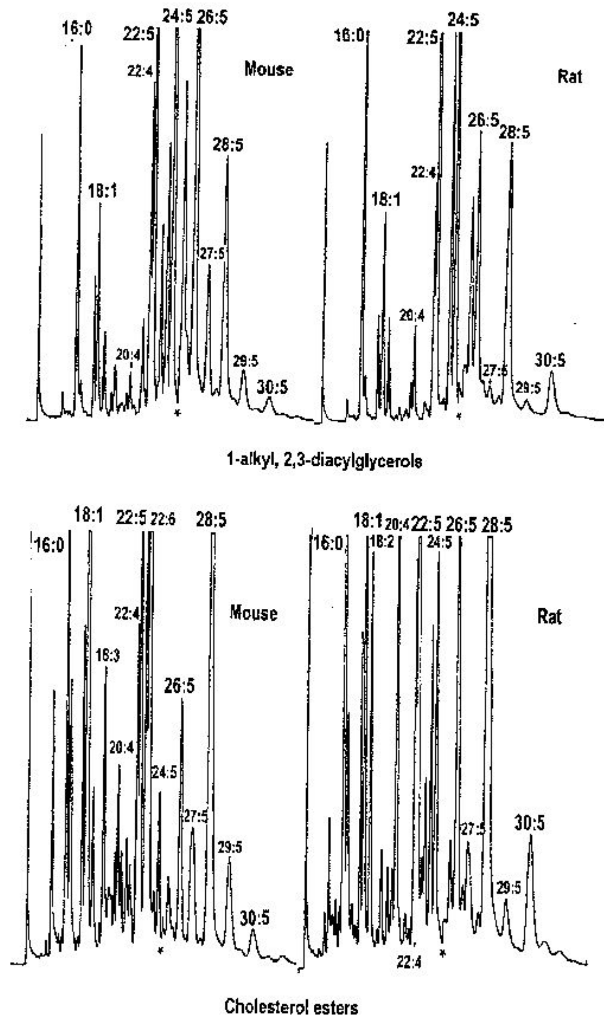

FIG. 1. FA from 1-O-alkyl-2,3-DAG (upper panel) and from cholesterol esters (lower panel) from mouse and rat testis. The FA, as methyl esters, were run on a set of conventional GC columns packed with a polar stationary phase as described in the Materials and Methods section. Total run time: 45 min. The asterisks denote a twofold increase in sensitivity.

with little or no change during aging, with no significant differences between young adults and rats aged 2.5 to 3 yr (against our expectations). Only in a group of rats that, incidentally, had been discarded due to reduced fertility (18 mon), were the VLCPUFA of these lipids significantly lower than in the normal fertile individuals. By contrast, all of the VLCPUFA shown in Tables 1 and 2 were absent altogether from the testicular CE and TG of 14-d-old rats. Moreover, both of these lipids had low levels of 22:5n-6 and other long-chain polyenes (data not shown) at that early age. Coincidentally, sexually immature rats and mice also lacked the 1-*O*-alkyl-2,3-DAG that carry most of the VLCPUFA associated with the TG class.

In the testis of other mammals, the proportion of VLCPUFA in CE and in total TG was smaller than in the two rodents of this study (Fig. 2). The total content of CE and TG per gram of testicular tissue (not shown) was lowest in bovine testis, intermediate in the two rodents studied here, and highest in cats and dogs. The epididymal tissue, examined in the same animals, contained vast amounts of TG, much more so at the caput than at the cauda regions, and negligible proportions of CE. Neither of these lipids contained VLCPUFA.

Changes induced by an n-3 PUFA-rich diet to the FA of mouse testicular lipids. After 2 wk of consuming a fish oil (FO)-rich diet, the major effect observed on the content of mouse testicular lipid classes was a significant decrease in TAG (6.6 ± 0.8 to 3.2 ± 0.2 mg/g of tissue), concomitant with a slight increase in the content of PL and CE, just as was the case with liver (18). The content of alkyl-DAG did not change significantly, as indicated by the amount of FA recovered from this lipid per gram of tissue. The FA modifications accompanying these lipid changes are shown in Tables 1 and 2, which disclose some differential effects of the n-3 diet on individual FA, and in Figure 3, which summarizes the effects on different groups of FA.

In the major membrane glycerophospholipids, PC and PE (Table 2), saturated and monounsaturated FA proportions remained practically unaltered, except for a slight increase of 18:1 in PC. In both lipids the proportion of the minor testicular n-3 PUFA increased with a concomitant reduction of the n-6 PUFA (changes in n-6/n-3 ratio: 5.8 ± 0.5 to 3.1 ± 0.1 for PC and 3.0 ± 0.2 to 1.7 ± 0.1 for PE, from controls to FO-fed mice, respectively). These changes were consistent with those seen previously in plasma and liver (18). However, in the testicular PL (Table 2), not all the n-6 PUFA were affected to the same extent. Within the n-6 PUFA group, the reduced FA were 18:2, 18:3, 20:2, 20:3, and 20:4n-6, whereas 24:4n-6,

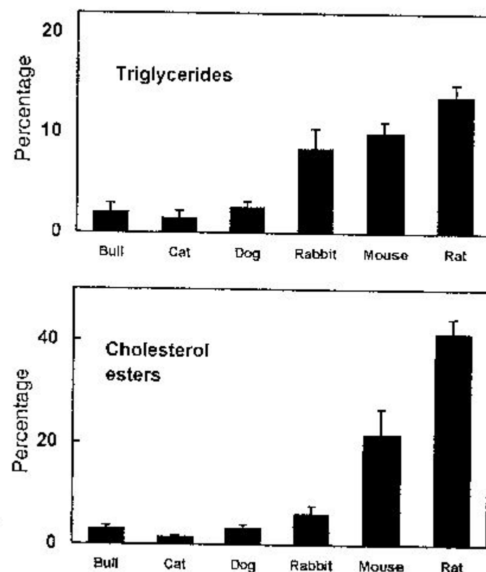

FIG. 2. Percentage of PUFA with very long chains in triglycerides and cholesterol esters in the testis from various mammals. The bars represent the sum of all FA with 24 to 32 carbon atoms. At least three animals per group were analyzed.

TABLE 2
FA of Testicular Glycerophospholipids and TG in Controls (C) and Fish Oil-Fed Mice[a] (FO)

	Choline glycerophospholipids		Ethanolamine glycerophospholipids		TAG		1-Alkyl-2,3-DAG	
	C	FO	C	FO	C	FO	C	FO
14:0	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.04	0.1 ± 0.03	0.8 ± 0.2	1.3 ± 0.6	0.8 ± 0.1	0.4 ± 0.2
15:0	0.1 ± 0.01	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.02	0.2 ± 0.04	0.2 ± 0.1	0.7 ± 0.1	0.1 ± 0.1
16:0	29.7 ± 1.0	30.6 ± 0.8	21.5 ± 3.2	20.4 ± 1.8	17.3 ± 1.5	20.0 ± 1.7	7.2 ± 2.2	7.2 ± 0.5
17:0	0.2 ± 0.03	0.3 ± 0.04	0.3 ± 0.02	0.3 ± 0.03	0.4 ± 0.1	0.4 ± 0.1	0.1 ± 0.1	0.1 ± 0.04
18:0	9.3 ± 1.2	8.4 ± 0.2	8.3 ± 0.4	8.0 ± 0.5	1.9 ± 0.4	3.2 ± 0.6	1.6 ± 0.4	2.2 ± 0.2
14:1	0.1 ± 0.01	0.03 ± 0.02	—	—	0.2 ± 0.03	0.03 ± 0.03	0.5 ± 0.02	0.03 ± 0.02
15:1	0.1 ± 0.1	0.1 ± 0.02	0.1 ± 0.1	0.1	0.1 ± 0.04	0.1 ± 0.02	0.2	0.02 ± 0.02
16:1	0.8 ± 0.1	1.2 ± 0.1	0.6 ± 0.04	0.6 ± 0.1	7.8 ± 0.6	5.2 ± 0.4	1.2 ± 0.3	0.7 ± 0.1
17:1	0.1 ± 0.02	0.1 ± 0.01	0.2 ± 0.1	0.1 ± 0.03	0.6 ± 0.03	0.3 ± 0.3	0.02 ± 0.1	0.02 ± 0.02
18:1	14.2 ± 0.3	17.5 ± 0.5	9.8 ± 0.5	10.9 ± 0.7	32.6 ± 1.7	25.6 ± 3.5	4.5 ± 1.4	5.2 ± 0.5
20:1	—	—	—	—	0.1 ± 0.01	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.2
18:2n-6	3.0 ± 0.2	1.7 ± 0.1*	1.9 ± 0.5	1.0 ± 0.1*	27.6 ± 1.3	19.5 ± 2.9*	0.9 ± 1.2	0.7 ± 0.2
18:3n-6	0.2 ± 0.01	0.1 ± 0.01	0.3 ± 0.1	0.1 ± 0.02	1.3 ± 0.5	1.1 ± 0.3	0.6 ± 0.1	0.6 ± 0.1
18:3n-3	0.3 ± 0.02	0.4 ± 0.02	0.3 ± 0.3	0.3 ± 0.03	0.9 ± 0.3	1.3 ± 0.2	1.3 ± 0.1	1.5 ± 0.2
20:2n-6	0.5 ± 0.02	0.4 ± 0.01	0.2 ± 0.1	0.2 ± 0.02	0.3 ± 0.1	0.2 ± 0.02	0.5 ± 0.1	0.4 ± 0.2
20:3n-9	0.2 ± 0.1	0.5 ± 0.01	0.3 ± 0.2	0.4 ± 0.01	0.1 ± 0.01	0.2 ± 0.05	0.2 ± 0.03	0.1 ± 0.03
20:3n-6	2.3 ± 0.1	1.4 ± 0.1*	0.9 ± 0.2	0.7 ± 0.03	0.3 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	0.4 ± 0.1
20:4n-6	15.0 ± 0.7	10.3 ± 0.4*	19.0 ± 1.0	16.2 ± 0.5	0.7 ± 0.2	1.3 ± 0.3	2.0 ± 0.1	0.9 ± 0.2
20:4n-3	—	—	—	—	0.2 ± 0.03	0.5 ± 0.04	0.4 ± 0.1	0.5 ± 0.2
20:5n-3, 21:4n-6	0.4 ± 0.05	1.2 ± 0.04*	0.4 ± 0.1	1.7 ± 0.1*	0.3 ± 0.1	0.2 ± 0.1	2.5 ± 0.3	2.5 ± 0.1
22:4n-9	0.1 ± 0.02	0.1 ± 0.01	0.3 ± 0.2	0.3 ± 0.01	0.1 ± 0.02	0.1 ± 0.02	0.5 ± 0.2	0.5 ± 0.05
22:4n-6	0.9 ± 0.04	0.6 ± 0.02*	2.4 ± 0.1	1.8 ± 0.1	0.6 ± 0.3	1.1 ± 0.2	8.1 ± 1.0	5.9 ± 0.3
22:5n-6	13.8 ± 1.1	13.9 ± 0.7	14.5 ± 1.6	13.6 ± 0.7	3.3 ± 0.5	8.1 ± 1.3*	14.5 ± 0.3	12.6 ± 0.8
22:5n-3, 23:4n-6	0.4 ± 0.05	0.6 ± 0.03	0.6 ± 0.1	1.0 ± 0.05	0.2 ± 0.2	1.3 ± 0.3	0.3 ± 0.03	0.7 ± 0.04
22:6n-3	5.4 ± 0.3	7.6 ± 0.4*	12.9 ± 0.3	18.2 ± 1.6*	1.3 ± 0.3	4.5 ± 0.6*	5.0 ± 0.7	5.9 ± 1.0
24:4n-6	0.8 ± 0.1	0.7 ± 0.02	1.1 ± 0.1	0.9 ± 0.1	0.2 ± 0.2	0.5 ± 0.1	7.9 ± 0.2	7.0 ± 0.5
24:5n-6	1.4 ± 0.1	1.2 ± 0.1	2.2 ± 0.3	1.8 ± 0.2	0.7 ± 0.3	1.7 ± 0.4*	25.0 ± 0.5	25.2 ± 0.4
25:4n-6	—	—	—	—	0.1 ± 0.02	0.1 ± 0.04	—	—
24:6n-3, 25:5n-6	—	—	—	—	0.2 ± 0.1	0.6 ± 0.2*	1.9 ± 0.51	3.6 ± 0.3*
26:4n-6	—	—	—	—	0.1 ± 0.04	0.1 ± 0.04	0.5 ± 0.03	0.7 ± 0.1
26:5n-6	—	—	—	—	0.3 ± 0.2	0.3 ± 0.1	5.6 ± 0.2	5.8 ± 0.4*
27:4n-6	—	—	—	—	0.01 ± 0.01	0.02 ± 0.02	0.1 ± 0.03	0.1 ± 0.02
27:5n-6, 26:6n-3	—	—	—	—	0.1 ± 0.1	0.2 ± 0.1	1.1 ± 0.3	2.2 ± 0.05*
28:4n-6	—	—	—	—	0.01 ± 0.01	0.01 ± 0.01	0.1 ± 0.03	0.2 ± 0.1
28:5n-6	—	—	—	—	0.2 ± 0.1	0.20 ± 0.05	3.5 ± 0.5	3.9 ± 0.6
29:4n-6	—	—	—	—	0.01 ± 0.01	0.01 ± 0.01	0.1 ± 0.01	0.05 ± 0.02
29:5n-6, 28:6n-3	—	—	—	—	0.03 ± 0.02	0.1 ± 0.1	0.5 ± 0.1	0.7 ± 0.2
30:4n-6	—	—	—	—	—	—	0.1 ± 0.02	0.03 ± 0.01
30:5n-6	—	—	—	—	0.2 ± 0.03	0.1 ± 0.01	0.4 ± 0.1	0.4 ± 0.02
31:4n-6	—	—	—	—	—	—	0.04 ± 0.05	0.03 ± 0.01
31:5n-6	—	—	—	—	—	0.03 ± 0.02	0.1 ± 0.04	0.04 ± 0.01
LC-PUFA	42.3 ± 0.6	38.9 ± 1.0	53.9 ± 1.0	55.6 ± 2.9	37.2 ± 0.6	39.7 ± 0.3*	37.2 ± 2.5	33.3 ± 1.4
VLCPUFA	2.2 ± 0.1	1.8 ± 0.1	3.3 ± 0.4	2.7 ± 0.2	2.1 ± 0.9	3.9 ± 1.1	46.9 ± 1.4	50.0 ± 1.5*

[a]Results are presented as in Table 1.

24:5n-6, and the major 22:5n-6 were not significantly altered by the dietary modification.

The FA profiles of testicular TG and CE (Tables 1 and 2), in general, were affected by 2 wk of FO intake in the same way as the glycerophospholipids. In the presence of unchanged proportions of saturates and monoenes, except for a small decrease of 18:1 in TG, the n-6/n-3 ratio decreased in both lipids.

In CE, among the FA that were increased by the diet were 20:5n-3, 22:5n-3, 22:6n-3, and some n-3 VLCPUFA; and among those reduced were 18:2n-6, 20:3n-6, 20:4n-6, and 22:4n-6 (Table 1). Interestingly, in CE, as in the other lipids, the proportion of the major PUFA, 22:5n-6, was the less affected. The sum of VLCPUFA remained high and quantitatively unaffected by the FO diet, the total VLCPUFA accounting for about 20% of the CE FA in both dietary conditions (Fig. 3).

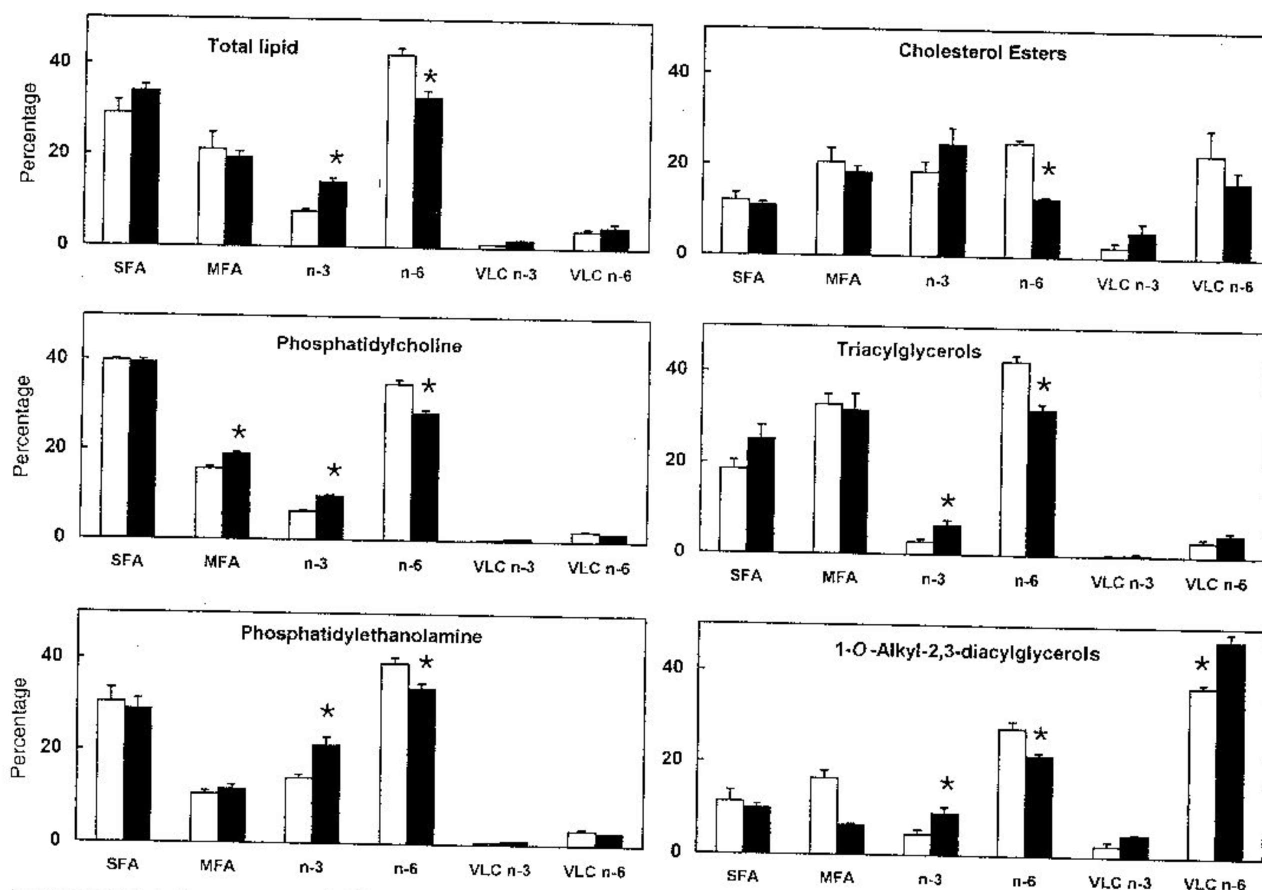

FIG. 3. Changes in the percentages of different groups of FA in mouse testicular lipids after 2 wk of consumption of an n-3 FA-rich diet. Open bars: controls, i.e., animals eating lab chow; solid bars: animals consuming 9% fish oil. The asterisks denote significant differences between both groups. The bars represent sums of FA as follows: SFA, saturated FA; MFA, monounsaturated FA; n-3 and n-6, PUFA with 18 to 22 carbon atoms; VLC n-3 and VLC n-6: PUFA with 24 to 32 carbon atoms. The sums of n-3 or n-6 PUFA and VLCPUFA were estimated after separating the FA by argentation TLC.

In focusing on the n-6 PUFA of TG, and especially considering that the amounts of testicular TAG were depleted after 2 wk on the FO diet, it was apparent that the 18:2n-6 available in this lipid was exhausted to a much larger extent than the longer and more highly unsaturated constituents (22:5n-6 and longer n-6 PUFA), whose proportions remained unchanged or even tended to increase in the abundance of n-3 dietary PUFA (Table 2). The depletion of 18:2 in TG was thus mostly responsible for the significant reduction in total n-6 PUFA seen in this lipid (Fig. 3). The 1-*O*-alkyl-2,3-DAG were the lipids whose FA composition changed less with the introduction of n-3 PUFA in the diet (Table 2). Instead of increasing the n-3 VLCPUFA, the diet resulted in a significant increase of total n-6 VLCPUFA in this lipid (Fig. 3).

DISCUSSION

The active and continuous formation and remodeling of cells that go along with spermatogenesis within the seminiferous tubule requires PL and free cholesterol for their membranes. The TG could play a role as intermediaries in which to temporarily store the polyenoic FA necessary as precursors (e.g., 18:2n-6 to be elongated and desaturated, 24:5n-6 to be chain-shortened) to give rise to PUFA with the "correct" length and unsaturation destined to be membrane PL (like 22:5n-6). The CE, after the action of CE hydrolases, could provide, besides PUFA, the necessary free cholesterol. This function, however, could be fulfilled by CE molecular species with much simpler acyl chains, not answering the question of why CE have PUFA with both high unsaturation and exceedingly long chains, such as 28:5n-6.

There is metabolic support to the interpretation that one possible role of the TG molecular species with PUFA and VLCPUFA could be to store preformed acyl chains, potential precursors of the major C_{22} PUFA that eventually constitute the membrane PL of tissues like testis. Thus, rat seminiferous tubules concentrate the highest levels of [^{14}C]-labeled PUFA into TG, whether exogenously provided (e.g., [^{14}C]24:4n-6) or endogenously produced from its desaturation ([^{14}C]24:5n-6) and elongation ([^{14}C]26:5n-6), suggesting that, of all seminiferous tubule lipid classes, it is TG that have a special role to play in the traffic and metabolism of testicular PUFA (6).

Something similar occurs in cell preparations from young rats testis, where most of the n-6 [^{14}C]22:4, 24:4, 24:5, and 22:5 formed after 24 h incubation with [^{14}C]20:4n-6 is concentrated in TG (24). Earlier work by Beckman and Coniglio (22) showed that when labeled 18:2n-6 and 20:4n-6 are injected *in vivo* in testis, they are taken up and metabolized primarily by Sertoli cells, PUFA like 22:5n-6 being synthesized and actively incorporated in TG, suggesting to these authors that TG may serve as a vehicle for transport of PUFA from Sertoli cells to germ cells, where they are incorporated into membrane PL.

The effects of the n-3-rich diet on testicular lipid FA support the interpretation that the acyl chains of the testicular neutral lipids may be used to sustain the PL with a minimum of change in their FA. Looking just as the components within the n-6 PUFA fraction of testicular TG as a class, it was evident that, whereas 18:2n-6 was considerably depleted, n-6 FA components with increasingly longer chains were much less affected by the diet. These TG may have acted not only as acceptors of PUFA (exogenously added, as in this case 22:6n-3, and endogenously synthesized, like 22:5n-6 from 18:2n-6) but also as donors of such acyl groups to the cellular PL, as suggested by the decreased TG concentrations. Moreover, the n-6 PUFA of TG not only decreased but also changed their quality, the synthesis of the longer n-6 PUFA of TG apparently being maintained at the expense of the shorter ones. Concurrently, the major testicular PL conserved the typical predominance of 22:5n-6, despite the drastic reduction in the availability of n-6 PUFA (18) caused by the dietary modification.

Although the structure, metabolism, and regulation of ether-linked neutral glycerolipids have been known for years (25), their cellular functions are still poorly understood. In rats and mice, 1-*O*-alkyl-2,3-DAG have been characterized as components of the lipid secreted by the Harderian gland of the eye (26) and of the lipid of skin (27). Alkyldiacylglycerols produced by acyl-CoA:alkylglycerol acyltransferase are characteristic markers in most tumors from animals and humans (25). They have also been described in the plasma membrane of goat epididymal sperm (28), but we have been unable to find previous references to the occurrence of these neutral lipids and their FA in testis. The present results show that testicular TAG differ from 1-alkyl-2,3-DAG not only in FA composition but also in the way they respond to a relative deficiency of n-6 FA in the diet. Whereas TAG were depleted of their PUFA moieties, 1-alkyl-2,3-diacyl-*sn*-glycerols retained their 22:5n-6 and longer n-6 PUFA, even increasing the latter. The ether-linked group may protect the acyl moieties at the 2- and 3-positions of the glycerol backbone from being hydrolyzed by the lipases that attack the TAG. The 1-*O*-alkyl, 2,3-DAG could function as a repository, temporarily storing the VLCPUFA that are potential precursors of 22:5n-6 (e.g., 24:5n-6).

Concerning CE, as is the case with the ether-linked neutral lipids, much more is known about the enzymes involved in their metabolism than about the CE themselves and their functions in most tissues, especially in relation to their FA. In rat testis, CE synthesis and hydrolysis are decreased as a result of hypophysectomy, the hydrolysis being reduced to a greater extent than the synthesis (29). There are two forms of testicular cytosolic CE hydrolase: The activity of one is inhibited with the elevation of testicular temperature above 37°C, and that of the other shows no temperature sensitivity (30,31). The temperature-stable form is present in Sertoli and Leydig cells and is induced by luteinizing hormone (LH), whereas the temperature-labile form is exclusive to Sertoli cells and is induced by follicle-stimulating hormone but not by LH. These enzymes have different M.W. and substrate specificities, with the temperature-stable form being more active toward CE with 18:1 and 20:4n-6, and the temperature-labile Sertoli cell form being more active toward CE with 18–24 carbons, especially with 24:1. The latter is not a component of testicular CE, but some of its properties probably resemble those of one the VLCPUFA described here (e.g., 28:5n-6). The Sertoli cell-specific, temperature-labile form has been purified and has been shown to be induced concomitantly with testicular maturation, in marked coincidence with the onset of testosterone synthesis and the beginning of spermatogenesis (32,33). A possible correlation between this enzyme and the CE with VLCPUFA described here is worth investigating, since these unusual lipids occur in cells located within the seminiferous tubules of fertile adult animals.

The lack of aging-related changes in the FA composition of testicular lipids in rats under a constant diet and the conservative changes shown by mouse testicular PL FA after consumption of fish oil suggest that one of the possible roles of the neutral lipids with VLCPUFA could be to protect testicular cells from eventual deficiencies, excesses, or inadequacies in the specific type of FA that is required for function, thus contributing to support spermatogenesis and to defend the genetic material.

ACKNOWLEDGMENTS

This study was supported by CONICET, Secretaría General de Ciencia y Tecnología, UNS, and Agencia Nacional de Promoción Científica y Tecnológica, Argentina. N.E.F. is a research fellow from the CONICET, Argentina. E.N.M. is a research fellow from the Colegio de Veterinarios, Provincia de Buenos Aires, Argentina.

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[Received April 18, 2002, and in revised form November 25, 2002; revision accepted December 30, 2002]