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Long-term biopermanence of ceramides, cholesteryl esters, and ether-linked triglycerides with very-long-chain PUFA in the cadmium-damaged testis

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ABSTRACT

Cadmium is known to harm rat testis by causing the dose-dependent apoptotic or necrotic death of seminiferous epithelium cells. Here we investigated how this affects the lipids with long-chain (C_{18}–C_{22}) and very-long-chain (C_{24}–C_{32}) polyunsaturated fatty acids (VLCPUFA) typical of spermatogenic and Sertoli cells. A severe acute inflammatory reaction resulted from the massive necrotic death of these cells two days after a single high (4 mg/kg) dose of $CdCl_2$. This led to the conversion of most testicular glycerophospholipids to diradylglycerols (DRG) and free fatty acids (FFA) and of most sphingomyelins to ceramides (Cer). By day 30 the testis weight had decreased three-fold. The DRG and FFA had been metabolized but, unexpectedly, ceramides persisted. Also slow to disappear were VLCPUFA-containing triacylglycerols from former germ cells and ether-linked triglycerides and cholesteryl esters (CE) from former Sertoli cells. Similar results were observed 30 and 45 days after administering repeated small non pro-inflammatory $CdCl_2$ doses (1 mg/kg). At day 30 after both treatments, an amorphous material replaced the original seminiferous tubules and the interstitium was populated by macrophages. Species of CE and ether-linked triglycerides containing fatty acids other than VLCPUFA steadily accumulated in the irreversibly damaged testis, a manifestation of the activity of these cells. The long-term permanence of original VLCPUFA-containing neutral lipids, especially ceramides, indicates that these phagocytes were slow to clear out the acellular material contained in seminiferous tubules, pointing to a form of silent chronic inflammation as an additional outcome of the multifactorial commotion caused in the testis by experimentally administered cadmium.

1. Introduction

Cadmium is a common environmental and occupational hazard whose adverse effects on male reproductive health are widely recognized. Cd-induced testicular injury results from a complex network of causes: it disrupts the testicular microvasculature, damages the integrity of the seminiferous epithelium by targeting specialized Sertoli-germ cell and inter-Sertoli protein junctions, is an endocrine disruptor, and is an inductor of significant oxidative stress [1]. During cadmium-induced oxidative stress highly reactive and short-lived oxygen and nitrogen species are produced that damage proteins, nucleic acids, and polyunsaturated fatty acids (PUFA) of cell lipids. Malondialdehyde and other thiobarituric acid-reactive substances (TBARS) are typical by-products of PUFA oxidation by these reactive species that have been extensively used in toxicological and pharmacological studies as tools to evaluate the extent of testicular oxidative stress in vivo and to assess the potential efficacy of a variety of substances in reducing and/or preventing this oxidation (see for example data in rat [2,3] and mouse [4] testis). Possible effects on the endogenous lipids of the testis have not yet been explored in this context, except for two early reports showing that cadmium administration to rats results in reduced total phospholipid content and decreased percentage of a major PUFA, docosapentaenoic acid (22:5n−6) [5,6].

The present study was designed to evaluate how cadmium exposure affects the level of rat testicular lipid classes that normally contain high levels of molecular species with long-chain (C_{18}–C_{22}) and very-long-chain (C_{24}–C_{32}) PUFA (VLCPUFA). In their non-hydroxylated (N) and 2-hydroxylated (2-OH) versions (see Scheme 1), the latter fatty acids were first described as components of sphingomyelin (SM) [7] and also of novel glycosphingolipids [8] in the rat and mouse testis, respectively. We have previously shown that the endogenous ceramides (Cer) of rat testis and spermatozoa are also exceedingly rich in N- and 2-OH VLCPUFA, mainly C_{28}–C_{32} (n−6) tetraenoic and pentaenoic fatty acids [9]. Our interest in these unique species stemmed from the fact that, just like the major 22:5n−6-rich species of glycerophospholipids (GPL) and triacylglycerols (TAG), these seminiferous epithelium

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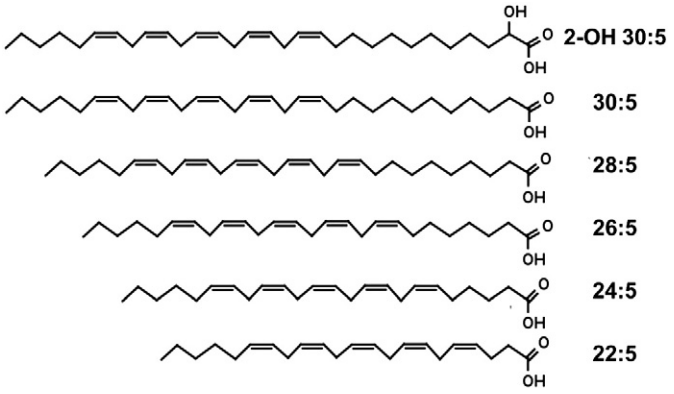

Scheme 1. Pentaenoic fatty acids that occur in different lipid classes of the rat testis.

sphingolipids are exclusive components of germ cells (spermatocytes, spermatids) [10]. The other two neutral lipid classes previously reported to collect 22:5n−6 and VLCPUFA in isolated seminiferous tubules from adult rat testis, namely cholesteryl esters (CE) and the ether-linked triglycerides 1-alkyl,2,3-diacylglycerols (ADG) [11], are mainly Sertoli cell products [10]. Knowing that cadmium damages both Sertoli and germ cells, we wondered how the testis disposes of their PUFA and VLCPUFA-rich lipids in vivo after treatments with the metal.

In this connection we anticipated that the effect of cadmium on adult rat testicular lipids would differ from that of cryptorchidism [12], doxorubicin treatment [13], X-ray irradiation [14] and moderate hyperthermia [15]. These four conditions were previously used as experimental tools to induce the death by apoptosis of vulnerable germ cell precursors, resulting in progressive depopulation of germ cells while sparing Sertoli cells in seminiferous tubules. The general outcome after a few weeks was a gradual germ cell egress, with progressive decrease in PUFA- and VLCPUFA-rich membrane lipids including ceramides, and a temporary build-up of 22:5n−6-rich CE and ADG in Sertoli cells. This build-up was consistent with the phagocytic and lipid metabolizing functions the latter cells normally display. However, these energy-requiring functions may be expected to cease in any condition that leads to the death of Sertoli cells.

Parenterally administered cadmium exerts its deleterious effects on the rat testis via necrotic and/or apoptotic mechanisms, depending on the dose. A single dose of 4 mg/kg $CdCl_2$ damages the testicular microvasculature and causes massive ischemia, leading to necrosis of all seminiferous tubule cells, followed a few hours later by an intense acute inflammatory reaction [16]. It was quantified that doses of 2 mg/kg (and larger) lead to more cell deaths by necrosis than by apoptosis in seminiferous tubules, whereas 1 mg/kg (and lower) is not lethal to all seminiferous tubule cells at once, just provoking focal areas of apoptosis a few hours later [17]. This may be related to the fact that cadmium dose-dependently targets the protein junctional complexes that maintain the connections among seminiferous epithelium cells; initially those between Sertoli cells and germ cells, then those between Sertoli cells, and lastly those between Sertoli cells and the basement membrane [18]. Thus, whereas a high Cd dose leads to massive cell detachment and death, the effects of a single 1 mg/kg dose are spermatogenic stage-specific, mostly affecting the last stages of spermiogenesis [19].

The aim of the present study was to assess the consequences on testicular lipids of massive cell death, followed by an inflammatory reaction, as it happens after necrosis induced by high Cd doses, in comparison with the effects of low, non pro-inflammatory doses. Because a single 1 mg/kg dose in our hands did not result in appreciable short- or long-term changes in testicular lipid levels, we decided to repeat this dose at intervals of 4 days in order to provoke a succession of pro-apoptotic events frequent enough to ultimately cause the death of all intra-tubular cells. Our results showed that the outcome of both treatments was similar: a permanent destruction of the cells originally populating the seminiferous tubules and an increased number of macrophages in the interstitium. While the major membrane GPL originally belonging to spermatogenic or Sertoli cells were readily hydrolyzed and eliminated from the testis, a relatively high part of the original neutral lipids of these cells – including Cer, triglycerides and CE – remained. Over this rather static picture, new species of CE and ADG were actively accumulated, pointing to a function of the new cohort of cells populating the interstitium.

2. Materials and methods

2.1. Animals and procedures

Male Wistar rats aged 4 months and housed under standard conditions with food and water ad libitum were given cadmium chloride as a single i.p. dose of 4 mg/kg, or as seven s.c. doses of 1 mg/kg each, one dose every four days, covering 28 days. At scheduled points in time, animals were sacrificed under CO_2 for testis removal. The protocols for animal experimentation were approved by an institutional Animal Care and Use Commission. Body weight gain with time (30 or 45 days after commencing the experiments) and food consumption were inferior ($p < 0.05$) in both cadmium-treated groups compared to their age-matched controls. Rats injected with saline and manipulated in a similar manner to the treated groups were used as the corresponding controls of each experimental condition. The histological and lipid results did not differ significantly from those of untreated animals. In all figures, the mean values from all of these controls are shown.

To confirm that Cd-treated rats exhibited the expected alterations in the testis, those of some animals were fixed in formaldehyde and reserved for histological examination. After embedding the fixed organs in paraffin, thin sections were prepared and stained with hematoxylin–eosin. The histochemistry for macrophage identification was performed in 5 μm-thick sections from control and treated testes, mounted on glass slides, deparafinized and rehydrated. After being blocked for 30 min with 1% BSA, all samples were incubated with appropriately diluted rabbit anti-murine F4/80 antibody, Phycoerythrin (PE) conjugate (kindly provided by Prof. Dr. Marta E. Roque, University of the South, Argentina). Slides were washed with PBS, counterstained with Hoechst for cell nuclei localization, and coverslipped. Samples incubated with PBS with no antibody were used as negative controls. Immunostaining was analyzed using a Nikon Eclipse E-600 microscope. Images were obtained with an SBIG Astronomical Instrument (Santa Barbara, CA) provided with a CCDOPS software package (version 5.02) to drive a model ST-7 digital charge-coupled device camera (765,510 pixels, 9.0-μm pixel size). Appropriate dichroic and emission filters were employed and images were analyzed using the ImageJ software.

2.2. Lipid class separation

Lipid extracts were prepared from control and treated testes by homogenization with chloroform–methanol [20], water-partitioned, taken to dryness and dissolved in chloroform–methanol. Aliquots were taken for total lipid phosphorus (P) and total lipid fatty acid analyses. The extracts were spotted (as bands) on TLC plates under N_2 for separation into lipid classes. Hexane/ethyl ether/acetone/acetic acid (30:40:20:1, by vol) up to the middle of the plates resolved the ceramides [9] into those containing nonydroxy and 2-hydroxy fatty acids. These same plates were dried and a second run with hexane/ether/acetic acid (80:20:1, by vol) up to the top separated CE from triglycerides (triradylglycerols), the latter directly resolving into TAG and ether-linked triglycerides [11] (see Supplementary Information 1, Figs. S1 and S2). The bands containing free fatty acids (FFA) and diglycerides (diradylglycerols, DRG) and that containing polar lipids – at the origin of the plates – were recovered for further separations. Major GPL classes and SM were separated essentially as previously described [13]. Care was taken to remove any potential lipid contaminant with

ester-bound fatty acids from samples of ceramides and SM by exposing them briefly to mild alkali [9], followed by a second TLC.

In some of the samples, the ether-linked triglycerides were observed to be made up by 1-O-*alkyl*, 2,3-diacyl-sn-glycerols and 1-*alk-1′-enyl*, 2,3-diacyl-sn-glycerols (the abbreviation ADG includes both). Their separation was achieved, as described earlier for plasmalogens [21], by exposing dried samples of ADG for 1 min with one volume of 0.5 N HCl in acetonitrile, followed by the addition of one volume of chloroform and one volume of aqueous sodium bicarbonate for neutralization. The chloroform phase was rapidly recovered, dried, and immediately subjected to TLC using hexane/ether mixtures. This separated the (unaffected) 1-O-alkyl, 2,3-diacyl-*sn*-glycerols from the fatty aldehydes *plus* diacylglycerols generated from 1-alk-1′-enyl, 2,3-diacyl-sn-glycerols (see Supplementary Information 1).

After separation, all lipid classes or subclasses were eluted from the silica support by thoroughly mixing it with water/methanol/chloroform (1:5:5, by vol), followed by partition of eluates with 4.5 volumes of water [20]. After adding appropriate internal standards, dried samples of the separated lipids in screw-capped tubes were dissolved in (N_2-saturated) anhydrous methanol containing 0.5 N H_2SO_4 and warmed at 45 °C overnight under N_2 to obtain the corresponding fatty acid methyl esters (FAME). After methanolysis, all FAME were subjected to TLC using pre-cleaned silica gel G plates and hexane:ether mixtures [9,13].

2.3. Fatty acid analysis

In addition to ordinary saturated, monoenoic and/or C_{18}–C_{22} PUFA, all rat testicular lipids contained different proportions of (nonhydroxy) VLCPUFA, typically tetraenes and pentaenes with 24 to 32 carbon atoms. Only SM and Cer contained nonhydroxy and 2-hydroxy VLCPUFA, mostly of 28 to 32 carbon atoms [9]. The latter were recovered as 2-OH FAME and converted into *O*-trimethylsilyl (TMS) ethers for their GC analysis [9,14]. The identity of both types of VLCPUFA was established by Poulos and colleagues more than 2 decades ago in testicular and spermatozoal SM of several mammals using a variety of lipid laboratory criteria and techniques that included mass spectrometry (MS) of the fatty acids themselves and of intact SM molecular species [7,22]. The two types of VLCPUFA were also previously characterized by our laboratory in the ceramides of rat testis using standard lipid analytical techniques including MS of intact and hydrogenated VLCPUFA derivatives [14,23]. The 2-OH VLCPUFA (e.g., 2-OH 30:5n−6) were also thoroughly identified by Sandhoff and colleagues in the mouse testis [8] as components of a novel series of glycosphingolipids.

All numerical data represent mean values from at least three different animals ± SD. The experimental samples were compared to the corresponding age-matched controls (cadmium- *vs* saline-injected animals subjected to the same manipulations). All Cd *vs* control lipid analytical procedures were performed in parallel. The significance of the differences in lipid amounts between animals at each time point was assessed using the two-tailed Student's *t* test. The figures represent the results for each lipid class as they were obtained, as amounts of fatty acids in each lipid per testis, with no further corrections.

3. Results

A similar long-term decrease in testicular weight was observed in animals treated with $CdCl_2$, whether given as a single high (4 mg/kg) i.p. dose or as smaller (1 mg/kg) but repeated and regularly spaced s.c. doses (Fig. 1).

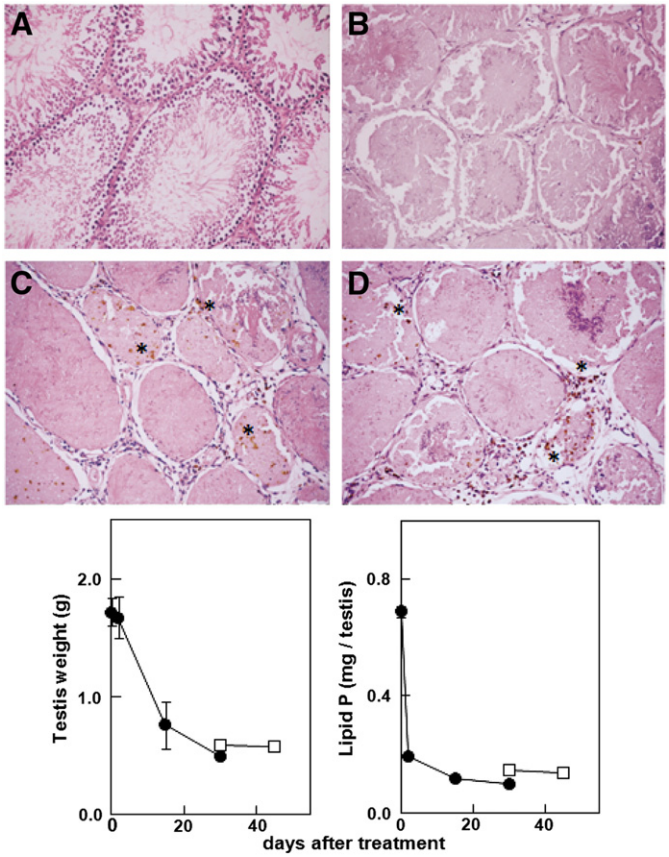

Fig. 1. Effects of cadmium administration on adult rat testicular histology, weight, and lipid phosphorus content. A representative section of control testes (A) is compared with samples obtained 2 days (B) and 30 days (C) after the animals received a single i.p. dose of 4 mg/kg $CdCl_2$, and 30 days (D) after commencing a regime of seven s.c. doses of 1 mg/kg administered at 4-day intervals. Asterisks indicate the presence of microcalcifications. The lower panels show the time-courses of the decreases in testicular weight and lipid phosphorus (P) from days 2 to 30 after the single dose (circles), and from days 30 to 45 after the repeated doses (squares) of cadmium.

Testicular size and weight as well as the diameter of seminiferous tubules decreased by as much as 70% 30 days after the commencement of each treatment. An amorphous acellular material of similar appearance filled the tubules, replacing the copious amount of cells that had originally populated them. An increased number of cells in the interstitium and an appreciable fibrotic thickening of testes were eventually observed in both cases.

The main difference between the two regimes occurred during the first few days: the 4 mg/kg dose had an acutely pro-inflammatory effect on the testis whereas the 1 mg/kg doses did not, in agreement with previous work in rats [24]. Two days after administration of the single high dose, the testes were severely congested, edematous and hemorrhagic, showing a red-purple discoloration. Compared to control testes (Fig. 1A), they showed an increased number of inflammatory cells in the interstitium (Fig. 1B). These changes were consistent with classical studies [16] showing that this dose of cadmium disrupts the inter-endothelial cell junctions in the peritubular vessel network, and causes extravasation of plasma to the interstitium and the formation of dense plugs of blood cells within testicular microvessels, this resulting in testicular ischemia. By 48 h this ischemia had caused the massive death of cells within seminiferous tubules, leading to the predictably severe acute inflammatory reaction. By day 30 no inflammation remained; the outline of tubules was still discernible and the number of cells had increased in the interstitium, where some microcalcifications appeared (Fig. 1C).

The lack of apparent testicular inflammation after each dose of 1 mg/kg was also in agreement with previous work showing that administration of just one such dose does not damage the testicular vascular endothelium [19], is not lethal to all seminiferous tubule cells but affects cells at specific stages of the spermatogenic cycle [19], and does not lead to any evident histological changes in rat seminiferous tubules or interstitium 1 week [24,25] or 56 days [25] after administration. It was repetition of this dose at intervals of 4 days, as we did here, what ultimately caused the death of all cadmium-vulnerable cells.

The dramatic decrease in tissue weight with time after both cadmium regimes (Fig. 1) was a direct macroscopic evidence of the activity of live cells, predictably those populating the interstitium, metabolizing part of the proteins, nucleic acids, and lipids from dead cells. That these cells were phagocytes was apparent in seminal work demonstrating that these cells actively incorporate carbon particles and endogenous serum albumin and globulins injected before the administration of cadmium to rats [26]. To identify them as macrophages we employed a specific antibody (Fig. 2). In increased numbers with respect to controls, these cells collected in the interstitium, where they persisted from day to 30 or 45 after commencing both treatments. They mostly surrounded, rather than pervading, former seminiferous tubules. In contrast to the myriad nuclei normally discernible within seminiferous tubules, the content of the latter structures had turned into an amorphous, acellular material.

The dystrophic microcalcifications in the interstitium observed at day 30 after both Cd treatments (Fig. 1) was consistent with a number of the interstitially located macrophages *also* having died in this period, some directly from Cd toxicity, some after phagocytosis of part of the Cd-contaminated tissue debris. However, even if they had been initially affected, these cells are potentially replaceable, either from pre-existing testicular macrophages, which are capable of undergoing mitosis [27], or from new cohorts of monocytes able to be recruited from the circulation [28].

3.1. Glycerophospholipids

Total cell membrane phospholipid content in the form of total lipid phosphorus (P) decreased concomitantly with testicular weight after the single 4 mg/kg Cd dose (Fig. 1), except at day 2, when lipid P had already fallen sharply though testicular weight was maintained, evidently at the expense of the intense inflammatory edema. This early steep drop in lipid P clearly showed that an intense hydrolysis of phospholipids was taking place. Because Sertoli and germ cells together represent more than 85% of the cells in adult rat testes, hydrolysis of their original membrane lipid constituents was a major contributor to this lipid P decrease. At day 30 the testicular weight had decreased to 29% and the lipid phosphorus content per testis to 14%, of their corresponding control values. Similar long-term results on lipid P were observed at day 30 with the regularly spaced 1 mg/kg doses (Fig. 1).

Figs. 3–6 show the comparative *amounts* of lipid classes analyzed, in terms of their fatty acids, under 5 different conditions: from left to right, the first bar represents the controls, the next two bars depict the early and late effects of the single (4 mg/kg) dose of $CdCl_2$ (days 2 and 30 post-injection, respectively), and the next two bars represent the effects of the repeated 1 mg/kg doses measured at days 30 and 45 after commencement of the administration.

After the single, high Cd dose, the acute (2 days) fall in lipid phosphorus (Fig. 1) was mostly accounted for by the massive and non-specific decrease in the amount of glycerophospholipids (GPL) per testis (Fig. 3). All

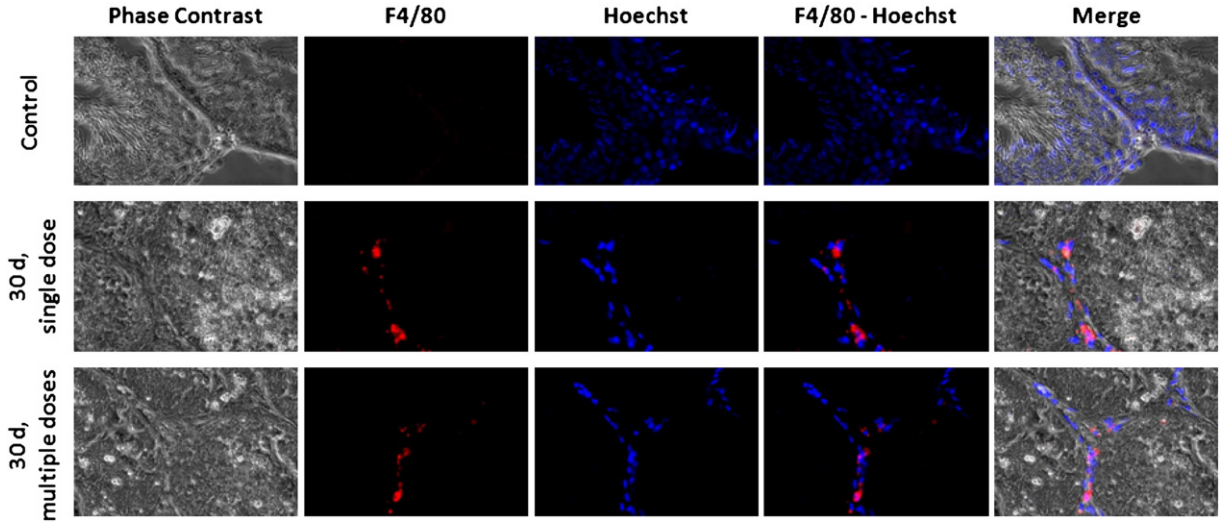

Fig. 2. Rat testis cells located within seminiferous tubules and in the interstitium before and after Cd treatments. The three conditions shown represent samples from controls, at day 30 after a single high $CdCl_2$ dose (4 mg/kg), and at day 30 after commencing the administration of multiple small but regularly repeated doses (1 mg/kg each). Thin sections from samples were incubated with an antibody conjugate able to detect macrophages (F4/80), washed with PBS, and counterstained with Hoechst for cell nuclei localization. Samples were observed with a 40× oil immersion objective and analyzed by fluorescence microscopy as described in Materials and methods. Note the absence of cells within seminiferous tubules, and the increased number of macrophages in the interstitium, 4 weeks after administration of cadmium.

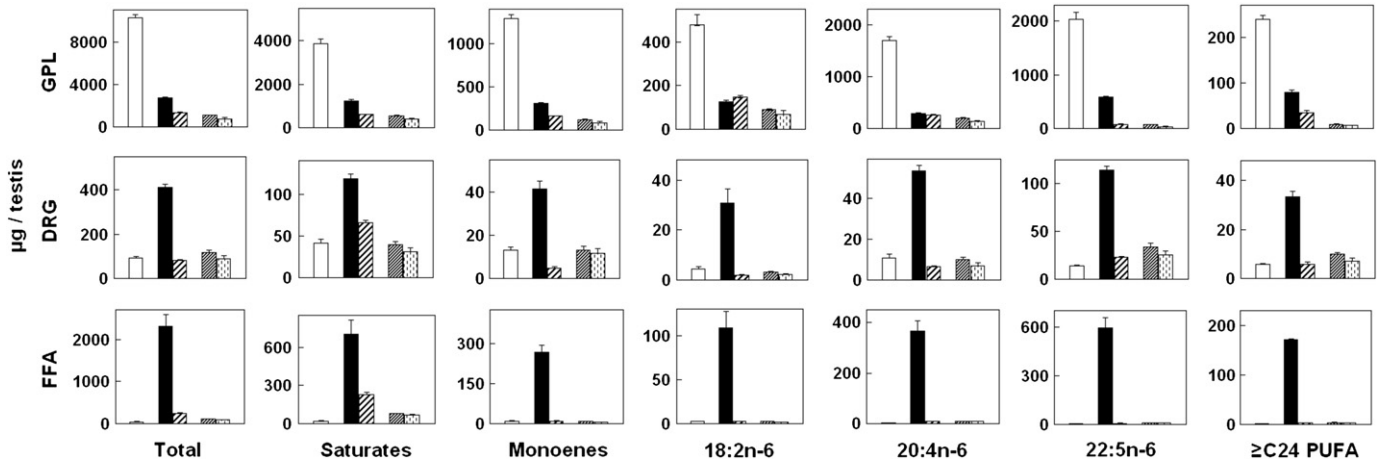

Fig. 3. Total glycerophospholipid (GPL), diradylglycerol (DRG) and free fatty acid (FFA) levels in rat testis after cadmium treatments, in terms of their fatty acids. This and the following figures show the comparative amounts of fatty acids in lipids under 5 different conditions: the first bar (white) shows the situation in control animals injected with saline; the next two bars (black and hatched) show the early (48 h) and late (30 days) effects, respectively, of a single (4 mg/kg) i.p. dose of $CdCl_2$; and the last two bars (gray and dotted) show the effects measured at days 30 and 45, respectively, after commencing the administration of seven (1 mg/kg) $CdCl_2$ doses at 4-day intervals. The word "Total" under the panels on the left represents the sum of fatty acids in each of the depicted lipids; $\geq C_{24}$ PUFA is the sum of polyenoic fatty acids having 24 to 32 carbon atoms.

of the original testicular GPL classes, rich in species with saturated fatty acids (18:0, 16:0) and long-chain PUFA (20:4n−6, 22:5n−6), including ethanolamine, serine, and inositol GPL (not shown), underwent a substantial decrease, as illustrated in Fig. 4 for the major choline glycerophospholipids (CGP).

Two days after the 4 mg/g $CdCl_2$ dose, an important part of the decrease in GPL was mirrored by the generation of large amounts of diglycerides (diradylglycerols, DRG) and free fatty acids (FFA) (Fig. 3). The increased DRG closely resembled in composition the original total GPL, both having saturates and 22:5n−6, followed by 20:4n−6, as their major polyenes. The FFAs that accumulated at day 2 were also saturates and 20:4n−6 followed by 22:5n−6 (Fig. 3). Consistently, this was accompanied by increased levels of lyso-GPL at day 2 (particularly lyso CGP) (Fig. S1). A form of phospholipase C to produce DRG and a form of phospholipase A to produce FFA and lysophospholipids from GPL were evidently responsible for these changes.

Although at a much slower rate compared to the first 2 days, the amount of GPL continued to decrease from days 2 to 30 after the high Cd dose (Fig. 3). The DRG and FFA accumulated at day 2 had decreased to control levels by day 30, suggesting further metabolism that may have included fatty acid oxidation. Thirty days after the successive 1 mg/kg $CdCl_2$ doses, the amounts per testis and fatty acid composition of GPL, DRG, and FFA were comparable to those present 30 days after the single 4 mg/kg dose. The level of these lipids was maintained low from days 30 to 45 (Fig. 3).

3.2. Triacylglycerols

The amount of fatty acids from total testicular lipids (Fig. 4) represented a good biological average between major polar lipids that decreased and minor neutral lipids that increased under the conditions of the present study. Triacylglycerols (TAG) levels increased more than two-fold 48 h after the 4 mg/g $CdCl_2$ dose.

With the exception of TAG species containing VLCPUFA, endogenous to seminiferous tubules, most of the TAG fatty acids, especially saturates, monoenes and 18:2n−6, increased significantly the second day after this dose (Fig. 4). Because these fatty acids abound in the TAG of rat plasma lipoproteins [29], which are poor in TAG with 20:4n−6 and 22:5n−6 (5% and less than 1% of TAG fatty acids, respectively), part of the increased TAG at day 2 can be attributed to lipids carried to the site by the inflammatory exudate (serum and cells) that infiltrated the testicular interstitium.

Another part of the increased TAG could have been biosynthesized in situ. The accumulation of saturates together with 20:4n−6 and 22:5n−6 in TAG (Fig. 4) suggests a relationship between the formation of these TAG and the increased DAG and FFA (Fig. 3). Since at late time points

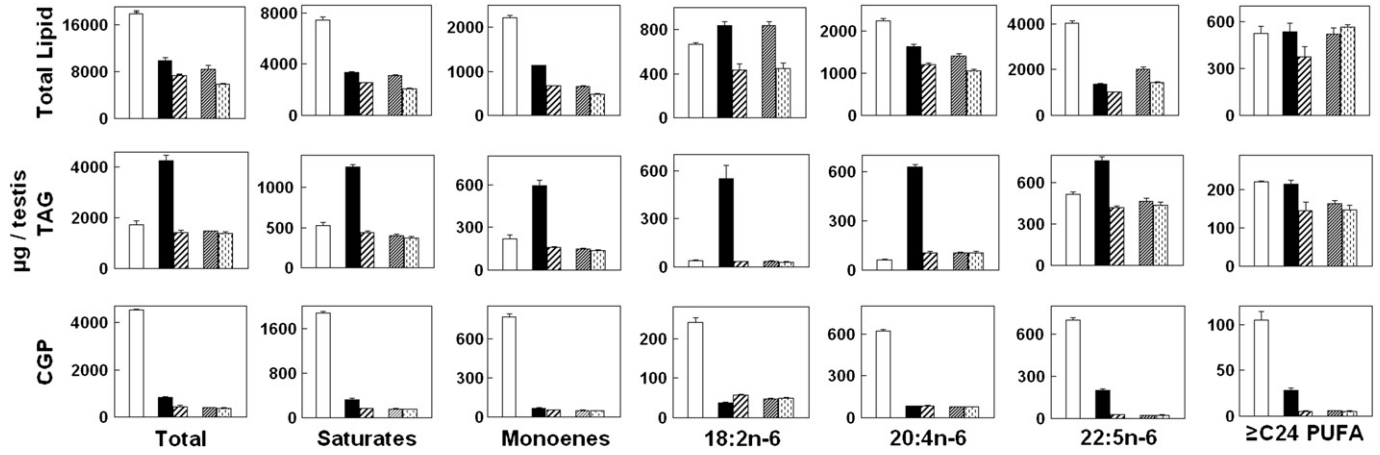

Fig. 4. Total lipid, triacylglycerols (TAG) and choline glycerophospholipids (CGP) of rat testis after cadmium treatments. The data are presented as described in the legend to Fig. 3. White bars: control animals; black and hatched bars: 2 and 30 days, respectively, after a single i.p. 4 mg/kg $CdCl_2$ dose; gray and dotted bars: 30 and 45 days, respectively, after commencement of a regime of 1 mg/kg s.c. doses of $CdCl_2$, each given every 4 days.

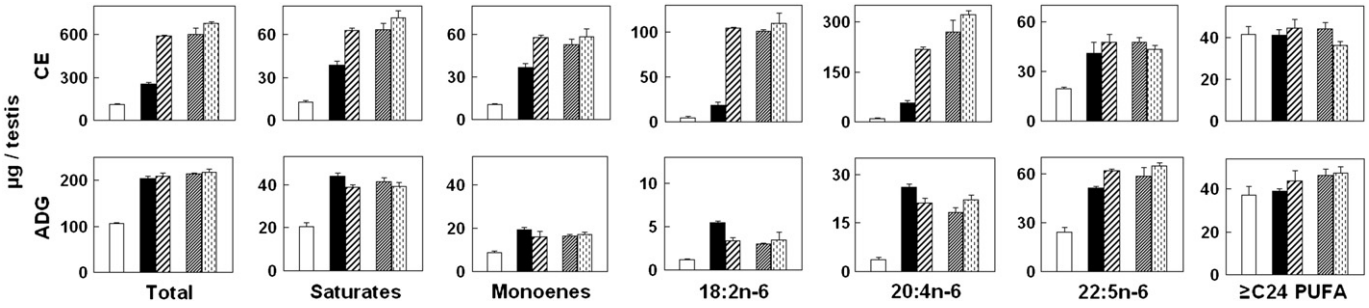

Fig. 5. Cholesteryl esters (CE) and alkyl plus alk-1′enyl diacylglycerols (ADG) of rat testis after cadmium treatments. The data are presented as described in Figs. 3 and 4. White bars: control animals; black and hatched bars: 2 and 30 days, respectively, after a single 4 mg/kg i.p. $CdCl_2$ dose; gray and dotted bars: 30 and 45 days, respectively, after commencement of a regime of 1 mg/kg s.c. doses of $CdCl_2$ every 4 days. Note the apparent lack of any significant change in the amount of total VLCPUFA in the two lipids.

there were no live cells remaining within seminiferous tubules (Figs. 1, 2), such biosynthesis must correspond to an activity of the cells populating the interstitium.

The build-up of TAG observed at day 2 after 4 mg/kg $CdCl_2$ was temporary, as TAG levels were similar to those of controls by day 30 after injection (Fig. 4). The TAG levels observed at day 30 after the multiple 1 mg/kg injections were similarly low (Fig. 4). From days 30 to 45 after this last treatment the amount per testis of TAG species rich in 22:5n−6 and 24–32 carbon VLCPUFA represented 80% and 70% of the initial values, respectively (Fig. 4), suggesting a slow but unremitting degree of metabolism of part of the TAG pre-existing in seminiferous tubules.

3.3. Cholesteryl esters

The total amount of CE per testis increased significantly with respect to controls 2 days after the 4 mg/kg cadmium injection (Fig. 4) but, in contrast to TAG, CE continued to increase at day 30, more than two-fold. At day 30 after commencing the 1 mg/kg Cd injections, the amount of CE per testis was as high as this one, and continued to increase between days 30 and 45 post-treatment.

As in the case of TAG, the CE species that increased most after both Cd treatments with respect to controls were saturates, monoenes, 18:2n−6 and 20:4n−6 (Fig. 4). All of these fatty acids, quantitatively minor in the CE of rat testis, continued to increase with time after both treatments, indicating that the new CE were synthesized in live cells of the testis, most likely the interstitial macrophages.

The amount of CE species containing 22:5n−6 increased approximately two-fold at day 2 but did not change much thereafter (Fig. 4). As a group, the initially abundant CE species containing VLCPUFA were the only CE species in the testis not to increase after the two treatments (Fig. 4).

3.4. Ether-linked triglycerides

The subclass of testicular ether-linked triglycerides (ADG, Fig. 5) markedly increased with respect to control values at day 2 after the single 4 mg/kg Cd dose, remaining equally high at day 30. The ADG level was also higher than that in controls at days 30 and 45 after the repeated 1 mg/kg Cd doses (Fig. 5).

The ADG subclass was found to be made up of the previously reported [11] 1-O-alkyl, 2,3-diacyl-sn-glycerols (alkyl-DAG), plus a relatively smaller but non-negligible proportion of 1-alk-1′-enyl, 2,3-diacyl-sn-glycerols (alkenyl-DAG) (mole ratio of about 1: 0.25 in control testes) (Supplementary Information 1, Fig. S3). Thus, as it happens with GPL, total triglycerides of rat testis are made up of three subclasses: TAG with fatty acids ester-bound to the 3 positions of the glycerol backbone and ADG with either a fatty alcohol or a fatty aldehyde at *sn-1* and fatty acids esterified at the other 2 positions of the glycerol backbone. The data for ADG in Figs. 5 and 6 represent the sum of these two subclasses.

Their separation allowed the observation that, in control testes, the two ADG subclasses had both negligible 18:2 and low 20:4n−6, and that the smaller 1-alkenyl-DAG had a relatively higher percentage of 22:5n−6 (and lower amount of 24–32 carbon VLCPUFA) than their relatively larger 1-alkyl-DAG counterparts.

After cadmium treatments, the amount of original ADG species that contained VLCPUFA changed little from days 2 to 30 or 45, in contrast to ADG species containing other fatty acids, which increased significantly

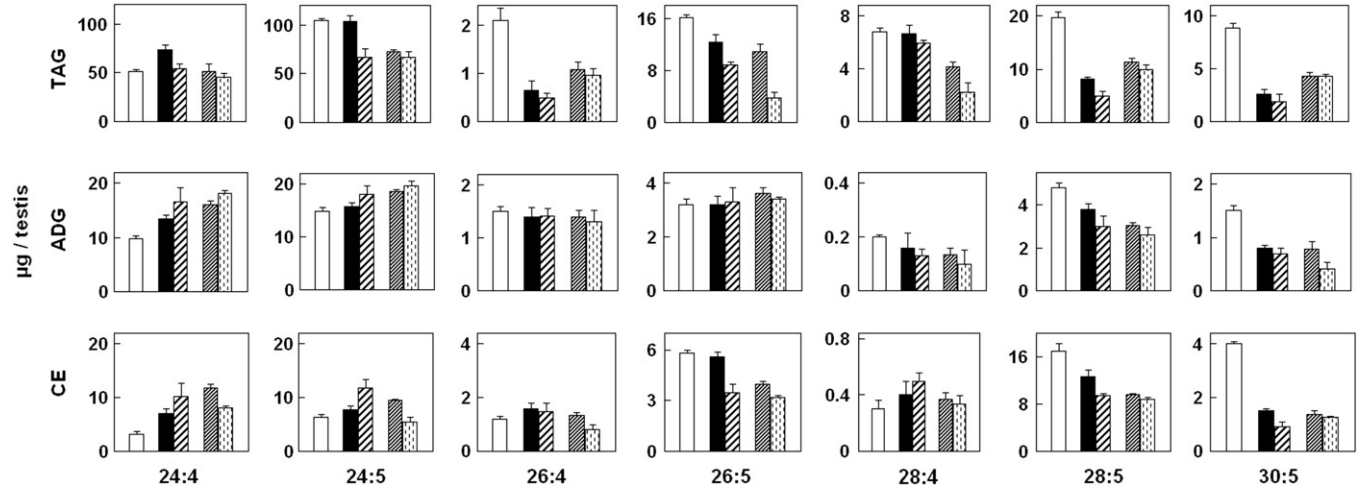

Fig. 6. Amount of representative VLCPUFA in rat testicular CE and ADG compared with major TAG. The data are presented as described in Figs. 3 to 5. White bars: control animals; black and hatched bars: 2 and 30 days, respectively, after a single 4 mg/kg i.p. $CdCl_2$ dose; gray and dotted bars: 30 and 45 days, respectively, after commencement of a regime of 1 mg/kg s.c. doses of $CdCl_2$ every 4 days. The results show that species with 24-carbon PUFA tended to increase while those with longer chain PUFA significantly decreased ($p < 0.05$) in ADG and CE.

in the same period. The "new" species of ADG that increased the most after both Cd treatments contained mainly saturated fatty acids, 20:4n−6, and 22:5n−6. Interestingly, the originally smaller total 1-alkenyl-DAG subclass increased relatively more (263%) than the original 1-alkyl-DAG subclass (45%) 30 days after Cd treatments (Supplementary information 1, Fig. S4).

3.5. VLCPUFA-containing species of TAG, ADG, and CE

A closer look at the amounts of the fatty acids making up the VLCPUFA group (labeled ≥ C_{24} PUFA in Figs. 3–5), revealed that in TAG, ADG and CE, the longest components of this group in fact decreased with time (weeks) after both Cd treatments while the "shorter" ones increased (Fig. 6). Thus, the decreases of VLCPUFA were compensated by increases of the comparatively shorter 24 carbon polyenes. Because the longest PUFA are minor but specific endogenous components of the depicted neutral lipids, they served to reveal that, although slowly, these lipids had started to be slowly metabolized with time.

3.6. Sphingomyelins and ceramides

Just as copious DRG were produced from GPL (Fig. 4), a significant hydrolysis of SM into Cer took place 2 days after the 4 mg/kg $CdCl_2$ dose (Fig. 7). Unexpectedly, whereas the amount of DRG had dropped again by day 30, that of the produced ceramides remained nearly as high at day 30 as at day 2. The amounts of all species of Cer were also higher than in controls at day 30 after the multiple 1 mg/kg doses, with no significant modifications between days 30 and 45. Taking into account that the amount of GPL and their hydrolysis products DRG and FFA had declined, these ceramides became a quantitatively significant component of the total residual lipid remaining in the testis for long periods after both Cd treatments.

The results shown in Fig. 7 suggest that an active form of sphingomyelinase (SM phosphodiesterase, SMPD) hydrolyzed into Cer most of the SM originally associated with seminiferous tubule cells. The fact that the level of such Cer was slow to decrease over the weeks indicated that it was not subsequently acted upon by a similarly active ceramidase.

Nonhydroxy and 2-hydroxy VLCPUFA-containing species of Cer evidently derived from the SM and Cer of spermatogenic cells, while a part of the 16:0- and 18:0-rich species were contributed by Sertoli cells [10], all of which died in situ because of cadmium. At day 2 after the pro-inflammatory 4 mg/kg $CdCl_2$ dose, most of the SM (and derived Cer) species that increased were of extra-tubular or extra-testicular origin, as indicated by the fact that they contained monoenoic and dienoic fatty acids that are minority components in endogenous SM and Cer from seminiferous tubules [10]. Part of these lipids may have derived from the SM carried to the site i) as components of the plasma lipoproteins of the inflammatory exudate; ii) as components of the blood cells trapped in the congested vessels (the SM of rat erythrocytes is rich in 24:0 and 24:1); and iii) as components of the extravasated inflammatory cells that infiltrated the testis. At days 2 and 30 after this condition the sum of SM + Cer fatty acids for saturated, monoenoic and dienoic fatty acid groups exceeded the respective sum of SM + Cer in the original controls. This was not the case at days 30 and 45 after the regularly provided 1 mg/kg doses, in agreement with the interpretation that there was no acute inflammation involved in this case.

The species of SM with nonhydroxy VLCPUFA were more readily hydrolyzed into Cer than those with 2-OH VLCPUFA (Fig. 7). Furthermore, the Cer with nonhydroxy VLCPUFA slowly decreased as the weeks post-treatments elapsed, whereas those with 2-OH VLCPUFA did not. On the contrary, they tended to increase beyond the level one would expect if they had only derived from hydrolysis of the corresponding SMs. This difference can be explained by the 2-OH VLCPUFA-containing glycosphingolipids described by Sandhoff et al. [8] being also partly hydrolyzed into Cer.

4. Discussion

The presented results add the complexity of lipid changes to the intricate network [1] of biochemical events that arise from experimental exposure of the rodent testis to cadmium. The unique set of long-chain and very-long-chain PUFA of rat testis cells served as biomarkers to reveal i) strong catabolic reactions that affected the major GPL, ii) catabolic reactions that unexpectedly occurred intriguingly slowly, such as the disappearance of endogenous and SM-derived ceramides; iii) an also indolent catabolism of pre-existing species of TAG, ADG and CE, as shown by their VLCPUFA; and iv) a steady biosynthesis of lipids, as manifested by the new species of CE and ADG accumulated with time.

In contrast to the virtual vanishing by day 30 of most of the original GPL and SM, hydrolysis and further metabolism of *neutral* lipids that had originally belonged either to germ cells (as was the case with the VLCPUFA-rich species of TAG and Cer [10]) or to Sertoli cells (as was the situation with the VLCPUFA-rich species of CE and ADG) was not completed one month after Cd-induced damage. Considering the severe loss of testicular weight, the concentration of all these neutral lipids increased substantially if expressed per gram of tissue.

The Cd-induced long-term persistence of the amorphous organic material filling the space originally occupied by cells within seminiferous tubules was not observed under experimental conditions that cause damage to spermatogenic cells but spare Sertoli cells [12–15] (see also Supplementary Information 2 for the effects of testicular ischemia). In all these cases, the eventual differentiation and egress from the testis of those germ cells that survive are possible, resulting in "Sertoli cell only", *patent* tubule lumena. In this case, Sertoli cells were themselves primary targets of $CdCl_2$: phagocytosis of germ cell corpses and seminiferous tubule fluid formation and secretion were absent, this

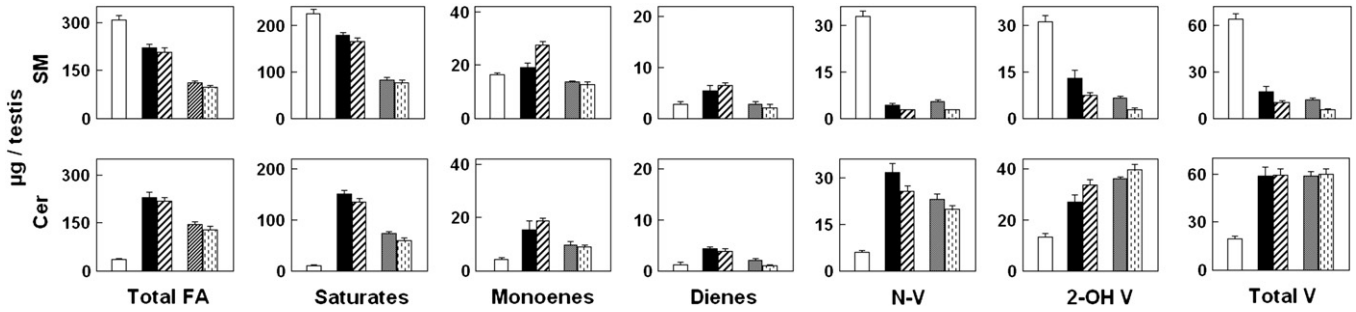

Fig. 7. Sphingomyelins (SM) and ceramides (Cer) in rat testis after cadmium treatments. The data are presented as in Figs. 3 to 6. White bars: control animals; black and hatched bars: 2 and 30 days, respectively, after a single i.p. 4 mg/kg $CdCl_2$ dose; gray and dotted bars: 30 and 45 days, respectively, after commencement of a regime of 1 mg/kg s.c. doses of CdCl2 each given every 4 days. The amounts of the different groups of fatty acids, including the two main types of VLCPUFA in both lipids, nonhydroxy and 2-hydroxy derivatives (here abbreviated N-V and 2-OH V, respectively), are depicted. Total V represents the sum of both types of VLCPUFA in each lipid.

explaining why cell remnants could not progress along the seminiferous tubules for their elimination.

In the present study the cells populating the interstitium in increased numbers after Cd treatments were identified as macrophages. Although these cells have not deserved much attention when appraising the myriad of biochemical reactions that occur in vivo in rodent testes at different times after Cd treatments, they must be credited as protagonists. Thus, they may be expected to orchestrate the acute inflammation that occurred during the first 48 h after the 4 mg/kg dose, as a normal pathophysiologic response to the ischemic-dependent necrosis of intra-tubular cells [16] that this dose provokes.

As in other tissues [30,31], different subsets of macrophages coexist in rodent testis, the two extremes of this continuum being the classically activated M1 and the alternatively activated M2 cells, discernible from one another by the surface antigens and immune mediators they produce [32,33]. The M1 subset that displays a cytotoxic, pro-inflammatory phenotype, is not normally active in healthy testes, but protects the testis against infection and allows for typical inflammatory responses [34]. These macrophages, rather than the damaged testicular cells, may be the source of the increased amounts of TNF-α, as well as the nitric oxide (NO) and highly reactive oxygen species (ROS) that have been shown to be produced in vivo 48 h after Cd treatments [35]. The mentioned short-lived pro-oxidant metabolites, known to damage proteins, nucleic acids and lipid PUFA, are to be blamed for part of the lipids previously belonging to intra-tubular cells being attacked at their highly unsaturated acyl chains, as revealed by the increased levels of TBARS detected in vivo 48 h after a 2 mg/kg dose of $CdCl_2$ in rat testis [2,3]. These macrophages may also in part be the source of the phospholipid hydrolases whose activity was overtly manifested in the present study by the rapid and massive degradation of testicular GPL and SM.

Albeit retaining phagocytic functions, M2 macrophages are functionally associated with scavenging cell debris, promoting tissue remodeling and repair, and being responsible for immune suppression [30,31]. Normally, the M2 subset prevails in the healthy rodent testis, where its anti-inflammatory/immunosuppressor profile is critical in protecting germ cells and spermatozoa from immune-mediated attack [36]. Rat testicular macrophages in culture do produce high levels of prostaglandins E_2 and $F_{2\alpha}$, which are able to *inhibit* lymphocyte proliferation [37]. The increased production of prostaglandin $F_{2\alpha}$ that occurs in the rat testis 48 h after treatment with a >2 mg/kg (but not with a <1 mg/kg) dose of $CdCl_2$ [38] could be an early manifestation of these cells increasing in numbers and/or activity trying to put a limit to Cd-induced damage. In this regard, administration of hemin, through its anti-inflammatory, antioxidant, and antiapoptotic effects, has been shown to protect rat testes against Cd-induced reduction in testosterone levels, to compensate deficits in antioxidant defense systems, and to suppress lipid peroxidation in testicular tissue resulting from cadmium administration [39]. Hemin is an inhibitor of heme oxygenase, precisely an enzyme known to be highly expressed in macrophages.

Chronic inflammation may follow acute inflammation or may start insidiously without any overt manifestation of the latter. The transition from acute to chronic inflammation involves an M1 to M2 phenotype change and occurs when the tissue damage fails to be resolved due to the persistence of the agent causing the damage or after insuperable interference with normal healing processes. At day 30 after commencing either of the two dose regimes of this study, irrespective of the initial form of cell demise and of the presence or absence of an initial phase of acute inflammation, the irreversibly devastated seminiferous tubules were surrounded by a cohort of mostly extra-tubular macrophages. This suggests a similar form of silent, chronic inflammation as the common outcome. The M2 macrophages normally prevailing in the testis may have played an important role in resolving the acute inflammation after the single high Cd dose and perhaps in preventing its development after the frequent low Cd doses. Walling off the potentially antigenic organic material (denatured protein, nucleic acids) contained in the damaged seminiferous tubules by anti-inflammatory/immunosuppressor macrophages seems to be a practical way of shielding the generated neo-antigens from exposure to reactive cells of the immune system.

4.1. Glycerophospholipids and triacylglycerols

The proportion of 22:5n−6 in GPL increases and that of 20:4n−6 decreases as germ cells differentiate from pachytene spermatocytes to spermatids and spermatozoa [10]. The fact that after Cd exposure most of the major GPL originally making up the different germ cell types of seminiferous tubule cells were rapidly hydrolyzed in situ, releasing massive amounts of FFA and DRG – mostly DAG – containing these PUFA, is a direct evidence that several phospholipases and related hydrolases were non-specifically activated after cell death: GPL → DRG; GPL → phosphatidic acid (PA); PA → DRG; DRG → FFA; and GPL → FFA. Part of these hydrolases may be endogenous to former Sertoli and/or germ cells undergoing autolysis, and part of them, in intracellular and secretory forms, may belong to the live cells that infiltrated the interstitium.

Even taking into account the additional amounts of neutral and polar lipids carried to the site in blood plasma and cells in the acute inflammatory exudate, the fatty acid content per testis in the *total lipid* was considerably lower than in controls at days 2 and 30 after the high Cd dose. This indicates that the fatty acids arising from hydrolysis of the total GPL and TAG were either exported or beta-oxidized in due course, evidently by day 30, by live and active cells of the testis, mostly testicular phagocytes that were able to overcome the toxic effects of cadmium.

Because these cells are normally capable of hydrolyzing TAG and utilizing their fatty acids, it was intriguing that an important part of the original TAG, those rich in 22:5n−6 and 24-carbon VLCPUFA, remained high at day 30 after both Cd treatments. These unique TAG are normally formed in spermatids in the last stages of their differentiation, as they reduce their cytoplasmic volume to become nascent spermatozoa [10]. Together with other materials left behind, these TAG are tightly packed in particles known as “residual bodies”. These bodies are normally released in areas proximal to the lumen and distal from the basal membrane of seminiferous tubules [10,14] to be engulfed, and their contents disposed of, by Sertoli cells. The persistence of these particular species of TAG for a month or more after cadmium treatments suggests that most of them remained out of the reach of the Sertoli or macrophage lipases, physically trapped within the stagnant organic material that replaced former tubules.

4.2. Cholesteryl esters and ether-linked triglycerides

Adult rat testis CE and ADG rich in 22:5n−6 and VLCPUFA are synthesized in Sertoli cells; during normal spermatogenesis these cells continuously phagocytize residual bodies and apoptotic bodies from germ cells and metabolize large amounts of the polar and neutral lipids thus incorporated [14]. The VLCPUFA-containing CE and ADG species we recovered after Cd treatments were thus, in part, an unmodified proportion of the original Sertoli cell CE and ADG. In contrast, the new species of CE and ADG that steadily accumulated with time well after cadmium treatments were obviously produced by live cells. Since Cd is toxic in vitro to Leydig cells [40], as corroborated by the fact that Cd administration to rats leads to a complete failure of steroidogenesis [3,38], the long-term survival of these cells under the present conditions is highly unlikely. After the disappearance of Leydig and Sertoli cells, interstitial macrophages are good candidates as responsible for the biosynthesis of the increasing CE and ADG species.

Following endocytosis or phagocytosis, CE may derive from materials containing excess free cholesterol and fatty acids, whether taken up from lipoproteins or from membrane lipids resulting from dead cells. After ester bond hydrolysis and conversion of fatty acids into acylCoA, acylation of cholesterol by acylCoA:cholesterol acyl transferase (ACAT) may be expected to occur in macrophages.

By the same token, DRG derived from (diradyl) GPL after phosporyl-base removal in phagocytized materials may be combined with a spare fatty acid at *sn-3* by a diglyceride acyl transferase (DGAT) to produce the corresponding triradylglycerols, namely TAG, 1-O-alkyl DAG, and alk-1-enyl-DAG subclasses. The fact that the latter subclass was detected in larger amounts 30 days after Cd exposures than in controls (see Supplementary Information 1, Fig. S4) points to diglycerides derived from original plasmalogens as their most likely source. Once formed, the ether-linked triglycerides may be temporarily stored in cell cytoplasmic lipid droplets, as is the case of other neutral lipids including TAG or CE.

Apart from an auto-protective function against excessive intracellular accumulation of DRG and FFA in phagocytes during GPL catabolism, which would apply to all three subclasses of triglycerides including TAG, the reason for the existence of three subclasses of triglycerides in cells remains as intriguing as the reason for the occurrence of three subclasses of membrane GPL.

A striking and infrequently recalled attribute of ADG is their high rate of production in a variety of spontaneously arising and transplantable solid animal and human tumors (see [41] and references therein), thus associating these neutral lipids with the increased cell turnover typical of cancer. Although the cell types within tumors in which these neutral lipids accumulate remain to be identified, it is currently accepted that a chronic inflammatory microenvironment of the type cadmium exposures provoke in the testis provides the fuel for solid tumor promotion, mostly facilitated by tumor-infiltrating macrophages that undergo a phenotypic 'switch' to the alternatively activated, M2 phenotype [42]. The notion that ADG could be produced in this particular type of chronic inflammatory cells in tumors is worth investigating in this context.

4.3. Sphingomyelins and ceramides

Among the cadmium-induced testicular lipid changes, the most unpredicted was the rapid and long-lasting increase in the levels of Cer. The fatty acids of the accumulated Cer species indicated that most of them arose directly from in situ hydrolysis of the SM formerly present in cells confined in seminiferous tubules. A lysosomal and a secretory form of acid sphingomyelinase (SMPD1) originating in a common protein precursor via divergent cellular trafficking have been identified [43]. The SM-derived ceramides produced in seminiferous tubules of Cd-treated rats may be ascribed in part to autolysis, to be catalyzed by a form of acid sphingomyelinase originally confined in lysosomes of Sertoli and germ cells and also in acrosomes of spermatids and spermatozoa. The importance of lysosomal acid SMase in normal gonadal function has been demonstrated in *smpd1*-null mice, a model of human Niemann–Pick disease [44,45]. These mice progressively accumulate SM as lipid inclusions in their organs including testes, mostly in Sertoli cells, and have reduced fertility because they produce and release structurally and functionally abnormal SM-rich – and Cer-poor – spermatozoa.

The long-term persistence of high levels of VLCPUFA-rich Cer under the present experimental conditions suggests that the ceramidase activity that should have hydrolyzed these species into sphingosine and FFA could have been too low, or present but strongly inhibited, by cadmium itself or by a lipid product formed because of its action.

Five ceramidases encoded by 5 different genes have been cloned in humans and mice [46], some of which occur in intracellular and in secretory forms. In favor of a low endogenous activity in the rodent testis is the fact that activity and expression (mRNA) of membrane-bound neutral ceramidase [47] and lysosomal acid ceramidase [48] are high in several mouse tissues but, intriguingly, barely detectable in the testis. In agreement with these findings, spermatids, the most numerous germ cell line elements in the rat testis, normally contain significant amounts of "endogenous" Cer with 2-OH VLCPUFA [10] as also do spermatozoa [10,49]. That these gametes have a vigorous sphingomyelinase but lack ceramidase activity is also indicated by the persistence of high Cer/SM ratios and unchanged levels of Cer in gametes that have undergone their capacitation and acrosomal reaction in vitro [9].

Even in the absence of germ cell ceramidases, the Cer molecules produced after Cd treatments in the testis could have been expected to be degraded by residual ceramidase from former Sertoli cells or by a ceramidase anticipated to be active in the macrophages present in the interstitium. Although numerically lesser than germ cells, Sertoli cells normally contain a measurable acid ceramidase activity [50], consistent with the fact that one of their physiological functions is to phagocytize and catabolize germ cell-derived residual bodies and apoptotic bodies that do contain SM [10]. In contrast to circulating monocytes, the long-lived macrophages that reside in tissues, like those of lung, do display high constitutive levels (mRNA, protein, and activity) of acid ceramidase [51], an activity that was deemed responsible, by maintaining low intracellular Cer levels, for the typically prolonged lifespan of these cells.

One possibility for explaining the persistently high Cer levels in the present conditions could be that the macrophages populating the interstitium had their ceramidase activity inhibited. In this context, $CdCl_2$ administration to rats results, 9 h later, in the accumulation in their testes of N-acylethanolamines, including N-palmitoyl, N-oleoyl, and N-arachidonoyl ethanolamine (anandamide) [52], of which N-oleoyl-ethanolamine is a well-known ceramidase inhibitor [51]. However, in addition to a potent acid ceramidase essential for their survival, macrophages also express an active N-acyl ethanolamine hydrolase [53]. Had these cells phagocytized the Cer-containing material present in seminiferous tubule remnants, such Cer should have vanished with time. These observations suggest that part of the Cer (as well as part of the original TAG, ADG and CE) of testis remained as residual components of this material, which was not readily phagocytized by the interstitially located macrophages.

A slow catabolism of Cer could in part arise from a ceramidase being normally active in the interstitially located macrophages but physically hindered from contacting its intra-tubular Cer substrates. This would explain that testicular Cer species that contain N-VLCPUFA decreased *before* species that contain 2-OH VLCPUFA (Fig. 7). As the former derive from SM that exclusively belong to less mature germ cells, like pachytene spermatocytes, they are expected to be located closer to the basal membrane of seminiferous tubules and extra-tubular phagocytes than the latter, which derive from SMs of more differentiated spermatids and nascent spermatozoa [10], originally located towards the center of seminiferous tubules.

Another possibility is that phagocytosis, metabolism, and efflux of molecules from the intra-tubular material was slow because the number of macrophages involved in these tasks was relatively small in comparison with the large mass of dead tissue to be eliminated. It is also possible that some of the intracellular lipids produced or accumulated in these cells could play a role in regulating the rate of these processes.

In support of this possibility, a surprisingly similar outcome to the present one in the testis after Cd treatment was previously observed in the lung parenchyma of rats, where cigarette smoke – a toxicant of which is precisely cadmium – induces alveolar epithelial and endothelial cell apoptosis, oxidative stress, and increase in Cer levels [54]. Although all these outcomes are accompanied by an increased number of alveolar macrophages, these cells are prevented from achieving the clearance of apoptotic cells expected from them [55], which was attributed to the high Cer levels. It is then possible that the Cer accumulated in rat tubules after Cd exposure could also play a role in regulating phagocytosis and/or hydrolysis of the ingested material.

The description of the long-term effects of cadmium on the testis, a persistent central necrotic core rich in neutral lipids, surrounded by active macrophages that progressively become anti-inflammatory as they convert into lipid-laden "foam" cell, resembles the familiar picture of an atheroma, a tuberculous granuloma and other silent forms of chronic

inflammation. Although the nature of the *lipid* confined in the central residual material typical of these lesions is barely given a second thought, it is worth investigating whether and to what extent it affects the functions of the macrophages that are supposed to eliminate such material, perhaps contributing to the long-term persistence of this type of lesions in the affected tissues.

Human macrophages in culture shift from pro-inflammatory to inflammation-anergic cells as they time-dependently phagocytize lipid-rich materials – like modified lipoproteins – and become lipid-laden "foam cells" [56]; in the process, these cells down-regulate the production of inflammatory cytokines and progressively become less responsive to pro-inflammatory stimuli. Myelin ingestion by human macrophages functionally converts them into lipid-laden "foamy" macrophages, involved in suppression of inflammation and unable to respond to prototypical inflammatory stimuli [57]. It is then possible that in the present cases as well, time-dependent accumulation of neutral lipids such as CE, ADG and especially Cer occurs in testicular macrophages as they change their phenotypic profile. Further studies are required not just in the testis but also in other tissues to elucidate which lipid components of phagocytized materials are capable of regulating macrophage function and the mechanisms involved.

Abbreviations

ADG	triglycerides with a fatty alcohol or a fatty aldehyde at *sn-1* (1-alkyl, 2,3-diacyl-*sn*-glycerols and 1-alk-1′enyl, 2,3-diacyl-*sn*-glycerols)
CE	cholesteryl esters
Cer	ceramides
CGP	choline glycerophospholipids
DRG	diradyl-glycerols (diglycerides with a fatty acid, a fatty aldehyde or a fatty alcohol at *sn*-1 and a fatty acid at *sn*-2)
FFA	free fatty acids
GPL	glycerophospholipids
PUFA	polyunsaturated fatty acids
SM	sphingomyelin
TAG	triacylglycerols

The fatty acids of lipids are abbreviated according to convention-number of carbon atoms: number of double bonds, with n−6 referring to the series of PUFA biosynthetically derived from linoleic acid (18:2n−6), such as 20:4n−6 or 22:5n−6. VLCPUFA, very-long-chain (C_{24}–C_{32} carbon atom) PUFA, composed of nonhydroxy (N) and 2-hydroxy (2-OH) versions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbalip.2013.09.014.

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