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Effects of chronic exposure to cadmium on prostate lipids and morphology

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Abstract

Cadmium is an environmental toxic metal implicated in human prostate carcinogenesis. The mechanism of its toxicity is not fully understood. Previously, we showed that cadmium exposure induces oxidative stress, especially lipid peroxidation. This study evaluates the effect of chronic exposure to 0.886 mM of cadmium (Cd) per liter in the drinking water on prostate lipid content and metabolism in Wistar rats. We determined the lipid profile and measured the expression of lipogenic enzymes: FAS, GPAT, LPL, DGAT-1, DGAT-2, ACO, CPT-1 and CT, and of certain factors involved in lipid regulation and fatty acid transporters: FAT/CD36, E-FABP, SREBP-2, PPAR-γ and PPAR-α by RT-PCR. Ultrastructure was analyzed by electron microscopy and, as prostate is an androgen controlled gland, AR expression was measured by RT-PCR and Western blot. Cd altered the prostatic lipid profile. Triglycerides (TG) and esterified cholesterol (EC) decreased, free cholesterol (FC) and phospholipids (PL) increased and total cholesterol (TC) did not change. FAS, MDH and IDH activities did not vary but G6PDH decreased significantly in Cd group. Regarding TG synthesis, DGAT-1 decreased while GPAT increased and FAS, LPL and DGAT-2 remained unchanged. Regarding beta oxidation, CPT-1 increased while ACO expression decreased in Cd group. In the PL pathway, CT expression was increased. All these results would justify the decrease of TG in Cd group when compared to control. In the cholesterol metabolic pathway, HMGCoAR and SREBP-2 increased. PPAR-α increased but PPAR-γ did not change. Regarding fatty acid transporters, FAT/CD36 decreased, while E-FABP increased. AR mRNA and protein expression decreased. Ultrastructural analysis showed a decrease in lipid droplets and signs of cellular damage in the Cd group.Cadmium exposure induces important changes in prostatic lipid profile and metabolism, confirmed by the morphology analyses, which also showed signs of cellular damage. These results could be important to further understanding the complex mechanism of cadmium toxicity in prostate and in the development of better treatments for people and animals exposed to the heavy metal.

Abbreviations: LD50 – medial lethal doses; FAS – fatty acid synthetase; HMGCoAR – hydroxymethyl-glutaryl coenzyme A reductase; GPAT – glycerol-3-phosphate acyltransferase; DGAT – diacyl glycerol acyl transferase; LPL – lipoprotein lipase; FC – free cholesterol; EC – esterified cholesterol; TG – triglycerides; AR – androgen receptor; CT – CTP-phosphocholine cytidylyltransferase; DAG – diacyl glycerol; ACO – acyl CoA oxidase; CPT-1 – carnitin palmitoil transferase; PC – phosphatidilcholine; FA – fatty acids; TBARS – thiobarbituric acid – reactive susbtances

Introduction

Cadmium (Cd), one of the most important environmental and occupational toxic metals, is widely dispersed in the environment. High level exposure to this toxic heavy metal is usually the result of environmental contamination from human activities, such as mining, smelting, fossil fuel combustion and industrial use (Nordberg 1972). Besides, it has been well established that Cd is one of the major contaminants of tobacco smoke (Li *et al*. 2000).

Cadmium has been classified by the International Agency for Research on Cancer as a category 1 (human) carcinogen (IARC 1993) because of its pulmonary carcinogenesis in humans. Exposure to cadmium has also been considered as a risk factor for human prostatic and testicular cancers (Waalkes *et al*. 1988; Ogunlewe & Osegbe 1989; Waalkes & Rehm 1994; Rhomberg *et al*. 1995). The mechanisms of its toxicity are far from fully understood, but it has been shown that cadmium exposure increases oxidative stress and lipid peroxidation in many organs (Caisová & Eybl 1997; Alvarez *et al*. 2004; Calderoni *et al*. 2005). It has also been shown that cadmium causes superficial irregularities on the plasma membrane and H_2O_2 accumulation that lead to changes on its permeability (Koizumi *et al*. 1996).

Cholesterol has an important role in modulating fluidity and phase transitions in the plasma membranes of animal cells. Recently, it has been implicated, together with sphingomyelin, in the formation of plasma membrane rafts or caveolae, sites where signaling molecules are concentrated. To perform these functions, membrane cholesterol content must be maintained at a constant level (Brown & Goldstein 1999). Regarding lipid metabolism in the prostate, it has been shown that androgens regulate many enzymes in the biosynthesis of fatty acids (e.g., ATP citrate lyase, acetyl-CoA carboxylase and malic enzyme) and in the cholesterol synthesis pathway, such as HMGCoA synthase, HMGCoA reductase and farnesyl diphosphate synthase (Swinnen *et al*. 1997). It has also been shown that androgens enhance the expression and nuclear contents of the sterol-regulatory element binding proteins (SREBP) in the maintenance of cholesterol homeostasis. However, all these studies have been made *in vitro* (Swinnen & Verhoeven 1998).

Little information is available on the effect of cadmium on lipid metabolism. For example, it has been shown that cadmium suppresses delta 9 desaturase activity, which converts 18:0 to 18:1 fatty acids in cultured hepatocytes (Kudo *et al*. 1990; Kudo & Waku 1996); another study showed that exposure of alveolar macrophages to Cd^{2+} causes a reduction in the rate of liberation of 20:4 fatty acids from cell lipids, as a possible result of the inhibition of PLA2 (phospholipase A2) activity by Cd^{2+} (Kudo *et al*. 1992).

It is known that lipids are the main component of cell membranes; therefore alterations in this complex structure could modify the permeability of the cell and generate inadequate nutrients entrance to the cell. One of the mechanism of cadmium toxicity could be through the alteration of lipid synthesis. Thus, the present work may be the first study that addresses the effect of cadmium on prostate lipid metabolism in *in vivo* models. Besides, the study of the effect of cadmium on lipid metabolism and content might help to explain previous findings, such as changes on cell permeability, in an attempt to understand cadmium toxicity.

There are few previous studies exploring chronic exposure to orally administered cadmium (Martin *et al*. 2001; Alvarez *et al*. 2004). For this reason, in the present work, we designed a model to mimic an environmental oral exposure to cadmium in order to find out its effect on the prostate, focusing our attention especially on ultrastructure and lipid metabolism. Since the medial lethal dose (LD50) of $CdCl_2$ when given orally to rats is reported to be 63–88 mg/kg (USAF 1990), we carried out these experiments using a dose of 0.886 mM per liter of Cd as $CdCl_2$, which would correspond to 1/12 LD50).

Materials and methods

Chemicals

Lipid standards were acquired from Sigma Chemical Co. All the other chemicals were of reagent grade and were obtained from Merck

Laboratory or from Sigma Chemical Co. Molecular Biology reagents were purchased from Promega Inc. and Invitrogen.

Experimental model

Twenty-one days old male Wistar rats were separated in two groups of eight rats: control group (Co) and cadmium (Cd) group. Cd group received 0.886 mM per liter of Cd, administered as $CdCl_2 \cdot 2H_2O$ in drinking water while Co group received tap water without Cd. The concentration of cadmium in tap water and in the Cd solution was confirmed by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES). Animals were housed individually in a controlled environment with 12 h light: 12 h darkness cycle at 21 °C, during three months and they were fed with rat chow (Cargill). Food and water were available *ad libitum*. Animal treatment protocols were previously approved by the local ethics committee, and are in accordance with care and treatment of rats recommended guidelines (US Public Health Service 1985).

The rats were sacrificed by decapitation after 3 months of exposure to Cd, prostates were removed, snap frozen in liquid nitrogen and stored at −70 °C until they were analyzed.

Quantification of Cd concentration on prostate

Prostate tissue was mineralized with 16N nitric acid as described by Clegg *et al*. (1981). Cadmium concentration was determined in the mineralized material by ICP-AES (Ihnat 1990) in an Atomic Emission spectrometer (BAIRD, Model "ICP-2070" – Bradford, USA). All the specimens were analyzed in duplicate.

Tissue preparation and enzymatic assays

Prostate portions (1 g for 4 ml of buffer) were homogenized in an Ultra Turrax T25 homogenizer in 0.5 M potassium phosphate buffer (pH 7) containing 10 mM EDTA, 10 mM D, L-dithiotreitol (DTT) and protease inhibitors. The homogenates were centrifuged at 100.000 × g for 1 h to yield the cytosolic fraction in a Beckman model L8-80M ultracentrifuge with a Ty-80 rotor.

Cytosolic FAS activity was determined spectrophotometrically by a modified version of the method of Alberts *et al*. (1974). The reaction mixture contained 0.5 M potassium phosphate buffer (pH 6.6), 1 μmol each of EDTA and DTT, 100 nmol of NADPH and 0.05 ml of the cytosolic fraction. The reaction was started by adding 100 nmol of malonyl-CoA and the final assay volume was 1.05 ml. The oxidation of NADPH at 30 °C was monitored at 340 nm. FAS activity was expressed as units/mg of cytosolic proteins.

To measure glucose-6-phosphate dehydrogenase (G6PDH), NADP-isocitrate dehydrogenase (IDH) and NADP-malic deshydrogenase (MDH) activity, prostates were homogenized with Tris-HCl buffer pH 7.4, containing 1 mM dithiotreitol (DTT). The homogenates were centrifuged at 100.000 × g for 1 h and the enzymatic activities were measured in the supernatant. G6PDH, IDH, and MDH were determined by the rate of NADPH formation at 340 nm, according to Glock & Lean (1953), Farrell (1980) and Ochoa *et al*. (1948), respectively.

Lipid determinations

The lipids from the prostate were extracted with chloroform:methanol (2:1) according to the method of Folch *et al*. (1968). Aliquots of the lipid extracts were used to determine total cholesterol and to separate the different lipid fractions by thin-layer chromatography with an n-hexane:diethyl ether:acetic acid (80:20:1, v/v/v) solvent system. The lipids were detected by exposing the plates to iodine vapors. The bands were scraped off, eluted and aliquots were used to determine the mass of the different lipids.

Free and esterified cholesterol were determined according to the method of Zack *et al*. (1954) after saponification (Abell *et al*. 1952). Triglycerides were quantified by the method of Sardesai & Manning (1968) and phospholipids were determined according to Rouser (1970).

RNA isolation and RT-PCR analysis

Total RNA was isolated by using TRIzol (Life Technologies). All RNA isolations were performed as directed by the manufacturers. Gel electrophoresis and ethidium bromide staining

confirmed the purity and integrity of the samples. Quantification of RNA was based on spectrophotometric analysis at 260/280 nm.

10 μg of total RNA were reverse-transcribed with 200 units of MMLV Reverse Transcriptase (Promega Inc.) using random hexamers as primers in a 20 μl reaction mixture, following the manufacturers instructions. RT-generated fragments coding for β-actin, FAS, glycerol-3-phosphate acyltransferase (GPAT), lipoprotein lipase (LPL), acyl CoA oxidase (ACO), carnitin palmitoil transferase-1 (CPT-1) (Zhou *et al*. 1999), diacylglycerol acyltransferase-1 and 2 (DGAT-1, DGAT-2) (Waterman *et al*. 2002), SREBP-2, FAT/CD36 (Guthmann *et al*. 1999), E-FABP (Guthmann *et al*. 1998), PPAR-γ, PPAR-α (Hoekstra *et al*. 2003), CT (Carter *et al*. 2003) and androgen receptor (AR) were amplified, using PCR.

PCR was performed in 35 μl of reaction solution containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.25 U of Taq polymerase, 50 pmol of each rat specific oligonucleotide primers and RT products (1/10 of RT reaction). The sequences of the different primers and the sizes of the expected PCR products for each reaction are shown on Table 1. The samples were heated to 94 °C for 2 min, followed by 38 temperature cycles. Each cycle consisted of three periods: (1) denaturation, 94 °C for 1 min; (2) annealing, 55 °C for LPL, FAS, SREBP-2, DGAT-1, DGAT-2, GPAT, ACO, CPT-1 and β-actin; 56 °C for FAT/CD36 and E-FABP; 60 °C for PPAR-γ, PPAR-α and CT and 65 °C for AR during 1 min; (3) extension, 72 °C for 1 min. After 38 reaction cycles, the extension reaction was continued for another 5 min.

The PCR products were electrophoresed on 2% agarose gel with 0.01% ethidium bromide, except those of PPAR-γ and PPAR-α, which where electrophoresed in 10% polyacrilamide gels. The image was visualized and photographed under UV transillumination. The intensity of each band was measured using NIH Image software and reported as the values of band intensity units. The relative abundance of each target band was then normalized according to housekeeping gene β-actin, calculated as the ratio of the intensity values of each target product to that of β-actin.

Histological studies

Light microscopy

The prostates were extracted and representative tissue samples were fixed in Bouin's solution. The samples were dehydrated in graded series of ethanol and embedded in paraffin. All sections were obtained from the same region of the prostate for effective comparison. Sections of 5–6 μm thickness were obtained using a Porter Blum Hn40 microtome and stained with hematoxylin–eosin. Photographs were obtained with a

Table 1. Sequences of the primers used to amplify different genes by RT-PCR and sizes of the fragments generated.

cDNA	Forward (5′- 3′)	Reverse (5′-3′)	Size (bp)
FAS	GTTTGATGGCTCACACACCT	TACACTCACTCGAGGCTCAG	515
GPAT	TGATCAGCCAGGAGCAGCTG	AGACAGTATGTGGCACTCTC	508
LPL	CCTGAAGACTCGCTCTCAGA	TTGGTTTGTCCAGTGTCAGC	454
DGAT-1	TTTCTGCTACGGCGGGTTCTTGAG	ACCGGTTGCCCAATGATGAGTGTC	329
DGAT-2	GGAGGCCACCGAAGTTAGCAAGAA	AGCCCCCAGGTGTCAGAGGAGAAG	453
SREBP-2	CACAATATCATTGAAAAGCGCTACC	TTTTTCTGATTGGCCAGCTTCAGCA	196
HMGCoAR	GTGATTACCCTGAGCTTAGC	TGGGATGTGCTTAGCATTGA	462
E-FABP	GCCATGGCCAGCCTTAAGGATC	CCAGTCCTCATTGTACCTTCTCATA	414
FAT/CD36	TGATTCTGCTGCACGAGGAG	AAGAATGGATCTTTGTAACCCCAC	583
CT	AGTGGAGGAGAAGAGCATCG	GGAAGTCTTGCCAGAGAAGG	232
PPAR-α	TCAAACTTGGGTTCCATGAT	TGAACAAAGACGGGATG	106
PPAR-γ	TTCTGAAACCGACAGTACTGACAT	CATGCTTGTGAAGGATGCAAG	131
ACO	GCCCTCAGCTATGGTATTAC	AGGAACTGCTCTCACAATGC	634
CPT-1	TATGTGAGGATGCTGCTTCC	CTCGGAGAGCTAAGCTTGTC	629
AR	CAGCCCCAGCCCAGCGACAGC	CAGGGTGAGGGGCGGGCAGTAGGA	423
β-Actin	CGTGGGCCGCCCTAGGCACCA	TTGGCCTTAGGGTTCAGAGGGG	243

Leitz Dialux microscope equipped with a Leica camera.

Electron microscopy

Histological studies were performed by light and transmission electron microscopy. The prostates were carefully excised from prostate-urethra-bladder complex from 8 rats in each group. In all instances, representative tissue samples were fixed in glutaraldehyde 2% (v/v, final concentration) buffered in PBS (PBS: phosphate buffer saline, pH 7.2) for 5 h. The samples were dehydrated in graded series of ethanol–acetone and embedded in Epon 812 (Pelco). All sections were obtained from the same region of the prostate for effective comparison.

Sections were cut for light microscopy, these plastic sections were used for a further histopathologic study. This routine ensured that representative cells were used to study specially the products of the prostate cell and storage vesicles among other things. Thin sections were obtained using an Ultracut Leica ultramicrotome. For all the morphological analyses, only the ventral prostate was used.

Western blot analysis for AR

Prostates were homogenized with 50 mM Tris-HCl (pH 7.8) containing 0.01% Triton X-100 and protease inhibitor cocktail, and centrifuged at 4 °C. Protein concentrations of the resulting supernatants were determined according to the method of Wang & Smith (1975), using BSA as standard. 40 μg of proteins were mixed with 10 μl of sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 3.5 mM DTT, 0.02% bromophenol blue and 20% glycerol), boiled for 2–3 min and loaded into a 10% SDS-PAGE gel. Protein molecular mass markers were always loaded on each gel. Separated proteins were transferred to PVDF membranes (Polyscreen NEF 1000, NEN Life Science Products) using a blot transfer system (BioRad Laboratories, Hercules, CA). After being blocked with 5% BSA–TBS solution (20 mM Tris, 500 mM NaCl, pH 7.5) overnight, at 4 °C with gentle agitation, membranes were incubated with a primary rabbit anti-AR polyclonal antibody solution (Santa Cruz Biotechnology Inc.) (1:1000 dilution) for 1 h, at room temperature. After washing three times with TTBS (0.1% Tween 20, 100 mM Tris-HCl, pH 7.5, 150 mM NaCl), membranes were incubated with a secondary goat anti-rabbit IgG antibody linked to biotin for 1 h at room temperature (1:2000 dilution). Membranes were washed again and the color was developed using a Vectastain ABC – detection system (Vector Labs). The intensity of the bands was scanned densitometrically with the image processing and analysis program *Scion Image* and expressed on arbitrary units.

Statistics

Values are expressed as mean ± standard error of mean (SEM). Significant differences were considered at $P < 0.05$, as determined by Student *t*-test.

Results

Cadmium content in mineralized prostate tissues, quantified by atomic emission spectrometry, was increased in Cd rats when compared to controls (Figure 1).

The general state of health of the animals exposed to cadmium was good, and there were no changes in the levels of different lipids concentrations, total proteins and albumin in serum (data not shown). In accordance to these results, and as a consequence of using just 1/12 of the LD50 of cadmium, there were no signs of morbidity nor deaths in the rats exposed to the metal. Prostate

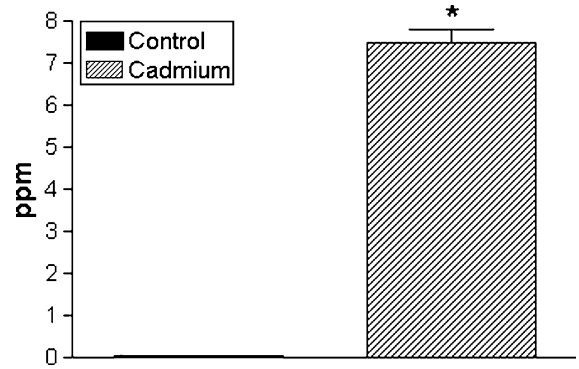


Figure 1. Quantification of cadmium concentration on prostate. A significant increase of Cd was found in the exposed group. Values are expressed as means ± SEM; $n = 8$ for each group. Asterisk indicates $P < 0.0001$ when compared to controls.

weight and total protein content were not modified, but important variations in the content of different lipids were found in Cd group (Table 2). Triglycerides (TG) and esterified cholesterol (EC) decreased while phospholipids (PL) and free cholesterol (FC) increased in Cd group. Nevertheless, neither the activity of the lipogenic enzyme FAS nor the activity of IDH, MDH and G6PDH, which provide NADPH for lipogenesis and production of total gluthatione (GSH), were modified in Cd group, except for G6PDH, which decreased (Table 2).

To determine whether the alterations observed in triglyceride content were produced by changes in the expression of lipogenic enzymes and/or factors involved in the uptake of lipids from plasma lipoproteins, the mRNA abundance of FAS, GPAT, DGAT-1 and 2, FAT/CD36, E-FABP and LPL was measured using semi-quantitative RT-PCR. Figure 2 shows that, in accordance with the enzymatic activity, FAS mRNA abundance remained unchanged in Cd group, suggesting that fatty acids synthesis is not changed. The expression of LPL was measured in order to analyze one of the ways of external contribution of fatty acids, but it did not change after the metal exposure. In contrast, the expression of the fatty acid translocase FAT/CD36, a membrane protein with high affinity for fatty acids and which is involved in the uptake of FA across the plasma membrane (Koonen *et al*. 2005), was decreased in Cd group. At the citosolic surface of the plasma membrane, FA can associate itself to FABP and mediate in that way the intracellular traffic of FA. The expression of E-FABP protein was increased in Cd group when compared to control group (Figure 2).

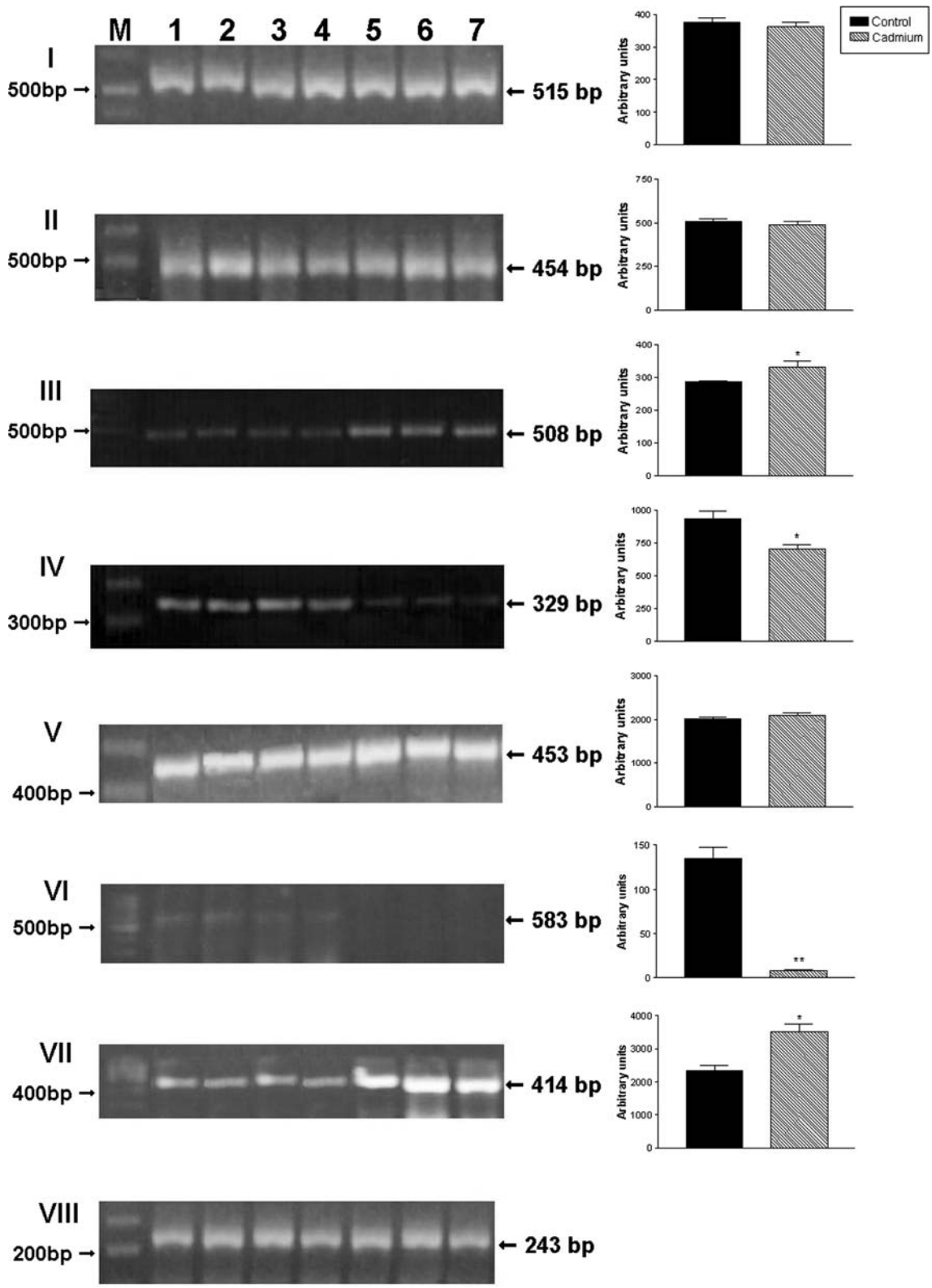
The decrease of TG seen in Cd group could be a consequence of different factors. Regarding lipid peroxidation, we have previously published that with 0.133 mM of Cd, there is an increased level of lipid peroxidation as it was shown by TBARS determination (Alvarez *et al*. 2004). We also found that TBARS level is even higher in rats exposed to 0.886 mM of Cd (data not shown). When we studied TG synthetic pathway, we found a decreased expression of DGAT-1 in Cd group and an increased expression of GPAT (Figure 2). Regarding beta oxidation, it is important to note that in the prostate, peroxisomes are very relevant (Zha *et al*. 2005). Then we studied the expression of CPT-1, the rate limiting enzyme of mitochondrial and peroxisomal beta oxidation, and the expression of ACO, one of the enzymes involved in the peroxisomal process (Figure 3). We found that CPT-1 increased and that ACO decreased in Cd group. Finally, in order to know if a part of the FA is being used in the synthesis of PL, we studied the expression of CTP-phosphocholine cytidylyltransferase (CT) that catalyzes a key step on the regulation of *de novo* synthesis of phosphatidylcholine (Shiratori *et al*. 1995). In Figure 4 we show that CT expression was significantly increased in Cd group.

On the other hand, the amount of free cholesterol increased significantly after Cd exposure.

Table 2. Effects of chronic exposure to 100 ppm of Cd on prostate triglycerides, phospholipids, and cholesterol concentrations and lipogenic enzyme activities.

	Control	Cadmium
Weight (g)	0.982 ± 0.03	0.949 ± 0.09
Total proteins (mg/dl)	32.65 ± 0.08	32.40 ± 0.88
Triglycerides (μg/mg prot)	148 ± 13.6	53.57 ± 1.89***
Total cholesterol (μg/mg prot)	4.73 ± 0.53	5.89 ± 1.85
Esterified cholesterol (μg/mg prot)	2.01 ± 0.25	0.62 ± 0.04*
Free cholesterol (μg/mg prot)	3.50 ± 0.52	6.06 ± 0.95*
Total phospholipids (μg P/mg prot)	6.67 ± 0.18	18.75 ± 3.75**
FAS (μmol NADPH/min/mg prot)	0.071 ± 0.021	0.063 ± 0.017
IDH (μmol NADPH/min/mg prot)	44.8 ± 3.6	56.5 ± 10.8
MDH (μmol NADPH/min/mg prot)	10.5 ± 4.5	12 ± 3
G6PDH (μmol NADPH/min/mg prot)	110.4 ± 21	130.1 ± 12

Abbreviations: FAS, fatty acid synthase; IDH, Isocitrate dehidrogenase; MDH Malic dehidrogenase; G6PDH, glucose-6-phosphate dehidrogenase; Cd, cadmium. Values are means ± SEM, $n = 8$ for each group.*$P < 0.05$, **$P < 0.005$, ***$P < 0.0001$ when compared to control.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VIII
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Control
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Figure 2. Effect of Cd exposure on the expression of proteins involved in the triglyceride synthetic pathway in control and exposed rats. I: Ethidium bromide-stained agarose gel of FAS PCR products. II: Ethidium bromide-stained agarose gel of LPL PCR products. III: Ethidium bromide-stained agarose gel of GPAT PCR products. IV: Ethidium bromide-stained agarose gel of DGAT-1 PCR products. V: Ethidium bromide-stained agarose gel of DGAT-2 PCR products. VI: Ethidium bromide-stained agarose gel of FAT/CD36 PCR products. VII: Ethidium bromide-stained agarose gel of E-FABP PCR products. VIII: Ethidium bromide-stained agarose gel of β-actin PCR products, used as an internal control. M: molecular weight marker. Lanes 1–4: control prostates. Lanes 5–7: Cd prostates. On the right, quantification of the abundances relative to β-actin, of each enzyme or factor mRNA. Values are expressed as means ± SEM for 3–4 rats on each group. One representative of three experiments is shown. Asterisks indicate: $*P < 0.05$; $**P < 0.005$ when compared to controls.

Then, the expression of HMGCoAR, the rate-limiting enzyme in cholesterol synthesis, was measured. Figure 5 shows that HMGCoAR increased its expression, which is concordant with the high levels of free cholesterol. Besides, the expression of Sterol Regulatory Element-Binding Protein-2 (SREBP-2), a transcription factor that regulates the expression of HMGCoAR (Brown & Goldstein 1999), was also increased in the exposed group (Figure 5).

An increased content of cholesterol might also modify the synthesis of phospholipids through the stimulation of CTP-phosphocholine cytidylyltransferase (CT), which was confirmed with the experiment shown in Figure 4.

Peroxisome proliferator-activated receptors (PPARs) are usually seen as monitors of intracellular NEFAs (non esterified fatty acids) and oxidized lipids (Nakamura *et al*. 2004). We studied the expression of PPAR-α and γ mRNA (Figure 6) and we found that PPAR-γ did not show modifications on its expression but PPAR-α was increased in the Cd group. In an attempt to determine whether the observed differences in lipids, mainly triglyceride content in prostate after cadmium chronic exposure affect the epithelium of the gland, we analyzed the morphology of the organ (Figure 7) using light and electron microscopy. The prostates of both groups were fixed, sectioned, stained and examined for evidence of injury. The prostate parenchyma of a control rat is shown in Figure 7A and C. The ventral prostates of Wistar rats appeared histologically normal and showed an active tall columnar epithelium. The prostatic acini were filled with a homogeneous secretion product. Significant morphological changes in prostate parenchyma were observed in the rats exposed to Cd when compared to control (Figure 7B and D). A significant reduction in the height of epithelial cells was noted in the atrophic epithelium. The prostates exposed lost the characteristic invaginations and showed lumen hypertrophy, which contained a different secretion than that observed in control prostates. This would suggest a decrease in the functionality of the gland, thus a reduced secretory capacity.

Using electron microscopy, we found that the exocrine cells of the control prostate epithelium

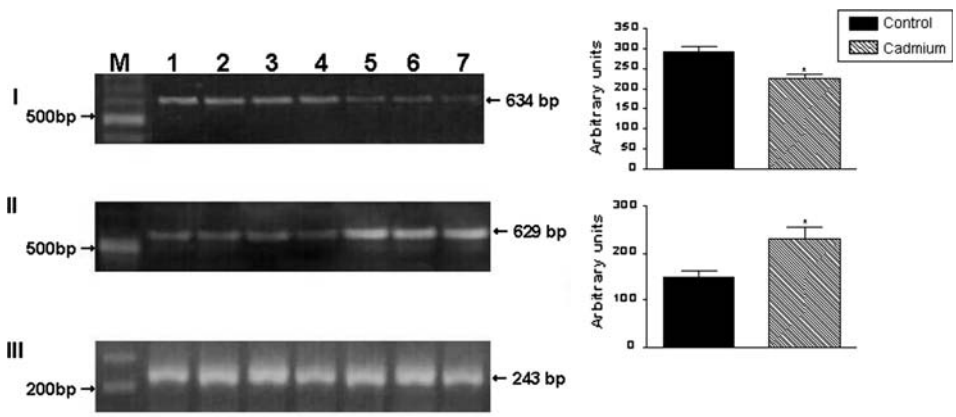

Figure 3. Effect of Cd exposure on the expression of genes involved in the beta oxidation of fatty acids. I: Ethidium bromide-stained agarose gel of CPT-1 PCR products. II: Ethidium bromide-stained agarose gel of ACO PCR products. III: Ethidium bromide-stained agarose gel of β-actin PCR products, used as an internal control. M: molecular weight marker. Lanes 1–4: control prostates. Lanes 5–7: Cd prostates. On the right, quantification of the abundances relative to β -actin, of each enzyme or factor mRNA. Values are expressed as means ± SEM. One representative of three experiments is shown. Asterisks indicate $P < 0.05$ when compared to controls.

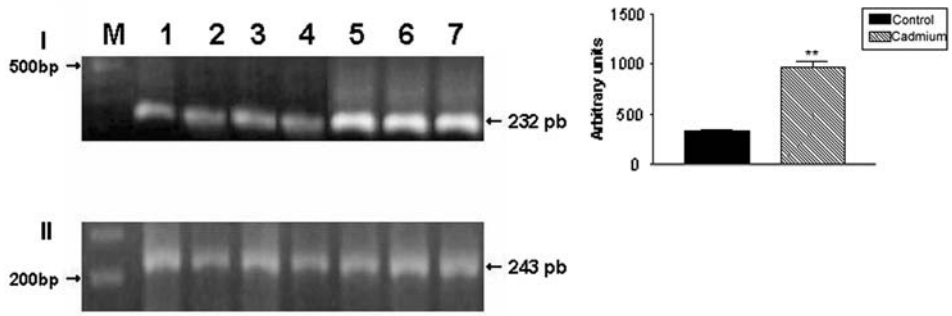

Figure 4. Effect of Cd exposure on expression of cytidylyltransferase (CT). I: Ethidium bromide-stained poliacrilamide gel of CT PCR products. II: Ethidium bromide-stained agarose gel of β-actin PCR products, used as an internal control. M: molecular weight marker. Lanes 1–4: control prostates. Lanes 5–7: Cd prostates. On the right, quantification of CT mRNA abundance relative to β-actin. Values are expressed as means ± SEM. One representative of three experiments is shown. Asterisks indicate $P < 0.005$ when compared to controls.

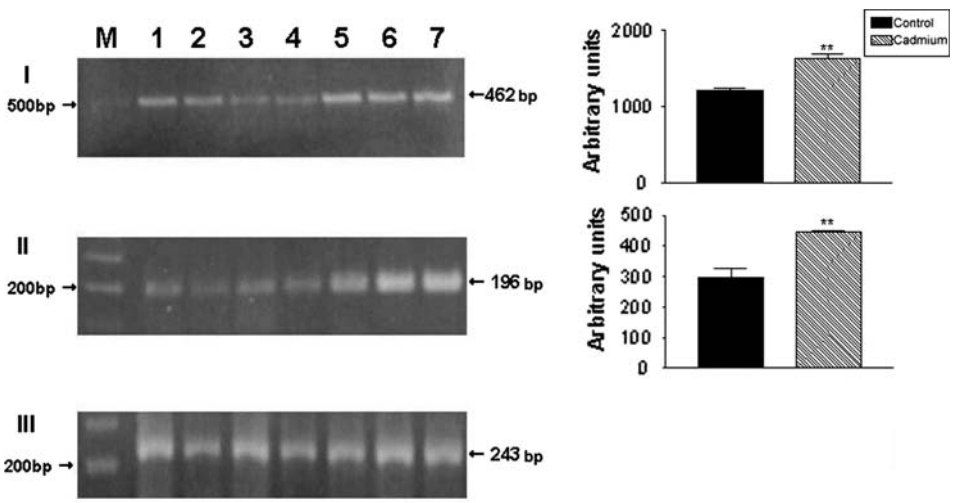

Figure 5. Effect of Cd exposure on the expression of genes involved in the cholesterol synthetic pathway. I: Ethidium bromide-stained agarose gel of HMGCoAR PCR products. II: Ethidium bromide-stained agarose gel of SREBP-2 PCR products. III: Ethidium bromide-stained agarose gel of β-actin PCR products, used as an internal control. M: molecular weight marker. Lanes 1–4: control prostates. Lanes 5–7: Cd prostates. On the right, quantification of the abundances relative to β-actin, of each enzyme or factor mRNA. Values are expressed as means ± SEM. One representative of three experiments is shown. Asterisks indicate $P < 0.01$ of significance when compared to controls.

had a varying number of supranuclear vesicles, so-called storage vesicles, filled with homogeneous and dark material (Figure 8A). In contrast, the cells from prostates of animals exposed to Cd showed on their apical regions, storage vesicles containing material that varied in amount and structure (Figure 8B). Lumen gland from control rats showed homogeneous content with many dense bodies and lipid droplets, while the luminal gland from Cd rats had a heterogeneous content and we did not observe many lipid droplets. Besides, some cells showed alterations compatible to cell death and/or apoptosis (Figure 8D).

Since androgens have described actions on lipid metabolism (Michaut *et al*. 1992; Ojeda *et al*. 1997) we measured the mRNA level of AR by RT-PCR, and we found that it was decreased in the Cd group when compared to control (Figure 9). Based on these results, we determined the protein expression of AR by western blotting and we found that it was also decreased (Figure 9B).

Discussion

The reports on the effects of Cd on lipid metabolism are relatively few. Cd has been shown to alter

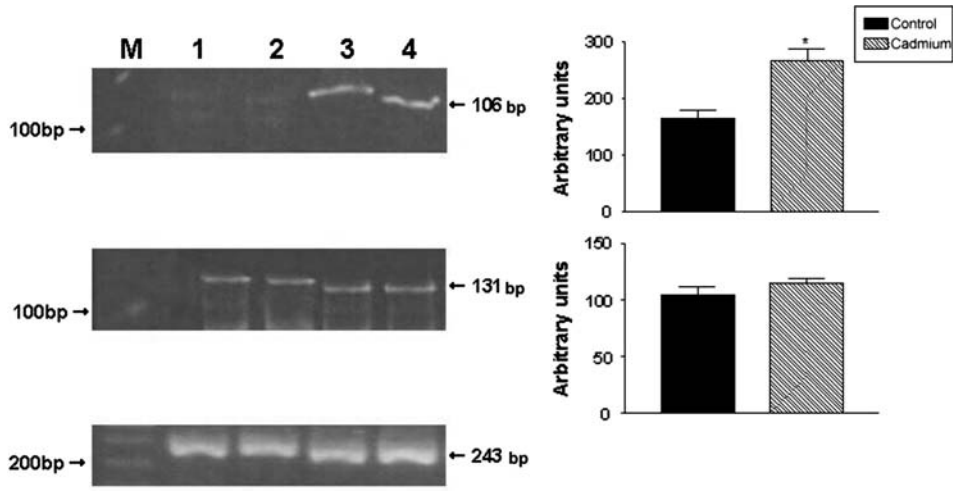

Figure 6. Effect of Cd on expression of transcription factors. I: Ethidium bromide-stained poliacrilamide gel of PPAR-α PCR products. II: Ethidium bromide-stained poliacrilamide gel of PPAR-γ PCR products. III: Ethidium bromide-stained agarose gel of β -actin PCR products, used as an internal control. M: molecular weight marker. Lanes 1–2: control prostates. Lanes 3–4: Cd prostates. On the right, quantification of the abundances relative to β -actin, of each factor mRNA. Values are expressed as means ± SEM. One representative of three experiments is shown. Asterisk indicates $P < 0.05$ of significance when compared to controls.

hepatic lipid metabolism, blood biochemistry and cytology and semen quality (El-Demerdash *et al.* 2004); a study from our laboratory showed effects on macrophage lipids (Ramírez & Gimenez 2002), but there are no reports on its effects on the prostate.

Many studies have been made in order to throw light on cadmium intoxication effects, but most of them have been made *in vitro* or by administration by injection (Terracio & Nachtigal 1986; Caisová & Eybl 1997; Martin *et al.* 2002). Our model, in which the animals were subjected to oral cadmium exposure for 3 months, has the advantage to be a closer mimic of the effects of chronic environmental Cd exposure and resulted in significant Cd accumulation in the prostates, as assessed by ICP-AES.

Profound changes in the lipid composition of prostates obtained from the exposed rats were found, particularly a decrease in triglycerides. This TG decrease could be a consequence of different factors, such as lower uptake, higher efflux, increased degradation, decreased synthesis or a combination of some of those factors. In our experimental model, the lower content of TG in Cd group when compared to control indicates that besides the effect of the lipid peroxidation on TG, the decrease of DGAT-1 and the increased expression of CTP-1 involved in the beta-oxidation process, there would be an extra factor acting on these compounds, as it is suggested by the increased expression of CT. This increased expression would lead to increased phospholipids (mainly PC), suggesting that a part of the fatty acids are shunted to the PL synthetic pathway at the expense of TG synthesis. Similar results (a decreased synthesis of triglycerides accompanied with increased phospholipids synthesis) has been reported in other models such as liver and lung of male castrated rats (Michaut *et al.* 1992); therefore, the changes in the Cd group could also be associated to the observed decrease of androgen receptors.

The lack of change in the activity and expression of FAS suggests that there was no variation in the amount of fatty acids produced. This conclusion is also supported by the unchanged or decreased activity of some dehydrogenases that provide NADPH for the activity of FAS. On the other hand, GPAT expression was increased in the Cd group; what would lead to an incremented amount of 1-acyl-*sn*-glycerol-3-phosphate, known to be an early substrate of both triglycerides and phospholipids synthesis pathways. The unchanged LPL expression may show that Cd does not alter the contribution of fatty acids from external sources to this organ, at least through this pathway. On the other hand, the increased expression of E-FABP and CPT-1 in Cd may be related to a higher mobilization of fatty acids to the beta-oxidation process.

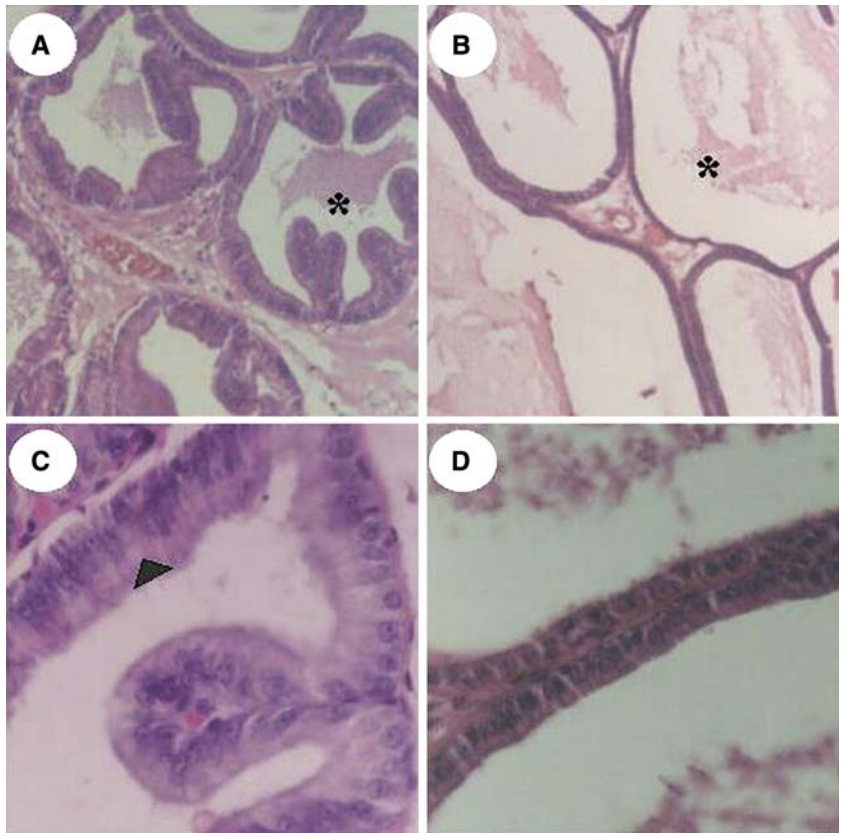

Figure 7. Effect of Cd on prostate morphology. (A) control rat (not exposed to Cd). Glandular epithelium consists of tall columnar cells with basal located nuclei. Abundant invaginations (asterisk). H–E 100 ×. (B) prostate exposed to 100 ppm of Cd. After 3 months of exposure to Cd, the prostate is highly damaged. Significant decrease of epithelium height and hypertrophy of the lumen diameter, where a non-homogeneous material without the colloidal normal characteristics is found (asterisk). Loss of the normal invaginations. Cellular atrophy and presence of picnotic nuclei. H–E 100 ×. (C) Detail of (A). The height of the epithelium indicates its secretory activity (arrowhead). H–E 400 ×. (D) Detail of (B). High magnification of the region showed in (B) exhibits cellular atrophy. H–E 400 ×.

The increased PL content found in the Cd group, in the presence of unchanged incorporation of exogenous fatty acids and no clear increase of endogenous ones, could be a consequence of a deviation of substrates toward this synthetic pathway, as suggested by the increased expression of CT (Figure 4).

It is known that phospholipids and triglycerides are metabolically interconnected by common lipid intermediates, such as diacilglycerol and fatty acids, which are exchanged in a recycling pathway that could be potentially relevant for membrane synthesis and lipid signal transduction (Igal *et al.* 2001). Enzymes for both pathways are subjected to common transcriptional regulation. However, little is known about the mechanism of potential coregulation of TG and phospholipid metabolism (Caviglia *et al.* 2004). Moreover, changes in the level of phospholipids would compromise the integrity and function of cell membranes, because these mainly depend on the lipid balance, especially on the cholesterol/phospholipids ratio (Finney *et al.* 2000).

Recently, Caviglia *et al.* provided evidence indicating that TG synthetic enzymes such as mitochondrial GPAT and DGAT, may indirectly regulate phospholipid synthesis by controlling the availability of lipid substrates (mainly fatty acids and DAG) for phospholipid formation. This would be a comparable situation with our experimental model, where there is not an increased synthesis but a redistribution of substrates, and where the synthesis of PC, molecule with both structural and potential signaling functions, seems

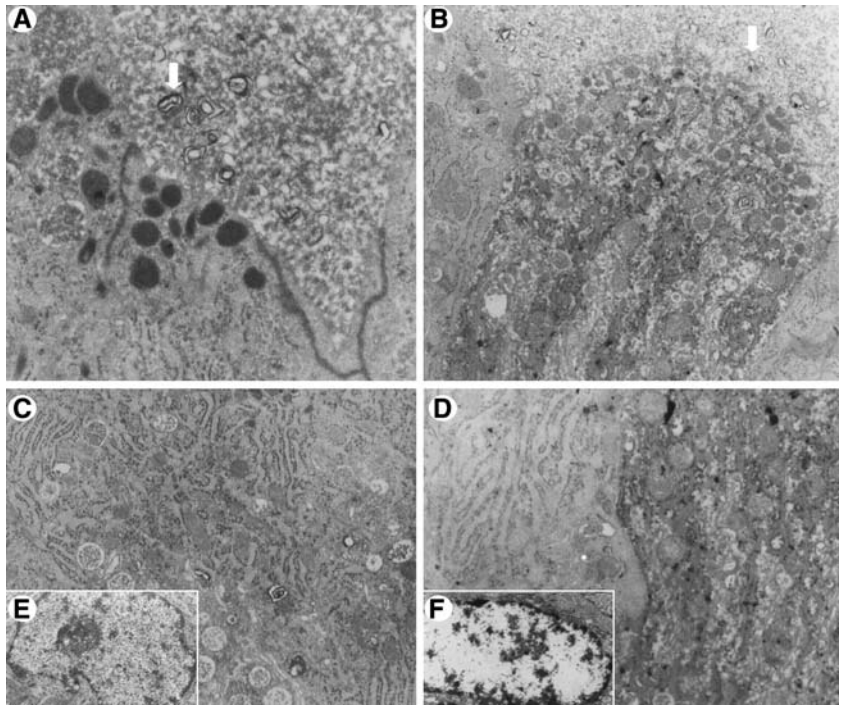

Figure 8. Effect of Cd on prostate ultrastructure. (A) Control rat (not exposed to Cd). Storage vesicles with a homogeneous content in the apical part of the cell. Presence of lipid droplets in the content of the lumen gland (arrow). × 12,000. (B) After 3 months of exposure to 100 ppm of Cd, the prostate is highly damaged. There is a dead cell with altered citoplasm and no apparent plasma membrane in the apical part. Fewer and smaller lipid droplets in the content of the luminal gland (arrow). The storage vesicles are not homogeneous and have less content when compared when the ones in the control cell. There are numerous vacuoles in the cytoplasm. × 12,000 (C) Higher magnification of a control cell shown in A. Presence of storage vesicles containing homogeneous secretion product and surrounding lipid droplets. Cytoplasm showing a developed RE and storage vesicles. (D) Cytoplasm portion of a normal and a dead cell from a Cd prostate. The cell on the right side shows a completely altered cytoplasm, without defined organelles and many vacuoles. On the left, it is possible to see a normal development of the RE and some storage vesicles. (E) Nucleus of the cell shown in A. Complex nucleolus characteristic of active cells. (F) Nucleus of the cell shown in B. Chromatin degradation and accumulation on the nuclear membranes. Absence of nucleolus.

to be prioritized, as shown by the increased CT expression.

The increase of phospholipids in Cd suggests a cellular alteration given the fact that maintaining a normal amount of membrane phospholipids, especially PC, is crucial for cell survival and replication (Caviglia *et al*. 2004). These alterations are observed in the ultrastructural study, supporting this idea.

On the other hand, Arienti *et al*. (1998) showed the importance of prostasome composition, where cholesterol would be the main component. Total cholesterol did not change in Cd group but the proportion of free cholesterol was increased, what could be produced by a lesser availability of fatty acids to esterify cholesterol, or to decreased cholesterol esterase activity. This also suggests that fatty acids may be mainly involved in phospholipid synthesis. Decreased esterified cholesterol would act as a signal to enhance the expression of SREBP-2, which leads to an increase of HMGCoAR expression, as it has been previously communicated (Brown & Goldstein 1999).

This decreased esterification could also be a consequence of the deviation of fatty acids toward an increased phospholipid synthesis. Based on our results, we can conclude that with this dose of cadmium the cholesterol/PC ratio is altered, which would lead to major changes in the composition of prostatic secretion, and might therefore affect spermatozoid viability.

We also studied the expression of transcription factors that regulate the lipid metabolism. PPARs are involved in diverse pathways, including lipid metabolism. PPAR-γ expression remained unchanged after Cd treatment, while PPAR-α

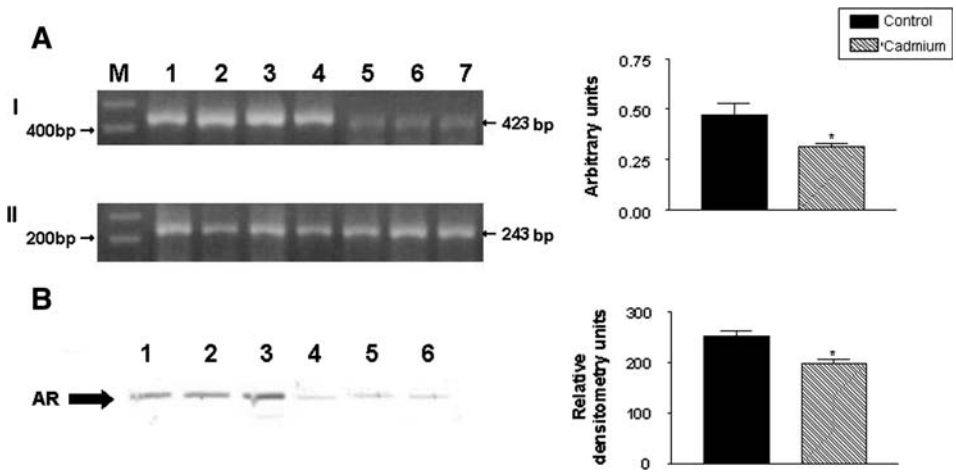

Figure 9. Effect of Cd exposure on prostate AR mRNA and protein abundances. (A) I: Ethidium bromide-stained agarose gel of AR PCR products. II: Ethidium bromide-stained agarose gel of β -actin PCR products, used as an internal control. M: molecular weight marker. Lanes 1–4: control prostates. Lanes 5–7: Cd prostates. On the right, quantification of the abundances of AR mRNA relative to β -actin. Values are expressed as means ± SEM for 3–4 rats on each group. Asterisk indicates $P < 0.05$ of significance when compared to controls. (B) Expression of AR protein in rat prostate. AR was detected by western blot. Densitometric analysis of the blots is represented as relative densitometry units. Bars represent mean ± SEM. One representative western blot of three experiments is shown. Asterisk indicates $P < 0.01$ of significance when compared to controls.

expression increased in the Cd group, what would justify the increased expression of SREBP-2, HMGCoAR and CT (Chawla *et al*. 2001).

These results suggest that there could be a change on membrane composition, that could lead to altered cell functioning and alter intrinsic membrane properties, such as fluidity, ion transport, enzyme activity and the aggregate state of cell surface determinant (Rong *et al*. 1996).

Martin *et al*. (2002) demonstrated that Cd induces a decrease on AR protein expression and on AR mRNA levels *in vivo*, what is coincident with our findings in Cd group. It is possible that part of the effects of Cd may be due to a diminished responsiveness of the prostate to androgens caused by reduced expression of AR. For example, the increased content of phospholipids could be a partial consequence of the diminished sensibility to androgens, as suggested by studies on castrated rats (Michaut *et al*. 1992; Ojeda *et al*. 1997). This is also consistent with the histological studies where we found an apparent state of involution in the prostates of rats exposed to Cd. Lipid composition changes can be correlated with the ultrastructural modifications in prostate cells exposed to Cd, which mainly show a change in the content of the lumen gland and a variation in the amount and content of the storage vesicles, which become few and heterogeneous.

In conclusion, the present findings suggest that chronic exposure to 0.886 mM of Cd produces changes in prostatic lipid metabolism regulation that result in increased fatty acid utilization for phospholipids synthesis in detriment of TG synthesis. These changes in lipids metabolism, in turn, could alter membrane composition and function as well as signaling through PC that result in deleterious histomorphological alterations in the gland. The modifications in prostate lipid metabolism and cellular ultrastructure might be mediated, at least in part, by the effects of Cd on the expression of androgen receptors and on factors that are known to regulate lipid balance and oxidative metabolism.

These results can be important to further understand the complex mechanism of cadmium toxicity in prostate and can be of help in the design of better treatments for people and animals exposed to the heavy metal.

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