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Cadmium exposure modifies lactotrophs activity associated to genomic and morphological changes in rat pituitary anterior lobe

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Abstract Cadmium (Cd) is widely used in industrial applications and is an important contaminant of agricultural products. As an endocrine disruptor, Cd modifies the hormone release of pituitary anterior lobe (PAL). This work was undertaken to evaluate a possible association between phospholipase D (PLD) and prolactin mRNA expressions and the activity of lactotrophs and folliculostellate cells (FSC) in PAL of Cd exposed adult male Wistar rats (Cd, 0.133 mM per liter for 2 months). The PALs were submitted to immunohistochemical and morphometric analysis to determine the percentage of lactotrophs (PRL-ir) and FSC (S-100-ir). Cultured PAL cells were stained with Hoechst 33258 to determine the presence of alterations in nuclear morphology consistent with apoptosis. The expressions of PLD and prolactin mRNA were assessed by RT-PCR. Cd treated rats showed a decrease of PLD mRNA levels that can be associated to both high number of apoptotic cells and increase of S-100 protein expression in FSC. Cd decreased prolactin mRNA expression, number of lactotrophs and percentage of PRL-ir suggesting a low availability of prolactin to be secreted from PAL. Cd modifies the lactotrophs activity of pituitary gland through biochemical, genomic and morphological changes and contributes directly or indirectly to the levels of serum prolactin.

Keywords Cadmium · Pituitary anterior lobe · Prolactin · Phospholipase D · Lactotrophs · Folliculostellate cells

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Introduction

Cadmium (Cd) has been shown to accumulate in various tissues such as kidney and liver (Kanwar et al. 1980) and more recently in hypothalamus, pituitary gland and gonads (Clark et al. 1985). The risk of Cd exposure comes from its high persistence in the tissues. The mean half-life of this metal is over 15 years in humans and over 200 days in rats (Kjellström and Nordberg 1978).

Cadmium exposure affects the pituitary gland and the reproductive function (Waalkes 2003), and is

considered an environmental endocrine disruptor that may play a role in the etiology of the pathology that involves the hypothalamic pituitary testicular axis (Lafuente and Esquifino 1999). In rats subchronically exposed to Cd during adulthood, an inhibitory effect of the metal on the pulsatile secretory pattern of prolactin from the pituitary gland has been observed, independently of the route of metal administration (Lafuente et al. 1997). Also, it has been reported that the acute, subchronic or chronic in vivo Cd administration reduces the plasma prolactin levels (Lafuente et al. 1997; Calderoni et al. 2005).

In mammalian cells, phospholipase D (PLD) is activated by various extracellular stimuli and is known to play an important part in signal transduction (Liscovitch et al. 2000; Morris 2007). The receptor-mediated PLD activation is considered to be implicated in a variety of cellular responses, including rapid responses such as secretion and cytoskeleton rearrangement, and long-term responses such as proliferation, differentiation, apoptosis and survival (Liscovitch et al. 2000; Lee et al. 2006; Jang et al. 2008). The PLD is involved in the regulation of the hormone secretion in endocrine cells (Chen et al. 1997). It has been found that the activity of PLD decreases in pituitary anterior lobe (PAL) of Cd exposed rats (Calderoni et al. 2005), which present low serum prolactin and growth hormone levels.

In addition to the secretory cells, the folliculostellate cells (FSC) have been reported in the pituitary of several mammalian species (Allaerts and Vankelecom 2005; Acosta and Mohamed 2009). They are star-shaped and follicle-forming cells in the anterior pituitary gland (Devnath and Inoue 2008) and are known to work as scavenger cells (Drewett et al. 1993). In particular, apoptotic endocrine cells are phagocytosed by FSC and digested by their lysosomal enzymes (Luziga et al. 2006; Devnath and Inoue 2008). It is known that Cd can bind to S-100 protein (Donato et al. 1991; Inoue et al. 2002), the main marker protein of FSC (FSC-S-100-ir) in the PAL (Itakura et al. 2007). However, the interaction between Cd and pituitary hormone is not well known.

To our knowledge there is not information about Cd toxicity on the mRNA expression of prolactin and PLD in PAL. The aim of this study was to determine a possible association of PLD and prolactin mRNA expressions with the activity of lactotrophs and FSC in PAL of Cd exposed rats, in order to explain part of the toxicity mechanisms of Cd in this pituitary lobe. Cd intoxication was induced in rats by administration (as $CdCl_2$) in the drinking water for 2 months.

Materials and methods

Chemicals

Cadmium chloride (as CdCl2 2.1/2 H2O) of 99.5% purity and organic solvents were obtained from Merck (Darmstadt, Germany). All the other chemicals were of reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Merck Laboratory (Buenos Aires, Argentina).

Animals and experimental model

Adult male Wistar rats of 300–350 g of body weight were maintained under standard laboratory conditions with ad libitum access to food (Cargill, Buenos Aires, Argentina) and water. They were housed and killed in accord to *The Guiding Principles in the Use of Animals in Toxicology* (Society of Toxicology 1999). All experiments were in compliance with the ANMAT No 6344/96 for Animal Care Guidelines, Argentina. Animals were kept in individual cages in a 22–25°C controlled environment with a light–dark cycle of 12 h each.

Cadmium intoxication was induced in 32 rats by administration in the drinking water of 0.133 mM per liter of Cd (as $CdCl_2$) for 2 months. Cadmium in drinking water and food of 32 control rats was not detectable (Calderoni et al. 2005).

The rats were sacrificed by decapitation under light anesthesia with diethyl ether to prevent changes in the histophysiology of the hormone producing cells which occurs with profound anesthesia. The pituitary gland was dissected, washed in a cold saline solution and the anterior lobe was removed. The samples were stored at −70°C until it was used for RNA extraction.

RNA isolation and reverse transcriptase polymerase chain reaction analysis for prolactin, phospholipase D2 and β-actin

Total RNA was isolated from anterior lobe of five pituitary glands in each case using TRIzol reagent

(Invitrogen, Carlsbad, CA, USA) within 1–2 week of obtaining the samples. All RNA isolations were performed as directed by the manufacturers. Electrophoresis on a 1% agarose gel and ethidium bromide staining confirmed the integrity of the RNA samples. Quantification of RNA was based on spectrophotometric analysis at 260/280 nm. Ten 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 manufacturer's instructions. RT-generated fragments code for prolactin, phospholipase (PLD2) and β-actin (Gen Bank accession no. NM012629, D88672 and M19533, respectively). Fragments coding for prolactin, PLD2 and β-actin were obtained by polymerase chain reaction (PCR) using the following primers: prolactin: (5′-CTTCTG TTCTGCCAAAATGTGC-3′ sense, and 5′-CGTTAG CAGTTGTTTTTATGGAC-3′ antisense); PLD2: (5′-CTATGGGGACCTGAACTC-3′ sense, and 5′-GAC TTTGTGTCTCTGGAGGTC-3′ antisense); and β-actin: (5′CGTGGGCCGCCCAGGCACCA-3′ sense, and 5′TTGGCCTTAGGGTTCAGAGGG-3′ antisense). PCR was performed in 35 μl of reaction solution containing 0.2 mmol/l of dNTPs, 1.5 mmol/l of MgCl2, 1.25 U of Taq polymerase, 50 pmol of each rat-specific oligonucleotide primer, and RT-generated cDNA (1/5 of RT reaction). The predicted sizes of the PCR-amplified products were 623 bp for prolactin, 338 bp for PLD2 and 243 bp for β-actin. 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, 60°C for prolactin, PLD2 and β-actin for 1 min; (3) extension, 72°C for 1 min. After 38 reaction cycles, the extension reaction continued for another 5 min (Thermal Cycler 2400, Perkin-Elmer, Shelton, CT, USA). Given that the number of cycles used was within the exponential phase of the PCR reaction, these conditions allowed detection of both cDNAs in the linear range of the assay. In all cases, aliquots of PCR products (5 μl) were run in triplicate on 1% (w/v) agarose gel with 0.01% (w/v) ethidium bromide. The amplified fragments were visualized under ultraviolet transillumination. Digitized images of the gels, which are presented in the negative form, were obtained with a Kodak EDAS 120 system. The intensity of each band was measured using the NIH Image software and reported as the values of band intensity units. The relative abundance of each band was normalized according to the housekeeping β-actin gene, calculated as the ratio of the mean of gray value of each product to that of β-actin.

Immunohistochemistry

The brain was rapidly exposed and the pituitary gland was excised, sagittally sectioned, fixed in Buin's fluid, processed for light microscopy, embedded in paraffin and serially sectioned in the horizontal plane. The immunohistochemistry technique was carried out as follows: serial sagittal sections (5 μm thick) were cut and carried through xylene and graded alcohols to water. Slides were incubated for 20 min in a solution of 3% H_2O_2 in water to inhibit endogenous peroxidase activity. Then, they were rinsed with distilled water and phosphate buffered saline (PBS; 0.01 M, pH 7.4). Non-specific binding sites for immunoglobulins were blocked by incubation for 15 min with 0.25% casein in PBS and rinsed with distilled water and PBS. Sections were then incubated overnight in a moist chamber at 4°C with the following primary antisera: monoclonal anti-prolactin hormone (DAKO, California, USA) and polyclonal anti-S-100 protein (BioGenex, San Ramon, California, USA). After the slides were rinsed with PBS for 10 min, the immunohistochemical visualization was carried out using the Super Sensitive Ready-to-Use Immunostaining Kit (BioGenex) at 20°C. The biotin-streptavidin amplified system was used as follows: sections were incubated for 30 min with diluted biotinylated anti-mouse IgG and, after being washed in PBS, they were incubated for 30 min with horseradish peroxidase-conjugated streptavidin, and finally washed in PBS. The reaction site was revealed by 100 μl of a 3.3′diaminobenzidine-tetrahydrochloride chromogen solution in 2.5 ml PBS and 50 μl of an H_2O_2 substrate solution. The sections were counterstained with hematoxylin for 1 min, dehydrated and mounted.

In order to confirm the specificity of the immunoreactive procedures, adjacent sections were stained according to the above described protocol, but incubation in the primary antiserum was omitted. In addition, normal rabbit serum was used instead of primary antiserum. No positive structures or cells were found in these sections (Fig. 1).

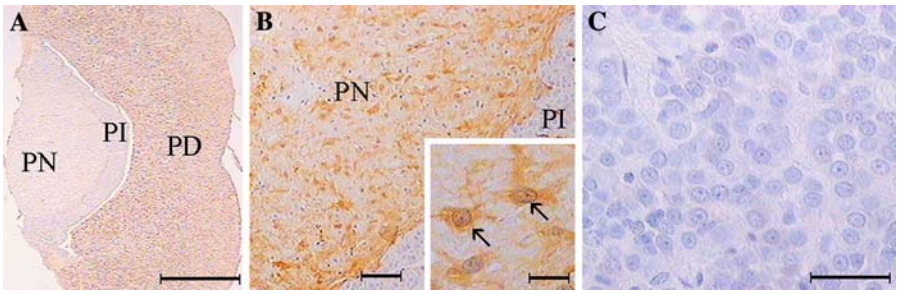

Fig. 1 **a** Pituitary gland of control rat immunostained with anti-Prl. *PN* pars nervosa; *PI* pars intermedia; *PD* pars distalis. *Scale bar*: 250 μm. **b** Positive control of immunostaining for S-100 protein in PN. *Scale bar*: 50 μm. Right inset: Pituicytes immunostained with anti-S-100. *Scale bar*: 12.5 μm. **c** Negative control of immunoperoxidase staining. *Scale bar*: 25 μm

Morphometric analysis

Computer-assisted image analysis system was used to measure the different morphometric parameters. The system consisted of an Olympus BX-40 binocular microscope, interfaced with a host computer, image processing and recording system. The images were captured by a Sony SSC-DC5OA camera and processed with Image Pro Plus 5.0 software under control of a Pentium IV computer. The software allowed the following processes: images acquisition, automatic analogous adjust, thresholding, background subtraction, distance calibration, area and diameter measuring, and diskette data logging. The images were displayed on a colour monitor, and the parameters were measured with the image analysis system. Before counting, a reference area (RA) of 18,141.82 μm^2 ($\times$40 objective) was defined on the monitor, and distance calibrations were performed using a slide with a micrometric scale for microscopy (Reichert, Austria). The lactotrophs and folliculostellate cells morphometric study was carried out as follows: six tissue sections from a pituitary gland were used, and all the microscopic fields captured with $\times$40 objective were analyzed in every section (70 microscopic fields according to the section). Therefore, 420 microscopic fields were analyzed in each gland, and 4 pituitary glands were studied in each group of animals. Finally, 1,680 microscopic fields or measures were carried out per group. The following morphometric parameters were determined.

Lactotrophs

Percentage of Prl-positive area (%IA-Prl) was calculated using the formula %IA-Prl = ΣAc/ΣRA $\times$ 100, where ΣAc was the sum of the area of immunolabelled cells for anti-Prolactin and ΣRA was the sum of the PAL area of every microscopic field.

The number of immunostained lactotrophs with a visible nucleus was counted in ten microscopic fields per section. The result was expressed as number of lactotrophs per reference area.

Folliculostellate cells

Percentage of S-100-positive area (%IA-S-100) was calculated using the formula %IA = ΣAi/ΣRA $\times$ 100, where ΣAi was the sum of the area of immunopositive cells for anti-S-100 and ΣRA was the sum of the PAL area of every microscopic field.

Apoptosis determination

In the present study fluorescence microscopy for confirming apoptotic bodies as a result of morphological change in the nucleus was employed. The cells were obtained from PALs by enzymatic (trypsin/DNase) and mechanical dispersion (extrusion through a Pasteur pipette) as described previously (Velardez et al. 2003). In all cases the cells were cultured for 3 h (37°C, 5% CO_2 in air) in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 μg/ml gentamicin and 50 μg/ml penicillin (DMEM-S-10% FBS). Cultured cells were stripped off from plastic plates by cell remover (Costar, Cambridge, MA) and fixed with 4% (wt/vol) of neutral buffered formalin for 30 min. After fixation, they were washed by PBS twice, stained with 0.6 mg/ml Hoechst 33258 (Wako Chemical Co.), and placed on glass slides. The

images were taken, at 400× magnification under a fluorescence microscope (NIKON Eclipse 50i) of 500 nuclei from control and experimental cells respectively, determining the percentage of apoptotic bodies (fragmented and condensed nuclei) in both cellular groups.

Statistical analysis

The results were expressed as mean ± standard error of the mean for all data sets. Differences between groups were evaluated using Student's *t*-test, $P < 0.05$ was assumed to be significant. The software GraphPad Prism (v. 3.02) was used for the statistical analysis of morphometric measurements.

Results

Effects of Cd exposure on the prolactin and phospholipase D mRNA expression

The expression of prolactin and PLD mRNA decreased in the PAL of Cd treated rats in relation to the controls (Fig. 2), suggesting that prolactin and PLD synthesis might be decreased.

Immunohistochemical and morphometric analysis

The percentage and the number of lactotrophs (Prl-ir) per reference area decreased significantly ($P < 0.01$ and $P < 0.001$; respectively) in the Cd treated group (Table 1; Fig. 3). Moreover, the percentage area FSC-S-100-ir increased significantly ($P < 0.01$) in Cd treated rats in relation to the controls (Table 1; Fig. 3). The distributions of values for the morphometric parameters (percentage of Prl-positive area, number of lactotrophs per reference area and percentage of FSC-S-100-positive area) in the control and cadmium groups are shown in the box plots of the Fig. 4.

Effects of Cd on the amount of apoptotic nuclei

The percentage of apoptotic cells (fragmented and condensed nuclei) of PAL was increased significantly ($P < 0.001$) in Cd exposed rats in relation to the controls (Table 1; Fig. 3).

Discussion

We have measured different endocrine parameters in PAL after administration of Cd in the drinking water to understand the interaction between this metal and

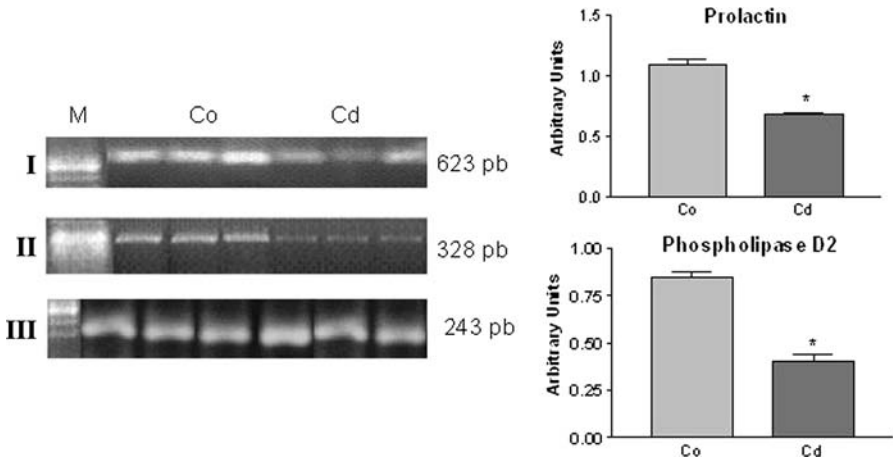

Fig. 2 Effect of Cd treatment on the mRNA expression of prolactin and phospholipase D2 in PAL. RT-PCR analysis for *I* prolactin; *II* phospholipase D2 and *III* β-actin, used as internal control. *M* molecular weight marker; *Co* control rats; *Cd* cadmium-treated rats. On the side, the quantification of the intensity of the fragment bands in relation to the intensity of the β-actin, is shown. Values are means with their standard errors depicted by *vertical bars* for three determinations using a pool of five PAL each. *$P < 0.001$ versus control

Table 1 Morphometric parameters of lactotrophs, folliculostellate cells (FSC) and percentage of apoptotic cells (fragmented and condensed nuclei; Hoechst 33258)

	Parameters	Control	Cadmium
Lactotrophs	%IA-Prl	16.17 ± 0.17	10.13 ± 0.13**
	n° cells/RA	18.29 ± 0.21	9.90 ± 0.10*
FSC	%IA-S-100	4.73 ± 0.13	8.29 ± 0.10**
Hoechst 33258 (%AC)		3.15 ± 0.03	15.20 ± 0.10*

The values are expressed as mean ± SEM. %IA-Prl: percentage of Prl-positive area. n° cells/RA: number of lactotrophs per reference area. %IA-S-100: percentage of FSC-S-100-positive area. %AC: percentage of apoptotic cells. Significant differences were determined by Student's *t*-test

* $P < 0.001$ and ** $P < 0.01$ versus control

the endocrine function of pituitary gland. Our results show that Cd modifies the mRNA expression of PLD and prolactin, and the number of lactotrophs and FSC cells in the PAL. The relation between them can explain part of the toxicity mechanisms of Cd, such as reduction of prolactin secretion and apoptosis induction.

Several researchers have done experiments using in vitro models with endocrine cultured cells exposed to Cd for different times (Yang et al. 2005; Poliandri et al. 2006). In the present work we used an in vivo model of Cd intoxication induced by administration in the drinking water of 15 ppm of Cd as $CdCl_2$ for 8 weeks. Under this condition we have previously

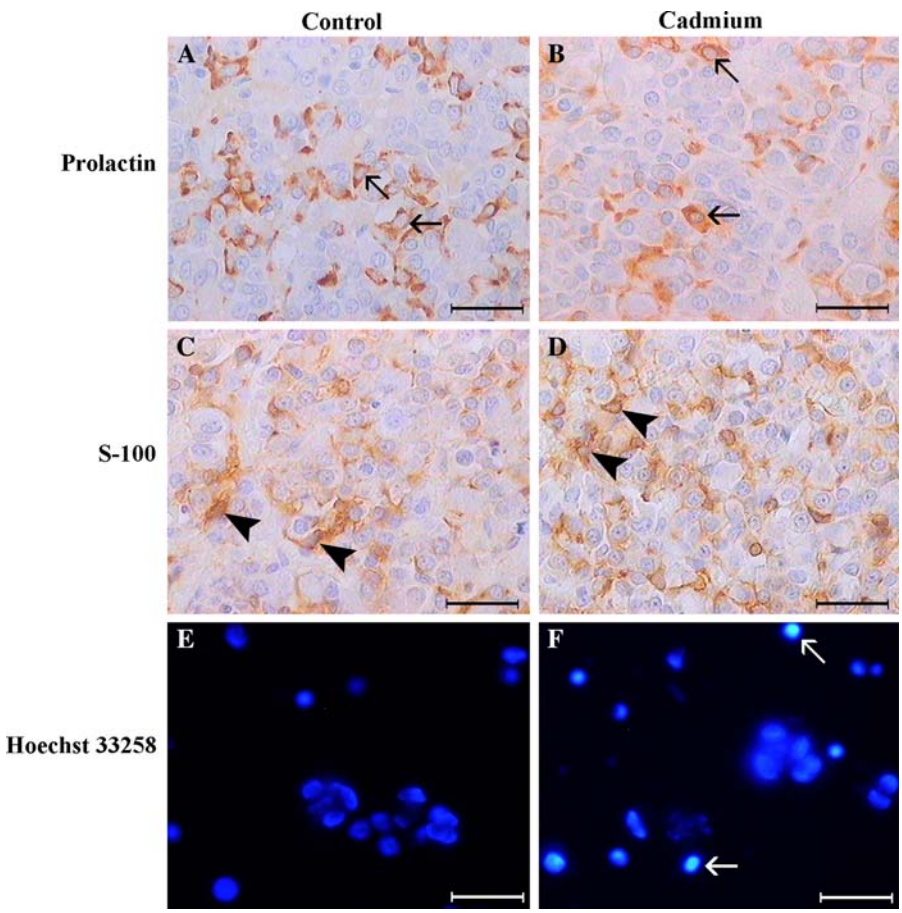

Fig. 3 **a**, **b** Pituitary pars distalis immunostained with anti-Prl. The percentage of Prl-positive area and number of lactotrophs (*arrows*) in the cadmium-treated group (**b**) are lower than control rats (**a**). **c**, **d** The cadmium treatment increases the S-100 protein expression (*arrowheads*) in the folliculostellate cells of the pituitary pars distalis. **e**, **f** Alterations in nuclear morphology consistent with apoptosis were observed under fluorescence microscope after staining with Hoechst 33258. The percentage of fragmented and condensed nuclei (*white arrows*) is higher in cadmium-treated (**f**) than control (**e**) rats. *Scale bars*: 25 μm

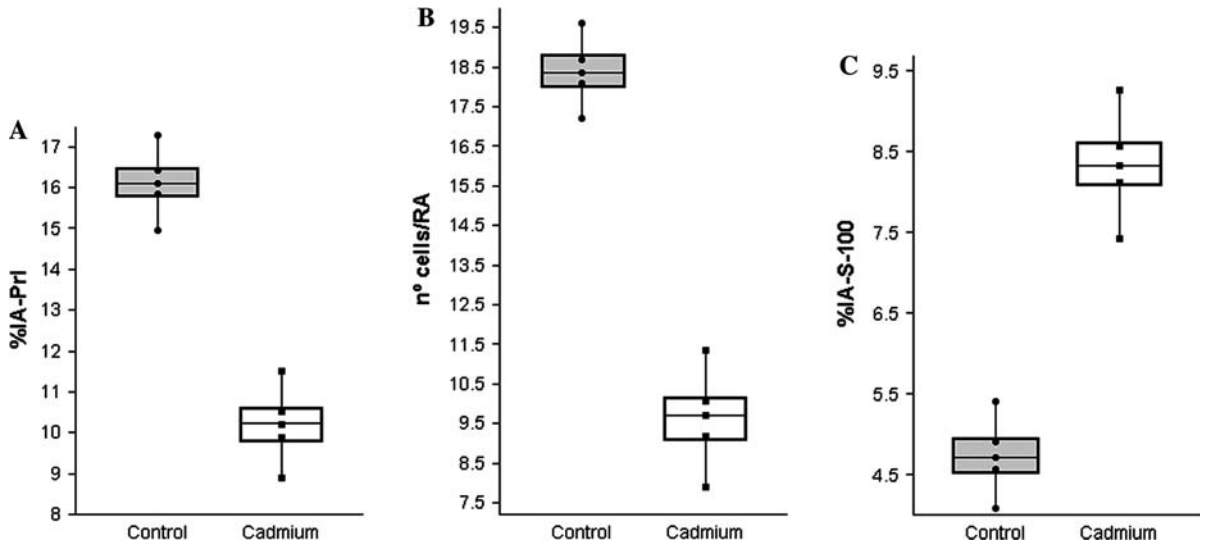

Fig. 4 *Box* Plots of the values for the morphometric parameters in the control and cadmium groups. **a** The percentage of Prl-positive area (%IA-Prl). **b** Number of lactotrophs per reference area (n° cells/RA). **c** Percentage of FSC-S-100-positive area (%IA-S-100)

informed that the level of Cd in plasma (Larregle et al. 2008) is similar to that observed in plasma of alkaline battery assemblers (Jakubowski et al. 1987). With the same experimental model we have observed an accumulation of the metal in PAL of 6.06 ± 0.02 μg/g of tissue while Cd is not detected in the control (Calderoni et al. 2005).

The decrease in the expression of PLD mRNA in PAL of Cd treated rats was coincident with a decreased of PLD activity previously reported both in PALs from Cd exposed rats and in PAL dispersed cells treated with Cd (Calderoni et al. 2005), suggesting that Cd could modify PLD at genomic and no genomic levels.

It is known that PLD is involved in the regulation of hormone secretion in endocrine cells (Chen et al. 1997). In mammalian cells, activation of a Golgi-associated PLD by ADP-ribosylation factor results in the hydrolysis of phosphatidylcholine to form phosphatidic acid. This reaction stimulates the release of nascent secretory vesicles from the trans-Golgi network of endocrine cells (Siddhanta et al. 2000; Riebeling et al. 2009). Furthermore, prolactin is synthesized in PAL and Cd decreases the percentage of Prl-ir, the number of lactotrophs and the expression of mRNA of prolactin, suggesting a reduced cellular activity. Considering all the above information and our previous results showing a decrease of serum prolactin in Cd treated rats (Calderoni et al. 2005) it is possible to associate the genomic effects of Cd with a reduced prolactin secretion from PALs. Thus, the activation of PLD could be one of the factors needed for secretion of prolactin. We have previously determined an alteration of cholesterol/phospholipids ratio in PALs of Cd treated rats compared with controls (Calderoni et al. 2005). Thus, we can not discard that changes in membrane fluidity of pituitary gland may directly or indirectly alter the hormone secretions in Cd exposed rats.

Moreover, we found that administration of Cd in the drinking water increased the number of apoptotic nuclei, as have been found by other authors in cell culture (Poliandri et al. 2006), and also the expression of S-100 protein in PAL. It is known that apoptotic endocrine cells are phagocytosed by FSC and digested by their lysosomal enzymes (Luziga et al. 2006; Devnath and Inoue 2008). Thus, we can suggest that the increment observed in the expression of S-100 protein is a response to the increment of apoptotic cells that are scavenger by FSC cells in the Cd treated animals. Probably, the increased apoptosis is related to the low mRNA expression/activity of PLD found in PAL of Cd intoxicated rats since in addition to the hormone secretion PLD has been reported to have an anti-apoptotic role in different cells (Lee et al. 2006; Jang et al. 2008).

To our knowledge this is the first time that chronic in vivo Cd administration is shown to affect the

mRNA expressions of PLD and prolactin in PAL. The results suggest that the decrease of the mRNA expression of PLD, and the decrease of PLD activity previously showed (Calderoni et al. 2005), could explain, at least in part, the apoptosis induced by Cd toxicity in PAL. Furthermore, Cd affects the pituitary gland by altering prolactin expression and number of lactotrophs suggesting a low availability of prolactin to be secreted from PAL. This could also be associated to the PLD changes induced by Cd. Finally, our results suggest that Cd modifies the lactotrophs activity of pituitary gland through biochemical, genomic and morphological changes, contributing directly or indirectly to the levels of serum prolactin.

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