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Alterations in the lipid content of pituitary gland and serum prolactin and growth hormone in cadmium treated rats

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Abstract

The present study was undertaken to assess whether chronic exposition to cadmium (Cd, 0.133 mM per liter for 2 months) through drinking water may affect the lipid contents in the pituitary anterior lobe (PAL) of adult male Wistar rats. As compared to metal non-exposed controls, PALs exposed to cadmium showed an increase in total phospholipid contents, which was associated to an increase of the incorporation of [1-^{14}C]-methyl choline into phosphatidylcholine and of [U-^{14}C]-glucose into total phospholipids. The incorporation of [1-^{14}C]-methyl choline into sphingomyelin was not changed. Incorporation of [1-^{14}C]-acetate into total fatty acids also increased but incorporation of [1-^{14}C]-acetate into cholesterol did not change. The activity of phospholipase D decreased both in PALs from Cd exposed rats and in PAL dispersed cells treated with Cd in the culture medium from Cd non-exposed rats. In PALS from Cd exposed rats, a decrease of serum prolactin and growth hormone concentrations was determined. The results shown that cadmium modifies the lipid contents of pituitary gland and directly or indirectly the levels of prolactin and growth hormone in serum.

Introduction

Cadmium (Cd) is one of the most toxic industrial and environmental metals, and represents a continuing health hazard (Hart *et al.* 1989; Trzcinka-Ochocka *et al.* 2002; Sorahan & Esmen 2004). In the human body, Cd has a biological half-life of over than 20 years (Sugita & Tsuchiya 1995). Also, it is well established that Cd is one of the major contaminants of tobacco smoke (Li *et al.* 2000).

Cd ions are taken up through calcium channels of the plasma membranes of various cell types, and are accumulated intracellularly due to their binding to cytoplasm and nuclear substances (Beyersmann & Hechtenberg 1997; Saderholm *et al.* 2000). This metal causes a number of lesions in different organs as liver, kidney and lung, depending of exposition dose, time and administration route (Shukla *et al.* 1989; Diamond *et al.* 2003). Cd has also been shown to affect cell physiology and growth (Ramirez & Gimenez 2000; Lafuente *et al.* 2003). However, little is known about the mechanisms that induce alterations in tissue metabolism.

Cadmium induces histopathological damage and oxidative stress through free radicals over production (Méndez-Armenta *et al.* 2003). Cd

exposure affects the pituitary gland and the reproductive function (Benoff *et al*. 2000; 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 *et al*. 2001).

There is considerable information about the effect of Cd on the amount of lipids in different organs. In rat brain Cd treatment decreases free cholesterol, phosphatidylethanolamine and other lipids implicated in myelinization (Kumar *et al*. 1996). Cadmium 10 ppm in the drinking water shows a significant reduction of total lipids in fetal brain, with a decrease of phosphatidylcholine and an increase of phosphatidylethanolamine (Gulati *et al*. 1987). In our laboratory we have observed that Cd decreases the amount of phospholipids in peritoneal macrophages (Ramirez & Giménez 2002).

The interaction between Cd and pituitary hormone is not well known (Lafuente *et al*. 2001, 2003). There is no information about its effect on the lipid compositions of this gland, particularly in the anterior lobe. The importance of the effect of Cd on lipids lies in the fact that they are components of cell membranes that act as a barrier between the cell and its environment. On the other hand, it is known that phosphatidic acid and phospholipase D (PLD) are involved in the regulation of the hormone secretion in endocrine cells (Chen *et al*. 1997). The pituitary anterior lobe (PAL) is characterized by having a specialized function and an heterogeneous cell population. Both prolactin and growth hormone are synthesized in this lobe. It is known that phosphatidic acid obtained from phosphatidylcholine is necessary for the secretion in endocrine cells (Athenstaedt & Daum 1999). The inhibition of phosphatidic acid synthesis alters the structure of Golgi apparatus and inhibits endocrine cell secretion (Sidhanta *et al*. 2000). In addition, PLD is a phospholipid hydrolyzing enzyme whose activation generates phosphatidic acid, which has been implicated in mediating signal transduction pathways, cell growth and membrane trafficking in mammalian cells (Senogles 2000).

The intracellular mechanisms involved in the action of Cd are not well understood yet and many pathways are probably involved in its cytotoxic effects (Poliandri *et al*. 2003, Ramirez & Giménez 2003). The aim of this paper is to determine if chronic administration of cadmium in drinking water modifies the lipid composition of the pituitary gland and also, to study the possible association of this effect with hormone secretion. The effect of Cd on the activity of PLD is established.

Materials and methods

Chemicals and radioisotopes

Cadmium chloride (as $CdCl_2$ 2.1/2 H_2O) of 99.5% purity and organic solvents were obtained from Merck (Darmstadt, Germany). Dulbeccós modified Eaglés medium (DMEM) and fetal calf serum (FCS) were purchased from Gibco BRL. [^{14}C]-*methyl*-choline, [^{14}C]-acetate, [1-^{14}C] glucose and [1-^{14}C] myristic acid were purchased from Amersham Co (Arlington, Heights, IL). Lipid standards and antibiotics were acquired from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All the other chemicals were of reagent grade and were obtained from 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 N^{o} 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.

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

Cadmium determination in PAL

Cd was determined by atomic absorption with an electrotermic atomizer (EAA-GF). The samples were dried at 60–70 °C for 24 h. After that, they were treated with nitric acid/perchloric acid for destruction of organic matter. The residue was dissolved in 5 ml of 1% nitric acid before applied or injected in the atomizer. The determinations were done by triplicate in an UNICAM 959, Grafite Furnae-90 equipment with autosampler and automatic dilutor, by the EPA-600/4-82-055 method. Optimum work range 0.25–10 μg/l. Detection limit 0.05 μg/l. Quantification limit 0.15 μg/l. (Crawford & Luoma 1993).

Lipid analysis

Four PAL were homogenized in 0.5 ml of buffer TRIS/HCL 0.1 mM pH 7.2; one aliquot was used for protein determination and the another one was added with 0.5 ml of hexane:isopropanol mixture (3:2, v/v) containing butylated hydroxytoluene as antioxidant. The extracted lipids were dried down in a stream of nitrogen and then dissolved in hexane. Aliquots were taken for determining phospholipids, measuring phosphorous (Rausser *et al.* 1970) and total cholesterol (Zack *et al.* 1954) after Abell saponification (Abell *et al.* 1952).

Fatty acid composition of PAL

Four PALs were homogenized and saponified by treatment with 10% (w/v) KOH in ethanol plus 500 μl of methanol in order to facility the subsequent extraction step. The fatty acids were recovered after acidification with HCl and extracted with petroleum ether (bp 30–40). Free fatty acids were esterified 1 h at 64 °C with boron trifluoride solution (20% in methanol). Fatty acids methyl ethers from these samples were analyzed by gas liquid chromatography (GLC) in a Varian 3300 Chromatograph (Barckeley, CA) equipped with a flame ionization detector: 250 °C and 10% SP-2330 columns. Outer diameter (OD): $1/8^{11}$. The injection temperature was 225 °C and the oven temperature was 185 °C. Chromatograph range: 10^{-11}. The amount of fatty acids methyl esters expressed as percentage of the total fatty acids.

Incorporation of [^{14}C]-methyl-choline into phosphatidylcholine and sphingomyelin

Two PALs were incubated for 10 min at 37 °C in a 95% air-5% CO_2 atmosphere in the DMEM red-phenol free supplemented with 1% FCS, 50 μg/ml gentamicin, 50 μg/ml penicillin, and 50 μg/ml fungizone. After that, the medium was replaced by 1 ml of fresh medium added with 1 μCi of [^{14}C]-*methyl*-choline and samples were incubated for 60 min. The reaction was stopped by addition of 0.2 ml of 6 N sulfuric acid and the PALs were washed with saline solution until no more radioactivity was detected in the wash water. Total lipids were extracted from PALs (as described above) and phospholipids were separated into component species by TLC using silica gel H plates and chloroform:methanol:water (65:25:4, v/v/v) as solvent system. Duplicate samples were used to separate the different phospholipids. The spots were detected using iodine vapors. Phosphatidylcholine and sphingomyelin spots were then scraped and its radioactivity were quantified in a Beckman LS 100 C Liquid Scintillation Counter. Duplicate samples were used for determining phosphorus.

Incorporation of [^{14}C]-glucose into phospholipids

Two PALs were incubated with 1 μCi of [^{14}C]-glucose in the culture medium for 60 min as indicated above. At the end of incubation time, the culture medium was taken off and the PALs were washed with saline solution until no radioactivity was detected in the wash water. Total lipids were extracted from PALs (as described above) and one aliquot was taken for measuring the total radioactivity incorporated into the tissue and other one to separate total phospholipids by TLC in chloroform:methanol:acetic acid:water (50:25:8:4, v/v/v/v) solvent system. Duplicate samples were used. The spots were detected using iodine vapors. One phospholipid spot was then scraped for quantifying its radioactivity in a Beckman LS 100 C Liquid Scintillation Counter, while the other one was used for determining phosphorus.

Incorporation of [^{14}C]-acetate into saponifiable and non-saponifiable lipid fractions

Two PAL were incubated with 1 μCi of [^{14}C]-acetate in the culture medium as was described

above, during 180 min. At the end of incubation time, the culture medium was taken off and the PALs were washed with saline solution until no more radioactivity was detected in the wash water. The tissue was submitted to saponification by treatment with 1 ml of 10% (w/v) KOH in ethanol plus 1 ml of methanol in order to facility the subsequent extraction step. The fatty acids were recovered from the saponifiable fraction, after acidification with HCl and extracted with petroleum ether (bp 30–40). The insaponifiable fraction was used for measuring the radioactivity incorporated in cholesterol. This fraction was dried down in a stream of nitrogen and then dissolved in hexane. The cholesterol was separated by TLC in a solvent system of *n*-hexane:diethyl ether:acetic acid (80:20:1, v/v/v). The spots were detected using iodine vapors. Duplicate samples were used. The radioactivity incorporated was quantified in a Beckman LS 100 C Liquid Scintillation Counter.

Determination of the activity of PLD in culture cells

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, μg/ml penicillin, and 25 μg/ml gentamicin (DMEM-S–10% FBS) and 1 μCi of [^{3}H]myristate/ml for labeling the phosphatidilcholine. The activity of the enzyme was determined on the basis of its transphosphatidylation activity which leads to production of [^{3}H]-phosphatidylethanol when cells containing [^{3}H]-phosphatidilcholine are incubated in the presence of ethanol (Martin *et al.* 1993). The radioactive medium was then aspirated and the cells washed twice with non-radioactive DMEM. Ethanol (1% final concentration) was added 5 min prior to the addition of Cd. The cells were incubated for 1 h, then washed once with ice-cold Ca-free PBS and extracted with chloroform:methanol as follows. Cells were scraped into 0.5 ml of methanol and wells were washed with further 0.5 ml of methanol. The two aliquots were combined and mixed with 0.5 ml of chloroform. Lipids were extracted as described above and separated by TLC using silica gel 60 coated glass plates. TLC plates were developed 50% of their lengths with chloroform:methanol:acetic acid (9:1:1, v/v/v) and then dried. The plates were then redeveloped up to their full length with petroleum ether:diethyl ether:acetic acid (60:40:1, v/v/v). The position of the lipids was identified after staining with I_2 vapor by comparison with authentic standards. Radioactive lipids were quantified after scraping from the plates by liquid scintillation counting. Results were calculated as a percentage of the radioactivity present in [^{3}H]-phosphatidylethanol compared to that in total lipids.

The activity of PLD measured in PALS from Cd exposed rats (*in vivo* experiment) was determined using the same procedure as in disperse cells.

Determination of prolactin and growth hormone in serum

PRL and GH were measured by double antibody radioimmunoassay, using materials generously provided by Dr. Parlow and the NHPP (National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA, USA). The hormones were radio-iodinated using the Chloramine T method and purified by passage through Sephadex G75. The results were expressed in terms of rat prolactin RP-3 or rat GH RP-2 standard preparations. Assay sensitivity was 0.5 μg l^{-1} serum and the inter- and intra-assay coefficients of variation were less than 10% for both hormones.

Protein assay

Proteins were determined by Lowrýs, using bovine serum albumin (BSA) fraction V as standard.

Statistical analysis

Results were expressed as mean ± SEM. Statistical differences were tested by Student's test for non-correlated data. Differences between groups were considered significant if $P < 005$.

Results

Cadmium content in PAL

The administration of cadmium through drinking water caused an accumulation of the metal in PAL

of 6.06 ± 0.02 μg/g of tissue. Cd was not detected in the control.

Phospholipid, cholesterol and protein contents in PAL

During the experimental period the body and pituitary gland weights of rats were not modified by Cd treatment. There was no difference in the amount of water drunk by Cd exposed and control rats. PALs from rats that drank water contaminated with Cd showed an increment of total phospholipid content in relation to control rats. No differences in the cholesterol concentration were observed between rats that drunk contaminated water and control rats. Consequently, the total cholesterol/phospholipids ratio was decreased. The amount of proteins was not modified by cadmium treatment (Table 1).

Incorporation of [^{14}C]-methyl-choline into phosphatidilcholine and sphingomyelin

The uptake of [^{14}C]-*methyl*-choline by PAL was not modified but its incorporation into phosphatidilcholine increased in PAL of cadmium treated rats as compared to controls. Cadmium did not modify the incorporation of [^{14}C]-*methyl*-choline into sphingomyelin (Table 2).

Incorporation of [^{14}C]-glucose into phospholipids

The uptake of [^{14}C]-glucose was not modified by Cd treatment but its incorporation into total phospholipids in PALs from Cd treated rats was significantly higher than in PALs from control rats. No modification in the label of free and esterified cholesterol was observed (Table 2).

Incorporation of [^{14}C]-acetate into fatty acids

The incorporation of [^{14}C]-acetate into fatty acids was increased in PALs from rat exposed to Cd compared with the controls, but was not modified in cholesterol (Table 2).

Determination of PLD in vivo *and* in vitro

The activity of PLD decreased in both *in vivo* and *in vitro* exposition to cadmium compared with the respective controls (Table 2).

Determination of fatty acid composition

The fatty acid composition of the lipid fraction of PAL is shown in Table 3. Palmitic, stearic, oleic, linoleic and arachidonic acids were determined. PALs of Cd exposed rats showed no significant differences in the quantity of fatty acid in relation to the controls, expressed as percentage of the total fatty acids.

Determination of PRL and GH in serum

The serum concentrations of PRL and GH from rats exposed to cadmium were decreased in relation to the controls (8 rats/group), 4.35 ± 0.20 vs.

Table 1. Effect of cadmium in the drinking water on body and pituitary weights, and lipid and protein contents of the PAL.

Parameters	Control	Cadmiun	
Pituitary weight (mg)[a]	4.6 ± 0.08	5.2 ± 0.19	n.s.
Rat body weight (g)[a]	320.56 ± 12.32	367.89 ± 15.14	n.s.
Drinking water daily (ml)[a]	28.50 ± 1.82	26.90 ± 2.04	n.s
Phospholipid content[b] (μg/mg tissue)	10.71 ± 0.17	27.50 ± 1.05	<0.001
Cholesterol content[b] (μg/mg tissue)	33.04 ± 1.33	37.62 ± 1.62	n.s.
Cholesterol/phospholipids[b]	3.25 ± 0.17	0.73 ± 0.02	<0.001
Protein content[b] (mg/mg tissue)	0.22 ± 0.03	0.22 ± 0.04	n.s.

The results are means ± SEM for [a]thirty two rats per group and [b]eight determinations using a pool of four PAL each. The *P* values indicate significant differences between Cd treated and non-treated animals (control group). n.s., not significant.

Table 2. Effect of cadmium in the drinking water on the labeling of lipids and PLD activity of rat PAL.

	Control (cpm/h/mg tissue)	Cadmium	*P*
Incorporation of			
[^{14}C]-choline into PC	1232.80 ± 90.50	1785.30 ± 100.20	< 0.001
[^{14}C]-choline into SM	204.96 ± 25.80	239.39 ± 10.15	n.s.
[^{14}C]glucose into Total Phospholipids	2376.89 ± 108.03	3585.45 ± 136.49	< 0.001
[^{14}C]acetate into Fatty acids	1282.50 ± 85.30	1479.30 ± 75.20	< 0.05
(Relative PLD activity, total lipid %)[a]			
PLD activity			
In vivo	23.30 ± 3.00	8.00 ± 2.00	< 0.001
In vitro	35.00 ± 3.00	12.00 ± 1.00	< 0.001

The results are means ± SEM for eight determinations using a pool of four PAL each. PC, phosphatidylcholine. SM, sphingomyelin.
[a]The enzyme activity is expressed as a percentage of the radioactivity present in [^{3}H]-phosphatidylethanol compared to that in total lipids. n.s., not significant.

Table 3. Effect of cadmium in the drinking water on fatty acids composition of PAL.

	Fatty acid[a] (mol%)		
	Control	Cadmium	
16:0	30.00 ± 0.75	30.67 ± 1.36	n.s.
18:0	25.37 ± 1.10	25.77 ± 0.35	n.s.
18:1 (*n*-9)	15.55 ± 0.22	14.97 ± 0.82	n.s.
18:2 (*n*-6)	5.44 ± 0.77	5.04 ± 0.30	n.s.
20:4 (*n*-6)	23.64 ± 1.17	23.55 ± 1.20	n.s.

Values are means ± SEM for four experiments using a pool of four PAL in each, control and cadmium treated rats.
[a]Number of carbon atoms:number of double bonds, followed by the position of the first double bond relative to the methyl end (*n*−) of the fatty acid. n.s., not significant.

17.5 ± 3.20 ng/ml and 3.03 ± 0.42 vs. 9.37 ± 1.28 ng/ml, respectively.

Discussion

Our results show that cadmium ingested through drinking water selectively modified the lipid composition of PAL. Simultaneously, a decrease in the PRL and GH concentrations in serum was observed. The increment in the amount of total phospholipids of PAL in presence of Cd can be related to the increased synthesis of phosphatidylcholine that was determined by the ^{14}C-choline incorporation and also, to the increased incorporation of [^{14}C]-glucose into total phospholipids. In addition, the increment in the incorporation of [1-^{14}C]sodium acetate into fatty acids suggests a high availability of these acids for phospholipid synthesis. The accumulation of phospholipids could be associated to the perturbation of PRL and GH release. It is known that phospholipid metabolism plays a key role in regulating intracellular vesicular transport, particularly in distal steps of the secretory pathway (Roth 1999). In addition, a decreased PLD activity caused by Cd was determined in PAL through *in vivo* and *in vitro* experiments. Mammalian phosphatidilcholine-specific PLD has been implicated in a wide range of physiological responses including metabolic regulation, cell proliferation, mitogenesis, oncogenesis, inflammation, secretion and other processes as exocytosis and endocytosis (Exton 1994; Liscovitch *et al.* 2000). In mammalian cells, activation of a Golgi-associated PLD activity by ADP-ribosylation factor results in the hydrolysis of phosphatidilcholine to phosphatidic acid. This reaction has been proposed to stimulate nascent

secretory vesicles budding from the trans-Golgi network. It has been demonstrated that in mammalian cells the accumulation of phosphatidic acid is a key step in regulating budding of secretory vesicles from the trans-Golgi network (Sidhanta & Shield 1998). On the other hand, the increment in the phospholipids synthesis here found in PALs of Cd treated rats can be related to findings reported by other researchers. It is known that Cd induces activation of c-fos expression in different cellular systems (Misra *et al*. 2003), and this activation has been associated with an increment of phospholipid synthesis (Bussolino *et al*. 2001). It addition, it has been shown that the activation of c-fos increases the activity of phosphatidic acid phosphatase, which decreases the availability of phosphatidic acid (Arriba Zerpa *et al*. 1999).

Cadmium in drinking water did not modify PAL cholesterol content or synthesis, measured by [^{14}C]-acetate incorporation. In consequence, the cholesterol/phospholipids ratio was lower in PALs of Cd treated rats compared with controls. More studies will be necessary to determine if changes in membrane fluidity of pituitary gland may directly or indirectly alter the hormone secretions in cadmium exposed rats.

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