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In vivo xenoestrogenic actions of cadmium and arsenic in anterior pituitary and uterus

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Abstract

Cadmium (Cd) and arsenic (iAs) are toxic metals ubiquitously present in the environment. Both pollutants exert nonmonotonic dose responses, being mostly cytotoxic at high concentrations but mimicking estrogen (E2) effects at low doses. Xenoestrogenic activity of Cd and iAs has been demonstrated in different hormone-dependent tumor cell lines; however, their actions *in vivo* remain largely unknown. Here, we investigated whether *in vivo* administration of low doses of Cd and iAs through drinking water would display xenoestrogenic effects in the anterior pituitary gland and uterus of ovariectomized rats. Cd (1 ppm) and iAs (0.1 ppm) exposure increased the wet weight of anterior pituitary gland and uterus and induced proestrus- and estrus-like vaginal smears. Both metals stimulate cell proliferation of these tissues as they increased the expression of proliferation markers. More importantly, they augmented soluble guanylyl cyclase α1 subunit expression, which has been linked to hormone-dependent tumor progression. Also, Cd and iAs modified protein levels of full-length estrogen receptor α and its truncated variants in an E2-like manner. Anterior pituitary hormone secretion was differentially affected by both metals. Luteinizing hormone synthesis and release were strongly diminished after Cd exposure and only mildly reduced by iAs. Both metals were able to increase prolactin synthesis, although only iAs augmented serum prolactin levels. This study shows for the first time that Cd and iAs exert strong xenoestrogenic effects on anterior pituitary gland at low doses. The differences between Cd and iAs E2-like behavior indicate that other Cd- and iAs-specific mechanisms could be involved. Altogether, these results contribute to the knowledge of reproductive disorders associated with Cd and iAs environmental contamination.

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Introduction

Cadmium (Cd) and arsenic (iAs) are extremely toxic environmental pollutants. In many areas around the world, humans are exposed to high concentrations of these metals. It has been widely described that both acute and chronic exposure to Cd or iAs are associated with endocrine imbalance and significant reproductive toxicity (ATSDR 1998, 1999).

Cd is a heavy metal widely dispersed throughout the environment primarily from mining, smelting, and electroplating and is found in consumer products such as batteries, pigments, and plastics (ATSDR 1998). Human Cd intoxication occurs primarily through dietary sources – food and water – and cigarette smoke (Koller 1998). This metal has a long biological half-life (~20 years) and is highly bioaccumulated over time in the blood, kidney, and liver as well as in the reproductive organs (Miler *et al.* 2010).

iAs is a naturally occurring metalloid with potent toxic and mutagenic effects (Klein *et al.* 2007). It is ubiquitously present in the environment and is released from both natural and man-made sources (Erbanova *et al.* 2008). Exposure to iAs from contaminated drinking water is one of the top environmental threats worldwide, based on the potential exposure of people to iAs and the numerous diseases with which it has been associated (Abernathy *et al.* 2003). Unlike Cd, iAs undergoes oxidative methylation following uptake, methylated metabolites being more easily excreted from cells (Styblo *et al.* 2000).

Disruption of the reproductive system of humans and wildlife has been attributed to exposure to environmental contaminants that mimic the effects of steroid hormones (Diamanti-Kandarakis *et al.* 2009). Moreover, it has been suggested that the high incidence of hormone-related cancers and diseases in the Western world is also due to the presence of endocrine disruptors (EDs). These compounds are defined as chemical substances that interfere with biosynthesis or activity of several endogenous hormones (McLachlan 2001). Among these, xenoestrogens (XEs) are non-steroidal organic or inorganic chemicals released into the environment from agricultural spraying, industrial activities, urban waste, and consumer products. They mimic the biological

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activity of sex hormones, including androgens and estrogens (Colborn *et al.* 1993). In the last decade, heavy metals and metalloids such as Cd, iAs, mercury, nickel, lead and zinc have been shown to trigger cell responses normally induced by estrogens, which consequently affect their signaling (Georgescu *et al.* 2011). Previous reports have shown that Cd and iAs may exhibit estrogenic mimicry in breast cancer cells by activating the estrogen receptor (ER) (Stoica *et al.* 2000*a,b*). Cd and iAs have also been shown to possess potent estrogen-like activity *in vivo* (Johnson *et al.* 2003, Jana *et al.* 2006, Höfer *et al.* 2009). However, xenoestrogenic effects at low doses of Cd or iAs *in vivo* through drinking water, a relevant route of exposure, have been poorly documented.

17β-Estradiol (E2) is the major female sex hormone mediating the differentiation, proliferation, development, and homeostasis of female reproductive functions (Morani *et al.* 2008). E2 stimulatory actions on hormone secretion and cell proliferation are principally mediated by estrogen receptor α (ESR1; ERα). The general structure of ER consists of an N-terminal activator function-1 domain (AF-1), followed by the DNA binding domain, a dimerization domain, and a ligand binding/AF-2 domain (Reid *et al.* 2002). In addition to the full-length 66 kDa ERα (ERα66) isoform, two truncated splice variants of 46 kDa (ERα46, lacking AF-1 domain) and 36 kDa (ERα36, lacking both AF-1 and AF-2 domains) have been reported (Wang *et al.* 2005). These splice variants were first detected in the pituitary gland (Mitchner *et al.* 1998) and then in other tissues including uterus, breast, endometrium, smooth muscle cells, and peripheral blood mononuclear cells (Pendaries *et al.* 2001, Shupnik 2002, Taylor *et al.* 2010).

E2 control of cell proliferation involves the regulation of target genes including cyclins, cyclin-dependent kinases (CDKs), proto-oncogenes, and negative cell cycle regulators. E2 induces expression of cyclins (A, D, and E) and other proteins such as the proliferating cell nuclear antigen (PCNA), which are involved in the control of cell cycle progression from $G_{0/1}$ to S and M (Matsuo *et al.* 1999, Butt *et al.* 2005).

E2-induced cell proliferation is also directly associated with upregulation of alpha 1 subunit of the soluble guanylyl cyclase (GUCY1A3). GUCY1A3, together with (GUCY1B3), forms Soluble guanylyl cyclase (sGC), the major intracellular receptor for nitric oxide (NO). NO binding to sGC activates this enzyme to catalyze the formation of cGMP from GTP (Krumenacker *et al.* 2004). Previous studies from our group demonstrated that E2 chronic and acute administration increases *Gucy1a3* mRNA and protein expression and decreases *Gucy1b3* levels in the anterior pituitary gland both *in vivo* and *in vitro* (Cabilla *et al.* 2006, 2009, 2011). Furthermore, Cai and coworkers have shown that androgen-driven GUCY1A3 upregulation correlates with cell proliferation in advanced prostate cancer, suggesting that GUCY1A3 could be an important mediator of procarcinogenic effects of androgens (Cai *et al.* 2007).

E2 are major regulators of anterior pituitary physiology, eliciting a plethora of processes including stimulation of lactotroph proliferation and upregulation of prolactin (PRL) gene expression, synthesis, and release (Freeman *et al.* 2000). Cd and iAs, at micromolar doses, have been shown to inhibit anterior pituitary secretion through feedback mechanisms or by directly affecting anterior pituitary cells (Lafuente & Esquifino 1999, Poliandri *et al.* 2003, 2006, Sarkar *et al.* 2003, Miler *et al.* 2010, Ronchetti 2014). However, we previously showed that Cd at nanomolar concentrations exerts E2-like activities on these cells in culture (Ronchetti *et al.* 2013). However, to date, iAs-mediated effects on the anterior pituitary gland have not been studied.

Considering previous knowledge, the aim of this study is to investigate whether *in vivo* administration of low doses of Cd and iAs through drinking water would display xenoestrogenic effects on the anterior pituitary gland. We also performed uterotrophic assays including histological analysis and E2-responsive gene expressions to further complete the landscape of E2-like effects of Cd and iAs.

Materials and methods

Ethics statement

All experimental procedures were approved by the Committee on Ethics of the School of Medicine (University of Buenos Aires, Res. (CD) No. 2831/10) and were carried out in compliance with the guidelines of the Institutional Committee for Care and Use of Laboratory Animals (CICUAL, School of Medicine, University of Buenos Aires) and the NIH Guide for the Care and Use of Laboratory Animals.

Drugs and reagents

Cadmium chloride ($CdCl_2.2½H_2O$) was purchased from Mallinckrodt Chemical Works (St. Louis, MO, USA). Sodium (meta) arsenite ($NaAsO_2$) was purchased from Sigma-Aldrich. Protease inhibitor cocktail, ACTB (β-actin), soluble guanylyl cyclase α1 (GUCY1A3), and β1 (GUCY1B3) antibodies were obtained from Sigma-Aldrich. Ketamine and xylazine were purchased from König (Buenos Aires, Argentina). Ketofen was obtained from Vetanco S.A. (Buenos Aires, Argentina). Proliferating cell nuclear antigen (PCNA), cyclin D3 (CCND3), and ERα antibodies were purchased from Santa Cruz Biotechnology. Anti-recombinant rat PRL and anti-rLH-11 antisera were provided by Dr A F Parlow (National Hormone and Pituitary Program, Torrance, CA, USA).

Animals and treatments

Adult female Wistar rats (180–200 g) were ovariectomized (OVX) under ketamine and xylazine anesthesia. After surgery, animals received an s.c. injection of 1% ketroprofen and were exposed to Cd or iAs treatment through drinking water for a month after a recovery period of 1 week. Animals were kept

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under controlled conditions of light (12 h light:12 h darkness cycle) and temperature (21–24°C) and received standard laboratory chow (Cooperación, Buenos Aires, Argentina) and tap water *ad libitum*. Neither iAs nor Cd levels were measured in the standard laboratory chow. Tap water (<10 ppb iAs, www.nutrired.org/agua/page/4) was filtered by PSA Senior System (PSA, Buenos Aires, Argentina), which retains most of the heavy metals including Cd.

Toxicant concentrations were selected after running a pilot experiment ranging from 0.01 to 5 ppm. Based on an uterotrophic assay, the minimum concentration showing significant xenoestrogenic effects was chosen for each metal. After surgery, animals ($N=6$ per cage) were randomly divided into four groups: control, Cd, iAs, and E2 (positive control). The control group drank filtered tap water without any metal. Treated groups were exposed to 1 ppm $CdCl_2.2½H_2O$ or 0.1 ppm $NaAsO_2$ corresponding to an average intake of 51 µg/kg/day and 6 µg/kg/day respectively. The positive control group was conformed by animals implanted s.c. with a silastic capsule containing 500 µg 17β-estradiol with free access to filtered tap water without any metal.

Samples of vaginal smears were collected daily. In order to avoid iAs oxidation, drinking water iAs solution was freshly prepared everyday. Body weight and water consumption were monitored periodically with no differences between control and treated groups. On day 30, rats were killed by decapitation. Anterior pituitary glands and uteri were removed, weighed, and stored in liquid nitrogen for further procedures.

Histological analysis

Uteri were fixed in Bouin solution for 20 h at room temperature (RT). Tissues were cut into 5 µm-thick sections and stained with hematoxylin–eosin (HE) for morphological analysis.

Immunohistochemistry

Histological uterus sections obtained from each animal and fixed in Bouin solution were paraffin-embedded. For deparaffinization, sections were placed at 60°C overnight and then treated for 5 min with xylene at RT. Rehydration was performed through incubation in a decreasing series of alcohols (ethanol 100, 96, 90, 70, and 40%) and a second incubation in distilled water for 5 min. Antigen exposure followed immersion of the tissues in 10 mM citrate buffer. Samples were heated twice for 3 min in a previously humidified microwave oven. Endogenous peroxidases were inactivated in a 3% H_2O_2–ethanol 70% solution for 10 min in darkness at 25°C. Uterus sections were incubated for 90 min in a blocking solution (2% BSA–PBS, 10% goat serum) after three washes with 0.1% PBS–Triton pH 7. Samples were incubated with anti-ERα antibody (1:100) diluted in 0.1% PBS–Triton/1% goat serum solution at 4°C in a humidified chamber overnight. After washing with 0.1% PBS–Triton pH 7.4, sections were incubated with anti-rabbit IgG-horseradish peroxidase (1:400) diluted in 0.1% PBS–Triton/1% goat serum. Immunoreaction product was visualized by Kit DAB (Vector Laboratories, Burlingame, CA, USA). Reaction was stopped with distilled water. After dehydration, samples were treated with xylene for 5 min and mounted with DPX.

Preparation of tissue homogenates for immunoblot analysis

Pituitaries were individually sonicated in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.1% SDS) containing protease inhibitor cocktail (1:500). Uteri were cut into small fragments and homogenized in lysis buffer (20 mM Tris, pH 7.4, 1 mM EDTA, and 0.1% Tween 20) containing protease inhibitor cocktail (1:500) in an IKA Ultra Turrax T18115 VAC (Cole Palmer, Vernon Hills, IL, USA). Homogenates were centrifuged for 20 min at 10,000 ***g*** (4°C) and post-mitochondrial fractions were used for the immunoblot analysis.

Protein measurement

The protein content of supernatants was measured by Bradford reagent (Bio-Rad), using bovine serum–albumin as a standard.

Immunoblot analysis

About 40–80 µg of total protein from each sample were boiled for 5 min in Laemmli sample buffer and fractionated on 15% SDS–PAGE. Resolved proteins were transferred to polyvinylidene difluoride membranes and blocked for 24 h at 4°C in blocking buffer (5% nonfat dry milk in 1% T-TBS). Then, membranes were co-incubated overnight at 4°C with primary antibodies: anti-CCND3 (sc-6283, 1:500), anti-PCNA (sc-56, 1:1000), anti-ERα (sc-542, 1:500), anti-PRL (Millipore, AB960, 1:25000), anti-LH (anti-rbetaLH-IC-2, 1:500), anti-GUCY1A3 (G4280, 1:1500), anti-GUCY1B3 (G4405, 1:500), and anti-ACTB (A2066, 1:1000), respectively, in blocking buffer. Blots were washed and incubated for 1 h at RT with secondary antibodies (1:2000), which were used depending on the primary antibodies: goat anti-rabbit IgG-horseradish peroxidase (111-035-003; Jackson, West Grove, PA, USA) and goat anti-mouse IgG-horseradish peroxidase (P0447; Sigma-Aldrich). Membranes were revealed using ECL detection kit (Kalium, Buenos Aires, Argentina).

Analysis of immunoblot data

Intensity of immunoblot signals was determined by digital image analysis using Gel Pro Analyzer software for Windows. For statistical comparison of results from different blots, levels were normalized to the value of the ACTB-immunoreactive band in each lane.

Hormone determination

PRL and luteinizing hormone (LH) were measured by a double-antibody radioimmunoassay (RIA) (Quinteros *et al.* 2007) using reagents provided by Dr A F Parlow (National Hormone and Pituitary Program, Torrance, CA, USA). Recombinant PRL (NIDDK-rPRL-RP-3) and recombinant LH (LHRP-3) were used as reference preparations. NIDDK-anti-rPRL-S-9 and anti-rLH-11 were used as antisera. Sensitivity of the assay was 0.1 ng/mL. Intra- and inter-assay coefficient of variation was under 10%. To prevent inter-assay variations, all samples were measured in the same assay.

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Statistical analysis

Results were expressed as mean ± s.e.m. and evaluated by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's test. Differences between groups were considered significant if $P<0.05$. Results were confirmed by at least three independent experiments.

Results

Cd and iAs exposure-induced changes in vaginal cytology of ovariectomized (OVX) rats

Estrous cycle analysis is a well-known method to determine xenoestrogenicity of environmental pollutants (Laws *et al.* 2000). Samples of vaginal smears from each experimental group were collected daily in the last 2 weeks of treatment. As expected, OVX control rats remained in diestrus because of estrogen withdrawal, whereas E2-treated rats (positive control) showed typical vaginal cytology of estrus. Smears corresponding to proestrus and estrus were found in both Cd- and iAs-exposed animals with a concomitant decrease in the relative frequency of diestrus (Fig. 1).

Cd and iAs exposure increased anterior pituitary and uterine wet weights

E2 regulates development and growth of many E2-responsive tissues. Anterior pituitary gland and sections of 3 cm from the uterus were carefully removed and weighed after killing animals. Similar to observations in E2-treated rats, exposure to Cd and iAs significantly increased wet weight of anterior pituitary gland by a 1.2- and 1.9-fold increase respectively (mg tissue/g body weight; control: 0.037 ± 0.002; Cd: 0.045 ± 0.003*; iAs: 0.07 ± 0.01**; E2: 0.137 ± 0.008***, ANOVA followed by Dunnett's test, *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control). Also, uterine wet weight showed a 1.9- and 2.5-fold increase respectively (mg tissue/g body weight; control: 0.15 ± 0.01; Cd: 0.29 ± 0.01*, iAs: 0.38 ± 0.06**; E2: 0.72 ± 0.09**, ANOVA followed by Dunnett's test, *$P<0.05$, **$P<0.01$ vs control). Altogether, these results suggest that the pollutants exert a trophic effect on these E2-responsive tissues.

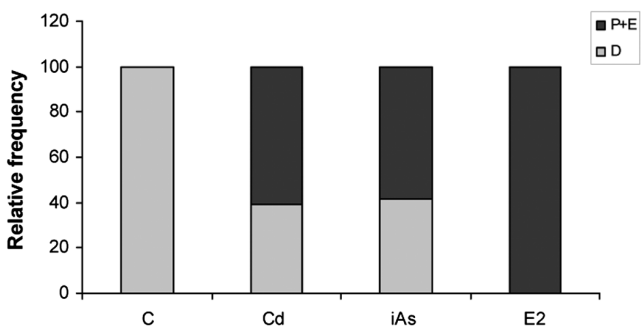

Figure 1 Cd and iAs exposure caused vaginal cytology of proestrus and estrus-like stages in ovariectomized rats. Samples of vaginal smears were collected daily in the last 2 wk of treatment. Bars represent the number of animals on proestrus + estrus (P + E) or diestrus (D) relative to the total number of animals per treatment ($n=6$).

Cd and iAs exposure stimulated cell proliferation marker expression in the anterior pituitary gland

Considering that Cd and iAs exposure increased the wet weight of the anterior pituitary gland, we determined whether this effect was a consequence of cell proliferation. We evaluated the expression of two classic cell proliferation markers involved in cell cycle progression such as proliferating cell nuclear antigen (PCNA) and cyclin D3 (CCND3) by western blot.

Exposure to both Cd and iAs significantly augmented protein expression levels of both markers to a similar extent to that observed in E2-treated rats (Fig. 2A and B).

Cd and iAs exposure upregulated soluble guanylyl cyclase alpha 1 (GUCY1A3) protein levels but not β1 (GUCY1B3) subunit expression in the anterior pituitary gland

Previously, we showed that E2 differentially regulates GUCY1A3 and GUCY1B3 subunit expressions in anterior pituitary gland by increasing GUCY1A3 levels, whereas GUCY1B3 expression decreased or remained unchanged (Cabilla *et al.* 2006, 2009). To further investigate whether Cd and iAs were able to mimic E2 effect on these subunits, protein levels were measured. As previously reported, E2 significantly augmented GUCY1A3 expression in the anterior pituitary gland but did not modify GUCY1B3 levels. Accordingly, Cd and iAs exposure mimicked E2 effects on sGC subunits to a similar extent, thus confirming that these metals reproduced the hormone's effects not only through classic E2-responsive genes but also by affecting the expression of other proteins not classically related to E2 proliferative signaling (Fig. 3).

Cd and iAs exposure modified estrogen receptor (ER) levels in the anterior pituitary gland

Previous evidence indicates that E2 regulates the synthesis of its own full-length receptor (ERα66) and truncated variants ERα36 and ERα46 (Mitchner 1998, Shupnik 2002). To examine the xenoestrogenic actions of Cd and iAs on ERα expression, protein levels were evaluated by western blot. Both Cd and iAs reproduced E2 effect by significantly augmenting ERα46 expression. iAs exposure induced a significant increase in ERα66, whereas Cd, such as E2, did not modify its expression (Fig. 4). In all cases, the protein band corresponding to ERα36 was undetected; therefore, it was excluded from further analysis.

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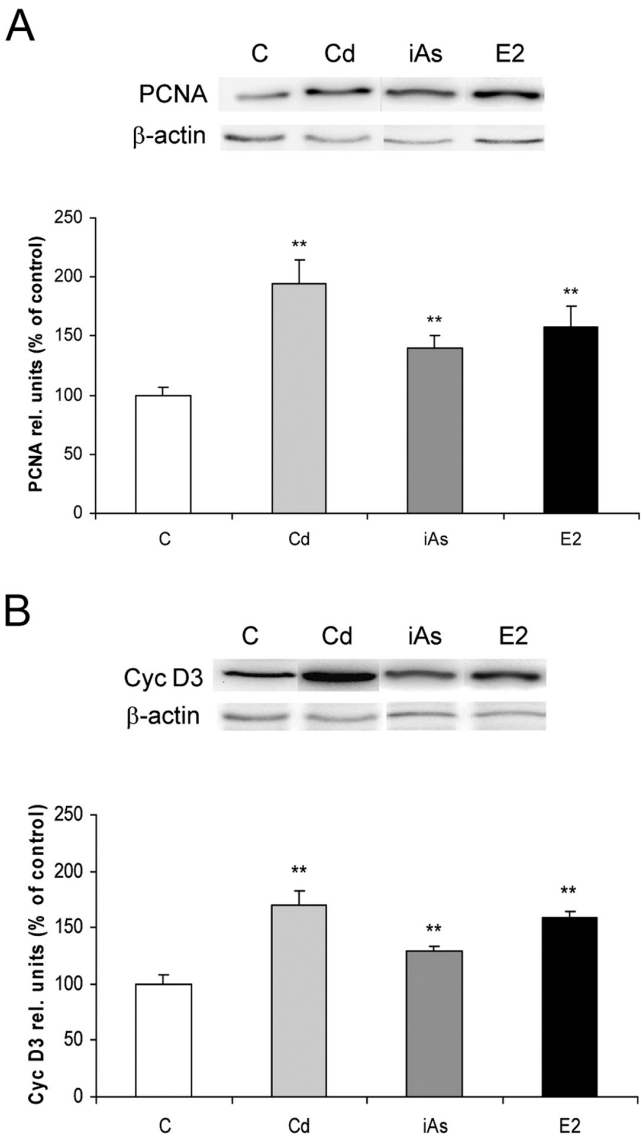

Figure 2 Cd and iAs exposure stimulated cell proliferation marker expression in the anterior pituitary gland. (A) Proliferating cellular nuclear antigen (PCNA) and (B) cyclin D3 (CCND3; Cyc D3) protein expression was determined by western blot. Bars represent mean ± s.e. of relative units corresponding to protein densitometric values relative to ACTB (β-actin) ($n=6$) expressed as percentage of control. ANOVA followed by Dunnett's test, $^{**}P<0.01$.

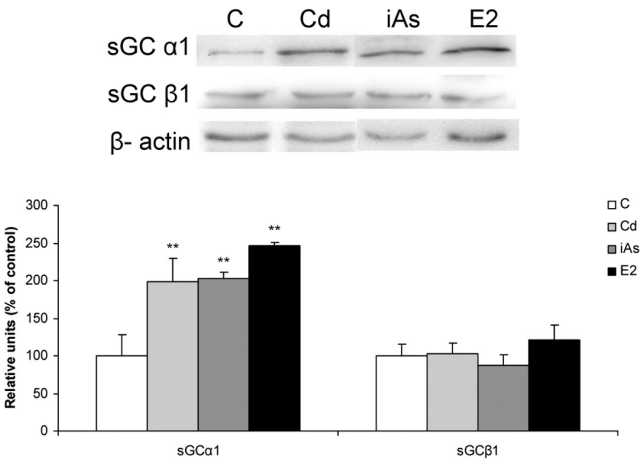

Figure 3 Cd and iAs exposure increased soluble guanylyl cyclase alpha 1 (GUCY1A3; sGCα1) subunit levels but not beta 1 (GUCY1B3; sGCβ1) expression in the anterior pituitary gland. sGCα1 and sGCβ1 protein levels were evaluated by western blot. Bars represent mean ± s.e.m. of relative units corresponding to protein densitometric values normalized to ACTB (β-actin) ($n=6$) expressed as percentage of control. ANOVA followed by Dunnett's test, $^{**}P<0.01$.

Cd and iAs exposure differentially affected prolactin (PRL) and luteinizing hormone (LH) secretion

Anterior pituitary hormones such as PRL and LH play a key role in the reproductive physiology control, their secretion tightly regulated by E2. To investigate whether Cd and iAs mimic E2 action on PRL and LH secretion in the anterior pituitary gland, protein expression and serum levels of these hormones were measured by western blot and RIA respectively.

As a consequence of gonadal steroid withdrawal after ovariectomy, control OVX rats exhibited significantly lower PRL and higher LH levels compared with intact random rats (PRL; intact: 14.1 ± 2.9 ng/mL; OVX: 4.29 ± 0.78 ng/mL*; LH intact: 0.74 ± 0.12 ng/mL; OVX: 22.7 ± 2.1 ng/mL***, $N=6$, Student's *t*-test, $^{*}P<0.05$, $^{***}P<0.001$). Exposure to Cd or iAs significantly increased PRL expression, suggesting that these metals can stimulate its synthesis. Tallying with iAs-induced PRL protein increase, iAs exposure augmented PRL release; however unexpectedly, Cd did not significantly modify it (Table 1).

Regarding LH secretion, only Cd was able to decrease both protein expression and serum levels, as iAs exposure did not significantly modify these parameters (Table 1).

In sum, these results show that Cd and iAs exert xenoestrogenic effects in the anterior pituitary gland by stimulating E2-regulated genes involved in cell proliferation, E2 canonic and noncanonic signaling, and by affecting hormone secretion.

Evaluation of Cd and iAs estrogenicity in uterus

Next, to further characterize the estrogenic potency of these pollutants, we determined Cd- and iAs-driven estrogenic responses by studying histological and molecular parameters in the uterus.

Morphological analysis

Uterus histological examination revealed that Cd and iAs exposure increased the number and size of endometrial glands and height of luminal and glandular epithelium. Cd exposure also increased the number of invaginations. According to previous reports (Johnson *et al.* 2003, Höfer *et al.* 2009), chronic E2 treatment caused endometrial hyperplasia and hypertrophy (Fig. 5).

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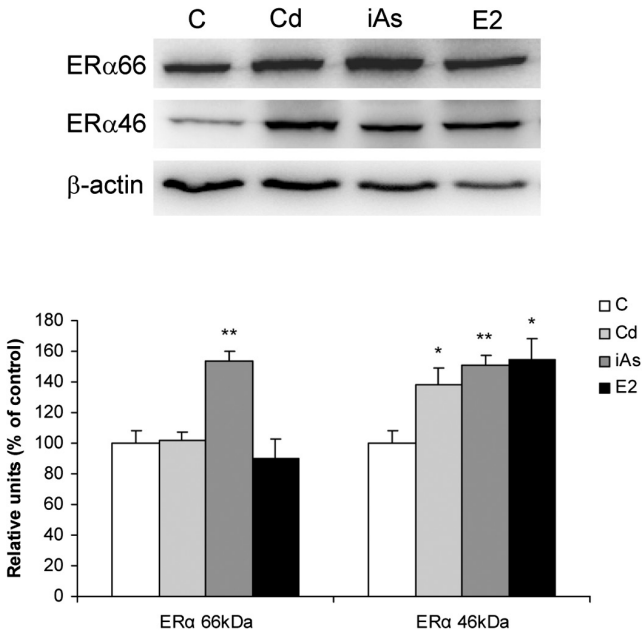

Figure 4 Cd and iAs exposure modified the expression of estrogen receptor α (ESR1; ERα66) and its truncated variant ERα46 in the anterior pituitary gland. ERα66 and ERα46 expressions were determined by western blot. Bars represent mean ± s.e.m. of relative units corresponding to ERα66 and ERα46 densitometric values relative to ACTB (β-actin) ($n=6$) expressed as percentage of control. ANOVA followed by Dunnett's test, $^{*}P<0.05$, $^{**}P<0.01$.

ERα expression

It has been widely reported that development and maintenance of the uterine endometrium are principally mediated by E2 through activation of ERα. Here, we studied ERα expression in the uterus by immunohistochemistry. Uteri from control rats showed weak staining localized in the nucleus of luminal and glandular epithelial as well as stromal cells. Cd and iAs exposure produced augmented immunoreactive nuclear ERα staining in luminal and glandular epithelial cells. Particularly, stromal cells showed the strongest ERα immunostaining intensity (Fig. 6A).

Table 1 Cd and iAs exposure differentially affected PRL and LH synthesis and release. Protein levels of intrapituitary PRL and LH were determined by western blot. Trunk blood was collected after killing animals to evaluate hormone content. Serum levels of PRL and LH were measured by radioimmunoassay (RIA).

	Serum hormone levels (ng/mL)		Intrapituitary hormone protein levels[a]	
	PRL	LH	PRL	LH
Control	4.29 ± 0.78	22.7 ± 2.1	100 ± 15	100 ± 17
Cd	3.8 ± 1.8	11.5 ± 2.4**	153 ± 7**	35.6 ± 9.9**
iAs	6.69 ± 0.79*	16.1 ± 0.6	165 ± 12*	96.6 ± 13.3
E2	455.9 ± 65.1***	0.75 ± 0.12**	220.2 ± 30.2***	10.9 ± 2.2**

Values represent mean ± s.e.m. from each experimental group ($n=6$).
[a]Relative units as % of controls. ANOVA followed by Dunnett's test, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

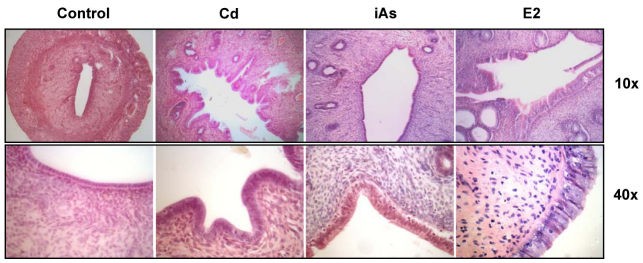

Figure 5 Cd and iAs exposure induced E2-like morphological changes in the uterus. Uteri were removed after killing and stained under hematoxylin–eosin (HE) technique for histological analysis. General view of uteri and endometrial glands (10×). Epithelium and stromal cells detail (40×).

In order to further validate the effects of these pollutants on full-length and variants of ERα expression, protein levels were evaluated by western blot. In concordance with results for anterior pituitary gland, exposure to Cd and iAs resulted in an increased expression of ERα46. Similar to observations in the anterior pituitary gland (Fig. 4), ERα66 expression was differentially affected by these pollutants being significantly upregulated by iAs but unchanged after Cd exposure (Fig. 6B).

Cell proliferation markers

To confirm Cd and iAs proliferative effects – evidenced by increased uterine wet weight and augmented cell number qualitatively observed by histological studies – PCNA and CCND3 expressions were evaluated. As we expected, Cd and iAs exposure, such as E2 treatment, significantly augmented protein levels of both markers (Fig. 7A and B).

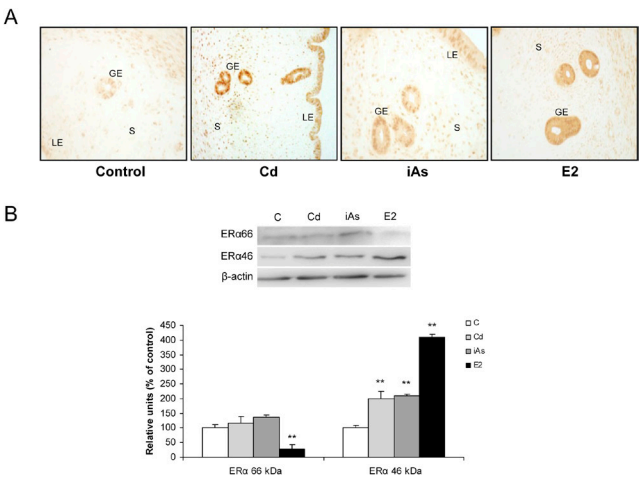

Figure 6 Cd and iAs exposure augmented ESR1 (ESR1 (ERα) expression in the uterus. (A) Uteri were removed after killing and processed for immunohistochemical staining for ERα expression. **LE**, luminal epithelium; **GE**, glandular epithelium, **S**, stroma (40×). (B) ERα66 and ERα46 protein expressions were evaluated by western blot. Bars represent mean ± s.e.m. of relative units corresponding to protein densitometric values relative to ACTB (β-actin) ($n=6$) expressed as percentage of control. ANOVA followed by Dunnett's test, $^{**}P<0.01$.

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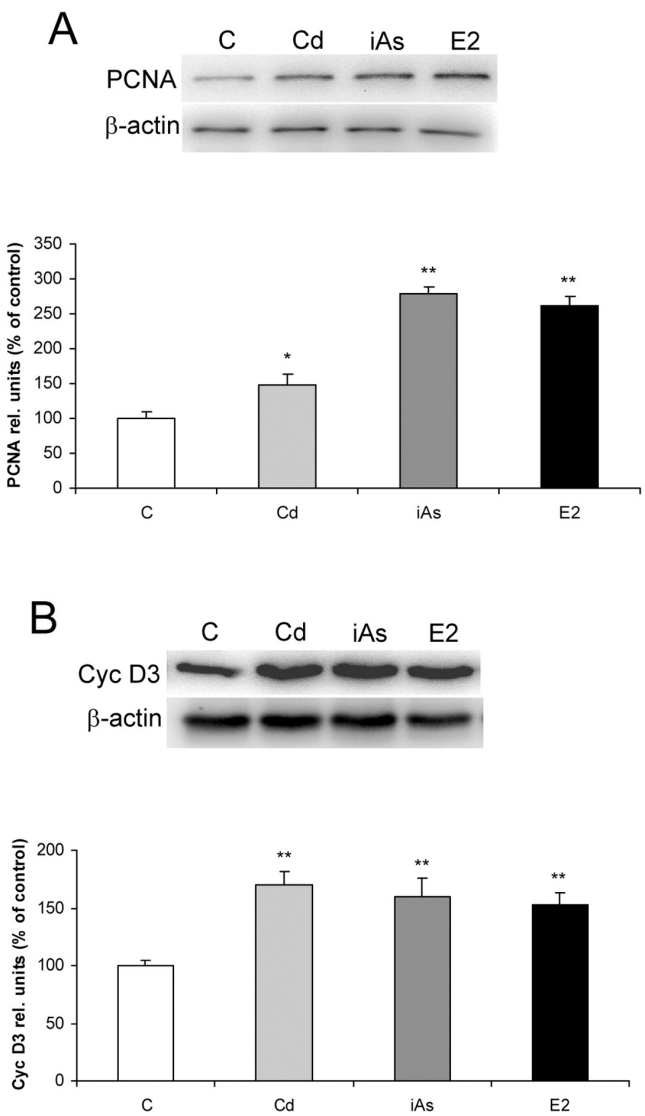

Figure 7 Cd and iAs exposure increased cell proliferation markers in the uterus. (A) PCNA, (B) CCND3 (Cyc D3) protein levels were evaluated by western blot. Bars represent mean ± s.e.m. of relative units corresponding to protein densitometric values relative to ACTB (β-actin) ($n=6$) expressed as percentage of control. ANOVA followed by Dunnett's test, $*P<0.05$, $**P<0.01$.

Finally, we analyzed the expression of GUCY1A3 and GUCY1B3 as an estrogen-responsive gene. Results showed that exposure to either metal significantly augmented GUCY1A3 expression and did not modify GUCY1B3 to a similar extent, as did E2 treatment (Fig. 8).

To sum up, similar to observations of the anterior pituitary, these results showed that Cd as well as iAs display uterotrophic E2-like effects evidenced by morphological and molecular changes.

Discussion

In this study, we investigated the xenoestrogenic effects of low doses of Cd and iAs exposure through drinking water in the pituitary gland and uterus.

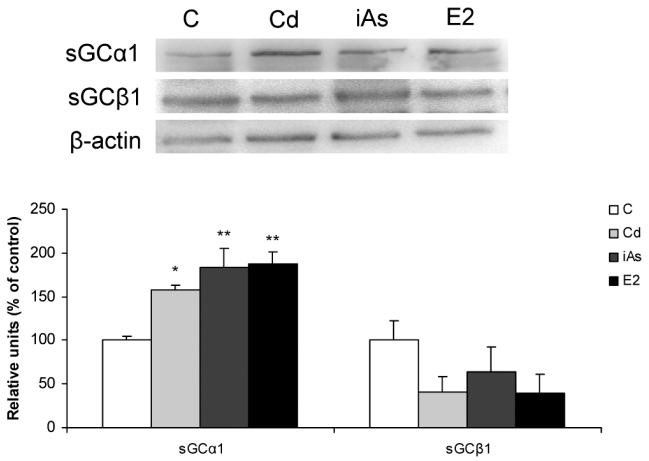

Figure 8 Cd and iAs exposure augmented soluble guanylyl cyclase alpha 1 (GUCY1A3; sGCα1) subunit levels but not beta 1 (GUCY1B3; sGCβ1) expression in the uterus. sGCα1 and sGCβ1 protein levels were evaluated by western blot. Bars represent mean ± s.e.m. of relative units corresponding to protein densitometric values normalized to ACTB (β-actin) ($n=6$) expressed as percentage of control. ANOVA followed by Dunnett's test, $*P<0.05$, $**P<0.01$.

Similar to many endocrine disruptors, Cd and iAs display non-monotonic actions depending on their concentrations. At high (micromolar) levels, these pollutants exert cytotoxic effects and induce oxidative stress leading to cell death (Poliandri *et al.* 2003, 2004, Ronchetti 2014), whereas at low (nanomolar) levels, mimic E2 actions by promoting cell proliferation and affecting hormone secretion (Johnson *et al.* 2003, Stoica *et al.* 2000*a*,*b*, Ronchetti *et al.* 2013, Ronchetti 2014).

This is the first report on E2-like effects of Cd and iAs on the pituitary gland *in vivo*. Here, we demonstrated that both toxicants stimulate cell proliferation, modify pituitary hormone secretion, and up-regulate E2-responsive genes. Our present data tally with previous results from our laboratory demonstrating that nanomolar Cd concentrations exert estrogenic activity in the anterior pituitary cells in culture and in lactosomatotroph GH3 cell line (Ronchetti *et al.* 2013). Regarding iAs, its xenoestrogenic effects on this gland have not been reported yet.

iAs effects are specific to species and tissue due to differential accumulation, methylation, and excretion. In rats, this metalloid binds to blood proteins higher than other mammalian species, which decreases both bioavailability and accumulation in other tissues. Comparatively, mouse, rabbit, and human, among other species, are more vulnerable to adverse iAs effects (Vahter 1999, Naranmandura *et al.* 2007). However, Cd toxicity does not substantially differ among species, being easily accumulated in all tissues (Bhattacharyya *et al.* 2000).

To date, *in vivo* studies of E2-like effects of Cd and iAs have been performed mainly by administration through i.p. or i.v. injections (Waalkes *et al.* 2000, Johnson *et al.* 2003, Höfer *et al.* 2009). However, we chose oral exposition to these pollutants through drinking water as it

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reproduces more plausible conditions of toxicant exposition and normal gastrointestinal absorption, which is by-passed by any non-oral route.

Here, we showed that exposure to iAs, at a dose as low as 0.1 ppm, displayed effects comparable to 1 ppm Cd regarding macroscopic indices of estrogenicity such as estrous cycle and wet weight of E2-responsive tissues. This finding could be due to differential absorption in the gastrointestinal tract, being about 95% for iAs and only 5% for Cd (ATDSR 1998, 1999).

By using the uterus, a well-known target of E2 and E2-like compounds (Rice *et al*. 2003, Kanno *et al*. 2003*a*,*b*), we confirmed that Cd as well as iAs exerted xenoestrogenic activities by increasing uterine wet weight and inducing molecular and histological changes similar to those observed following E2 stimulation.

Here, we showed that both, Cd and iAs, were able to increase classical proliferation markers such as CCND3 and PCNA in the anterior pituitary gland and uterus. More importantly, both were able to augment GUCY1A3 expression and did not modify GUCY1B3 levels. These results concord with previous evidence obtained after E2 chronic and acute administration in the anterior pituitary gland (Cabilla *et al*. 2004). Regarding uterus, to date, E2 effects on GUCY1A3 expression were associated only with sGC enzymatic activity. In this sense, Murad *et al*. reported acute E2-triggered *Gucy1a3* mRNA and protein down-regulation and decreased sGC activity (Krumenacker *et al*. 2001). Contrary to this evidence, we found that long-term E2 or E2-like compound exposure resulted in significant GUCY1A3 upregulation without affecting GUCY1B3 expression.

Considering that both sGC subunits are required in a 1:1 stoichiometry to shield enzymatic activity, this Cd- and iAs-driven imbalance in their expression is likely related to other sGC functions independent of its cGMP-producing activity. In this sense, GUCY1A3 has been elegantly linked to cell proliferation and tumor progression in other hormone-responsive tissues (Cai *et al*. 2008, 2012). Our observations open a new field as GUCY1A3 might be an important mediator of the procarcinogenic effects exerted by any E2-like compound in hormone-dependent tissues, a line currently under investigation.

Anterior pituitary expresses full-length ERα and two truncated variants, ERα36 and ERα46, which play a key role in E2-regulated feedback in this gland (Mitchner *et al*. 1998). In uterus, many ERα-splicing variants have been reported (Varayoud *et al*. 2005), including the aforementioned truncated variants. Noticeably, only iAs was able to augment full-length ERα expression in both tissues, whereas Cd did not modify it. In this sense, it seems that iAs cannot fully modulate E2 feedback loop on its receptor, which indicates that iAs and Cd might differ in their respective estrogenic potencies or in the activation of alternative E2-related regulatory mechanisms. E2 treatment decreased full-length ERα in the uterus. This observation tallies with previous reports on the long-term E2 administration to OVX rats (Mohamed & Abdel-Rahman 2000).

In uterus, our studies showed that exposure to both toxicants induced strong ERα immunoreactive staining localized in luminal and glandular epithelial as well as in stromal cells. Similar results were observed in mouse uterus after repeated i.v. 0.5 mg/kg iAs injections (Waalkes *et al*. 2000). Molecular analysis revealed that the rise in ERα expression is mainly due to an increase in ERα46 levels. Concordantly, Cd and iAs exposure also increased ERα46 protein levels in the anterior pituitary gland in a manner similar to E2. These observations could be due to negative regulation in response to long-term E2 or E2-like compound stimulation. Several endocrine disruptors are able to activate ERα; however, the effects on ERα variant expression remain largely unknown. Recently, the phytoestrogen genistein was reported to increase ERα46 expression in brain cortex from OVX rats (Morán *et al*. 2013).

Regarding hormone secretion, our findings showed that Cd as well as iAs mimicked E2 by increasing PRL synthesis, whereas unexpectedly, only iAs was able to stimulate PRL release. To date, little is known about low-dose Cd and iAs effects on hormone pituitary secretion. The present data partially contrast with previous *in vitro* results by showing that Cd at nanomolar concentrations for 48 h increases synthesis and release of PRL in primary anterior pituitary cultures, probably due to differences in the two experimental designs.

Both toxicants exerted differential effects on LH secretion. Cd reduced both intrapituitary LH protein levels and release, possibly by reproducing hypothalamic E2 regulatory actions on GNRH release. Also, we cannot rule out that under subchronic *in vivo* exposure, LH and PRL release could be affected by other Cd-triggered actions independent of its xenoestrogenic activity, a possibility that needs to be investigated.

However, iAs produced a slight but non-significant LH decrease. At the hypothalamic level, lower iAs accumulation due to the brain–blood barrier and enhanced iAs excretion via methylated metabolites (Sanchez-Peña *et al*. 2010) could explain this mild iAs xenoestrogenic action on LH.

Considering the central role of anterior pituitary hormone in reproduction and the ubiquitous nature of these toxicants, more detailed understanding of the endocrine effects of Cd and iAs exposure carry great relevance. In this direction, we have provided the first *in vivo* approach to Cd and iAs xenoestrogenic activity in the anterior pituitary gland and broadened knowledge of their proliferative actions in the uterus.

This information is essential for knowledge of exposure-related reproductive disruptions as well as preneoplastic and neoplastic pathologies associated with these pollutants, thereby contributing to better understanding of the etiology of conditions that represent grave risks to human and animal health.

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Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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