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Dose- and route-dependent hormonal activity of the metalloestrogen cadmium in the rat uterus☆

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ABSTRACT

The toxic heavy metal cadmium (Cd) is regarded as a potential endocrine disruptor, since Cd exerts estrogen-like activity *in vitro* and can elicit some typical estrogenic responses in rodents upon intraperitoneal (*i.p.*) injection. But estrogenic effects have not been documented in vivo with other more relevant routes of exposure, although it is known that Cd absorption and distribution in the body is strongly affected by the application route. Therefore, we investigated its hormonal activity in ovariectomized Wistar rats after oral administration of $CdCl_2$ (0.05–4 mg/kg b.w. on 3 days *by gavage* and 0.4–9 mg/kg b.w. for 4 weeks in drinking water) in comparison with *i.p.* injection of $CdCl_2$ (0.00005–2 mg/kg b.w.). Uterus wet weight, height of uterine epithelium, and modulation of estrogen-regulated gene expression, *i.e.* uterine complement component 3 (C3), were determined, and also Cd-levels in uterus and liver were measured by atomic absorption spectrometry. The analysis revealed pronounced differences in Cd tissue levels and hormonal potency for the two routes of administration: a single *i.p.* injection of Cd increased dose-dependently uterine wet weight and thickness of the uterine epithelium. Interestingly, C3 mRNA expression in the uterus was down regulated at low doses of $CdCl_2$ (0.00005–0.05 mg/kg b.w.), but strongly stimulated at the highest dose of 2 mg/kg b.w. Other than *i.p.* injection, oral treatment with Cd, by gavage or in drinking water, did neither increase uterine wet weights nor epithelial thickness. But, both 3-day- and 4-week oral Cd administration resulted in a dose-dependent stimulation of C3 expression in the uterus, significant at and above 0.5 mg/kg b.w. In summary, our data demonstrate an estrogenic effect in the uterus upon *i.p.* injection of Cd, but considerably lower hormonal potency with oral administration: short and long-term oral treatment with Cd did not affect uterus weight or histology, whilst on the molecular level, an induction of estrogen sensitive uterine gene expression was observed, albeit at dose levels far exceeding those of dietary exposure in humans.

1. Introduction

Cadmium is an important environmental and industrial pollutant, and one of the most toxic heavy metals (IPCS, 2007; Järup and Åkesson, 2009). Cadmium can adversely affect human health, primarily acting on kidneys, but also on bone and lung (DFG, 2004; Godt et al., 2006; Bernard, 2008; Nordberg, 2009). In addition to its well-known toxic effects, cadmium is now also regarded as potential endocrine disruptor (Takiguchi and Yoshihara, 2006; Iavicoli et al., 2009). The exact mechanisms by which cadmium may interfere with the endocrine system remain to be elucidated. But, *in vitro* studies have shown that it can activate the estrogen-receptor α (ERα) *via* high-affinity interaction with the hormone-binding domain (Stoica et al., 2000), and cadmium triggered estrogenic effects in MCF-7 breast cancer cells that are typical for 17β-estradiol, *e.g.* increases in progesterone receptor mRNA levels (Garcia-Morales et al., 1994), and stimulation of cell proliferation (Brama et al., 2007). Studies in rats and mice have shown that intraperitoneal (*i.p.*) injection of cadmium ($CdCl_2$) increased uterine wet weights and promoted the growth and development of mammary glands (Johnson et al., 2003; Alonso-González et al., 2007). But, so far estrogenic effects have not been documented *in vivo* with other more relevant routes of exposure, although it is known that Cd absorption and distribution in the body is strongly affected by the application route

Abbreviations: AAS, atomic absorption spectrometry; b.w., body weight; C3, complement component 3; $CdCl_2$, cadmium chloride; E_2, 17β-estradiol; EE, 17α-ethinylestradiol; ERα/β, estrogen-receptor alpha/beta; *i.p.*, intraperitoneal; OVX, ovariectomized; PTWI, provisional tolerable weekly intake.

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(WHO/IPCS, 1992; Elsenhans et al., 1994b; Järup and Åkesson, 2009).

It may be argued that *i.p.* administration is a worst case scenario resembling cadmium exposure by inhalation of tobacco smoke or in occupational settings with exposure to Cd-containing fumes. But, for non-smokers in the general population, oral exposure to cadmium with food and beverages is more important. The dietary daily cadmium intake assessed in several countries, *e.g.* in diet duplicate studies (ATSDR, 2008; Ryan et al., 2001; Wilhelm et al., 2002; Ysart et al., 2004), can be rather high compared to the provisional tolerable weekly intake (PTWI) of 7 μg/kg b.w. per week as still recommended by JECFA (FAO/WHO, 2006).

This study in ovariectomized Wistar rats investigated estrogenic activity of cadmium chloride ($CdCl_2$) after oral short- and long-term exposure at several dose levels, and also after an *i.p.* injection, since this route of administration was used in previous rodent studies with cadmium (Johnson et al., 2003; Alonso-González et al., 2007; Zhang et al., 2008). As in other uterotrophic assays with xenoestrogens, the uterine wet weights, height of uterine epithelium and expression of estrogen-regulated complement component C3 served as biomarkers for estrogenicity (Diel et al., 2002, 2004; Schmidt et al., 2006). To better understand route- and dose-dependent differences in the estrogenic activity of cadmium, its concentrations were analyzed by atomic absorption spectrometry (Wilhelm et al., 1995, 2002) in blood, uterus and liver of all groups.

2. Materials and methods

2.1. Animals and experimental treatments

Female Wistar rats (101–125 g) were obtained from Janvier (Le-Genest St-Isle, France) and acclimatized for 1 week before they were ovariectomized (OVX). Then 14 days after the endogenous hormonal decline the animals were treated with the test compounds (see below). Rats were kept under controlled conditions (temperature 20 ± 1 °C, humidity 50–80%, illumination 12L/12D) and had free access to tap water and a diet low in phytoestrogen content (R/M-H, from Ssniff GmbH, Soest, Germany).

The OVX rats were randomly allocated to treatment or vehicle groups (*i.p.* and drinking water experiment: $n = 6$ animals per group; gavage experiment: $n = 8$ animals per group). The doses of $CdCl_2$ were based on a pilot study with gavage and *i.p.* administration; those of 17β-estradiol (E_2) and 17α-ethinylestradiol (EE) were based on previous experiments with E_2 and EE as reference estrogens (Diel et al., 2002, 2004). The compounds were purchased from Sigma–Aldrich (Taufkirchen, Germany).

2.1.1. Uterotrophic assay—intraperitoneal injection

$CdCl_2$ was dissolved in sterile water to achieve doses of 0.00005 mg/kg, 0.0005 mg/kg, 0.005 mg/kg, 0.05 mg/kg, 0.5 mg/kg, or 2 mg/kg body weight (b.w.) in a total application volume of 1 ml/kg b.w. Animals of the negative control group (OVX) were injected with the vehicle water. E_2 was dissolved in propanediol/ethanol (80:20, v:v) and injected at a dose level of 0.5 mg/kg b.w.

2.1.2. Uterotrophic assay—short-term oral administration

$CdCl_2$ was dissolved in tap water and administered by gavage at doses of 0.05 mg/kg, 0.5 mg/kg, 2 mg/kg, or 4 mg/kg b.w. on 3 consecutive days. OVX controls received the vehicle water. The reference estrogen (EE dissolved in propanediol/ethanol, 80:20) was given at a dose of 0.1 mg/kg b.w. on 3 days by gavage.

2.1.3. Subchronic (28 days) oral administration with drinking water

EE (1 mg) was dissolved in a small amount of ethanol and then filled up to 1 L drinking water. $CdCl_2$ was dissolved in drinking water at concentrations of 5 mg per liter, 50 mg per liter or 150 mg per liter (*i.e.* 5 ppm, 50 ppm, or 150 ppm). The OVX group received tap water. Since rats consumed about 20 ml water per day this corresponds to $CdCl_2$ dose levels of 0.4 mg/kg, 4 mg/kg, or 9 mg/kg b.w. × day and 0.08 mg/kg b.w. × day for EE.

All animal husbandry and handling conditions were according to the Institutional Animal Care and Use Committee guidelines, and legal permission to conduct the animal experiments was obtained from the local responsible authorities.

2.2. Tissue collection and preparation

At the end of the experiments body weights of the rats were determined and the animals were sacrificed by decapitation. Blood samples were collected and frozen in liquid nitrogen. The wet weights of freshly excised liver and uterus were determined, and pieces of liver and uterus were snap-frozen for mRNA preparations and analysis (see below). In addition, a piece of uterus was fixed in 4% formaldehyde and later embedded in paraffin for histological analysis.

2.3. Histological analysis

Seven micrometer tissue sections of paraffin-embedded uterine sections were mounted on slides precoated with polylysine (Menzel Gläser, Hilden, Germany), then cleared, hydrated, and stained with hematoxylin and eosin (Fischer et al., 2006). Sections were examined with a light microscope (Axiophot, Zeiss, Jena, Germany), and the height of the uterine epithelium was gauged according to Diel et al. (2002, 2004).

2.4. RNA isolation and real-time RT-PCR

RNA was isolated from frozen tissues by the method of Chomczynski and Sacchi (1987). TRIzol® (Invitrogen), a solution of phenol and guanidine for the isolation of total RNA from cells and tissues, was used followed by first-strand cDNA synthesis with SuperScript® Kit (Invitrogen). Real-time RT-PCR was performed in a MX3005P thermal cycler (Stratagene) or in iCycler (Bio-Rad). The protocol comprised 5 min at 95 °C followed by 45 cycles of 94, 60 and 72 °C for 1 min each. For subunit 1A of cytochrome c oxidase we used the forward 5′-CGTCACAGCCCATGCATTCG-3′ and the reverse 5′-CTGTTCATCCTGTTCCAGCTC-3′ and for complement C3 we used forward primer 5′-CAGCCCGCAGAGTGCCAGTAGTC-3′ and as reverse primer 5′-CCATCCTCCTTTCCATCAACTGC-3′. The data were relatively normalized for 1A expression via the delta–delta method of Pfaffl (2001), whereas subunit 1A of cytochrome c oxidase served as reference gene.

2.5. Cadmium measurements

The Cd-content in tissue and blood samples was analyzed by atomic absorption spectrometry (AAS) after appropriate sample preparation. Blood samples were diluted 1:4 with HNO_3 (65%, puriss. p.a., Fluka, Buchs, Switzerland) to decompose the proteins. After centrifugation for 5 min with 3 g the supernatant (or an adequate dilution) was taken for the Cd measurements by atomic absorption spectrometry. The detection limit was 0.4 μg/L reference material ClinChek Level 1 #8840 Lot 545 (Recipe, Munich, Germany) was used for quality control.

Tissues were dried in a Speed Vac RC 1022 Jouan (Thermo Scientific) for 4 h at 60 °C. The lyophilized organ samples (uterus about 25–120 mg, liver about 220–620 mg and kidney about 80–190 mg) were weighed in 90 ml PTFE-tubes. After addition of 3.0 ml HNO_3 (65%, puriss. p.a., Fluka, Buchs, Switzerland), the samples were digested with a high performance microwave system (MLS 1200 mega from MLS GmbH, Leutkirch, Germany) using the following temperature program: 200 W (2 min), 0 W (1 min), 300 W (2 min), 0 W (1 min), 530 W (2 min), 0 W (1 min), 680 W (2 min). The digested sample was filled up to 5.0 ml with bidistilled water in a polypropylene tube. This solution or an adequate dilution of it was used for determination of cadmium by atomic absorption spectrometry. The detection limit was 5.0 μg/kg dry weight when using 100 mg lyophilisate. For internal quality control together with each digestion run also reference material "Bovine Liver", BCR No.185R Lot 0940(LGC-Standards, Wesel, Germany) was analyzed.

2.6. Atomic absorption spectrometry

Concentrations of Cd were determined by graphite furnace atomic absorption spectrometry (GFAAS) using a PerkinElmer model SIMAA 6000 or SIMAA 6100 spectrometer equipped with an autosampler AS-72 or AS-800, respectively. Matrix modifiers were 0.1% $Mg(NO_3)_2$ and 0.15% $Pd(NO_3)_2$ (Merck, Darmstadt, Germany). Instrument conditions were: electrodeless discharge lamp at 450 mA, wavelength 228.8 nm, spectral slid width 0.7 nm, transverse-heated graphite tubes with integrated platform, gas flow rate 250 ml/min and 0 ml/min during atomization, Zeeman background correction and 20 μL sample volume. The temperature program (temperature [°C], time ramp [s]/hold [s]) was: 110, 1/15; 130, 10/40; 700, 10/15; 1800 (atomization), 0/6; 2450, 1/3. Calibration standards ranged between 0.1 and 2.5 μg Cd/L.

2.7. Statistical analysis

Data on body-, liver-, uterine weight, uterine epithelium height and cadmium content in blood and organs were expressed as mean ± SD. We used two-way analysis of variance followed by pair-wise comparison of selected means with the Mann–Whitney *U*-test. Differences in uterine gene expression were assessed by one-way ANOVA followed by Tukeyĭs *a posteriori* test. Significance was set at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

The estrogenic response to cadmium and reference substances was assessed in two 3-day uterotrophic assays (with intraperitoneal and oral administration) and after subchronic oral Cd