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Estrogen-like effects of diet-derived cadmium differ from those of orally administered CdCl₂ in the ERE-luc estrogen reporter mouse model

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ABSTRACT

Cadmium (Cd), an environmental and dietary contaminant, has been described to mimic the effects of 17 β -estradiol (E₂) in selected model systems when studied as an inorganic salt. However, inorganic Cd salts do not represent the main form of Cd exposure in general human populations. The aims of this study were to compare the estrogen-like effects and the bioavailability of dietary Cd to inorganic CdCl₂. Adult ovariectomized ERE-luc reporter mice were administered two bread based diets containing different concentrations of Cd (17.57 and 49.22 μ g/kg, corresponding to oral intakes of 1.8 and 5.1 μ g/kg body weight (bw) per day, respectively), inorganic CdCl₂ (1 μ g/kg bw per day by gavage) or E₂ (5 μ g/kg bw per day pellet) for 21 days. The effects on estrogen signaling were investigated by studying the uterine weights, luciferase activation, and expression of endogenous estrogen target genes. The uterine weight was significantly increased by both CdCl₂ and E₂ but not by the Cd containing diets. All treatments modulated the expression of luciferase and the endogenous estrogen target genes; however, there was no consistent overlap between the responses triggered by the bread diets and the responses stimulated by CdCl₂ or E₂. Oral exposure to Cd was calculated and the concentrations in liver and kidneys quantified to estimate the amount of absorbed Cd retained in tissues. The results suggest significantly lower absorption and/or tissue retention of dietary Cd compared to CdCl₂ following oral exposure. Altogether, our results support previous reports on *in vivo* estrogenicity of CdCl₂ but do not suggest the same activity for diet bound Cd. This study calls for caution when extrapolating results from pure compound studies (e.g. estrogenicity of CdCl₂) to dietary exposure scenarios (e.g. estrogenicity of diet bound Cd). Further basic research is needed on the mechanisms of interaction between Cd and the estrogen signaling, biologically active species of Cd, and biomarkers of estrogen-like effects of Cd *in vivo* before human health risk assessment on the hormone disruptive effects of Cd can be carried out.

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Abbreviations: BLI, bioluminescence imaging; ATP, adenosine 5'-triphosphate; E₂, 17 β -estradiol; s.c., subcutaneous; i.p., intraperitoneal; WAT, white adipose tissue; ER, estrogen receptor; ERE, estrogen responsive element; KPO₄, potassium phosphate; RLU, relative light unit; OVX, ovariectomized; PTWI, provisional tolerable weekly intake; IARC, International Agency for Research on Cancer; TWI, Tolerable weekly intake; EFSA, European Food Safety Authority; WHO, world health organization; FAO, Food and Agriculture Organization; JECFA, Joint FAO/WHO Expert Committee on Food Additives; ICPMS, Inductively coupled plasma mass spectrometry; bw, body weight; MT, metallothionein; PTMA, prothymosin.

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1. Introduction

Cadmium (Cd) is ubiquitously present in the environment, due to its natural occurrence and as a pollutant from industrial and agricultural sources. Animal and epidemiological studies have confirmed that Cd is toxic to the gonads (Henson and Chedrese, 2004; Thompson and Bannigan, 2008), kidney (Lind et al., 1997; Min et al., 1996), liver (Rikans and Yamano, 2000.) and bone (Bhattacharyya et al., 1988). In 1993 the International Agency for Research on Cancer (IARC) classified Cd as a human carcinogen (Group 1) based on cancer incidence data derived from occupationally exposed adults (IARC, 1993). Atmospheric pollution, phosphate fertilizers and sewage sludge appear to be the major contributors to Cd deposition in agricultural soils. Cd is present in virtually all foods at variable concentration depending on the type of food and concentration of Cd in the soil (Mor and Ceylan, 2008; Satarug et al., 2003). Diet represents the main source of Cd exposure in non-occupationally exposed humans. Primarily because of their high consumption, the food groups that contribute to the daily Cd intake in humans the most are cereal products, rice, vegetables, nuts, roots and tuber (FAO/WHO, 2005; EFSA, 2009). Bread, accounting for 36% of the weekly Cd intake, has been identified as the largest single source of Cd in diet in Sweden (Olsson et al., 2002).

The provisional tolerable weekly intake (PTWI) for Cd is set to 7 µg/kg bw by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1988, and re-evaluated in 2005 without a change (FAO/WHO, 1988, 2005). European Food Safety Authority (EFSA) estimated in 2009 that the median of average intake values across European countries is 2.3 µg/kg bw per week (range from 1.9 to 3.0 µg/kg bw per week) (EFSA, 2009), and set the tolerable weekly intake (TWI) as 2.5 µg/kg bw. The current population exposure levels are very close to the limits set by JECFA and EFSA. Specific groups in risk of consuming higher dietary Cd than recognized as safe are children and vegetarians (EFSA, 2009).

Dietary Cd is mainly absorbed in the proximal part of the small intestine (Reeves and Chaney, 2008). It enters the systemic circulation complexed to proteins, such as albumin, metallothionein and cysteine, and is taken up by tissues (Zalups and Ahmad, 2003). Cd accumulates in particular to liver and kidneys (Henson and Chedrese, 2004; Thompson and Bannigan, 2008), and the amount of Cd in these organs is estimated to account for over 70% of total body burden (Andersen, 1989). The half-life of Cd varies from 6 to 38 years in the human kidney and from 4 to 19 years in the human liver (Kostial et al., 1979; Kjellstrom and Nordberg, 1985). This very long biological half-life makes Cd a cumulative toxin (Järup and Akesson, 2009).

Several independent reports suggest that Cd interacts with the estrogen receptor alpha (ERα) (Stoica et al., 2000; Martin et al., 2003). ERs are ligand-inducible transcription factors that mediate the responses to the female sex steroids estrogens for instance in reproductive, skeletal and nervous systems. Dysregulation of ER activity by environmental chemicals has been suggested to be involved in the development of reproductive disorders and cancer. CdCl₂ was reported to bind ERα with high affinity (Stoica et al., 2000), activate it in transient transfection assays, induce the expression of estrogen target genes and promote proliferation of estrogen dependent cells *in vitro* (Stoica et al., 2000; Garcia-Morales et al., 1994; Martínez-Campa et al., 2006; Wilson et al., 2004). In ovariectomized (OVX) rats CdCl₂ at a concentration similar to the PTWI (single i.p. injection 5 µg/kg bw) induces uterine growth and up-regulates estrogen target genes in the uterus and mammary gland (Johnson et al., 2003). In some studies the *in vitro* and *in vivo* effects of CdCl₂ were blocked by antiestrogens suggesting a direct involvement of the ERα pathway (Stoica et al., 2000; Johnson et al., 2003; Brama et al., 2007). In most *in vivo* studies to date, the estrogen effects of Cd were estimated after exposure to inorganic

Table 1

Analyzed composition of the custom-made breads per fresh weight.

Component	Wheat bread	Flax bread	Unit
Carbohydrates	48.9	38.1	g/100 g
Glucose	0.10	0.16	g/100 g
Fructose	0.33	0.33	g/100 g
Saccharose	0.09	0.22	g/100 g
Lactose	0.04	<0.04	g/100 g
Protein	10.0	10.2	g/100 g
Fat	3.0	5.4	g/100 g
Saturated fatty acids	26.2	12.0	%
Monounsaturated fatty acids	33.2	37.1	%
Polyunsaturated fatty acids	39.3	50.1	%
Energy content	263	242	kcal/100 g
Total dietary fibre	3.1	6.9	g/100 g
Ash	2.0	2.1	g/100 g
Moisture	33.0	37.3	g/100 g
Sodium	540	490	mg/100 g
Cadmium	20.5	85.5	µg/kg

Cd salts dissolved in a buffer or saline (Johnson et al., 2003; Höfer et al., 2009; Ali et al., 2010). It is not known whether diet-bound Cd would induce similar effects.

The aim of the present study was to investigate the effects of whole food items that intrinsically contain varying concentrations of Cd on estrogen signaling and tissue Cd concentrations *in vivo*, comparing the results to those of CdCl₂ and in parallel to E₂ as an estrogen control. Since cereal products are among the most significant contributors to the daily Cd intake in humans (EFSA, 2009; Olsson et al., 2002), and bread has been identified as an important cereal contributor (Olsson et al., 2002), we designed two bread-based diets with differing intrinsic concentrations of Cd. The effects were studied in estrogen reporter ERE-luc mice as changes in the uterine weight and expression of luciferase and endogenous estrogen target genes and as Cd concentration in intestine, liver and kidneys after 21 days of exposure.

2. Materials and methods

2.1. Chemicals

17β-Estradiol (E₂) was administered to the mice as a long-term release pellet (Innovative research of America) that can release 5–6 µg/kg bw per day. CdCl₂ was purchased from Sigma–Aldrich (Pomezia, Italy), ketamine (Imalgene 500) from Merial (Toulouse, France), xilazine (Rompun) from Bayer (Shawnee Mission, Kansas, USA), and Beetle luciferin potassium salt from Promega (Milan, Italy).

2.2. Experimental animals

Animal colonies were housed according to the Guidelines for Care and Use of Experimental Animals. The Italian Ministry of Research (DM124/2003-A) and Milan University approved all animal studies, after approval by the expert committee at the Department of Pharmacological Sciences, University of Milan. The studies were carried out in heterozygous 4–6 months adult female ERE-Luc mice (Ciana et al., 2001) in the C57BL/6 genetic background. The mice were ovariectomized (OVX) 15 days before the experiment to eliminate the production of ovarian estrogens. The animal room was maintained within a temperature range of 22–25 °C and relative humidity of 50 ± 10% with a 12-h light–dark cycle (lights on at 07:00 a.m.). In each experimental group, mice were matched for age, weight and background luciferase expression.

2.3. Preparation of the breads-based diets

To obtain bread with differing Cd concentrations, white wheat toast bread with and without flaxseed supplement were custom made. Flaxseed is an oilseed that efficiently accumulates Cd from the soil, and flaxseeds are a common ingredient in multigrain breads. The flaxseed-supplemented bread was based on the same recipe as the white wheat toast but it was modified to contain 10% milled flaxseed. The breads were analyzed for macronutrient composition by Lantmännen Analyzen (Tampere, Finland) with accredited methods (Table 1). Cd concentration in the breads was analyzed in duplicates with graphite furnace atomic absorption spectrometry using standard addition quantification method as described before (Khan et al., 2007).

The rodent diets were designed and manufactured by Special Diet Services (Whitham, Essex, UK) so that highest feasible amount of bread (42.92%) was added

Table 2
Calculated macronutrient composition of the rodent diets.

	AIN93G	Wheat bread diet	Flax bread diet
Moisture	10.0	10.0	10.0
Crude oil	6.9	6.7	6.7
Crude protein	16.4	16.0	16.6
Crude fibre	3.3	3.3	3.3
Ash	3.2	3.9	4.0
Carbohydrates	59.6	59.6	58.5
Energy (kcal/g)	3.7	3.6	3.6

to the purified AIN93G rodent diet without compromising the macronutrient composition or pellet quality of the diets. The Cd concentration in the rodent diets was analyzed by inductively coupled plasma mass spectrometry as described before (Ali et al., 2010). The final calculated macronutrient compositions of the experimental diets are shown in Table 2.

2.4. Study design

To lower the background exposure to dietary estrogens the mice were switched to purified AIN93G diet at the time of the ovariectomy, 2 weeks prior to the start of the experimental period.

Initially, we tested whether the selected animal model is applicable to study estrogenic activity of inorganic Cd by treating adult intact ERE-Luc female mice with a single dose of CdCl₂ (1 µg/kg bw) by gavage. Tissues were dissected 6 h post exposure and luciferase activity quantified in uterus, liver, kidney, lung, thymus, WAT and skeletal muscle as described before (Rando et al., 2010).

For the 21-day study, the baseline luciferase activity before the start of the experimental period was measured in all animals by *in vivo* imaging and the mice were allocated to the different experimental groups so that the average background luciferase activity at start was comparable in all groups. Each group consisted of five animals and all animals had free access to feed and tap water throughout the experiment. Food, water and body weights were recorded once a week throughout the experiment. Since animals were maintained in cages of five mice each, we measured the entire amount of food and water used daily by the group and normalized to single animal.

The five different experimental groups were: (1) control group (purified AIN93G diet); (2) CdCl₂ group (1 µg/kg bw per day by gavage); (3) E₂ group (s.c. long term release pellet 5 µg/kg bw per day to resemble the physiological concentration at diestrus); (4) wheat bread diet (low Cd content 17.57 µg/kg); and (5) flax bread diet (high Cd content 49.22 µg/kg). In the CdCl₂ group, CdCl₂ dissolved in saline was administered as a single 100 µl bolus every 24 h. The mice were maintained in the experimental groups for 21 days.

Bioluminescent imaging (BLI) sessions were carried out on days 0, 1, 7, 14 and 21 at 2:00 p.m. before the daily CdCl₂ administration, as described before (Ciana et al., 2003). For quantification, photon emission was counted in the regions of interest and the signals obtained were integrated from each anatomical area as previously described (Biserni et al., 2008; Rando et al., 2010). Photon emission is defined as the number of counts per second per square centimeter (cts/cm²s). Quantifications were done using WinLight32 imaging software (Berthold Technologies). Normalization was performed using an external source of photons enabling to measure the instrumental efficiency of photon counting (Glowell, Luxbiotech, Edinburgh, UK).

On the last day of the experiment (day 21) the animals were sacrificed and tissues were dissected and equally divided for the quantitative analysis of the content of Cd, luciferase and specific mRNAs. All tissues were handled with acid washed material to avoid Cd contamination and the small intestine was washed with 0.9% NaCl containing 5 mM EDTA (pH 7) to remove any undigested food.

2.5. Cd in tissues

We quantified the concentration of Cd in the major storage organs, liver and kidneys, and in the small intestine (duodenum and jejunum) with the method described before by Ali et al. (2010). For the bioavailability estimation, the absolute quantity of Cd in these organs was calculated based on the tissue weights. To account for the background Cd concentration at the start of the experiment, we subtracted the control group values from the flax bread, wheat bread, and CdCl₂ group values. The tissue Cd values were then compared to the oral intake to estimate the absorption of the orally consumed Cd into the gut epithelium, and deposition into the liver and kidneys.

2.6. Luciferase enzymatic assay

Luciferase extraction from frozen tissues was carried out as previously described (Rando et al., 2010).

Fig. 1. Acute effects of CdCl₂ on luciferase activity in adult cycling ERE-luc mice. Luciferase activity in different tissues *ex vivo* 6 h after a single oral dose of 1 µg/kg bw CdCl₂. The luciferase activity was related to the protein concentration in each sample and expressed as RLU/µg protein. Values represent mean ± SEM (n = 5). The data was analyzed by one-way ANOVA and Bonferroni test. *p < 0.05, **p < 0.01, treatment versus control. RLU, relative light units.

2.7. Gene expression analysis

Real time PCR experiments were performed using RNA extracted with RNeasy® Mini kit (Qiagen, Milan, Italy). The preparation of cDNA was carried out as described (Biserni et al., 2008) using TaqMan technology with pre-made primers from TaqMan Gene Expression assays (Applied Biosystems, Milan, Italy) for the endogenous gene *Esr1* (Mm00433149.m1), *progesterone receptor* (Mm00435625.m1) *prothymosine* (Mm02342432.g1), and as a reference gene assay 18S rRNA VIC-MGB-PDAR. The amplification reaction was carried out according to the manufacturer's protocol using 7900HT fast real-time PCR system (Applied Biosystems, Milan, Italy) and data were analyzed using the 2^{-ΔΔCt} method (Livak and Schmittgen, 2001).

2.8. Statistical analysis

Normally distributed data was analyzed with one-way or two-way ANOVA and p < 0.05 was considered as statistically significant difference. The differences between the groups were then analyzed with Bonferroni *post hoc* test, comparing the treatments to the control group. All analyses were carried out with GraphPad Prism version 5.01 for Windows, GraphPad Software (San Diego California USA). In tables and figures, data is presented as mean ± SEM unless stated otherwise, and statistical differences are marked as * for p < 0.05, ** for p < 0.01 and *** for p < 0.001. All probabilities are two-tailed.

3. Results

3.1. Reporter gene activity in the ERE-Luc mouse model after acute exposure to CdCl₂

The sensitivity of the selected animal model was tested by treating adult cycling females with a single oral dose of 1 µg/kg/bw of CdCl₂. Significant luciferase induction was detected 6 h later in the uterus and thymus (Fig. 1) demonstrating that the reporter gene system in the ERE-Luc mouse model is sensitive to oral Cd treatment in low exposure concentrations.

3.2. Food and water intake and organ weights

OVX adult female mice were maintained on the experimental diets for 21 days and the food and water consumption was recorded once a week. The average daily food intake was 2.5 g/mouse and water consumption 5.5–6 g/mouse in all treatments except the E₂ group (7.6 g/mouse). All groups consumed similar amounts of food, and the food and water consumption did not significantly change during the 21-day experimental period within each respective group. Treatments did not affect the body weight of the control, wheat bread, flax bread and E₂ groups, but by the second week of the study in the mice treated with CdCl₂ we observed a significant weight loss (Table 3).

The weights of livers and kidneys did not differ between control, wheat and flax bread groups (Fig. 2). CdCl₂ administration

Table 3
Body weights of the ERE-Luc mice during the experiment.

Treatment	Day of treatment				% difference at d21 (treatment versus control)
	1	7	14	21	
Control	23.8 ± 0.6	24.0 ± 0.4	24.2 ± 0.4	25.0 ± 0.5	–
CdCl ₂	23.2 ± 0.3	22.6 ± 0.4	22.3 ± 0.7*	22.7 ± 0.5*	–9
Wheat bread	23.5 ± 0.2	24.0 ± 0.3	24.2 ± 0.3	24.4 ± 0.3	–3
Flax bread	23.9 ± 0.9	24.3 ± 0.9	24.6 ± 0.9	25.7 ± 1.0	+3
17β-Estradiol	23.0 ± 0.6	24.0 ± 0.8	25.1 ± 0.8	25.7 ± 0.6	+3

The weights are expressed in grams as mean ± SEM (n = 5).

* p < 0.05 control versus treatment and time by two-way ANOVA and Bonferroni post hoc test.

Fig. 2. Organ weights at sacrifice. The wet weights of uterus, liver and kidneys were recorded at sacrifice. Values represent mean ± SEM (n = 5). The data was analyzed by one-way ANOVA and Bonferroni test. *p < 0.05, **p < 0.01, treatment versus control.

caused a significant decrease in the liver weight and E₂ significantly increased liver weight, but these treatments did not change kidney weights (Fig. 2).

The wet weight of the uterus has long been utilized as a hallmark of estrogen action, and the OECD uterine growth assay has become the standard guideline in the assessment of estrogenic activities of chemicals. In our study, E₂ significantly increased uterine weight, as expected. A smaller but significant increase in the uterine weights was observed in the CdCl₂ group. No modulation of uterine weight was observed in the wheat and flax diet groups (Fig. 2).

3.3. Cd intake and concentration in gut, liver and kidneys

The wheat bread diet was analyzed to contain 17.57 µg/kg Cd, and the diet based on the same bread supplemented with 10% flaxseed contained 49.22 µg/kg. The Cd concentration in the control diet (AIN93G) was 9.22 µg/kg. All diets were comparable to each other in terms of macronutrient composition (Table 2).

The total oral intake of Cd per kg bw was calculated in each group based on the recorded body weights (Table 3) and food consumption data. During the 21-day study, the mice consuming control diet ingested a total of 483 ng Cd (≈0.95 µg/kg bw per day); the group treated with CdCl₂ 966 ng (≈2.0 µg/kg bw per day, consisting of 1.0 µg/kg bw per day as CdCl₂ and 1.0 µg/kg bw per day as dietary Cd originating from the control diet); the mice consuming wheat bread diet 924 ng (≈1.8 µg/kg bw per day) and the mice consuming flax bread diet 2580 ng (≈5.1 µg/kg bw per day) (Table 4). Significant increase in Cd concentration was observed in the small

intestine and liver of the animals in the inorganic CdCl₂ and flax diet groups (Fig. 3). No accumulation over control was found in animals fed with the wheat bread diet, and none of the treatments significantly affected Cd content in the kidney (Fig. 3).

We then estimated the amount of Cd accumulated into the intestine, liver and kidneys during the 21-day experimental period. Since the study was conducted with adult mice, there was a substantial background concentration of Cd already present in the tissues at the start of the experiment (as indicated in the control group values in Fig. 3). Therefore we decided to use the control group tissue Cd values as a measure of the Cd that had accumulated into the tissues before the start. Hence, we subtracted the average control group tissue concentrations (14 ng in liver, 40 ng in kidneys and 8 ng in intestine) from the corresponding values in the wheat bread, flax bread and inorganic CdCl₂ groups to obtain an estimation of the amount of Cd taken up by the mice during the 21-day exposure. The highest amount Cd, 56 ng, was absorbed to the small intestine, liver and kidneys in the flax bread group, followed by 44 ng in the inorganic CdCl₂ group and 15 ng in the wheat bread group (Table 4). In the flax bread and inorganic CdCl₂ groups, the majority of the Cd was present in the small intestine, followed by kidneys and then liver. In the wheat bread group, Cd was more evenly distributed between the three tissue types (Table 4).

3.4. Spatio-temporal luciferase activity in vivo

We measured luciferase catalyzed photon emission at 0, 1, 7, 14, and 21 days of treatment and quantified the response in the chest,

Table 4
Cd intake and tissue accumulations in the 21-day exposure.

	Oral intake (ng)	Exposure (µg/kg/day)	TOT Cd ^a in tissues ^b , ng (% intake)	Cd in intestine ^a , ng (% intake)	Cd in liver ^a , ng (% intake)	Cd in kidneys ^a , ng (% intake)
Control	483	0.95	–	–	–	–
Wheat bread group	924	1.8	15 (1.6)	6 (0.6)	4 (0.4)	5 (0.5)
Flax bread group	2580	5.1	56 (2.2)	36 (1.3)	7 (0.3)	13 (0.5)
CdCl ₂ group	966 (483 ng from the CdCl ₂ and 483 ng from the AIN93G diet)	2.0 (1.0 from the CdCl ₂ and 1.0 from the AIN93G diet)	44 (9.1) ^c	32 (6.6)	5 (1.0)	7 (1.4)

^a The average background concentration (i.e. average concentration in the control group, 14 ng in the liver, 40 ng in the kidney, and 8 ng in the intestine) has been subtracted from the values.

^b Intestine, liver and kidneys.

^c % intake value is based on the intake of CdCl₂.

Fig. 3. Concentration of Cd in liver, kidneys and intestine. The concentration of Cd in small intestine, liver and kidneys was measured by inductively coupled plasma mass spectrometry. Exposure to Cd during the 21-day period in the different groups: control 483 ng, wheat bread diet 924 ng, flax bread diet 2580 ng, and CdCl₂ 966 ng (483 ng as CdCl₂ and 483 ng from control diet). Values represent mean $\pm$ SEM ($n=5$). The data was analyzed by one-way ANOVA and Bonferroni test. * $p < 0.05$, ** $p < 0.01$, treatment versus control.

genital area, abdomen and limbs separately to reflect the activities in the liver, reproductive tract, gut and bone (Biserni et al., 2008; Rando et al., 2010). The luciferase activity did not significantly vary in time in the control group (Fig. 4). In the E₂ group significantly increased activity was measured in the chest, genital area and abdomen (Fig. 4c–e). CdCl₂, wheat and flax diets affected luciferase expression only in the chest area (Fig. 4a).

3.5. Luciferase activity ex vivo in tissue homogenates

Although the *in vivo* imaging is a good starting point when studying estrogen activity, it cannot give detailed information on responses in inner organs. Therefore we carried out luciferase analysis in tissues dissected on the last day of the experiment (day 21). In E₂ group, significant luciferase induction was detected in the small intestine (jejunum), kidney, brain, pituitary gland, WAT, and uterus, and significant repression in thymus (Fig. 5). Inorganic CdCl₂ significantly affected luciferase activity only in WAT (Fig. 5). The bread diets modulated luciferase activity in a wider range of tissues, namely in liver, pituitary gland, thymus and WAT. Both inhibitory and inducing effects were observed with the bread diets, but the effects did not systematically mimic those of CdCl₂ or E₂ (Fig. 5). To control that the lack of similarity to E₂ effects in the CdCl₂ and bread diet groups was not dependent on repression of ER α expression by the treatments, we quantified the expression of ER α in uterus, WAT and thymus. The expression did not differ from the baseline, suggesting that the lack of similarities in the estrogen responses were not due to altered receptor expression (Supplementary Fig. 1).

3.6. Expression of endogenous estrogen target genes

We quantified the expression of two endogenous markers of estrogen signaling, progesterone receptor (PR) and prothymosine A (PTMA), in uterus, WAT and thymus. As predicted by the luciferase data, E₂ induced both PR and PTMA expression in the classical estrogen target tissues, uterus and WAT (Fig. 6). Similarly, inorganic CdCl₂ displayed estrogen activity in the WAT by inducing PR expression, as predicted by the luciferase response (Fig. 6). However, the responses to the bread diets were more difficult to predict based on the luciferase response: wheat bread induced luciferase in WAT but none of the endogenous genes, and both bread diets induced PTMA in uterus without affecting luciferase. The correlation between luciferase and endogenous gene responses in the thymus was even more complicated. Inorganic CdCl₂ did not affect any measured parameters in this organ, while bread diets increased luciferase and PTMA, flax bread in addition decreased PR, and E₂ decreased luciferase and PR but increased PTMA (Fig. 6).

4. Discussion

The present study investigated the effect of a 21-day oral administration of Cd in different forms on markers of estrogenicity and tissue deposition of Cd in ERE-Luc reporter mice. Our results confirm previous reports showing that CdCl₂ may possess estrogen-like activity *in vivo* (Johnson et al., 2003; Höfer et al., 2009; Ali et al., 2010), and we identify WAT as a potential novel target tissue. However, bread bound Cd did not mirror the effects of CdCl₂. Our data suggests that caution should be taken when pure compound results are extrapolated to dietary exposure scenarios.

4.1. Effects of the treatments on estrogen signaling

We used three different marker genes to assess genomic estrogen signaling after Cd exposure: luciferase, PR and PTMA. The luciferase gene is driven by a dimerized consensus estrogen responsive element and functions as a sensitive indicator of estrogen activity (Ciana et al., 2003). Both chosen endogenous genes harbor estrogen responsive elements in their promoter regions and respond to estrogens in different tissues (Garnier et al., 1997; Tsitsilonis et al., 1998; Martini and Katzenellenbogen, 2001; Bianco and Montano, 2002). We first measured the expression of luciferase in a wide variety of tissues, and then completed the data with the two endogenous markers in uterus, a classical estrogen target tissue; in WAT, an estrogen target tissue where CdCl₂ increased luciferase activity; and in thymus, a non-classical estrogen target tissue where both bread diets modulated luciferase activity.

In the E₂ treated mice, luciferase, PTMA and PR expression were all significantly up-regulated in typical estrogen target tissues, uterus and WAT (Figs. 5 and 6). In thymus, however, E₂ repressed luciferase and PR while promoted PTMA expression. Although all three marker genes are regulated directly by E₂, PTMA also affects ER α signaling on another level: it binds to co-factors involved in estrogen signaling affecting their availability for ER α (Bianco and Montano, 2002). Perhaps in thymus, where the biological function and genomic targets of estrogens might differ from the classical target tissues such as uterus and WAT, also the regulation of the receptor activity in terms of co-factors is different, leading to the discrepancy in the expression between PTMA and the two other markers in our study.

The mice receiving inorganic CdCl₂ displayed a narrower range of effects than the mice receiving E₂. For instance, none of the genes was significantly regulated in the uterus. Previously, inorganic Cd has been shown to up-regulate C3 and PR in the rat uterus (Johnson et al., 2003; Höfer et al., 2009). In another reporter mouse model, CdCl₂ did not affect luciferase expression; instead it promoted markers of membrane-bound estrogen signaling (Ali et al., 2010). Strains differ in their capacity to produce metallothionein to neutralize Cd, which could lead to different biological

Fig. 4. Spatio-temporal luciferase expression in the treatment groups during the study. Luciferase activity was measured by *in vivo* imaging as photon emission in the chest, genital area, abdomen and limbs on days 0, 1, 7, 14 and 21 of treatment. Daily exposure to Cd in the different groups as $\mu\text{g/kg bw}$: control 0.95, wheat bread diet 1.8, flax bread diet 5.1 and CdCl₂ 2.0 (1.0 from CdCl₂ and 1.0 from control diet). The results are expressed as cts/cm² and the bars represent mean $\pm$ SEM ($n = 5$). The data was analyzed by two-way ANOVA and Bonferroni test. * $p < 0.05$, ** $p < 0.01$, treatment versus control.

outcomes (Shaikh et al., 1993). For example, in Sprague-Dawley rats up-regulation of C3 is obtained after a single 5 $\mu\text{g/kg bw}$ dose, while in Wistar rats 2 mg/kg bw dose is required (Höfer et al., 2009; Johnson et al., 2003). Our study was conducted in C57BL6 mice, as was the study of Ali et al. Although the length of their treatment period was only 3 days compared to our 21 days, the results are similar: no significant regulation of genomic markers of estrogen signaling was observed in the uterus, suggesting that C57BL6 mice differ from rats. Interestingly, when we treated intact cycling female mice with CdCl₂ in our sensitivity test, significant induction of luciferase in the uterus was observed (Fig. 1). These observations suggest that both the strain and the hormonal milieu of the animals might affect the bioactivity of Cd. More studies are needed to define the circumstances under which Cd can promote estrogen signaling.

Interestingly, CdCl₂ promoted the expression of PR and luciferase in WAT. Moreover, the body weight of the CdCl₂ treated mice was significantly lower than control by the end of the experiment, despite similar food consumption in all groups. Recently, CdCl₂ was shown to reduce both body weight and WAT weight in adult male mice (Kawakami et al., 2010). Although in that study relatively high doses were used (0.5–2 mg/kg bw), the results together with ours suggest that CdCl₂ might be active in the adipose tissue, and this activity could involve estrogen signaling.

The bread diets had diverse effects, modulating at least one of the markers in all three tissues (WAT, thymus and uterus). In the uterus, the bread diets did not regulate luciferase or PR, which is in agreement with the literature (Penttinen-Damdimopoulou et al., 2009). PTMA expression in the uterus, however, was up-regulated by the diets. As discussed above, the more complex involvement of

Fig. 5. Effects of the treatments on tissue specific luciferase expression. Luciferase activity in different tissues *ex vivo* after the 21-day long treatment period. The luciferase activity was related to the protein concentration in each sample and expressed as RLU/μg protein. Daily exposure to Cd in the different groups as μg/kg bw: control 0.95, wheat bread diet 1.8, flax bread diet 5.1, and CdCl₂ 2.0 (1.0 from CdCl₂ and 1.0 from control diet). The bars represent mean ± SEM (*n* = 5). The data was analyzed by one-way ANOVA and Bonferroni test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 treatment *versus* control. RLU, relative light units.

PTMA in estrogen signaling might make this gene a more general marker of estrogen modulation than luciferase and PR.

In WAT, where both E₂ and CdCl₂ induced ER signaling, the high Cd flax bread diet was devoid of such activity but the low Cd wheat bread promoted luciferase expression. Although the oral intake of Cd was similar in wheat and CdCl₂ groups (1.8 μg/kg bw from diet *versus* 1.0 μg/kg as CdCl₂ + 1.0 μg/kg from diet, respectively), the total tissue deposition of Cd was more comparable between the flax bread and CdCl₂ groups (56 ng *versus* 44 ng, respectively). It is therefore difficult to understand why the flax bread diet did not affect ER activity in WAT while wheat bread diet did. Similarly, in thymus, the effects induced by the bread diets did not systematically mirror those of E₂ or CdCl₂ and there was no apparent dose-dependency. Here, the presence of other bioactive substances in the bread matrix has to be considered. For instance wheat contains alkylresorcinols and flaxseed lignans. The influence of these compounds on estrogen signaling in the presence of Cd could be addressed in future studies.

Luciferase activity was also measured in other tissue types by *ex vivo* analysis as well as in different body areas by *in vivo* imaging. In prolonged exposure to ER modulators, the reporter gene activity in our mouse model oscillates with compound/treatment specific amplitudes and frequencies (Rando et al., 2009; Rando et al., 2010). The functional consequences of such oscillation are not clear, however, to reliably detect ER modulation in long exposure the reporter gene activity should be measured at different time points. If we summarize the tissue targets of the treatment in our study based on both *in vivo* and *ex vivo* analysis, it seems that the bread diets target liver, pituitary gland, thymus and WAT. Inorganic CdCl₂, on the other hand, only targets WAT and liver. E₂ is active in a wider range of tissues (liver, reproductive tissues, intestine, kidney, brain, pituitary gland, thymus, and WAT), as expected based on our earlier studies (Rando et al., 2010; Ciana et al., 2003). In our study, liver was the only tissue type where modulation of estrogen signaling

was observed after all treatments (CdCl₂ and E₂ *in vivo*, bread diets *in vivo* and *ex vivo*). Since many absorbed dietary compounds are first transported to the liver, and this organ responds to estrogen modulators, biomarkers of estrogen signaling in the liver could be used as a quick screen for estrogen modulation by dietary factors.

The traditional screen for estrogen-like activity *in vivo* is the rodent uterine growth test. We therefore measured the uterine weights in our study, and as expected, E₂ significantly increased the weight. Interestingly, also CdCl₂ increased uterine weight although none of the markers of genomic signaling were regulated. In response to estrogens, the expression of hundreds of genes in the uterus is regulated in preparation to embryo implantation and pregnancy, and both genomic and non-genomic signaling pathways are involved. In a recent study in another estrogen reporter mouse model, a 3-day s.c. exposure to 5–500 μg/kg bw CdCl₂ activated markers of non-genomic signaling in the absence of ERE-response or uterine growth effects (Ali et al., 2010). In the present study, membrane associated signaling was not measured, but it is possible that this pathway was stimulated by CdCl₂ throughout the 21-day experimental period, finally leading to increased uterine weight. It is noteworthy that the increase we measured was significantly lower than in the E₂ group, suggesting only partial estrogen effects. The bread diets did not modulate uterine weight. Similar results were obtained with wheat bran and flaxseed diets in another reporter mouse model (Penttinen-Damdimopoulou et al., 2009) and with flaxseed diet in an immature rat uterotrophic test (Khan et al., 2007).

4.2. Bioaccumulation of dietary Cd

The mice in all groups, including control, ingested Cd in amounts exceeding the new EFSA recommendation (2.5 μg/kg bw in a week): the control mice ingested 2.7-fold, wheat bread mice 5.0-fold, CdCl₂ group 5.6-fold (both dietary background and gavage)

Fig. 6. Effects of the treatments on endogenous estrogen target genes. The expression of progesterone receptor and prothymosin mRNA was quantified against ribosomal 18S RNA in selected tissues. Daily exposure to Cd in the different groups as $\mu\text{g/kg bw}$: control 0.95, wheat bread diet 1.8, flax bread diet 5.1, and CdCl₂ 2.0 (1.0 from CdCl₂ and 1.0 from control diet). The bars represent mean $\pm$ SEM ($n=5$). The data was analyzed by one-way ANOVA and Bonferroni test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ treatment versus control.

CdCl₂ counted), and flax bread group 14.3-fold more than considered safe by EFSA. These intake values are not exaggerated compared to humans in that they reflect exposures in polluted areas (WHO, 1992; Järup et al., 1998). As planned, a clear gradient in the oral intake of Cd was achieved between the groups: the wheat bread and CdCl₂ mice ingested 2-fold and the flax bread mice over 5-fold more Cd than the controls. When Cd deposition to intestine, liver and kidneys were compared between the groups, a pattern different from the oral intake was observed. Although wheat bread and CdCl₂ mice had similar oral intakes of Cd (924 ng versus 483 ng as CdCl₂ + 483 ng from the diet, respectively), the CdCl₂ mice accumulated markedly more Cd into the tissues (44 ng) than the wheat bread mice (15 ng) (Table 4). Indeed, the total tissue accumulation in the CdCl₂ group was more comparable to the flax bread group (56 ng), although the oral intake in the flax group (2580 ng) was over twice as high as in the CdCl₂ group. This suggests higher bioavailability of gavaged CdCl₂ compared to diet bound Cd, as observed in previous studies (Chan et al., 2000; Lind et al., 1998).

Studies conducted with ¹⁰⁹Cd have shown that over 70% of absorbed dietary Cd is deposited into liver and kidneys (Andersen, 1989). However, in our study majority of the Cd seemed to accumulate to the intestine (Table 4). The absorption of Cd has been extensively studied, and the metal is known to rapidly accumulate within the intestinal mucosa (Zalups and Ahmad, 2003; Reeves and Chaney, 2008). In contrast, the transport of Cd into the systemic circulation occurs with a low rate (Zalups and Ahmad, 2003). It is

therefore possible that the Cd bound to mucosa sheds away with the renewing enterocytes before further absorption. Some support for this hypothesis comes from a study by Phillpotts (1979). He orally administered adult rats a low dose of ¹⁰⁹Cd and studied the retention in various tissues. The majority of ¹⁰⁹Cd was detected in the duodenum within the 24 h post challenge, but as the concentration in the duodenum rapidly fell between 24 and 48 h post Cd administration, the concentration in the inner organs did not rise (Phillpotts, 1979). Similarly, House et al. (2003) has shown that the whole body retention of ¹⁰⁹Cd declines dramatically within the first week after ingestion, suggesting an initial shorter term retention for this metal than the long term storage in liver and kidneys (House et al., 2003). In agreement with Andersen (1989), also Phillpotts detected the majority of absorbed Cd (i.e. Cd present in inner organs) in the kidneys and liver. Hence, we can use the amount of Cd in liver and kidneys as a proxy of the absorbed Cd when estimating the bioavailability in our study.

When the quantity of Cd in liver and kidneys is compared to the oral intake, the highest retention was observed in the CdCl₂ group, where a quantity corresponding to 2.4% of the oral CdCl₂ intake was detected. In the bread groups, markedly less Cd accumulated to these organs; in both wheat and flax bread groups the quantity of Cd accumulated to the liver and kidneys corresponded to less than 1% of the orally ingested dose. These values are in agreement with the literature, showing that the presence of wheat in diet decreases Cd accumulation in tissues (Chan et al., 2000; House et al., 2003). The

factors believed to contribute to the Cd uptake from the gut most are trace element deficiencies and dietary fibre (Reeves and Chaney, 2008). In all our diets, the same standard amount of AIN93G mineral mix was added (Reeves et al., 1993), so deficiencies are not likely. However, there are differences in the makeup of the dietary fibre in the diets. All diets contain 3.3% fibre. In the control diet, this was entirely composed of cellulose. In the bread diets the fibre fraction contained both fibre naturally present in the breads and cellulose that was added to balance the fibre fraction to 3.3% in all diets. The presence of complex plant fibre, known to inhibit Cd absorption, probably partially explains the lower bioavailability from the bread diets (Andersen et al., 2004).

In spite of similar oral intakes in the wheat and CdCl₂ groups, and similar absolute tissue levels of Cd in flax bread and CdCl₂ groups, we did not observe any systematic similarities in estrogen responses in these groups. This suggests that the form in which Cd is ingested affects the absorption as discussed above, but also that the form in which Cd is absorbed affects subsequent biological activity. When CdCl₂ is administered by oral gavage, Cd is probably rapidly available for absorption in ionic form at the low pH of proximal duodenum. In the flax bread diet, Cd is mainly bound to the flaxseed protein fraction (Lei et al., 2003), that requires digestion before Cd is released. Interestingly, mercury can be absorbed through amino acid transporters in complex with cysteines (Cannon et al., 2001). Cd can also complex to cysteines, but the involvement of amino acid transporters in the absorption of Cd from intestine lumen has not been studied to our knowledge. Intriguingly, the form of Cd is known to affect its toxicity: in the case of kidney damage, metallothionein complexed Cd causes nephrotoxicity at 8-fold lower doses than cysteine complexed Cd (Min et al., 1986). We hypothesize that the form of absorbed Cd may also affect subsequent estrogenic activity, which could help to explain the differences between CdCl₂ and bread diets in the present study. Another explanation to the differences could be additional bioactive substances present in the flax bread diet. For instance, flaxseed is the richest known dietary source of lignans, plant secondary metabolites that are converted by gut microbes to enterolactone. We have shown that enterolactone selectively regulates estrogen signaling, and this compound might thereby contribute to the net results measured in this study (Penttinen et al., 2007; Adlercreutz, 2007). Furthermore, the effect of food matrix on the *in vivo* activity of estrogenic compounds has been demonstrated in several other studies (Rando et al., 2009; Sirtori et al., 2005; Sirtori et al., 2009; Penttinen-Damdimopoulou et al., 2009).

4.3. Cd as an endocrine disruptor: critical issues and conclusions

The current classification of Cd as type I human carcinogen is based on data from occupationally exposed adults, and the health guidance values on biomarkers of nephrotoxicity. The recent discovery of the endocrine effects of Cd *in vivo* in experimental animals calls for re-evaluation of human health effects, and data supporting risk assessment, such as identification of biomarkers, should be a priority of future Cd studies.

To date the estrogenicity of Cd *in vivo* has been studied by administering inorganic Cd salts by injections or gavage, although human populations are exposed to Cd mainly through diet. Our study is the first one to compare the estrogen-like activity of inorganic CdCl₂ to the activity of a major dietary source of Cd, bread. The lack of overlap in effects of the bread diets, CdCl₂ and E₂ on estrogen signaling in the various different parameters measured is intriguing and illustrates the challenges in extrapolating the observed effects of a pure compound, such as the E₂-like activity of inorganic CdCl₂, to the effect of a Cd-containing bread diet. It seems that when the biological activity of dietary substances is investigated, pure compound studies should be accompanied by approaches, which

take into consideration the natural food context of the substance in question to account for possible matrix effects.

The quantification of Cd in tissues revealed that the diet-bound Cd is poorly absorbed compared to inorganic Cd. Furthermore, even in the presence of similar tissue Cd concentrations, diet-derived Cd does not possess the same estrogenic activity as CdCl₂. Taken together, our results support previous findings of estrogen-like activity of inorganic CdCl₂ *in vivo*, but do not suggest that diet-bound Cd would possess the identical activity. The future research efforts should be focused on understanding the mechanism of interaction between Cd and estrogen signaling; the identification of biologically active Cd species *in vivo*; and the characterization of biomarkers of the estrogenic effects of Cd *in vivo*.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxlet.2010.12.022.

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