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Lack of activity of cadmium in in vitro estrogenicity assays

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Abstract

Prompted by reports about strong estrogenic effects of cadmium, attempts were made to reproduce these observations using the yeast estrogen screen (YES) and the E-Screen assays. For the first time, possible activation of the Src/MAPK pathway was also investigated. In the YES, only a slight activation (10% of a maximal effect) of the estrogen receptor alpha (ERα) was observed at cadmium concentrations between 5×10^{-7} M and 5×10^{-6} M. In the E-Screen assay, carried out by two laboratories, the heavy metal was without observable cell proliferative effects when tested in the range between 6×10^{-11} M and 1×10^{-5} M. However, in both assays, cadmium led to a reduction of the effects of 17β-estradiol (E2). Treatment of MCF-7 human breast cancer cells with 1×10^{-7} M cadmium failed to induce phosphorylation of Src and the MAP kinases Erk1 and Erk2—effects shown to occur with E2 and epidermal growth factor (EGF). In summary, we were unable to confirm the strong estrogenicity of cadmium reported recently by a number of laboratories. This apparent absence of effects in our hands is not due to a lack of uptake of the metal or to effective protection against cadmium by high levels of glutathione or metallothionein, since toxicity and an antagonism of E2 responses were observed both in the YES and the E-Screen.

Keywords: Cadmium; Heavy metal; Estrogen; Endocrine disrupter; E-Screen; YES; Signalling pathways; MAP kinases; Estrogen receptor

Introduction

Cadmium is a ubiquitous contaminant, which is released into the environment from industrial activities, such as mining, smelting and electroplating, and from the extensive use of consumable items such as batteries, pigments and plastics (Jarup, 2003). The inherent persistence of this heavy metal is a reason for concern, as it bioaccumulates throughout ecosystems and can be found in high levels in soil, surface water, sediments, and, consequently, up the food chain (ATSDR, 1999; Nasreddine and Parent-Massin, 2002).

Human exposure to cadmium occurs primarily through food ingestion, corresponding to an average total dietary exposure of 0.12 μg/kg body weight/day in the UK (FSA, 2004). Tobacco smoking is also a very significant source of exposure, with a single cigarette containing approximately 1–3 μg of the heavy metal. The very long biological half-life of 10–30 years in humans allows cadmium to accumulate over time in kidneys and liver, as well as in reproductive organs, such as the placenta, testis, ovaries and breast. The concentration of cadmium in the kidneys of non-smokers has been reported as 15–20 μg/g wet weight, whereas in smokers, it can reach 30–40 μg/g wet weight (ATSDR, 1999). Although the human mammary gland is not a primary target, the cadmium concentrations in this organ can reach 31 μg/g wet weight (Antila et al., 1996).

The toxic effects of cadmium have been documented extensively. In humans, exposure to the metal has been associated with renal disease, osteoporosis, impairment of

Abbreviations: YES, yeast estrogen screen; MAPK, mitogen activated protein kinase; E2, 17β-estradiol; ER, estrogen receptor; hER, human estrogen receptor; EGF, epidermal growth factor; $CdCl_2$, cadmium chloride; CPRG, chlorophenol red-β-D-galactopyranoside; DMEM, Dulbecco's modified Eagle's medium; FBS, Fetal bovine serum; MEM-NEAA, non-essential aminoacids; CDHS, charcoal–dextran stripped human serum; TCA, trichloroacetic acid; SRB, sulfurhodamine-B; PE, proliferative effect; MT, metallothionein

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lung function and hypertension, as well as effects on the immune and nervous systems (ATSDR, 1999). Also, based on human and in vivo experimental evidence, cadmium has been linked to reproductive disorders and to a number of cancers, such as lung, kidney, bladder, pancreas, breast and prostate (IARC, 1993; Satarug and Moore, 2004; Takenaka et al., 1983).

Like many other heavy metals, cadmium toxicity is associated with its high reactivity with thiol groups of proteins. This means that it can interact and disrupt the activity of enzymes, transcription factors and nuclear receptors (Asmuss et al., 2000; Hanas and Gunn, 1996; Kawai et al., 2002). Additionally, it is able to compete with calcium both at Ca^{2+} channels and at intracellular calcium binding proteins (Nelson, 1986; Richardt et al., 1986).

Several studies have reported that heavy metals can also interact with steroid receptors, inhibiting binding of the cognate ligands. In 1992, Predki and Sarkar showed that zinc in the zinc fingers of the DNA binding domain of the human estrogen receptor (hER) can be replaced by several other metals such as copper, cobalt, nickel and cadmium (Predki and Sarkar, 1992). Subsequent in vitro and in vivo studies have demonstrated that cadmium is not only able to compete with 17β-estradiol (E2) for binding to the hER, but can also activate this receptor, exerting effects characteristic of the endogenous hormone (Stoica et al., 2000). These effects include induction of cathepsin D, progesterone receptor and pS2, reduction of ERα levels (Garcia-Morales et al., 1994), induction of human breast cancer cell proliferation (Choe et al., 2003) and increases in uterine weight and mammary development in rats (Johnson et al., 2003). In many of these studies, the potency of the metal was considerably higher than that of most organic xenoestrogens.

The possibility that cadmium is a potent endocrine disrupter has alarmed the scientific community. The publication of the paper by Johnson and colleagues prompted Safe (Safe, 2003) to draw attention to the fact that cadmium showed estrogenicity at doses that were comparable to the provisional tolerable weekly intake recommended by the World Health Organisation (WHO). Based on these data, Safe suggested the need for revision of WHO cadmium standards.

However, a number of research groups have failed to observe an activation of the ER by cadmium, although competition of cadmium with E2 for the binding to the receptor is not under dispute. Le Guevel and colleagues (2000) observed that the heavy metal inhibited the transcriptional activity of E2 activated ER in yeast cells transfected with the rainbow trout ER. The same observation was made when cells were transfected with hER. When tested alone, cadmium did not induce activation of either receptor. More recent work carried out on rainbow trout has shown an inhibition of E2-stimulated vitellogenin expression following treatment with the metal (Vetillard and Bailhache, 2005). This appears to be linked to a decrease in available ER for the binding of the hormone.

The contradictory findings reported in the literature have prompted us to further investigate the potential estrogenicity of cadmium. The ability of the metal to activate the hERα was evaluated in a receptor based assay, the yeast estrogen screen (YES). The E-Screen assay, which exploits the principle that MCF-7 breast cancer cells proliferate in the presence of chemicals that directly or indirectly activate the ER, was also employed. Studies with the E-Screen were carried out in two laboratories, using cadmium sourced from the same supplier.

For the first time, we have explored the effects of cadmium on the Src/Ras/Erk signalling pathway in human breast cancer cells. This pathway is primarily involved in the cellular response to growth factors, such as the epidermal growth factor (EGF) and has been shown to be rapidly and transiently activated by E2 (Migliaccio et al., 1996; Migliaccio et al., 1998). It is thought that the activation of this pathway by the hormone is mediated by the ER (Cheskis, 2004; Migliaccio et al., 2002). Following activation, the receptor promotes the phosphorylation of the target kinase Src and, consequently, the mitogen-activated protein kinases (MAPK) Erk1 and Erk2. Therefore, if cadmium is able to activate the ER to the extent reported by other groups, it should be able to cause phosphorylation of Src and Erks in a way similar to E2.

Materials and methods

Chemicals. Cadmium chloride ($CdCl_2$, 98%) and 17β-estradiol (E2, 98%) were purchased from Sigma-Aldrich (Dorset, UK). $CdCl_2$ was also obtained from Acros Organics (99.99%, Loughborough, UK) and VWR (99.99%, Dorset, UK). Chlorophenol red-β-D-galactopyranoside (CPRG) was purchased from Boehringer Mannheim, East Sussex, UK. Dulbecco's modified Eagle's medium (DMEM), phenol-red free DMEM, fetal bovine serum (FBS) and MEM nonessential amino acids (MEM-NEAA) were obtained from Invitrogen Corporations, Paisley, UK. FBS used by the Granada group was from BioWittaker, Walkersville, MD. Rabbit polyclonal anti-phospho-Erk1/Erk2, anti-Erk1/Erk2 and anti-phospho-Src (Tyr 416) antibodies, anti-rabbit and anti-mouse secondary HRP-conjugated antibodies and enhanced chemiluminescence detection (ECL) system were all purchased from New England Biolabs, Hitchin, UK. Mouse monoclonal anti-v-Src (clone 327) antibody was from Merk Biosciences, Nottingham, UK. Bradford reagent was from Bio-Rad, Hertfordshire, UK. All remaining chemicals were purchased from Sigma-Aldrich.

Stock solutions of $CdCl_2$ (5×10^{-1} M) were prepared in ultra high quality water, filter-sterilised and serial dilutions prepared in sterile water. Stock solutions were kept at 4 °C and dilution series prepared freshly before every experiment. E2 stock solutions (5×10^{-4} M) were made up and diluted in HPLC-analysed ethanol and stored at −20 °C.

The recombinant yeast estrogen screen. The yeast estrogen screen (YES) was carried out following the protocol developed by Routledge and Sumpter (1996). Briefly, 50 ml of growth media were inoculated with 125 μl of 10× concentrated yeast stock and grown overnight in an orbital shaker at 28 °C until turbid (absorbance at 640 nm of 1.0). The assay medium consisted of 50 ml of growth medium, CPRG (10 mg/l) and 2 ml of the overnight yeast culture. Aliquots of 10 μl of the dilutions of E2 were transferred to 96-well, optically flat bottom microtiter plates and allowed to evaporate to dryness. The aqueous solution of $CdCl_2$ (10 μl) was transferred to the microtiter plates shortly before the addition of the yeast cell suspension. Final tested concentrations of E2 and $CdCl_2$ ranged between 5×10^{-12} and 2.5×10^{-9}, and 2.5×10^{-11} and 1×10^{-4} M, respectively. For that, stock solutions at concentrations 20 times higher were prepared.

All plates included a row of ethanol controls (i.e. no test agent) and a row of assay medium without yeast cells (blanks). To each well, except the blanks, a volume of 200 μl of yeast-seeded assay medium was added. To minimise evaporation during the subsequent incubation time, the outer wells were not used, instead being filled with sterile water.

Plates were sealed and shaken vigorously for 2 min on a microtiter plate shaker before incubating at 32 °C, in a humidified box for 72 h. During this period, they were again shaken at 24 h and 71 h. Plates were then analysed