Effect of Cadmium and Mercury on the Nuclear Retinoic Acid Receptors

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Abstract. The actions of retinoic acids (RA) are mediated by their cognate nuclear receptors – ligand inducible transcription factors (retinoic acid receptors (RAR)). Possible interactions of toxic heavy metals on the RAR system are of interest due to involvement of the RAR system in multiple systemic processes. We assayed cadmium chloride and mercury chloride for their influence on the RAR system in rat and in cell culture. Mercury chloride was observed to decrease the maximal binding capacity *in vitro* of RARs for all-trans RA in liver nuclear fraction containing sets of nuclear receptors by seventy percent at a concentration of 0.1 mmol/l, though not cadmium chloride. Neither mercury chloride nor cadmium chloride induced any changes with respect to mRNA levels of RAR and binding properties of nuclear receptor fraction for RA or retinoic acid responsive elements (RARE) in male Wistar rats receiving tap water with cadmium chloride (9.7 mg/l) or mercury chloride (11.5 mg/l) for six weeks. In rat pituitary GH_4C_1 cells, neither mRNA levels nor binding properties for RARE in cell culture were affected by non-toxic concentrations of these heavy metals. From the data obtained it is suggested that, *in vivo*, cadmium or mercury have no significant impact on RA nuclear receptor system.

Key words: Retinoic acid — Heavy metals — Cadmium — Mercury — Retinoic acid receptors

Introduction

Retinoids are well known regulators in a wide range of body functions, ranging from embryonal development to immune system status and the central nervous system

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(Dev et al. 1993; Goodman 1996; Krezel et al. 1998; Wolf 1998). Their effects are mediated by receptors of the thyroid/steroid receptor superfamily, the retinoic acid receptors. There are two main classes of receptor chains, RAR and RXR, that bind all-trans retinoic acid (RA) and 9-*cis* retinoic acid, respectively. Each class consists of three members (alpha, beta and gamma). RXR may combine to homodimers, while RAR forms heterodimers with another RXR chain. In addition, RXR is also known to assemble heterodimers with other members of the thyroid/steroid receptor superfamily (Jacobs and Moxham 1991; Pfahl 1993; Carlberg 1996). In general, not so much the status of local amount of all-trans RA, but the expression patterns of the retinoic acid receptors define the action of RA response. RA receptors are nuclear receptors that reside in the nucleus and act via direct interaction with DNA and are considered to be retinoic acid inducible transcription factors. They bind to retinoic acid receptor responsive elements (RARE), both as repressors without bound ligand and activators when joined with ligand (Minucci and Pelicci 1999). RAREs occur as direct repeats of two motifs separated by two or five nucleotides. The possible number of RAREs is at least as high as the number of receptors, but it is possible to determine consensus sequences for the constructing motifs, like AGGTCA (Lazar 1993; Gudas 1994). The high number of possible receptors and possible RAREs point to the immense impact of RA in its global use in the organism and to the problems when differentiating between the singular effects of the hormonal network.

Cadmium and mercury are heavy metals that are known for their toxicity in biological systems (Domingo 1994). Contrary to selenium, another metal that exhibits toxicity in high doses, there are no biochemical processes known that may depend on the presence of cadmium or mercury. However, both metals induce several effects. Cadmium is able to affect the homeostasis of other bivalent cations such as zinc (Brzoska and Moniuszko-Jakoniuk 2001) and iron (Crowe and Morgan 1997; Goyer 1997). This may have influence on biochemical systems depending on these cations, as can be seen on the reduced macrophage activity in the molybdenum induced copper deficiency (Cerone et al. 2000). Cadmium also leads to systemic symptoms like proteinuria due to its enrichment in the kidney (Choi et al. 1999; Jarup 2002), disturbs several cellular processes like DNA synthesis and repair (Beyersmann and Hechtenberg 1997; Hartwig 1998; Hartwig and Schwerdtle 2002) and may even induce DNA damage directly by producing free radicals (Sarkar 1995; Stohs and Bagchi 1995; Blasiak et al. 2000). These carcinogenic properties of cadmium may be one reason for the correlation of cadmium intake and cancer risk (Antila et al. 1996; Schwartz and Reis 2000; Waalkes 2000; Nakamura et al. 2002), though in the case of breast cancer, cadmium mimics the effect of estrogen (Stoica et al. 2000). But cadmium also shows immunomodulating action, both suppressing (Cook et al. 1984a,b; Grazia Cifone et al. 1989; Descotes 1992; Lee et al. 2001) and enhancing the immune response, here most probably due to induction of an immuno-alert state (Dong et al. 1998). Cadmium exposure induces a systemic increase in metallothionein, whose gene regulation includes a promotor sequence reacting specific to heavy metals (Karin et al. 1984). Cadmium bound to

metallothionein is then adsorbed in the kidney and forms a depot there. A continuous circle of cadmium release from metallothionein in lysosomes and rebinding to freshly synthesized metallothionein prevents cadmium excretion through urine and leads to the high biological half life of cadmium poisoning. Before metallothionein levels raise, cadmium is mainly bound to serum albumin and deposited in the liver (Nordberg 1984). Other cellular effects include decrease of testosterone production by Leydig cells (Laskey and Phelps 1991), reduction of binding capacity of glucocorticoid receptors in rat liver (Dunderski et al. 1992; Dundjerski et al. 2000), stimulated glucose transport in rat adipocytes (mimicking insulin action) (Ezaki 1989), a decrease in estrogen receptor levels but simultaneously an increase of progesterone receptor levels in a human breast cancer cell line (Garcia-Morales et al. 1994), reduction of LDL-receptors in human trophoblast cells (and by that reduction of progesterone production) (Jolibois et al. 1999), induction of apoptosis (Li et al. 2000) and mobilization of free intracellular calcium (Smith et al. 1989).

Mercury causes autism-like symptoms (Bernard et al. 2001) and other toxic effects on the nervous system (Carpenter 2001) (best known as Minamata disease (Harada 1995)), induces renal autoimmunity (Bigazzi 1999), shows mutagenicity on nucleotide and chromosomal level (De Flora et al. 1994; Stohs and Bagchi 1995), stimulates glucose transport in rat adipocytes (Ezaki 1989), pushes $CD4^+T$ cell blast formation *in vitro* (Loftenius et al. 1997) and in general it shows an immunosuppressive effect (Moszczynski 1997).

The study we present here was aimed on the *in vitro* and *in vivo* effects of cadmium and mercury on the retinoic acid receptor status in rat liver nuclei and GH_4C_1 rat pituitary cell line.

Materials and Methods

Animals

Six male Wistar rats (initial body weight 150.8 ± 3.6 g S.E.) (Charles Rivers Laboratories) received cadmium chloride and mercury chloride at a concentration of 9.7 mg/l (0.05 mmol/l) and 11.5 mg/l (0.04 mmol/l), respectively, in their tap water *ad libitum* for a period of 6 weeks, or no heavy metal at all as control. The rats were sacrificed by decapitation and tissue samples from the liver were immediately frozen in liquid nitrogen. Samples were then subject to RNA preparation, electrophoretic mobility shift assay (EMSA), "Supershift" assay and Scatchard plot. The animals were treated according to local guidelines.

Cell culture

The rat pituitary gland cells GH_4C_1 were cultivated in F12-Ham Medium (Sigma) supplemented with 12.5 % horse serum (GibcoBRL/Life Technologies), 2.5 % fetal calf serum (GibcoBRL/Life Technologies) and 1× Penicillin/Streptomycin (Sigma). For the experiment, 10^5 cells were passaged *per* well of a Falcon 24-well-cell culture plate in 1 ml medium and allowed to rest for 24 h. Then either cadmium chloride

or mercury chloride was added in different concentrations ranging from 0.1 μmol/l to 10 μmol/l at least in duplicate wells. After a time span varying from 15 min to 96 h the cells were harvested for RNA preparation. As control, cells incubated for the same time without any heavy metal were used. For EMSA and Supershift Assay, 1.5×10^6 cells were passaged *per* 25 ml Falcon cell culture flask and allowed to rest 24 h. Then cadmium chloride or mercury chloride in a concentration of 0.5 μmol/l, 1 μmol/l and 2 μmol/l or no heavy metal at all for control was added for 72 h. Cells were harvested by PBS supplemented with 2 mmol/l EDTA and the cell solution was used for preparation of nuclear extract.

Scatchard plot of RAR binding properties

Liver tissue was minced and homogenized in 0.32 mol/l sucrose, 1 mmol/l $MgCl_2$, 0.1 mmol/l PMSF and 1 mmol/l dithiothreitol (DTT). The homogenate was centrifuged at $1000 \times g$, the crude pellet was washed and then mixed with 2.3 mol/l sucrose containing 1 mmol/l $MgCl_2$, 1 mmol/l DTT, 0.1 mmol/l PMSF, and treated by isopycnic ultracentrifugation at $220{,}000 \times g$ for 30 min. Nuclei were then washed twice in ice-cold SMCT buffer (0.32 mol/l sucrose, 10 mmol/l Tris-HCl, pH 7.4, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 1 mmol/l DTT, 0.1 mmol/l PMSF) once in the presence of 0.25 % Triton X-100 and once without. The nuclear protein fraction containing retinoic acid receptors was obtained directly from purified nuclei with a high ionic strength buffer containing 0.3 mol/l KCl, 1 mmol/l $MgCl_2$, 10 mmol/l Tris-HCl buffer, (pH 7.0) at 0 ℃ for 1 h and by subsequent ultracentrifugation at $135{,}000 \times g$ (Torresani and DeGroot 1975; Brtko 1994). As a result of this treatment, this fraction was totally devoid of cytoplasmic protein (Torresani and DeGroot 1975), including RA-binding molecules like CRBP and CRABP. The assays on labeled all-trans retinoic acid binding to nuclear proteins were performed in a high ionic strength buffer (0.3 mol/l KCl, 1 mmol/l $MgCl_2$, 10 mmol/l Tris-HCl buffer, pH 7.0) at 20 ℃ in dark. Samples were incubated with 0.66 nmol/l of [11,12–^{3}H(N)] retinoic acid (1 mCi/ml, NEN Research products; 3 μl ethanol solution per 0.5 ml sample) for 2 h and increasing concentrations of all-trans retinoic acid (0.33–3.96 nmol/l; 3 μl ethanol solution *per* 0.5 ml sample). Nonspecific binding of the labeled ligand was determined by simultaneous incubation with 0.396 μmol/l all-trans retinoic acid. After incubation, 0.5 ml of charcoal-dextran suspension (4.5 mg Norit $A + 0.45$ mg dextran/ml) in the high ionic strength buffer (pH 7.0) was added to each sample in dark at 0–4 ℃. After short vortexing, the suspension was placed on an ice bath for 10 min, then vortexed and the supernatant was collected after 10 min centrifugation at $1500 \times g$. Then radioactivity of 0.5 ml of the supernatant was assayed (Brtko 1994). This method for determination of RAR binding characteristics has been employed successfully in previous works (Brtko et al. 1997, 1998; Schmutzler et al. 1998). Previous data has also clearly confirmed that by both the RAR saturation binding assay and the RAR displacement binding assay, the same maximal binding capacity $B_{\max}$ and the association constant K_a values have been achieved (Brtko 1994). Samples were either livers from animals pretreated with cadmium chloride or mercury chloride or nuclear extractions of liv-

ers from untreated control animals supplemented with these heavy metals during the assay at a concentration of 10^{-6} mol/l to 10^{-4} mol/l.

RNA preparation

RNA from cells and tissues was performed following the manufacturers protocol using the TriZol Kit (GibcoBRL/Life Technologies). In the case of the cell culture experiment, the cells were lysed directly in the well after aspiration of the medium by adding 250 μl of TriZol.

cDNA transcription

cDNA was prepared from 2 μg RNA using the Ready-To-Go You-prime First-Strand Beads (Amersham Pharmacia Biotech) following the manufacturers protocol with $pd(N)_6$ primers (Amersham Pharmacia Biotech).

Reverse transcription-PCR (RT-PCR)

PCR was performed on 1 μl cDNA in a PCR volume of 25 μl with 1.5 mmol/l $MgCl_2$, 0.5 U Taq DNA Polymerase (Promega) and 200 μmol/l dNTP (Promega). Shortly, PCR was performed at 94℃ for 3 minutes, than 35 cycles at 94, 57, and 72℃ for 45, 30, and 90 s, respectively, followed by an ending step at 72℃ for 10 min. Primer sequences and used amounts are listed in Table 1, the primers for RAR-alpha, -beta and -gamma were described elsewhere (Ohata et al. 2000). The cDNA was probed with each primer pair in a separate PCR. For evaluation, the PCR products were placed on 2% agarose gel and band intensity was measured using the STS 6220I Documentation System (Ultralum, Claremont, CA, USA). Finally, the band intensity for every cDNA was normalized to the band intensity of the PCR product corresponding to the housekeeper gene beta-actin.

Table 1. Primer for RT-PCR

Receptor subchain		Sequence	product	pmol/PCR
RAR-alpha	fw	CAGATGCACAACGCTGGC	397 bp	20
	rv	CCGACTGTCCGCTTAGAG		20
RAR-beta	fw	ATGCTGGCTTCGGTCCTC	470 bp	20
	rv	CTGCAGCAGTGGTGACTG		20
RAR-gamma	fw	GTGGAGACGGAATGGACC	521 bp	20
	rv	GACAGGGATGAACACAGG		20
RXR-alpha	fw	CTCTACCCAGGTGAACTCTT	293 bp	5
	rv	TGCTGCTCACAGGGTTCATG		5
RXR-beta	fw	CCAGACAGCTCCTCCCCAAA	342 bp	5
	rv	TGCACAGAGCCGTTTGCCAG		5
beta-actin	fw	GAGGCCCAGAGCAAGAGAGG	843 bp	3.75
	rv	GTACTTGCGCTCAGGAGGAGC		3.75

fw, forward; rv, reverse.

Electrophoretic mobility shift assay (EMSA) and "Supershift" assay

EMSA and "Supershift" assay followed the protocol that has already been described (Brtko et al. 2002). Nuclear preparation of cells has already been described as well (Andrews and Faller 1991). For EMSA, nuclear preparations were incubated with ^{32}P labeled oligonucleotides and then applied on a non-denaturating SDS-gel for 2.5 h at 17 mA and 4 ℃. For "Supershift" assay, nuclear preparations were additionally incubated with 1 μl antibody for 30 min before running the SDS-gel for 2.5 h at 20 mA and 4 ℃. Gels were fixated in a solution containing 30 % acetic acid and 10 % methanol for 60 min and dried completely. The gel bound radioactivity was measured by exposing the gels to X-OMAT AR5 X-Ray films (Kodak) for five days. Developed films were scanned using the STS 6220I Documentation System (Ultralum, Claremont, CA, USA). The successful implementation of this protocol for "Supershift" assay was demonstrated by us in other works (Schmutzler et al. 1998). The antibodies used were: anti-RAR-alpha, anti-RAR-beta, anti-RAR-gamma, anti-RXR-alpha and anti-RXR-beta (Santa Cruz Biotechnology, CA, USA), the oligonucleotide (Amersham Pharmacia Biotech) which resembled the sequence ATCTGAGGTCAGGAGTTCAAGACC was derived from iodothyronine 5′-deiodinase promoter sequence.

Results

Scatchard plot allows to determine the maximal binding capacity $B_{\max}$ and the equilibrium association constant K_{a} for all-trans RA of RA-binding proteins in nuclear fractions. These values were evaluated by us for *in vitro* action of mercury and cadmium. Mercury chloride, added directly to the assay together with nuclear fraction of control animals, reduced $B_{\max}$ in a linear way starting at a concentration of 10^{-5} mol/l of mercury chloride, while cadmium chloride did not show any effect (Fig. 1). In contrast, looking for possible *in vivo* effects we could not find any differences in the Scatchard plots of liver samples from animals pretreated with these heavy metals. For the case of cadmium, the amount of cadmium in liver was determined to be 6.2 ± 0.62 μg/g wet tissue (in contrast to less than 0.02 μg/g for the untreated control). This corresponds to 3.38×10^{-5} mol/kg wet tissue and is well within the effective range of these heavy metals.

Furthermore, RT-PCR was used to assay changes in mRNA levels in response to cadmium and mercury exposure. *In vivo* effects were tested on RNA from livers of groups of six rats exposed to cadmium chloride and mercury chloride with respect to the subchains RAR-alpha, RAR-beta and RXR-alpha (Fig. 2). In cell culture, GH_4C_1 cells were exposed to concentrations of 0, 1, 5, 10 and 20 μmol/l for 24 h and then assayed for RAR-alpha, RAR-beta, RAR-gamma and RXR-alpha, then for concentrations of 0, 0.1, 0.5, 1, 5 and 10 μmol/l for 48 h and assayed for RAR-alpha, RAR-beta and RAR-gamma. In addition, a time course was performed for the concentration of 10 μmol/l of heavy metal at the time points of 0, 24, 48, 72 and 96 h for the subchains RAR-alpha, RAR-beta and RAR-gamma, and for

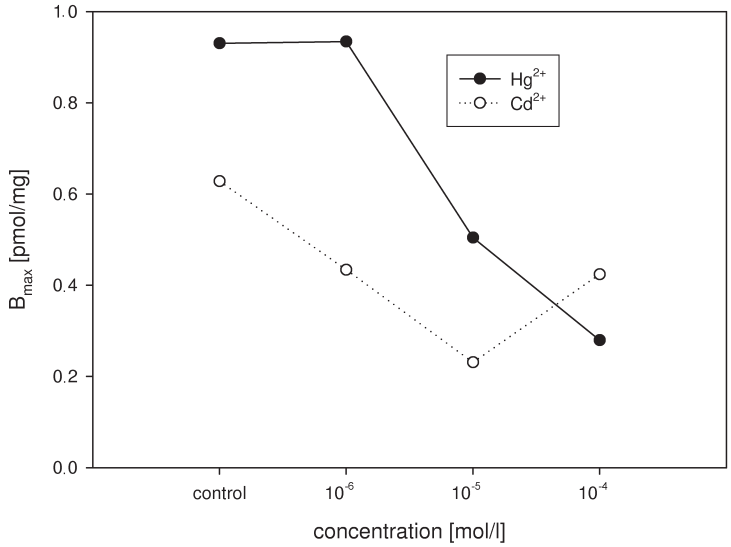

Figure 1. *In vitro* effects of Cd^{2+} and Hg^{2+} on maximal binding capacity ($B_{\max}$) of RAR in liver nuclear fraction. $CdCl_2$ and $HgCl_2$ were added at different concentrations during determination of $B_{\max}$ of liver nuclear fraction of untreated control rats by Scatchard plot.

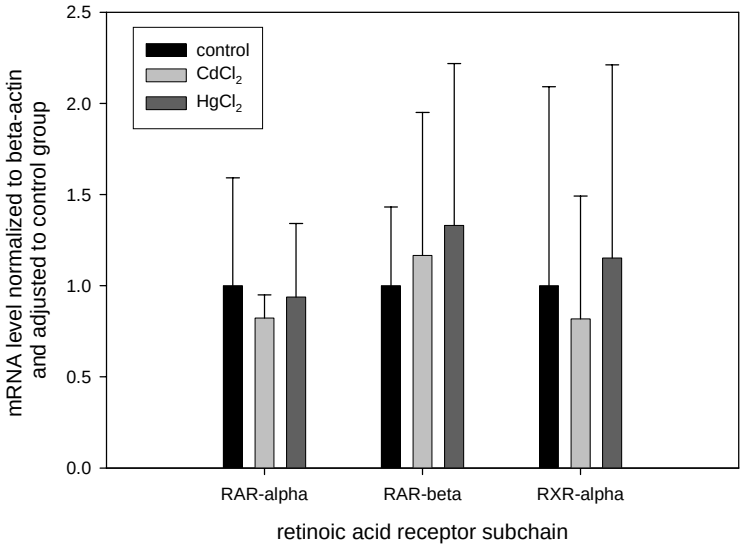

Figure 2. *In vivo* effect of $CdCl_2$ or $HgCl_2$ on RAR mRNA in rat liver. mRNA levels of RAR-alpha, RAR-beta and RXR-alpha in liver of six animals either untreated, or pretreated with $CdCl_2$ or $HgCl_2$ were evaluated by RT-PCR, the results were normalized to beta-actin content of the respective sample and then adjusted to mean of the control groups.

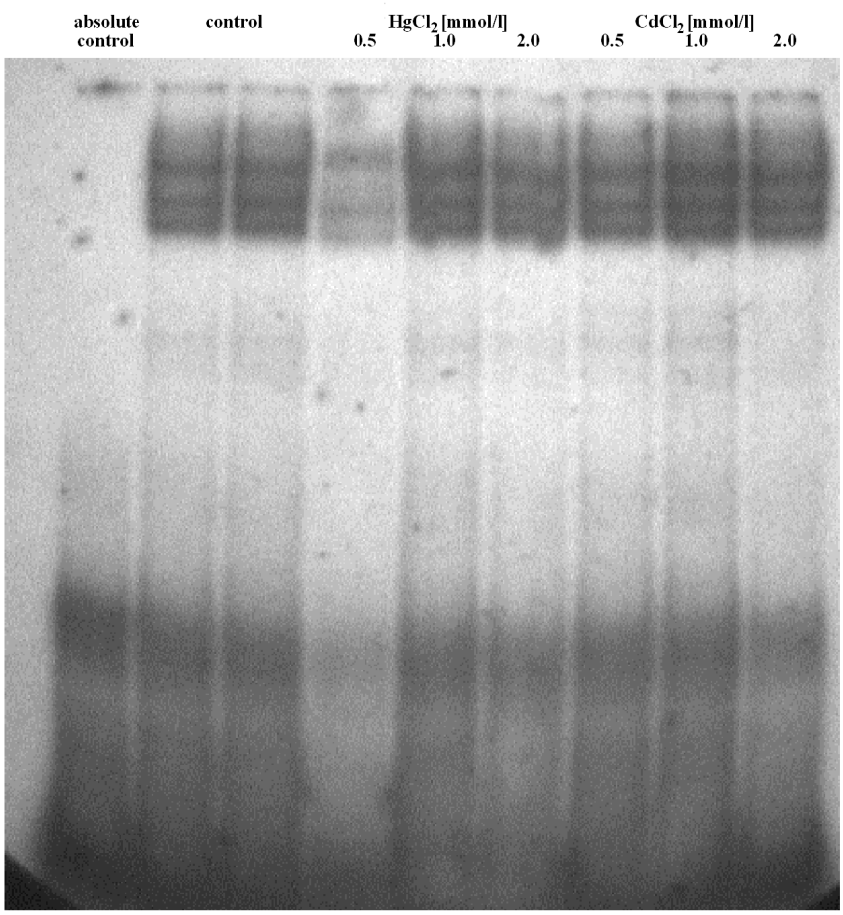

Figure 3. Effect of $CdCl_2$ or $HgCl_2$ on RAR content in GH_4C_1 cell line. GH_4C_1 cells were incubated with different concentrations of $CdCl_2$ or $HgCl_2$ and then subject to EMSA for determining the amount of RAR-specific RARE binding proteins in nuclear fraction. Lanes (from left to right): absolute control without nuclear fraction; nuclear fraction of GH_4C_1 cells without heavy metal incubation (2 lanes); GH_4C_1 cells incubated with 0.5 mmol/l, 1.0 mmol/l and 2.0 mmol/l $HgCl_2$; GH_4C_1 cells incubated with 0.5 mmol/l, 1.0 mmol/l and 2.0 mmol/l $CdCl_2$.

the concentration of 1 μmol/l for the time points 0, 15, 30, 45, 60 and 120 min for the subchains RAR-alpha, RAR-gamma, RXR-alpha and RXR-beta (data not shown). We could not detect any significant changes in the mRNA levels of any RAR subchain, neither in the livers of treated animals nor in GH_4C_1 cell culture.

By the use of EMSA, the amount of RARE binding protein in a nuclear fraction preparation can be estimated. This was used for studying the nuclear fraction

of liver of three animals pretreated with cadmium chloride or mercury chloride. In addition, Supershift assays (where incubation with specific antibodies allows evaluating the amount of a certain receptor subchain) for the subchains RAR-alpha, RAR-beta and RXR-alpha were performed (data not shown). The effects of cadmium chloride and mercury chloride at concentrations of 0, 0.5, 1 and 2 μmol/l for 72 h on GH_4C_1 cell culture were tested by EMSA (Fig. 3) and Supershift assay with respect to RAR-beta, RAR-gamma and RXR-beta. We could not detect any significant changes in the amount of labeled oligonucleotide bound to protein fraction in the assayed samples of livers of pretreated animals and GH_4C_1 cell culture.

Discussion

Heavy metals induce a broad spectrum of symptoms in an organism. These effects range from disturbance of systemic metal homeostasis over immunomodulation to carcinogenicity. Their targets on molecular level are proteins – for example, cadmium binding to serum metallothionein disturbs the zinc homeostasis and induces renal malfunction due to deposit building in the kidney. Heavy metals also migrate into the cells and interact with intracellular proteins.

Prominent members of cadmium target proteins are DNA and RNA polymerases (Hartwig 1998) and glucocorticoid receptor (GR) (Dunderski et al. 1992), those actions are disturbed by cadmium. Here, cadmium replaces the zinc of these zinc-finger proteins thus abolishing the DNA binding center. A side effect is the formation of radicals by Fenton reaction, which may be a part of the carcinogenic action of cadmium (Sarkar 1995). But the zinc-finger sites are not always the reason for a cadmium effect. In the case of estrogen receptor, the metal interactions with the estrogen binding site (blocking estradiol binding) lead to a raised estrogen receptor activity (Stoica et al. 2000). For the cases of GR and RAR-alpha, cadmium-113 was used to label the zinc-finger domains for NMR studies of the protein structure (Kellenbach et al. 1991; Knegtel et al. 1993), proving the principal possibility of cadmium to substitute zinc in these proteins. Still, while cadmium was shown by others to influence GR activity, our experiments did not show any kind of influence of cadmium on the RAR system. Neither of (1) DNA binding capacity (tested by EMSA), (2) amount of a specific RAR subclass (tested by "Supershift"), (3) retinoic acid binding activity, and (4) mRNA levels were altered in any test group. It seems that the specific biochemistry of the retinoic acid receptor chains prevented cadmium from taking any action upon them. A similar effect has been described with zinc *in vitro* with respect to the epidermal growth factor receptor (erbA): here, zinc diminished the binding of T3 to erbA alpha, but had no influence on the binding of T3 to erbB beta, while the bivalent metals cadmium, manganese and copper decreased binding to both receptors and magnesium had no influence on any erbA subclass (Lu et al. 1990). But cadmium was shown to inhibit the DNA binding of the transcription factor

IIIA, while mercury did not show any effect (Hanas and Gunn 1996). In summary, following available literature, cadmium in principle is able to bind to steroid receptors and to influence both the ligand binding and DNA binding properties. Though, in the specific case of RAR, there is no effect of cadmium, neither *in vivo*, nor *in vitro* or in cell culture system. It seems, as if the principally possible exchange of zinc with cadmium in the zinc-finger site of RAR did not influence the DNA binding activity, though it may contribute to the carcinogenic properties of cadmium.

Hardly any data exist on intracellular activities of mercury with respect to DNA binding receptors. Its carcinogenicity depends more on the production of radicals (Stohs and Bagchi 1995) than on interference with the DNA repair mechanisms. One study shows an ambivalent influence of mercury on inositol polyphosphate receptor binding. Here, mercury decreased *in vitro* binding of [^{3}H]inositol 1,4,5-trisphosphate (IP3) and [^{3}H]inositol 1,3,4,5-tetrakisphosphate (IP4) to cerebral membranes, while methyl mercury stimulated the binding. *In vivo* no effect of mercury was found, while methyl mercury increased binding of IP4 after a short-time treatment (3 or 24 h), and of IP3 and IP4 after a long-time treatment (24 h) (Chetty et al. 1996). A similar work describes the reversible inhibition of progesterone receptor binding and vitamin D receptor binding (both affected receptors belong to the steroid receptor family) (Coty 1980). Another affected receptor may be the acetylcholine receptor, as seen in the electric ray (*Torpedo ocellata*) (Eldefrawi et al. 1977), where methyl mercury inhibits acetylcholine binding, and in transfected *Xenopus* oocytes, where mercury both enhances and suppresses binding activity of the receptor, depending on its subchain types (Mirzoian and Luetje 2002). Mercury increased the receptor binding of GABA(A) receptor in cell culture of cerebral cells (Fonfria et al. 2001). Sani et al. purified RAR from chicken skin and showed that the gained protein preparation lost its RA binding capacity in the presence of mercury (Sani and Banerjee 1978; Sani et al. 1990). This negative effect of mercury was reproduced by us, proving a dose-dependent inhibition of RA binding to nuclear fraction preparations of liver (containing RAR) *in vitro* by Scatchard plot. No other effect has been observed, neither on protein nor on mRNA levels.

Liver is well known as one of the main target tissues of heavy metals mercury and cadmium. We could demonstrate that both these heavy metals have no influence *in vivo*, though the RAR themselves were clearly affected *in vitro* by mercury. Therefore we hypothesize a mechanism preventing mercury to exhibit this effect *in vivo*. It seems likely that this general mechanism lies somewhere in the detoxification of heavy metals, thus affecting the whole organism, not just specific tissues. In summary, the effect of mercury on the ligand binding activity of RAR *in vitro* does not play an important role *in vivo*, perhaps due to other mercury binding proteins which prevent the nucleus from higher levels of mercury.

Our work shows that cadmium and mercury do not have any adverse effects on the RAR system *in vivo* and their overall toxicity has no effect on the retinoic acid network.

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