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Inhibitory Effects of Heavy Metals on Cytochrome P4501A Induction in Permanent Fish Hepatoma Cells

B. J. Brüschweiler[1]*, F. E. Würgler[2], K. Fent[1]

[1] Swiss Federal Institute for Environmental Science and Technology (EAWAG) and Swiss Federal Institute of Technology (ETH), Überlandstrasse 133, CH-8600 Dübendorf, Switzerland

[2] Institute of Toxicology, Swiss Federal Institute of Technology and University of Zürich, Schorenstrasse 16, CH-8603 Schwerzenbach, Switzerland

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Abstract. The interactions *in vitro* of heavy metals Cd(II), Co(II), Cu(II), Ni(II), Pb(II), and Zn(II) with cytochrome P4501A (CYP1A) induction response and enzyme activity were studied in fish hepatoma cells PLHC-1. Cells were simultaneously exposed to heavy metals and to 3-methylcholanthrene (3-MC), an inducer of CYP1A. Heavy metals were added to the cells in different concentrations. Cytotoxicity were measured in the neutral red (NR) assay, relative CYP1A protein contents in an enzyme-linked immunosorbent assay (ELISA), and CYP1A activities in the ethoxyresorufin-*O*-deethylase (EROD) assay. All metals had a more pronounced effect on EROD activity than on CYP1A protein content and cytotoxicity. For the most active metal Cd(II), a 50% inhibition of EROD activity was observed at significantly lower concentrations ($2.2 \cdot 10^{-5}$ M) than a 50% reduction of CYP1A protein ($5.3 \cdot 10^{-5}$ M), and a 50% cytotoxicity ($1.4 \cdot 10^{-4}$ M). The inhibitory potency of the metals had the following order: Cd(II) > Ni(II) > Cu(II) > Co(II) = Zn(II) > Pb(II). In a second set of experiments, lysates of 3-MC–induced cells were exposed to heavy metals. Cd(II) and Cu(II) caused a 50% inhibition of EROD activity at significantly lower concentrations than in the experiments with living cells, at $8.2 \cdot 10^{-6}$ M and $1.3 \cdot 10^{-5}$ M, respectively, whereas the effect by Co(II) occurred at a significantly higher concentration ($8.2 \cdot 10^{-4}$ M). The results indicate that Cd(II) and Cu(II) in particular may affect the CYP1A system of the liver of fish at low concentrations through direct inhibition of the CYP1A enzyme activity. CYP1A induction response in fish liver is increasingly being used in biomonitoring programs. In the environment, interactions of CYP1A-inducing and CYP1A-inhibiting components (such as heavy metals) can be expected and must be taken into consideration.

Correspondence to: K. Fent

**Present address:* University of Connecticut, Storrs, Connecticut 06268, USA

Heavy metals are widespread and persistent in the aquatic environment, accumulate and show various adverse effects in fish (Sorensen 1991) and other aquatic organisms (Mance 1987). Several mechanisms that may account for their toxicity at the cellular level have been described (Viarengo 1989). Particularly, interactions between heavy metals and SH-containing proteins that may affect their function, thus compromising several enzyme activities (Viarengo and Nicotera 1991). Metals such as Cd(II), Cu(II), and Pb(II) are known to inhibit the cytochrome P450 enzyme activities (Alvares *et al.* 1972; Hadley *et al.* 1974) and to reduce the microsomal cytochrome P450 content (De Matteis 1978) in mammals. This may be relevant for the metabolism of xenobiotics and endogenous substrates in fish, in which several cytochrome P450 subfamilies have been described (Stegeman and Hahn 1994). Cytochrome P4501A (CYP1A) is induced by various polycyclic aromatic hydrocarbons (*e.g.*, benzo[*a*]pyrene) and halogenated aromatic hydrocarbons (*e.g.*, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 3,3′,4,4′-tetrachlorobiphenyl) mainly in fish liver, but other organs as well. Induction of CYP1A is increasingly being used as biomarker for such compounds in fish liver (Bucheli and Fent 1995) and for assessing the induction potential of environmental samples *in vitro* (Tillitt *et al.* 1991). To our knowledge, Cd(II) is the only heavy metal that has been reported to inhibit CYP1A in fish (Fair 1986; Förlin *et al.* 1986; George and Young 1986). Inhibition of CYP1A induction response has been observed after intraperitoneal co-administration of Cd(II) and a CYP1A-inducer in fish (Fair 1986; George and Young 1986). This inhibition appears to be dependent upon time (George and Young 1986), route of administration (Förlin *et al.* 1986), and dose (George 1989).

Several mechanisms may be responsible for the effects of metals on the CYP1A system. Cd(II), Co(II), Cu(II), Ni(II), Pb(II), Zn(II), and other metals such as Hg(II) and Sn(II) stimulate the turnover of heme by the induction of heme oxygenase accompanied by the loss of cytochrome P450 *in vivo* (Maines and Kappas 1977). Pb(II) is an inhibitor of the 5-aminolaevulinate (ALA) dehydratase, Co(II) an inhibitor of the ALA synthetase, and both Pb(II) and Co(II) inhibit the ferrochelatase (De

Matteis 1978; Sassa 1978; Tephly 1978). Furthermore, direct inhibition of benzo[*a*]pyrene hydroxylase by Cd(II) in mammalian microsomes after short-time exposure has been reported at low concentrations of 10^{-6} M and higher (Fukuhara and Takabatake 1982). Recently, Pb(II) has been found to preferentially inhibit the expression and induction of CYP1A2 enzyme at both the mRNA and the protein level in mammals (Degawa *et al.* 1993a). It is probable that heavy metals affect the expression of cytochrome P450 isozymes due to specific interactions with DNA-binding proteins (Degawa *et al.* 1993b; Goering 1993). For the above reasons, more information about the sensitivity and relevance of these mechanisms is needed, when heavy metals and CYP1A-inducers are present in fish. These effects may be important in environmental monitoring, when CYP1A is used as biomarker in heavy metal polluted waters.

The aim of this study was to investigate *in vitro* interactions of Cd(II), Co(II), Cu(II), Ni(II), Pb(II), and Zn(II) with the CYP1A system. *In vitro* methods are useful tools to study interactions of CYP1A-inducing and CYP1A-inhibiting compounds (Brüschweiler *et al.* 1996a). The permanent fish hepatoma cell line PLHC-1 is derived from topminnow (*Poeciliopsis lucida*) (Hightower and Renfro 1988; Ryan and Hightower 1994) showing sensitive and prompt CYP1A-induction response (Hahn *et al.* 1993; Brüschweiler *et al.* 1996b). In a first set of experiments, the effects of these metals on EROD activity, relative CYP1A protein content, and cytotoxicity were analyzed under different conditions. Effects were investigated in cultured living cells after simultaneous exposure of a CYP1A-inducer and of metals for 3 d. In a second set of experiments, 3-MC–induced cell lysates were treated for a short period of time with different metals and the inhibition of EROD activity was measured. The latter allowed the analysis of the direct effects on the enzyme activity alone and to exclude the indirect effects, *e.g.*, on heme metabolism or on CYP1A protein synthesis. The data obtained are compared with the available *in vivo* and *in vitro* information in fish.

Materials and Methods

Chemicals

$CdCl_2$, 3-MC, and bovine serum albumin (BSA) were obtained from Fluka AG (Buchs, CH). $Co(NO_3)_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, $Pb(NO_3)_2$, and $ZnCl_2$ were purchased from Merck (Darmstadt, D). 7-Ethoxyresorufin (7-ER) was obtained from Molecular Probes (Eugene, OR, USA). The monoclonal mouse anti-scup antibody 1-12-3 was a gift from J.J. Stegeman (Woods Hole Oceanographic Institution, USA). The peroxidase-conjugated goat anti-mouse antibody was purchased from Dako (Glostrup, Denmark). Peroxidase substrate kit and the dye reagent for the protein assay were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Other chemicals and reagents were obtained as before (Brüschweiler *et al.* 1995; Brüschweiler *et al.* 1996b).

Cell Culture

Fish hepatoma cells PLHC-1 were supplied by L.E. Hightower (University of Connecticut, USA) and were grown and subcultivated as described previously (Hahn *et al.* 1993; Brüschweiler *et al.* 1995). The cells were maintained as monolayer cultures at 30°C in a 5% CO_2 atmosphere in Eagle Minimum Essential Medium (MEM) with Earle's salts, supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 25 mM sodium hydrogen carbonate, 100 units/ml penicillin G and 100 µg/ml streptomycin in 75 cm^2 flasks. PLHC-1 cells ($8 \cdot 10^4$) in 100 µl medium containing 10% (v/v) FCS were seeded in wells of a 96-well tissue culture microtiter plate (Nunclon™). Cells were allowed to attach, and were grown in the incubator for 24 h at 30°C and 5% CO_2 atmosphere.

Treatment with 3-Methylcholanthrene and Metals

3-Methylcholanthrene (3-MC) was dissolved in dimethyl sulfoxide (DMSO) in a concentration of 5 mM and diluted with MEM containing 10% (v/v) FCS to a concentration of 10^{-4} M. $CdCl_2$, $Co(NO_3)_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, $Pb(NO_3)_2$, and $ZnCl_2$ were dissolved in distilled water in a concentration of 0.5 M and filtered through a 0.2-µm filter (Schleicher & Schuell). These metal stock solutions were stored in glass vials at 4°C for no longer than two weeks. To minimize the possible loss of metals by sorption to containers, glassware was approriately handled (rinsing). Concentrations of metals in this paper refer to these metal salts, and not to the free metal ion concentrations. The common following terminology was used for the metals (Me) in the medium: Me(II), which is the sum of all different species of the metal with oxidation number +II.

Stock solutions were diluted with MEM containing 10% (v/v) FCS for dilution series. When the color of the medium changed, the pH was adjusted to 7.4 with 0.5 N NaOH. After removal of the old medium from the wells, 50 µl of the medium containing 3-MC and 50 µl of medium containing metals was added to the wells and incubated at 30°C for 72 h. The final concentration of 3-MC was $5 \cdot 10^{-6}$ M, which has been shown to cause maximal induction after a 3-d exposure (Brüschweiler *et al.* 1996b).

Neutral Red Assay, ELISA, Total Proteins

The neutral red assay (NR assay) and enzyme linked immunosorbent assay wih cells (ELISA) were performed as described previously (Brüschweiler *et al.* 1995, 1996b). The total protein content per well was determined using the Bio-Rad protein assay with Commassie blue dye (Brüschweiler *et al.* 1996b).

EROD Assay

The EROD assay was carried out as described previously (Brüschweiler *et al.* 1996a). By freezing and thawing, cells were lysed and the lysate used for EROD analysis. After 72 h exposure, the medium was removed and the plates were transferred to a freezer (−80°C). After thawing plates for 10 min at room temperature, 20 µl BSA (5.32 mg/ml in 50 mM Tris, pH 7.8) and 40 µl 7-ER (4 µM in 50 mM Tris, pH 7.8) were added to each well containing cell lysate. The plates were shortly shaken on a plate shaker and incubated for 15 min at room temperature. Afterwards, 20 µl NADPH (6.7 mM in 50 mM Tris, pH 7.8) were added to each well. The final reaction volume was 80 µl per well and final concentrations of BSA, 7-ER, and NADPH were 1.33 mg/ml, 2 µM, and 1.67 mM, respectively. Fluorescence was measured every 60 s for 15 min on a plate reader (Dynatech Fluorolite 1000) with a 530 nm excitation filter and a 590-nm emission filter at a lamp voltage of 11 V. The fluorescence data were fitted to a resorufin standard curve.

For the analysis of short-term effects of metals on EROD activity in cell lysate, cells were exposed to 3-MC ($5 \cdot 10^{-6}$ M) for 3 d. Plates were transferred to a freezer and allowed to thaw for 10 min at room temperature. Thereafter, cell lysates were exposed to 20 µl of a series

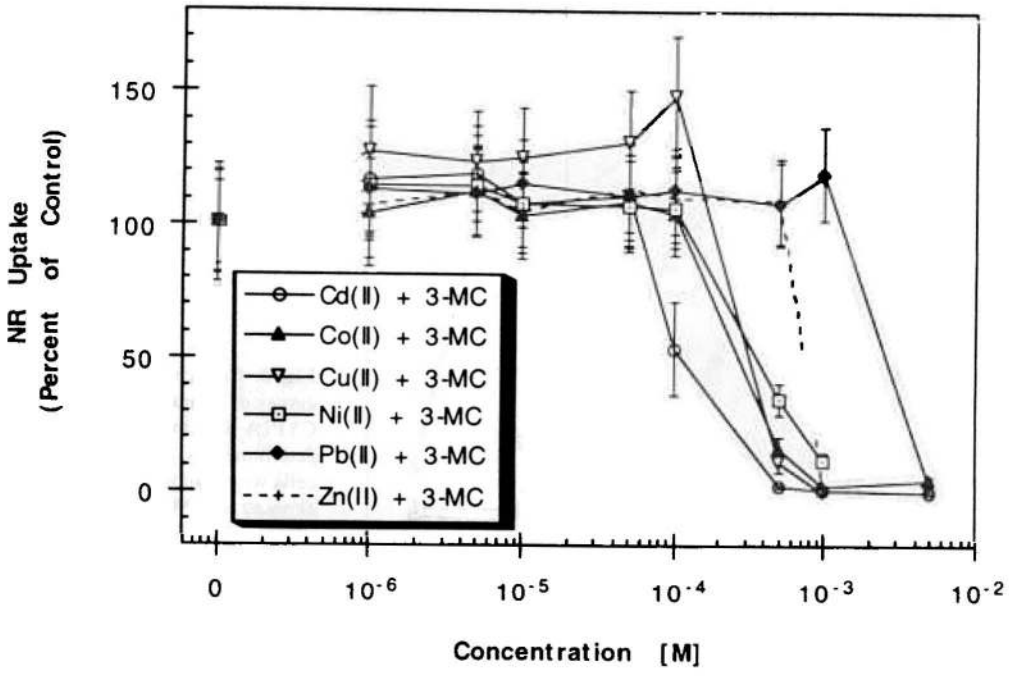

Fig. 1. Cytotoxicity of metals in PLHC-1 cells. Cells were simultaneously incubated with noncytotoxic concentrations of 3-MC ($5 \cdot 10^{-6}$ M) and a series of dilutions of metals for 3 d. Cytotoxicity was determined by NR uptake assay. Data are shown as mean percentage of control ± S.E.M. (six values)

of metals in Tris buffer (50 mM Tris, pH 7.8), instead of BSA solution for 15 min following the described protocol.

Data Analysis and Statistics

Each experiment was performed at least three times. Data in different groups were reduced to the arithmetic mean ± standard error of the mean (S.E.M.). Concentrations, at which the enzymatic activity and the relative protein content of CYP1A as well the cytotoxicity were one-half maximal (EC50), were determined by regression analysis using a logarithmic curve fit model or a Michaelis-Menten model (Brüschweiler *et al.* 1995). Means of EC50 values were compared with Student's t-test with significance limits $\alpha < 0.05$.

Results

Simultaneous Exposure to a CYP1A-Inducer and Metals for Three Days

Cells were simultaneously exposed to a non-cytotoxic 3-MC-concentration ($5 \cdot 10^{-6}$ M) and different metals for 3 d. Heavy metals were added to the cells in different concentrations. Cytotoxic effects were measured at similar concentrations as in earlier studies, in which metals were incubated alone for 1 d (Babich *et al.* 1986; Segner and Lenz 1993; Ryan and Hightower 1994). Figure 1 shows that the sequence of cytotoxicity is Cd(II) > Co(II) = Ni(II) = Cu(II) > Zn(II) > Pb(II) with midpoint cytotoxicity values (EC50) of $1.4 \cdot 10^{-4}$ M, $2.8 \cdot 10^{-4}$ M, $2.9 \cdot 10^{-4}$ M, $3.2 \cdot 10^{-4}$ M, $7.2 \cdot 10^{-4}$ M, and $2.6 \cdot 10^{-3}$ M, respectively. Cu(II) tended to increase NR uptake at subcytotoxic concentrations at 10^{-4} M.

The relative amount of CYP1A protein was determined in the ELISA assay and compared to the CYP1A amounts induced by 3-MC alone (Figure 2). At concentrations higher than 10^{-5} M, the metals led to a concentration-dependent decrease of CYP1A protein content with the sequence Cd(II) > Ni(II) = Cu(II) = Co(II) > Zn(II) > Pb(II). The respective EC50 values were $5.3 \cdot 10^{-5}$ M, $2.5 \cdot 10^{-4}$ M, $3.0 \cdot 10^{-4}$ M, $3.2 \cdot 10^{-4}$ M, $7.8 \cdot 10^{-4}$ M, and $2.9 \cdot 10^{-3}$ M. In the case of Pb(II), an increase of CYP1A protein content at 10^{-3} M and $5 \cdot 10^{-3}$ M was detected.

As observed in an earlier study (Brüschweiler *et al.* 1996b), EROD activities between 35 and 45 pmol/min/mg protein were measured, when cells were exposed to 3-MC ($5 \cdot 10^{-6}$ M) alone for 3 d. For standardizing the inhibitory effects by the metals, the concurrent control value was set to 100%. The most sensitive effect was observed for Cd(II) (EC50 = $2.2 \cdot 10^{-5}$ M) followed by Ni(II) ($8.1 \cdot 10^{-5}$ M), Cu(II) ($1.6 \cdot 10^{-4}$ M), Co(II) ($2.0 \cdot 10^{-4}$ M), Zn(II) ($2.3 \cdot 10^{-4}$ M), and Pb(II) ($8.0 \cdot 10^{-4}$ M) (Figure 3). Contrary to the other metals, a slow decrease of EROD activity for Pb(II) has been found showing a statistically significant inhibitory effect at $5 \cdot 10^{-6}$ M.

Effects of Metals on CYP1A-Induced Cell Lysate

In addition to the previous experiments, interactions of the metals with cytochrome P450 were investigated in lysed cells. Cells, which had been induced by 3-MC and thereafter frozen, were exposed to the metal ions for 15 min. The EROD activity was inhibited in a concentration-dependent manner (Figure 4). An EC50 value was found for Cd(II) at $8.2 \cdot 10^{-6}$ M, for Cu(II) at $1.3 \cdot 10^{-5}$ M, and for Co(II) at $8.2 \cdot 10^{-4}$ M. In contrast to Cd(II) and Cu(II), the EROD activity decreased slowly with the Co(II), Ni(II), and Pb(II) concentrations. The solubility of Ni(II) and Pb(II) was limited at approximately $7 \cdot 10^{-4}$ M in Tris, where EROD activity was about 60% of the control value in both cases. For that reason, no EC50 was determined for Ni(II) and Pb(II). No effects were observed for Zn(II) at concentrations up to $6.7 \cdot 10^{-3}$ M.

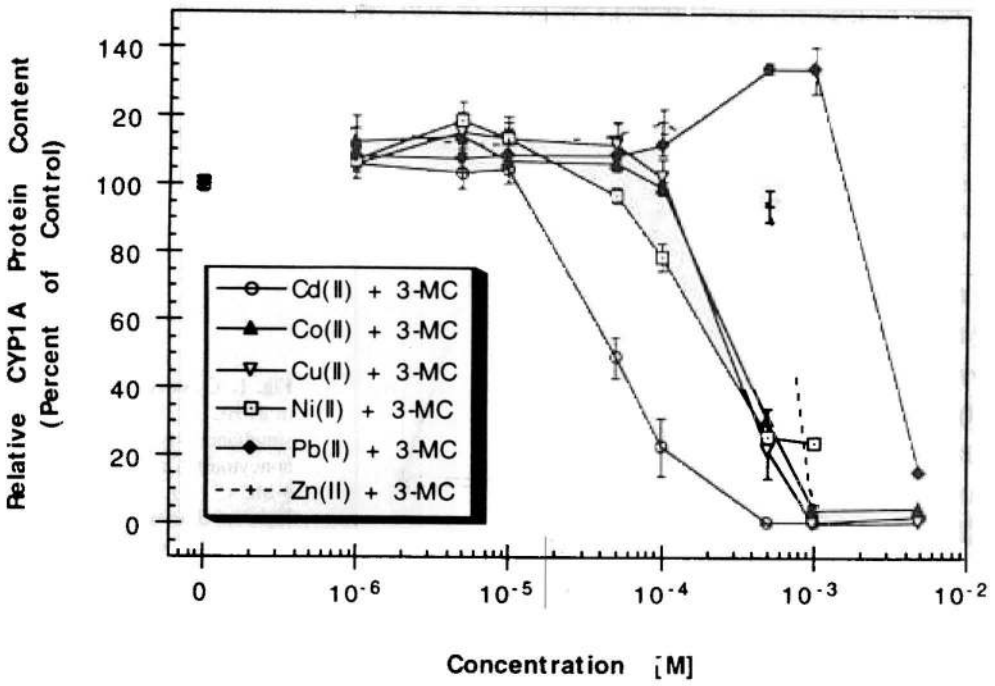

Fig. 2. Concentration–response curves of metals on relative CYP1A protein content determined by ELISA. PLHC-1 cells were simultaneously exposed to 3-MC ($5 \cdot 10^{-6}$ M) and a series of dilutions of metals for 3 d. CYP1A amount is expressed as precent of control value. Data are shown as mean percentage of control ± S.E.M. (six values)

Comparison of EROD Activity, Relative CYP1A Protein Content and Cytotoxicity

A comparison of the EC50 values of the metals obtained in the different assays is shown in Figure 5. Apart from Cd(II), the cytotoxic effect and a decrease of CYP1A protein content occurred at the same concentration. The inhibition of EROD activity was the most sensitive reaction in all cases. This indicates that heavy metals inhibit the CYP1A activity prior to affecting the CYP1A protein and causing cytotoxicity. Inhibitory concentrations of Cd(II) and Cu(II) on EROD activity were significantly lower after 15 min than after a 3-d exposure (2.7 times and 12.3 times, respectively). In contrast, Co(II) inhibited CYP1A enzyme activity at a 4.1 times higher concentration after 15 min than after 3 d, suggesting that heme metabolism is likely the most sensitive target for this metal.

For Cd(II), differences between the effect concentrations measured in the four assays were most obvious. The reaction as determined by the EC50 value obtained in the EROD assay after 15 min exposure of the cell lysate was 2.7 times more sensitive than after 3-d simultaneous exposure in living cells, 6.4 times more sensitive than the EC50 measured in the ELISA, and 16.8 times more sensitive than the EC50 measured in the NR assay. Interestingly, Pb(II) showed striking differences in the shape of the concentration-response curves between the assays. In contrast to a slow decrease of the EROD activity starting at $5 \cdot 10^{-6}$ M in both EROD assays (Figures 3, 4), an increase of the relative CYP1A protein content at 10^{-3} M and $5 \cdot 10^{-3}$ M was found in the ELISA (Figure 2).

Discussion

Simultaneous treatment of PLHC-1 cells with 3-MC, a CYP1A-inducer, and various heavy metals for 3 d resulted in a concentration-dependent inhibition of CYP1A activity, reduction of relative CYP1A protein content and cytotoxicity. Induction of CYP1A protein and activity in the presence of both an inducer (3-MC) and low concentration of inhibitors (heavy metals) has been observed. Therefore, heavy metals seem not to interfere with the binding of the inducer to the aryl hydrocarbon (Ah) receptor, but act at the CYP1A protein level. The EC50 values in the assays were between 10^{-5} and 10^{-3} M, which are at significantly higher concentrations than those measured for tri-substituted and disubstituted organotins in a previous study (Brüschweiler *et al.* 1996a). All metals were found to affect EROD activity at significantly lower concentrations than the relative CYP1A protein content, whereby Cd(II) was most active (EC50 = $2.2 \cdot 10^{-5}$ M). Preliminary immunological studies in fish indicated that the inhibition of CYP1A by Cd(II) is due to a decrease in CYP1A protein rather than a direct inhibition of the enzyme activity (George 1989). In the present study, however, Cd(II) affected EROD activity at a significantly lower concentration (EC50 = $2.2 \cdot 10^{-5}$ M) than the relative CYP1A protein content (EC50 = $5.3 \cdot 10^{-5}$ M). Reasons for these differences are not known.

Short-term treatment of cell lysate with Cd(II) and Cu(II) led to an inhibition of EROD activity at even lower concentrations than in the 3-d experiment. Inhibitory concentrations in the present study coincide with those from a study with mammalian microsomes, in which a direct inhibition of benzo[*a*]pyrene hydroxylase has been found at low Cd(II) concentrations of 10^{-6} M and higher (Fukuhara and Takabatake 1982). Two reasons may account for the higher sensitivity of the cell lysate compared to the cultured living cells. First, in the experiment with cell lysate, the buffer does not contain any serum that may bind free metal ions, which are the most biologically active species. Second, experiments *in vivo* in fish and *in vitro* in trout hepatoma cells have shown that Cd(II), Cu(II) and Zn(II) induce metallothionein B, which is capable of sequestering metal ions by binding via thiolate bonds of cysteine residues (Zafarullah *et al.* 1989). In contrast to the other metals tested, Co(II) affected

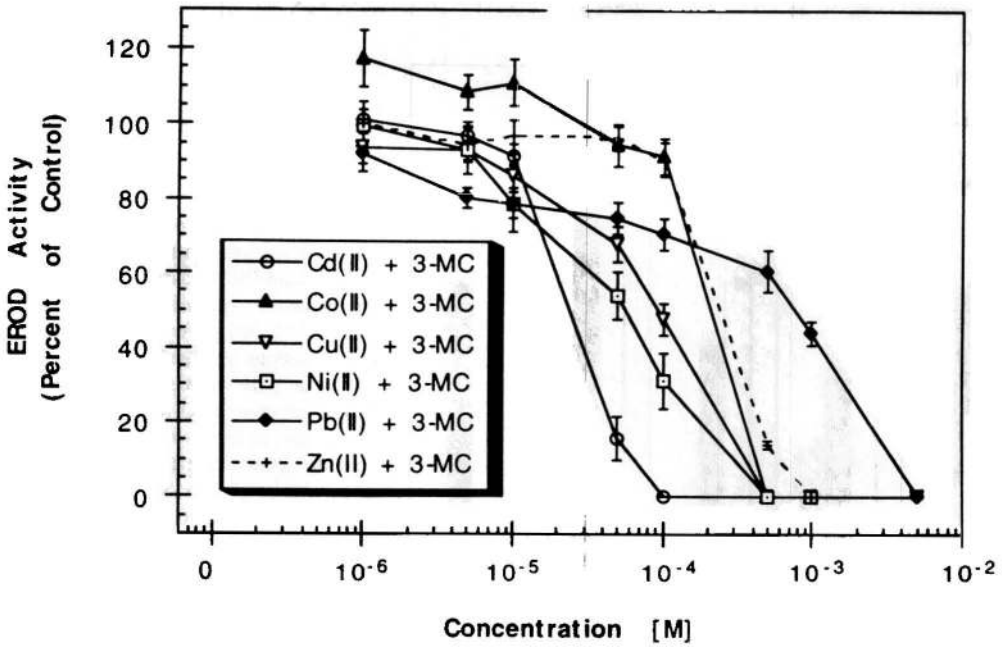

Fig. 3. Concentration–response curves of metals on EROD activity. PLHC-1 cells were simultaneously exposed to 3-MC ($5 \cdot 10^{-6}$ M) and different concentrations of metals for 3 d. Data are shown as mean percentage of control ± S.E.M. (six values)

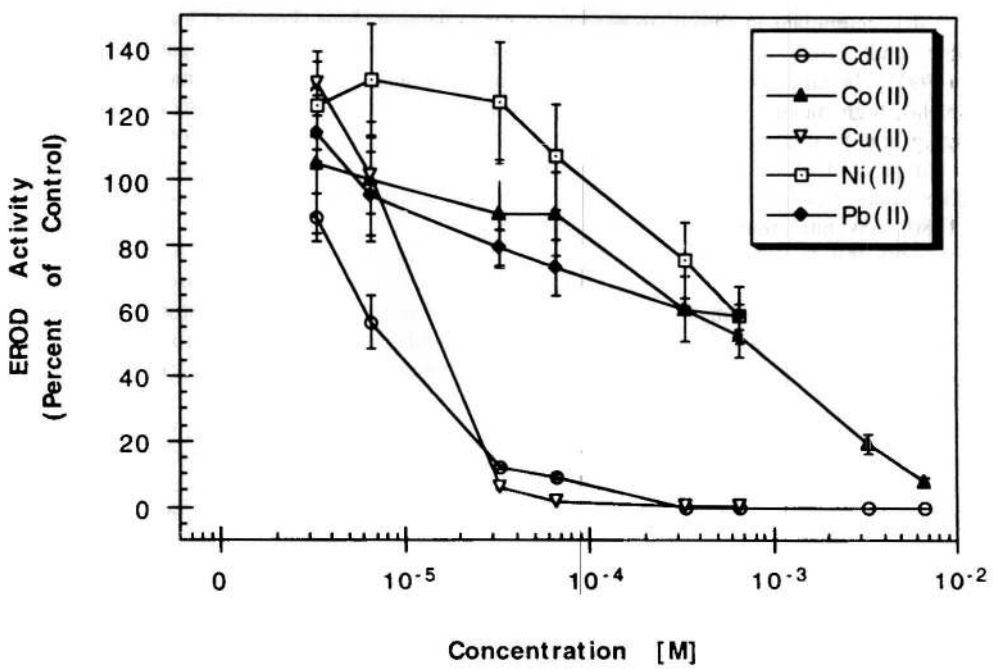

Fig. 4. Effects of metals on EROD activity on lysates of 3-MC–induced cells. Cells were exposed to 3-MC ($5 \cdot 10^{-6}$ M) for 3 d, frozen, and exposed to a series of dilutions of metals for 15 min after thawing. Data are shown as mean of percentage of control ± S.E.M. (three values). No effects were observed for Zn(II) up to $6.7 \cdot 10^{-3}$ M (curve not shown)

CYP1A enzyme activity in cell lysate less than in living cells. These findings suggest that Co(II) may disturb CYP1A enzyme activity rather in an indirect fashion, probably by inhibition of heme biosynthesis and/or induction of heme oxygenase.

Apart from Cd(II), relative CYP1A protein content was only decreased at cytotoxic concentrations of the heavy metals. Similar effects have been observed for organotins, particularly for dibutyltin, at even lower concentrations in PLHC-1 cells (Brüschweiler *et al.* 1996a). Thereby, the EC50 in the EROD assay was at $1.2 \cdot 10^{-6}$ M, while those in the ELISA and NR assay were in the same concentration range ($9.0 \cdot 10^{-6}$ M and $8.7 \cdot 10^{-6}$ M, respectively). These results give support for CYP1A immunodetection as complementary method to the EROD assay. In case of Cd(II), also a higher susceptibility of the relative CYP1A protein content than in the cytotoxicity was observed. It can be concluded that in addition to the direct effects on EROD activity, indirect effects of Cd(II) resulting in the decrease of CYP1A protein have also to be taken into consideration. Thereby, the interference of Cd(II) with heme metabolism via induction of heme oxygenase may be most probable.

Whole-body metal levels found in marine fish are in the range of 0.03–4.2 mg/kg (dry weight) for Cd(II), 0.07–1.45 mg/kg for Co(II), and 2–42 mg/kg for Cu(II) (Depledge *et al.* 1994).

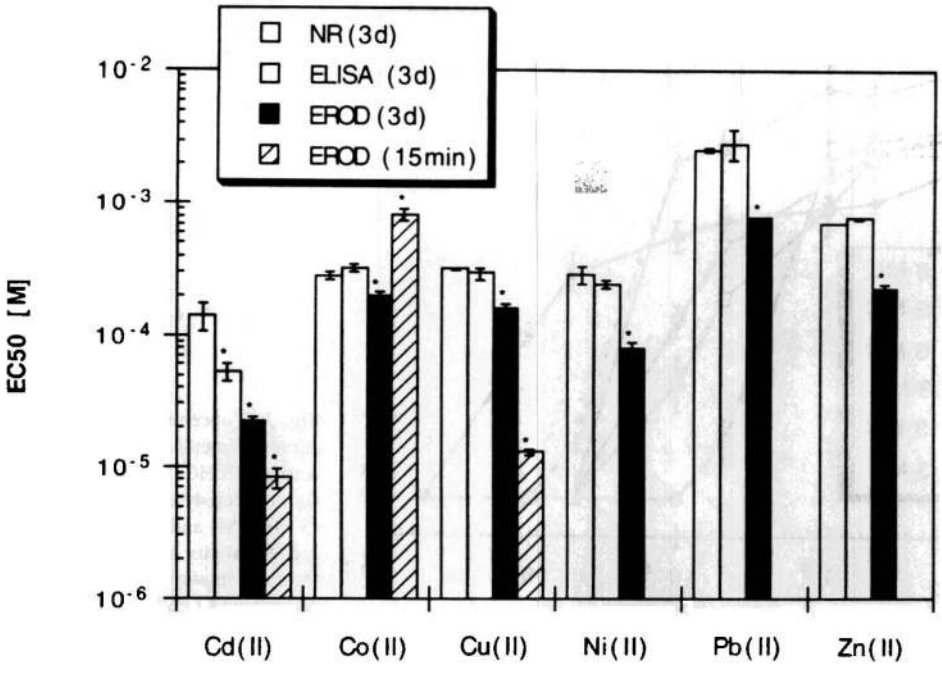

Fig. 5. Comparison of the EC50 values determined in the NR assay, ELISA, and EROD assay after 3-d simultaneous exposure to 3-MC ($5 \cdot 10^{-6}$ M) and different concentrations of metals. Additionally, EROD activity was determined after a 15 min exposure of 3-MC–induced cell lysates to metals (EROD 15 min). Data are shown as mean of percentage of control ± S.E.M. *Significantly different from the left neighboring bar at $p < 0.05$. No effects were measured for Ni(II), Pb(II), and Zn(II) due to their limited solubility; for that reason, no bars for these metals are shown

Cd(II) and Cu(II) are known to primarily accumulate in the liver and to a lesser extent in the kidney and skeletal muscle of fish [for overview, see Sorensen (1991)]. In lakes near a base metal smelter at Flin Flon (Canada), high mean Cd(II) and Cu(II) concentrations of 10.1 mg/kg and 84 mg/kg (dry weight), respectively, were measured in the liver of white suckers (*Catostomus commersoni*) and northern pikes (*Esox lucius*), respectively (McFarlane and Franzin 1980). Assuming that the liver dry weight is 20% of the fresh weight, this would give Cd(II) and Cu(II) concentrations in the liver of $1.8 \cdot 10^{-5}$ M and $2.6 \cdot 10^{-4}$ M, respectively. This is in the range of the inhibitory effects on the EROD activity measured in the present study after 3-d exposure in living fish hepatoma cells (EC50(Cd(II)) = $2.2 \cdot 10^{-5}$ M, EC50(Cu(II)) = $1.6 \cdot 10^{-4}$ M). Mean Cd(II), Co(II) and Cu(II) levels in fish would however be below these effect concentrations. The comparison of the environmental tissue concentrations in fish and the effects measured *in vitro* underscores the environmental relevance of the possible inhibitory activity of metals on the CYP1A system at highly polluted sites.

In vivo experiments in fish have shown that the influence of heavy metals on CYP1A induction response is complex. Inhibition of EROD and benzo[*a*]pyrene hydroxylase activity has been found after intraperitoneal co-injection of Cd(II) (1 and 1.4 mg/kg body weight) and a CYP1A-inducer in plaice (*Pleuronectes platessa*) (George and Young 1986), and in black sea bass (*Centropistis striata*) (Fair 1986) after 2-d exposure. No changes in the liver cytochrome P450 monooxygenase activities, a slightly elevated liver cytochrome P450 content, and even a marked increase of 7-ethoxycoumarin-*O*-deethylase activity in the kidney have been observed after four weeks exposure of rainbow trout to sublethal aqueous Cd(II) concentration (100 µg/L) compared to the control without Cd(II) preexposure (Förlin *et al.* 1986). Even a doubling of the benzo[*a*]pyrene hydroxylase activity and microsomal cytochrome P450 protein content have been measured, when eels *Anguilla anguilla* were exposed to Cd-containing (5 µg/L) seawater for 24 d following a benzo[*a*]pyrene injection (20 mg/kg body weight), compared to benzo[*a*]pyrene injection alone (Lemaire-Gony and Lemaire 1992). Tolerance development of Cd(II) on hepatic drug oxidation has been shown in rats, whereby metallothionein induction and other unknown factors were suggested to be responsible for this tolerance phenomenon. The reasons for the enhancing effect of Cd(II) on the CYP1A induction response in eel remain unclear. Differences may occur among species, after short-term simultaneous exposure and long-term preexposure of Cd(II), and after uptake via intraperitoneal injection and via contaminated water. Modulatory effects on CYP1A by heavy metals, especially Cd(II) and Cu(II), have to be investigated in further studies in order to elucidate their significance for indirect toxicity and for the application of CYP1A as biomarker in environmental monitoring.

Cytotoxicity in terms of EC50 values of the mixture of metals and 3-MC were found between $1.4 \cdot 10^{-4}$ M (for Cd(II)) and $2.6 \cdot 10^{-3}$ (for Pb(II)). This is in accordance with other *in vitro* studies, in which fish cell lines have been used and exposed to the metals alone (Babich *et al.* 1986; Segner and Lenz 1993; Ryan and Hightower 1994). With mammalian cells *in vitro*, the following biochemical targets are primarily involved in heavy metal cytotoxicity. Cd(II) and other heavy metals such as Hg(II), Cu(II), and Zn(II) are known to bind to Ca^{2+} channels, Ca^{2+}/Mg^{2+}-ATPase, and calmodulin leading to the disruption of Ca^{2+} homeostasis and following loss of cell viability [for review, see Viarengo and Nicotera (1991)]. Other important enzymatic and structural SH-containing proteins, such as Na^+/K^+-ATPase, DNA- and RNA-polymerase, tubulin, and actin are affected by Cd(II) interactions (Viarengo and Nicotera 1991). Determination of the most sensitive and relevant biochemical targets in heavy metal cytotoxicity awaits further experimental evaluation *in vitro* and *in vivo*.

Conclusions

Inhibitory effects of heavy metals on the cytochrome P4501A induction in fish hepatoma cells PLHC-1 were analyzed. During

the induction response in living cells, the EROD activity was more strongly affected by metals than the relative CYP1A protein content indicating a direct inactivation of CYP1A catalytic activity. These findings are corroborated by the inhibitory concentrations of Cd(II) and Cu(II) on EROD activity, which are significantly lower in the experiments with cell lysate than in those with cultured living cells. Effects on CYP1A protein synthesis and heme metabolism are less sensitive than enzyme activity after the 3-d exposure. The results lead to the conclusion that Cd(II) and Cu(II) with their higher potential to affect CYP1A enzyme activity than other metals have to be taken into consideration in CYP1A biomarker monitoring. This is of special importance, because Cd(II) and Cu(II) are widespread environmental pollutants. The modulating effect of heavy metals on CYP1A corroborate previous findings with organotins (Fent and Stegeman 1993; Fent and Bucheli 1994; Fent 1996; Brüschweiler *et al.* 1996), thus underscoring the inclusion of additional environmental pollutants in the interpretation of biomarker findings. Since the CYP1A protein level is less susceptible than enzyme activity to inhibitory actions of environmental pollutants (such as heavy metals and organotins) during CYP1A induction response, CYP1A biomarker monitoring should be relied on both measures (Bucheli and Fent 1995).

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