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Aluminium and Cadmium Interfere with the Estrogen Receptor Level in the Primary Culture of Hepatocytes in the Rainbow Trout *Oncorhynchus mykiss*

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Al and Cd-induced inhibition of vitellogenin (VTG) production was examined at the estrogen receptor (ER) level in rainbow trout *Oncorhynchus mykiss* hepatocytes. The binding of [^{3}H] estradiol-17β (E_2) to hepatocytes reached a plateau 3 days after addition of E_2 (2×10^{-6} M) to the medium. The binding activity was linearly reduced with the increased concentrations ($\sim10^{-5}$ M) of 4-hydroxy-tamoxifen (4-OHT) and specific binding linearly increased with the increased doses of [^{3}H]E_2, indicating that the radioligand bound to ER. Al (10^{-4} M) and Cd (10^{-6} M) as well as 4-OHT (10^{-6} M) significantly reduced the [^{3}H]E_2-binding activity by 30~40%, while they completely inhibited VTG production. Al and Cd had no effect on E_2-human ERα binding activity at any concentrations used ($\sim10^5$ nM each). These results suggested that Al and Cd inhibited VTG production in part by interfereing with the ER level. Inhibitory effects of these metals on the E_2-dependent upregulation of ER activity are also discussed.

Key words: Aluminium, Cadmium, Estrogen, Estrogen receptor, Hepatocyte culture, Vitellogenin, Tamoxifen, Rainbow trout

Introduction

Water pollution in the environment has induced various types of physiological dysfunction in fish. Metal contaminants such as Al and Cd were found to impair oogenesis as shown by poor accumulation of egg yolk (Lee and Gerking, 1980; Victor et al., 1986; Pereira et al., 1993) and a reduction in egg number (Runn et al., 1997), resulting in recruitment failure.

Vitellogenin (VTG), a precursor molecule of egg yolk, is synthesized in the liver under stimulation of estrogen, e.g. estradiol-17β (E_2), released into the circulation, and transported into oocytes for accumulation as yolk proteins after cleaving into lipovitellin, phosvitin, and β'-component (Matsubara and Sawano, 1995). The first step of VTG synthesis is initiated by binding of E_2 to its receptors (ER) in hepatocytes, which receptors are upregulated by E_2 itself. In a subsequent step, the hormone-receptor complex activates the specific target DNA sites and sequent expression of VTG mRNA (Lazier and MacKay, 1993). Although Hwang et al. (2000) reported that Al and Cd inhibited VTG synthesis at the transcriptional level in rainbow trout, it remains unknown how these metals affect VTG synthesis at the ER level.

The aim of the present study was to clarify the effects of Al and Cd on the ER level in the primary culture of hepatocytes in rainbow trout. The binding of [^{3}H]E_2 to ER (whole cells) was examined in the presence of these metals or 4-hydroxy-tamoxifen (4-OHT), antiestrogen. Effects of Al and Cd on E_2-ER binding affinity were also examined using an enzyme-linked immunosorbent assay (ELISA).

Material and Methods

Rainbow trout, *Oncorhynchus mykiss*, weighing 150~450 g were obtained from a commercial dealer

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and kept in outdoor concrete ponds with running water at 14~16℃. They were fed trout food pellets once a day but starved on the last day before sampling. Maturing females were not included.

Hepatocyte were isolated using collagenase and incubated in 3 mL of William's medium E (Life Tech.) at 15℃ under 5% CO_2. The whole medium was changed every day throughout a 2-day preculture and subsequent experimental periods. Spent media were analyzed for VTG production by SDS -polyacrylamide gel electrophoresis (PAGE). Details of these methods and VTG identification were described by Kwon et al. (1993) and Hwang et al. (2000). Hepatocyte were subjected to ER studies as in the following.

[^{3}H]E_2-binding experiments

Temporal sequence of ER upregulation

The time course of ER expression by E_2 was examined in the present culture system. After a 2-day preculture, E_2 (2×10^{-6} M, Sigma) and [2, 4, 6, 7-^{3}H(N)]E_2 ([^{3}H]E_2, 5 kBq; New England Nuclear) in ethanol each were simultaneously added to the incubation medium and hepatocytes were incubated for 5 more days, during which time hepatocytes were harvested daily and analyzed for [^{3}H]E_2 activity.

Harvested hepatocytes were washed three times with ice-cold phosphate buffer, solubilized with 0.3 mL of 0.3 N NaCl at room temperature, and added to Sintizol-EX (Dojindo Chem). Radioactivity was counted with a liquid scintillation counter (LS 6000, Aloka) and expressed as dpm per proteins. Proteins were measured by the method of Bradford (1976) using bovine serum albumin as a standard.

4-OHT effects

4-OHT (Sigma) was added to culture media to confirm the competitive binding of [^{3}H]E_2 and the antiestrogen to ER. Hepatocytes were precultured for 2 days and then E_2, [^{3}H]E_2, and 4-OHT were simultaneously added to the dishes. 4-OHT was dissolved in 3 μL of dimethyl sulfoxide (DMSO) and added to final concentrations of 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M. Control cultures received the equivalent amount of the solvent only. After a 3-day culture, spent media and hepatocytes were analyzed for VTG production and [^{3}H]E_2 activity, respectively.

Al and Cd effects

Effects of Al and Cd on the binding activity of [^{3}H]E_2 to ER were examined to clarify whether these metals interfere with VTG synthesis at the ER level. Hepatocytes were precultured for 2 days and then [^{3}H]E_2, E_2, and Al (10^{-4} M), Cd (10^{-6} M), or 4-OHT (10^{-6} M) were added to the culture media. The culture continued for 3 more days and then spent media and hepatocytes were analyzed for VTG production and [^{3}H]E_2 binding, respectively.

ELISA

Effects of Al and Cd on the binding of E_2 to ER was examined with ELISA using an ER competitor screening kit (Wako Pure Chem.). This kit consists of a 96-well microplate coated with human ER α (hER α) recombinant, reaction solution including fluorescence-labeled E_2, and other necessary reagents. The assay was conducted according to the kit instructions.

Al, Cd, E_2, and 4-OHT were dissolved in 6 μL of DMSO, added to the reaction solution in hER α -coated wells, and incubated for 2 h at room temperature. Final concentrations of these additions were 0, 0.1, 1, 10, 10^2, 10^3, 10^4, and 10^5 nM. Control was incubated with the reaction solution containing DMSO only. Fluorescence intensity in each well was measured with a microplate photometer (MTP -22, Corona) at 490 nm (excitation) and 530 nm (emission).

Statistical analysis

Data were analyzed by one-way ANOVA (Fisher PLSD test). Fisher's r-test was also used to examine the significance of correlation coefficients. Significance was accepted at $P<0.05$. Percentage data were statistically analyzed after being arcsine transformed.

Results

The temporal sequence of [^{3}H]E_2 binding to E_2 -treated hepatocytes was examined to clarify how ER is upregulated by E_2 during incubation. Hepatocyte radioactivity increased sharply until day 2 after incubation with E_2 and reached its maximum on day 3 (Fig. 1). This level remained almost unchanged until at least day 5. On the basis of these results, the following experiments on [^{3}H]E_2-ER binding

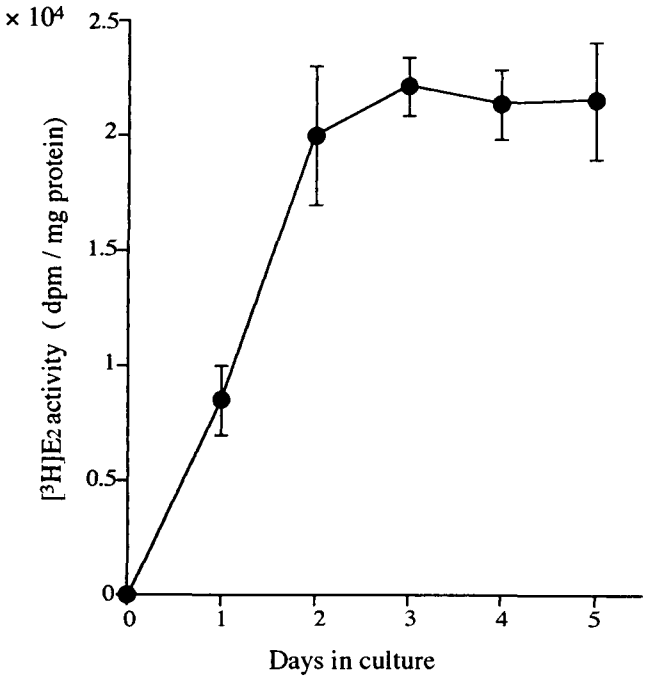

Fig. 1. Temporal sequence of [^{3}H]E_2 binding to hepatocytes in cultures with E_2 (2×10^{-6} M) in rainbow trout. Each point represents the mean ± SE of three fish.

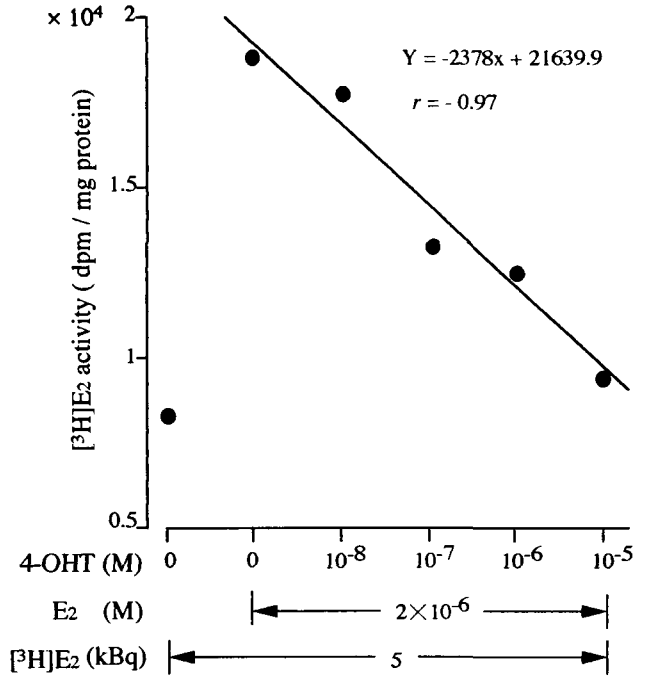

Fig. 2. Concentration-dependent inhibition of [^{3}H]E_2 binding to hepatocytes by 4-OHT in cultures with E_2 and [^{3}H]E_2 in rainbow trout. [^{3}H]E_2 activity was estimated on day 3 in culture. The regression was significant ($P<0.05$).

were conducted 3 days after E_2 and [^{3}H]E_2 addition.

The addition of 4-OHT to the incubation medium reduced the binding of [^{3}H]E_2 to hepatocytes in a concentration-dependent way and the binding was almost completely inhibited to the background level without E_2 at a concentration of 10^{-5} M (Fig. 2). Spent media in this experiment were also examined for VTG production by SDS-PAGE. VTG production decreased linearly with an increase of 4-OHT concentrations (data not shown).

The effects of Al and Cd on VTG production and the binding of [^{3}H]E_2 to estrogenized hepatocytes were examined using spent media and hepatocytes, respectively. SDS-PAGE showed that E_2 induced a definite VTG band with 175 kDa, but the addition of 4-OHT, Al or Cd inhibited expression of this band (Fig. 3).

The VTG bands on the gel were quantified by integrated optical density (IOD) and expressed as a percentage of the IOD of total proteins including VTG (Fig. 4). VTG accounted for 3.5% of the total proteins in the control culture with E_2 alone, but the addition of Al, Cd, or 4-OHT decreased the value to almost 0% (background level).

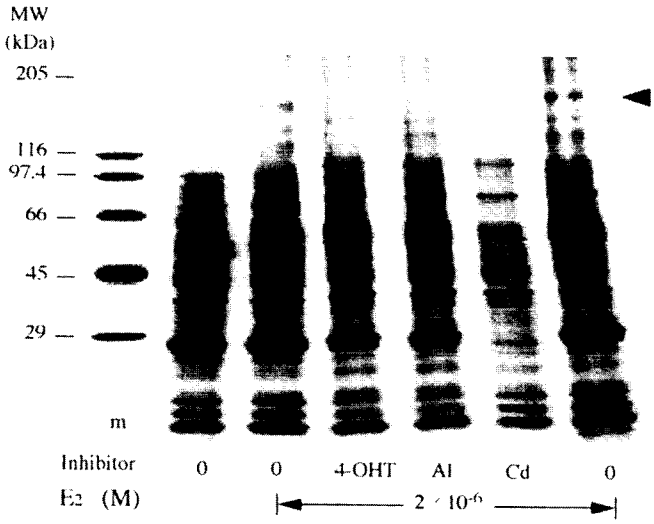

Fig. 3. SDS-PAGE showing the inhibition of VTG (arrowhead) production by 4-OHT (10^{-6} M), Al (10^{-4} M), or Cd (10^{-6} M) in hepatocyte cultures with E_2 in rainbow trout. Spent media were analyzed on day 3 in culture. m: molecular weight (MW) markers. CBB stain.

In the same culture, the binding of [^{3}H]E_2 to estrogenized hepatocytes was significantly reduced

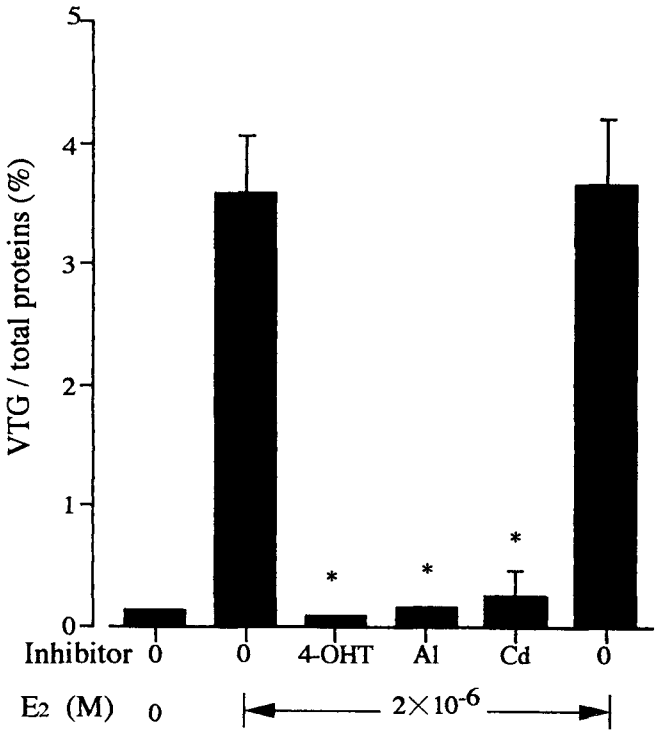

Fig. 4. Effects of 4-OHT (10^{-6} M), Al (10^{-4} M), or Cd (10^{-6} M) on VTG production in hepatocyte cultures with E_2 in rainbow trout. The activity of VTG production was estimated for the relative optical density of VTG to total proteins after SDS-PAGE on day 3 after 4-OHT, Al, or Cd addition. Vertical bars represent the mean ± SE of three individuals. *P<0.01 for control (E_2 only).

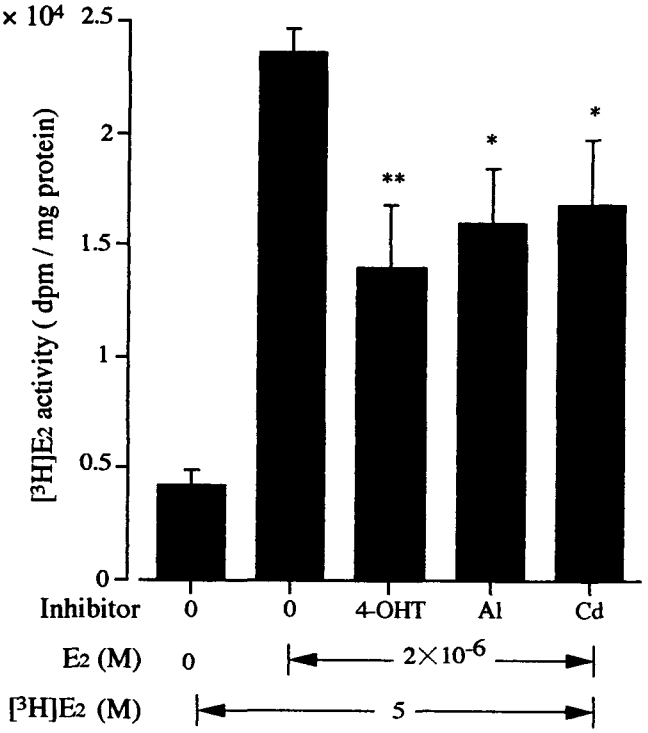

Fig. 5. Effects of 4-OHT (10^{-6} M), Al (10^{-4} M), or Cd (10^{-6} M) on [^{3}H]E_2 binding to hepatocytes in cultures with E_2 and [^{3}H] E_2 in rainbow trout. Radioactivity was determined on day 3 in culture. Vertical bars represent the mean ± SE of three individuals. *P<0.05 and **P<0.01 for control (E_2 only).

by 4-OHT (P<0.01), Al (P<0.05), or Cd (P<0.05) (Fig. 5). However, the rates of reduction were about 40% of the control for 4-OHT, 33% for Al and 30 % for Cd, in contrast to almost 100% inhibition of VTG production.

The effects of Al and Cd on the binding affinity of fluorescence-labeled E_2 to hER α were examined with ELISA. The addition of E_2 or 4-OHT to the wells inhibited the binding of the labeled E_2 to hER α in a sigmoid fashion (Fig. 6). It was inhibited by 98% and 100% at 10^5 nM of 4-OHT and E_2, respectively. On the contrary, Al and Cd had no effect on the binding at any concentrations used.

Discussion

It is well known that ER in the liver is upregulated by the ligand, E_2 itself (MacKay et al., 1996; Pakdel et al., 1997). Indeed, *in vitro* ER mRNA levels were increased by E_2 to 15 times in hepatocyte culture in rainbow trout (Flouriot et al., 1996; Pakdel et al., 1997). In the present experiment, the binding of [^{3}H]E_2 to hepatocyte was maximized 3 days after E_2 addition and this level was maintained until at least day 5. Valotaire et al. (1993) also reported that the maximum level of ER mRNA expression was obtained 2 to 3 days post-injection in rainbow trout. Therefore, it seems reasonable to think that full expression of ER by E_2 takes about 3 days under both *in vivo* and *in vitro* conditions in rainbow trout.

To confirm that the binding of [^{3}H]E_2 to hepatocytes is associated with ER in the present experiments, we conducted competitive experiments with 4-OHT, a competitor with E_2 for binding sites on ER (Donaldson and Hunter, 1983), and with radiolabeled E_2. Increases in 4-OHT concentrations linearly decreased the radioactivity of estrogenized

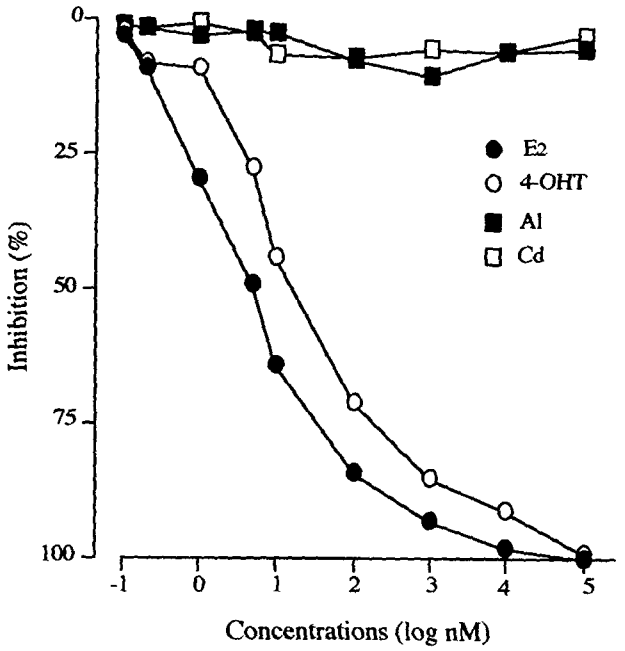

Fig. 6. Inhibitory effects of E_2, 4-OHT, Al, and Cd on the binding activity of fluorescence E_2 to hER α coated on a microplate. Each point represents the mean of six determinations. Error bars are omitted.

hepatocytes as well as VTG production (data not shown). On the contrary, an increase in specific activity of the radioligand linearly increased specific binding to the cells in the present culture. These results indicate that [^{3}H]E_2 competed with E_2 for binding to ER. The results of the ER upregulation experiment also indicate that the hepatocyte radioactivity essentially reflects the status of ER in the cells.

Although 4-OHT was reported to be an agonist for VTG induction in some cases (Lazier and MacKay, 1993), it consistently acted as an antagonist in the present study. Al and Cd as well as 4-OHT significantly inhibited the binding of [^{3}H]E_2 to hepatocytes. However, inhibition rates were as much as 30~40%, while VTG production was completely inhibited in the same culture. These facts led us to the interpretation that Al and Cd impair VTG production not only at the ER level but also at another level such as transcription, because these metals inhibited the E_2-induced expression of VTG mRNA in hepatocyte culture of rainbow trout (Hwang et al., 2000). Similarly, an *in vitro* experiment showed that Cd interfered with transcriptional activation of VTG in the trout (Olsson et al., 1995).

Although the present results clearly demonstrated that Al and Cd interfered with the ER level, it remains unclear whether these metals depressed the upregulation of ER expression by E_2 or the binding affinity of E_2 to the receptors. If these metals impair E_2-binding sites on ER, E_2-binding affinity should be reduced. To examine this interaction, we used a hER α assay kit, because the structure of rainbow trout (rt) ER genes resembles that of hER genes (Ponglikitmongkol et al., 1988) and the fundamental process of rtER interaction with E_2 response elements is similar to that found in birds, frogs, and mammals (Lazier and MacKay, 1993). In the affinity experiment, Al and Cd had no effect on E_2-ER binding activity at any concentrations used, while 4-OHT and E_2 characteristically reduced the activity. Considering these results, the inhibitory effect of these metals on the ER level is probably due to the depression of E_2-induced upregulation of ER expression. Le Guevel et al. (1998) reported that Cd did not affect E_2 binding affinity to ER and only the hormone-dependent ER expression was affected by the metal in rainbow trout.

In conclusion, Al and Cd interfered with the ER level in estrogenized hepatocytes not by affecting E_2-ER binding affinity but probably by depressing the E_2-dependent upregulation of ER activity.

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