Interaction of cadmium and oestradiol-17β on metallothionein and vitellogenin synthesis in rainbow trout (*Oncorhynchus mykiss*)

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The induction of metallothionein and vitellogenin synthesis in rainbow trout liver was studied after injection of oestradiol-17β alone or in combination with cadmium or zinc. Intraperitoneal injection of oestradiol-17β increased the liver somatic index, with subsequent induction of vitellogenin synthesis. Oestradiol-17β did not induce metallothionein synthesis. Injection of cadmium induced the synthesis of metallothionein mRNA and metallothionein. Injection of oestradiol-17β in combination with cadmium resulted in inhibition of transcription and translation of both vitellogenin and metallothionein. Chromatography of liver cytosols revealed that cadmium, when co-injected with oestradiol-17β, did not bind to metallothionein but would initially bind to high-molecular-mass (HM_r) cytosolic proteins. In fish injected with cadmium in combination with oestradiol-17β, cadmium was gradually redistributed from HM_r proteins to metallothionein. This resulted in induction of metallothionein synthesis and in binding of most of the cadmium to metallothionein. Induction of vitellogenin mRNA was observed 15 days after injection, as cadmium was being redistributed to newly synthesized metallothionein. These findings indicate that cadmium inhibits the transcription of vitellogenin. The binding of cadmium to these non-metallothionein proteins represses the induction of metallothionein and results in increased toxicity of the metal. Preinduction of metallothionein by zinc injections resulted in decreased cadmium sensitivity of the fish and a decrease in the repression of vitellogenin mRNA. Furthermore, a role for metallothionein in the detoxification of cadmium is indicated by the induction of vitellogenin synthesis that occurs once metallothionein has begun sequestering cadmium.

INTRODUCTION

Metallothioneins (MTs) are a family of heavy-metal-binding proteins that are involved in the maintenance of trace-metal homoeostasis and the detoxification of heavy metals in a wide variety of organisms (Olsson, 1993; Zafarullah et al., 1989a). These roles for MT have been indicated by the observations that heavy-metal ions induce MT gene transcription and then bind to MT (Zafarullah et al., 1989b; Durnam and Palmiter, 1981). Exposure of rainbow trout (*Oncorhynchus mykiss*) to cadmium via the water results in transcriptional activation of MT (Olsson et al., 1989a; Norey et al., 1990).

During the period of sexual maturation in most egg-laying vertebrates, large amounts of the yolk protein precursor, vitellogenin (VTG), are synthesized in the liver (Wallace, 1985). After synthesis, VTG is secreted into the blood and transported to the growing oocytes, where it is incorporated and processed to form the major yolk proteins used to nourish the embryo and larvae until the beginning of feeding. The synthesis of VTG is under the endocrine control of oestradiol-17β (E_2) (Wallace, 1985; Mommsen and Walsh, 1988). In response to E_2 the liver undergoes major ultrastructural changes, such as proliferation of the Golgi apparatus, increase in cisternae of the nuclear envelope, increased synthesis of ribosomes, increase in rough endoplasmic reticulum and an increase in metabolic enzymes, all in order to synthesize and secrete VTG (van Bohemen et al., 1981; Peute et al., 1985; Wallace, 1985; Mommsen and Walsh, 1988).

When an organism is exposed to cadmium, severe toxic symptoms may occur (Olsson, 1993). It has been argued that metal toxicity occurs when the rate of metal accumulation exceeds the rate of MT synthesis (McCarter et al., 1982). Cadmium poisoning causes a multitude of effects, many of them disrupting the normal calcium and zinc metabolism. Cadmium interferes with calcium and zinc metabolism by occupying the calcium- and zinc-binding sites in proteins (Webb, 1979; Verbost et al., 1987). It has been proposed that the most sensitive periods to cadmium poisoning are the periods of embryonic and larval development. Previous studies have shown that chronic cadmium exposure results in lower fecundity and decreased overall reproductive success in fish (Benoit et al., 1976; Sephar and Brewer, 1976). Cadmium also decreases the plasma levels of VTG in vitellogenic rainbow trout (*Oncorhynchus mykiss*) and winter flounder (*Pleuronectes americanus*) as well as in E_2-treated flounder (*Platichtys flesus* L.) (Haux et al., 1988; Povlsen et al., 1990; Pereira et al., 1993). A role for MT during the period of sexual maturation as well as during the early embryo development has previously been shown in rainbow trout (Olsson et al., 1989b, 1990).

Zinc is required for the stability of membranes and polyribosomes, for many components of the protein-synthesizing systems, and possibly for the allosteric regulation of some enzymes (Vallee and Wacker, 1970; Sandstead, 1975). During vitellogenesis in rainbow trout, the hepatic zinc levels increase drastically, followed by up-regulation of MT at the end of vitellogenesis (Olsson et al., 1987). MT's role during late vitellogenesis has been suggested to be to prevent possible toxic effects caused by the zinc that is being released at the end of the vitellogenic period. MT is up-regulated in response to a re-

Abbreviations used: MT, metallothionein: VTG, vitellogenin: LSI, liver somatic index: E_2, oestradiol-17β; HM_r, high molecular mass.

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distribution of zinc from high-molecular-mass (HM_r) proteins to MT once the VTG synthesis has finished in E_2-injected fish (Olsson et al., 1989b).

The objective of the present study was to determine the possible role of MT in the cadmium-mediated decrease in VTG in rainbow trout. Determination of transcriptional and translational activation of both the MT and the VTG genes, as well as the cytosolic distribution of cadmium, copper and zinc, was performed in order to investigate the influence of cadmium on MT regulation during E_2-induced VTG synthesis.

MATERIALS AND METHODS

Fish holding conditions and sampling

Juvenile rainbow trout, with a body weight of about 100 g, were obtained from a local fish hatchery near Göteborg (Antens Laxodling AB, Sweden). The fish were acclimated in the laboratory for 1 week before the experiment, in basins with filtered, aerated and recirculating tap-water at a temperature of 10 °C. The fish were not fed during the experiment.

The experiment was started by giving 48 fish an intraperitoneal injection of E_2 (10 mg of E_2/kg body wt.) (Sigma Chemical Co.) dispersed in peanut oil. A total of 16 E_2-injected fish were immediately co-injected with $ZnCl_2$ (0.5 mg of Zn^{2+}/kg body wt.) and another 16 E_2-injected fish were co-injected with $CdCl_2$ (0.2 mg of Cd^{2+}/kg body wt.): 16 control fish received peanut oil only. After treatment the fish were transferred to 50-litre aquaria, eight fish in each, supplied with a continuous flow of water at 10 °C. The fish were sampled 1, 5, 10 and 15 days after the injections. At sampling, the fish were stunned with a blow on the head and weighed. Blood was collected from the caudal vessels with a heparinized syringe and directly centrifuged. The resulting plasma was immediately frozen and stored at −80 °C until analysed. The liver of each fish was excised and weighed, and was immediately frozen and stored at −80 °C until analysed.

In a separate experiment, one group of 20 fish received an intraperitoneal injection of $ZnCl_2$ (5 mg of Zn^{2+}/kg body wt.) to preinduce MT. Then 3 days later these fish and a separate group of 20 untreated fish received an intraperitoneal injection of E_2 (10 mg of E_2/kg body wt.). After the E_2 injection, groups of five fish received different doses of $CdCl_2$ (2.0, 1.0, 0.5 or 0.2 mg of Cd^{2+}/kg). After treatment, the fish were transferred to 50-litre aquaria, five fish in each, supplied with a continuous flow of water at 10 °C. The effect of zinc-mediated preinduction of MT was then monitored during 5 days.

In a third experiment, 35 fish were divided into seven groups and received the following treatments: group 1, control; group 2, 1.0 mg of Zn^{2+}/kg; group 3, 0.2 mg of Cd^{2+}/kg; group 4, 5.0 mg of Zn^{2+}/kg (in order to preinduce MT), followed after 3 days by co-injection of 10 mg of E_2/kg and 0.2 mg of Cd^{2+}/kg; group 5, co-injection of 10 mg of E_2/kg and 0.2 mg of Cd^{2+}/kg; group 6, injection of 10 mg of E_2/kg, followed after 24 h by injection of 0.2 mg of Cd^{2+}/kg; group 7, injection of 10 mg of E_2/kg, followed after 48 h by injection of 0.2 mg of Cd^{2+}/kg. At sampling, the fish were stunned with a blow on the head and weighed. The liver of each fish was excised and weighed, and was immediately frozen and stored at −80 °C until analysed.

Liver somatic index (LSI) and plasma protein determinations

The LSI was calculated as the percentage of the total body weight (100 × liver weight/body weight). Total protein in plasma was determined by the biuret method (Henry et al., 1957) with BSA as standard.

MT determination

A 100 mg sample of each liver was homogenized in 5 ml of ice-cold saline (0.9 % NaCl), followed by heat denaturation at 95 °C for 5 min. The denatured homogenate was cooled on ice, and flocculent material was removed by centrifugation at 10000 *g* for 10 min at 4 °C. MT was determined by differential pulse polarography at 20 °C (PARC model 174 analyser, PARC/EG & G model 303 SMDE) as previously described (Olafson and Olsson, 1991).

Chromatography

Liver samples from two or three fish were pooled and homogenized in ice-cold 10 mM Tris/HCl buffer, pH 8.0, in a Teflon/glass homogenizer. The homogenates were centrifuged at 20000 *g* for 30 min at 4 °C. The supernatant was subsequently chromatographed on a Sephadex G-75 column (1.5 cm × 50 cm) equilibrated with the same buffer. Fractions of 2.5 ml were collected, and the contents of cadmium, copper and zinc were determined by atomic absorption spectrophotometry (Olsson et al., 1987).

Protein gel electrophoresis

SDS/PAGE was performed as described by Laemmli (1970), in discontinuous polyacrylamide (30 % total monomer concentration/2.7 % cross-linking monomer concentration) gels with a 4 % stacking gel and a 9 % separating gel. Electrophoresis was carried out at 25 mA (constant current) per gel. Before electrophoresis, plasma was diluted 1:20 with sample buffer (60 mM Tris/HCl, pH 6.8, 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol, 0.001 % Bromphenol Blue) and heated at 95 °C for 4 min. A total of 4 μl of diluted plasma, in sample buffer, was subjected to electrophoresis. The gels were stained with Coomassie Brilliant Blue R-250. The molecular mass was determined by comparing the mobility of the unknown proteins with those of standard proteins (Bio-Rad molecular-weight standards, broad range).

Isolation of total RNA, Northern-blot and slot-blot analysis

For the measurement of MT mRNA and VTG mRNA, total RNA was extracted by the method described by Chomczynski and Sacci (1987). To determine the intactness of the RNA and the specificity of the probes, 1.2 %-agarose-gel electrophoresis was performed. For Northern-blot analysis, 10 mg of total RNA was subjected to electrophoresis on formaldehyde gels (Maniatis et al., 1982) and transferred to Hybond N membranes by vacuum transfer (Bio-Rad model 785 vacuum blotter). Equal loading of RNA was determined by ethidium bromide staining of the gels. Before transfer, the gel was soaked for 10 min in 50 mM NaOH and the membrane was wetted in 10 × SSC (1 × SSC is 0.15 M NaCl/15 mM sodium citrate buffer, pH 7.0). Transfer was performed at 7 mmHg for a duration of 90 min. After transfer, the membrane was washed in 2 × SSC and UV-cross-linked, 10 min per side. UV exposure of both sides was used to minimize background during subsequent hybridization with digoxigenin probes. Slot-blot analysis was performed to quantify MT and VTG mRNA. Hybond N membranes were soaked in 6 × SSC. RNA samples (10 mg) were made up to 6 × SSC/7.5 % formaldehyde and applied in duplicate to the filters. The slot-blots were then washed twice with 3 × SSC and UV-cross-linked as described above.

Single-strand RNA probe, labelled with digoxigenin, complementary to the protein-encoding region of rainbow trout MT-B cDNA (kindly given by Dr. L. Gedamu, University of Calgary, Canada) was used for detection of total MT mRNA levels. Nick-

translated DNA probe complementary to rainbow trout VTG (kindly given by Dr. M. P. R. Tenniswood, University of Ottawa, Canada) was used to detect VTG mRNA. Filter hybridization using the MT-B RNA probe was performed at 68 °C in standard hybridization buffer (50% formamide, 5 × SSC, 2% blocking reagent, 0.1% *N*-laurylsarcosine, 0.02% SDS). Filter hybridization using the nick-translated VTG cDNA probe was performed at 45 °C in high-SDS hybridization buffer (50% formamide, 5 × SSC, 2% blocking reagent (Boehringer Mannheim), 0.1% *N*-laurylsarcosine, 7% SDS). After hybridization the filters were washed twice at room temperature in 2 × SSC/0.1% SDS, followed by two washes at 68 °C in 0.1 × SSC/0.1% SDS. Detection was performed with Lumigen PPD and the DIG luminescent detection kit (Boehringer Mannheim). Hybridization was revealed on Kodak XAR5 film: overnight exposure was used for MT determinations, and 3 h exposure for VTG determinations.

RESULTS

Injection of E_2 or a combination of E_2 and zinc ($E_2 + Zn^{2+}$) resulted in elevated LSI levels after 5 days as compared with control fish (Figure 1). At day 15 the LSI levels had declined in the E_2- and $E_2 + Zn^{2+}$-injected fish. When E_2 was co-injected with cadmium ($E_2 + Cd^{2+}$), the response was delayed and increased LSI levels were first detected after 10 days. In the $E_2 + Cd^{2+}$-injected fish the LSI levels were further increased on day 15.

The analysis of total plasma protein levels revealed that injection of E_2 or $E_2 + Zn^{2+}$ resulted in a gradual increase in plasma protein (Figure 2). Contrary to this, co-injection of cadmium with E_2 resulted in decreased plasma protein levels on day 5 and day 10 after injection. The plasma protein levels in $E_2 + Cd^{2+}$-injected fish returned to initial values at the final sampling occasion on day 15. Gel electrophoresis showed that one major protein, with a molecular mass of 170 ± 2 kDa ($n = 6$), appeared in plasma of E_2- and $E_2 + Zn^{2+}$-injected fish after 5 days, and increased further during the experimental period (Figure 3). This major protein has previously been identified as VTG on the basis of its molecular mass and inducibility by E_2 (Silversand et al., 1993). Determination of plasma protein composition (Figure 3) showed that the increase in plasma protein observed after E_2 or $E_2 + Zn^{2+}$ injection was specifically due to increased levels of VTG in the plasma. Although the decrease in plasma protein levels in the $E_2 + Cd^{2+}$-injected fish was due to a general decrease in plasma protein, it was found that VTG appeared to be the main protein responsible for the return of plasma protein to initial levels in this group.

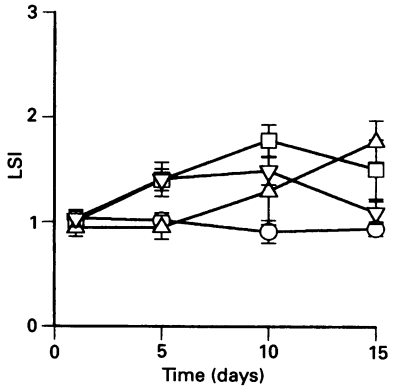

Figure 1 Liver somatic index

The LSI of control (○), E_2-treated (□), $E_2 + Zn^{2+}$-treated (▽) and $E_2 + Cd^{2+}$-treated (△) juvenile rainbow trout are shown as means ± S.D. ($n = 4$).

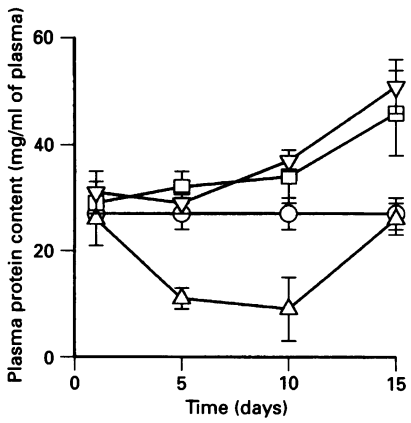

Figure 2 Plasma protein content

The plasma protein levels of control (○), E_2-treated (□), $E_2 + Zn^{2+}$-treated (▽) and $E_2 + Cd^{2+}$-treated (△) juvenile rainbow trout are shown as means ± S.D. ($n = 4$).

There was no mortality in the control group, the E_2 group, the $E_2 + Zn^{2+}$ group, or when only cadmium was injected. Different concentrations of cadmium were tested in combination with 10 mg of E_2/kg, and it was found that 0.2 mg of Cd^{2+}/kg gave no mortality. Therefore this concentration of cadmium was chosen for the experiment. Other concentrations of cadmium were tested in combination with E_2, and it was found that 0.5 mg of Cd^{2+}/kg resulted in 20% mortality, 1.0 mg of Cd^{2+}/kg resulted in 60% mortality and 2.0 mg of Cd^{2+}/kg resulted in 100% mortality after 5 days. After pretreatment of fish with 5.0 mg of Zn^{2+}/kg 3 days before co-injection of E_2 with different doses of cadmium, all fish survived, except in the group injected with 2.0 mg of Cd^{2+}/kg, where a 40% mortality was observed. In the present study, and in previous studies, rainbow trout survive 1.0 mg of cadmium/kg if cadmium is injected alone (Zafarullah et al., 1989b).

Hepatic VTG mRNA levels were measured in order to determine whether the inhibitory effects of cadmium on VTG synthesis occurred at the transcriptional level (Figure 4). There was no VTG mRNA present in the livers of control fish. Injection of E_2 or $E_2 + Zn^{2+}$ resulted in the appearance of VTG mRNA on day 1, and the VTG mRNA levels then remained high throughout the experimental period. In $E_2 + Cd^{2+}$-injected fish, low levels of vitellogenin mRNA were observed on day 1, but on day 5 and day 10 there was no VTG mRNA detected in the livers, indicating that cadmium interfered with the transcriptional activation of VTG. However, on day 15 hepatic VTG mRNA re-appeared in the $E_2 + Cd^{2+}$-injected fish, and this time the levels were comparable with those observed for the E_2-injected fish.

The induction and accumulation of MT after different treatments was determined at the transcriptional and translational level. There was no apparent increase in MT mRNA, and no or only a slight increase in MT during the experimental period in the control fish, E_2-injected fish or $E_2 + Zn^{2+}$-injected fish (Figures 5 and 6). The MT mRNA levels were clearly inducible on day 1 after injection of zinc, cadmium, or cadmium in the presence of E_2 (Figure 5 and Table 1). The induction observed after co-injection of E_2 and cadmium was, however, followed by a transient decrease in MT mRNA 5 days after injection. The MT

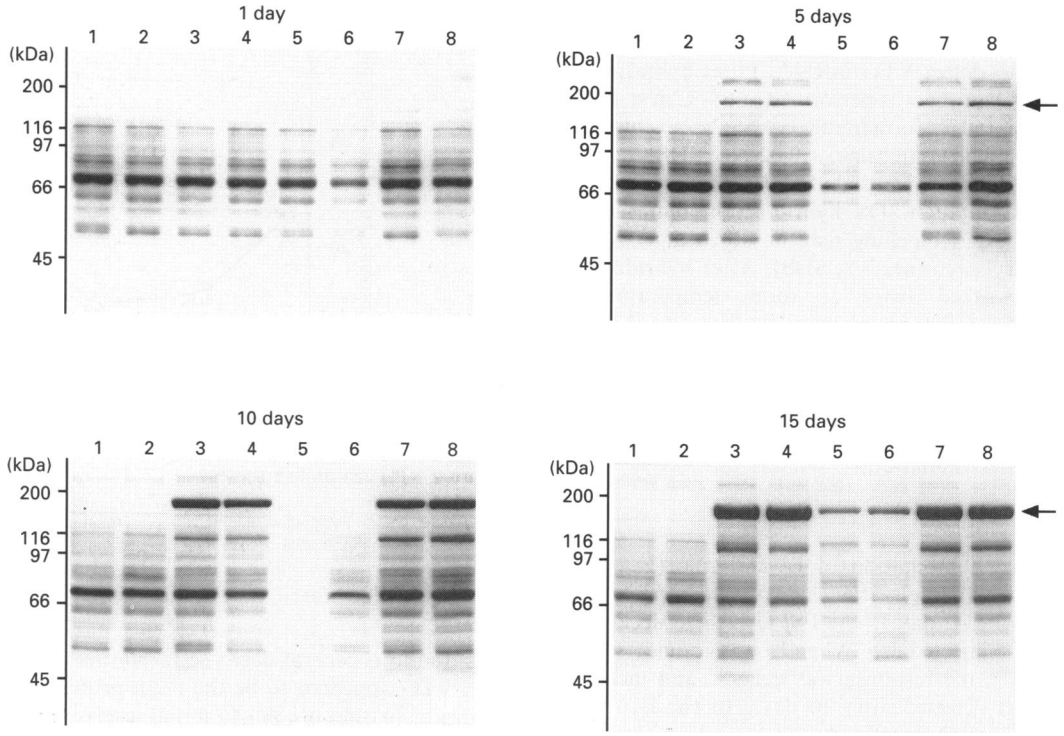

Figure 3 Gel electrophoresis of plasma

Coomassie Brilliant Blue-stained SDS/PAGE using discontinuous polyacrylamide gels with 4% stacking gel and 9% separating gel was performed on plasma sampled 1, 5, 10 and 15 days after injection. Lanes 1 and 2, plasma from control rainbow trout; lanes 3 and 4, plasma from E_2-treated rainbow trout; lanes 5 and 6, plasma from $E_2 + Cd^{2+}$-injected rainbow trout; lanes 7 and 8, plasma from $E_2 + Zn^{2+}$-injected rainbow trout. A 0.2 μl portion of plasma was added to each lane. The molecular masses of the standard proteins (kDa) are indicated on the left of each panel. The arrow on the right indicates the position of the VTG band. Two fish from each group were analysed in parallel on each gel.

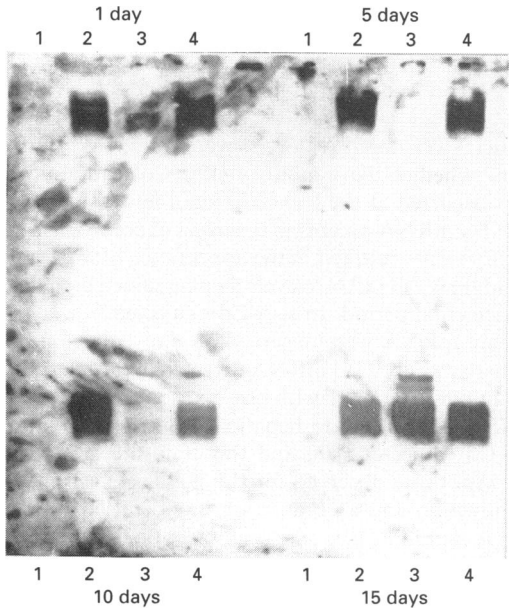

Figure 4 VTG mRNA levels in liver

VTG mRNA levels were determined 1, 5, 10 and 15 days after injection. Lanes: 1, control; 2, E_2; 3, $E_2 + Cd^{2+}$; 4, $E_2 + Zn^{2+}$. Total RNA (10 mg) was run on a 1.2%-agarose gel. The filters were hybridized and washed under stringent conditions.

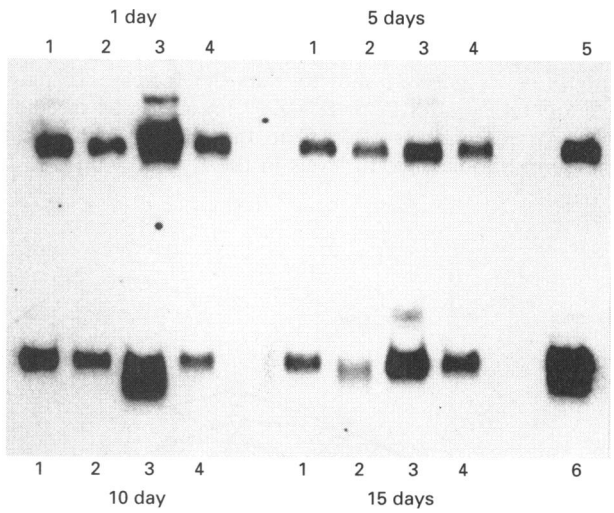

Figure 5 MT mRNA levels in liver

MT mRNA levels were determined 1, 5, 10 and 15 days after injection. Lanes: 1, control; 2, E_2; 3, $E_2 + Cd^{2+}$; 4, $E_2 + Zn^{2+}$. MT mRNA was also determined in control (lane 5) and cadmium-treated (lane 6) fish 5 days after injection. Total RNA (10 mg) was run on a 1.2%-agarose gel. The filters were hybridized and washed under stringent conditions.

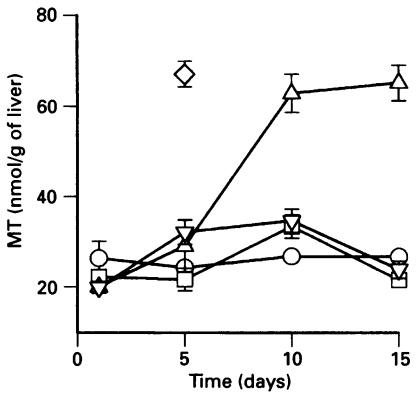

Figure 6 MT levels in liver

Hepatic MT concentrations in control (○), E_2-treated (□), E_2+Zn^{2+}- (▽) and E_2+Cd^{2+}- (△) treated juvenile rainbow trout were determined by differential pulse polarography of heat-denatured samples. After cadmium injection on its own, the MT induction was determined after 5 days (◇). The results are presented as means ± S.D. ($n = 4$).

mRNA levels were then again elevated on day 10 and day 15 after injection of E_2+Cd^{2+}. The MT levels did not increase significantly above basal levels on either day 1 or day 5, even though there was an initial increase in MT mRNA transcription on day 1 (Figure 6). However, when the MT mRNA levels increased on day 10, there was a corresponding increase in MT levels. Since injection of cadmium alone resulted in elevated MT mRNA and MT levels on day 5, the discrepancy between MT mRNA and MT levels observed on day 1 and the lack of elevated levels of MT mRNA or MT on day 5 after co-injection of E_2+Cd^{2+} appeared to be due to inhibited MT synthesis (Figures 5 and 6).

In order to determine whether the initial decrease in VTG mRNA was caused by cadmium, a separate experiment was performed. It was found that preinduction of MT by zinc injections resulted in elevated levels of VTG mRNA one day after co-injection of E_2+Cd^{2+} (Table 1). To determine whether the decrease in MT mRNA at day 5 was due to E_2, fish were either co-injected with E_2+Cd^{2+} or injected with E_2 1 or 2 days before injection of cadmium. It was found that the MT mRNA levels were significantly decreased if E_2 was injected 1 or 2 days before injection of cadmium (Table 1). The effect of E_2 was monitored by measuring the increase in the LSI levels. These levels were

Table 1 LSI, MT mRNA and VTG mRNA levels after different metal and E_2 treatments

The fish were treated by intraperitoneal injections with Zn^{2+} and Cd^{2+} alone or in different combinations with E_2. The following concentrations were used: E_2, 10 mg/kg; Cd^{2+}, 0.2 mg/kg; Zn^{2+}, 1.0 mg/kg. Pre-induction of MT was performed with 5.0 mg of Zn^{2+}/kg 3 days before E_2 injection. Cd^{2+} was either co-injected with E_2 or injected 24 h or 48 h after E_2 was given. The fish were sampled 24 h after the last treatment. Values are given as means ± S.D. ($n = 4$). The mean MT mRNA level in the control group was arbitrarily set to 1.00, whereas the mean VTG mRNA level in the E_2+Cd^{2+} (0 h after E_2 injection) group was set to 1.00. A dash indicates that no VTG was detected.

Treatment	LSI	MT mRNA	VTG mRNA
Control	0.93 ± 0.11	1.00 ± 0.04	–
Zn^{2+}	0.95 ± 0.10	1.74 ± 0.07	–
Cd^{2+}	0.97 ± 0.26	5.51 ± 0.41	–
E_2+Cd^{2+} (pre-induced with Zn^{2+})	1.23 ± 0.18	6.23 ± 2.15	4.68 ± 1.44
E_2+Cd^{2+} (0 h after E_2 injection)	1.02 ± 0.11	5.92 ± 0.95	1.00 ± 0.16
E_2+Cd^{2+} (24 h after E_2 injection)	1.27 ± 0.13	3.42 ± 1.58	4.93 ± 0.67
E_2+Cd^{2+} (48 h after E_2 injection)	1.51 ± 0.18	1.37 ± 0.23	6.04 ± 1.35

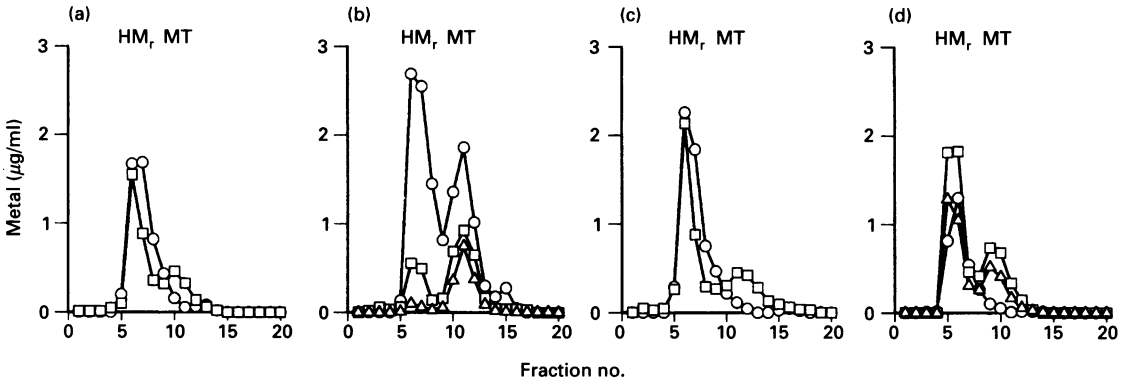

Figure 7 Cytosolic distribution of cadmium, copper and zinc

Representative Sephadex G-75 gel-chromatography elution profiles of liver samples collected from control (**a**), cadmium- (**b**), E_2- (**c**) and E_2+Cd^{2+}- (**d**) treated fish 5 days after injection. Cadmium (△), copper (□) and zinc (○) were determined by atomic absorption spectrophotometry. No cadmium was detected in control and E_2-treated animals. For each chromatogram, a total of 2 ml of supernatant was applied to a 1.5 cm × 50 cm column and eluted in 2.5 ml fractions. The column void volume was about 10 ml; HM$_r$ proteins were eluted at approx. 15 ml, and MT was eluted at approx. 25 ml.

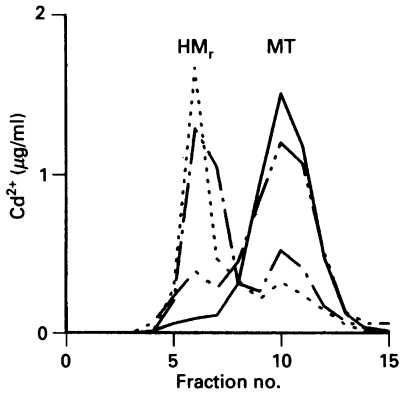

Figure 8 Cytosolic redistribution of cadmium

The distribution of cadmium between HM$_r$ proteins and the MT-containing fraction after Sephadex G-75 chromatography is shown for day-1 (·····), day-5 (–·–·–), day-10 (–··–··–) and day-15 (—) livers of $E_2 + Cd^{2+}$-treated rainbow trout. For each chromatogram a total of 2 ml of supernatant was applied to a 1.5 cm × 50 cm column and eluted in 2.5 ml fractions. The column void volume was about 10 ml; HM$_r$ proteins were eluted at approx. 15 ml, and MT was eluted at approx. 25 ml.

increased after pre-induction of MT and when E_2 was injected before cadmium (Table 1).

Figure 7 shows the cytosolic distribution of cadmium, copper and zinc between HM$_r$ protein and the MT peak 5 days after injection. As seen for both the control group and the E_2 group, MT is mainly a copper-containing protein in the absence of MT induction. When MT is induced by cadmium, there is a subsequent increase in both cadmium and zinc in the MT peak, and most of the cytosolic cadmium is sequestered by MT. Contrary to this, after $E_2 + Cd^{2+}$ injection most of the cadmium was found in the HM$_r$-peak. These results are in agreement with the data for MT mRNA and MT and indicate that MT is not induced, nor does it bind cadmium in the initial stages of vitellogenesis when fish are injected with E_2.

Since MT mRNA and MT levels increased 10 days after injection of $E_2 + Cd^{2+}$, and since this was followed by an induction of VTG mRNA and VTG on day 15, it was decided to study the time course of cadmium distribution in the liver cytosol of $E_2 + Cd^{2+}$-injected fish (Figure 8). On day 1, most of the cytosolic cadmium was present in the HM$_r$ peak, and this was followed by a slight increase in cadmium in MT on day 5. As shown in Figure 7(c), there was no increase in zinc in the MT peak on day 5. We find that when MT is induced there is always an increased content of zinc in MT. The lack of zinc in the MT peak thus indicates that cadmium is displacing copper from MT. On day 10 most of the cytosolic cadmium was redistributed to the MT peak, and this increase in cadmium bound to MT coincided with activation of transcription and translation of MT (Figures 5 and 6). As shown in Figures 3 and 4, VTG synthesis was apparent first on day 15, when almost all of the cytosolic cadmium was bound to MT.

DISCUSSION

The results of the present study demonstrate that cadmium treatment, but not zinc treatment, of E_2-injected fish leads to transcriptional down-regulation of VTG synthesis. The cadmium-mediated decrease in plasma VTG appears to be due to preferential binding of cadmium to non-MT proteins in the liver of E_2-treated rainbow trout. Binding of cadmium to these non-MT proteins also result in decreased cadmium induction of MT mRNA and MT. The binding of cadmium to non-MT macromolecules, instead of to MT, resulted in increased sensitivity of the fish to cadmium, as shown by the high mortality of $E_2 + Cd^{2+}$-treated fish.

Since E_2 induces a multitude of different hepatic systems involved in the synthesis, processing and secretion of VTG and other E_2 induced hepatic proteins (van Bohemen et al., 1981; Peute et al., 1985; Mommsen and Walsh, 1988; Hyllner et al., 1991), and since zinc is needed in these systems (Olsson et al., 1987), there must be an abundance of sites available for interaction with both zinc and cadmium. Elevated VTG mRNA levels were observed already on day 1 in E_2-injected fish. The induction of VTG mRNA is rapid, and others have shown that VTG mRNA is detectable already within a few hours after E_2 treatment (Le Guellec et al., 1988). Although zinc is essential for the function of the E_2-induced systems, and therefore should not interfere with vitellogenesis, it has been shown previously that cadmium treatment of vitellogenic fish results in lowered LSI and lowered VTG levels in plasma (Haux et al., 1988; Pereira et al., 1993). This was also the case in the present study, where only low levels of VTG mRNA could be observed 1 day after injection of $E_2 + Cd^{2+}$. However, the cadmium-mediated inhibition of VTG mRNA on day 1 could be suppressed by pre-induction of MT by zinc before the co-injection of $E_2 + Cd^{2+}$. Thus it appears that the presence of high levels of MT, which is able to bind cadmium, counteracts the inhibitory effects of cadmium on vitellogenesis.

The decrease in VTG synthesis observed in $E_2 + Cd^{2+}$-treated fish correlated both with binding of cadmium to non-MT protein and with a lack of MT induction. Although both zinc and cadmium were able to induce MT mRNA synthesis on their own, this was not the case after co-injection of E_2 with either metal. Cadmium was found to be more toxic to E_2-treated fish than to control fish. The increased toxicity is suggested to be a result of the inhibition of MT synthesis. Consequently, this sensitivity could be reversed by pre-induction of MT before co-injection of $E_2 + Cd^{2+}$. The decrease in VTG observed in $E_2 + Cd^{2+}$-injected fish and the increased mortality seen in the present study may therefore be a result of this decreased likelihood of MT to sequester and detoxify cadmium.

Preinduction of MT by zinc injections before $E_2 + Cd^{2+}$ injection resulted in protection of the fish, as seen by lowered mortality. However, when co-injected with E_2 the ability of zinc to induce MT was diminished. Although the zinc-inducibility of MT is decreased by the addition of E_2, it is not expected that zinc will disrupt the vitellogenic processes, since it may function as a cofactor in many of the enzymes required for the activated metabolic processes during vitellogenesis.

It has been argued that E_2 induces MT transcription in fish (Overnell et al., 1987; Povlsen et al., 1990). However, in previous studies we have shown that MT is not primarily induced by E_2, but by zinc, which is released from HM$_r$ proteins once the vitellogenic period is over (Olsson et al., 1987, 1989b, 1990). We have further shown that E_2 does not induce MT in tissue cultures of rainbow-trout cells (Olsson et al., 1989c). No induction of MT was seen in either E_2-treated or $E_2 + Zn^{2+}$-treated fish in the present experiment. The initial induction of MT mRNA by cadmium on day 1 in the $E_2 + Cd^{2+}$ group indicates that the non-MT proteins that compete with MT for cadmium require more than 24 h to accumulate and that they are not present before E_2 treatment. Before the appearance of these proteins, cadmium will be available for binding to the metal-responsive transcription factors, thus activating transcription of the MT genes. The delayed inhibitory effect of E_2 treatment on cadmium-mediated induction of MT mRNA became apparent when cadmium was

injected after E_2. This treatment resulted in 43 % lower induction of MT mRNA when cadmium was injected 1 day after E_2 and no induction when cadmium was injected 2 days after E_2, indicating that the E_2-activated systems require several days to reach full activity.

As shown by the hepatic MT levels, there was a small but noticeable increase in MT on day 5 after co-injection of $E_2 + Cd^{2+}$. This increase also correlated with a slight increase in MT-bound cadmium. Further, the induction of MT mRNA on day 1 after injection of $E_2 + Cd^{2+}$ indicates that E_2 has no direct inhibitory effect on MT at the gene level. Thus E_2 does not itself appear to repress MT transcription in rainbow trout. The metabolic activation of the liver after E_2 induction appears to result in a net increase in binding sites for trace metals. These binding sites sequester most of the cytosolic cadmium that is actively accumulated by the liver. Once sequestered by the E_2-induced gene products, the trace metals are no longer available for induction of MT. We therefore suggest that the decrease in MT that was observed in the present study is due to lack of availability of cadmium for induction and binding to MT. On the other hand, the inhibition of VTG synthesis that results from the cadmium treatment appears to be acting at the transcriptional level, since very little or no VTG mRNA is produced after this treatment. The combined effects of E_2 and cadmium on the two systems studied give a possible explanation of why the reproductive stages are highly sensitive to cadmium. Further studies are, however, necessary to determine at what level cadmium inhibits VTG transcription. It would be interesting to determine whether it does this by binding to E_2 or the E_2 receptor, thereby interfering with the transcriptional control of vitellogenesis.

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