Disruption of Metallothionein Expression with Antisense Oligonucleotides Abolishes Protection Against Cadmium Cytotoxicity in Molluscan Hemocytes

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Received June 27, 2000; accepted August 31, 2000

The relationship between metallothionein (MT) induction and cytotoxicity was examined in isolated oyster hemocytes exposed in vitro to cadmium, copper, and zinc. In all cases MT induction increased to peak levels with increased metal dose, then declined with continued increases in dose. The effectiveness of these metals as inducers of MT was cadmium > copper > zinc, with cadmium the most effective inducer in magnitude of induction and sensitivity to dose. Cytotoxicity of the metals, based on decreases in lysosomal neutral red retention times, was copper > cadmium > zinc. Zinc was relatively nontoxic and a poor inducer of MT. Cadmium, which was intermediate in toxicity, required concentrations in excess of that causing peak induction of MT to have substantial effects on lysosomal membrane integrity. In contrast, copper was highly cytotoxic at concentrations below that which resulted in peak MT induction. This reversal in the rank order of cadmium and copper suggested that the toxicity of cadmium was tempered by the protective effects of MT. This was verified by disrupting MT expression with antisense phosphorothioate-substituted oligodeoxynucleotides. Administration of 2.5 µM CdCl₂, which induced MT but had no effect on lysosomal membrane stability when administered alone, reduced neutral red retention time to 41% of control levels when administered in the presence of antisense. The resulting toxicity was greater than that caused by a comparable concentration of copper. The findings represented evidence that expression of MT protects against cadmium toxicity in an estuarine mollusk.

Key Words: metallothionein; cadmium; copper; zinc; antisense; phosphorothioate; oligodeoxynucleotide; lysosome; hemocyte; mollusc; oyster.

Metal detoxification was recognized as a possible function for metallothionein (MT) in early studies describing characteristics associated with its high metal content and metal affinity (Kågi and Vallee, 1960, 1961). A specific role in protection against cadmium toxicity was later proposed (Piscator, 1964). There is now a considerable body of evidence in support of such a function (reviewed recently in Klaassen et al., 1999), although the significance remains a topic of discussion (Klaassen et al., 1999; Palmiter, 1998; Vallee, 1995). Direct evidence for a role in protection against metal toxicity is derived from gene deletion experiments showing that targeted disruption of MT expression in yeast and mice compromises resistance to copper, cadmium, or zinc toxicity, yet does not otherwise alter the phenotype of the affected organisms under nonstressed conditions (Hamer et al., 1985; Kelly et al., 1996; Masters et al., 1994; Michalska and Choo, 1993; Thiele et al., 1986). Furthermore, transgenic mice that overexpress MT exhibit greater resistance to cadmium toxicity (Liu et al., 1995), and insertion of a human MT gene rescues the copper-resistant phenotype of MT-disabled yeast (Thiele et al., 1986).

While studies on the biology and chemistry of MT in invertebrate species are numerous (reviewed by Roesijadi, 1992), there is still little direct evidence of a role for MT in protection against metal toxicity in the varied taxa represented in this group. Extension of such function to species of lower phylogenetic status could shed light on the general question of metal detoxification as a function of MT. Class I MTs similar to those of vertebrates have been reported in several crustacean and molluscan species (Brouwer et al., 1995; Dallinger et al., 1993; Lerch et al., 1982; Pedersen et al., 1994; Unger et al., 1991). Putative metal regulatory elements exhibiting homology with mammalian counterparts have also been reported in an insect, sea urchin, nematode, and a mollusc (Freedman et al., 1993; Harlow et al., 1989; Khoo and Patel, 1999; Otto et al., 1987). Furthermore, the notion that MT is a protein involved in metal detoxification remains central to the field of ecotoxicology, despite the current lack of direct evidence. Thus, it is of interest to investigate the function of MT in species directly relevant to this field. MTs in aquatic species such as fish, molluscs, arthropods, and annelids are induced by and, in turn, bind metals such as cadmium, copper, mercury, or zinc (reviewed by Roesijadi, 1992). Individuals in metal-contaminated aquatic environments often possess elevated concentrations of MT, and MT is considered a potential biomarker of effects or exposure to metals (Stegeman et al., 1992). However, its use as a practical measure for assessing metal exposure has yet to be

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realized, due in part to unresolved questions relating to function and factors that affect induction.

We examined the effect of cadmium on hemocytes, the blood cells involved in the molluscan nonspecific defense response (reviewed by Cheng, 1996), in the eastern oyster Crassostrea virginica, hypothesizing that MT expression is necessary to protect against cadmium toxicity. *C. virginica* (Unger et al., 1991), like other molluscan species studied to date (Barsyte et al., 1999; Berger et al., 1995; Dallinger et al., 1997; Engelken and Hildebrandt, 1999; Khoo and Patel, 1999; Mackay et al., 1993), possesses class I MT resembling those in higher organisms. This species is commercially and ecologically important and has been the subject of monitoring programs for metal contamination of the coastal environment (Lauenstein et al., 1990; NOAA, 1987). Phosphorothioate-substituted oligodeoxynucleotides antisense to the oyster MT mRNA (aMT-S-ODN) were used to determine whether disruption of MT expression would influence the toxicity of cadmium. Both basal and cadmium-induced MT expression have been described for these cells (Roesijadi et al. 1997a). We now report that treatment of hemocytes with the aMT-S-ODN inhibited levels of both basal and cadmium-induced MT mRNA and that low cadmium concentrations that normally induce MT, but do not elicit symptoms of sublethal cytotoxicity, became toxic when MT expression was disrupted by administration of aMT-S-ODN. These findings represent direct evidence that induction of MT by cadmium confers protection against cadmium toxicity in cells of this mollusc.

**MATERIALS AND METHODS**

**Animals.** Cultured oysters (2–3 years old) were purchased from Pemaquid Oyster Co., Damariscotta, ME, and maintained in 25 parts per thousand (ppt) salinity artificial seawater (Instant Ocean, Aquarium Systems, Mentor, OH) at 18°C ± 1. The seawater was recirculated through a carbon filter and biological filter (Bio Chem Beads, Aquarium Pharmaceuticals Inc., Chalfont, PA) and monitored for ammonia, nitrite, nitrate, and pH. Oysters were fed resuspended algal paste composed of 45% Isochrysis galbana, 35% Skeletonema sp., and 5% *Chlorella vulgaris* (Diet C-5, Coast Seafood Company, Quinique, WA) at a ration of approximately 0.05 g per oyster per day. This was supplemented every other day with 1 liter each of live, 10- to 21-day-old cultures of *T. weisfloggi* and *T. galbana*.

**Isolation of hemocytes.** The procedure for isolation of hemocytes was based on a previously described protocol (Anderson et al., 1992). Hemolymph was withdrawn from the adductor muscle sinus using a syringe with a 23-gauge needle and transferred to plastic petri dishes. After 30 min, adherent hemocytes were washed and resuspended in culture medium composed of 25 ppt artificial seawater, 0.5% antibiotic/antimycotic solution (penicillin, streptomycin, and amphotericin B; Sigma A-9909), and 2 mg/ml glucose. Hemocytes were pelleted at 250 × g, resuspended in culture medium and diluted to a density of 4 × 10^6 cells/ml, then aliquoted to various experimental treatments. Cells isolated from 12 to 16 oysters were pooled to provide sufficient numbers of cells to conduct experiments in which MT mRNA was determined by RT-PCR or in which antisense was used to disrupt MT expression; numbers of replicate pools are noted with the results of individual experiments.

**Metall exposure.** Hemocytes were exposed for 20 h to cadmium, copper, or zinc in 1.5 ml polypropylene microcentrifuge tubes at room temperature (24–26°C) in darkness. Stock solutions of CdCl₂, CuCl₂, and ZnCl₂ dissolved in 0.01 N nitric acid were first diluted in culture medium to make fresh working stocks. Volumes of the working stocks added to samples were the same in all cases, and the small volumes added had no detectable effect on pH.

**Cytotoxicity assays.** Hemocyte viability was measured by supravital staining with neutral red (Borenfreund and Puerner, 1985, 1987) during a 20-min incubation or by trypan blue exclusion.

Sublethal cytotoxicity was measured as reduced lysosomal neutral red retention time, which is diagnostic of lysosomal membrane instability of stressed cells (Lowe et al., 1992). Measurement of lysosomal neutral red retention time was conducted according to a procedure developed for isolated mussel hemocytes (Lowe et al., 1995), modified by omitting dimethylsulfoxide in the solvent for neutral red. The neutral red incubation solution was prepared in culture medium just prior to use. Cells were monitored under oil immersion at 1000× magnification. Following uptake and concentration of neutral red in lysosomes, the proportion of cells exhibiting loss of dye from lysosomes was recorded until loss was observed in greater than 50% of the cells. Observations for neutral red retention in cells exposed to cadmium or zinc were recorded at 20-min intervals. With copper, observations were made every 5 min for the initial 20 min, due to the more rapid release, then at 20-min intervals thereafter. The neutral red retention time was estimated as the time at which 50% of hemocytes released their accumulated neutral red (ET₅₀). Measurements were made on cells of individual oysters.

**Total RNA isolation and quantification.** Total RNA was isolated using Tri Reagent (Molecular Research Center, Cincinnati, OH) and quantified fluorometrically (Schmidt and Ernst, 1995) using SYBR Green II RNA dye (Molecular Probes Inc., Eugene, OR). RNA was incubated with dye in white microtiter plates (Packard Optitplate) for 30 min at room temperature, then analyzed on an FCA fluorescence plate reader (IDEXX, Portland, ME) at 485 nm excitation/535 nm emission. Quantification was based on standard curves derived from fluorescence of yeast transfer RNA standards.

**Antisense oligodeoxynucleotides.** An antisense phosphorothioate-substituted oligodeoxynucleotide (5'-GGACCGGTGACCTTCCTCCCCCA-3') corresponding to nucleotides 201 to 221 of the oyster MT cDNA (Unger et al., 1991) was synthesized by Oligos Etc. (Wilsonville, OR) and further purified in our laboratory by anion exchange high-performance liquid chromatography (HPLC) (Warren and Vella, 1993). The sample was applied to a TSK DEAE-5PW column (7.5 mm × 7.5 cm; Toyoda Soda) at initial buffer conditions (73% buffer A [25 mM Tris, pH 8, 10% acetonitrile], 27% buffer B [3 M NaCl in buffer A]), and aMT-S-ODN was eluted with a 45-min gradient from 27 to 100% buffer B at 1 ml/min. HPLC-purified material was recovered by ethanol precipitation overnight at ~20°C and centrifugation for 15 min at 12,500 × g, 4°C. The pellet was washed twice in 80% ice-cold ethanol, resuspended in water, quantified by measuring absorbance at 260 nm, aliquoted, lyophilized, and stored at ~70°C.

Antisense-S-ODN was administered to the cells 2 h prior to initiation of 20-h metal exposures. A fully phosphorothioate-substituted oligonucleotide of identical length and similar nucleotide composition (5'-GCCGAGGTCATGTCGTACGC-3') was used as a control for potential nonspecific S-ODN toxicity.

**Quantitative RT-PCR.** MT mRNA was quantified by competitive RT-PCR (Becker-Andre and Hahlbrock, 1989; Wang et al., 1989), using an external standard derived from a cDNA deletion clone (Δ6 bases) of the oyster MT coding region. This Δ6 clone was created by in vitro mutagenesis (ExSiteTM PCR-based Site-Directed Mutagenesis Kit, Stratagene, LaJolla, CA) of the native cDNA sequence cloned in pGEM-3Z (Fuentes et al., 1994). The external standard was synthesized using T7 RNA polymerase (Ausbel et al., 1993).

For the competitive RT-PCR, 0.025 μg total sample RNA was co-reverse transcribed with serial dilutions of the Δ6 standard. Reverse transcription was conducted in 1 × Tris buffer solution (10 mM Tris–HCl pH 9, 50 mM KCl, 0.1% Triton X-100), 5 mM MgCl₂, 1 mM dNTP mix, 5 μM reverse primer (5'-CATCTTCTGGACGTCGCCG-3'), and 0.33 U Prime RNase Inhibitor (Prime-3cPrime Inc., Boulder, CO) in a final volume of 10 μl. For PCR, samples were supplemented with additional 1 × Tris buffer, MgCl₂, to 1.5 mM final concentration, 5 μM biotinylated (*) forward primer (5'-*TGATTGAGACTGTCGGACCTG-3'), and 1.25 U Taq polymerase, adjusted to a final
volume of 50 μl and amplified for 21 cycles. Amplification conditions were denaturation for 5 min at 94°C initially, 1 minute thereafter; annealing for 2 min at 50°C; elongation for 3 min at 72°C, with a 10-min elongation step in the last amplification cycle.

RT-PCR product detection was based on a microtiter plate-based chemiluminescent assay. Duplicate, amplified, biotinylated cDNA subsamples were bound to streptavidin-coated microtiter plates, denatured, and hybridized to fluorescein-labeled oligonucleotide probes specific for either the native MT mRNA or the external standard. The hybridization buffer was 6 × SSC, 5 × Denhardt’s solution, 0.1% Tween-20, 100 μg/ml sheared, denatured, calf thymus DNA. Alkaline phosphatase-conjugated antifluorescein antibody (Tropix Inc, Bedford, MA) was bound to the hybridized probes and analyzed for chemiluminescence after addition of substrate (CSPD; Tropix Inc.) and enhancer solution (Sapphire II, Tropix Inc.). A Top Count liquid scintillation counter (Packard Instrument Co.) in single photon counting mode was used for chemiluminescence detection. MT mRNA was estimated at log(T/S0) = 0, the point of equivalence of target and standard, in a plot of log(T/S0) versus logS0 (Raeymaekers, 1993). T/S0 is the ratio of target to standard signal after RT-PCR, and S0 is the initial amount of each external standard in the standard dilution series.

Data analysis. Data were analyzed using ANOVA. Post hoc comparisons used Student-Newman-Keul’s test. The level of significance was set at p ≤ 0.05.

RESULTS

It was of interest to conduct experiments at sublethal cytotoxic concentrations. Cell viability was not appreciably affected by exposure to CdCl2 up to 100 μM, CuCl2 up to 100 μM, or ZnCl2 up to 1 mM, the maximum concentrations used in this study. Viability of metal-exposed hemocytes was within 91.5–111.2% of control values in cadmium exposures, 94.3–116.8% in copper exposures, and 94.2–105.3% in zinc exposures. A small decrease in viability to 91% of controls at 100 μM CdCl2 was the only consistently observed, statistically significant reduction in viability in the various experiments. In some cases, small but significant increases in viability over control levels suggested that hormesis may have occurred at times at the lowest metal concentrations of 2.5 μM CdCl2 and CuCl2 and 10 μM ZnCl2.

The relative effectiveness of cadmium, copper, and zinc as inducers of MT expression and cytotoxicants was determined for oyster hemocytes. Dose-response curves indicated large differences in the intracellular levels of metal ions (Fig. 1). With all three metals, dose-response curves exhibited an increase in MT mRNA to maximal levels as dose increased. Increasing dose above these levels attenuated induction. Cadmium was the most effective inducer of the metals investigated, followed by copper and zinc. This was evident in both magnitude of induction and sensitivity to dose: induction by cadmium reached higher levels and required lower doses. Estimations of the effectiveness of MT mRNA induction by the three metals, which took into account both the magnitude of induction and sensitivity to dose, supported a rank order of Cd > Cu > Zn (Table 1). When both were taken into account, cadmium was 13.7-fold more effective than copper and 258-fold more effective than zinc in inducing MT mRNA. In this case, maximal levels of induction in response to CdCl2 reflected a 9.3-fold increase in MT mRNA over basal levels, in comparison with 5.9- and 3.8-fold over basal levels in response to CuCl2 and ZnCl2, respectively.

Neutral red retention measurements showed the rank order of adverse effect on lysosomal membrane stability was Cu > Cd > Zn (Fig. 2), with corresponding EC50 values of 3.9, 180.4, and 1389 μM, respectively, for CuCl2, CdCl2, and ZnCl2 (Table 1). Copper was highly toxic to hemocytes when compared to cadmium and zinc. The relatively low effect of zinc on neutral red retention mirrored the effect of zinc on cell viability.

It was also instructive to consider the effectiveness of MT induction by the three metals in relation to their toxicities. Of particular interest was the reversal observed in relative cytotoxicities of cadmium and copper when compared to their effectiveness in inducing MT (Table 1). Copper was highly toxic to lysosomes at metal concentrations lower than those inducing substantial MT. The reverse was true for cadmium, with maximal MT induction occurring below the cadmium EC50 for lysosomai neutral red retention. MT was fully induced by cadmium at doses much lower than those causing toxicity. These observations suggested that the greater resistance of hemocytes to cadmium may be due to the ability of MT to confer protection against cadmium toxicity. Thus, the effect of cadmium on lysosomal neutral red retention in hemocytes was examined in cells administered antisense prior to initiation of metal exposure.

Fully substituted aMT S-ODN was used in this study. As antisense at concentrations used here had no adverse effect on viability (data not shown), any changes in lysosomal membrane stability would reflect sublethal cytotoxic effects.
rather than nonspecific responses associated with cell death. Partially substituted antisense S-ODN for MT (i.e., 4 sulfur substitutions at each end of the oligonucleotide) was also tested and found to be ineffective in downregulating MT, causing little or no reduction of MT mRNA at the concentrations found to be effective with the fully substituted form. Treatment with 5 and 10 \( \mu M \) aMT-S-ODN resulted in dose-dependent reductions in MT mRNA induction (not shown), with no additional reduction when the dose was increased to 20 \( \mu M \). Thus, 10 \( \mu M \) aMT-S-ODN was selected for routine disruption of MT expression. This concentration of control and aMT S-ODN did not affect viability of untreated and cadmium-exposed cells. Treatment of cells with 10 \( \mu M \) control S-ODN affected neither basal nor cadmium-induced MT mRNA levels (Fig. 3).

### TABLE 1

Maximal Levels of MT mRNA Induction, the Corresponding Metal Dose, Effectiveness of Induction, and the Respective EC\(_{50}\) for Neutral Red Retention Time (NRR) for Cadmium, Copper, and Zinc Exposures

<table>
<thead>
<tr>
<th>Metal</th>
<th>Basal*</th>
<th>Max induced*</th>
<th>Metal concentration at max MT induction (( \mu M ))</th>
<th>Effectiveness of MT induction</th>
<th>EC(_{50}) for NRR (( \mu M ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CdCl(_2)</td>
<td>39</td>
<td>364</td>
<td>10.5</td>
<td>31.0</td>
<td>180.4 ± 25.2</td>
</tr>
<tr>
<td>CuCl(_2)</td>
<td>28</td>
<td>164</td>
<td>60</td>
<td>2.27</td>
<td>3.9 ± 0.7</td>
</tr>
<tr>
<td>ZnCl(_2)</td>
<td>30</td>
<td>113</td>
<td>682</td>
<td>0.12</td>
<td>1388.7 ± 330.2</td>
</tr>
</tbody>
</table>

* Measured concentrations of MT mRNA in control hemocytes of respective treatments.

\(^{1}\) Estimated from induction curves of Figure 1. Effectiveness of MT induction reflects MT mRNA induced per \( \mu M \) metal exposure concentration at the metal concentration inducing the maximum level. This value is calculated by normalizing the maximum induced minus basal MT mRNA level to the inducing metal concentration and incorporates both the magnitude of maximal induction and the metal dose inducing this level.

\(^{2}\) EC\(_{50}\)s were determined from regression curves of probits of NRR (expressed as percent of control) versus log metal dose. Values are mean ± standard error of the mean, \( n = 3 \); individual EC\(_{50}\)s were determined for cells obtained from a single oyster.

FIG. 2. Dose-response curves for neutral red retention time, a measure of lysosomal membrane stability, in hemocytes of *Crassostrea virginica* exposed to cadmium, copper, or zinc. Three replicate measurements were made for each metal, with each replicate based on cells from a single oyster. The neutral red retention time, expressed as percent of the control ET\(_{50}\), was estimated for each replicate. All data points are shown in this figure; the regression lines are shown only to emphasize the differences in slopes for the three metals. The EC\(_{50}\) concentration, defined as the metal concentration reducing lysosomal neutral red retention to 50% of control levels, and its standard error are presented for each metal in Table 1. Reductions in neutral red retention time followed the order copper > cadmium > zinc.

FIG. 3. Effect of phosphorothioate-substituted oligodeoxynucleotides (S-ODN) antisense to the MT mRNA (aMT) on basal and cadmium-induced MT mRNA levels in hemocytes of *Crassostrea virginica*. The cadmium dose was 15 \( \mu M \) CdCl\(_2\). Differences among groups were highly significant (\( p < 0.0001 \), ANOVA on log transformed data due to unequal variance among groups). Both basal (*) and cadmium-induced MT mRNA (**) were significantly reduced by administration of antisense (\( p < 0.05 \), Student-Newman-Keuls test). The 95% confidence intervals are shown above bars. Four hemocyte pools, each pool composed of cells from 12 oysters, were analyzed for treatments with 10 \( \mu M \) control S-ODN (C-ODN in figure) and antisense S-ODN (aMT in figure); five hemocyte pools were analyzed for the remaining treatments.
induced MT mRNA concentrations by approximately 55 and 50%, respectively (Fig. 3).

To test whether MT induction conferred protection against the toxicity of cadmium, the stability of lysosomal membranes was examined in hemocytes whose ability to induce MT was compromised by treatment with aMT-S-ODN. Destabilization of lysosomal membranes was expected to be observed if disrupting MT expression also disrupted a protective function associated with its expression. Cells were tested at 2.5 µM CdCl₂, shown earlier to induce MT mRNA, but having little adverse effect on hemocyte lysosomes. Neutral red retention time was defined as the time for 50% of hemocytes to release neutral red from lysosomes. Differences among groups were highly significant (p < 0.005, ANOVA), and administration of the antisense prior to cadmium exposure resulted in significantly reduced neutral red retention time at a cadmium concentration that normally causes little or no effect (* (p < 0.05, Student-Newman-Keuls test)). (Mean ± 1 SEM; n = 3, except n = 2 in 10 µM aMT treatment in which one of the samples was lost during processing.)

FIG. 4. Effect of phosphorothioate-substituted oligodeoxynucleotides antisense to the MT mRNA (aMT) on neutral red retention time of hemocytes of Crassostrea virginica exposed to 2.5 µM CdCl₂, a concentration that induced MT (see Fig. 1) but had little effect on hemocyte lysosomes. Neutral red retention time is defined as the time for 50% of hemocytes to release neutral red from lysosomes. Differences among groups were highly significant (p < 0.005, ANOVA), and administration of the antisense prior to cadmium exposure resulted in significantly reduced neutral red retention time at a cadmium concentration that normally causes little or no effect (* (p < 0.05, Student-Newman-Keuls test)). (Mean ± 1 SEM; n = 3, except n = 2 in 10 µM aMT treatment in which one of the samples was lost during processing.)

DISCUSSION

Cadmium is a potent inducer of MT in hemocytes of the oyster Crassostrea virginica (present study and Roesijadi et al., 1997a). Copper and zinc were relatively poor inducers in comparison with cadmium. Copper was the most toxic of the three metals, decreasing the EC₅₀ for lysosomal neutral red retention times roughly 46-fold over that of cadmium and 356-fold over that of zinc. The rank orders for MT induction (Cd > Cu > Zn) and metal cytotoxicity (Cu > Cd > Zn) were consistent with rank orders for MT induction (Roesijadi et al., 1997b) and toxicities of these metals (Calabrese et al., 1973, 1977) to larval stages of this oyster. A role for MT in protection against cadmium toxicity can be inferred by comparing EC₅₀s for neutral red retention times and metal concentrations eliciting maximal MT induction in Table 1. In the case of cadmium, MT mRNA is maximally induced at concentrations substantially lower than the cadmium EC₅₀. With copper, the reverse was true; copper toxicity occurred at concentrations much lower than those needed to induce MT. Disrupting MT gene expression with the antisense oligonucleotide at a cadmium concentration not normally cytotoxic resulted in a high level of cadmium cytotoxicity and reversed the rank order of cytotoxicity, so cadmium was now more toxic than copper. Thus, the cytotoxicity of cadmium was tempered by induction of MT. These findings represented strong evidence that MT expression conferred protection against cadmium toxicity. Upper limits for protection of MT against cadmium toxicity were also noted: complete protection was seen only at a low level of cadmium exposure. At exposures that exceeded 2.5 µM CdCl₂, reductions in neutral red retention times and induction of MT were concurrent.

The increase in cadmium toxicity that resulted from disruption of MT mRNA concentrations with antisense oligodeoxynucleotides was consistent with the large numbers of studies implicating MT in protection against cadmium toxicity in higher animals (reviewed in Klaassen et al., 1999). For example, induction of MT by pretreatment with low doses of cadmium results in adaptive tolerance to the toxicity of higher concentrations (Klaassen and Liu, 1998); overexpression of MT as a result of gene amplification results in increased cadmium resistance in cultured mammalian cells (Beach and Palmiter, 1981; Gick and McCarty, 1982; Hildebrand et al., 1979); overexpression of MT in MT transgenic mice results in increased cadmium resistance (Liu et al., 1995); and disruption of MT expression in knockout mice results in loss of resistance to cadmium toxicity (Masters et al., 1994; Michalska and Choo, 1993). Increased sensitivity to cadmium in MT knockout mice is the strongest evidence to date that loss of protection against cadmium toxicity is an important phenotype associated with MT gene disruption in animals. Recent studies also suggest that MT induction by cadmium may be a specific cellular response not mediated by pathways involving zinc. They describe cadmium-specific regulation of MT gene expression in cells lacking the zinc-responsive metal transcription factor MTF-1 (Chu et al., 1999) and a signal transduction pathway that involves the upstream regulatory factor (USF) (Andrews, 2000; Datta and Jacob, 1997; Li et al., 1998). Such observations lend credibility to the notion that induction of MT by cadmium follows...
pathways specific for this metal and that the induction functions to protect against cadmium toxicity.

A recent study describes the first reported genomic MT sequences of a mollusc, MT-Ia1 and MT-Ia2 of the green mussel Perna viridis (Khoo and Patel, 1999). MT-Ia1 possesses a TATA box and putative sequences for two metal responsive elements, two glucocorticoid receptor sites, and three AP1 binding sites. MT-Ia2 has a TATA box and putative sequences for an MRE, two AP1 sites, and an enhancer E box promoter region. This complexity mirrors that seen in higher animals (Palmiter, 1999) and suggests responsiveness of MT expression to zinc and factors other than metals, such as hormones and reactive oxygen species. However, the significance of such responsiveness remains to be clarified in higher animals (Klaassen et al., 1999; Palmiter, 1998, 1999), and has yet to be investigated in molluscs.

Our findings contribute to a mechanistic justification for continued evaluation of molluscan MT as a biomarker for potential biological effects in cadmium-contaminated aquatic environments: where MT-bound cadmium is detected in organisms such as oysters, individuals are likely protected from this fraction of accumulated cadmium. Thus, the biological significance of such binding would be the lack of adverse effects from this MT-bound fraction of cadmium, a notion that has value for interpreting responses of individuals in metal-contaminated environments (reviewed in Roesijadi, 1992). Our findings of a strong inverse relationship between MT expression and the toxicity of cadmium will need to be extended to an understanding of how MT is expressed and functions in natural populations.

Our study showed that, of the metals examined, cadmium was the most effective in inducing MT in isolated oyster hemocytes and that expression of MT conferred protection against cadmium toxicity. The latter conclusion was deduced from a loss-of-function experiment in which cadmium concentrations normally not cytotoxic became highly toxic after MT induction and the toxicity of cadmium will need to be extended to an understanding of how MT is expressed and functions in natural populations.

Our study showed that, of the metals examined, cadmium was the most effective in inducing MT in isolated oyster hemocytes and that expression of MT conferred protection against cadmium toxicity. The latter conclusion was deduced from a loss-of-function experiment in which cadmium concentrations normally not cytotoxic became highly toxic after MT expression was disrupted with antisense oligodeoxynucleotides. Thus, in molluscs, as in higher animals, MT protects against cadmium toxicity. However, the finding that MT is important in protection against cadmium does not preclude the possibility that MT is also involved in other important biological functions.

ACKNOWLEDGMENTS

Support from the following organizations are acknowledged: U.S. Environmental Protection Agency Exploratory Research Grant R-822282–01; NIH Training Grant no. T32 ES-7263 to the Program in Toxicology, University of Maryland Baltimore; Chesapeake and Potomac Chapter of the Society for Environmental Toxicology and Chemistry; and Melbourne R. Carricker Student Research Grant, National Shellfisheries Association. Ms. Regina Macatangay conducted measurements for neutral red retention times in the initial dose-response experiments.

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