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Effects of cadmium on cellular protein and glutathione synthesis and expression of stress proteins in eastern oysters, *Crassostrea virginica* Gmelin

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SUMMARY

Cadmium (Cd) is an important toxicant in estuarine and coastal environments that can strongly affect energy balance of aquatic organisms by increasing the organism's basal energy demand and reducing its aerobic capacity. Mechanisms of cadmium-induced increase in basal metabolic costs are not well understood and may involve elevated detoxification costs due to the synthesis of cellular protective proteins and glutathione. We studied the short-term effects of cadmium exposure (4 h) on protein and glutathione (GSH) synthesis and expression of stress proteins (heat shock proteins HSP60, HSP70 and HSP90) and metallothioneins in isolated gill and hepatopancreas cells of the eastern oyster, *Crassostrea virginica*. Our study showed that exposure to cadmium resulted in a dose-dependent increase in the rate of protein synthesis in oyster cells, which reached 150% of the control at the highest tested Cd level (2000 μmol l^{-1}). GSH synthesis was significantly inhibited by the highest Cd concentrations, especially in hepatopancreas, which resulted in a slight but significant decrease in the total GSH concentrations. Elevated protein synthesis was associated with the increased expression of metallothioneins and heat shock proteins. Interestingly, stress protein response differed considerably between gill and hepatopancreas cells. In hepatopancreas, expression of metallothionein mRNA (measured by real-time PCR) increased 2–8-fold in response to Cd exposure, whereas no significant increase in metallothionein expression was found in Cd-exposed gill cells. By contrast, HSP60 and HSP70 protein levels increased significantly in Cd-exposed gill cells (by 1.5–2-fold) but not in hepatopancreas. No change in HSP90 expression was detected in response to Cd exposure in oyster cells. These data indicate that metallothionein expression may provide sufficient protection against Cd-induced damage to intracellular proteins in hepatopancreas, alleviating the need for overexpression of molecular chaperones. By contrast, Cd detoxification mechanisms such as inducible metallothioneins and GSH appear to be insufficient to fully prevent protein damage in gill cells, thus necessitating induction of HSPs as a secondary line of cellular defense. Therefore, gills are likely to be among the most Cd-sensitive tissues in oysters, which may have important implications for impaired oxygen uptake contributing to energy misbalance and reduced aerobic scope in Cd-exposed oysters.

Key words: heat shock protein, metallothionein, protein synthesis, glutathione, cadmium, bivalve.

INTRODUCTION

Heavy metals such as cadmium (Cd) are important stressors in aquatic environments due to their persistent nature and ability to bioaccumulate in organisms. In the marine realm, estuaries and coastal waters are particularly prone to Cd pollution, which can be released from both anthropogenic (e.g. mining, production and use of pigments and batteries) and natural sources such as run-off from cadmium-rich soils or diatom deposition in marine sediment (GESAMP, 1987; Roesijadi, 1996; Frew et al., 1997). Eastern oysters, *Crassostrea virginica*, are common estuarine inhabitants and a useful model to study the mechanisms of Cd toxicity and tolerance. Oysters are exposed to varying Cd concentrations in their habitats and have an ability to accumulate Cd burdens exceeding environmental levels by orders of magnitude (Roesijadi, 1996; Frew et al., 1997). According to some estimates, up to 40% of total Cd load in estuarine ecosystems can be contained in oyster soft tissues (Pigeot et al., 2006), making these mollusks an important vector of Cd transfer to higher levels of the food chain, including humans.

Cadmium is a toxic metal with no known biological function in animals and can strongly affect organisms' physiology, survival and performance. Earlier studies have shown that energy misbalance is implicated in Cd-induced stress and toxicity in aquatic organisms such as oysters. Mitochondrial dysfunction is an important aspect of Cd toxicity in oysters, leading to impaired ATP production, reduced aerobic capacity and elevated oxidative stress (Sokolova, 2004; Cherkasov et al., 2007). On the other hand, exposure to Cd results in elevated basal metabolic demand in oysters, thus resulting in a mismatch between energy demand and supply and greatly limiting the aerobic scope of an organism (Lannig et al., 2006). Reduced aerobic scope due to the toxin exposure may negatively affect the organism's fitness due to physiological tradeoffs that can divert energy from essential processes such as growth, reproduction or locomotion towards maintenance (Sibly and Calow, 1989; Calow and Forbes, 1998; Pörtner, 2001; Roff 2002; Notten et al., 2006; Lannig et al., 2006), although some empirical studies indicate that energetic burden imposed by detoxification costs may be weak (Van Straalen and Hoffmann, 2000). Currently, the mechanisms of the elevated basal energy demand in Cd-exposed oysters are not fully understood, and further studies are needed to identify the processes contributing to the Cd-induced energetic burden.

Protein synthesis cost is one of the major components of cellular energy demand, accounting for 10–20% of standard metabolic rate (Hand and Hardewig, 1996; Hulbert et al., 2002). Exposure to pollutants, including heavy metals, can lead to an increase in protein synthesis and associated energy costs by switching ribosomes to the selective translation of protective proteins (Pytharopoulou et al., 2006). By contrast, under conditions of acute stress, an organism may limit protein synthesis by reversible inactivation of ribosomes to conserve energy, which would result in an overall decline of protein synthesis rates (Pytharopoulou et al., 2006). Our earlier studies using indirect methods show that oxygen consumption associated with protein synthesis increases along with the overall increase in the basal metabolism during Cd exposure of oysters (Cherkasov et al., 2006; Lannig et al., 2006). This increase was proposed to reflect the cost of the *de novo* synthesis of cellular protection proteins and the replacement of the proteins damaged by Cd. However, in order to test this hypothesis, direct measurements of the protein synthesis rates and expression of cell protection proteins in response to Cd exposure are needed.

Metallothioneins and heat shock proteins (HSPs) are among the primary candidates for cellular protection, which may contribute to the elevated protein synthesis costs in Cd-exposed oysters. Metallothioneins are low-molecular-mass (6–7 kDa), cysteine-rich proteins. They have been shown to participate in free-radical scavenging and antioxidant protection (Andrews, 2000; Coyle et al., 2002), but their primary function is binding of essential and non-essential metal ions in cytoplasm for storage and/or detoxification and eventually targeting them to lysosomes for deposition and/or disposal (Klaassen et al., 1999; Andrews, 2000; Tanguy et al., 2001). Metallothioneins are strongly induced by heavy metals such as Cd, and elevated expression of these proteins is a hallmark of metal exposure (Amiard et al., 2006). In addition to metallothioneins, glutathione (GSH) plays an important role in metal detoxification. GSH is a short polypeptide containing a cysteine residue that serves as a primary nucleophile in detoxification reactions, including metal binding (Meister and Anderson, 1983). Up to 10% of total metals in bivalve cytoplasm may be bound to GSH (Giguere et al., 2003). Overexpression of GSH can thus increase metal tolerance of an organism, while GSH depletion results in an increased susceptibility to heavy metal toxicity (White et al., 1999; Conners and Ringwood, 2000; Ringwood and Conners, 2000; White and Cappai, 2003).

Heat shock proteins are involved in protection against a wide range of environmental stressors, and their expression is triggered by oxidatively modified or partially denatured/misfolded proteins in the cell. Cytosolic chaperones HSP70 and HSP90 are among the most abundant cellular proteins protecting against stress-induced damage. HSP70 is the most ubiquitous and universal cytosolic chaperone family involved in folding/refolding of newly synthesized and damaged proteins as well as sequestering and degradation of proteins that are damaged beyond repair (Mayer and Bukau, 2005). HSP90 is another general chaperone of the eukaryotic cytosol, orchestrating the folding of many proteins; however, HSP90 alone is insufficient to assist in refolding of partially denatured proteins and requires other chaperones such as the HSP70 chaperone family to complete this task (Csermely et al., 1998; Mayer and Bukau, 2005). By contrast, HSP60 is predominantly found in mitochondria and chloroplasts of eukaryotes, assisting with the protein folding and stress protection in these organelles (Cechetto et al., 2000). Given that, in oysters, Cd localizes to mitochondria and cytosol (Sokolova et al., 2005), one can expect a strong impact of Cd exposure on expression of these molecular chaperones.

To date, the effects of Cd exposure on protein synthesis have not been extensively studied in aquatic ectotherms, and the relationship between the global proteosynthetic response and expression of different cytoprotective mechanisms (such as metallothioneins, HSPs and GSH) during Cd stress is poorly understood. It is also not known whether tissue-specific variations in protein synthesis and expression patterns may affect the response of different tissues to Cd exposure. In this study, we used a direct method to determine the effects of Cd exposure on the protein and glutathione synthesis rates in isolated gill and hepatopancreas cells of eastern oysters. We tested a hypothesis that Cd exposure results in elevated rates of protein and/or glutathione synthesis and analyzed relationships between these rates and the expression of key components of cellular protection including GSH, metallothioneins, and cytoplasmic and mitochondrial HSPs.

MATERIALS AND METHODS

Animal collection and maintenance

Adult oysters (70–120 mm shell length) were collected in June 2006 from Stump Sound, North Carolina, USA. Water temperature during the week of collection varied between 21 and 32°C, salinity was 29–32‰. The study site has very low background concentrations of heavy metals and organic pollutants [(Mallin et al., 1999); James Schwarzenberg, J & B Aquafood, personal communication]. Animals were transported to the University of North Carolina at Charlotte within 5 h and acclimated at 20±1°C and 30‰ in recirculating aerated tanks with artificial seawater (Instant Ocean®; Kent Marine, Acworth, GA, USA) for at least 3 weeks prior to experimentation. They were fed *ad libitum* with a commercial algal blend (1 ml per oyster every other day) containing live *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella* spp. (2–20 μm) (DT's Live Marine Phytoplankton, Premium Reef Blend; DT's Plankton Farm, Sycamore, IL, USA).

Cell isolation

Gills or hepatopancreas (or digestive gland) tissues from 2–5 oysters were pooled on ice in 5 ml of digestion buffer containing 24.72 g l^{-1} NaCl, 0.68 g l^{-1} KCl, 1.36 g l^{-1} $CaCl_2.2H_2O$, 0.18 g l^{-1} $NaHCO_3$ and 30 mmol l^{-1} Hepes at pH 7.5. Tissues were minced and washed with an additional 10 ml of digestion buffer. Tissue fragments were digested for 15 min at room temperature in 0.125% trypsin in balanced Hank's solution (Fisher Scientific, Suwanee, GA, USA) adjusted to 720 mOsm with sucrose, carefully triturated to release cells and washed twice with digestion buffer. The suspension was filtered through 100 μm sterile nylon mesh and centrifuged for 15 min at 900 ***g*** to pellet the cells. Cells were washed in cell suspension medium (CSM) containing 24.72 g l^{-1} NaCl, 0.68 g l^{-1} KCl, 1.36 g l^{-1} $CaCl_2.2H_2O$, 0.18 g l^{-1} $NaHCO_3$, 4.66 g l^{-1} $MgCl_2.6H_2O$, 6.29 g l^{-1} $MgSO_4.7H_2O$, 30 mmol l^{-1} glucose and 30 mmol l^{-1} Hepes at pH 7.5, centrifuged for 10 min at 900 ***g*** and re-suspended in 4 ml of the same medium. Cell count was performed with a Beckman Coulter Z2 cell counter, 100 μm aperture diameter (Beckman Coulter, Inc., Fullerton, CA, USA). The particle size range window was set to 10–30 μm, corresponding to the known size of gill and hepatopancreas cells from oysters (Eble and Scro, 1996). Viability of isolated cells, determined by the Trypan Blue exclusion assay, was >90–95% throughout the experiment. Our earlier studies also show that isolated oyster cells maintain viability for over 48 h in culture with no appreciable increase in the levels of apoptosis or necrosis in

millimolar concentrations of Cd (Sokolova et al., 2004). Sample size (*N*) reported in the figures and throughout the paper refers to the number of individual cell isolates, each obtained from 2–5 oysters as described above.

Cadmium determination

Isolated gill and hepatopancreas cells were exposed to different Cd levels (0–2000 μmol l^{-1}) for 4 h at 20°C. After the exposures, cell suspensions were centrifuged (2000 ***g*** for 15 min), washed twice in Cd-free CSM to remove surface-associated Cd, and re-suspended in Cd-free CSM. Washed cell suspensions were mixed 1:1 with 70% nitric acid and digested in a 60°C water bath for 24 h. Cd concentrations were determined with an atomic absorption spectrometer AAnalyst 800 (Perkin Elmer, Waltham, MA, USA), equipped with a graphite furnace and Zeeman background correction. National Institute of Standards and Technology (NIST) oyster tissue (1566b) was analyzed with the samples to verify the metal analyses; the percent recoveries over all batches were 94.6±6.6% (mean ± s.d.). Cellular Cd levels are expressed as ng Cd 10^{-6} cells.

Determination of protein and glutathione synthesis rates

Protein and glutathione synthesis rates were determined by incorporation of radioactively labeled L-leucine (^{3}H-Leu) or L-glycine (^{3}H-Gly), respectively (Bonifacino, 1999), in control and Cd-exposed cells at 20°C. Briefly, 300 μl of cell suspension (0.2–1.7×10^7 cells ml^{-1}) was incubated with 1 μCi (1 μCi= 37 kBq) of labeled amino acid ([^{3}H]Leu or [^{3}H]Gly) for 4 h at 20°C with different concentrations of Cd (0–2000 μmol l^{-1}) and cycloheximide (0 or 2 mmol l^{-1}). After incubation, 20 μl of sample were placed on Whatman binder-free glass microfiber filters (type GF/A; Fisher Scientific), air dried and exposed to 10% ice-cold trichloroacetic acid (TCA) containing 5 mmol l^{-1} of the respective unlabeled aminoacid (L-Leu or L-Gly) for 10 min. The filter was then washed twice with 5% TCA (15 min each wash) and dehydrated in 100% ethanol (three times 10 min each) at room temperature. After dehydration, filters were air dried and placed into glass scintillation vials containing 5 ml of ScintiSafe Econo 1 scintillation cocktail (Fisher Scientific, Suwanee, GA, USA). Pilot studies with filters spiked with radiolabeled amino acids showed that these washes were sufficient to remove all unincorporated label. Counting was performed with a Beckman Coulter LS6000SC scintillation counter. Counts per minute (c.p.m.) were recalculated to disintegrations per minute (d.p.m.) with internal counter software. Addition of cycloheximide (2 mmol l^{-1}) routinely inhibited 95–98% of [^{3}H]Leu incorporation, confirming that most of the incorporated leucine was due to the protein synthesis. A similar degree of inhibition of [^{3}H]Leu incorporation was obtained with another specific inhibitor of protein synthesis, emetine (300 μmol l^{-1}) (data not shown). The glutathione synthesis rate in the presence and absence of cadmium was calculated as [^{3}H]Gly incorporation rate when co-incubated with 2 mmol l^{-1} cycloheximide under the assumption that most of the labeled glycine was incorporated into glutathione under these conditions.

Glutathione concentrations

Total glutathione (GSH) concentrations were determined enzymatically by the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) – glutathione disulfide (GSSG) reductase recycling assay following the rate of formation of 5-thio-2-nitrobenzoic acid (TNB) (Griffith, 1980; Anderson, 1985). Gill and hepatopancreas cells were incubated at 20°C without Cd (control) or in the presence of 2000 μmol l^{-1} Cd (Cd-exposed). After the predetermined exposure times (0, 4, 8 and 12 h), the cells were homogenized in equal volumes (1:1) of 10% sulfosalicylic acid (SSA), sonicated for 20 s (output 30 W; Sonicator 3000; Misonix Inc., Farmingdale, NY, USA) and centrifuged (16 000 ***g*** for 5 min at 4°C). Total glutathione was measured in the supernatant with a UV-Vis spectrophotometer (VARIAN Cary 50 Bio; Cary, NC, USA) equipped with a temperature-controlled cuvette holder at 30°C (±0.1°C) at 412 nm. The calibration curve was prepared using GSH standards dissolved in a 1:1 mixture of CSM and 10% SSA. Results are expressed as total GSH content in nmol 10^{-6} cells.

Expression of HSPs determined by immunoblotting

Cell suspensions isolated from gill and hepatopancreas were incubated for 4 h without Cd (controls) or in the presence of 50, 500 and 2000 μmol l^{-1} Cd. Cells were collected by centrifugation and homogenized in ice-cold homogenization buffer [100 mmol l^{-1} Tris, pH 7.4, 100 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA, 1% Triton-X100, 10% glycerol, 0.1% sodium dodecylsulfate (SDS), 0.5% deoxycholate, 0.5 μg ml^{-1} leupeptin, 0.7 μg ml^{-1} pepstatin, 40 μg ml^{-1} phenylmethylsulphonyl fluoride (PMSF) and 0.5 μg ml^{-1} aprotinin] at a concentration of 4×10^6 cells ml^{-1} of buffer. HSPs were detected using standard immunoblotting methods. Briefly, the homogenate was sonicated three times for 10 s each (output 69 Watts; Sonicator 3000, Misonix Inc.) and centrifuged at 14 000 ***g*** for 5 min at 4°C. Protein content was measured in the supernatant using the Bio-Rad Protein Assay kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Bovine serum albumin (BSA) was used as a standard. 30 μg of sample protein per lane was loaded onto 8% polyacrylamide gels and run at 100 mA for 2 h at room temperature. The resolved proteins were transferred onto a nitrocellulose membrane in 96 mmol l^{-1} glycine, 12 mmol l^{-1} Tris and 20% methanol (v/v) using a Trans-Blot semi-dry cell (Thermo Fisher Scientific Inc., Portsmouth, NH, USA). To verify equal protein loading, membranes were stained with Amido Black Stain Solution (Fisher Scientific; 1 g l^{-1} Amido Black in 10% methanol, 10% glacial acetic acid) for 30 s. The membranes were then destained by washing in several changes of water and blocked overnight in 5% non-fat milk in Tris-buffered saline, pH 7.6 (TBST). Blots were probed with primary monoclonal antibodies against HSP70, HSP90 and HSP60, respectively (MA3-007, Affinity Bioreagents, Golden, CO, USA; SPA-835 and SPA-805, Stressgen Bioreagents, Ann Arbor, MI, USA). After washing off the primary antibody, membranes were probed with the respective polyclonal secondary antibodies conjugated with horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA, USA), and proteins were detected by enhanced chemiluminescence according to the manufacturer's instructions (Pierce, Rockford, IL, USA). Densitometric analysis of the signal was performed by GelDoc 2000™ System with Quantity One 1-D Analysis Software (Bio-Rad). Each blot included a control sample as an internal standard, and expression of HSPs in Cd-exposed cells was calculated as percent of the respective control.

RNA extraction and RT-PCR amplification

Expression of metallothionein mRNA was measured by quantitative real-time PCR (QRT-PCR). There are no commercially available antibodies specific for molluskan metallothioneins, and several commercial antibodies developed against mammalian metallothioneins failed to cross-react with oyster metallothioneins in our pilot studies (data not shown).

Therefore, we have used mRNA expression as a proxy for metallothionein expression, as it has been previously shown that it is primarily regulated at the transcription level (Rose et al., 2006).

RNA was extracted by homogenization in Tri Reagent (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's protocol. RNA concentration and quality were verified by UV spectroscopy. Reverse transcription was performed using 200 U μl^{-1} SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), 50 $\mu mol\ l^{-1}$ of oligo$(dT)_{18}$ primers and 5 μg total RNA to obtain single-stranded cDNA.

For QRT-PCR, specific primers were designed to amplify cDNA for metallothionein I (MTI), total metallothioneins (MTI and MTII) and β-actin using *C. virginica* sequences published in GenBank (NCBI accession numbers AY331695.1, AY331705.1 and X75894.1, respectively). Because of the high sequence similarity and the duplicated β-domain in MTII (Tanguy and Moraga, 2001), we were unable to design specific primers to MTII that would amplify a single product; therefore, metallothionein II expression could not be measured separately. Instead, we designed consensus primers that amplified a single-length PCR product from either MTI or MTII and thus determined expression of both metallothioneins simultaneously. Expression of β-actin was used as a housekeeping gene to normalize expression of metallothioneins. In preliminary studies, several housekeeping genes, including β-actin, ubiquitin, ribosomal protein L13a and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were tested because earlier research indicates that some housekeeping genes may show deviant expression, thus potentially skewing experimental results if used as a reference (Khimani et al., 2005). Our pilot study showed that in oysters, the expression of all four housekeeping genes varied concordantly between samples (data not shown), suggesting that any of these is a suitable reference gene. Therefore, in all subsequent analyses, metallothionein expression was normalized to β-actin. The following primers were used for QRT-PCR. For metallothionein I: MT I-FW, 5′-cca cct gca aat gtg gat ca-3′; MTI-RV, 5′-atc aat aca taa aag aaa cat cac tcg-3′. Consensus primers for metallothioneins I and II: MTI+II-FW, 5′-ggc tgt aaa tgt ggg gag aa-3′; MTI+II-RV, 5′-gag aac gcc tct cat tgg tc-3′. For β-actin: Act-Cv-F437, 5′-cac agc cgc ttc ctc atc ctc c-3′; Act-Cv-R571 5′-ccg gcg gat tcc ata cca agg-3′.

Quantitative RT-PCR was performed using a LightCycler® 2.0 Real Time PCR System (Roche Applied Science, Indianapolis, IN, USA) and QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturers' instructions. The reaction mixture consisted of 5 μl of 2× QuantiTect SYBR Green master mix, 0.3 $\mu mol\ l^{-1}$ of each forward and reverse primer, 1 μl of 10× diluted cDNA template and water to adjust to 10 μl. Ten microliters of reaction mixture were loaded into LightCycler 20 μl capillaries (Roche Applied Science) and subjected to the following cycling: 15 min at 95°C to denature DNA and activate Taq polymerase; 40–55 cycles of 15 s at 94°C, 20 s at 55°C and 15 s at 72°C. SYBR Green fluorescence (acquisition wavelength 530 nm) was measured at the end of each cycle for 2 s at the read temperature of 78°C. The read temperatures of the amplified fragments were determined in the pilot experiments and set at the value to melt all primer dimers but not the amplified gene product. At the end of each run, melting temperature profiles were run between 50 and 99°C with continuous fluorescence acquisition to confirm the expected melting temperatures for the amplified fragments. In each run, serial dilutions of a cDNA standard were amplified to determine amplification efficiency (Pfaffl, 2001), and an internal standard was included to test for amplification variability between the runs. Dilutions of the experimental cDNA samples were selected so that their crosspoints for fluorescence fell within the range of the crosspoints for cDNA standards. Amplification efficiencies (E) were 2.05+0.04 (N=7), 1.84+0.06 (N=8) and 2.03+0.04 (N=8) for consensus metallothioneins I and II, metallothionein I and β-actin, respectively. Expression of the target genes (metallothioneins) were calculated relative to the expression of β-actin and normalized against the internal standard as proposed by Pfaffl (Pfaffl, 2001):

$$R = \frac{E_{\mathrm{t}}^{\Delta \mathrm{CP_t}}}{E_{\mathrm{ref}}^{\Delta \mathrm{CP_{ref}}}},$$

where E_t and E_{ref} are amplification efficiencies for the target gene and the reference (β-actin), respectively, and ΔCP_t and ΔCP_{ref} are differences between crosspoints for fluorescence of the sample and the internal standard for the target gene and β-actin, respectively.

Chemicals

All other chemicals were purchased from Sigma Aldrich, Fisher Scientific or GE Healthcare (Buckinghamshire, UK) and were of analytical grade or higher. Enzymes were purchased from Sigma Aldrich.

Statistical analysis

Repeated-measures ANOVAs were used to test the effects of Cd on the studied variables after testing the assumptions of normality of data distribution and homogeneity of variances. Dunnett's tests were used for *post-hoc* comparisons of sample means against the respective controls, and one- or two-tailed LSD (least squared difference) tests were used for planned comparisons of sample means as appropriate. Individual sample (i.e. cell isolate) was used as a repeated-measures variable, thus allowing us to separate the sample-to-sample variability from the factor effect (Cd exposure). For the sake of clarity, data for protein and glutathione synthesis are shown as % of the respective controls. However, all statistics for protein and glutathione synthesis were done on the original data (i.e. the rate of incorporation of [^{3}H]Leu or [^{3}H]Gly for protein and glutathione, respectively). Because of high sample-to-sample variation, statistical tests for mRNA and protein expression were performed on normalized data (i.e. expression levels in Cd-exposed cells normalized to the respective controls from the same cell batch). Statistical analyses were performed using SAS 9.1.3 software (SAS Institute, Cary, NC, USA). Factor effects and differences between the means were considered significant if the probability of Type II error was less than 0.05. Values are given as means ± standard error (s.e.m.).

RESULTS

De novo protein and glutathione synthesis

Exposure to Cd resulted in a dose-dependent accumulation of this metal in gill and hepatopancreas cells (Fig. 1), with hepatopancreas cells accumulating nearly twice as much Cd as the gills at the same exposure level. At the highest Cd exposures (1000–2000 $\mu mol\ l^{-1}$), intracellular Cd concentrations leveled off in gill cells, indicating saturation of uptake and/or storage mechanisms, whereas Cd burden in hepatopancreas cells continued to rise with increasing exposure concentrations.

Protein synthesis rate, as estimated by the rate of incorporation of [^{3}H]Leu, was nearly 2-fold higher in hepatopancreas cells than in gills, although this difference was only marginally significant (P=0.07) due to the high variation (Fig. 2A). Exposure to Cd

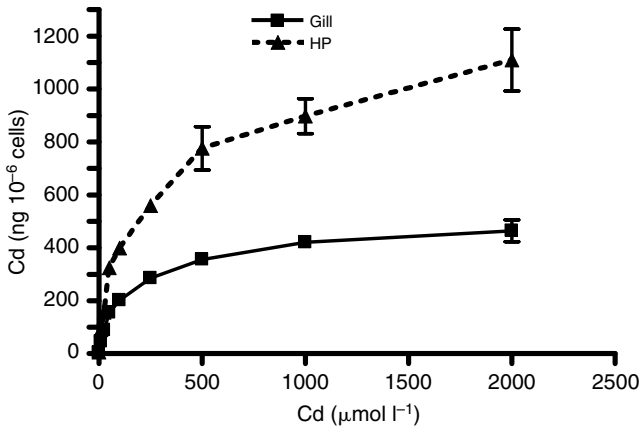

Fig. 1. Dose-dependent accumulation of cadmium (Cd) in isolated gill and hepatopancreas (HP) cells of *C. virginica*. Mean Cd burdens (*y*-axis) accumulated after 4 h exposure in varying Cd concentrations (*x*-axis) at 20°C are shown. Vertical bars represent s.e.m. (*N*=5).

resulted in a dose-dependent increase in the rate of *de novo* protein synthesis in gill and hepatopancreas (Fig. 2B). At the highest studied Cd exposures (1000–2000 μmol l^{-1}), protein synthesis rates were approximately 50% higher than in the control.

By contrast, the rate of GSH synthesis, estimated by cycloheximide-insensitive incorporation of [^{3}H]Gly, declined in Cd-exposed cells in a dose-dependent manner (Fig. 3). At the highest studied Cd exposure (2000 μmol l^{-1}), GSH synthesis rate in oyster cells declined by 25–35% compared with the respective controls. GSH synthesis accounted for 63 and 52% of the total [^{3}H]Gly incorporation in gill and hepatopancreas cells, respectively, and this percentage did not significantly change with Cd exposure. The rate of GSH synthesis estimated by cycloheximide-insensitive incorporation of [^{3}H]Gly tended to be higher in gill cells (Fig. 3A) but this trend was only marginally significant (*P*=0.07). Notably, GSH synthesis rate was more strongly suppressed by Cd exposure in hepatopancreas cells than in gills. This was also reflected by a decrease in GSH content in response to Cd exposure, which was significant in hepatopancreas but not in gill cells (ANOVA; *P*=0.95 and 0.02 for the overall effects of Cd exposure in gills and hepatopancreas, respectively) (Fig. 4). However, GSH depletion was minimal under the experimental conditions of this study, probably reflecting the short-term exposures (4–12 h), and the total GSH concentration dropped by only 4–9% after 4–12 h of exposure of hepatopancreas cells to 2000 μmol l^{-1} Cd.

Expression of metallothioneins and HSPs

Metallothionein mRNA was detected in considerable amounts in the control cells, indicating that these genes are constitutively transcribed in the absence of a stressor. The constitutively expressed metallothionein (MTI) mRNA levels normalized to actin tended to be lower in control (non-Cd-exposed) hepatopancreas cells compared with the gills (1.05±0.42 and 0.28±0.08 in gills and hepatopancreas, respectively, *P*<0.05), whereas the differences in total (MTI+II) mRNA expression were not significant between the two tissues under control conditions (*P*>0.05). Cadmium exposure resulted in a significant dose-dependent increase in metallothionein levels in the hepatopancreas but not in the gill cells (Fig. 5). Expression levels of total metallothioneins (MTI and II) in hepatopancreas cells were 2.5–4-fold elevated after 4 h of exposure

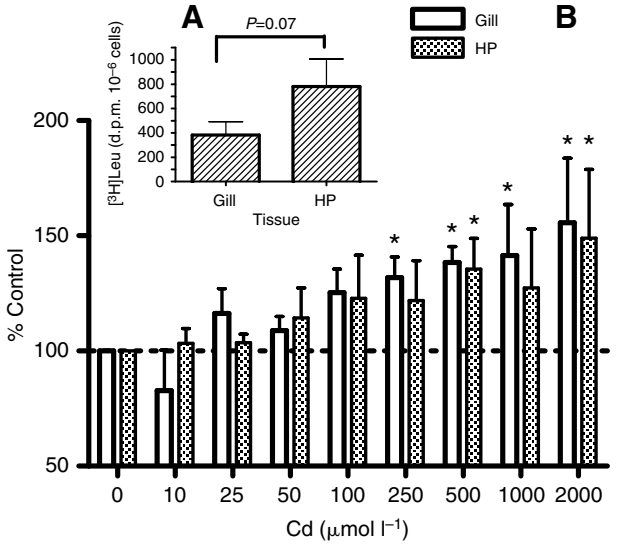

Fig. 2. Effects of cadmium (Cd) exposure on protein synthesis rates in gill and hepatopancreas (HP) cells of oysters. (A, insert) Protein synthesis rates in control gill and HP cells estimated by the rate of incorporation of [^{3}H]Leu. Vertical bars represent s.e.m. (*N*=8). (B) Changes in [^{3}H]Leu incorporation in response to Cd exposure for 4 h at 20°C expressed as % of the respective controls. Vertical bars represent s.e.m. Asterisks denote values significantly different from the respective controls (*P*<0.05). *N*=7–8 except for the lowest (10–25 μmol l^{-1}; *N*=3) and the highest (1000–2000 μmol l^{-1}, *N*=4–5) Cd levels.

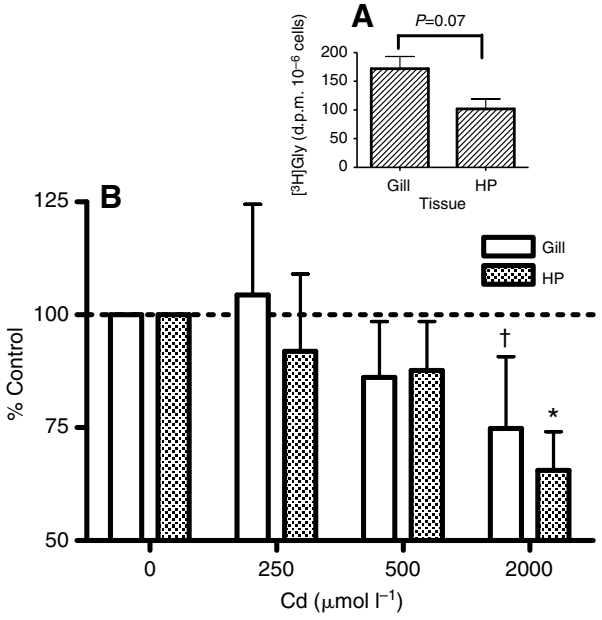

Fig. 3. Effects of cadmium (Cd) exposure on glutathione synthesis rates in gill and hepatopancreas (HP) cells of oysters estimated as the rates of cycloheximide-insensitive incorporation of [^{3}H]Gly. Mean values of glutathione synthesis in the cells exposed to different Cd levels (*x*-axis) for 4 h at 20°C are expressed as % of the respective controls. Vertical bars represent s.e.m. The asterisk denotes values significantly different from the respective control [*P*<0.01; the dagger denotes a marginally significant difference (*P*=0.09)]. *N*=5.

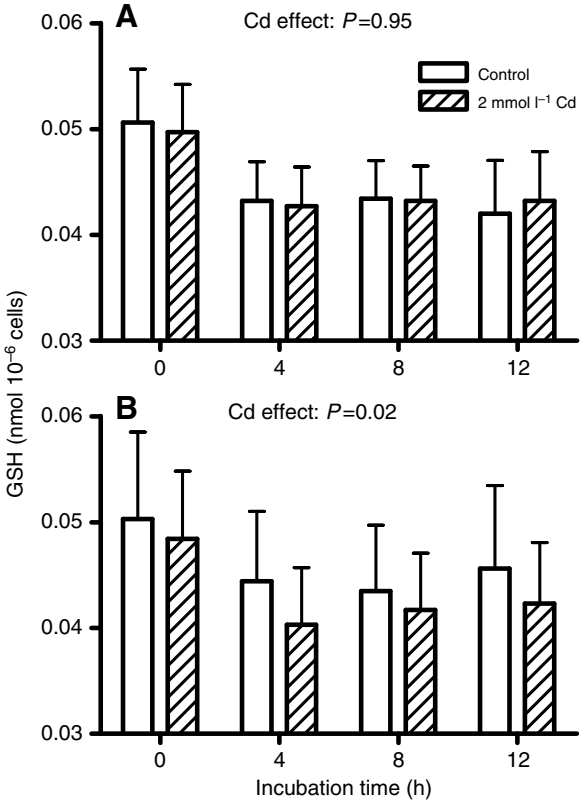

Fig. 4. Effects of cadmium (Cd) exposure on total glutathione (GSH) concentrations in gill (A) and hepatopancreas (B) cells of oysters. Cells were incubated for varying periods of time (*x*-axis) with 0 μmol l^{-1} (control) or 2000 μmol l^{-1} Cd at 20°C, and their total (reduced + oxidized) GSH levels were determined. *P*-values for the effect of Cd on GSH levels, as determined by ANOVA, are given. At each exposure time, differences in GSH content between control and Cd-exposed cells were not significant ($P>0.05$) but the overall effect of Cd estimated by ANOVA was significant in hepatopancreas cells (see *P*-values in the figures). Vertical bars represent s.e.m. ($N=5$).

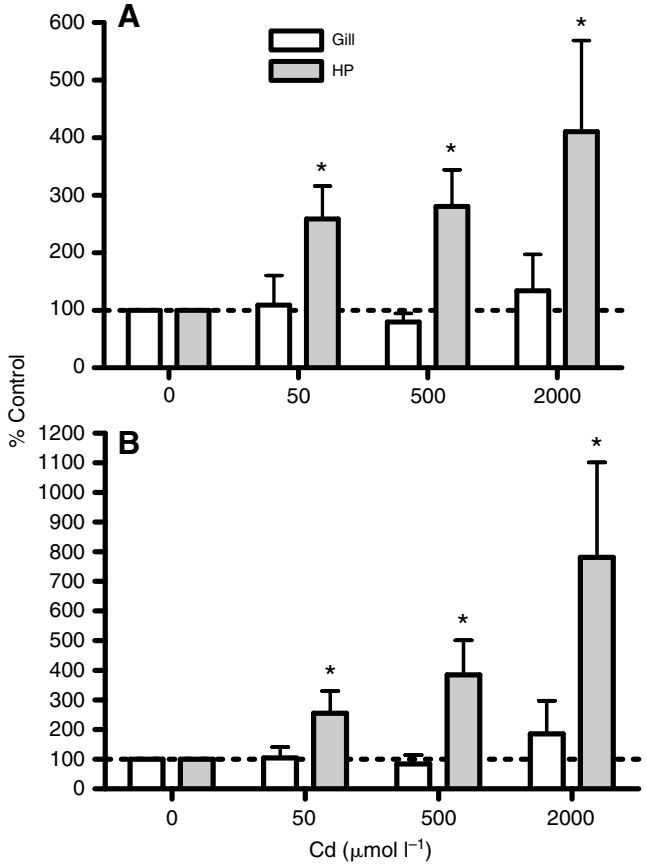

Fig. 5. Effects of cadmium (Cd) exposure on expression of metallothionein mRNA in gill and hepatopancreas (HP) cells of oysters. Metallothionein levels were measured using real-time PCR, normalized to the actin mRNA levels and expressed as % of the respective controls. Cells were exposed to varying Cd levels (*x*-axis) for 4 h at 20°C. (A) mRNA for total metallothioneins and (B) for metallothionein I only. Vertical bars represent s.e.m. Asterisks denote values significantly different from the respective controls ($P<0.05$). $N=9$.

to 50–2000 μmol l^{-1} Cd (Fig. 5A), whereas mRNA levels of MTI alone rose by 3- to nearly 8-fold at the same exposures (Fig. 5B).

Analysis of HSP60, HSP70 and HSP90 expression using western immunoblotting indicated a strong dose-dependent increase in the mitochondrial HSP60 and cytosolic HSP70 protein levels (by up to 1.5–2-fold) in gills but not in hepatopancreas (Fig. 6A,B). No induction of HSP90 in response to Cd exposure was detected in isolated oyster cells (Fig. 6C).

DISCUSSION

Our study clearly shows that Cd exposure elicits a strong proteosynthetic response as well as induction of transcription and/or translation of cytoprotective proteins in isolated gill and hepatopancreas cells of oysters. Interestingly, stress response to Cd strongly differed between the two studied tissues, likely reflecting the tissue-specific differences in Cd accumulation and inducibility of different protective systems.

Overall, oyster hepatopancreas cells accumulated considerably higher (2–2.5-fold) Cd loads than gill cells, indicating faster uptake and/or greater retention of Cd. This is not likely to be a simple consequence of different metabolic rates because our earlier studies indicated that oyster gill and hepatopancreas cells have similar rates of basal metabolism (Cherkasov et al., 2006). Given that the main cellular uptake route for Cd is through membrane ion carriers for essential metals such as Zn and Ca (reviewed in Roesijadi and Robinson, 1993; Bressler et al., 2004), higher rates of uptake may reflect higher density and/or activity of these carriers in hepatopancreas cells. Our present data can shed no light on this hypothesis; however, the fact that bivalve gills are the primary site of metal ion uptake from aqueous phase and are characterized by a high density of active and passive ion transport systems (Prosser, 1973; Marigomez et al., 2002; Apeti et al., 2005) makes this explanation less plausible. Notably, the high rates of Cd accumulation and a greater Cd burden in hepatopancreas cells go hand-in-hand with higher rates of overall protein synthesis and a significantly greater expression of Cd-induced metallothioneins in these cells. In mollusks, the majority of metal ions (including Cd) in cytoplasm are bound to metallothioneins, although at high and/or prolonged exposures, metals may ‘spill over’ onto the high-molecular-mass proteins (Roesijadi, 1982; Roesijadi, 1996; Roesijadi and Klerks, 1989; Roesijadi et al., 1995; Giguere et al., 2003). In mammalian cells, high intracellular levels of inducible metallothioneins enhance Cd uptake into the cells, possibly by maintaining a transmembrane gradient of Cd through binding of

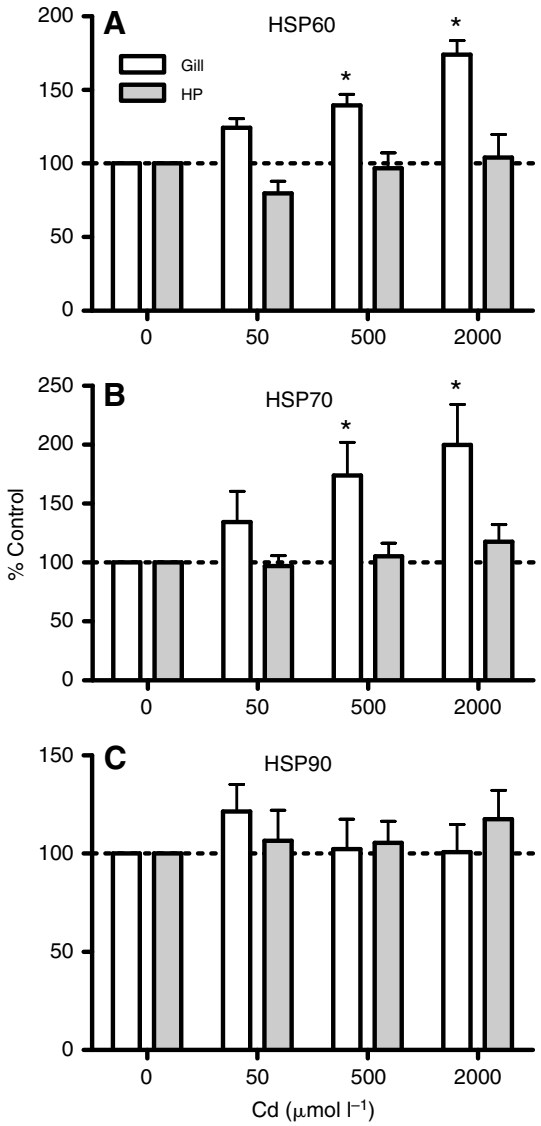

Fig. 6. Effects of cadmium (Cd) exposure on expression of cytoplasmic (HSP70, HSP90) and mitochondrial (HSP60) heat shock proteins in gill and hepatopancreas (HP) cells of oysters. HSP levels were measured using immunoblotting and expressed as % of the respective controls. Cells were exposed to varying Cd levels (*x*-axis) for 4 h at 20°C. Vertical bars represent s.e.m. Asterisks denote values significantly different from the respective controls (P<0.05). N=5.

intracellular free Cd^{2+} (Blais et al., 1999). Thus, it is very likely that the higher level of inducible metallothioneins and a higher overall rate of protein synthesis provide more Cd binding sites and are responsible for higher Cd accumulation in hepatopancreas cells.

It is worth noting that the faster Cd accumulation in isolated hepatopancreas cells found in this study contrasts some earlier reports on whole-organism Cd exposures, which indicated higher rates of Cd accumulation in gills (Roesijadi and Klerks, 2005; Sokolova et al., 2005; Cherkasov et al., 2006; Cherkasov et al., 2007). This may reflect differences in Cd exposure regimens between *in vitro* and *in vivo* studies. During *in vivo* exposures to waterborne Cd, gills are likely to be the primary uptake site for this metal, whereas Cd accumulation in hepatopancreas occurs at a later stage following Cd transport by the circulatory system from gills to the internal organs (Cherkasov et al., 2007). Similarly, earlier studies in other aquatic organisms demonstrated that the highest accumulation *in vivo* (at least initially) occurs in the organ that first comes into contact with Cd and is capable of active ion exchange – i.e. in the gills during exposure to waterborne Cd and in the gut during exposure to dietary Cd (Chowdhury et al., 2005). Therefore, our finding of higher Cd accumulation in hepatopancreas cells compared with gills indicates differences in intrinsic cellular capacities of uptake and retention of Cd; by contrast, *in vivo* rates of Cd accumulation will depend on exposure routes and Cd availability to different organs.

Our study indicates that increased protein synthesis rate is among the earliest cellular responses to Cd and was evident in gill and hepatopancreas cells already after the first few hours of exposure. This increase was dose-dependent and paralleled by the elevated expression of stress response proteins such as metallothioneins or HSPs, suggesting that the observed response at least partially reflects *de novo* synthesis of stress proteins. This agrees with previous findings indicating that acute exposure of cells to Cd^{2+} triggers a preferential increase in stress protein synthesis (Somji et al., 2000; Coyle et al., 2002; Madden et al., 2002; Urani et al., 2007). Currently, there are very few published data on the quantitative contribution of stress proteins to the global protein synthesis rates. Earlier studies showed that synthesis rates of HSPs can be quite high even under non-stress conditions and are comparable to those of some housekeeping genes such as actin (Lanks, 1983). According to some estimates, stress proteins can represent up to 7% of the total protein pool (Kultz, 2003). Given that stress protein expression increases several fold during stress exposure, their contribution to global protein synthesis will also increase. Since protein synthesis in general (irrespective of the nature of produced proteins) and the functioning of stress proteins such as HSPs are ATP-dependent processes (Feder and Hofmann, 1999), a Cd-induced increase in protein synthesis and stress protein expression may represent a significant cost to the organism, diverting energy from essential functions such as growth, reproduction and immunity towards metal detoxification and damage repair.

Interestingly, stress protein response to Cd greatly differed between gill and hepatopancreas cells. Basal levels of constitutive MTI expression were higher in gill cells compared with hepatopancreas. This may reflect the role of oyster gills as the key sites of gas and ion exchange that form an interface between the organism and its environment and thus are among the first organs to be exposed to environmental insults including variations in oxygen levels, pH, pollutants, etc. Metallothioneins with their high sulfhydryl content can function as antioxidants by reacting with free radicals and reactive oxygen species (Basu and Lazo, 1990; Andrews, 2000; Coyle et al., 2002), and high levels of constitutive metallothioneins may thus provide protection against this background environmental stress to the gills. Moreover, constitutive metallothioneins can play an important role in transport, metabolism and storage of essential metals (Coyle et al., 2002); therefore, high levels of constitutive metallothioneins in gill cells may reflect their role in uptake and handling of essential metals in oysters. Cd exposure of isolated gill cells did not result in an induction of metallothionein expression above the background constitutive levels whereas HSP60 and HSP70 were significantly overexpressed with increasing Cd levels. By contrast, in hepatopancreas cells, Cd exposure resulted in a dramatic induction of metallothioneins whereas expression of HSPs was unchanged.

The observed tissue-specific patterns of HSP expression may be explained by differential inducibility of metallothioneins in gills and hepatopancreas. Metallothioneins can be considered the first line of defense against intracellular toxic metals, including Cd, because the metal's binding to metallothioneins significantly reduces its toxicity, preventing interactions with critical cellular components such as enzymes, structural proteins, DNA and membrane lipids. According to different estimates, 75–80% of cytosolic Cd is bound to metallothioneins in mollusks (Roesijadi et al., 1995; Giguere et al., 2003) and only 20–25% is found in non-thionein pools, presumably accounting for toxicity. At higher exposures, the levels of non-thionein-bound Cd substantially increase as the metallothionein binding capacity is exhausted (Roesijadi et al., 1995). High levels of metallothionein induction by Cd in hepatopancreas appear to provide sufficient protection to cellular proteins to prevent modifications such as oxidation, denaturation and/or misfolding, thus alleviating the need for expression of molecular chaperones. By contrast, gill cells are less protected from elevated Cd levels because of the absence of a significant metallothionein induction, which may lead to the spill-over of Cd into the non-metallothionein pool, proteotoxic stress and expression of HSPs required for the repair of the damaged proteins and/or their targeting for degradation (Vayssier and Polla, 1998). Notably, earlier studies indicate that inducible metallothionein levels in oyster gills increase during prolonged (days to weeks) exposure to Cd [Roesijadi (Roesijadi, 1996); and references therein]. The absence of metallothionein induction in gill cells during short Cd exposures (4 h) in the present study suggests that the transcriptional response is slower in these cells, possibly related to their lower overall biosynthetic activity.

It is also noteworthy that among the studied HSPs, HSP60 and HSP70 but not HSP90 showed a significant increase in response to Cd exposure in gill cells. HSP60 is a stress protein constitutively expressed in mitochondria and may serve as a specific biomarker for mitochondrial injury. Our earlier studies have shown that mitochondria are target organelles of Cd accumulation and toxicity in oyster gills (Sokolova, 2004; Sokolova et al., 2005; Cherkasov et al., 2006; Cherkasov et al., 2007). In mitochondria, Cd can affect the electron transport chain and ATP synthesis, stimulate production of reactive oxygen species and damage matrix enzymes (Sokolova, 2004; Cherkasov et al., 2007); thus, HSP60 expression can be expected to be induced early during Cd exposure. HSP60 is also increased in gill and mantle cells of marine mussels and in nematodes exposed to copper (Sanders et al., 1991; Sanders and Martin, 1993; Kammenga et al., 1998) and in rat kidney cell lines exposed to arsenic (Madden et al., 2002), indicating that other metals and metalloids targeting mitochondria may have similar effects on induction of the protective HSP60 response.

Unlike HSP60, HSP70 and HSP90 families belong to cytoplasmic chaperones that assist in a wide range of protein folding processes including refolding of misfolded and aggregated proteins in response to cellular stress (Bensaude et al., 1996; Söti and Csermely, 2000; Urani et al., 2005). In mammalian systems, Cd exposure has been found to induce stress proteins of 72 and 90 kDa in human keratinocytes and in fibroblasts, 70 and 90 kDa in primary rat hepatocytes, and 32, 72, 90 and 110 kDa in human melanoma cells (Bauman et al., 1993; Madden et al., 2002; Urani et al., 2005). In chick embryos, exposure to Cd enhanced *de novo* synthesis of HSP70, whereas HSP24 and HSP90 were unaffected (Papaconstantinou et al., 2003). Data for the Cd-induced chaperone response in aquatic organisms are limited. However, an earlier study in European oysters, *Ostrea edulis*, exposed to 500 μg l^{-1} Cd for 7 days *in vivo* showed upregulated HSP70 expression in gills and hepatopancreas (other HSPs were not tested) (Piano et al., 2004). By contrast, expression levels of another general cytosolic chaperone, HSP90, did not change in response to Cd exposure in oyster cells. Possibly, high levels of expression of HSP70 in response to Cd provide sufficient protection for cytoplasmic proteins in these cells, alleviating the need for elevated HSP90 expression. Interestingly, some studies also indicate that divalent cations greatly suppress the chaperone activity of HSP90, increasing the possibility that HSP90 can be less effective in Cd-exposed cells (Jakob et al., 1995). Overall, it appears that expression of HSP70 is a general feature of the cytosolic response to Cd across a variety of taxa, reflecting the ubiquitous nature of this HSP family and their broad specificity towards a wide range of intracellular proteins.

Elevated synthesis of GSH can serve as an additional cytoprotective mechanism through direct metal binding, detoxification of reactive oxygen and the maintenance of cellular redox status (Meister and Anderson, 1983; Ringwood and Conners, 2000). Our earlier study has shown that long-term exposure to Cd resulted in a significant increase in the GSH level at 20° and 24°C but not 28°C (Lannig et al., 2006). However, in the present study, Cd exposure resulted in a significant decrease in the rates of GSH synthesis in hepatopancreas cells correlated with a small but consistent depletion of GSH levels. This may reflect higher Cd exposure levels of isolated cells in our current study compared with the *in vivo* exposures of whole oysters in the earlier study (Lannig et al., 2006). Indeed, previous research indicates that exposures to high Cd levels typically result in GSH depletion (Regoli and Principato, 1995; Ringwood et al., 1998; Mitchelmore et al., 2003; Toplan et al., 2003), whereas at lower Cd concentrations no change or an increase in GSH levels can be observed (Viarengo et al., 1990; Ringwood et al., 1999; Mitchelmore et al., 2003; Regoli et al., 2004; Lannig et al., 2006). The present study shows that inhibition of glutathione synthesis is a likely mechanism for its depletion at high Cd levels, at least in hepatopancreas cells. It is worth noting that despite a significant (by nearly 35%) decrease of the rate of GSH synthesis in hepatopancreas cells at 2000 μmol l^{-1} Cd, a decrease in total GSH levels was much smaller (less than 10%) and only significant when all exposure times were taken into account. In gills, Cd exposure had a less pronounced effect on the rate of GSH synthesis, resulting in an ~25% decrease at the highest Cd concentration (2000 μmol l^{-1}), and no effect of Cd on total GSH levels was detected. It suggests that longer, chronic exposures may be needed for the reduced rates of GSH synthesis to be significantly reflected in the size of the intracellular GSH pool.

As a corollary, this study directly demonstrates, for the first time, that Cd exposure results in a strongly elevated protein synthesis in isolated gill and hepatopancreas cells of oysters associated with elevated expression of cellular protective proteins. Elevated protein synthesis rates likely reflect the need for detoxification and cellular protection and may contribute to the observed increase in the basal maintenance costs of Cd-exposed oysters (Cherkasov et al., 2006; Lannig et al., 2006). Interestingly, stress protein response significantly differed between gill and hepatopancreas cells despite a similar degree of overall stimulation of protein synthesis by Cd. In hepatopancreas, overexpression of metallothioneins appeared to be a major protective response that was also the likely cause of the observed high levels of Cd accumulation in these cells. No increase in HSP expression in hepatopancreas indicates that levels of damage to mitochondrial and cytosolic proteins are not significantly above the background and suggests that

metallothioneins provide adequate protection of critical cellular proteins against Cd stress. This further emphasizes the high efficiency of metallothioneins as a protective mechanism against heavy metals. By contrast, metallothionein induction was not observed in response to Cd in gill cells, resulting in protein damage and a compensatory increase in molecular chaperones, HSPs, despite the lower overall levels of Cd accumulation. GSH synthesis was suppressed in Cd-exposed cells and no compensatory elevation of GSH levels was observed in response to Cd. These data clearly indicate that cytoprotective response against Cd stress can vary greatly among different tissues and may result in their differential sensitivity to metal exposure. Based on our data, we suggest that gills would be among the most Cd-sensitive tissues in oysters. High sensitivity of gill cells to Cd stress will have important implications for whole-organism physiology and may result in functional hypoxia and energy misbalance due to the elevated metabolic maintenance cost, on one hand, and the impaired ability for oxygen uptake, on the other. Indeed, our recent studies show that Cd exposure results in impaired oxygen uptake in oyster gills whereas oxygen delivery through the circulation system is not affected (G. Lannig, C. Bock, A. Cherkasov, H.-O. Poertner and I. Sokolova, in review). This leads to a reduction in the aerobic scope of the organism, resulting in a lower scope for growth and/or reproduction, reduced tolerance to other environmental stressors such as elevated temperatures and thus reduced fitness of an organism in polluted environments.

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